Protein Post Translational Modifications in Human Diseases:

Bacterial Glycosylation Profiling by Peptide Microarray

Protein Phosphorylation Analysis in High Risk Neuroblastoma

by

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ABSTRACT

Post Translational Modifications (PTMs) are a series of chemical modifications with the capacity to expand the structural and functional repertoire of proteins. PTMs can regulate protein-protein interaction, localization, protein turn-over, the active state of the protein, and much more. This can dramatically affect cell processes as relevant as gene expression, cell-cell recognition, and cell signaling. Along these lines, this Ph.D. thesis examines the role of two of the most important PTMs: glycosylation and phosphorylation.

In chapters 2, 3 and 4, a 10,000 peptide microarray is used to analyze the glycan variations in a series lipopolysaccharides (LPS) from Gram negative bacteria. This research was the first to demonstrate that using a small subset of random sequence peptides, it was possible to identify a small subset with the capacity to bind to the LPS of bacteria. These peptides bound to LPS not only in the solid surface of the array but also in solution as demonstrated with surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) and flow cytometry. Interestingly, some of the LPS binding peptides also exhibit antimicrobial activity, a property that is also analyzed in this work.

In chapters 5 and 6, the role of protein phosphorylation, another PTM, is analyzed in the context of human cancer. High risk neuroblastoma, a very aggressive pediatric cancer, was studied with emphasis on the phosphorylations of two selected oncoproteins: the transcription factor NMYC and the adaptor protein ShcC. Both proteins were isolated from high risk neuroblastoma cells, and a targeted-directed tandem mass spectrometry (LC-MS/MS) methodology was used to identify the phosphorylation sites in each protein. Using this method dramatically improved the phosphorylation site detection and

increased the number of sites detected up to 250% in comparison with previous studies. Several of the novel identified sites were located in functional domain of the proteins and that some of them are homologous to known active sites in other proteins of the same family. The chapter concludes with a computational prediction of the kinases that potentially phosphorylate those sites and a series of assays to show this phosphorylation occurred in vitro.

To Michelle and Sam: you are my love, strength and my inspiration.

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CHAPTER 1

INTRODUCTION

From the sequencing of the human genome, we know that approximately 20 to 25,000 genes can be found inside the human DNA(1). From this amount of material, it has been estimated that up to 100,000 different mRNA transcripts can be generated by means of different mechanism such as alternative promoters, differential transcription termination, alternative splicing of the transcripts, and others.(2) Human cells have the capacity of expanding this number even further to generate more than 1,000,000 different variations of proteins by using a series of chemical modifications known as Post Translational Modifications (PTMs)(3, 4). These modifications have the capacity of to expand not only the diversity but the functionality of the proteome as one single protein can be utilized for different purposes by means of these chemical changes.

Some of these gained functions are, in fact, only possible because of specific characteristics of the modification. For example, the reversible addition of a phosphate, also known as phosphorylation, is the key mechanism behind intracellular cell communications. This process, which requires the fast transmission of chemical signals, is only possible because of the rapid addition and removal of phosphorylations by kinases and phosphatases.

Table 1.1. Some of the most common and relevant post translational modifications (adapted from Mann and Jensen 2003)

PTM	Function and notes
Phosphorylation	Reversible, activation/inactivation of enzyme activity, modulation of molecular interactions, signaling
Acetylation	Protein stability, protection of N terminus. Regulation of protein–DNA interactions (histones)
Methylation	Regulation of gene expression
Glycosylation	Excreted proteins, cell–cell recognition/signaling O-GlcNAc, reversible, regulatory functions
Sulfation	Modulator of protein–protein and receptor–ligand interactions
Disulfide bond formation	Intra- and intermolecular crosslink, protein stability
Deamination	Possible regulator of protein–ligand and protein–protein interactions, also a common chemical artifact
Ubiquitination	Destruction signal. After tryptic digestion, ubiquitination site is modified with the Gly-Gly dipeptide

Some of the most common PTMs and their associated functions are presented in Table 1.1. From this variety of modifications, the present work focuses on two of them: glycosylations and phosphorylations.(5)

Section 1.1 of this introduction focuses on the role of glycosylation in bacteria and its role as a determinant of bacterial serotype. It also covers the use of affinity-based technologies for the analysis of glycosylations. This introduction forms the foundation

for Chapters 2, 3 and 4 where the analysis of glycans on the surface of gram negative bacteria is further expanded.

Section 1.2 of this introduction focuses on the role of phosphorylation in the modulation of cell signaling with an emphasis on neuroblastoma, a severe type of pediatric cancer. The introduction preludes Chapters 5 and 6 where the development of a mass spectrometry methodology to identify novel phosphorylations in neuroblastoma proteins is presented.

1.1 Glycans

"Glycans" is the common designation of mono- or polysaccharides attached to proteins or lipids. In contrast to glyco-polymers, like glycogen that functions as energy storage, glycans perform multiple functions as structural units, enzymes, hormones, transporters, immunological agents, etc. Three major categories of glycans have been established: O-linked (i.e. bound to serine or threonine in proteins), N-Linked (i.e. bound to asparagine in proteins) and lipid bound. Glycans are structurally one of the most diverse molecules in biology. A typical glycan is composed of multiple carbohydrate moieties bound to each other through glycosidic linkage. This covalent binding can take place in multiple positions for each unit, which results in numerous possible combinations. Figure 1.1 presents a typical N-lined glycan visualized as a chemical structure, a word representation and a symbolic representation for simplicity.(6)

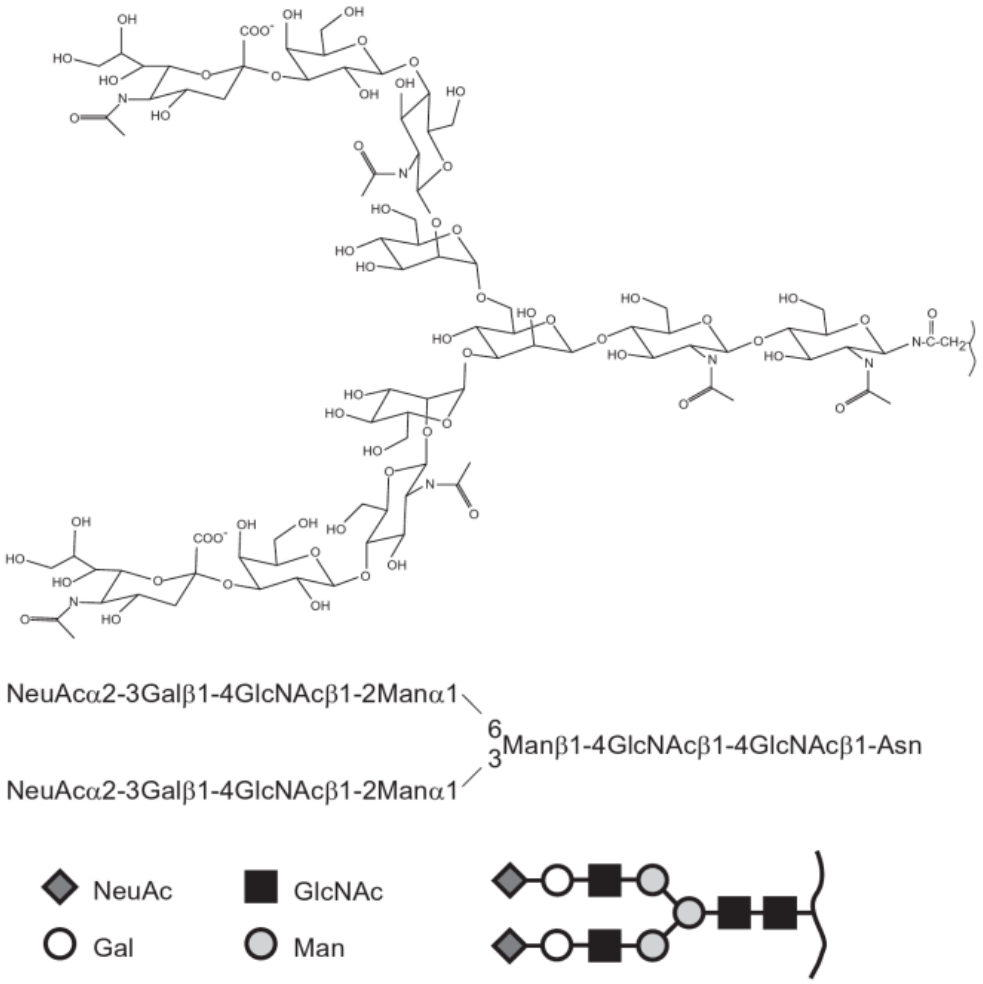

Figure 1.1. Representation of an N-linked glycan in its chemical, condense and symbolic form. (Taylor, Maureen E. Drickame. Kurt. Introduction to Glycobiology, Oxford University Press, 2011)

1.1.1 Glycosylation in Bacteria

Glycosylation in bacteria can take place in different forms depending on the bacteria sub-type.

Gram positive bacteria. The cell wall of gram positive bacteria is coated with teichoic acid. This polysaccharide provides structure and rigidity to the cell wall in the

absence of a second membrane, such as that present in gram negative bacteria.(7) The structure is illustrated in Figure 1.2.

Gram negative bacteria. This type of bacteria has an inner and an outer cell membrane, and the space between them is connected by a dense peptidoglycan. The outer membrane is associated with capsular polysaccharides and lipopolysaccharides(8,9).

Mycobacteria. The cell membrane of this bacteria is composed of a thick layer of peptidoglycans interwoven with lipoarabinomannan and arabinogalactan and covered with trehalose-linked lipids(10) (Figure 1.2). This combination confers mycobacterium with unique permeability properties that allow them to elude a large set of antibiotic molecules.(11)

The glycans on the surface of the three types of bacteria are closely linked to bacterial survival and pathogenicity. It has been observed that Gram positive bacteria lacking teichoic acid are also unable to colonize or infect their host(12). Gram negative bacteria with a truncated LPS lack the capacity to infect their host as they do not survive its immune system.(13). Gram (+) or gram (-) bacteria lacking peptide glycans do not survive osmotic changes(14). In a similar manner mycobacteria with a trehalose-deficient cell wall are unable to proliferate and enter the stationary phase, and they are also incapable of resisting thermic stress.(15)

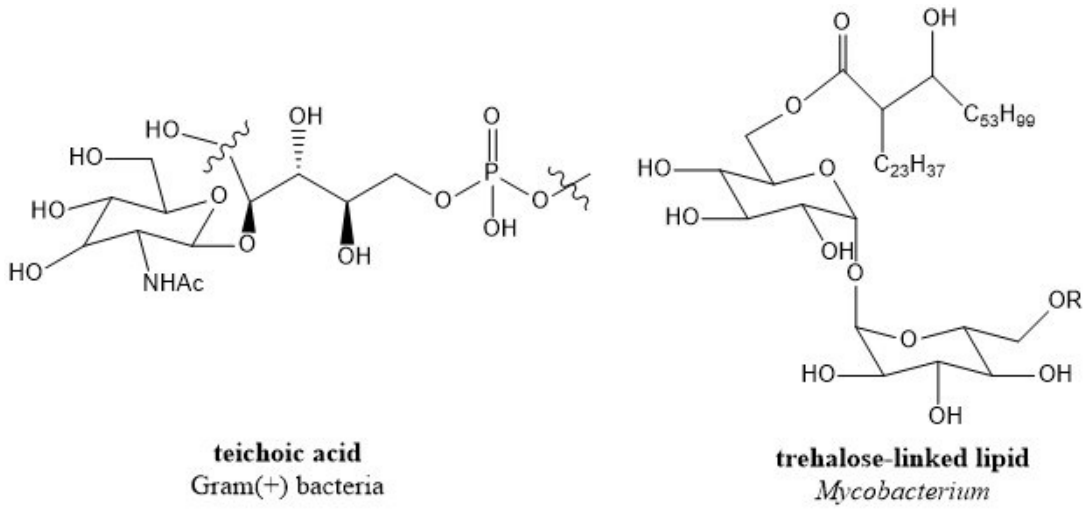

Figure 1.2. Structure of the major glycolipids on the surface of bacteria. Gram positive bacteria contain teichoic acid and mycobacterium contain a trehalose-linked lipid. Gram negative bacteria contain a large lipopolysaccharide (LPS) of a more complex structure (presented in detail in the next figure).

1.1.2 Lipopolysaccharides

Bacterial lipopolysaccharides (LPS) are the major component of the outer membrane of gram negative bacteria(16). LPS has multiple functions: it protects bacteria from the immune system, it provides rigidity to the cell wall, and it induces host damage as a major component of toxic shock.

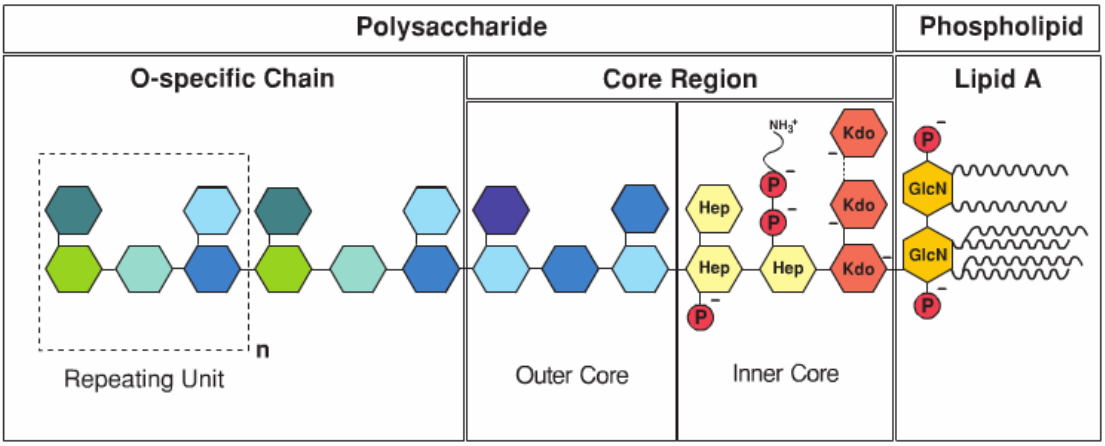

Figure 1.3. Lipopolysaccharide structure. From right to left, the innermost to the outermost components of LPS are the membrane embedded Lipid A, the core polysaccharide and the O-specific antigen. (From Alexander and Rietschel, 2001)

As shown in Figure 1.3, LPS's are constituted of 3 main units: 1) a lipid component embedded in the bacterial membrane that is commonly termed Lipid A. 2) a core region consisting of a polysaccharide unit of relatively conserved composition and 3) a hyper variable polysaccharide region named O-antigen. Each of the three units has different characteristics and functions as detailed below:

The lipid A is the main entity responsible of generating a strong immune response in humans. This response is mediated by Lipid A binding to Toll-like receptor 4, MD-2 and CD14. This event is the major component in the development of toxic shock syndrome.(17)

The core carbohydrate unit, named core-antigen, is composed of 2 sections: the inner and the outer core. The inner core is the closest to the lipid A and is a much conserved unit composed of two uncommon pentoses: 3-deoxy-D-manno-octulosonic

acid (Kdo) and L-glycero-D-manno heptose (Hep). Kdo is an irreplaceable unit as it bridges the lipid A with the rest of the carbohydrates. The outer core is composed of more common hexoses and although its composition may change, it is mainly conserved between families of bacteria.(7)

The O-antigen is the outer most section of LPS and is the most abundant component of the bacteria exposed to the outside. It is composed of multiple copies of a single polysaccharide unit that repeats up to 50 times. The O-antigen is composed of multiple possible hexoses arranged in different combinations or modified by rings, substitutions or chemical linkage etc. Each strain of bacteria has a unique O-antigen composition but the divergence is smaller in strains belonging to the same families. In Figure 1.4, the O-antigen composition of three bacteria is compared side by side. Strains belonging to the same family *(E coli*) resemble each other in carbohydrate composition but differ in branching. In contrast *P aeruginosa* differ prominently in sugar composition.(18)

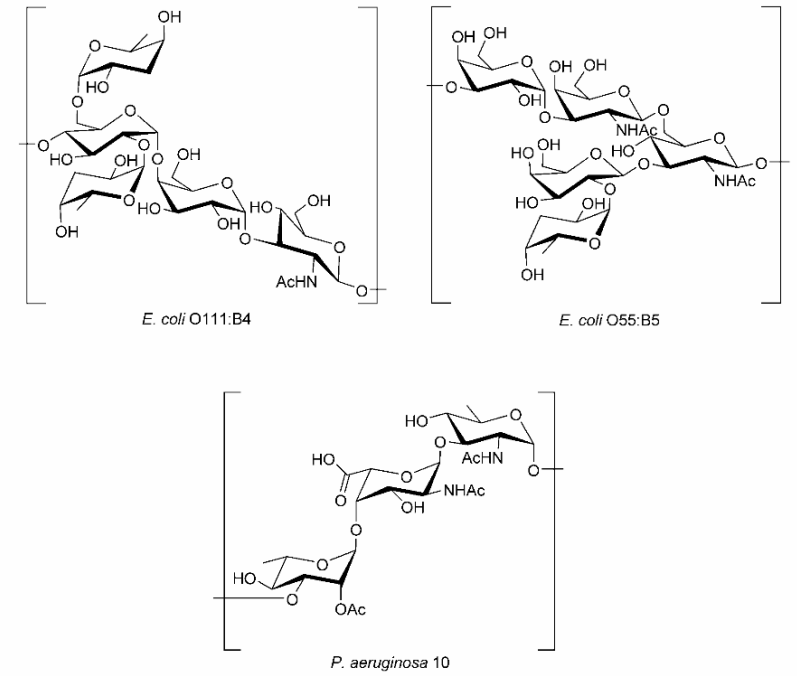

Figure 1.4. O-antigen composition of *E. coli* O111:B4, *E. coli* O55:B5 and *P. aeruginosa* 10. The repeating unit of both *E. coli* is composed of five neutral monosaccharides: glucose (Glc), galactose (Gal), N-acetyl-galactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and colitose (Col; 3,6-dideoxy-l-galactose). Both structures slightly vary in the branching but the overall sugar composition is the same. The repeating unit of *P. aeruginosa* consists of three unusual sugars: 2-O-acetyl-l-rhamnose (RhaAc), 2-N-acetyl-l-galacturonic acid (GalNA), and 2-N-acetyl-2,6-dideoxy-d-glucos-amine (QuiN). (From Morales Betanzos, C. et al 2009)

The O-antigen has several unique characteristics and functions. It is essential for the survival of the bacteria in the host serum.(19) It protects the bacteria from phagocytes intake and from the activation of the complement system.(20,21) It protects the bacteria against bile acids and lipid A recognition antibodies.(22) In some commensal bacteria

like *H Pylori*, *N meningitis* and *H influenza*, the O-antigen composition of the bacteria mimics the sugar composition of the host, which allows it to escape the immune system.(23-25)

1.1.3 Glycosylation as a Serotyping Determinant in Bacteria

Serotypes are groups within a single species of bacteria that share distinctive surface antigens. The variety and composition of these antigens depends on the bacteria subtype. Some of the most common surface antigens are O-antigen (polysaccharide) and H antigen (flagella protein) in gram negative bacteria, and teichoic acid and other polysaccharides in gram positive bacteria. Serotypes are useful to differentiate different bacteria strains, for instance, highly pathogenic from non-pathogenic strains. For example, The Center for Control Disease has determined that from the 2,500 known serotypes of *Salmonella*, only 100 serotypes are infectious to humans. Serotyping is conducted through the use of antibodies recognizing specific antigens in the surface of bacteria. Glycans are the mayor determinants of bacterial serotypes. In gram negative bacteria, the antibody reaction to the O-antigens determine the serotype. In some cases the glycoproteins on the surface of the flagella (H-antigen) are used to complement the serotype distinction.

1.1.4 Multiplexed Analysis of Glycan Variations

Because polysaccharides are so diverse in composition and structural organization, the analysis of glycan variations is not an easy task. The structural analysis of glycans is conducted with a combination of enzymatic digestion and mass spectrometry. The first is directed to simplify the glycan into units of lower complexity

that can be then analyzed with MS. This process is, in the best case, laborious and requires the use of specialized enzymes for the different glycosydic bond types and the use of costly instrumentation. The data analysis is also hard as special attention should be taken to distinguish not only the components of the glycan but the structural information that accompanies the branching of the glycan as well. Because of the difficulties of this type of analysis, different techniques to approach glycan analysis have been pursued. Affinity based approaches have emerged as promising alternatives with higher throughput. In these approaches, a set of affinity reagents are immobilized in a solid platform (i.e. microarray) and use to interrogate glycol-conjugates of different composition or structures. The binding events between the affinity probes and the glycan are then quantified using fluorescence dyes, and the differences are catalogued. Different platforms containing lectins or antibodies have been created with this purpose.

Lectin microarrays, illustrated in Figure 1.5, have been used successfully to analyze glycosylation patterns in glyco-proteins(26,27), to demonstrate changes in the glycoprotein composition during cell development and cancer (28-30) and to discriminate pathogenic from non-pathogenic bacteria(31). Antibody-based microarrays have been used to identify glycosylation changes in 2 serum proteins relevant for pancreatic cancer.(32) Some limitations of this approach are the small current repertoire of available lectins and carbohydrate binding antibodies. It has been estimated that although the number of glycan motifs range around 7000, only about 100 lectins are currently available.(27) The use of recombinant lectins, antibodies and other affinity reagents has been proposed as an alternative to cover this deficiency.

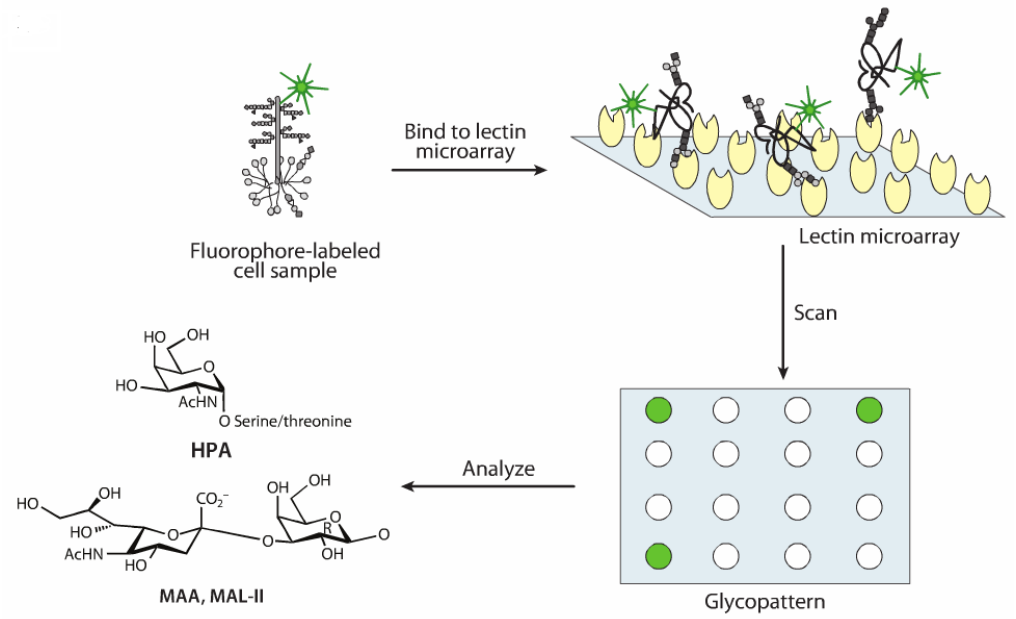

Figure 1.5. Lectin microarray. A lectin microarray is interrogated with a labeled polysaccharide. Because of the promiscuous nature of lectins, several spots light up. Using a computational approach the binding affinities of the identified lectins are analyzed to deduce the identity of the polysaccharide. (From Rakus and Mahal, 2011)

1.1.5 Peptide Microarray

As an addition to the field of affinity-reagents-based platforms, the Center for Innovations in Medicine (CIM) at Arizona State University developed a novel peptide microarray. This platform consists of 10,000 random sequence peptide microarrays immobilized on a glass slide. The library of 20-mer peptides was synthesized using standard solid-phase methods utilizing 19 of the 20 natural amino acids, excluding cysteine. A terminal –GSC sequence was incorporated at the end of each peptide as a way to link the library to the glass slide using an NHS-ester and maleimide cross linker.

As part of the CIM-microarray program, we evaluated the capacity of the peptide microarray to discriminate small and large glycan variations. We utilized glycans from bacteria as an experimental model for 2 main reasons: 1) the biological key relevance of glycans for the pathogenicity and survival of bacteria. 2) The composition of glycans in the surface of bacteria is very similar between strains of the same family and diverges in members of different families. This property allowed us to study similar and very different glycan compositions by virtue of using close or distant family members of bacteria.

1.1.6 Hypothesis

The working hypothesis of this study was that owing to the large chemical diversity of the random peptide structures with no pre-conceived specificity, we could utilize the CIM-peptide microarray as an approach to identify novel lectin mimics with affinity toward bacterial glycans.

As a proof of concept we labeled gram negative lipopolysaccharides (LPS) with highly fluorescent quantum nano-particles. Then we utilized these novel fluorescent probes to interrogate the peptide microarray in an attempt to identify novel LPS binding peptides. The synthesis of the novel LPS-nano particles is documented in Chapter 2 of this work, and the use of the fluorescent conjugates on the peptide microarray is presented in Chapter 3.

1.1.7 Specific Aims

The specific aims of the project were:

1. Synthesize a fluorescent probe using LPS from Gram negative bacteria and fluorescent nano particles.
2. Using a 10,000 peptide microarray, identify a set of peptides that bind to three selected lipopolysaccharides from bacteria: two from *E coli* with similar glycan composition and one from a *Pseudomonas Aeruginosa* of divergent composition.
3. Evaluate the binding affinity of the identified peptides using orthogonal binding assays (Surface Plasmon Resonance (SPR), isothermal calorimetry (ITC) and flow cytometry).
4. Describe the biological properties of the selected list of peptides.

1.2 Phosphorylations in Neuroblastoma

1.2.1 Phosphorylation Chemistry

Protein phosphorylation is one of the most important post-translational modifications. Phosphorylation is closely involved in the regulation of many cellular processes including growth, differentiation, apoptosis and several other signal transduction pathways. It has been calculated that as many as one third of the of the human proteins are phosphorylated at some point of their life cycle.(33) In eukaryotes reversible phosphorylation takes place in the side chain of three amino acids: Serine, Threonine and Tyrosine. All of them contain a nucleophilic group (-OH) that reacts with the terminal phosphate of ATP to transfer the phosphate into the amino acid residue. This

process is facilitated by protein kinases, which catalyze the reaction in the presence of magnesium (Figure 1.6).

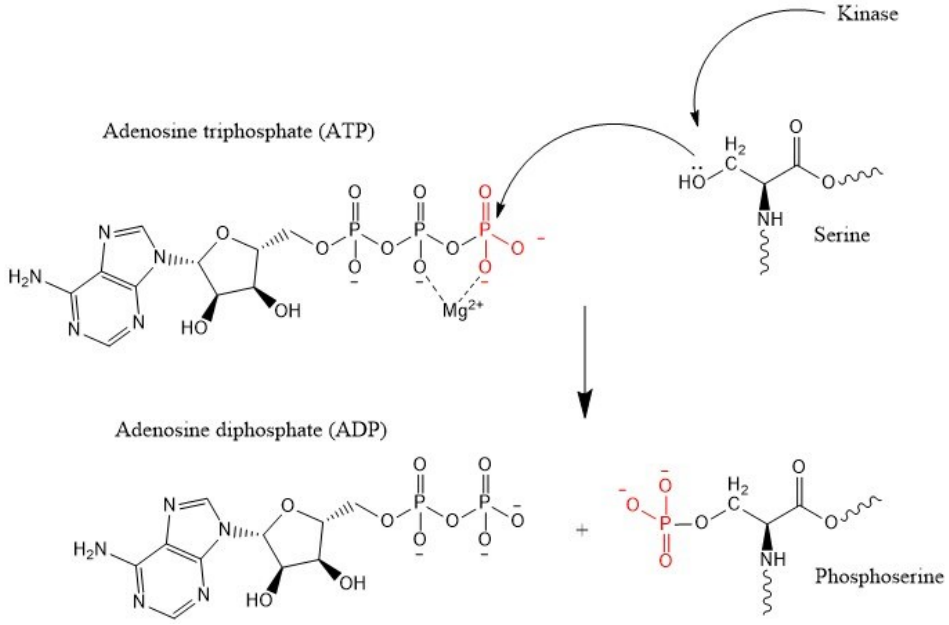

Figure 1.6. Protein phosphorylation reaction. The kinase initiates the reaction by transferring a proton to the Serine. Then the nucleophilic attack from the Serine group (-OH) to the gamma-phosphate of ATP results in the phosphate transfer to the serine. ADP is produced as a by-product.

The transfer of a phosphate group can results in a profound effect on the function of a protein. The addition of a double negative charge and the gained capacity to form hydrogen bonds are two of the major changes that this modification can produce.

The results of this addition diverge greatly depending of the protein context. Phosphate groups can act as steric blocking groups(34,35), allosteric activators(36-38) or allosteric inhibitors(39,40). Phosphorylations can also regulate the protein recognition by

other protein domains such as SH2 or PTB (41,42) or induce the association(43,44) or dissociation(45,46) of protein-protein interactions.

Phosphorylation addition is mediated by a group of proteins known as kinases that facilitate the transfer of the phosphate group from ATP to the proteins. To date, more than 500 human kinases have been identified. The collection of human kinases is commonly referred as the kinome has been divided in subfamilies, according to their catalytic domain. For example kinases phosphorylating tyrosine or serine/threonine residues(47).

The counterpart to protein kinases are another group of proteins named phosphatases that are responsible for removing the phosphorylation group from selected protein residues. This group of approximately 150 proteins in humans takes the role of removing the phosphorylation from serine and threonine residues or from tyrosines using direct hydrolysis of the phosphorous atom by means of a Fe/Zn center (in Ser/Thr phosphatases) or using a covalent thiophosphoryl intermediate to facilitate the removal of the phosphate (in tyrosine phosphatases).(48-50)

Kinases and phosphatases are thus the two balancing elements modulating the addition and removal of protein phosphorylation. Both of these reactions can occur at very fast rates, which makes protein phosphorylation a very dynamic process. (51-53) The versatility and the fast dynamics of protein phosphorylation make this post translational modification a recurring mechanism for the processing and communication of cell information, which is also designated as cell signal transduction.

Signal transduction is characterized by a series of molecular events, commonly called cascades, which mediate the sensing and processing of cellular stimuli. These

molecular cascades detect, amplify and communicate diverse signals that result in cellular responses such as cell growth, gene expression, cell death and others. The process typically starts when a sensing protein is activated by a stimulus (i.e. growth factor, hormone, drug, etc.) This initial signal is then communicated in the form of a kinase-mediated phosphorylation, protein-protein interaction or dissociation that can activate even more downstream signals until the specific response is reached.

This cascade of events can be rather complex as many cell events can be triggered by a single stimulus. For example, a single growth factor can initiate the activation of a whole cell proliferation program (54,55) or a single death factor can induce the release of an apoptosis program responsible for cell death(56-58). Figure 1.7 exemplifies the complexity of a single signaling pathway. In this case, the circuitry regulating the cell processes of growth and differentiation through the MAPK/ERK pathway is presented.

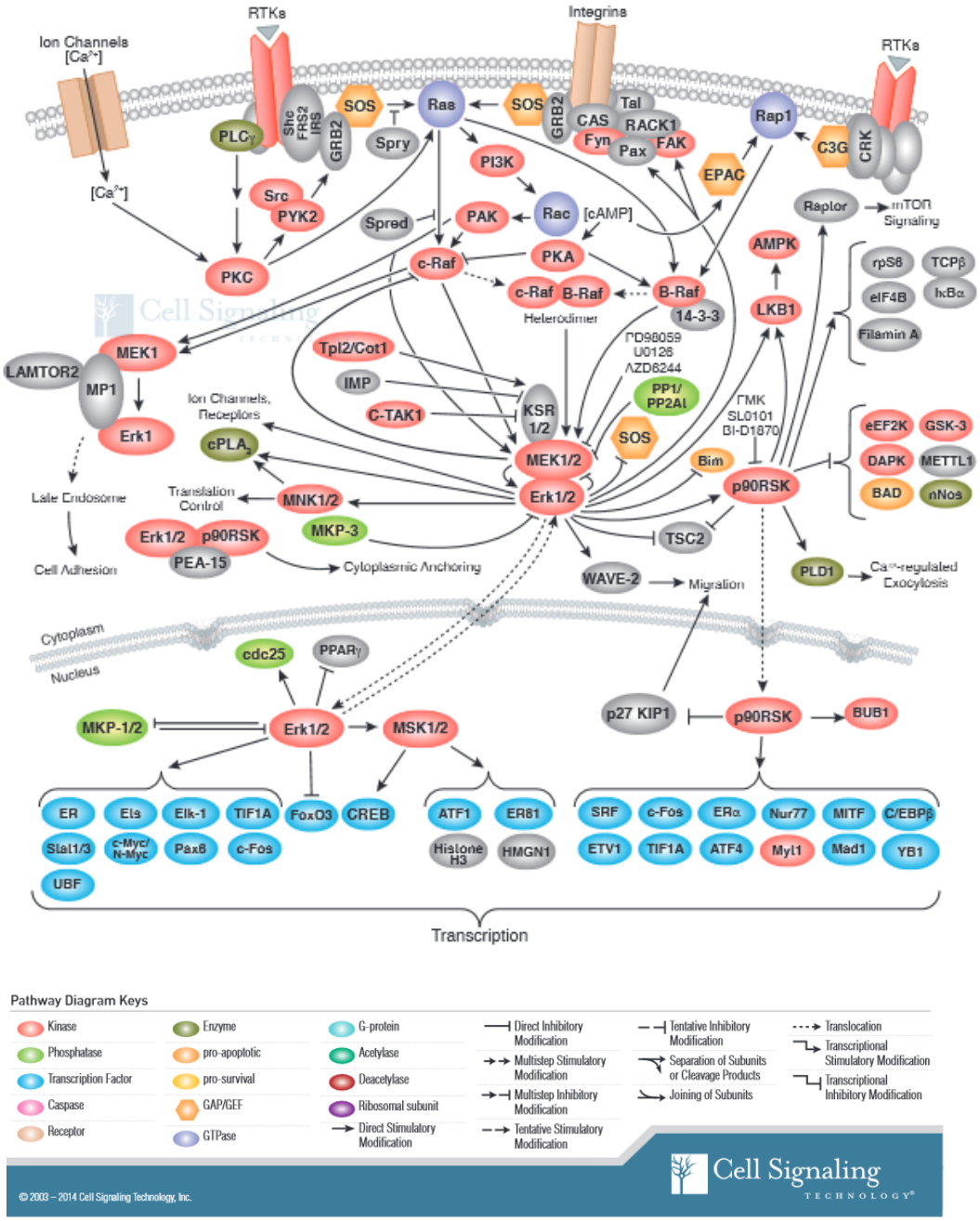

Figure 1.7. MAPK/ERK signaling pathway in cell growth and differentiation. The MAPK/Erk signaling cascade is activated by a wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases (RTKs), integrins, and ion channels. The specific components of the cascade vary greatly among different stimuli, but the architecture of the pathway usually includes a set of adaptors (Shc, GRB2, Crk,

etc.) linking the receptor to a guanine nucleotide exchange factor (SOS, C3G, etc.) transducing the signal to small GTP-binding proteins (Ras, Rap1), which in turn activate the core unit of the cascade composed of a MAPKKK (Raf), a MAPKK (MEK1/2), and MAPK (Erk). An activated Erk dimer can regulate targets in the cytosol and also translocate to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression. Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).

The protein elements involved in the transduction of this signaling pathway can be categorized in several subtypes including receptors (brown), kinases (red), phosphatases (green), transcription factors (blue) and many other proteins covering multiple functions (grey). All of these elements interact in many different forms by inhibiting, activating or cooperating with each other.

For the purpose of the present work, we focus on the role of two subtypes of cell signaling proteins: the transcription factors (i.e. c-MYC, NMYC, c-Fos, shown as blue in Figure 1.7) and the adaptor proteins (i.e. Shc, Grb2, shown as gray in Figure 1.7).

Transcription factors (TF) are a group of proteins responsible for regulating gene expression. They do so by binding to DNA by themselves or as part of protein complexes and by promoting or repressing the binding of RNA polymerase to a specific gene sequence. Transcription factors can be classified using their DNA binding motif(59). Large, well-established families include helix-turn-helix (HTH) proteins, homeodomains, zin finger proteins, leucine zipper proteins and helix-loop-helix (HLH) proteins. The regulation of TF takes place through several mechanisms including protein

synthesis, nuclear localization, ligand binding activation, phosphorylation, dimerization and others.(60,61) Cell signaling molecules commonly interact with TF to regulate their activity and though them induce a change in gene expression leading to a cell response. In Figure 1.8, the transcription factor TFIID binds to a promoter to start the formation of the transcription complex that in turn initiates gene expression (a). The initial step of the process is the binding of one of the TFIIB subunits: the TATA binding subunit (TBP) to DNA. This creates a morphological change of DNA that initiates the complex formation(b). It has been documented that the phosphorylation state of TBP is a potent regulator of TBP binding to DNA and of the overall transcription process.(62,63)

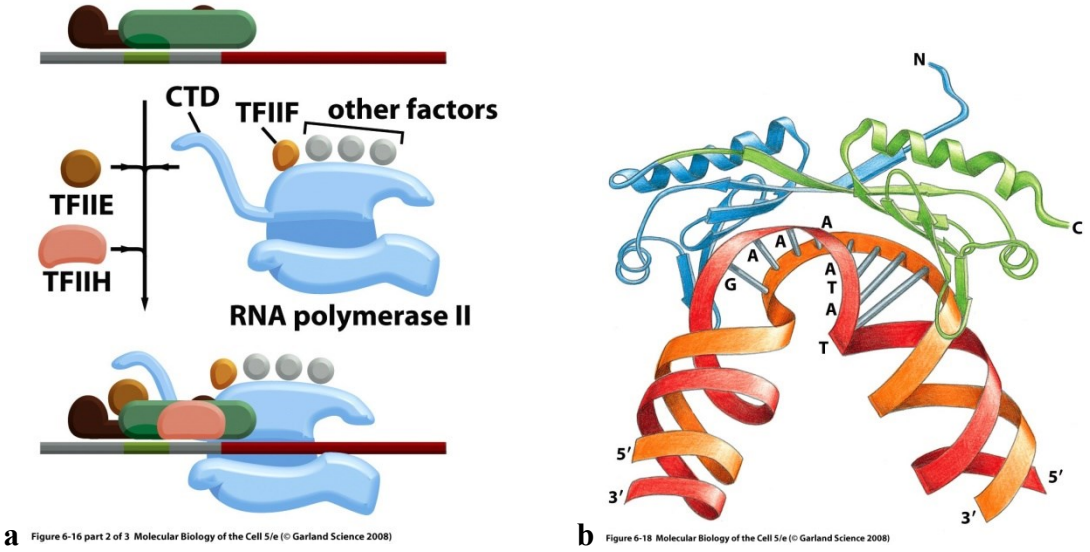

Figure 1.8. (a) After binding to the gene promoter, the transcription factor TFIID (green) triggers a large distortion of the DNA that serves as a physical landmark to assemble the transcription initiation complex. This assembly is composed of other transcription factors and the RNA polymerase. (b) TATA-Binding sub-unit (TBP) of the transcription factor

TFIID binding to DNA. Alberts, Bruce. *Molecular Biology of the Cell*. New York: Garland Science, 2008.

Adaptor proteins are a group of molecules that participate in the midpoints of several cell signaling pathways. This group of proteins lacks in many cases enzymatic domains of their own. Instead, it contains several protein binding domains to support multiple protein-protein interactions. The main function of adaptor proteins is to interact with a cohort of multiple proteins to assemble an organized signaling complex.(64) These complexes can propagate and elicit appropriate responses that the individual members would not be able to do by themselves. The specificity of the signal is dictated by adaptor protein identity, its binding sequence, binding partners and cellular localization.(65) Adaptor proteins usually contain multiple binding domains within one single protein. These domains can bind to phosphorylated proteins like Phosphotyrosine Binding domain (PTB), Src Homology 2 and 3 (SH2, SH3), or to other molecules like Pleckstrin homology domain (PH) that binds to phosphatidylinositol in the cell membrane.(66) In addition to these common domains, several adaptor proteins contain other amino acid sequences and phosphorylation sites to harbor other protein interactions like SH3 and WW recognition sequences(64). In Figure 1.9, the adaptor protein Grb2 directs the interaction of three cell signaling proteins by means of its three binding domains. In addition to protein-protein interactions, Grb2 mediated cell signals can be modulated through phosphorylations.(67-69)

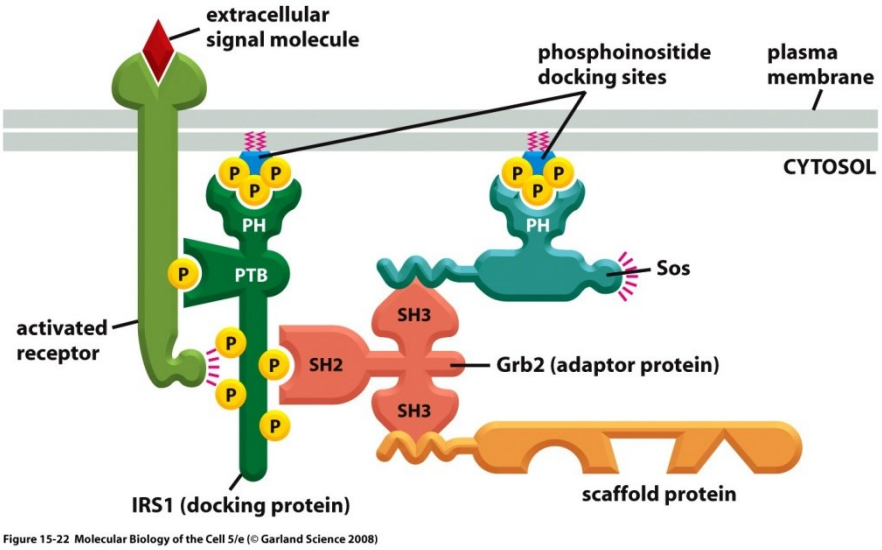

Figure 1.9. Adaptor protein Grb2 participates in the midst insulin cell signal. Grb2 contains three distinctive domains that allow it to interact with IRS1 docking protein, GTPase-activating protein, Sos and a scaffold protein. Grb2 interactions can be also modulated via phosphorylation. Alberts, Bruce. *Molecular Biology of the Cell*. New York: Garland Science, 2008.

1.2.2 Abnormal Cell Signaling in Cancer

Because cell signal transduction is involved in the regulation of the most important cellular processes, it should not be surprising to point out these signals are altered in cancer, a disease characterized by abnormal cell growth. The ways in which the cellular circuitry can be can be modified to induce tumors are relatively large. Abnormal cascades conferring self-sufficient cell growth are a classic example and a common find in several types of cancer(70-72). Other signaling pathways commonly altered in cancer include apoptosis evasion, migration, limitless replication, and sustained angiogenesis.(73,74)

1.2.3 Neuroblastoma

Neuroblastoma is a malignant tumor that arises from neural crest tissues, located mainly in the sympathetic chain and adrenal glands. It is the most common extracranial solid childhood tumor.

Neuroblastoma is the most common extra cranial tumor in children. In the USA alone, it accounts for one case in every 70,000 live births.(75) According to the NCI the incidence of the disease is 10.54 cases per million per year in children younger than 15 years.

Neuroblastoma originates in early nerve cells called neuroblasts, which are part of the sympathetic nervous system (SNS). The SNS is part of the autonomous nervous system and includes:

- The nerve fibers running along the spinal cord.
- The clusters of nerves called ganglia present along the nerve fibers.
- The inner nerve-like cells of the adrenal glands. These glands which produce a large number of hormones are located in the top of the kidneys.

Neuroblastoma is accordingly found localized along any of these areas although, for the most aggressive cases, metastases are commonly found in other tissues. The image in Figure 1.10 shows one example of a localized neuroblastoma tumor as visualized by MIBG scan and CT scan.(76)

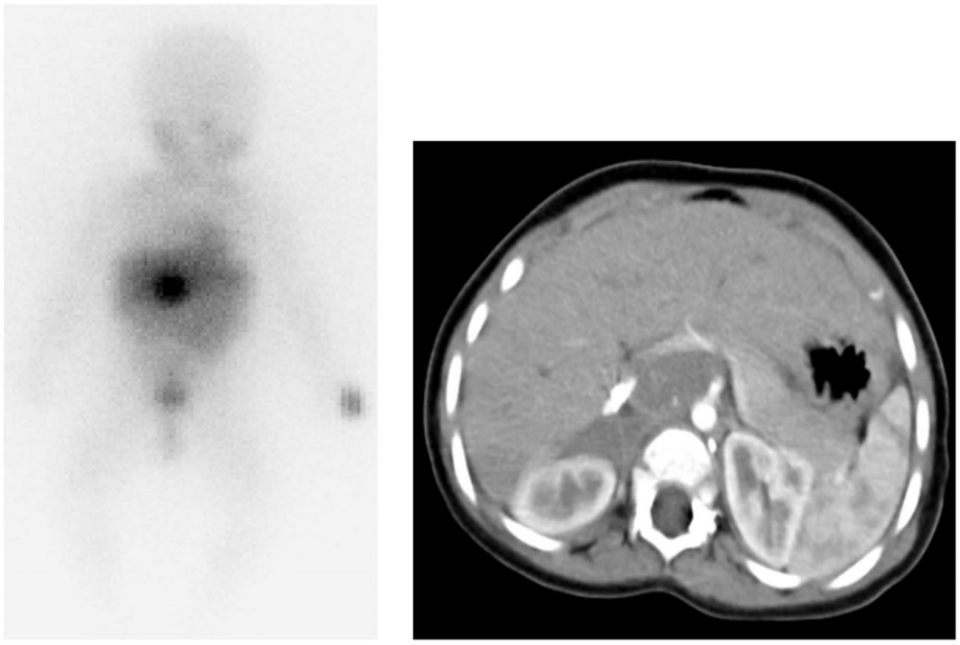

Figure 1.10. Supra-renal neuroblastoma tumor visualized with radio-labelled meta-iodobenzylguanidine (MIBG) Scintigraphy (left) and with a CT scan (right). MIBG is commonly utilized for treatment and visualization of neuroblastoma. Copyright © 2014 Raef Riad, Magdy Kotb, Walid Omar, Ahmed Zaher, Emad Ebied, Alexander Gregory Pitman, Hussein Abdel Dayem et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

One of the hallmarks of Neuroblastoma is its heterogeneity. A patient with the disease can present very different scenarios ranging from a localized tumor that regresses spontaneously to a metastatic highly aggressive form that requires multi-modal treatment.

Several studies have focused on understanding the molecular features that characterize the different forms of the disease. One of the most important markers of tumorigenicity was first identified by Kohl(77) and Schwab(78) in 1983 in the form of a

transcription factor named NMYC that was amplified in certain neuroblastomas. Soon after that, other groups linked the presence of the amplification with the most aggressive forms of the disease and this observation remains until now as the most important neuroblastoma marker.(79,80) Since then, the discovery of other pathological and molecular features have complemented NMYC and helped to stratify the diverse forms of the disease. The Children's Oncology Group (COG) neuroblastoma risk stratification system is the scheme followed in North America for the classification of patients and selection of treatment.(81-84) This system takes into consideration the age of the patient, pathological evaluation (INPC), tumor extension (INSS), ploidy (number of chromosomes) and NMYC amplification to classify the neuroblastoma patients. The classification is defined in three groups, low, medium and high risk, which describes an increasing level of cancer aggressiveness and is treated more intensively.(85) Table 1.2 shows the complete COG stratification system in low, medium and high risk neuroblastomas.(81-84) The International Neuroblastoma Staging System (INSS) score describes the propagation of the tumor in a range from 1 to 4 with the exception of 4S that represents a specific type of dissemination. The International Neuroblastoma Pathologic Classification (INPC) scores the tumor according to its degree of differentiation as evaluated by a pathologic examination. It is noticeable that from all the markers, NMYC amplification is the predominant factor that differentiates high risk neuroblastoma. This amplification invariably results in the most aggressive type of tumors, with the exception of a few localized tumors (see low risk INSS 1, 2 and 4S).

Table 1.2. Children's Oncology Group (COG) Neuroblastoma risk stratification system. INPC = International Neuroblastoma Pathologic Classification; INSS = International Neuroblastoma Staging System. aDNA Ploidy: DNA Index (DI) > 1 is favorable, DI = 1 is unfavorable; hypodiploid tumors (with DI < 1) will be treated as a tumor with a DI > 1 (DI < 1 [hypodiploid] to be considered favorable ploidy) Stage 1 Localized tumor with complete gross excision, with or without microscopic residual disease. Stage 2A Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically. Stage 2B Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically Stage 3 Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column. Stage 4 Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs, except as defined for stage 4S. Stage 4S Localized primary tumor, as defined for stage 1, 2A, or 2B, with dissemination limited to skin, liver, and/or bone marrow (by definition limited to infants younger than 12 months). Marrow involvement should be minimal (i.e., <10% of total nucleated cells identified as malignant by bone biopsy or by bone marrow aspirate). More extensive bone marrow involvement would be considered stage 4 disease. The results of the mIBG scan, if performed, should be negative for disease in the bone marrow.

Low Risk	**INSS Stage**	**Age**	**MYCN Status**	**INPC Classification**	**DNA Ploidy[a]**
	1	0–21 y	Any	Any	Any
	2A/2B	<365 d	Any	Any	Any
		≥365 d–21 y	Nonamplified	Any	-
		≥365 d–21 y	Amplified	Favorable	-
	4S	<365 d	Nonamplified	Favorable	>1
Medium Risk					
	3	<365 d	Nonamplified	Any	Any
		≥365 d–21 y	Nonamplified	Favorable	-
	4	<548 d	Nonamplified	Any	Any
	4S	<365 d	Nonamplified	Any	=1
		<365 d	Nonamplified	Unfavorable	Any
High Risk					
	2A/2B	≥365 d–21 y	Amplified	Unfavorable	-
	3	<365 d	Amplified	Any	Any
		≥365 d–21 y	Nonamplified	Unfavorable	-
		≥365 d–21 y	Amplified	Any	-
	4	<365 d	Amplified	Any	Any
		≥548 d–21 y	Any	Any	-
	4S	<365 d	Amplified	Any	Any

High risk neuroblastoma. High risk neuroblastoma is the most aggressive form of neuroblastoma. In contrast to low and medium risk, which have a >95% survival rate

with minimum therapy, high risk neuroblastoma has a low survival rate and requires very intensive therapy.

Therapy for high-risk neuroblastoma has 4 components: chemotherapy, local control with surgery and radiation therapy, consolidation chemotherapy with stem cell rescue and treatment of minimal residual disease with biologic agents. Despite this intensive therapy associated with significant side effects, the five-year event free survival for children with high-risk neuroblastoma is a dismal 20-30%.(86-89). New therapies are urgently needed.

Target specific treatments for neuroblastoma. In the case of high risk neuroblastoma, therapeutic targets are starting to be identified. In a randomized trial conducted by the Children's Oncology Group, patients treated with retinoic acid, a potent differentiating agent, had improvements in both event free survival and overall survival.(90-92) In another randomized trial conducted by the same group, patients treated with ch14.18 (a chimeric antibody to GD-2, an antigen found on neuroblastoma cells) had significantly better survival than patients who did not receive the antibody.(93) An understanding of the molecular biology of neuroblastoma will drive the rational development of new therapeutic agents to improve survival of this disease.

The next frontier in oncologic therapy is the use of agents that target specific pathways of tumor growth and development. The classical example of this rational approach is imatinib, a tyrosine kinase inhibitor that revolutionized the treatment of Philadelphia positive acute lymphoblastic leukemia and chronic myeloid leukemia.(86,94) Imatinib (Gleevec) was discovered as a selective tyrosine kinase inhibitor of Bcr-Abl, a fusion protein that activates cell proliferation enzymes through

phosphorylation. This example illustrates the importance of target discovery as a prelude to finding new therapeutic agents.

1.2.4 Molecular Factors Associated with Poor Prognosis in Neuroblastoma

After more than 30 years of research since the identification of NMYC amplification, several other proteins have been correlated with the occurrence of high risk neuroblastoma; however NMYC has remained as the most important prognosis factor in neuroblastoma.

Some of the most interesting proteins that have been identified as prognosis markers and/or potential therapeutic targets in neuroblastoma include the neutrophin receptor TrkA (NTRK1), the Anaplastic Lymphoma kinase (ALK), and (Src Homology 2 Domain Containing) Transforming Protein 3 (ShcC).

The expression of NTRK1 was correlated with good neuroblastoma prognosis. This receptor is believed to act through its involvement in apoptosis and cell differentiation.(95,96) In contrast, high expression of TrkB (NTRK2) correlated with poor prognosis and high risk neuroblastoma. TrkB is involved in the autocrine survival pathway.(97,98)

ALK has been found either mutated or amplified in 10-15% of high risk neuroblastoma cancers. Its action mechanism in neuroblastoma has not been fully elucidated, but it has been hypothesized that it may act in a similar fashion to anaplastic large cell lymphoma (ALCL), where it is also amplified.(99,100)

The expression of ShcC has been also correlated with poor outcome in high risk neuroblastoma. ShcC inhibition in cells induced the differentiation of high risk neuroblastoma cells and decreased their growth rates. In addition, xerograph models of

ShcC neuroblastoma knockouts resulted in smaller tumors than wild type neuroblastoma cells. (101-103)

In the present work, we focus our attention in the phosphorylation analysis of two of the aforementioned proteins: the transcription factor NMYC and the modulator protein ShcC. The selection of these two proteins was based in the following criteria: the expression of each protein correlates with the worst outcome cases of neuroblastoma, and both proteins have shown to be key modulators of neuroblastoma tumorigenicity. Additionally, both molecules belong to families of proteins where phosphorylation plays a central role in the regulation of their function. This is evident with the fact that several active phosphorylations have been identified in homologue proteins within their families. The characteristics of both families of proteins are described in the next sections.

1.2.5 NMYC Transcription Factor

The transcription factor NMYC (V-myc myelocytomatosis viral related oncogene) is a member of the MYC family of transcription factors that shares a common helix-loop-helix (bHLH) DNA binding domain. NMYC is localized in the nucleus of the cells and is highly expressed in neuronal tissue.

NMYC amplification is the most important neuroblastoma oncoprotein. Its amplification was initially observed in 20% of neuroblastoma patients was soon linked to the most aggressive tumors (79,80). Since then, NMYC has been extensively described in literature as a major component of the tumorigenic cell signaling of neuroblastoma(104,105), medulloblastoma(106,107), rhabdomyosarcoma(108), neuroendocrine prostate cancer(109) and human acute myeloid leukemias(110).

MYC family of proteins. The MYC family of transcription factors is composed of three members: c-MYC, NMYC and MYCL1. The amplification of each of the three members of the family has been linked to different types of cancer. C-MYC amplification has been observed in colon cancer and leukemia cells (111,112). NMYC amplification is a notable hallmark of high risk neuroblastomas(113,114), and the MYCL1 amplification is consistent in lung cancer and ovarian carcinomas(115,116) .

Mechanism of the MYC family of proteins. c-MYC is the prototypical MYC family member. Despite having been studied extensively for almost 30 years, the extent of its action mechanism was found to be very complex and is still being understood. Because c-MYC is the best described member of the MYC family, we use it to illustrate the most important features known to this group of proteins. It is currently well-established that c-MYC can act as both a transcriptional activator and a repressor, although the former has been studied in more detail(117).

As an activator of transcription, c-Myc depends on the formation of a protein complex with the protein binding partner MAX (Myc-associated factor X). c-MYC-MAX interaction takes place through the dimerization of the HLH-LZ domain present in the c-terminal portion of both proteins. The MYC-MAX heterodimer has the capacity of binding to the CACGT E-box sequences found upstream of several promoter regions(118). The heterodimer binding to E-boxes is essential for the MYC mediated transformation (117) and generally (but not always) corresponds with the activation of gene expression(119). The c-MYC MAX complex activates transcription by different mechanisms that include several possible binding partners, with some of the most well-described being TRRAP, INI1 and GCN-5. TRRAP (Transformation/ transcription

domain associated protein) is an adapter protein essential for MYC transcription activation and concomitant cellular transformation(120). INI1(Integrase interactor 1), which is part of the SWI-SNF ATP-dependent chromatin remodeling model, also interacts with MYC-MAX and induces the ATP-dependent chromatin remodeling leading to changes in gene expression(121). C-MYC has been also identified to recruit GCN-5, which acetylates histone tails resulting in an open chromatin configuration and an increase in gene transcription(122). MYC has also been found to directly promote the elevation of RNA polymerase II c-terminal domain levels, which may have also potential effects on gene transcription(123).

As a transcriptional repressor, the MYC-MAX complex interacts with transcriptional activators bound directly to DNA and either recruits co-repressors or displaces co-activator proteins. Some of the known targets of this repression mechanism include nuclear factor Y (NFY)(124), the transcription factor SP1(125) and the MYC-interacting zinc finger 1 (MIZ1) through the inhibition of nucleophosmin (NPM)(126).

In contrast to other classical transcription factors that bind to a limited number of well-defined targets, c-MYC gene targets are broad. With the use of ChIP-PET and ChIP-seq in combination with expression array data, several hundred MYC targets have been identified (127-130). It has been estimated that MYC can bind to approximately 10-15% of the genome and regulate encoding proteins and non-coding RNA products of several functions(127, 128). Several criteria have been stipulated to differentiate true MYC transcription targets from indirect binding targets. These results have been summarized by Dang et al in a MYC target database that utilizes those criteria and furthers separates

those MYC targets in functional classes, allowing the visualization of the whole MYC genetic program(131).

MYC family function is mediated by phosphorylation. Of all the members of the MYC family, c-MYC is the protein that has been described in most detail. 27 phosphorylation sites have been identified in c-MYC, and at least 15 of them have been linked to function(132). For example, In Burkitt's lymphoma the phosphorylation of c-MYC at T58 increases the half-time of the protein in two- to six-fold by inhibition of the ubiquitin-proteasome pathway(133). Phosphorylations at MYC-T58 and S62 also have been identified as essential for transactivation of gene expression by c-MYC, as mutation of these sites to alanine dramatically reduced transcription of downstream genes(134, 135). Phosphorylations at MYC-S62 and S71 have been also identified as pro-apoptotic signals by JNK signaling(136). In leukemia, lymphoma prostate and pancreatic cancer c-MYC is dramatically stabilized by Pim-1 and Pim-2. This stabilization takes places trough the phosphorylation at MYC-S329 by Pim-2 and at T58 by Pim-1(137). MYC-S71, S81, T343, S344, S347 and S348 have been also identified as sites of negative regulation in c-MYC mediated cell transformation(138). Pak-2 a Ser/Thr kinase that participates in cell stress interference with c-MYC binding to DNA by blocking the dimerization of c-MYC to MAX through MYC-S373 and T400 phosphorylation or by interfering with c-MYC binding to DNA trough T358 phosphorylation(139).

Phosphorylation site comparison between MYC and NMYC. Although c-MYC and NMYC share a 47% homology, hold the same protein domains and both are amplified in different cancer types(140-144), NMYC has not been studied in the same

detail. This difference is probably a result of the ubiquitous nature of c-MYC and the restricted expression of NMYC to few neuronal tissues.

To date, only 6 phosphorylations sites have been identified in NMYC(132). From those, only two have been linked to function: NMYC-T58 and NMYC-S62 phosphorylations increase the turn-over of NMYC in neuroblastoma cells by hindering the ubiquitin mediated degradation of the protein, thus stabilizing NMYC in neuroblastoma cells. This specific phosphorylation has been linked to the kinase Aurora A(145).

Because several of the c-MYC phosphorylation sites have been directly linked to function in cancer and some of them even selected as therapeutic targets(138), it is very possible to imagine a similar scenario for NMYC where many other phosphorylation sites may be present in the protein with potential function in the tumorigenicity of neuronal cancers.

1.2.6 ShcC Scaffold Protein

ShcC has been recently associated with poor prognosis in neuroblastoma. ShcC is a scaffold protein normally involved in cell proliferation and development of normal neurons. In 2005, Terui et al. analyzed 52 neuroblastoma tumors from patients using semi-quantitative reverse transcription PCR and found that patients with high levels of ShcC mRNA had poor prognosis compared with patients with lower levels(103). In 2008, Miyake et al. also correlated the presence of ShcC with poor prognosis using western blot(102). The latter study evaluated the effect of ShcC knockdown in neuroblastoma differentiation and xenograft tumors in mice using RNA interference (RNAi) and showed that neuroblastoma cells treated with ShcC RNAi differentiated into neurites and were

less tumorigenic when injected into nude mice. This study suggests that ShcC inhibits neuroblastoma cell differentiation into neurites and may represent a potential target in the treatment of the disease.

The Shc Family of Proteins. ShcC is one of the members of a family of 4 scaffold proteins -- ShcA, ShcB, ShcC and ShcD -- that have very similar structures and functions but are present in different tissues. ShcA is ubiquitously expressed except in the central nervous system (CNS), whereas ShcB and ShcC are only found in the CNS. ShcD has been found only in adult brain and skeletal muscle.(146)

Mechanism of the Shc Family of Proteins is Closely Mediated by Phosphorylation

The Shc family of adaptor proteins connects and assembles the protein components of numerous signaling pathways. The individual members of the family ShcA, B, C or D may connect similar or totally different signaling cascades depending on the cell context. ShcA is the prototypical family member; it has been studied for almost 20 years and is the member of the family for which the most signaling pathways have been described. Because the other family members have not been described in close detail, we utilize ShcA to exemplify the Shc family pathways; however, caution should be taken before extending these observations to the other family members.

ShcA has been observed as a component of multiple signaling pathways, for example, the receptor tyrosine kinase signal (RTKs), specifically the EGFR/MAPK and the PI3K/AKT pathway. ShcA also mediates the EGFR independent activation of the RAS/MAPK in T-cells and B-lymphocytes. In a divergent side, ShcA is also involved in the signaling of Focal Adhesion Kinase (FAK) and the response of oxidative stress in apoptosis(147).

As part of the EGFR/MAPK, EGFR is auto-phosphorylated in less than 5 seconds of ligand stimulation(148). From the multiple resulting phosphorylations, EGFR-Y992, Y1148 and Y1173 interact with ShcA via its SH2 or PTB domains(149, 150). This interaction induces the phosphorylation of ShcA-Y317 that leads to the formation of a Grb2 binding motif (151). The ShcA-GrB2 complex then bind to SOS, a RAS exchange factor, via two SH3 domains and activates the MAPK cascade(152). ShcA also participates in a RAS independent activation of the MAPK pathways via phosphorylations at Y239/240, which stimulates the c-MYC transcription by an unknown mechanism (153). As an example of the non-EGFR activation of RAS cascade, ShcA connects the antigen recognition in T-cells to the RAS signaling pathway. This takes place after the T-cell receptor Complex (TCR) engages with antigen. CD3, a TCR subunit, phosphorylates and recruits the kinase ZAP-70. CD3 binds to the SH2 domain of ShcA while ZAP-70 binds the ShcA PTB domain, thus activating the RAS signaling(154). ShcA also participates in the signal transduction to MAPK induced by insulin in fibroblasts. This activity is mediated by ShcA binding to the PTP-_PEST phosphatase, which in turn is mediated by ShcA phosphorylation at Serine 129.(155). In a MAPK independent pathway, ShcA also participates in the pro-survival EGFR/ERK pathway in liver epithelia where it allows liver cells to survive under severe oxidative damage. This activity requires the phosphorylation of tyrosine ShcA residues Y349 and Y350 and is dominantly primarily regulated by the phosphorylation in the Serine 36(156).

Phosphorylation site comparison between ShcA and ShcC. The well-studied ShcA participates in several signaling pathways, and many of its phosphorylation sites

have been identified already. In contrast, much less is known about ShcC, for which only a few tyrosine phosphorylation sites have been identified. It is noticeable that no Serine or Threonine sites have been described yet. Presumably, the absence of good phosphoserine- or phosphothreonine-specific antibodies, compared to the availability of robust and specific phosphotyrosine antibodies, has made the study of these phosphorylation sites more difficult to study. It is for the same reason that the study of these two phosphorylation sites requires more sophisticated methods like mass spectrometry. Because ShcA and ShcC are very similar in structure (47% homology) and because they share similar functions as signal transducers, we propose that they may also share similar phosphorylation sites. Some of the most interesting phosphorylation sites in ShcA with sequence homologous is ShcC include ShcA S36 S54 Y315 S139 Y349 Y350 (all of them with described function) which are homologous of S24, S35, S145, Y308 Y341 Y342 (most of them not previously identified).

Known ShcC phosphorylation sites. To date, several tyrosine phosphorylation sites in ShcC (Y341, Y342, Y379, Y380, Y406, Y424)(157, 158) and one kinase that phosphorylates them have been identified. This kinase, known as ALK, was measured in a set of 85 neuroblastoma samples but was only abnormally amplified in one of them(102). This finding suggests that other kinases may be involved in the activation of ShcC. In contrast to the tyrosine phosphorylations, the Serine and Threonine phosphorylation sites have not been studied in detail, homology studies between ShcA and ShcC suggest that there is still many possible active sites that remain unidentified, some of them located inside functional domains of the protein(132).

1.2.7 Hypothesis

The working hypothesis of this work is that, similar to their well-described family members ShcA and c-MYC: 1) ShcC and NMYC are regulated by protein phosphorylations and that 2) by identifying these novel phosphorylation sites we can also identify novel mechanisms regulating high risk neuroblastoma. These mechanisms can in turn help to elucidate potential targets in the treatment of high risk neuroblastoma. To test our hypotheses, we divide our study into two specific aims:

1.2.8 Specific Aim 1

Specific Aim 1 is to identify all the amino acid sites in NMYC and ShcC that can be phosphorylated in high risk neuroblastoma human cells and tissue samples. We pursued this goal by using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to characterize and compare all the phosphorylation sites in the proteins extracted from neuroblastoma cells and human tissue samples.

The mass spectrometry experiments consisted of two different approaches. In a general approach, all the phosphoproteins from high-risk neuroblastoma cells were separated and digested, and their phosphopeptides concentrated and analyzed. In a target specific approach, NMYC and ShcC were immunoprecipitated first and purified through SDS-PAGE. Gel bands were then digested and phosphopeptides enriched and analyzed. A schematic of both methods is presented in Figure 1.11.

The description of the technical development of this method and its application to detect novel phosphorylations in NMYC and ShcC is presented in Chapter 6.

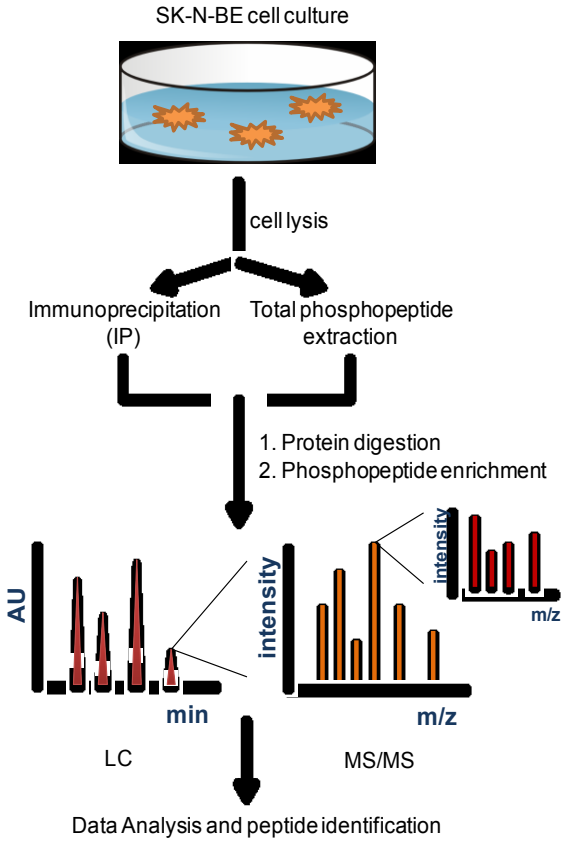

Figure 1.11. Mass spectrometry strategy used to identify NMYC and ShcC phosphorylation in high risk neuroblastoma cells. A general approach and a targeted approach are alternatively utilized.

1.2.9 Specific Aim 2

Specific Aim 2 of our project is to describe the biological relevance of each phosphorylation site identified in NMYC and ShcC. This was accomplished by means of a homology comparison between the novel identified phosphorylation sites and the well-described family members ShcA and c-MYC. The analysis of the novel phosphorylation sites identified in both proteins is described in the Chapter 6.

In Chapter 5 a more detailed analysis of the novel NMYC phosphorylations is presented. In the same chapter we also conducted a series of computational predictions complemented with i*n vitro* assays to identify the kinases involved in the phosphorylation of the novel NMYC sites identified.

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CHAPTER 2

FACILE LABELING OF LIPOGLYCANS WITH QUANTUM DOTS

Carlos Morales Betanzos, Maria Gonzalez-Moa, Stephen Albert Johnston, Sergei A. Svarovsky

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2.1 Contributions

The following chapter describes the synthesis of a novel fluorescent conjugate of Lipopolysaccharides and Quantum dots. Carlos Morales Betanzos designed the strategy for the conjugation of LPS-Quantum dots based on the in-situ formation of a ternary solvent system to allow the interaction between hydrophobic Quantum dots and hydrophilic LPS. Maria Gonzalez-Moa conducted the micelle size characterization by means of Dynamic Light Scattering (DLS). Carlos Morales Betanzos conducted the monocytes binding assays. Dr. David Lowry from the School of Life Sciences Bioimaging Facility obtained all the TEM images. Dr. Sergei Svarovsky and Dr. Stephen Johnston advised in the conduction of the experiments. Dr. Svarovsky , Dr. Gonzalez and Mr. Morales Betanzos wrote the manuscript. The results of this work were published in Biochemical and Biophysical Research Communications.

2.2 Abstract

Bacterial endotoxins or lipopolysaccharides (LPS) are among the most potent activators of the innate immune system, yet mechanisms of their action and in particular the role of glycans remain elusive. Efficient non-invasive labeling strategies are necessary for studying interactions of LPS glycans with biological systems. Here we report a new method for labeling LPS and other lipoglycans with luminescent quantum dots. The labeling is achieved by partitioning of hydrophobic quantum dots into the core of various LPS aggregates without disturbing the native LPS structure. The biofunctionality of the LPS–Qdot conjugates is demonstrated by the labeling of mouse

monocytes. This simple method should find broad applicability in studies concerned with visualization of LPS biodistribution and identification of LPS binding agents.

2.3 Introduction

Bacterial lipopolysaccharides (LPS), also known as endotoxins, are the major constituents of the outer surface of Gram-negative bacteria [1]. They occupy up to 90% of the bacterial cell surface and are responsible for septic shock that kills nearly 200,000 of critically ill patients in the US alone [2]. Not surprisingly, there is a great deal of interest in understanding mechanisms of LPS action for the developing of antisepsis drugs. The development of such agents depends on the availability of efficient labeling strategies for LPS molecules [3]. Ideally such labeling should be the least disruptive to the LPS functionality.

LPSs are complex, negatively charged lipoglycans composed of three distinct regions: (a) a fatty acid region called Lipid A; (b) a core region oligosaccharide composed of approximately 10 monosaccharides; and (c) a highly variable O-antigenic polysaccharide responsible for much of the bacterial pathogenicity and immunospecificity. Most labeling strategies rely on chemical modification of LPS molecules with organic dyes and normally require complex manipulations and purification steps [4,5] due to the aggregative tendencies of LPS molecules [6]. The chemical modification is not site-specific and depends on the availability of reactive groups that are not always accessible or available in LPS [1]. If such groups are not present, they are chemically introduced by oxidation of the Oantigenic glycans [5,7]. By introducing additional moieties to the LPS molecule these methods perturb its physical properties and biomolecular recognition events [8], making such probes unlikely candidates for elucidating the roles of glycan interactions.

Nanometer-sized crystals of semiconductors known as quantum dots (Qdots) have recently emerged as useful luminescent labeling agents [9]. These nanoprobes have significant benefits over organic dyes including long-term photostability, high luminescent intensity, and multiple colors with single-wavelength excitation that open up possibilities for multiplex detection. Coating of hydrophobic quantum dots with phospholipids [10] and synthetic amphiphilic polymers have been described [11]. Both methods rely on phase transfer of hydrophobic quantum dots from an organic solvent to an aqueous solution of amphiphilic molecules. Here, we report an application of hydrophobic quantum dots to non-covalent labeling of LPS and its derivatives. We show that this method may be broadly applicable to other lipoglycans as well. This method takes advantage of the universal amphiphilic nature of lipoglycans and does not introduce any chemical modalities to the LPS structure, making it ideally suitable for studying glycan interactions.

2.4 Materials and Methods

Unless otherwise noted, all chemicals were purchased from Sigma, Inc. (Milwaukee, WI) and used without further purification. Smooth type LPS from *Escherichia coli* (serotypes O111:B4 and O55:B5) and *Pseudomonas aeruginosa* (type 10) were supplied by Sigma, Inc. (Milwaukee, WI). Lipid A was purchased from Avanti Polar Lipids (Alabaster, AL). FITC-labeled LPS from *E. coli* O111:B4 was from Sigma (cat# F3665). (Caution! LPS and Lipid A are pyrogens that may cause fever. It may be harmful if inhaled, ingested, or absorbed through skin. Good laboratory practice should be employed. Wear a lab coat, gloves, safety glasses and a respirator mask). Deionized water was obtained from a Millipore ultrapure water filtration unit. Organic Qdots were purchased from Invitrogen (Carlsbad, CA). Sepharcyl HiPrep 16/60 (S-200 HR) was from GE Healthcare. Transmission electron microscopy (TEM) was done on a Philips CM12S electron microscope operated at an accelerating voltage of 80 kV. Samples were deposited onto carbon-formvar mesh grids and images were recorded using a Gatan model 791 digital camera. In solution nanosizing and zeta potential measurements were done on Zetasizer® Nano-ZS instrument (Malvern Instruments, UK). Spectrophotometric measurements were carried out on NanoDrop® ND-1000 instrument.

Labeling LPS with Qdots. The supplied solution of organic Qdots (QDot® 605 ITK™, catalog #Q21701MP, Invitrogen, Inc.) in decane (1 μM) was evaporated to dryness on SpeedVac at room temperature and re-dissolved in equal amount of chloroform. A 100 μL aliquot of the chloroform solution was diluted to 500 μL with chloroform and mixed with 100 μL of 10 mg/mL aqueous solution of corresponding lipoglycan (*E. coli* O111:B4, *E. coli* O55:B5, *P. aeruginosa* 10, or Lipid A). Methanol

was added dropwise with occasional vortexing until complete mixing of both phases was achieved (400 μL of MeOH). This homogeneous mixture of 5/4/1 chloroform/methanol/water was then evaporated to dryness on a SpeedVac and the solid residue was re-suspended in 100 μL of ddH_2O. A saturated solution of tetramethylammonium hydroxide pentahydrate ($Me_4N^+OH^-5H_2O$) was added until pH 11 (ca. 25 μL). The solution was sonicated for 30 min and then passed through two consecutive Zeba columns (2 mL, Pierce) to remove salts and excess of free LPS. We further purified the LPS-coated Qdots by size-exclusion chromatography on Sepharcyl HiPrep 16/ 60 (S-200 HR) column (50 x 1 cm). The LPS–Qdots eluted in a narrow color band and were stored in the dark at 4 ˚C. Under these conditions, the LPS–Qdots are stable for at least one month without any visible signs of flocculation or deterioration in fluorescent intensity. In a control experiment, the above procedure was repeated without a lipoglycan; no solubilization of organic Qdots was observed in this case.

Preparation of control PEG20K-QDots. 17.2 μL of 8 μM solution of amino QDots (QDot® 605 ITK™ amino(PEG) Quantum Dots, catalog #Q21501MP, Invitrogen, Inc.) were diluted with 200 μL of 100 mM sodium borate buffer pH = 8.5. To this solution, 27.5 μg of Traut's reagent were added and the mixture was shaken at 750 rpm for 2 h at rt. Then, the buffer was exchanged with 300 μL of 1 x PBS by using a Zeba column (2 ml, Pierce). To the solution obtained, 2 mg of mPEG-maleimide 20 K (NEKTAR) was added and the mixture was reacted overnight at room temperature. Excess of mPEGmaleimide was eliminated by filtering the mixture through an Amicon Ultra-4 100 K centrifugal filter and washing two times with 1 x PBS. The solution obtained was used immediately as a control to label monocytes.

Monocytes culture. Mouse monocytes from the cell line RAW 264.7 (American Type Culture Collection, ATCC) were provided by Dr. Yung Chang from the Center of Infectious Diseases and Vaccinology at The Biodesign Institute at ASU. Monocytes were cultured in DMEM (Dulbecco Modified Eagle medium; GIBCO, Grand Island, NY, USA) with 10% (v/v) Fetal Calf Serum (FCS) at 37 ˚C in a 5% CO_2 atmosphere and 95% humidity until confluence.

Monocytes labeling. Monocytes were detached using non-enzymatic cell dissociation agent, (Cellstripper™ Cat # 25-056-CI, Mediatech Inc.) and washed twice with 1 x PBS. Elutriated monocytes (1 x 10^6) were incubated in polypropylene tubes with 100 μg/mL of FITC conjugated *E. coli* O111:B4 LPS or the LPS equivalent of Biomimetic probe or QDots-PEG20K control, suspended in a final volume of 300 μL of HBSS (Hank's Balanced Salt Solution, GIBCO, Grand Island, NY, USA). Incubation time was 1 h at 37 ˚C [12]. After incubation cells were washed twice, fixed with 1% paraformaldehyde solution and analyzed by flow cytometry (FACS Caliber, BD Biosciences Inc.). The same cell suspension was poured on to a poly(L-lysine)-coated glass slide, incubated 30 min at room temperature and analyzed using a fluorescence microscope (BX51, Olympus America Inc.).

2.5 Results and Discussion

2.5.1 Labeling of Smooth-Type LPS

Due to its amphipathic nature, LPS has strong tendency to form aggregates in solution [13]. The aggregation behavior depends on the concentration and the nature of the LPS molecule. Smooth-type LPS is believed to self-assemble into micellar structures of over 1 MDa [6]. This self-aggregation behavior is a function of the lipid A component of LPS molecule that also confers its ability to bind to hydrophobic surfaces. We exploited this ability for labeling LPS with luminescent quantum dots (Qdots) as shown schematically in Figure 2.1.

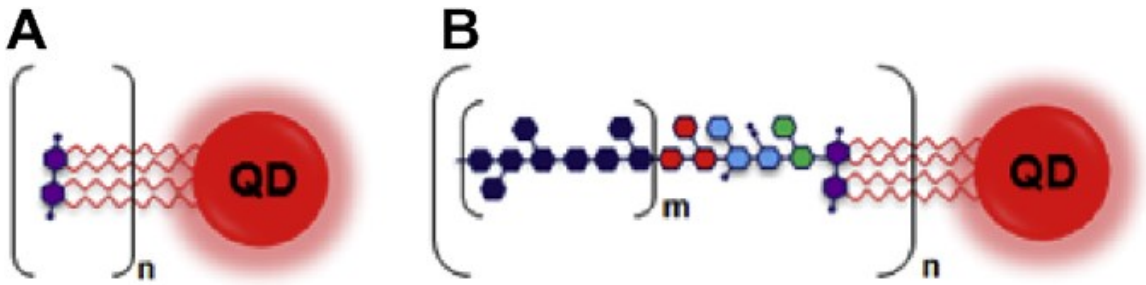

Figure 2.1. (A) Lipid A-Qdots; (B) LPS–Qdots. m designates number of O-antigen repeating units; n, number of LPS molecules encapsulating the Qdot.

Aqueous solution of LPS was mixed with a solution of organic Qdots in chloroform-methanol-water, evaporated, and re-suspended in basic aqueous solution. These steps resulted in the homogenous mixing of the hydrophobic constituents and incorporation of the Qdots into the LPS micelles. We followed the formation of Qdot–LPS conjugates by the Dynamic Light Scattering (DLS). As an example, Figure 2.2

shows the size distribution during the labeling process of smooth-type LPS from *E. coli* O55:B5. The size of LPS micelles prior to labeling is represented by a broad peak with a hydrodynamic diameter of 100 nm, which agrees with previously reported values [13]. After mixing with Qdots and adding the base, the size of the newly formed Qdots–LPS aggregates is reduced to 50 nm. The basification process makes the LPS monomeric [4] favoring the access of the Qdots to the lipidic part of the LPS. Under the UV light, the Qdot particles can be seen quickly transitioning into the aqueous solution, which is only possible if the particles are taken up by the amphiphilic LPS. No luminescence is observed in solution in the absence of LPS even after sonication. The sonication of the mixture of Qdot–LPS for 30 min makes the aggregate more compact as the diameter is reduced to 38 nm and the size distribution becomes narrower, indicating increasing homogeneity of the Qdot–LPS conjugates.

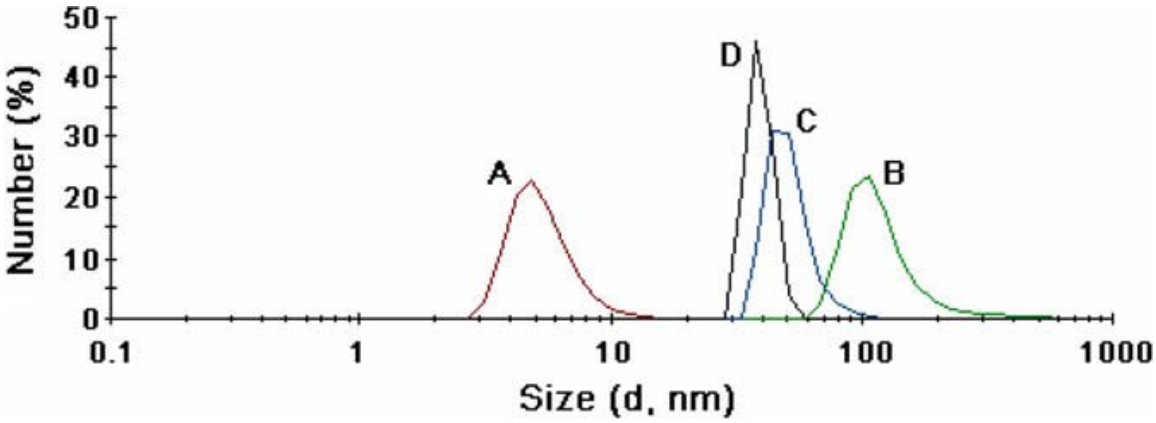

Figure 2.2. Size distribution by number of particles obtained from the DLS analysis of the LPS *E. coli* O55:B5 serotype labeling process. Three key steps in the labeling process are included in the DLS study: (A) Qdots alone; (B) LPS alone; (C) after basification step,

and (D) after sonication step. X-axis shows the diameter in nanometer and the Y-axis shows the percentage of particles of each specific diameter.

The Qdot–LPS conjugates were purified by size-exclusion chromatography and studied by Transmission Electron Microscopy (TEM). Figure 2.3 shows the sizes of the Qdots (ca. 5 nm) and the purified Qdot–LPS conjugates (ca. 50 nm) obtained in the TEM experiment. No free Qdots were observed in the aqueous solution of Qdot–LPS conjugate by TEM. These results agree with the data obtained by DLS shown in Figure 2.2.

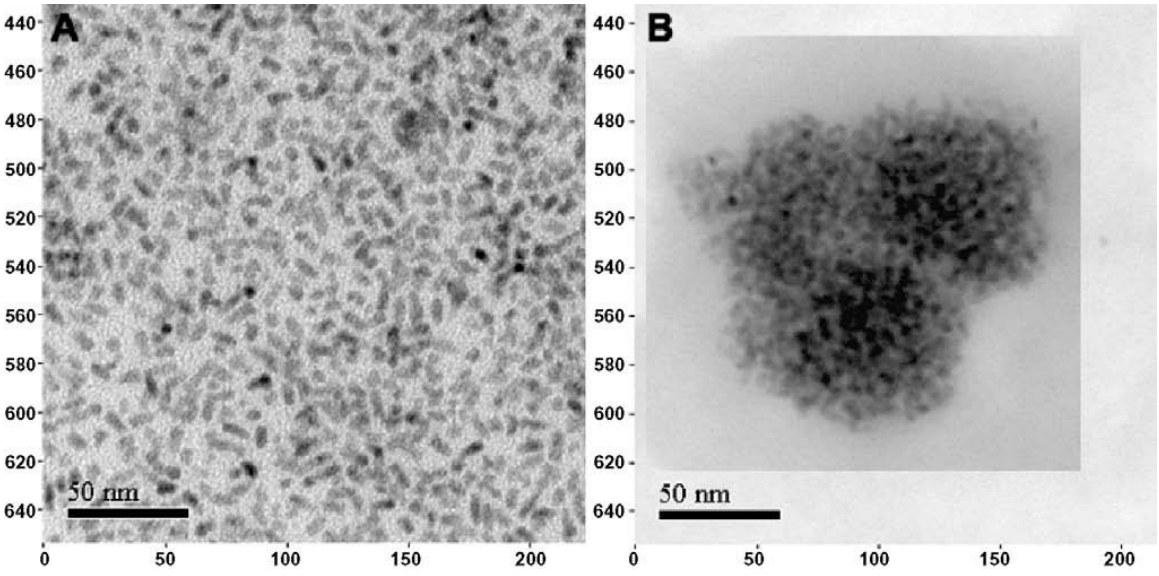

Figure 2.3. Transmission Electron Microscopy image of (A) the organic Qdots from CHCl3 solution; and (B) the purified Qdot-LPS *E. coli* O55:B5 conjugates in water.

2.5.2 Labeling of Other Lipoglycans

Similarly, we labeled Lipid A, LPS from *P. aeruginosa, E. coli* O111:B4, and lipoteichoic acid (LTA)—a LPS equivalent in gram-positive bacteria. Interestingly, despite differences in molecular sizes, all of these conjugates had similar hydrodynamic diameters (centered around 30 nm) after conjugation to the Qdots (Supplementary Fig. S1). Even the complex formed by the smallest Lipid A molecule presents a size comparable to the one formed by the much larger smooth-type LPS. Since similar responses to basification and sonication were observed with the LPS alone, we conclude that presence of Qdots in the lipophilic core of the lipoglycans causes minimal disturbance to their native micellar structures. This highlights an important advantage of our labeling strategy over conventional labeling of individual LPS molecules in the developing assays for the binding analysis of endotoxin-receptor interactions. While in the case of individually labeled LPS molecules a possibility for false-positive binding, e.g. via membrane incorporation [14], is high, this is not the case with the Qdot-labeled LPS micelles since any molecule detached from the micelles is photosilent. Hence, only relevant saccharidic interactions can be detected with the Qdot–LPS probes.

2.5.3 Biological Functionality of Qdot–LPS Probes

To demonstrate that the Qdot labeled LPS keeps the integrity and biofunctionality of the bacterial LPS, and that it can bind as efficiently as the conventional FITC labeled LPS, we studied the recognition of LPS from *E. coli* O111:B4 by its natural cellular membrane receptors. A number of mammalian cells respond to LPS stimulation, with mononuclear phagocytes (monocytes) being the primary targets of the LPS action [15]. Although the exact mechanism of this interaction is not fully understood, it is evident that

in order to elicit a response, LPS must first bind to the surface of the cells. We used monocytes as a model to study the interaction of Qdot-labeled LPS and compare it to the commercially available FITC-labeled LPS [8]. Also, to determine if that the LPS part of the complex is responsible for monocytes binding, we used Qdot-PEG20K as a control, where the Qdots were conjugated to a linear 20 kDa polyethyleneglycol (PEG) molecule. Figure 2.4 shows fluorescent microscope images of the monocytes treated with the different probes, using two different filter sets for each. Figure 2.4A and Figure 2.4B correspond to FITC–LPS labeling, Figure 2.4C and Figure 2.4D depict the Qdot–LPS labeling, and Figure 2.4E and Figure 2.4F relate to the Qdot- PEG20K labeling. The LPS labeled with FITC and Qdots show binding to the surface of the monocytes, whereas no binding is observed with PEG20K QDots. The monocytes labeling was also followed by flow cytometry to get a more complete picture of the binding process (Supplementary Fig. S2). Both FITC- and Qdot-labeled LPS efficiently stained the monocytes, while the control Qdot-PEG20K did not.

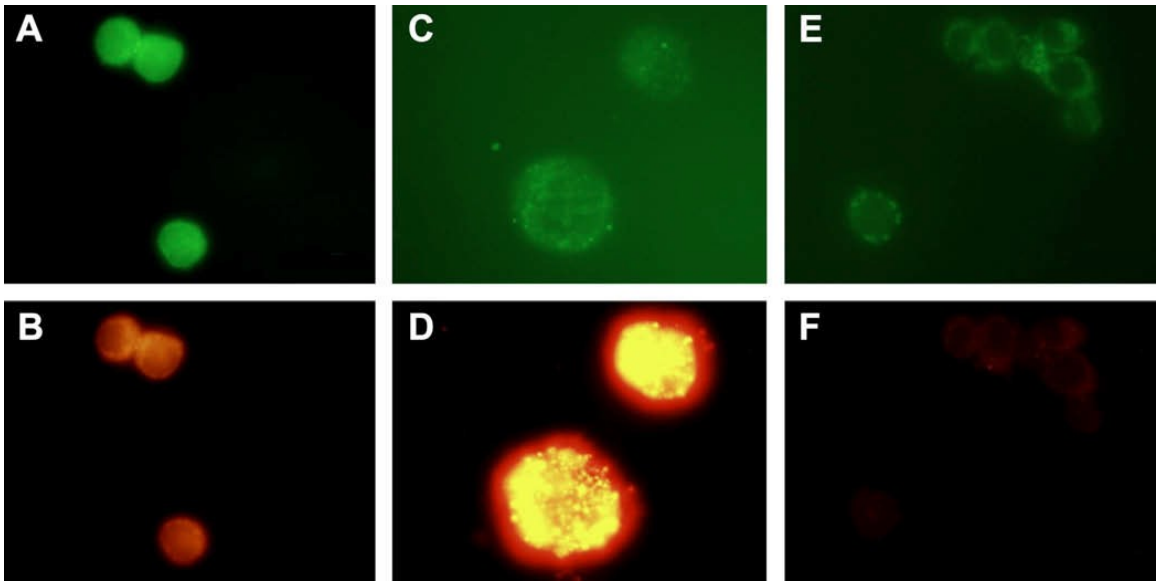

Figure 2.4. Fluorescent microscope images of the labeling of mouse monocytes with (A, B) FITC-LPS; (C, D) Qdot-LPS; (E, F) Qdot-PEG20K. The top panel shows images taken using a filter with a wavelength range of 480–600 nm. The bottom panel shows images taken using a filter with a wavelength range of 530–650 nm.

In summary, we developed a new unobtrusive method for labeling lipoglycans with luminescent quantum dots. The labeling takes place by incorporation of lipophilic quantum dots into the native LPS aggregates. These highly luminescent LPS–Qdot complexes were formed and found to be stable in aqueous solution. Since the lipid functionality is concealed, LPS–Qdot constructs are ideally suited for studying interactions of the polysaccharide moiety of LPS in micellar presentation.

2.6 Acknowledgements

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2.7 Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbrc.2008.12.167.

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CHAPTER 3

BACTERIAL GLYCOPROFILING BY USING RANDOM SEQUENCE PEPTIDE MICROARRAYS

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3.1 Contributions

The following chapter describes the use of fluorescent conjugates of Lipopolysaccharides and Quantum dots to interrogate a 10,000 peptide microarray with the objective of identifying peptides binding to the glycans on the surface of bacteria. Carlos Morales Betanzos and Dr. Maria Gonzalez-Moa conducted the screening of the LPS-Qdots on the peptide microarray and conducted the analysis of the information. Together with Dr. Sergei Svarovsky, they identified the best peptide candidates. Using a protein blast tool, Mr. Morales Betanzos identified the sequence homology of target to natural occurring antimicrobial peptides and performed all the inhibitory assays. Dr. Kathryn Boltz performed the ITC experiments. Dr. Svarovsky performed the ITC data analysis. Mr. Brian Vander Werf performed the SPR experiments and data analysis. Mr. Morales Betanzos conducted the flow cytometry experiments and data analysis. Dr. Gonzalez-Moa conducted the DLS experiments and data analysis. Dr. Svarovsky , Dr. Gonzalez and Mr. Morales Betanzos wrote the manuscript. Dr. Svarovsky and Dr. Stephen Johnston oversaw the research methods and advised in the conduction of the project. The results of this work were published in Chembiochem: a European Journal of Chemical Biology.

3.2 Abstract

Current analytical methods have been slow in addressing the growing need for glyco-analysis. A new generation of more empirical high-throughput (HTP) tools is needed to aid the advance of this important field. To this end, we have developed a new HTP screening platform for identification of surface-immobilized peptides that

specifically bind O-antigenic glycans of bacterial lipopolysaccharides (LPS). This method involves screening of random sequence peptide libraries in addressable high-density microarray format with the newly developed luminescent LPS–quantum dot micelles. Screening of LPS fractions from O111:B4 and O55:B5 serotypes of E. coli on a microarray consisting of 10 000 20-mer peptide features revealed minor differences, while comparison of LPS from *E. coli* O111:B4 and *P. aeruginosa* produced sets of highly specific peptides. Peptides strongly binding to the *E. coli* LPS were highly enriched in aromatic and cationic amino acids, and most of these inhibited growth of *E. coli*. Flow cytometry and isothermal titration calorimetry (ITC) experiments showed that some of these peptides bind LPS in-solution with a K_d of 1.75 μM. Peptide selections against *P. aeruginosa* were largely composed of hydrogen-bond forming amino acids in accordance with dramatic compositional differences in O-antigenic glycans in *E. coli* and *P. aeruginosa*. While the main value of this approach lies in the ability to rapidly differentiate bacterial and possibly other complex glycans, the peptides discovered here can potentially be used off-array as antiendotoxic and antimicrobial lead compounds, and on-array/on-bead as diagnostic and affinity reagents.

3.3 Introduction

The increasing awareness of the importance of glycosylation to biological systems has led to recognition of the need to develop better tools for the analysis of protein–carbohydrate interactions. In contrast to template-driven nucleic acid and protein sequences, which aid function assignments, the need for a more empirical, high-throughput analysis of potential carbohydrate patterns has resulted in a variety of

approaches.[1] The two key newcomers in the area of functional glycomics thus far have been glycan[2] and lectin microarrays,[3] in which glycans or lectins are immobilized on glass slides for investigating the specificity of glycan-binding proteins or glycoconjugates, respectively. In lectin microarrays, carbohydrate-binding proteins, such as lectins and anticarbohydrate antibodies, are immobilized on a solid support in high spatial density. Interrogation of these arrays with fluorescently-labeled samples creates binding patterns (glycosignatures) that depend on the carbohydrate structures present, and provide a method for rapid characterization of glycans on glycoproteins,[4] bacteria,[5] or mammalian cells.[6] The microarray format allows rapid parallel analysis of multiple carbohydrate–protein interactions with a minimal amount of sample. Notwithstanding the advantages, lectin microarrays are intrinsically handicapped by restricted availability,[3] and the limited and often unexpected specificities of natural lectins.[7] Only about 60 lectins are commercially available, and they have the ability to recognize only a fraction of glycans present on mammalian and especially on microbial cells.[3] The common problems inherent to other protein arrays,[8] such as linking chemistry, orientation-dependent binding activity, and storability, are also important factors that strongly argue in favor of alternative approaches.

Proteins are not the only molecules that bind carbohydrates. Cyclic tricatechol[9] and terphenyl[10] constructs, acyclic pyridine, pyrimidine, and naphthyridine units,[11] self-assembled structures and various boronic acid derivatives[12, 13] have been described. Also, aptamers and peptides have been explored.[14] Synthetic peptides have long been known as highly versatile molecules for a variety of biological applications. Unlike proteins, which unfold readily and subsequently lose their biological activities,

peptides are functionally stable and capable of retaining their activities under most reaction conditions; this makes them the preferred molecules for facile and robust screening assays, especially in microarray-based formats.

We have an ongoing program applying addressable random sequence peptide microarrays, as an alternative to phage display, to the analysis of various biomolecular interactions. As a part of this program, we have tested these microarrays for their ability to detect carbohydrate interactions. We hypothesized that owing to the large chemical diversity of peptide structures with no preconceived specificity, the microarray could provide an expedient approach to de novo discovery of artificial lectin mimics with engineered specificities towards glycans of interest. As a proof-of-concept, we chose to use the microarray to analyze the saccharidic portion of bacterial lipopolysaccharides (LPS). The reason for choosing such a complex target was threefold. First, the diversity of glycan structures unique to bacteria would allow the widest dynamic range of molecules to be tested and therefore let us evaluate the limitations of this approach. Second, from a practical standpoint LPS have been implicated in the systemic inflammatory response and septic shock, which have claimed more than 200 000 lives each year in the U.S. alone.[15] Hence, there is a great deal of interest in developing therapeutic agents that can efficiently bind LPS.[16] Third, whereas the therapeutic strategy directed against viral glycans was a success, a similar approach to antibacterial therapies has not been systematically explored due to difficulties in finding molecules that can selectively bind to bacterial glycans.[17]

Herein, we report our findings in screening of 10 000 random 20-mer peptide sequences printed on a glass slide with newly developed luminescent LPS glycoprobes

for potential lectinomimetic activity. A set of specific lectinomimetic antagonists of LPS molecules have been discovered that can be used as a new class of diagnostic, antiendotoxic, and antimicrobial peptide leads in both on- and off-array formats. To our knowledge, this is the first application of peptide microarrays to studying carbohydrate interactions.

3.4 Results and Discussion

3.4.1 General Experimental Set-Up

The peptide microarrays were constructed by spotting 10 000 random 20-mer sequences in duplicates on a maleimide-functionalized microscope glass slide by using a robotic pin spotter. The random peptide library was produced by conventional solid-phase synthesis based on computer-generated random sequences of 19 amino acids, excluding cysteine, for the first 17 amino acids. A C-terminal –GSC sequence was incorporated into each peptide to facilitate coupling to the array surface. These arrays were probed directly with fluorescently labeled LPS.

3.4.2 General Considerations in the Design of LPS Glycoprobes

LPS is a complex, negatively charged lipoglycan composed of three distinct regions: 1) a fatty acid region called lipid A that has very low variability ; 2) a conserved glycosidic “core” consisting of approximately ten monosaccharides; and 3) a highly variable region called O-antigen, consisting of repetitive sub-units of one to eight monosaccharides repeated up to 100 times.[18] The O-antigen region defines the strain, serotype, and even the virulence of the bacteria; this makes it a very attractive target to study. Probing the peptide microarray with directly labeled LPS allowed us to avoid the

use of secondary detection reagents, which complicate the interpretation and later deconvolution of the data. This consideration is especially important for the random peptide microarrays because each peptide on the array de facto is not specific and serves as a putative ligand for any target of choice. Not only can the secondary probe bind to the array, but it could also compete with the primary probe. In the case of carbohydrates, the binding affinities of which are typically weak, the latter can present a serious problem. For these reasons, the arrays were probed directly with conventional organic dye-labeled and the newly developed quantum dot (Qdot)-labeled LPS in order to specifically single out carbohydrate interactions.

3.4.3 LPS Labeling with Qdots

The existing LPS labeling strategies rely on chemical modification of LPS molecules with organic dyes (Figure 3.1A). This method requires complex manipulation and purification steps, is not site-specific, and depends on the availability of reactive groups in the LPS molecule.[19] When such groups are unavailable, an extra functionality is introduced into the saccharidic branch of LPS, which might affect its physical properties and biomolecular recognition events; thus it is not ideally suitable for the purposes of this study. For this reason, we have developed an alternative labeling strategy that takes advantage of the amphipathic nature of LPS molecule and does not introduce any new chemical modalities into the structure.

We used nanometer-sized crystals of semiconductors known as quantum dots (Qdots) that have recently emerged as useful luminescent labeling agents.[20] Coating of hydrophobic Qdots with phospholipids[21] and synthetic amphiphilic polymers have been previously described.[22] Both methods rely on phase transfer of hydrophobic

Qdots from organic solvent to an aqueous solution of an amphiphile. Using a similar approach, we conjugated smooth-type LPS from E. coli and P. aeruginosa to hydrophobic Qdots (Figure 3.1B). In this case, the lipid A, which is responsible for self-aggregation, also confers the ability of LPS to bind to hydrophobic surfaces of Qdots. Since the lipid functionality is attached directly to the label, Qdot–LPS constructs are especially useful for studying the saccharidic moiety of LPS.

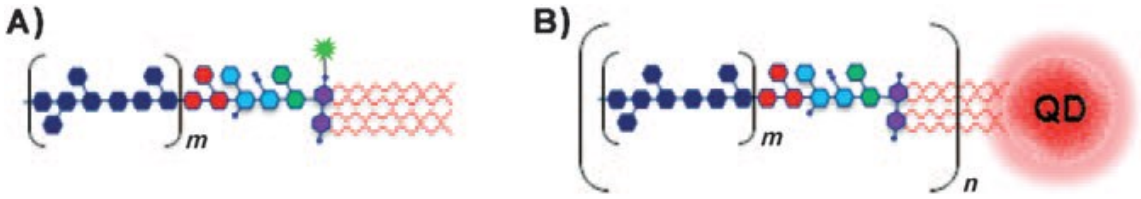

Figure 3.1.A cartoon depicting: A) organic dye labeled LPS; B) Qdots-labeled LPS; m designates the number of O-antigen repeating units, n designates the number of LPS molecules attached to the Qdot.

3.4.4 Comparison of Qdot- and Organic Dye-Labeled LPS

Although many peptides have been shown to bind LPS in solution,[23] at the outset of this work it was not clear if peptides in the microarray format would also be able to bind LPS specifically and reproducibly. In order to demonstrate that the peptides on the microarray indeed bind LPS and the interaction is not dye- or lipid-induced, we conducted several experiments. In the first of these experiments, identical concentrations of LPS from *E. coli* O111:B4 (EC_{O111}) labeled with FITC (FITC–EC_{O111}) and with Qdots (QDot–EC_{O111}) were used to probe the microarray slides. The "unblocked" sample was used as is, while the "blocked" sample was spiked with 100-fold excess of unlabeled

EC_{O111} during the binding step. For both FITC–EC_{O111} and QDot– EC_{O111} probes (Figure S1 in the Supporting Information) blocking with unlabeled LPS localized the specific interactions, as most of the top binders to LPS become low binders when excess unlabeled LPS is used to inhibit the specific peptide– LPS interactions. This simple test effectively eliminated any unspecific dye-induced interactions. The same test was applied to all binding experiments described below.

To test the applicability of the Qdot–LPS probes for specifically detecting carbohydrate-binding events, we used scatter plots as previously reported by Reddy and Kodadek,[24] and compared the results with those obtained with conventionally labeled LPS. This representation helped us to focus our attention only on peptides with high expression profile for both experiments (FITC–EC_{O111} and Qdot–EC_{O111}). Figure 3.2 shows reasonable correlation (R=0.824) between the two experiments. Although some unique hits were present both in Qdot–LPS and FITC–LPS binding peptides, they can be attributed to differences in the probe construction and photophysical properties of the labels. The LPS is presented on the multivalent Qdots in a defined orientation, since the hydrophobic Qdots only exist in aqueous solution when they are enclosed in the hydrophilic environment created by the lipid portion of the LPS molecules. This orientation exposes the saccharidic branch of the LPS, similar to their orientation in the micellar (or cellular) state of LPS. In contrast the monovalent FITC–LPS probe can potentially detect saccharidic-, dye-, and lipid-induced interactions. It has been found that aggregated micellar FITC– LPS has strongly diminished fluorescence due to quenching, while the disaggregation of single FITC–LPS molecules from micelles leads to enhancement in fluorescence.[25] So, it is likely that oriented micellar LPS molecules

would not be observable, and the most significant signal detected would come from the single LPS molecules, which include nonsaccharidic components. Due to the well-known cluster glycoside effect,[26] the interaction of multiple sugars is also stronger than a single LPS molecule. These observations highlight the utility of Qdot–LPS for studying variable saccharidic components.

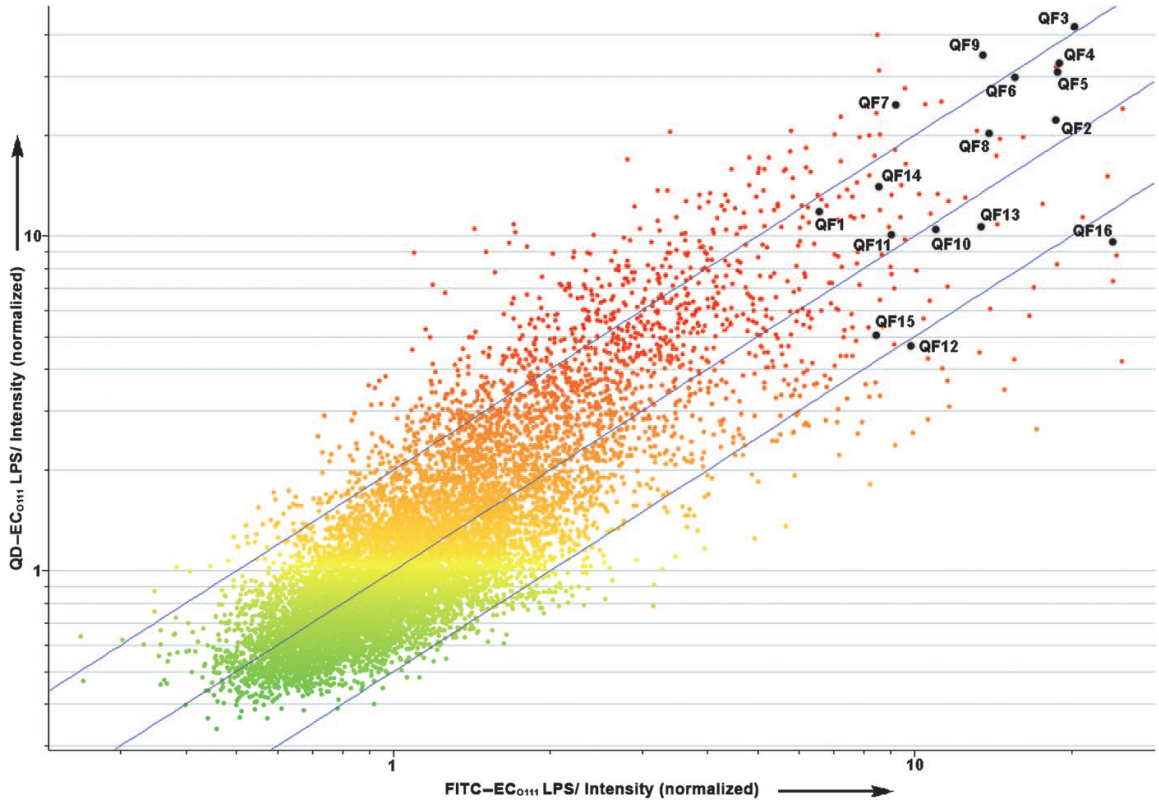

Figure 3.2. FITC-labeled versus Qdot-labeled *E. coli* O111:B4 LPS correlation (R=0.824). Annotated black dots indicate selected LPS-binding peptides shown in Table 1. Both axes show normalized signal in a logarithmic (log2) scale. Blue lines delimit the twofold change.

3.4.5 Selection of Peptides Binding the Saccharidic Branch of LPS

Since the Qdot labels were novel for LPS, we argued that only peptides that bind both FITC–LPS and Qdot–LPS with high intensity were the most reliable saccharidic LPS-binding peptides. Such peptides were identified by statistical analysis by using image-processed data, and visualized as a scatter plot.[24] We have selected only high intensity binders with a minimal standard deviation ($s<0.2$) that were present in both Qdot– LPS and FITC–LPS experiments (Figure 3.2). Autofluorescent peptides were filtered as described in the Experimental Section and each hit was independently confirmed by careful visual inspection of the slides.

The data revealed 16 peptides, QF1–QF16, that bound with high affinity to E. coli O111:B4 LPS (Table 3.1). Most of these peptides contain noticeably abundant cationic arginine, lysine, and histidine, along with clusters of aromatic hydrophobic tryptophan and phenylalanine and/or tyrosine. Since many of the existing LPS-binding peptides are also antimicrobial,[27] we hypothesized that if our selections were valid then at least some of the peptides should share sequence similarity with the existing antimicrobial peptides (AMPs). To test this hypothesis, we compared the selected sequences against several AMP databases. In particular, we found that the above amino acids were also abundant in indolicidin- like AMPs.[28] Moreover, using the Antimicrobial Peptide Database,[29] we found that some of these peptides (QF1–8) shared 30 to 40% similarity to human histatins-2, -6, or -9, which are histidine-rich AMPs found in oral cavities. Finally, we applied a recently developed algorithm that predicts antibacterial sequences based on similarity to the existing 486 AMPs.[30] The higher the antibacterial peptides prediction (APP) score, the more probable the antibacterial activity, while negative scores

suggest no antibacterial activity. The APP scores shown in Table 3.1 predicted that 11 out of 16 selected peptides had potential antibacterial properties.

Table 3.1. *E. coli* O111:B4 LPS binding peptides arranged by isoelectric point (pI), number of negative residues (NR), number of positive residues (PR), aliphatic index (AI), and antibacterial peptides prediction score (APP).[27]

ID	Peptide sequence	pI	NR	PR	AI	APP
QF1	RHWRKPRKWHKKWPPHRGSC	12.0	0	8	0	1.904
QF2	HRKHWRKRHKKHWKKRKGSC	12.0	0	11	0	2.673
QF3	HWKRRHKHKWPKRHPHKGSC	11.8	0	8	0	2.035
QF4	HFRKWHKRRWKHHKKWKGSC	11.8	0	9	0	2.155
QF5	WKKKRKHRHKKHWHPWRGSC	11.8	0	9	0	1.616
QF6	WKFRHRHHRHHWHKKWKGSC	11.8	0	7	0	2.167
QF7	WFWKHKKWRRHPRKWHWGSC	11.8	0	7	0	1.567
QF8	HRKPKFRHHHFKWKHWKGSC	11.2	0	7	0	1.529
QF9	WWHHKWFKHKKFWRHKFGSC	10.6	0	6	0	2.121
QF10	RVFKRYKRWLHVSRYYFGSC	10.6	0	6	49	1.296
QF11	VLKHHRVKAFKFWHEYIGSC	9.6	1	4	73	0.724
QF12	TWTQQMHHFRFSHKLERGSC	9.5	1	3	20	−0.566
QF13	THRPHNWYLFKNILFSHGSC	9.3	0	2	59	−0.964
QF14	GTNERYNMRKYHWWYWYGSC	9.0	1	3	0	−0.348
QF15	FQTAKLFFGYHNHTESSGSC	6.9	1	1	25	−0.036
QF16	EWHHIWINNQHYNHASHGSC	6.6	1	0	44	−0.795

3.4.6 Antimicrobial Properties of the LPS Binding Peptides

We assayed the ability of the LPS-binding peptides to inhibit E. coli growth, and compared them to 142 LPS nonbinding peptides (Table S1). Figure 3.3 shows that nearly 70% of the LPS-binding peptides demonstrated some growth inhibition activity against *E. coli* DH10B, while none of the 142 nonbinding peptides inhibited growth by more than 20% (Figure S2). Interestingly, the peptides QF12, -13, -14, and -16 demonstrated enhancement of bacterial growth (Figure 3.3). In agreement with the APP scores peptides QF1–10 displayed antibacterial activity (Table 3.1). An evident outlier, QF15, which departs from the conventional cationic amphipathic motifs associated with AMPs, was also identified. Further testing through kinetic growth curves showed that these peptides

are bacteriostatic, not bactericidal. This agrees with recent work demonstrating that the biophysical properties required to kill bacteria differ from those to bind LPS.[31] In addition to affinity for LPS, bactericidal activity requires the abilities to traverse the LPS layer and to disaggregate LPS micelles. Our concentration-dependent studies (data not shown) demonstrated that even at 10 mm concentration, peptides QF7, -8, and -10 retained their ability to inhibit up to 50% of *E. coli* growth.

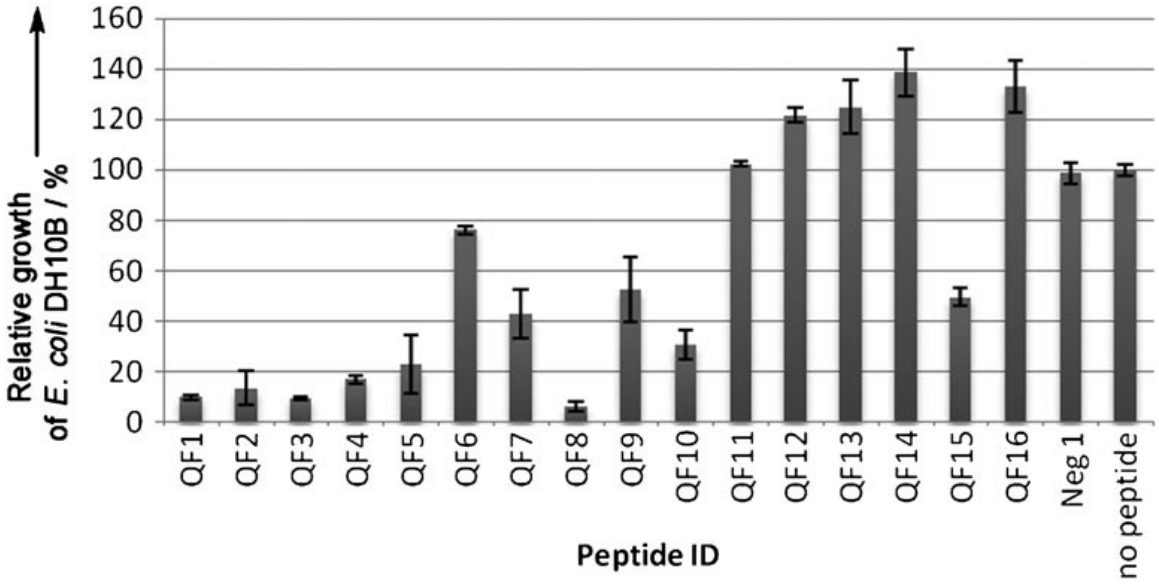

Figure 3.3. Relative growth inhibition activities of peptides QF1–16 tested against *E. coli* DH10B. The error bars are standard deviations of triplicate measurements; Neg1 is a negative control peptide.

3.4.7 Flow Cytometry Studies of the LPS-Binding Peptides

Intrigued by the high incidence of antimicrobial activity of the selected peptides, we conducted flow cytometry studies to quantify the in vivo binding abilities of the

selected peptides to *E. coli*, and the ability of preincubation with LPS to block binding.[32] The LPS nonbinding peptide, Neg1, was used as a negative control. The peptides were biotinylated and their specificities were compared through quantifying the cell surface staining of *E. coli* DH10B cells with AlexaFluor488-labeled streptavidin. Cells labeled only with streptavidin were used as controls, and fluorescent intensity greater than that associated with streptavidin only labeled cells was quantified as the M1 region. Peptides QF1 through QF10 bound the cells almost completely in the M1 region; this indicates that these peptides bound the cells with higher affinity than would be expected from streptavidin-only binding (Table S2). The results for streptavidin, the negative control peptide Neg1, QF5, and QF8 are summarized in Figure 3.4. Both QF5 and QF8 bound to DH10B cells, and their cell-surface binding was nearly eliminated after preincubation with EC_{O111} LPS. While these results do not elucidate the nature of the target on the DH10B cell surface, they do show that the peptides bind to and are sequestered by the interaction with their target EC_{O111} LPS.

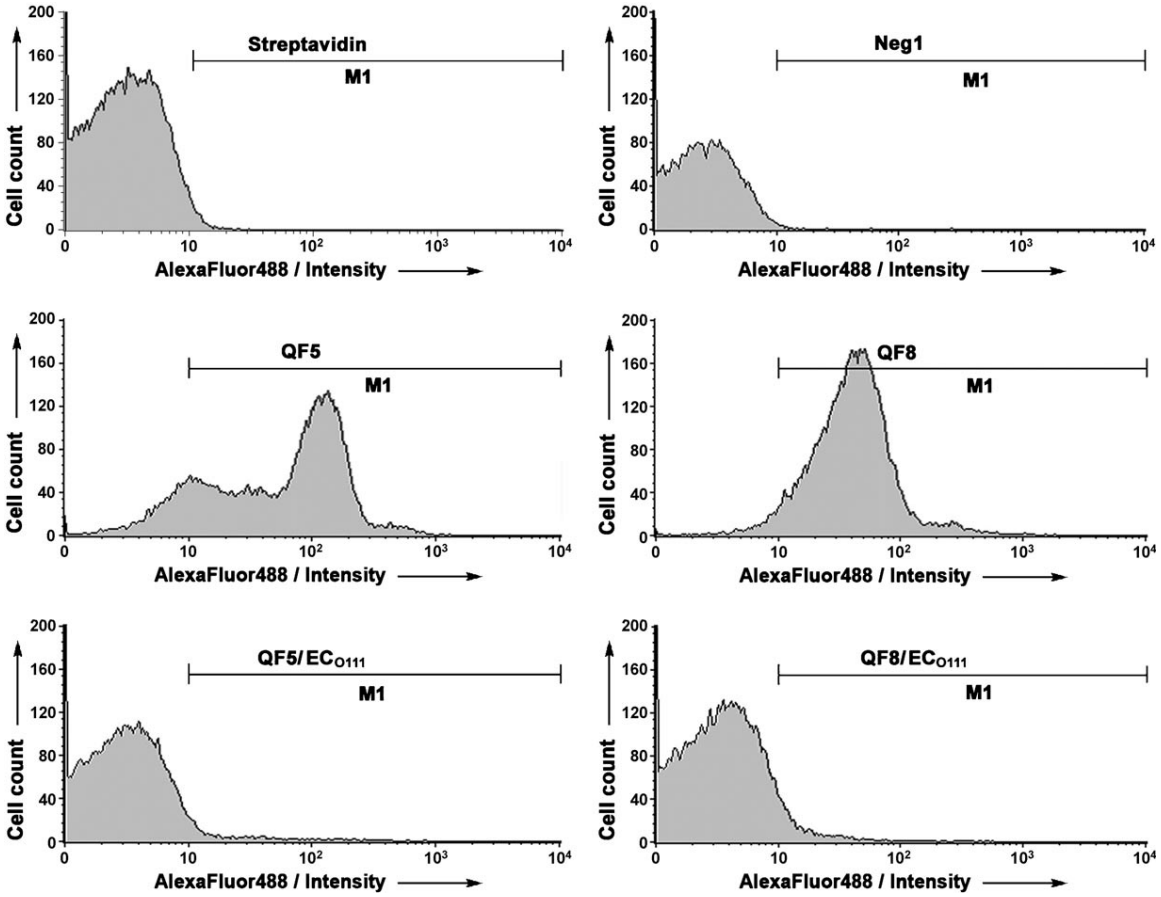

Figure 3.4. Flow cytometry of AlexaFluor488-labeled streptavidin, Neg1 control peptide, QF5, QF8, and QF5 and QF8 after 1 h preincubation with 100-fold excess of E. coli O111:B4 LPS. The y axes show the cell count, and the x axes show the AlexaFluor488 intensity.

3.4.8 Surface Plasmon Resonance (SPR)

High resolution differential SPR was used to compare the binding of QF5, QF8, and Neg1 to EC_{O111} (Figure 3.5 and S3). This technique has sufficient sensitivity to detect direct binding of free glycans to lectins immobilized on a sensor chip and allows the evaluation of sugar–lectin dissociation constants in the nm range.[33] Both QF5 and QF8

are strong antimicrobial candidates, while Neg1—a peptide showing no binding to LPS on the peptide microarrays—was used as a negative control. The relative responses of these peptides were compared by using normalization based on the immobilization density and molecular weight of the respective peptides. Both QF5 and QF8 peptides had similar abilities to bind LPS, while Neg1 had negligible binding (Figure S3 A).

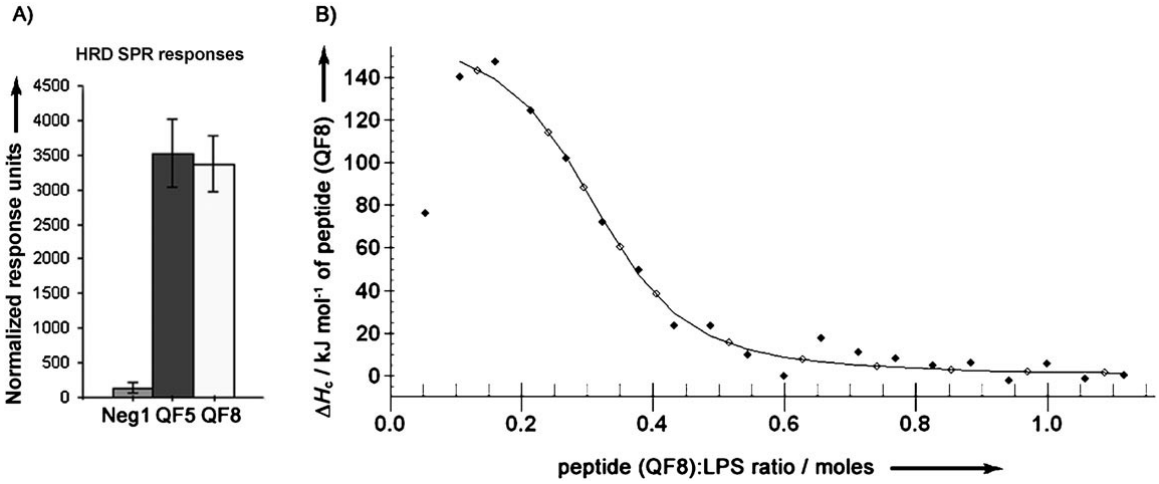

Figure 3.5. A) High resolution differential (HRD) SPR responses of peptides Neg1, QF5, and QF8 to *E. coli* O111:B4 LPS. B) ITC titration curve of the LPS EC_{O111} with peptide QF8.

3.4.9 Isothermal Titration Calorimetry (ITC)

To estimate the ability of peptide QF8, which exhibited maximal antimicrobial activity, to bind LPS in solution, we conducted the microcalorimetry titration of LPS EC_{O111} with QF8. The integrated heats in Figure 3.5B represent the net heats of each injection after subtraction of the heat of dilution of QF8 into pure buffer. The upward position of the ITC titration peaks (Figure S3 B) and the resultant positive integrated

heats indicate that the association between QF8 and LPS is an endothermic process.[34] With a single site independent binding model, the enthalpy (ΔH) of association between QF8 peptide and LPS is 7.8 $kcalmol^{-1}$ with an equilibrium association constant (K_a) of 568 731M^{-1} (K_d=1.75 μM) and a stoichiometry of 0.2–0.4 QF8/LPS (due to heterogeneity of LPS) obtained at pH 7.4. This ratio likely corresponds to the net charge compensation between anionic LPS (2–4 negative charges) and cationic QF8 (seven positive charges).[35] The free energy (ΔG) and entropy (ΔS) changes of binding are estimated to be -7.8 $kcalmol^{-1}$ and 52.6 $calmol^{-1}$ deg^{-1}, respectively.

3.4.10 Differentiation of *E. coli* Serotypes

Gram-negative bacteria are classified by serological types (serotypes) based on the composition of the LPS O-antigen domains. Thus, the O-antigen, which is responsible for much of the immunospecificity of the bacterial cells, essentially serves as the "glycosignature" of a bacterium.[18] To test whether we can distinguish among different serotypes of a bacterium using the peptide microarray, we screened Qdot-labeled LPS derived from two different serotypes of *E. coli*: O111:B4 (EC_{O111}) and O55:B5 (EC_{O55}). Figure 3.6 shows the 2D scatter plot corresponding to these experiments. Overall, an excellent correlation (R=0.907) was observed between the two serotypes; this indicates that there are only marginal differences detectable by the microarray. The high correlation coefficient is in agreement with the compositional similarity of the LPS molecules derived from the two serotypes (Figure 3.7). The O-antigen repeating units of EC_{O111} [36] and EC_{O55} [37] LPS are composed of five neutral monosaccharides, which include glucose (Glc), galactose (Gal), *N*-acetyl-galactosamine (GalNAc), *N*-

acetylglucosamine (GlcNAc), and colitose (Col; 3,6-dideoxy-L-galactose). Although both structures differ in branching and sequence, the overall sugar content remains similar.

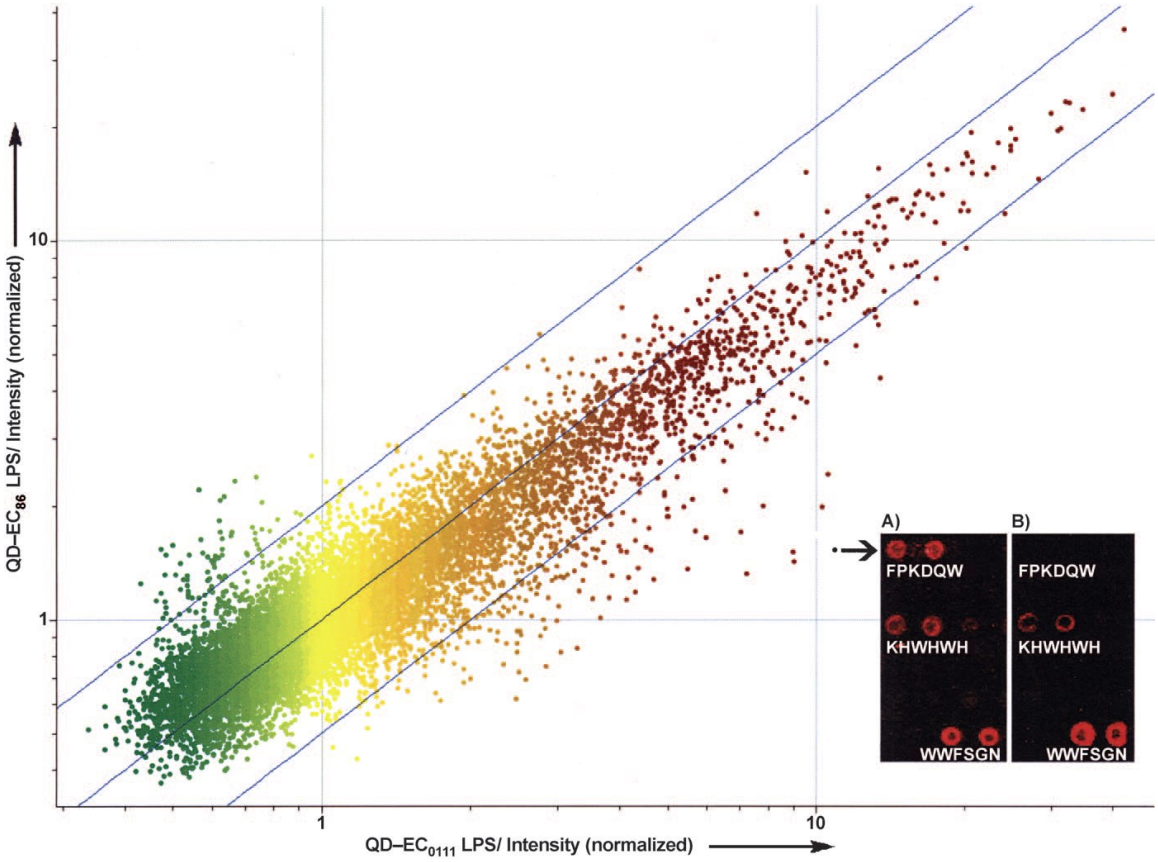

Figure 3.6. *E. coli* O111:B4 versus *E. coli* O55:B5 Qdot–LPS correlation for triplicate experiments of each (R=0.907). The black dot corresponds to the EC_{O111} specific peptide FPKDQW (shown in the insert, with EC_{O111} on the left and EC_{O55} on the right). Both axes show normalized signal in a logarithmic (log2) scale. Blue lines delimit the twofold change. Insert shows close-up of the peptide microarray binding patterns of: A) EC_{O111} and B) EC_{O55}. The first six (of 20) amino acids are shown. (For a full sequence see the Supporting Information.)

Despite the negligible statistical differences, a close visual inspection of the slides revealed several distinct hits that are unique to EC_{O111} (Figure 3.6, insert) and to EC_{O55}. In all the cases, for a hit to be statistically significant it must be reproduced in all replicate slides with a standard deviation of less than 0.2.

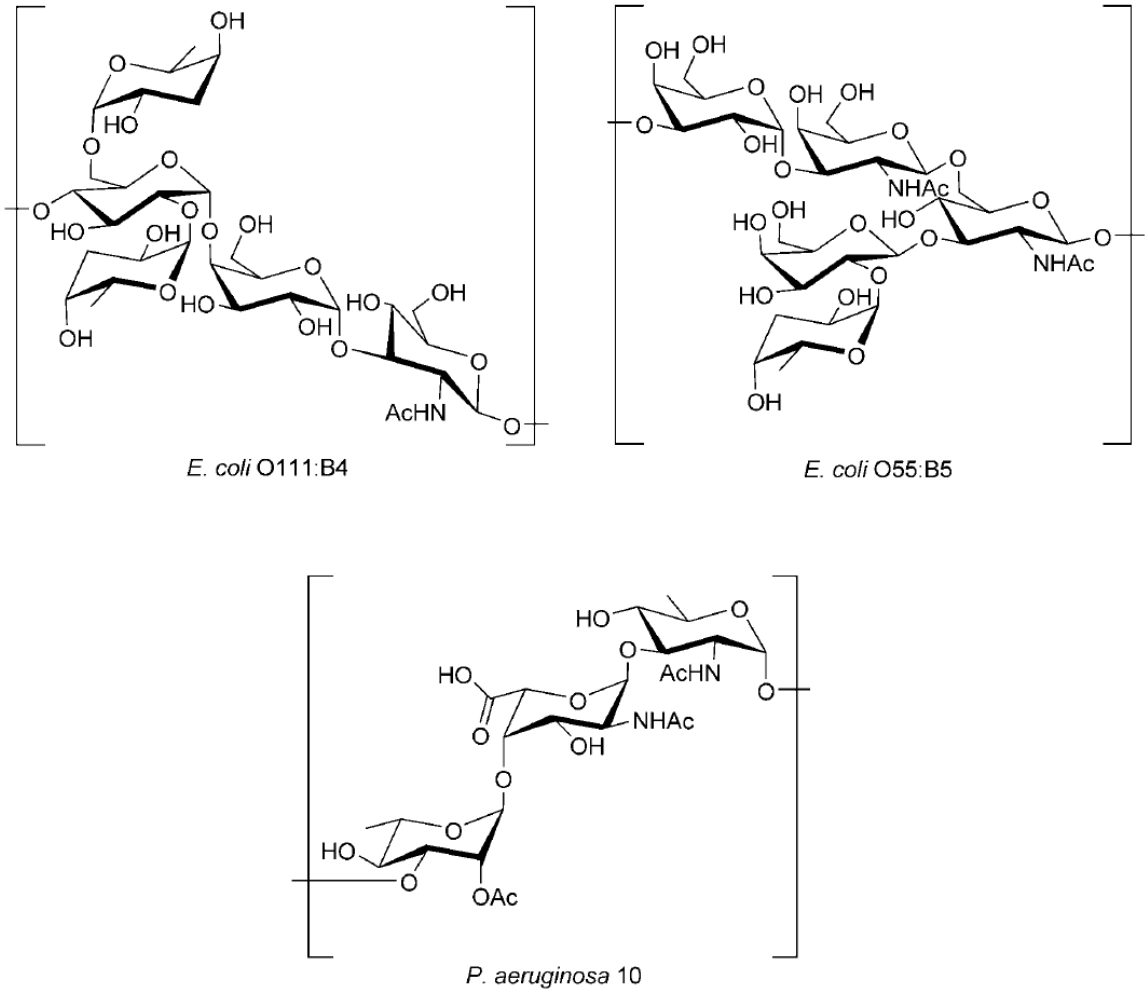

Figure 3.7. Chemical structures of the repeating units of LPS used in this work.[36–38]

3.4.11 Differentiation Between *E. coli* and *P. aeruginosa*

While the two *E. coli* serotypes have subtle compositional differences, more prominent differences are apparent when the LPS structures of *P. aeruginosa* 10 (PA_{10})

and EC_{O111} are compared (Figure 3.7). The repeating unit of PA_{10} consists of three unusual sugars: 2-*O*-acetyl-L-rhamnose (RhaAc), 2-*N*-acetyl-L-galacturonic acid (GalNA), and 2-*N*-acetyl-2,6-dideoxy-D-glucosamine (QuiN).[38] One of these sugars (GalNA) contains a carboxylic acid group that can carry negative charge and form strong hydrogen bonds. Screening of the PA_{10} LPS labeled with Qdots and statistical correlation of the results with EC_{O111} revealed a number of distinct hits for EC_{O111} and PA_{10}. Indeed, even a superficial visual inspection of the slides immediately shows differences in binding patterns between the two experiments (Figure 3.8).

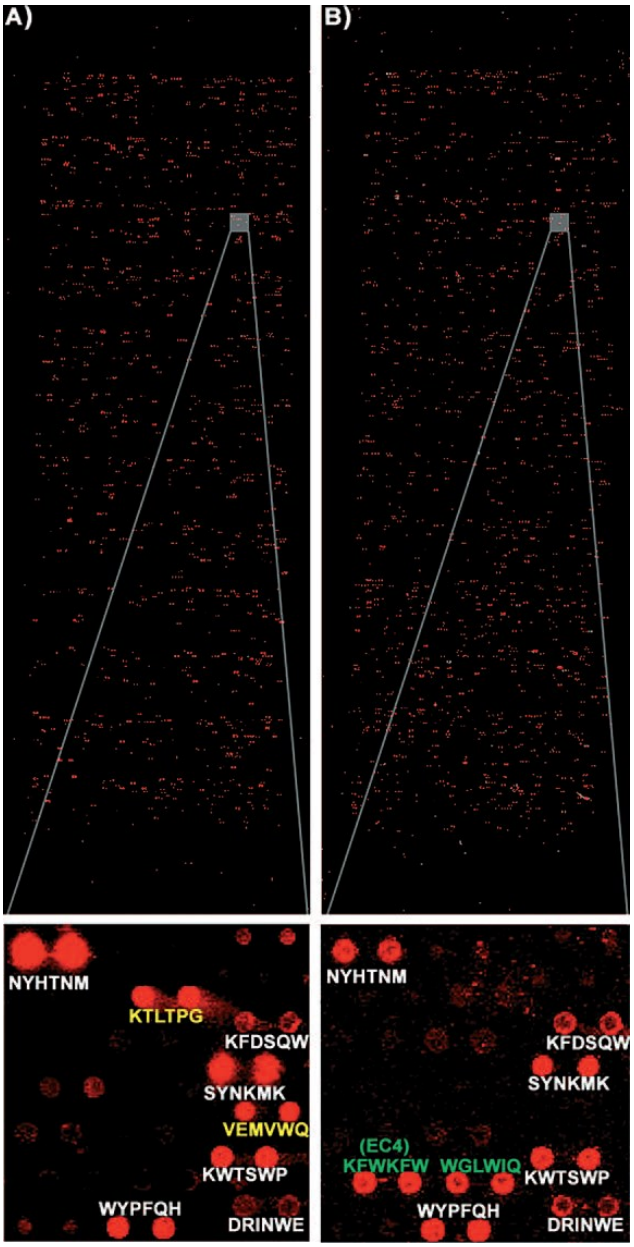

Figure 3.8. LPS binding patterns ("glycosignatures") on the microarray of: A) *P. aeruginosa* 10 LPS and B) *E. coli* O111:B4 LPS. Sequences in yellow indicate peptides unique to *P. aeruginosa*; sequences in green are unique to *E. coli*; sequences in white are common binders. Only the first six (of 20) amino acids are shown. (For a full sequence see the Supporting Information.)

Figure 3.9 shows the statistical correlation between Qdot–PA_{10} and Qdot–EC_{O111} experiments as a scatter plot.[24] The correlation coefficient is far lower (R=0.630) than in the case of EC_{O111} versus EC_{O55} (R=0.907; Figure 3.7). Peptides EC1–8 (Table 3.2), which specifically bind EC_{O111} but not PA_{10}, were identified by minimizing the error (standard deviation $\sigma < 0.2$) while maximizing the ratio of normalized EC_{O111} to PA_{10} signals. These comparisons independently validate the first selection of EC_{O111} binding peptides QF1–16 (Table 3.1) which are annotated in blue in Figure 3.9. A similar selection strategy seeking peptides that specifically bind PA_{10} but not EC_{O111} yielded peptides PA1–8 (Table 3.2). The heat map shown in Figure 3.9 graphically demonstrates the expression levels of each of the EC1–8 and PA1–9 peptides in Qdot–PA_{10} and Qdot–EC_{O111} experiments. All EC peptides present a high expression in the EC_{O111} experiment, while the expression in the PA_{10} experiment is low. The opposite is true for PA peptides, which have high expression in the PA_{10} experiment, but low in the EC_{O111} experiment.

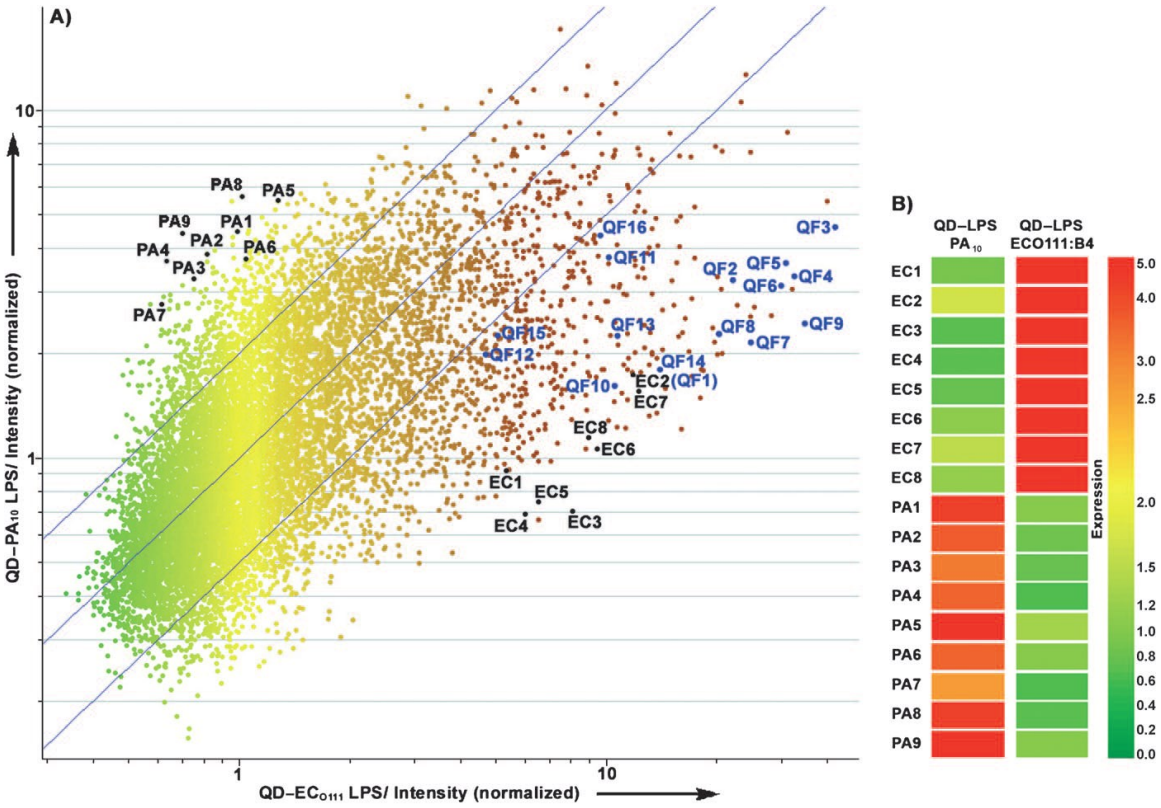

Figure 3.9. A) *E. coli* O111:B4 versus *P. aeruginosa* 10 LPS correlation (R=0.630). Annotated blue dots correspond to peptides shown in Table 3.1; annotated black dots correspond to the peptides shown in Table 3.2. Both axes show normalized fluorescence signal at 605 nm on a logarithmic scale. Blue lines delimit the twofold change. B) Heat map compares the level of expression (luminescent intensity, log2) for the EC and PA peptides (green: low; red: high).

3.4.12 Structural Considerations

As seen in Table 3.2, most of the peptides unique to EC_{O111} are enriched in aromatic tryptophan and cationic arginine, lysine, and histidine, while peptides specific to PA_{10} tend to contain aliphatic amino acids, anionic aspartic, and glutamic acids, and

especially hydrogen bond forming glycine, proline, serine, and threonine (Figure S4). These differences are reflected in the consistent differences in pI values and aliphatic indices (AI) of the selected peptides (Table 3.2). This can be explained by the prominent compositional differences between EC_{O111} and PA_{10} LPS. Interestingly, Cherkasov et al.[39] recently found the same kind of amino acid distribution by using artificial intelligence in the design of peptide antibiotics.

Table 3.2. Peptides specific to EC LPS versus PA_{10} LPS. Column headings indicate the isoelectric point (pI), negative residues (NR), positive residues (PR), and aliphatic index (AI).

ID	Peptide sequence	Qdot–EC_{O111}	Qdot–EC_{O55}	Qdot–PA_{10}	pI	NR	PR	AI
EC1	KFWHHKWWHWFKWRRRRGSC	+	+	–	12.0	0	7	0
EC2	RHWRKPRKWHKKWPPHRGSC	+	+	–	12.0	0	8	0
EC3	HHFKHHRHWKRRRHWFWGSC	+	+	–	12.0	0	6	0
EC4	KFWKFWHKHRHRHRWHRGSC	+	+	–	12.0	0	7	0
EC5	HRWWFKKKHRFRWWKRWGSC	+	+	–	12.0	0	8	0
EC6	WRHWRRRKHFWWKRRWHGSC	+	+	–	12.3	0	8	0
EC7	GWAREHHWPRIIYGVLRGSC	+	+	–	9.5	1	3	78
EC8	HHPRHWWWKRWHPFRFFGSC	+	+	–	11.7	0	4	0
PA1	VPTPNDQGKQWVNSVNAGSC	–	–	+	5.8	1	1	49
PA2	RKHDYEEVESEFHPRKGGSC	–	–	+	6.0	5	4	15
PA3	SHPRITTSDDHGDSPKGGSC	–	–	+	5.9	3	2	20
PA4	VPVHDKTRKTAPAEEIVGSC	–	–	+	6.7	3	3	73
PA5	GSSMHHHPLWPTPEPHTGSC	–	–	+	6.4	1	0	20
PA6	RGMFHSPGDVMETEPHVGSC	–	–	+	5.3	3	1	29
PA7	WIEVEKTMDSGSGPKGHGSC	–	–	+	5.5	3	2	34
PA8	MTGIWSAMPYHNIESHNGSC	–	–	+	5.9	1	0	44
PA9	SHGNNQSHPEAYPGPWTGSC	–	–	+	5.9	1	0	5

As shown in Figure 3.7, the O-antigens of EC_{O111} and EC_{O55} LPS are dominated by neutral galactose-like structures, such as colitose, galactose, and galactosamine. The aromatic amino acids, W, F, or Y, are known to interact with the nonpolar b-face of galactose to provide a common binding motif residue for most galactose-binding proteins.[40, 41] So, it is not unusual that the EC_{O111} binding peptides show a high incidence of aromatic amino acids, such as tryptophan, phenylalanine, and tyrosine.

These observations are further supported by two independent investigations. In one study, peptides were selected to bind components of the bacterial cell membrane devoid of polysaccharides. This selection led to peptides containing only cationic arginine and lysine, but no aromatic residues.[42] In a second study, peptides that bind LPS from *S. enterica* (LPSs from *E. coli* and *S. enterica* are closely related)[37] were identified by screening phage displayed peptide libraries against bead-immobilized LPS.[43] All of these peptides were found to be enriched in aromatic hydrophobic residues, such as tryptophan and phenylalanine, along with cationic residues. These peptides were capable of discriminating between various bacterial species, which strongly supports their ability to target the distinctly variable O-antigenic domains.

In contrast to the neutral EC_{O111} and EC_{O55} repeating units, the repeating unit of PA_{10} consists one third of negatively charged galacturonic acid (Figure 3.7), which can form strong hydrogen bonds with the aspartic and glutamic acids,[44] as well as with hydrophilic glycine, proline, serine, and threonine, which are prominently over-represented in the selected PA_{10}- specific peptides (Figure S4).

3.4.13 Electrostatic Contributions

To test the contribution of electrostatic interactions to LPS binding to microarray peptides, we measured the zeta potential (ζ-potential) of EC_{O111} and PA_{10} LPS. The zeta potential is the overall charge a particle acquires in a specific medium and is a measure of the potential at the slipping plane, which is the layer just past the bulk solution layer of ions surrounding the particle. Under conditions identical to those used in the microarray probing experiments, the EC_{O111} LPS had a charge of $\zeta = (-6.7 \pm 1.4)$ mV, while the PA_{10} LPS was also negative and of significantly greater magnitude at $\zeta = (-25.7 \pm 3.1)$ mV,

which is consistent with the presence of negatively charged galacturonic acid. Since the EC_{O111} LPS has only hydroxyls in the structure and thus lacks the ability to form strong hydrogen bonds in aqueous solutions,[45] its interactions are dominated by CH–π interactions[45] and by electrostatic attraction, which drive the selection towards hydrophobic aromatic and cationic amino acids. On the other hand, the galacturonic acid in the repeating unit of PA_{10} LPS has a strong propensity to form hydrogen bonds, which overpowers the electrostatic forces and drives the selection towards hydrogenbond-forming amino acids.

We conclude that specific interactions of peptides with LPS on microarrays are not driven by electrostatic forces alone, but involve far more specific molecular interactions, such as hydrogen bonds and hydrophobic forces. This makes the peptide microarray a suitable tool for studying carbohydrate interactions.

3.5 Conclusions

In summary, we have developed a LPS screening technology to quickly identify LPS binding peptides that are specific to the variable saccharidic branch of LPS. We demonstrated that such a platform, which consists of only 10 000 random 20-mer sequences, is capable of differentiating between Gram-negative bacterial strains based on differences in their LPS structures. We also demonstrated that the parallel analysis platform, inherent to the microarray format, allows rapid and, most important, direct identification of multiple LPS interactions at the same time. In contrast to screening of phage displayed or other solution-based combinatorial peptide libraries, microarray format allows systematic analysis and statistical deconvolution of postselection data. In

the future, as peptide microarray technology matures and the number of features increases, this platform could enable direct discovery of high-affinity and protease-resistant peptidomimetics since peptides can be readily synthesized with unnatural functionalities, for example, damino acids, cyclic structures, and unnatural side chains, to facilitate the transition of discovered leads into the clinic. Finally, this technology paves the way for systematic investigation of disease-associated changes in other poorly defined complex glycobiomolecules, such as mucins and glycosylaminoglycans that currently present insurmountable challenges to the available analytical methods.

3.6 Experimental Section

Materials and methods: Smooth-type LPS from *E. coli* serotype O111:B4 was obtained from Fluka (Cat# 62325), serotype O55:B5 was from Sigma (Cat# 62326); *P. aeruginosa* 10 was from Sigma (Cat# L8643). FITC-labeled LPS from *E. coli* O111:B4 was from Sigma (Cat# F3665). ***CAUTION!*** LPS molecules are highly pyrogenic and can cause severe fever in humans if inhaled, ingested, or absorbed through skin. Good laboratory practices should be employed. Wear a lab coat, gloves, safety glasses, and a respiratory mask while handling LPS.

Unless noted otherwise, all chemicals were purchased from Sigma– Aldrich, Inc. (Milwaukee, WI, USA) and used without further purification. Deionized water was obtained from a Millipore ultrapure water filtration unit. PEPscreen® peptides were synthesized by Sigma–Genosys, Inc., with 100% quality control and used as received for initial screens. Lead peptides were resynthesized inhouse by using Fmoc chemistry and purified to 95% by HPLC. Organic Qdots® were purchased from Invitrogen (Carlsbad,

CA, USA; Cat# Q21701MP). Sephacryl HiPrep 16/60 (S-200 HR) was from GE Healthcare. In-solution nanosizing and zeta potential was measured by using a Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK). Spectrophotometric measurements were carried out by using a NanoDrop® ND-1000 instrument.

Labeling LPS with Qdots: The supplied solution of organic Qdots (QDot 605 ITK™, Cat# Q21701MP, Invitrogen, Inc.) in decane (1 μM) was evaporated to dryness by using a SpeedVac® at room temperature and redissolved in equal amount of chloroform. An aliquot (100 μL) of the chloroform solution was diluted to 500 μL with chloroform and mixed with an aqueous solution of corresponding LPS (100 μL of $10 mgmL^{-1}$; *E. coli* O111:B4, *E. coli* O55:B5, and *P. aeruginosa* 10). Methanol was added dropwise and the sample was occasionally vortexed until both phases were completely mixed (about 400 μL of MeOH). The mixture was then evaporated to dryness by using a SpeedVac and the solid residue was suspended in ddH_2O (100 μL). A saturated solution of tetramethylammonium hydroxide pentahydrate ($Me_4NOHx5H_2O$) was added until the mixture was at pH 11–12 (about 25 μL). The latter basification step is critical as it allows the transfer of the Qdots into the aqueous phase; no transfer occurs in nonbasified solutions. The mixture was sonicated for 30 min, and the colored solution was then passed through two consecutive Zeba columns (2 mL; Pierce) to remove salts and excess free LPS. We further purified the LPScoated Qdots by size-exclusion chromatography using Sephacryl HiPrep 16/60 (S-200 HR) column (50x1 cm). The Qdot–LPS constructs eluted in a narrow color band and were stored in the dark at 4 ˚C. Under these conditions, the Qdot–LPS are stable for at least one month without any visible signs of deterioration. In a control experiment, the above procedure was repeated without LPS. No

solubilization of Qdots was observed without LPS as determined by measuring absorbance of Qdots in the supernatant.

Peptide microarray design and construction: The peptide microarray consisted of 10000, 20-residue peptides of random sequence, with a C-terminal linker of -Gly-Ser-Cys-COOH. All peptides were synthesized by Alta Biosciences Ltd. (Birmingham, UK) based on amino acid sequences provided by in-house custom software (Hunter, Preston, and Uemura, Yusuke, CIM, The Biodesign Institute). Nineteen amino acids (cysteine was excluded) were selected at random for each of the first seventeen positions with -GSC as the carboxy-terminal linker. The synthesis scale was 2–5 mg total at ≥ 70% purity and 2% of the peptides were tested at random by mass spectrometry as quality control. Dry peptides were dissolved in *N,N'*-dimethylformamide (100 %), then diluted 1:1 with purified water at pH 5.5 to a master concentration (2 $mgmL^{-1}$). The original 96-deep-well plates were robotically transferred to 384-well spotting plates, and the peptides were diluted to a final spotting concentration (1 $mgmL^{-1}$) in phosphate buffered saline at pH 7.2. High-quality precleaned Gold Seal glass microscope slides were obtained from Fisher (Fair Lawn, NJ, USA; Cat# 3010). Each slide was treated with amino-silane, activated with sulfo-SMCC (Pierce Biotechnology, Rockford, IL, USA; Cat# 22622) to create a maleimideactivated surface, and the quality was checked for coating efficiency. During spotting, we employed a Telechem Nanoprint 60 using 48 Telechem series SMP2 style titanium pins. Each pin spots approximately 500 pL of peptide (1 $mgmL^{-1}$) per spot—an estimate based on pin trajectory, surface dwell time, and the amount of liquid each pin holds. The spotting environment was at 25 ˚C and 55% humidity. The maleimide-activated surface reacts with the sulfhydryl group on the peptide's terminal

cysteine. Each peptide was spotted twice per array. The arrays were spotted in an orangecrate packing pattern to maximize spot density. Six fiducials were applied asymmetrically by using AlexaFluor647, -555, and -488 labeled peptides. The fiducials were used to align each subarray during image processing. The printed slides were stored under an argon atmosphere at 4˚C until used. Quality control included imaging the arrays by laser scanner (Perkin–Elmer ProScanArray HT, Perkin–Elmer, Wellesley, MA, USA) at 647 nm to image the spot morphology. If the batch passed this test, further testing of randomly selected slides with known proteins and antibodies was carried out for the quality control of precision spot intensity. Array batches that failed to meet an array-to-array variability of 30% CV (coefficient of variation) were discarded.

Microarray probing: Each microarray probing was performed in triplicate. The slides were placed in a humidified chamber and blocked for 1 h at room temperature with BSA (650 µL of 3% solution) and methoxytetraethyleneglycol thiol ($mPEG_4$-SH; 1 mM)[46] in 1xPBS with Tween-20 (TBS-T; 0.05 %). The slides were washed with 1xTBS-T (3x30 inversion in a Coupling jar) and ddH_2O (3x30 inversion in a Coupling jar). The slides were then dried by centrifugation at 1500 rpm for 3 min, with the barcode label at the bottom to avoid the spread of the label glue onto the slide surface. The slides were then scanned at the appropriate wavelength to note any peptide autofluorescence. An AbGene frame was then attached to the surface of each slide to confine the solution (260 µL) of labeled LPS in 1xPBS (0.154 $mgmL^{-1}$ for FITC–LPS or AF488–LPS and 0.630 $mgmL^{-1}$ for Qdot–LPS) that was added to the printed area. A plastic coverslip was used to spread the solution on the surface of the slide and seal the frame while avoiding bubbles. The slides were incubated for 1 h in the dark at room temperature in a

humidified chamber. The coverslips and AbGene frames were then removed, and the slides were washed by being dipped two times in ddH_2O, then incubated for 5 min in ddH_2O, and then dipped two more times in ddH_2O; the solution was changed each time. Finally the slides were dried by centrifugation at 1500 rpm for 3 min at room temperature and scanned.

Microarray scanning and image analysis: Microarrays were scanned by using a Perkin–Elmer ProScanArray HT Microarray Scanner with the 488 and 543 nm excitation lasers at 100% power and 70% photomultiplier tube gain. Detection was done at 605 nm for Qdot probes and at 543 nm for FITC probes. All scanned images were analyzed by using GenePix Pro 6.0 software (Axon Instruments, Union City, CA, USA). Upon careful visual inspection, bad spots were eliminated by flagging them "absent". Median spot intensities were used in further analyses. Statistical analysis comparison of microarray data was done with GeneSpring 7.2 (Agilent, Inc., Palo Alto, CA, USA) by importing image-processed data from GenePix Pro 6.0 (Molecular Devices, Inc.). Median signal intensities were used in the calculations. For statistical comparisons, each slide was normalized to 50^{th} percentile. Measurements of less than 0.01 were set to 0.01; per "gene" normalization were not included since it tends to overemphasize differences in peptide expression, even when the intensity value is almost negligible for all the experiments. However, the goal of this work is to differentiate peptides that show a distinct behavior towards different probes, not only by statistical means, but also by visual inspection of the slides. Autofluorescent peptides were identified by scanning the slides prior to binding with LPS, peptides which had fluorescent intensities comparable to the postbinding intensity were eliminated from the final selections. The results collected

for each experiment were represented by using scatter plots as previously reported by Reddy and Kodadek.[24] This representation helped us focus our attention only on peptides with the right expression profile, that is, either with high expression against one of the LPSs tested and low expression for the other LPS, or with high expression for both.

Antimicrobial assays: DH10B *E. coli* cells (MAX Efficiency® DH10BTM Competent Cells, Cat# 18297-010, Invitrogen Inc.) were grown, overnight, at 37˚C at 270 rpm rotation in LB medium with streptomycin (0.1%) to a cell density of 2000x106 $CFUmL^{-1}$. An aliquot (1 μL) of these cultured cells was then mixed with fresh medium (1 mL) containing an individual peptide at concentrations of 25, 50 and 100 μM, and allowed to grow, overnight. The McFarland turbidity scale for *E. coli*[47, 48] was used to quantify the overnight growth of the cells by comparing the optical density of the cells at 600 nm to the turbidity equivalent of $BaCl_2$ (1%)/H_2SO_4 (1%) in the microplate reader (Spectra MAX 190, Molecular Devices, Inc.). In control experiments, the above procedure was repeated with no peptide in the culture and with nonbinding peptide (Neg1, sequence EFSNPTAQVFPDFWMSDGSC) as a negative control.

Flow cytometry: Peptides were conjugated to biotin by incubation with heterobifunctional maleimide-PEO_2-biotin linker (Pierce Biotechnology, Inc.; Cat# 21901) in 1xPBS at pH 7.2, overnight, at room temperature. Excess biotin was removed by overnight dialysis by using 1 kDa cutoff membrane (Spectrum Laboratories, Inc.). *E. coli* DH10B (MAX Efficiency® DH10B™ Competent Cells, Cat# 18297-010, Invitrogen Inc.) were cultured under routine conditions, pelleted by centrifugation and washed (3x1xPBS) to remove traces of media. The harvested cells were resuspended in blocking buffer (1xPBS, 0.05% FBS). Experiments with LPS preincubation involved mixing

biotinylated peptide solution (400 μM; 10 μL) with LPS solution (200 μM; 20 μL) for 1 h at room temperature. Then, $10x10^6$ cells were mixed with biotinylated peptides (400 μM; 10 μL), biotinylated peptides preincubated with LPS, or biotinylated wheat germ agglutinin (WGA) lectin (EY Laboratories, Inc.; 10 μL of 1 $mgmL^{-1}$ solution) and incubated for 1 h on ice. The cells were then washed three times with blocking buffer (1 mL) to remove unbound peptides or lectins. For detection of bound peptides and lectins, streptavidin–AlexaFluor488 (4 μgmL^{-1}, 100 μL; Invitrogen, Inc., Cat# S-11223) was added to the cells and incubated for 1 h. Cells were washed three times in blocking buffer, resuspended in blocking buffer (300 μL) and analyzed for cell-surface staining by using the FACS Caliber machine (BD Biosciences, Inc.). Cells stained with streptavidin–AlexaFluor488 only, were used as controls.

Surface plasmon resonance: Bare gold SPR sensor chips (Biosensing Instruments, Tempe, AZ, USA) were functionalized by adding 8- amino-1-octanethiol (1 mM; Dojindo Molecular Technologies, Inc., Cat# A424) in ddH_2O to the ethanol prewashed gold surface and incubating for 2 h at room temperature in a humidified chamber. The surface was then washed with ddH_2O and dried by using ultrapure argon gas. A solution of sulfo-SMCC linker (1 mM; bio- WORLD, Dublin, OH, USA) in 1xPBS was added to the gold surface, incubated for 30 min at room temperature in a humidified chamber, then washed and dried as above. The SPR instrument sensitivity was calibrated by using the response from ethanol (1%) in water on a bare gold sensor chip as a standard. The AOT/sulfo- SMCC modified chip was mounted on the instrument, and the peptide was immobilized in the sample channel by injecting a solution of peptide (100 μM) in TBS-T. A solution of sodium dodecylsulfate (0.01 %;

SDS) in TBS-T was injected to dissociate any peptide aggregates. The SPR response after the SDS wash was used to calculate the immobilization density, where 1 RU=1 $pgmm^{-2}$. To abrogate possible unspecific interactions, a solution of $mPEG_4$-SH (1 mM)[46] was used to block unreacted maleimide groups on the sample channel and to act as a nonbinding control on the reference channel. A solution of LPS (1 $mgmL^{-1}$) from *E. coli* serotype O111:B4 was injected at 20 $\mu Lmin_{-1}$ flow rate in the TBS-T analyte solution. Regeneration was accomplished by using SDS (0.05%) followed by an injection of glycine (10 mM; pH 2.5).

LPS zeta potential (ζ-potential) and size measurements: The zeta potential of the LPS was determined by electrophoretic mobility by using a Zetasizer Nano-ZS (Malvern Instruments). Measurements were performed at 25˚C in clear disposable Zeta cells (Malvern Instruments). LPS concentrations were the same as the ones used in the microarray probing experiments. All measurements were done in triplicate.

ITC measurements:[34] LPS was dissolved in PBS, pH 7.4, to give a 75 μM solution (assumed M_W=10000 kDa) equilibrated by dialysis, overnight, and degassed under vacuum. The QF8 peptide was dissolved in the same buffer at 1 mM and degassed under vacuum. Isothermal calorimetric titrations were performed by using the Nano ITC (TA Instruments, New Castle, DE, USA) at 23.5 ˚C. Degassed LPS (0.075 mM) was loaded into the sample cell (volume 950 μL), the reference cell was filled with water, and the degassed QF8 peptide (1 mM) was loaded into the injection syringe. Aliquots of QF8 (20x5 μL) were titrated into the LPS in the reaction cells at an interval of 300 s while being stirred at 150 rpm. Raw data were corrected for the heat of dilution of QF8 into buffer and integrated by using NanoAnalyze 1.1.0 software. The independent binding

model allowed the determination of the binding stoichiometry (n), association constant (K_a), and enthalpy change (ΔH). The free energy (ΔG) and enthalpy (ΔS) changes were calculated through the fundamental equations of thermodynamics: $\Delta G = -RT \ln K_a$ and $\Delta S = (\Delta H - \Delta G)T^{-1}$, respectively. All titration curves were repeated at least three times.

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3.8 Keywords

bacterial lectins · microarrays · mimetics ·peptides · polysaccharides

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CHAPTER 4

A METHOD FOR UNOBTRUSIVE LABELING OF LIPOPOLYSACCHARIDES WITH QUANTUM DOTS

Carlos Morales-Betanzos, Maria Gonzalez-Moa and Sergei A. Svarovsky

4.1 Contributions

The following chapter is a book chapter by invitation originally published in Methods in Molecular Biology. The goal of the chapter was to describe in detail the synthesis of fluorescent conjugates of Lipopolysaccharides and Quantum Dots with emphasis on the experimental protocol. Carlos Morales Betanzos and Dr Maria Gonalez-Moa prepared the LPS-Qdot micelles. Dr. Gonzalez- Moa characterized them by DLS. Mr. Morales Betanzos conducted the experiments with monocytes and conducted the fluorescent imaging. Mr. Morales Betanzos, Dr. Sergei Savrosky and Dr. Maria Gonzalez-Moa prepared the manuscript.

4.2 Abstract

Bacterial endotoxins or lipopolysaccharides (LPS) are among the most potent activators of innate immune system, yet mechanisms of their action and, in particular, the role of the glycans remains elusive. Efficient noninvasive labeling strategies are necessary for studying interactions of LPS glycans with biological systems. Here, we describe a new method for labeling LPS and other lipoglycans with luminescent quantum dots (QDots). The labeling is achieved by the partitioning of hydrophobic quantum dots into the core of various LPS aggregates without disturbing the native LPS structure. The biofunctionality of the LPS-QDot conjugates is demonstrated by labeling of mouse monocytes. This simple method will find broad applicability in studies concerned with visualization of LPS biodistribution and identification of LPS-binding agents.

Key Words: Endotoxin, Lipopolysaccharide, Labeling, Monocytes, Quantum dots, Fluorescence imaging.

4.3 Introduction

Bacterial lipopolysaccharides (LPS), also known as endotoxins, are the major constituents of the outer surface of Gram-negative bacteria [**1**]. They occupy up to 90% of the bacterial cell surface and are responsible for septic shock, which kills nearly 200,000 critically ill patients in the USA alone [**2**]. Not surprisingly, there is a great deal of interest in understanding mechanisms of LPS action for the developing of antisepsis drugs. The development of such agents depends on the availability of efficient labeling strategies for LPS molecules [**3**]. Ideally, such labeling should not be disruptive to the LPS functionality.

LPS are complex, negatively charged lipoglycans composed of three distinct regions: (a) a fatty acid region called Lipid A; (b) a core region oligosaccharide composed of up to 15 monosaccharides; and (c) a highly variable O-antigenic polysaccharide responsible for much of the bacterial pathogenicity and immunospecificity. Most labeling strategies rely on chemical modification of LPS molecules with organic dyes and normally require complex manipulations and purification steps [**4, 5**] due to the aggregative tendencies of LPS molecules [**6**]. The chemical modification is not site-specific and depends on the availability of reactive groups that are not always accessible or available in LPS [**1**]. If such groups are not present, they are chemically introduced by oxidation of the O-antigenic glycans [**5, 7**]. By introducing additional moieties to the LPS molecule, these methods perturb its physical properties and biomolecular recognition events [**8**], making such probes unlikely candidates for elucidating the roles of glycan interactions.

Nanometer-sized crystals of semiconductors known as quantum dots (QDots) have recently emerged as useful luminescent labeling agents [**9**]. These nanoprobes have significant benefits over organic dyes including long-term photostability, high luminescent intensity, and multiple colors with single-wavelength excitation that opens up possibilities for multiplex detection. Coating of hydrophobic QDots with phospholipids [**10**] and synthetic amphiphilic polymers have been described [**11**]. Both methods rely on phase transfer of hydrophobic QDots from an organic solvent to an aqueous solution of amphiphilic molecules. Here, we describe an application of hydrophobic QDots to non-covalent labeling of LPS and its derivatives. We show that this method may be broadly applicable to other lipoglycans as well. This method takes advantage of the universal amphiphilic nature of lipoglycans and does not introduce any chemical modifications to the LPS structure, making it ideally suitable for studying glycan interactions.

4.4 Materials

4.4.1 Labeling of LPS with QDots

1. Organic Quantum Dots (QDot® 605 ITK™, Invitrogen, Inc) (*see* **Note 1**).
2. Smooth-type LPS from *Escherichia coli* serotypes O111:B4 and O55:B5 (Sigma Inc.) and *Pseudomonas Aeruginosa* (Sigma Inc.), as well as Lipid A (Avanti Polar Lipids, Inc.), Kdo_2-lipid A (Avanti Polar Lipids, Inc.), and lipoteichoic acid (LTA) from *Bacillus subtilis* (Sigma, Inc.) (*see* **Note 2**) (Fig. 1).
3. Chloroform and methanol.

4. A saturated solution of tetramethylammonium hydroxide pentahydrate ($Me_4NOH+5H_2O$)
5. Deionized water (18 mΩ) was obtained from a Millipore ultrapure water filtration unit.
6. Zeba desalting spin columns (Pierce Inc.).
7. Speed Vacuum concentrator (Thermo Scientific, Inc.).

4.4.2 Cell Culture

1. Mouse monocytes (ATCC RAW 264.7, American Type Culture Collection, Manassas, VA).
2. Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS).
3. Dulbecco's phosphate-buffered saline 1x (DPBS, GIBCO, Grand Island, NY).
4. Nonenzymatic cell dissociation agent (Cellstripper^{TM} Mediatech Inc.).
5. Hank's Balanced Salt Solution (HBSS, GIBCO, Grand Island, NY).
6. Fixing solution - paraformaldehyde 1% in PBS (Invitrogen Inc.).

4.4.3 Flow Cytometry

1. Flow cytometer (FACS Caliber, BD Biosciences Inc) (*see* **Note 3**) .
2. 5 mL polystyrene round-bottom tube 12 x 75 mm style.

4.4.4 Fluorescence Microscope

1. Fluorescence microscope (for example, BX51, Olympus America Inc) (*see* **Note 4**).
2. Poly(L-lysine)-coated glass slides (*see* **Note 5**).

4.4.5 Dynamic Light Scattering

1. Zetasizer NanoZS (Malvern Instruments).
2. 100 μL disposable plastic cuvettes (Malvern Instruments, model ZEN0117).

4.4.6 Transmission Electron Microscopy

1. Electron microscope (Philips CM12S) operated at an accelerating voltage of 80 kV.
2. Carbon-formvar mesh grids.
3. Digital camera (e.g., Gatan model 791).

4.5 Methods

The described method for LPS labeling is compatible with several types of LPS varying in the glycan component, as well as for other lipoglycans with a similar amphipatic behavior. The resulting bioluminescent probes in the form of micelles are better evaluated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Although the protocol as described was designed for complete LPS, we have also used it for labeling monophosphoryl Lipid A, which is the amphipatic component of LPS responsible for its toxicity, Kdo_2-lipid A and LTA. Although Lipid A does not contain the glycan elements present in complete LPSs, it also forms micelles in solution, which prove to be necessary for our labeling method.

4.5.1 Labeling of LPS in suspension

1. Evaporate the solution of the Organic QDots (Invitrogen, Inc) until dry using the Speed Vac at room temperature (23˚C). Resuspend the solid residue in equal amount

of chloroform. In our knowledge, the QDs suspended in chloroform are stable for several months without any visible sign of flocculation.

2. Take a 100 µL aliquot of the chloroform solution and dilute it to 500 µL with chloroform. Prepare 100 µL of a 10mg/mL aqueous solution of the corresponding lipoglycan (i.e. *E. coli* 0111:B4, *E. coli* 055:B5, *P. aeruginosa* LPS, or Lipid A).
3. Mix together the QDots in chloroform and the lipoglycan solution. At this point a very distinctive two-layer water-chloroform system will be formed. Add methanol dropwise with occasional vortexing until complete mixing of the two phases (ca. 400µL of methanol). The homogeneous mixture chloroform/methanol/water will have an approximate ratio of 5:4:1 (*see* **Note 6**).
4. Evaporate the homogeneous mixture until dry and resuspend the solid residue in 100 µL of ddH_2O. Add dropwise tetramethylammonium hydroxide pentahydrate ($Me_4NOH.5H_2O$) solution until pH 11 (ca. 25 µL). The latter basification step is critical as it allows transfer of the QDots into the aqueous phase. No transfer occurs when no lipoglycan is present in the mixture.
5. Then, sonicate the solution for 30 min. Pass the solution through two consecutive Zeba columns to remove salts and excess of free LPS (*see* **Note 7**).

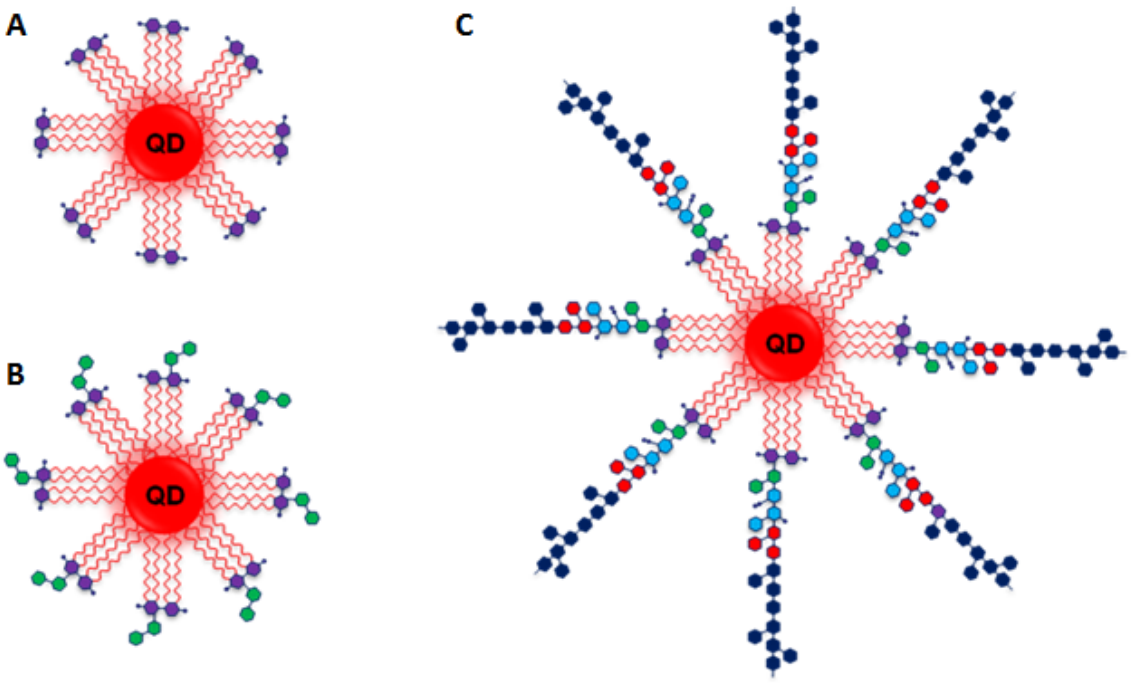

Fig. 1. Schematic of the lipoglycans-QDot micelles formed with (a) lipid A, (b) Kdo_2-lipid A, and (c) LPS.

4.5.2 DLS Analysis of Lipoglycan-QDot Micelles

1. Place 100μL of the labeled LPS solution in a plastic disposable cuvette.
2. Load the cuvette in the Zetasizer, close the lid, and run the size measurement of the lipoglycans-QDot micelles (see **Note 8**) (**Fig. 2**).

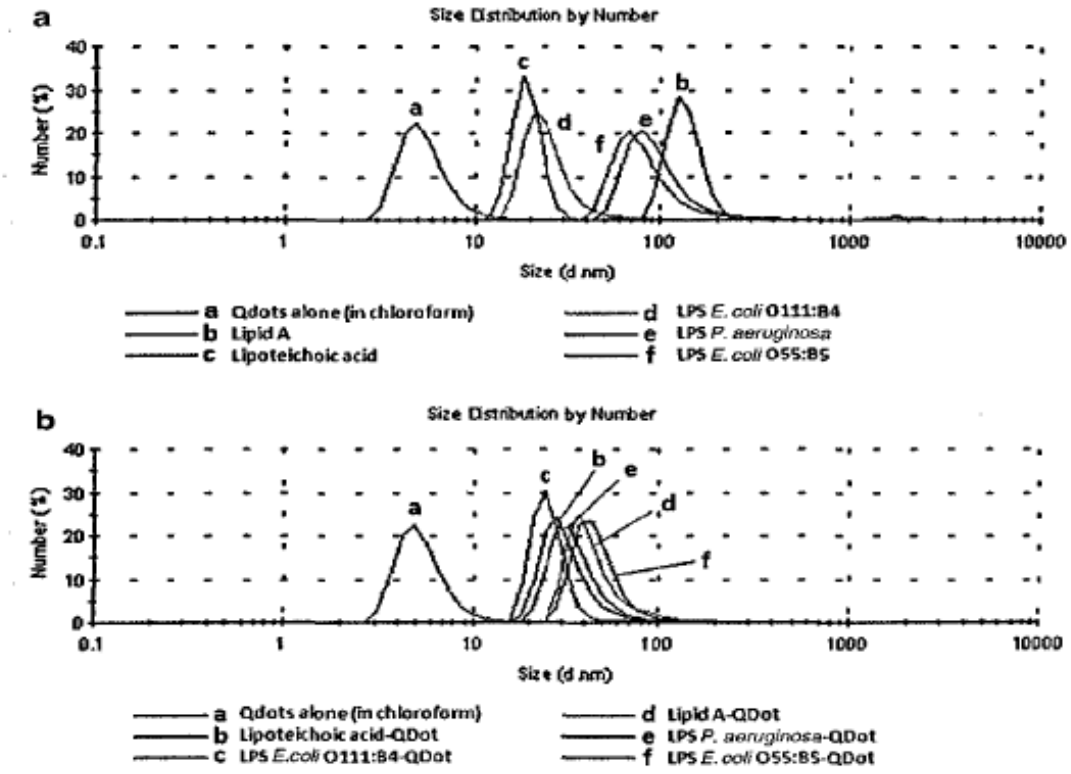

Fig. 2. Size distribution by number of particles obtained from the DLS analysis of the LPS-QDot nanoprobes. The x-axis shows the particles diameter in nm, and the y-axis shows the percentage of particles of each specific diameter. (a) Isolated Qdots and lipoglycans (without Qdots) showing various sizes due to unilamellar and multilamellar arrangements in aqueous solution. (b) Isolated QDots and QDots complexed with LPS from *P. aeruginosa* 10, *E. coli* O111:B4, *E. coli* O55:B5, and lipid A are included. After

mixing with QDots and adding base, the size of the newly formed QDots-LPS aggregates is around 50 nm. The basification process makes the LPS monomeric, favoring the access of the QDots to the lipid part of the LPS. Under UV light, the QDot particles are seen in the aqueous solution, which is only possible if the particles are taken up by the amphiphilic LPS. No luminescence is observed in solution prior to adding the base. The sonication of the mixture of QDots-LPS for 30 minutes makes the aggregate more compact as the diameter is reduced to 38 nm and the peak distribution becomes narrower, indicating increasing homogeneity of the QDot-LPS conjugates. The excess of non-solubilized QDots can be observed under UV light on the walls of the flask.

4.5.3 TEM Analysis of Lipoglycan-QDot Micelles

1. 2 µl of the labeled LPS solution was applied to a glow-discharged mesh copper grid coated with carbon-formvar.
2. The sample was allowed to adhere for 1 min and excess liquid was removed. Contrasting stain was not required due to the inherent electron density of the QDots (Fig. 3).
3. The sample was analyzed at an accelerating voltage of 80kV in the TEM, and images were recorded using magnification-calibrated digital acquisition.

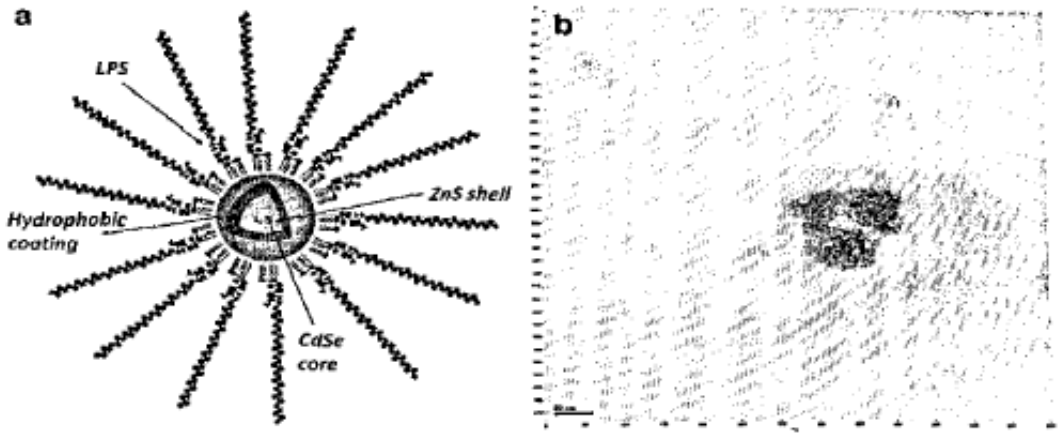

Fig. 3. (a) Schematic of PS-QDot miscelles and (b) TEM image of the LPS-QDot micelles synthesized using this protocol.

4.5.4 QD-LPS Labeling of Monocytes.

1. Culture mouse monocytes until confluence in DMEM supplemented with 10% FCS at 37°C in a 5% CO_2 atmosphere and 95% humidity.
2. Detach the monocytes from the flask using Cellstripper™ and wash twice with 1 x PBS. Resuspend the pellet in HBSS 1x. Count the monocytes in the solution and separate them in aliquots of 1 x 106 CFU in 5-mL polypropylene round-bottom tubes. Add LPS pre-labeled with QDots to a final concentration of 100 µg LPS/mL, for a final volume of 300 µL. Incubate for 30 min at 37°C.
3. After incubation, wash the cells twice with PBS and fix them in 1% paraformaldehyde solution; wash two more times with PBS to remove the excess of fixing solution (Fig. 4).

4.5.5 Flow Cytometry

1. Place an aliquot of 1 x 10^6 CFU pre-labeled and fixed in a 5-mL polypropylene round-bottom tube. Place the tube in the flow cytometer's sample injection port.
2. Set the appropriate beam laser for your sample (*see* **Note 4**). Set the appropriate filter for the emission of your QDots (*see* **Note 3**).
3. Adjust the FSC and SSC detector levels to position the population properly on the graph.
4. Adjust the threshold to eliminate low-level signals.
5. Use the gate tool to draw a region around the cell population of interest.
6. Adjust the fluorescence detector voltage for the channel (color) of interest.
7. Acquire data.

4.5.6 Fluorescence Microscopy

1. Place 60μL of the fixed cells solution on a poly-L-lysine-coated glass slide and set a cover slide on top. Allow the cells to attach for 30 minutes at room temperature (23˚C).
2. Use the fluorescence microscope to visualize the cells: Set the laser beam and filter in a short visible wavelength, such as blue or green, and view the cells using the corresponding filter for the emission of your QDots (*see* **Note 4**). Adjust the exposure time to maximize the signal above the background and acquire images.

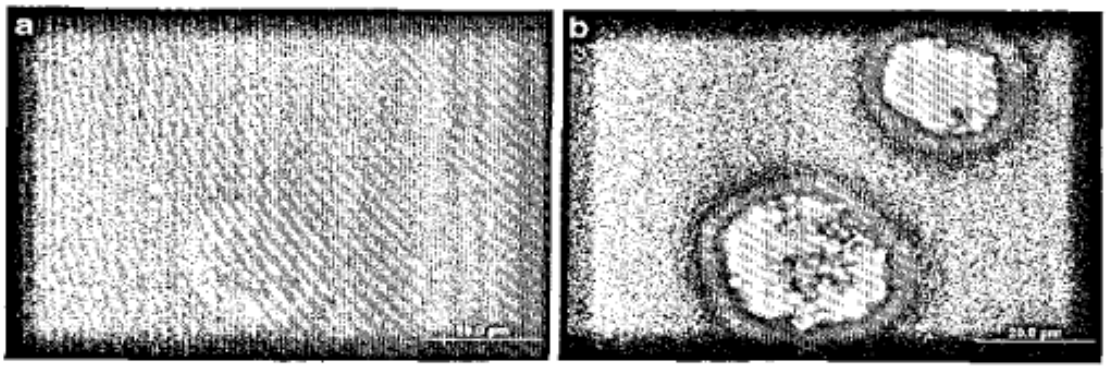

Fig. 4. Fluorescence microscope image of mouse monocytes labeled with QDot-LPS. (a) Image taken using a filter with range 480-600 nm. (b) Image taken using a filter with a wavelength range of 539-650. QDot 605 nm labeling is only observed in (b).

4.6 Notes

1. We used QDot® 605 ITK™ organic quantum dots from Invitrogen with a peak emission wavelength of 605 nm. These QDots have a lipophilic coating required for this protocol. Alternatively, other organic QDots can be used with a different emission wavelength (Invitrogen offers organic QDots with 7 different emission wavelengths: 545, 565, 585, 605, 655, 705, 800 nm)
2. Note that LPS, lipid A, Kdo_2-lipid A, and LTA are pyrogens that may cause fever. It may be harmful if inhaled, ingested, or absorbed through skin. Good laboratory practice should be employed. Wear a lab coat, gloves, safety glasses and a respirator mask. LPSs from numerous Gram-negative bacteria are available from a large number of vendors. In the method described here, LPS from three different bacteria was used. Additionally, other lipoglycans with a similar amphipatic behavior can be

used with our method. For example, monophosphoryl Lipid A (PHAD, Avanti Inc.) was used to synthesize labeled micelles with similar properties to the LPSs.

3. We used a Flow Cytometer (FACS Caliber, BD Biosciences Inc) equipped with a 488 nm Argon laser. The Instrument has three band-pass emission filters. FL1: 530 ± 30 nm (green), FL2: 585 ± 42 nm (yellow - orange), FL3> 650 nm (red). Since the emission of the QDots we used is 605nm, FL2 was used.
4. We used a Fluorescence microscope (BX51, Olympus America Inc) equipped with a 100-W mercury lamp, two separate filter wheels for fluorescence and excitation emission, and an Olympus DP70 12.5 megapixel cooled digital camera (Olympus America Inc). The samples labeled with QDot® 605 ITK™ were exited using a 488nm beam, and emission was registered through an orange filter. (QDots are characterized by a broad excitation band that precedes the emission peak and are better excited using UV light. However, for cell samples, it is recommended to use short visible wavelengths such as blue or green to avoid damaging the samples.)
5. Poly-L-lysine-coated glass slides were prepared as described elsewhere by incubating acid-washed slides in poly-L-lysine 0.1% solution for 30 min and drying them at 115°C for 30 min. Alternatively poly-L-lysine-coated slides and cover slides can be purchased from different vendors (Polysciences Inc, BD Biosciences).
6. Although the 5:4:1 ratio works well for most LPSs, Lipid A solution attained homogenization with less methanol. In general, the predominant factor to observe is the complete mixing of the phases independently of the ratio of solvents.
7. If necessary, further purification can be achieved through size exclusion chromatography. We used a Sephacryl HiPrep 16/60 (S-200 HR) column (50 x 1 cm)

(GE Healthcare) for this purpose. The LPS-QDots conjugate was eluted in a narrow color band and stored in the dark at 4 ºC. Under these conditions, the LPS-QDots are stable for at least one month without any visible signs of flocculation or deterioration of fluorescent intensity. If such flocculation occurs, the constructs can be reconstituted by sonication.

8. DLS measures the size of particles, emulsions and molecules in suspension, by means of the fluctuation of their Brownian motion when the particles or molecules are illuminated with a laser. The intensity of the scattered light fluctuates at a rate that is dependent upon the size of the particles. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence, the particle size using the Stokes-Einstein relationship.

Figure 2 shows the size distribution during the labeling process of smooth-type LPS from *E. coli* O55:B5, measured at 173° scattering and at room temperature (23ºC). The experiment was performed with the following settings: material profile "protein", refractive index = 1.450, absorption = 0.001; dispersant water: temperature = 25°C, viscosity = 0.8872 cP, and refractive index = 1.330. The Mark-Houwink parameters are as follows: A parameter = 0.428, K parameter (cm^2/s) = $7.65.10^{-5}$; equilibration time = 2 min, and cell: ZEN0117-disposable micro-cuvette (100µL).

4.7 Acknowledgements

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CHAPTER 5

IDENTIFICATION AND ANALYSIS OF NOVEL NMYC PHOSPHORYLATION SITES IN HIGH RISK NEUROBLASTOMA

5.1 Abstract

The transcription factor NMYC is a proto-oncogene associated with several types of cancer, of which the most notable are neuroblastomas. Amplification of NMYC is the most important feature of high risk neuroblastoma, the most aggressive form of this disease. Despite the central role of NMYC in neuroblastoma tumorigenicity, its biochemical regulation is still incomplete. To date, only 6 NMYC phosphorylation sites have been found, which contrasts dramatically with the 27 sites identified in c-MYC, another member of the MYC family and a close homologue that also regulates cancer. In this paper, we describe the purification of endogenous NMYC from five high risk neuroblastoma cell lines and the use of a 3-step LC-MS/MS methodology to identify 11 NMYC phosphorylations, including 8 novel sites. The new phosphorylations shared an almost identical sequence homology and relative position to other active sites within the MYC family responsible for regulating cell transformation, increasing protein stability and protein turn-over in cancer. We conducted a bioinformatics analysis using the amino acid sequence of the novel phosphorylation sites and identified Chk1, CK1 and CK2 as plausible upstream kinases of the novel phosphorylations. We then confirmed the capacity of the three kinases to phosphorylate NMYC using in vitro assays, which suggests that those kinases may be important in regulating NMYC in high risk neuroblastoma through the novel identified sites.

5.2 Introduction

Neuroblastoma accounts for 7.5% of all cancers in children younger than 15 years old and 15% of all pediatric oncology deaths.(1) It is the most common extra cranial solid tumor in childhood affecting 1 case per 7,000 live births in the USA.(2)

Neuroblastoma is very heterogeneous, with a wide spectrum of clinical presentations and outcomes depending on several factors including age at the time of diagnosis, extent and biology of the tumor. Using a combination of these factors, neuroblastoma is clinically classified into three distinctive risk levels: low, medium and high risk (LR-, MR- and HR-NB). Each level describes increasing cancer aggressiveness and is treated more intensively.(3) LR-NB has a very good prognosis with a 5-year survival rate of > 95%. MR-NB is treated with surgery and chemotherapy with generic cancer agents (carboplatin, cyclophosphamide, doxorubicin or etoposide) and has a 5-year survival rate of 90-95%. In striking contrast, HR-NB is treated in a very intense, 3-step treatment consisting of: (a) Induction, which aims to destroy as much of the tumor as possible using chemotherapy and surgery; (b) Consolidation with high doses of chemo- and radiotherapy, which is followed in many cases follow by stem cell transplantation to help patients recover; and (c) Maintenance, which focuses on decreasing the chances of recurrence. This is accomplished using retinoic acid and the only known target-specific agent for neuroblastoma, the antibody ch14.18 directed toward GD-2, a ganglioside highly expressed in neuroblastoma. Despite the very intensive treatment, the 5-year survival for HR-NB is only 40-50%.(4) New treatments for HR-NB that are more effective and less intense for the patients are urgently needed.

One attractive area to explore in the search for treatments for HR-NB is the identification and targeting of abnormal cell signals in the tumor. This approach, which has been successfully used to treat 80% of children with acute lymphoblastic leukemia(5, 6), aims to inhibit the key protein phosphorylations that modulate the abnormal cell signals in tumors. The target of such approaches should be proteins that are key for the tumorigenicity of the cancer and whose mechanism of action relies on the phosphorylation state of the protein.

The transcription factor NMYC is the most important neuroblastoma oncoprotein known to date. It was first linked to neuroblastoma by Kohl(7) and Schwab(8) in 1983 when multiple copies of the gene were identified in certain neuroblastoma cases. It was soon after this discovery that amplification of the gene was linked to the worse prognosis in 20% of patients (9, 10). Since then, NMYC has been extensively described in literature as a major component of the tumorigenic cell signaling of neuroblastoma(11, 12), medulloblastoma(13, 14), rhabdomyosarcoma(15), neuroendocrine prostate cancer(16) and human acute myeloid leukemias(17).

Despite its relevance in several neuronal tumors, NMYC-mediated cell signaling has not been described to nearly the same extent as its non-neuronal counterpart c-MYC, which is also a well-documented proto-oncogene that shares 47% sequence homology with NMYC. C-MYC is also involved in the tumorigenic signal of many types of cancer of non-neuronal origin (18-22). This disparity in understanding the cell signaling of the two oncogenes is evident when comparing the number of phosphorylation sites currently described in NMYC (6 sites) and those described in c-MYC (27 sites)(23).

Because several of the c-MYC phosphorylation sites have been directly linked to function in cancer and even selected as therapeutic targets(24), a similar scenario may hold for NMYC where many other phosphorylation sites may be present in the protein with potential function in the tumorigenicity of neuronal cancers.

In this paper, we describe the identification of eleven phosphorylation sites in endogenously expressed NMYC using five high risk neuroblastoma cell lines. This more than doubles the number of known phosphorylation sites for NMYC. All of the novel sites located next to MYC family functional domains, and some sites have a direct homolog in c-MYC with known oncogenic signaling involvement. The increase in phosphorylation detection was accomplished through the use of immunoaffinity purification (IP) followed by in-gel trypsin digestion (TD) and phosphopeptide enrichment (TiO_2) before LC-MS/MS. We further conducted the identification of a series of kinases with the capability of phosphorylating the novel NMYC sites using two computational models and then experimentally confirmed the phosphorylation events using *in vitro* kinase assays.

5.3 Materials and Methods

Cell culture and reagents - High risk neuroblastoma cell lines CHP134 and Kelly were purchased from Sigma Aldrich. SK-N-BE(2), IMR-32 and SK-N-DZ were purchased from ATCC. Cells were grown in either RPMI or DMEM (Life Technologies) supplemented with 10% Fetal Bovine Serum (Hyclone) according to cell repository instructions. All cells were maintained in a humidified atmosphere at 37°C in 5% CO_2.

The anti-NMYC rabbit antibody used for immunoprecipitation was purchased from Santa Cruz Biotechnology, Inc.

Immuno-affinity purification (IP) - for each experiment, high risk neuroblastoma cells were plated in eight 145 cm^2 plates, grown to 90% confluency and lysed using 2mL of cold RIPA buffer supplemented with EDTA-free HALT protease and phosphatase inhibitor (Thermo Scientific). The cell lysate was collected and centrifuged at 11,000 x g for 15 minutes at 4°C and the pellet discarded before the IP. For a single immunoprecipitation experiment, 30-50 mg of total protein were regularly utilized. This amount or protein was extracted routinely from 8 plates of cells. For the immunoprecipitation, 600ug of antibody was immobilized in 1 mL of protein-G dynabeads (Invitrogen) according to manufacturer instructions. Antibody conjugates beads were then added to the clarified lysates and incubated overnight at 4°C. Target proteins were eluted from the beads using 4x SDS and resolved via SDS-PAGE on 4-20% Tris/Glycine gels (Bio-rad) and stained with Commassie protein stain following manufacturer instruction (Invitrogen). NMYC bands were photographed and then cut inside of a laminar flow cabinet following standard practices for MS sample preparation.

MS sample processing - Target protein bands (which typically comprised two bands, one of slightly slower migration than the other around 50 KDa) were de-stained in 20% ethanol overnight, washed for 2 hours and diced into small 1 mm^3 gel fragments. In-gel protein digestion was performed according to the classic Shevchenko method modified by Mann et al(25). Briefly, samples were dehydrated in acetonitrile, incubated in 10mM dithiothreitol (Bio-Rad) in 100mM ammonium bicarbonate, acetylated with 55mM Iodoacetamide (Sigma Aldrich) and digested overnight in 13ng/ul trypsin in

10mM ammonium bicarbonate+10% acetonitrile. Digested peptides were extracted using a solution of formic acid 5% and acetonitrile in a 1:2 ratio. Peptide solution was evaporated to dryness in a vacuum concentrator (Eppendorf) and peptides either analyzed as is using LC-MS/MS or further processed with titanium oxide (TiO_2).

TiO_2 phosphopeptide enrichment - Titansphere centrifuge pipette tips (3mg, 200uL) were purchased from GL Sciences. Tips were conditioned according to manufacturer instructions using Buffer A (TFA 2% and acetonitrile 1:4 ratio) and Buffer B (Solution B + Buffer A 1:3 ratio). Dehydrated peptides from the previous step were re-suspended in Buffer B and re-circulated inside the tip three times using a benchtop centrifuge (Beckman Coulter) at slow speed (~1500 rpm) for 12 minutes. The tips were washed once in Buffer B and three times in Buffer A. Elution was performed in three steps using 1%NH_4OH in water, 1% NH_4OH in acetonitrile and 5% pyrrolidine in water. The purpose of each step was to elute hydrophilic, hydrophobic and poly phosphorylated peptides from the TiO_2 column. The three elutions were pooled together and neutralized with acetic acid 50% before evaporating to dryness in a vacuum concentrator for 4 hrs.

LC-MS/MS Analysis of phosphopeptides - MS analyses of phosphopeptides were performed by Michael Ford Ph.D. from MS Bioworks (Ann Arbor, Michigan). In brief, samples were solubilized in 70 uL of 0.01% trifluoroacetic acid (TFA) in water. Each gel digest was analyzed by nano LC-MS/MS in a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75µm analytical column at 350nL/min. Both columns were packed with Jupiter Proteo resin (Phenomenex), and the injection volume was 30µL. The mass spectrometer was operated in data dependent mode, with the Orbitrap operating at 60,000

FWHM and 17,500 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS.

MS Data Processing - Data were searched using a local copy of Mascot with the following parameters: Enzyme: Trypsin/P, Database: SwissProt Human, Fixed modification: Carbamidomethyl (C), Variable modifications: Oxidation (M), Acetyl (N-term), Deamidation (N,Q), Phospho (S,T,Y), Mass values: Monoisotopic, Peptide Mass Tolerance: 10 ppm, Fragment Mass Tolerance: 0.02 Da, Max Missed Cleavages: 3. Mascot DAT files were parsed into Scaffold software for validation, filtering and to create a non-redundant list per sample. For protein identification, the data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein. Ascore-derived PTM site localizations were calculated and the fragmentation spectra alignments were manually inspected using the Scaffold PTM software v 2.0 (Proteome Software).

Identification of kinase substrate motifs - A bioinformatics analysis was performed to correlate the sequence of the novel phosphorylation sites with known kinase substrate motifs using two independent algorithms (26, 27). A series of eleven kinases were identified using both methods. NMYC peptide sequence and kinase motifs are presented together in Table 5.1. The list of kinase candidates was further analyzed using an exhaustive literature search for the terms “kinase name” and “neuroblastoma” in PubMed and SciFinder (April 2014). Results were parsed looking for papers describing a correlation between the kinase protein expression levels or kinase inhibition and neuroblastoma invasion, proliferation or growth. From the original list of 11 kinases,

several kinases were identified as linked to cancer but only three were linked specifically to high risk neuroblastoma.

Table 5.1. Identification of kinase motifs correlating with the novel NMYC sites.

Kinase	aa site	NMYC peptide	kinase substrate motif
AMP-PK	s352	IKSEAsPRPL	[M/V/L/I/F][R/K/H]XXX[**pS/pT**]XXX[M/V/L/I/F]
CaMKII	S131,S315	LERAVsEK, RAQs	[M/V/L/I/F]X[R/K]XX[**pS/pT**]XX, RXXpS
CaMKIV	S131	LERAVs	[M/I/L/V/F/Y]XRXX[**pS/pT**],
CK1	T142, T149, S352, S371	tAGS, tAQs, SEAS, sPRNS	[**pS/pT**]XX[S/T], pSPXX[**pS*/pT***]
CK2	T142, T149, S315, S352, S375	tAGS, tAQS, sSE, sEAS, sDS	[pS/pT]XX[E/D/pS*/pY*], pSX[E/**pS*/pT***], pSXX[E/**pS*/pT***], pSX[E/**pS*/pT***]
Chk1	S131	LERAVs	[M/I/L/V]X[R/K]XX[**pS/pT**]
DNA-PK	T142	PtA	P[**pS/pT**]X
ERK1, ERK2,	T149, S355, S371	sP	**pS**P
GRK1 (RHOK)	S131, T142, S315, S352, S371, S375, S371	AVsE, PtAGST, QSsE, IKsE, LsPRNS, SDsE, sPR	XX[**pS/pT**]E, X[**pS/pT**]XXX[A/P/S/T], XX[**pS/pT**]E, XX[**pS/pT**]E, X[**pS/pT**]XXX[A/P/S/T], XX[**pS/pT**]E, [**pS/pT**]P[R/K]
GSK3	T149, S371	sTAQS, sPRNS	pSXXX**pS***
MAPKAPK2	T149,S371	sTAQs, sPRNs	pSXXX[**pS/pT**]
PKA	S131,S315,S352	RAVs, RAQs, KKIKs, sPR, RNs	RXX**pS**, KXXX[**pS/pT**], [**pS/pT**]X[R/K], RX**pS**
PKC	S131,S315,S352	RAVs, RAQs, KIKs, sPR, RNs	[R/K]XX[**pS/pT**], [**pS/pT**]X[R/K], [R/K]X[**pS/pT**]

***In-vitro* kinase assays -** NMYC protein expression plasmid was acquired from the DNASU plasmid repository(28) (http://www.dnasu.org). The NMYC gene was located inside the *pLP-DS3xFlag* vector for in-vitro transcription and translation (IVTT) with mammalian extract. The plasmid also contains a C-terminal 3xFlag tag for immunoprecipitation. IVTT reaction was performed using the 1-step coupled Human IVT Protein Expression Kit (Thermo Scientific) according to manufacturer instructions. Proper expression of the full-length protein was characterized by western blot with both a-Flag antibody (Sigma-Aldrich) and a-NMYC (cell signaling) antibodies (Figure 5.1).

NMYC-Flag protein was immunoprecipitated using a-Flag antibody (Sigma-Aldrich) immobilized in protein-G magnetic beads (Invitrogen). The Protein-antibody-bead complex was washed in PBTS 0.2% and incubated with a solution of Lambda Protein Phosphatase (New England Biolabs) for 1 hr at 30^0C to remove non-specific phosphorylations occurring during the IVTT reaction. De-phosphorylated NMYC was then incubated with a solution of Chk1, CK1 or CK2 active kinases (Millipore) in kinase buffer containing 200uM of ATP. The concentration of each kinase was adjusted according to the activity plot provided by the manufacturer. Mixtures were incubated for 1 hour at 30^0C and washed intensively with TBS buffer. Phosphorylated NMYC was eluted using 4x SDS sample solution, resolved using a SDS-PAGE gel and the NMYC band cut and processed for MS/MS using the same protocol previously described.

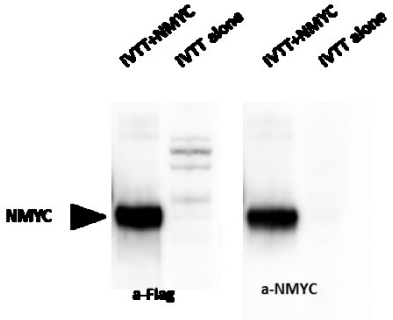

Figure 5.1. Characterization of correct NMYC-Flag expression using western blots.

5.4 Results

5.4.1 Isolation of Endogenous NMYC from High Risk Neuroblastoma Cell Lines

To analyze the phosphorylations of endogenously expressed NMYC in detail, we grew five different high-risk neuroblastoma cell lines (CHP134, Kelly, SK-N-BE(2) , IMR-32 and SK-N-DZ), all of which expressed high levels of NMYC (Figure 5.2). Eight 90% confluent plates of each cell line were required to generate enough material for the immunoprecipitation and subsequent phosphorylation analysis. IP was performed in 30-50mg of total protein (cell lysate), and NMYC was eluted from the beads and loaded into a SDS-PAGE gel. Figure 5.3 shows the purified NMYC protein stained with Coomassie dye. The SDS gel was run inside the electrophoretic chamber for a few minutes, which was just enough time to resolve most of the proteins while allowing the two bands corresponding to the phosphorylated NMYC to run together. NMYC protein bands were then processed as described in material and methods.

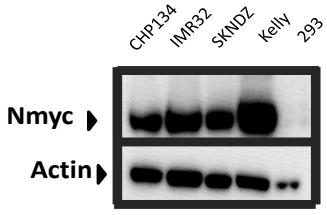

Figure 5.2. NMYC expression in 4 HR-NB cell lines and 1 negative control.

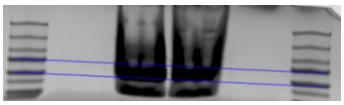

Figure 5.3. SDS gel stained with Coomassie dye showing the Immunoprecipitation of a large amount of NMYC protein.

5.4.2 Identification of Novel NMYC Phosphorylation Sites Using a 3-Step Approach

Liquid Mass Chromatography tandem mass spectrometry (LC-MS/MS) is the most common method used to identify novel post-translational modifications including phosphorylations. A well-known limitation of this technology is the difficulty to detect less abundant analytes in the presence of more abundant proteins that can mask the signals from the first, which can be compared to finding the mythical needle in the haystack where phosphopeptides are the needle and the non-phosphorylated peptides of both target and non-target proteins are the haystack. This problem has been addressed by separating complex mixtures of proteins (i.e., whole cell lysate) into fractions of lesser complexity. This is accomplished by isoelectric point separation, organelle specific isolation or other similar techniques. However, the problem still remains for the analysis of protein phosphorylation because most cell signaling proteins are not only low in abundance, but only a fraction of them are modified. This scarcity within a scarcity (a haystack inside of another haystack) makes the analysis of protein phosphorylation very challenging.

Other groups have previously approached this problem using different methods (29-34). Here, we used a 3-step approach to sequentially immunoprecipitate (IP) NMYC from cells, produce tryptica digests (TD), and finally, enrich for only the phosphorylated

peptides (Ser, Thr and Tyr) using titanium dioxide (TiO_2). Using a combination of these methods (**IP-TD-TiO_2**), we increased the number of total phosphopeptides identified in the target protein up to 4-fold. Figure 5.4a and Figure 5.4b show the difference between a standard LC-MS/MS run (A) and the IP-TD-TiO_2 approach (B). It is noticeable that although the protein coverage of sample A was large, only few phosphorylation sites were identified. In contrast, in sample B the protein coverage was much lower, but the number of phosphorylations identified was 4 times higher. This significant difference resulted from the addition of a phosphopeptide enrichment step in sample B which eliminated the non-phosphorylated peptides from the tryptic digest and allowed only the phosphorylated species to reach the MS detector. The phosphopeptide enrichment step resulted in a decrease of overall coverage (non-phosphorylated peptides), but also an increase of phosphopeptide concentration and a subsequent increase in phosphorylation site detection.

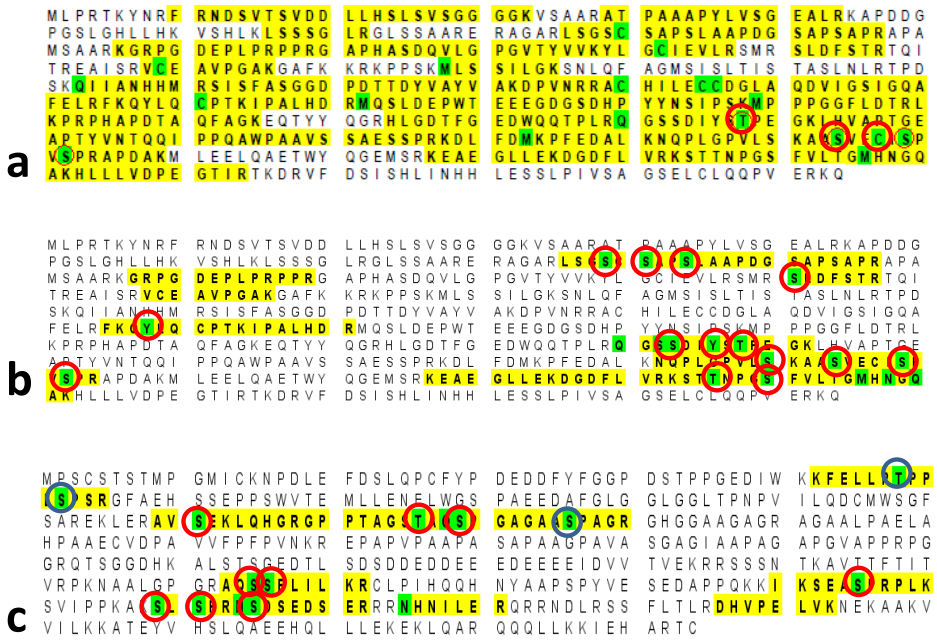

Figure 5.4. a) The application of a standard LC-MS/MS run resulted in 81% protein coverage, however only four phosphorylations were identified. b) A similar MS analysis conducted after Immunoprecipitation, in-gel trypsin digestion and TiO2 phospho enrichment (IP-TD-TiO) resulted in 21% protein coverage and fourteen S,T and Y phosphorylation sites were identified. c) The same process applied to NMYC extracted from 5 high risk neuroblastoma cells resulted in the identification of 11 phosphorylation sites including 8 novel sites. Amino acid protein sequences are represented with letters, MS identified peptides are highlighted in yellow, posttranslational modifications are labeled in green, phosphorylation sites are highlighted in red.

5.4.3 LC-MS/MS Identification and Assignment of Novel NMYC Phosphorylation Sites

Using the IP-TD-TiO_2 approach, we identified eight novel and three of the six reported phosphorylation sites in endogenous NMYC expressed in SK-N-BE high risk neuroblastoma cells (Figure 5.4c). The three most highly documented sites, T58, S62 and S156, were all observed with our approach; however, S64, S261 and S263, which have been only seen in few studies, were not observed. All identified sites were evaluated by Ascore and confirmed manually before confidently assigning the modification to the site. To eliminate potential phosphorylation artifacts because of the use of only one cell line, we confirmed the novel phosphorylations in five independently established high risk neuroblastoma cell lines: SK-N-BE(2), SK-N-DZ, IMR-32, Kelly and CHP-134 cells. Table 5.2 shows the sites that were identified in each cell line and confirms that most of the novel sites were present in multiple lines. A representative spectrum for each novel phosphorylation site is presented in Figure 5.5, and the best Ascore values for each peptide are presented in Table 5.3.

Table 5.2. NMYC phosphorylation sites identified in 5 high risk neuroblastoma cells using LC-MS/MS. The table shows the phosphorylations sites identified in each cell line in blue (known sites) and red (novel sites).

high risk NB cell line	Kelly	SK-N-DZ	IMR-32	CHP-134	SKNBE
T58	Y	Y	Y	Y	Y
S62	Y	Y	Y	Y	Y
S131	Y	Y	Y	Y	Y
T142	-	Y	-	-	-
S149	Y	Y	Y	Y	Y
S156	Y	Y	Y	Y	Y
S315	Y	Y	-	-	Y
S352	Y	-	-	-	-
S355	Y	-	Y	-	Y
S371	Y	Y	-	Y	Y
S375	Y	Y	-	Y	Y

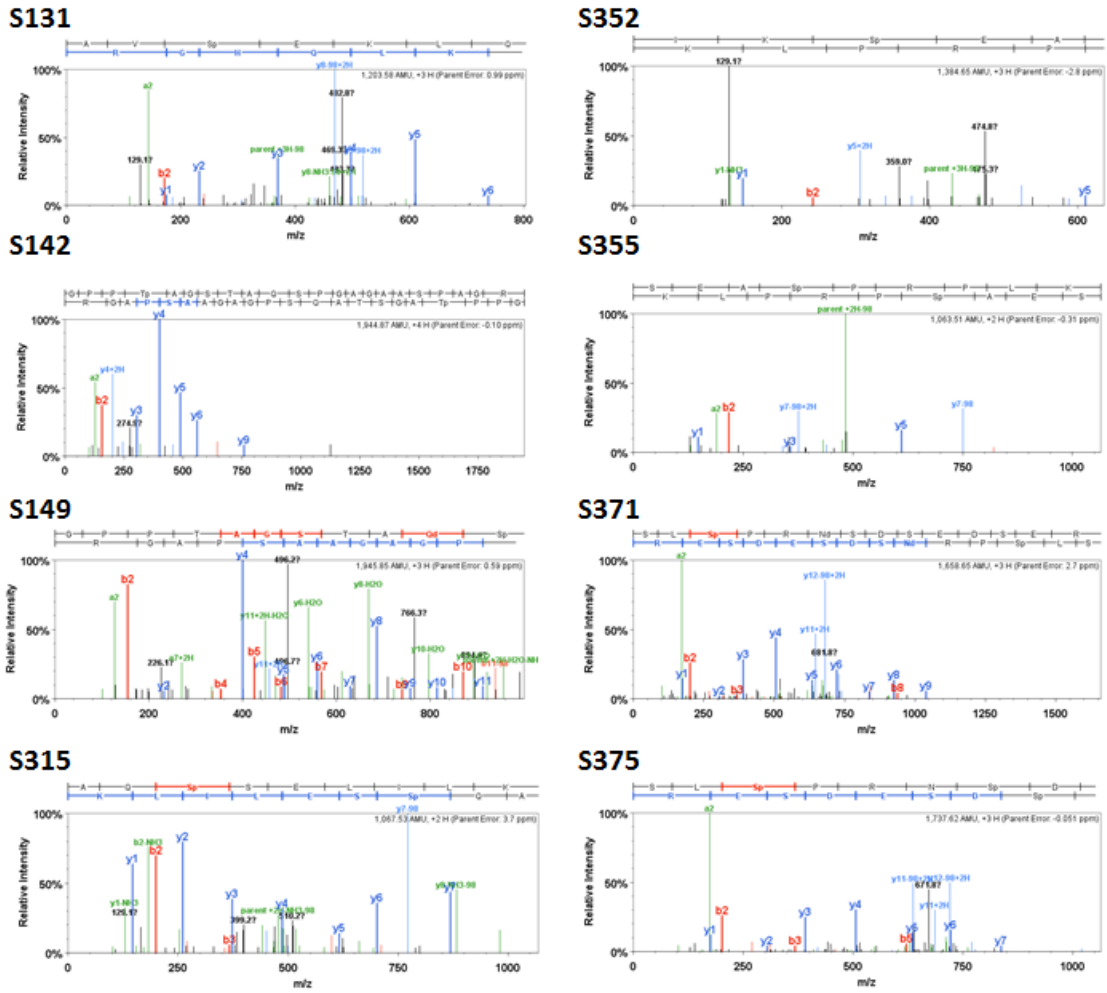

Figure 5.5. Representative MS/MS spectra of NMYC novel phosphorylation sites. Peptides prepared from IP-TD-TiO2 were subject to nano LC-MS/MS using a Waters NanoAcquity HPLC system interfaced to a Thermo Fisher Q Exactive as described in experimental procedures. Shown are representative examples of tandem mass spectra for each novel phosphorylation site displaying all PTM-bearing peptides fragment ion assignments.

Table 5.3. Novel phosphorylation sites identified in endogenous NMYC from high risk neuroblastoma cell lines. The table shows the phosphorylation site, best Ascores for each site, localization probability and peptide sequence.

Site	Modification	Best Ascore*	Localization Probability	Peptide Sequence	Start	Stop	Peptide Score	Charge	Actual Mass
T58	Phospho	102.07	100%	KFELLPtPPLsPSR	52	65	136.47	3	1740.825942
S62	Phospho	27.96	100%	KFELLPTPPLsPSR	52	65	178.52	2	1660.858008
S131	Phospho	1,000.00	100%	AVsEKLQHGR	129	138	141.74	2	1203.577248
T142	Phospho	21.44	98%	GPPtAGSTAQSPGAGAASPAGR	139	160	127.99	4	1944.869096
S149	Phospho	112.12	100%	GPPTAGSTAQsPGAGAASPAGR	139	160	395.49	2	1944.874128
S156	Phospho	238.84	100%	GPPTAGSTAQsPGAGAAsPAGR	139	160	261.45	3	2024.840112
S315	Phospho	53.98	100%	AQsSELILK	313	321	189.22	2	1067.530488
S352	Phospho	1,000.00	100%	IKsEAsPRPLK	350	360	58.51	3	1384.648002
S355	Phospho	1,000.00	100%	IKsEAsPRPLK	350	360	58.51	3	1384.648002
S371	Phospho	50.19	100%	SLsPRnSDSEDSER	369	382	213.97	3	1658.646582
S375	Phospho	70.16	100%	SLsPRNsDSEDSER	369	382	180.94	3	1737.621792
*Note that an Ascore of 1,000.00 means that there is no uncertainty in the position of the modification.									
This happens when there is only one amino acid where the modification could go									

5.4.4 Identification of Kinase Substrate Motifs Correlating with Novel NMYC Phosphorylation Sites

Using two independent literature-curated databases of kinase substrates, The Human Protein Reference Database- motif finder (26) and Phosida-motif finder (27), we searched for serine or threonine kinases with substrate motives correlating with the novel phosphorylation sites identified in NMYC. In both databases, we identified 11 kinases with motifs matching the sequence of the novel sites as depicted in Table 5.4. After an exhaustive literature search with this list of kinases, three of them stand out as significant: Chk1, Ck1 and CK2 have been linked directly to high risk neuroblastoma tumorigenicity (35, 36)_ENREF_27 (37, 38). Chk1 is particularly interesting because we identified its substrate motif (NMYC-Ser131) in all five neuroblastoma cell lines and because Cole K.

et al (35) found that Chk1 inhibition had the strongest inhibitory effect on high risk neuroblastoma cell survival from a panel of 529 kinases shRNAs.

Table 5.4. In-vitro phosphorylation of novel NMYC sites by CK1, Ck2 and Chk1.

	NMYC	+	+	+	+	+
	Pptase	-	+	+	+	+
	Kinase	-	-	CK1	CK2	Chk1
novel phosphorylation site	131	Y	-	Y	Y	Y
	142	Y	-	Y	Y	Y
	149	Y	-	Y	Y	Y
	315	Y	Y	Y	Y	Y
	352	-	-	Y	Y	Y
	355	Y	-	Y	-	-
	371	-	-	Y	Y	Y
	375	Y	-	-	Y	-

5.4.5 In-Vitro Kinase Assay

To further characterize the effect of ChK1, CK1 and CK2 in the phosphorylation of NMYC, we conducted a series of *in vitro* kinase assays. We cloned NMYC into an *in vitro* translation vector and expressed the Flag tagged NMYC using a human IVT protein expression system. We immunoprecipitated NMYC and removed the non-specific phosphorylations occurring during the IVTT reaction using a protein phosphatase. The non-phosphorylated protein was then incubated with active Chk1, Ck1 or Ck2 in presence of ATP for 1h at 30˚C. The phosphorylated NMYC was eluted and further analyze by LC-MS/MS as previously described. The NMYC phosphorylation identified at each step of the IVT process are reported in Table 5.4. It is noticeable that each kinase induced the phosphorylation of a slightly different subset of sites. According to the bioinformatics analysis we performed and previous publications, CK2 was the only

kinase capable of phosphorylating S375, and CK1 was the only kinase capable of phosphorylating S355 which was confirmed experimentally. However, the remaining 6 novel sites were phosphorylated in a similar manner by all three kinases.

5.5 Discussion

Despite being one of the most common solid tumors in children and accounting for ~1 of 7 of pediatric cancer deaths, neuroblastoma treatment still relies on cytotoxic agents. To date, the monoclonal antibody Ch14.18, which targets GD2, is the only neuroblastoma-specific agent used in clinics. New therapeutic agents that target high risk disease are especially needed because of the poor success even after intensive therapy. Targeting abnormal cell signaling in cancer has proven highly successful, especially targeting kinases; however, many of the neuroblastoma-specific phosphorylations are still unknown. In this paper, we utilized a combination of techniques to describe in detail the phosphorylations of the most important neuroblastoma onco-protein: the transcription factor NMYC. Our analysis identified eight novel phosphorylation sites next to key functional domains of the MYC family of proteins (MBII, MBIV, and BR). Furthermore, several of these NMYC sites are homologous to oncogenic regulatory phosphorylations in the c-MYC oncoprotein.

5.5.1 NMYC Novel Phosphorylation Sites Have Homologous Positions in c-MYC

Based on its selective expression, NMYC is considered the neuronal counterpart of its famous sibling, the proto-oncogene c-MYC. Both proteins belong to the same family of transcription factors, are 47% homologous to each other and share the same functional domains. However, they are expressed in different subsets of cells as NMYC

is predominantly present in neuronal cells (39) whereas c-MYC is expressed ubiquitously. The cell signaling pathways mediated by c-MYC have been studied extensively; to–date, 27 phosphorylation sites have been described in c-MYC. However, only 6 phosphorylation sites are currently known for NMYC. Using a phosphorylation-targeted approach, we increased the identification of NMYC phosphorylation sites to 14 sites. Most of the new identified sites have either a direct sequence or a relative position homologous in c-MYC. Figure 5.6 shows the homology comparison between the novel NMYC sites and the previously described sites in c-MYC. The figure also shows that some of the c-MYC homologous sites have been previously linked to an increment of c-MYC turn over (A), an increase in c-MYC stabilization (B) and an increase in cell transformation and tumorigenicity (B and C).

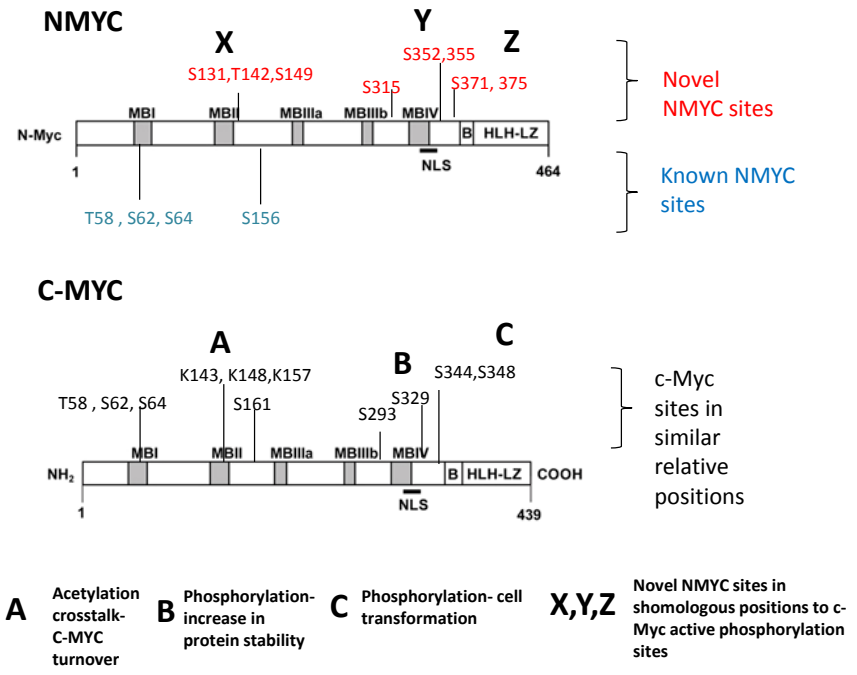

Figure 5.6. Homology comparison of novel (red) and known (blue) phosphorylations sites identified in NMYC and the protein family member c-NMYC. Novel sites were confirmed in five high risk neuroblastoma cell lines.

5.5.2 Novel NMYC Phosphorylation Sites Are Located Next to Functional MYC Domains

It is remarkable that the novel phosphorylation sites we identified in NMYC are situated next to well-described functional domains of the MYC family: The Myc Homology Box II and IV (MBII and MBIV) and the Basic Region (BR). In c-MYC, MBII is necessary for interaction with the transactivation/transformation associated protein (TRRAP) and other co-factors involved in cancer transformation. MBIV and BR are also key for transformation of primary and immortal cells, especially the latter through its interaction with c-MYC binding partner MAX(40). As shown in Figure 5.6

X,Y and Z, the novel NMYC sites S131 and T142 are located next to the MBII, S352, S355 are next to MBIV, and S371 and S375 are next to the BR domain. All these sites have homologous sites in similar positions in c-MYC as described in Figure 5.6 A, B and C. Some of those sites have even more remarkable similarities as described below.

5.5.3 Novel NMYC Sites Are Homologous to Known c-MYC Sites Regulating Cell Transformation

Using Clustal Omega(41), a multiple sequence alignment program for proteins, we aligned the amino acid sequences of NMYC and c-MYC. As reported, the homology between both proteins was 47%. Using the analysis software Jalview2(42), we visualized the alignment of both proteins side-by-side to compare the novel phosphorylation sites identified in NMYC against the analogous sites in c-MYC. The side-by-side comparison can be appreciated in the Figure 5.7. From this analysis, we observe that NMYC novel sites S371 and S375 have an almost identical sequence homology to c-MYC sites S344 and S348. These two c-MYC sites were recently identified by Wasylishen et al (24) as two regulators of cellular transformation in MCF10A cells and SH-EP cells. Their study utilized anchorage independent growth in combination with gene expression profiling to evaluate the role of several c-MYC phosphorylation site mutants. The investigation concluded that S344 and S348 together with other two sites modulate MCF10A transformation through transcriptional regulation of c-MYC as they increase the protein binding to selected promoters. Within that list, genes involved in non-coding RNA processing and metabolism were significantly enriched. Because of the similarity in both protein sequence and relative position to functional domains, it is conceivable that S371

and S375 in NMYC may also affect cellular transformation and anchorage independent growth – a hypothesis that should be evaluated by site directed mutagenesis studies.

Figure 5.7. Sequence homology comparison between c-MYC (top) and NMYC (bottom). Novel NMYC phosphorylation sites are highlighted in red, c-MYC analogous sites are highlighted in blue (phosphorylation) and green (acetylation).

5.5.4 Novel NMYC Sites Are Homologous to Known c-MYC Site Regulating Protein Stability

The novel NMYC sites S352 and S355 are also remarkably similar to Serine 329 in c-MYC. They share the same relative position next to the functional MBIV domain (Figure 5.6) and share analogous amino acid vicinity as depicted in Figure 5.7. Serine 329 in c-MYC has also been well characterized, and phosphorylation in this position has been linked to increased c-MYC stability in B-cell lymphoma. Zhang et al (43) previously reported that phosphorylation of S329 regulates c-MYC endogenous levels by stabilizing c-MYC in vivo. This group also showed that the mutant S329D, which mimics the phosphorylation state of S329, was much more stable than wild type c-MYC and produced a large number of colonies during anchorage independent growth assays. In contrast the c-MYC mutant S329A, which cannot be phosphorylated, was less stable when transfected into cells and produced fewer colonies than wild type c-MYC. The combination of these results point to the relevance of c-MYC phosphorylation at S329 to c-MYC stability and induced tumorigenicity. Because the novel NMYC phosphorylation sites S352 and S355 share a similar relative position and peptide sequence to S329 in c-MYC, both of these sites may play a role in stabilizing NMYC. It is not clear if there is an advantage to having two possible phosphorylation sites in the corresponding position where c-MYC has only one site. Could this imply a possible redundant mechanism related to stabilizing NMYC?

5.5.5 Novel NMYC Sites Surround Homologous c-MYC Stabilization Sequence Regulated By Acetylation

As depicted in the Figure 5.7, the novel NMYC phosphorylations at S131, T142 and S149 are located adjacent to the functional domain MBII. This area comprising 25 amino acids has a sequence homolog in c-MYC (KL-SEKL--YQA-RK) that is also flanked by 3 lysines (K143, K148 and K157). Martinez and colleagues reported that the acetyl transferases P300 and GCN5 regulate c-MYC abundance through acetylation of these lysines (44-46). Faiola et al described the mechanism of this system, which involves the acetylation of the three residues reducing c-MYC turnover through the proteasome, and they proposed that acetylation of the three sites may compete with c-MYC ubiquitination and ubiquitination-mediated proteosomal degradation. This interpretation is consistent with the identification of ubiquitinated K143, K148 and K157 on multiple proteome-wide MS analyses (47-49). In our study with NMYC, we identified that this regulatory sequence was flanked by three novel phosphorylations at S131, T142 and S149, all of them within a close range of the same segment. Phosphorylation has been also documented as a plausible competitor of ubiquitin by means of steric hindrance of the E3 ligase responsible of the ubiquitination(50-52). It is therefore plausible that these phosphorylations in NMYC may play a similar role to the prior mentioned acetylations in c-MYC, acting as a negative regulator of ubiquitination in NMYC and thus eluding NMYC proteosomal degradation, but the confirmation of this model would require further testing.

5.5.6 CK1, CK2 and ChK1 Phosphorylate Novel Sites During In Vitro Assays

Using two different literature curated databases of kinase motifs, we identified several kinases with the potential to phosphorylate the novel sites identified in NMYC. Using in vitro kinase assays we verified that these kinases can phosphorylate the novel sites in different combinations. It is noticeable that two of the novel sites identified in NMYC (S371 and S375) have direct contiguous homologous sites in c-MYC (S344 and S348). For c-Myc, it has been reported that only S348 is phosphorylated by CK2. Similarly, we found during our in vitro kinase assays with NMYC that only S375 but not S371 was phosphorylated by CK2(53). When using an *in silico* approach, we identified that S352 and S355 in NMYC are also plausible targets of CK2, and we conducted an *in vitro* kinase assay to show that was the case. Using the same method, we identified that the other novel phosphorylation sites we identified in NMYC were plausible targets of the Chk1 and CK1 kinases. The *in vitro* assays conducted with those kinases again showed the phosphorylation of several of the novel sites. This observation is interesting in light of the observation that both ChK1 and CK1 kinases have been directly linked to high risk neuroblastoma tumorigenicity in two separate studies where inhibition of each kinase caused a significant decrease in NMYC amplified neuroblastoma cell survival(35, 36).

Check point kinase 1 (ChK1) was recently identified as a novel therapeutic target in neuroblastoma. This protein, which is highly expressed in some neuroblastoma cells, was the top hit of a recent high through put RNAi kinome screening to identify therapeutic neuroblastoma targets(35). The study also highlighted that Chk1 expression was significantly higher in NMYC amplified cells and that the effect of Chk1 inhibition

directly correlates with the total expression level of NMYC. It is therefore possible that Chk1 and NMYC modulate each other through a direct mechanism. Because we observed the in vitro phosphorylation of NMYC by Chk1, it is plausible that this modulation may take place through a phosphorylation mediated process; however, the characterization of this mechanism and its relationship to neuroblastoma tumorigenicity is still to be tested.

Casein Kinase 1 (CK1) is also overexpressed in NMYC amplified neuroblastoma cells(54). It has been reported that the inhibition of CK1 either by siRNA or kinase inhibitors (IC261) prevents the growth of NMYC amplified xenografts and that a double KO of CK1 and NMYC is synthetically lethal for NMYC amplified cells (36). The precise relationship between CK1 and NMYC has not been yet characterized. In this paper, we identified NMYC as a substrate of CK1 *in vitro* and showed that several of the novel sites we identified in NMYC can be phosphorylated by CK1, which opens the possibility that this relationship may take place via phosphorylation.

5.6 Conclusions

The novel phosphorylation sites identified in NMYC are summarized in Figure 5.6. In this diagram the analogous c-MYC sites and their known biological activity is also shown. We conducted a homology comparison between the sequence of NMYC and c-MYC and concluded that the position and sequence of the novel NMYC phosphorylations closely resembles known c-MYC active sites involved in abnormal protein stability, increased protein turn over and regulation of tumorigenicity. Therefore, we speculate that the novel NMYC phosphorylations will play a similar role in NMYC mediated cancers like neuroblastoma, where NMYC is overexpressed and plays a critical

role in tumorigenicity. This proposed model, which is shown in Figure 5.6, is presented to create testable scenarios to evaluate each novel phosphorylation site through site directed mutagenesis or site-specific antibodies.

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CHAPTER 6

PHOSPHORYLATION TARGETED MS ANALYSIS OF NMYC AND SHCC, TWO KEY ONCOPROTEINS HIGHLY EXPRESSED IN HIGH RISK NEUROBLASTOMA

6.1 Abstract

Phosphorylation is one of the most important protein post-translational modifications involved in cell regulation. The analysis of phosphorylated proteins is fundamental to understand abnormal cell processes like cancer. However, because of the low abundance and low stoichiometry of phosphorylated proteins, their analysis is still challenging. This paper describes an alternative method to increase phosphorylation identification using LC-MS/MS using a combination of immunoaffinity, SDS purification, in-gel trypsin digestion and TiO2 phosphoenrichment. This sequential methodology decreased the complexity of biological mixtures at a protein level and at a peptide level and brought out phosphopeptide information not previously available. The process was successfully used to analyze two key neuroblastoma oncoproteins, the scaffold protein ShcC and the transcription factor NMYC. Using cells and human tissue samples, we applied our methodology to identify 5 and 8 novel phosphorylation sites in ShcC and NMYC respectively. Many of the novel sites were located in functional protein domains or were homologous to known active sites in other protein family members.

6.2 Introduction

Serine, Threonine and Tyrosine phosphorylations are one of the most important mechanisms that eukaryotic cells utilize to regulate cell signaling. In diseases characterized by abnormal cell signaling like cancer, the analysis of the phosphorylation events in key tumorigenic proteins is an important step in understanding the mechanism and dynamics of such proteins in the context of the disease. Presently, the amino acid position of phosphorylation events is identified mainly through the use of tandem mass

spectrometry (MS/MS). The execution of such analyses is, however, a challenging task limited by the low abundance and low stoichiometry of phosphoproteins as well as other sample-specific considerations.

The primary difficulty presented by phosphoproteins participating in cell signaling is the relatively low abundance in comparison with structural or house-keeping proteins within the cell. This unfavorable ratio, which resembles a needle (phosphoprotein) within a haystack (structural proteins), makes the discrimination of phosphoproteins signals very challenging even for the most powerful mass spectrometers. This is further complicated by the standard MS sample processing in which phosphoproteins are digested into peptides suitable for MS analysis using proteolytic enzymes. For a single phosphoprotein, this process renders a mixture of phosphorylated and non-phosphorylated peptide digests. Because the number of modified sites in most phosphoproteins is usually small, the ratio of phosphorylated to non-phosphorylated peptides is in most cases unfavorable for the MS detection of this modification.

Previously, techniques or combinations of techniques such as isoelectric point separation, immunoprecipitation or phopho-specific antibodies have been successfully used to identify phosphorylation sites in large sets of samples.(1-6) These methods, however, have not achieved in-depth phosphorylation site identification of individual proteins because of the difficulties to detect low abundant phosphopeptides in a large number of proteins simultaneously. In the present work, we report the application of immunoaffinity purification, SDS electrophoresis, in-gel digestion and titanium oxide (TiO2) phosphopeptide enrichment to sequentially protein purify, digest and enrich the phosphopeptides of two key neuroblastoma proteins, ShcC and NMYC. By using a

combination of four methodologies we were able to detect a significant increase in the identification of phosphorylation sites, which included several novel phosphorylation sites, in ShcC and NMYC. The methodology was tested in endogenous protein expressed in cells and in clinical human tissue samples.

Two neuroblastoma oncoproteins were selected for the application of this study because of their relevance to abnormal cell processes. (*Src homology* 2 domain containing) transforming protein 3 (ShcC) is a modulator protein that has been documented as an important regulator in glioblastoma(7), ependymomas(8) and neuroblastoma(9, 10). It has been specifically linked to the persistent undifferentiated state and tumorigenicity of high-risk neuroblastoma cells. (9, 11) v-myc myelocytomatosis viral related oncogene (NMYC) is a transcription factor described as the most important neuroblastoma oncoprotein(12-14). Its amplification and expression is the single most important marker of neuroblastoma aggressiveness(15-17), and it is clinically utilized to stratify patients for treatment(18, 19). NMYC-amplified patients exhibit the most aggressive type of tumors and present the lowest survival rates.

6.3 Experimental Section

Cell culture and reagents - Neuroblastoma cell lines SK-N-BE(2) were purchased from ATCC. Cells were grown in RPMI (Life Technologies) supplemented with 10% Fetal Bovine Serum (Hyclone). Cells were maintained in a humidified atmosphere at 37°C in 5% CO_2. The rabbit antibodies NMYC (sc-53993) ShcC (sc-28833) used for immunoprecipitation were purchased from Santa Cruz Biotechnology. Eight plates of neuroblastoma cells were plated in 145 cm^2 plates (5663-9160Q, USA

Scientific), grown to 90% confluence and lysed using cold RIPA buffer containing Tris-HCl 50 mM, NaCl 150 mM, Igepal CA 630 1% and 1/100 EDTA-free HALT protease and phosphatase inhibitor (Thermo Scientific). Cells were washed twice with 1x PBS and placed on an ice bath. 2 mL of RIPA buffer was added for 3 minutes before mechanically disrupting the cell layer using a plastic spatula. The cell lysate was collected and centrifuged at 11,000 xg for 15 minutes at 4°C, and the cell pellet was discarded.

Human frozen tissue samples collection - IRB approval from Arizona State University and the Phoenix Children's Hospital was obtained. Annual continuing reports were submitted and approved by both IRBs in a timely manner. Frozen tissue samples were obtained from the Hospital historical repository and transported frozen in large amounts of dry ice. The average tissue size was 230 mg. Patient information was blinded by a hospital representative and only unidentified sample information provided including: individual sample code, tissue anatomic source, patient gender, age at collection, collection year, storage temperature, tissue weight and tissue time lapse to processing. Samples were stored in liquid nitrogen vapor until lyses.

Human frozen tissue samples processing - Tissue samples were placed on a dry ice cooled metal surface and cut to small $1mm^2$ pieces with a cold blade. 1 mL of cold RIPA buffer supplemented with inhibitors was used for each 10mg of tissue. Lysis was conducted inside a 30 mL Dounce Tissue Homogenizer (DTH) equipped with a PTFE head (89026-388, VWR). Homogenization was conducted at 700 rpm in 10 strokes of 30 seconds each. Using these conditions, a yield of 70-150 ug of protein/mg of tissue was routinely obtained. After lysis, homogenates were centrifuged at 11,000 x g for 15

minutes at 4^0C. The resulting pellets were discarded and the supernatants retained and stored at -80^0C until used. Any subsequent sample preparation was performed at 4^0C.

Protein and phosphorylation stability - Protein integrity and the degree of phosphorylation was measured by western blots. Antibodies of species different to those used for immuno-affinity purification (IP) were purchased for NMYC (9405S, Cell signaling) and ShcC (610642, BD Biosciences) and used at 1:1000 dilution for all western blots.

Immuno-affinity purification (IP) -. For a single immunoprecipitation experiment, ~40 mg of total protein extracted from cells or tissue lysate were regularly utilized. This amount of protein was obtained routinely from 8 plates of cells or 230 mg of tissue. For the immunoprecipitation, 600 ug of antibody were incubated together with 1 mL of protein-G magnetic dynabeads (100-04D, Invitrogen) in 5 mL of PBST 0.02% and spun for 1 hr at room temperature. Antibody-bead conjugates were washed 3 times in PBST 0.02% and added to the clarified lysates for overnight incubation at 4°C. The next day, beads were separated from the lysate using a magnet and washed with RIPA buffer, PBST 0.02% and PBS. Immunoprecipitated proteins were eluted using 4x SDS sample buffer and resolved via SDS-PAGE on a Criterion TGX 4-20% Tris/Glycine gel (567-1093, Bio-rad). Proteins were stained using Commassie protein stain following manufacturer instruction (LC6065, Invitrogen). ShcC bands were photographed and then cut inside of a laminar flow cabinet following standard practices for MS sample preparation.

MS sample processing - Target protein bands were de-stained in 20% ethanol overnight, washed for 2 hours in water and diced into small 1 mm^3 gel fragments. In-gel

protein digestion was performed as previously described(20). Briefly, samples were dehydrated in acetonitrile for 10 minutes, incubated in 10 mM dithiothreitol (Bio-Rad) in 100 mM ammonium bicarbonate for 30 min at 56^0C. Samples were dehydrated again and then acetylated with 55 mM Iodoacetamide (Sigma Aldrich) for 20 min at RT and digested overnight in 13 ng/ul trypsin in 10 mM ammonium bicarbonate+10% acetonitrile. Digested peptides were extracted from the gel using a solution of formic acid 5% in acetonitrile in a 1:2 ratio. The peptide solution was evaporated in a vacuum concentrator (Eppendorf) until dry and peptides were analyzed either as is using LC-MS/MS (QExactive, Thermo-Fisher) or further processed with titanium oxide (TiO_2).

TiO_2 phosphopeptide enrichment - Titansphere centrifuge pipette tips (3mg, 200uL) were purchased from GL Sciences. Tips were primed using Buffer A (TFA 2% and acetonitrile 1:4 ratio) and Buffer B (manufacture's Solution B + Buffer A 1:3 ratio). Dehydrated peptides from the previous step were re-suspended in Buffer B, added to the tip and centrifuged at slow speed (~1500 rpm) for 12 minutes using a benchtop centrifuge (Beckman Coulter). To wash out the non-phosphorylated peptides, tips were washed once in Buffer B and three times in 50 uL Buffer A. Elution of phospho peptides was performed in three steps using 25 uL of 1%NH_4OH in water, 25 uL of 1% NH_4OH in acetonitrile and 50 uL of 5% pyrrolidine in water. The purpose of each step was to elute hydrophilic, hydrophobic and poly phosphorylated peptides from the TiO_2 column. The three elutions were pooled together and neutralized with acetic acid 50% before evaporating in a vacuum concentrator for 4 hrs until dry.

LC-MS/MS Analysis of phosphopeptides - MS analyses of phosphopeptides was performed by Michael Ford Ph.D. from MS Bioworks (Ann Arbor, Michigan). In brief,

samples were solubilized in 70 uL of 0.01% trifluoroacetic acid (TFA) in water. Each gel digest was analyzed by nano LC-MS/MS in a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75μm analytical column at 350nL/min. Both columns were packed with Jupiter Proteo resin (Phenomenex), and the injection volume was 30μL. The mass spectrometer was operated in data dependent mode, with the Orbitrap operating at 60,000 FWHM and 17,500 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS.

MS Data Processing - Data were searched using a local copy of Mascot with the following parameters: Enzyme: Trypsin/P, Database: SwissProt Human; Fixed modification: Carbamidomethyl (C), Variable modifications: Oxidation (M), Acetyl (N-term), Deamidation (N,Q), Phospho (S,T,Y); Mass values: Monoisotopic, Peptide Mass Tolerance: 10 ppm; Fragment Mass Tolerance: 0.02 Da; Max Missed Cleavages: 3. Mascot DAT files were parsed into Scaffold software for validation, filtering and to create a non-redundant list per sample. For protein identification, the data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein. Ascore-derived PTM site localizations were calculated and the fragmentation spectra alignments were manually inspected using the Scaffold PTM software v 2.0 (Proteome Software).

6.4 Results

6.4.1 Isolation of Target Proteins from High Risk Neuroblastoma Cells

We initially attempted to identify novel phosphorylation sites in two key neuroblastoma proteins using standard Liquid Chromatography tandem mass spectrometry (LC-MS/MS) directly on whole neuroblastoma cell lysate. The lysate was fractionated using Immobilized pH gradient strips. Then, individual fractions were digested and phosphopeptides were concentrated using TiO_2 columns. From the list of 578 proteins identified (data not shown), neither ShcC, NMYC, ALK, BCL-2 nor any other of the major neuroblastoma oncoproteins were present. This was surprising especially for proteins like NMYC, which are not only amplified but also overexpressed in high risk neuroblastoma cells. Instead, our list was mostly occupied by several families of structural and housekeeping proteins. In a second approach, both proteins were immunoprecipitated using protein specific antibodies before analyzing them by LC-MS/MS. Approximately 40 mg of high risk neuroblastoma cell lysate was utilized to immunoprecipitate either the ShcC or NMYC protein as described in experimental methods. The target proteins were then run in a SDS gel and stained with Coomassie dye for 1 hr. Using a dilution series of BSA protein standard along the gel, it was estimated that between 5-10 ug of protein was obtained from this amount of lysate (Figure 6.1).

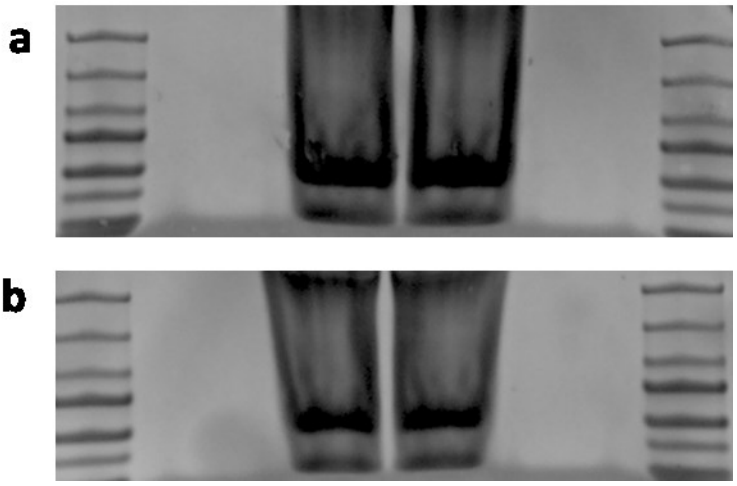

Figure 6.1. SDS-PAGE stained with Coomassie dye showing the immunoprecipitation of ShcC (a) and NMYC. The concentration of the precipitated fraction was calculated between 5-10 ug using an FBS standard.

6.4.2 Characterization of Phosphorylation Sites in Key Neuroblastoma Proteins

Using this methodology, 63 unique spectra from 38 exclusive ShcC peptides were identified. The peptides accounted for 71% coverage of the ShcC sequence with a False Discovery Rate of 1.0% (Figure 6.2a). The result, although encouraging, seemed incomplete as only 3 phosphopeptides containing 4 putative phosphorylation sites were identified (Figure 6.2b). This number was much lower than the number of phosphorylation sites previously identified in other members of the Shc family. For example, the analysis of ShcA (the prototypical protein of this family) identified 14 phosphorylation sites, eight of which has been found functional for protein stabilization(21), apoptosis induction(22-24), apoptosis inhibition(25, 26) and abnormal cell proliferation(27-29). This disparity between ShcA and ShcC phosphorylation

inspired us to look for technical improvements to further increase phosphorylation detection. The problem was addressed by the use of titanium oxide columns which, under acidic conditions, hold a positive charge that selectively binds to phosphorylated species. Therefore, our next approach was conducted using a combination of immunoprecipitation, SDS gel separation, in-gel digestion and TiO2. The method resulted in the overall reduction in protein coverage of ShcC to 24%; however, the total number of phosphorylation sites identified increased to 15 putative phosphorylations sites (Figure 6.2c).

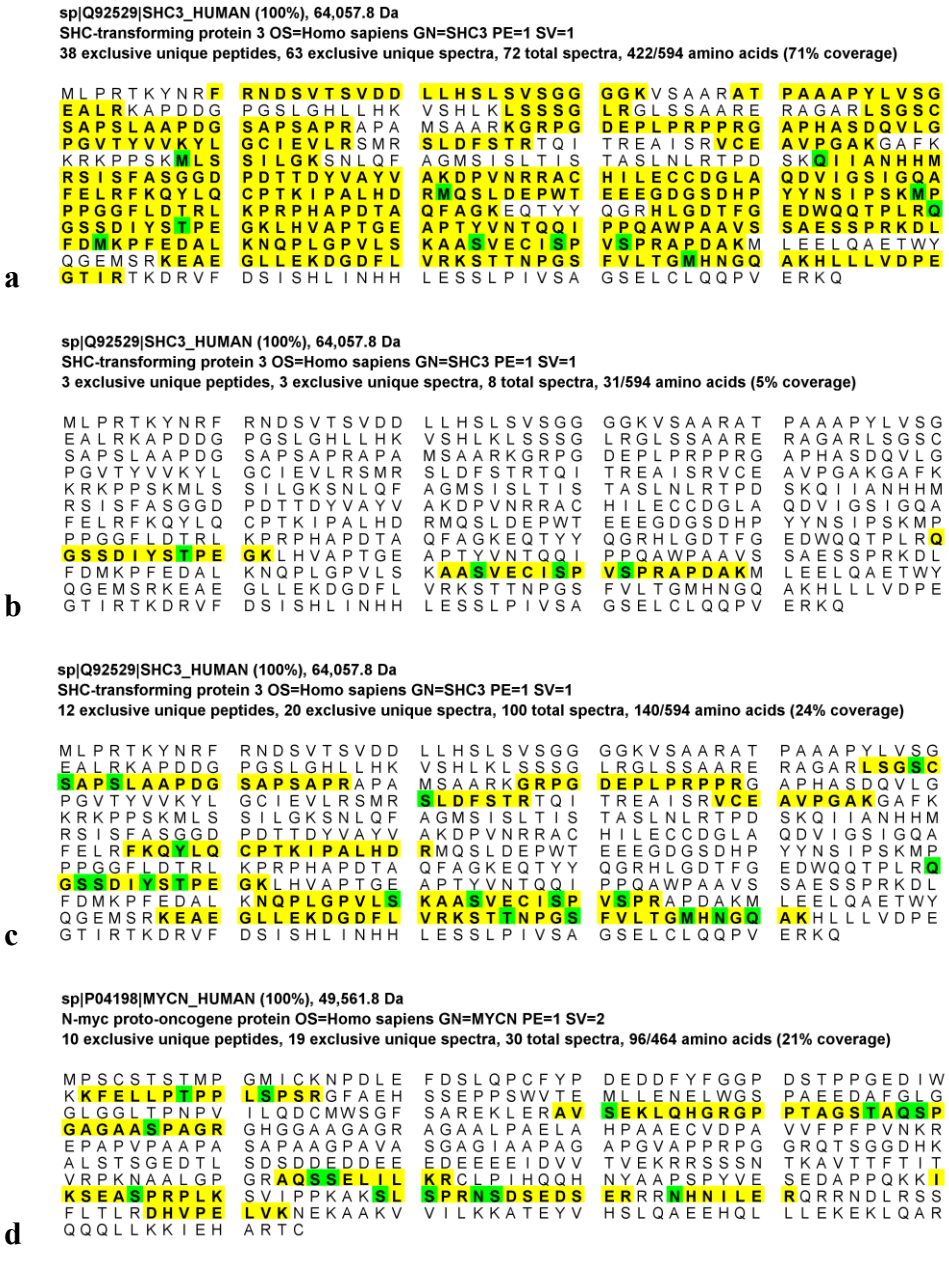

Figure 6.2. (a) Protein coverage of ShcC scaffold protein isolated from high risk neuroblastoma cells by immunoprecipitation and analyzed by MS/MS. 63 unique spectra belonging to 38 exclusive peptides were identified. From those, only 3 phosphopeptides were identified as presented in (b). (c) After using a combination of IP, SDS gel purification, in-gel digestion and titanium oxide, the ShcC protein coverage decreased to

24%; however, all 12 identified peptides were phosphorylated and included several putative phosphorylation sites. (d) Using the same methodology on endogenous NMYC isolated from high risk neuroblastoma cells, a total of 10 unique peptides were identified containing 12 putative phosphorylation sites.

To unambiguously assign the phosphorylation modification to specific sites, we relied on the Ascore algorithm developed by Beausoleil(30). This method measures the probability of correct phosphorylation site localization based on the presence and intensity of site-determining ions in MS/MS spectra. An Ascore value ≥19 was considered localized with near certainty of >99%. Peptides containing unambiguous phosphorylation assignment (i.e. only one site in the peptide can be phosphorylated) hold a score of 1,000. We calculated the Ascores for all the putative sites and filtered out the peptides with low localization probability. The validated phosphopeptides and their calculated Ascores are presented in

Table 6.1. A total of eight phosphorylation sites were assigned with confidence for endogenous ShcC in cells, including 3 phosphorylation sites that have never been previously reported.

Table 6.1. Summary of the Ascore validated ShcC phosphorylation sites identified in high risk neuroblastoma cells. The table indicates the position of the validated phosphorylation sites, the Ascore of the best peptide, the localization probability and the novelty of the identified site.

Protein Accession	Protein Name	Site	Modif	Best Ascore	Localization Probability	Novelty
sp|Q92529|SHC3_HUMAN	SHC-transforming protein 3 (ShcC) OS=Homo sapiens GN=SHC3 PE=1 SV=1	S171	Phospho	73.91	100%	novel
		S402	Phospho	32.22	100%	novel
		S470	Phospho	1,000.00	100%	novel
		S474	Phospho	1,000.00	100%	known
		S479	Phospho	1,000.00	100%	known
		S482	Phospho	1,000.00	100%	known
		Y308	Phospho	47.92	100%	known
		Y406	Phospho	27.45	100%	known

The same approach was utilized to precipitate and analyze the transcription factor NMYC, also from high risk neuroblastoma cells. For this protein, 11 Ascore-validated phosphorylations were identified including 8 novel sites (

Table 6.2). This number accounts for almost twice the number of sites that have been previously identified for NMYC.

Table 6.2. Summary of the Ascore validated NMYC phosphorylation sites identified in high risk neuroblastoma cells.

Protein Accession	Protein Name	Site	Modif	Best Ascore	Localization Probability	Novelty
sp|P04198| MYCN_ HUMAN	N-myc proto-oncogene protein (NMYC) OS=Homo sapiens GN=MYCN PE=1 SV=2	S62	Phospho	29.21	100%	known
		T58	Phospho	95.01	100%	known
		S156	Phospho	233.41	100%	known
		S355	Phospho	101.21	100%	novel
		S131	Phospho	1,000.00	100%	novel
		S315	Phospho	32.22	100%	novel
		S371	Phospho	43.67	100%	novel
		S375	Phospho	21.44	99%	novel
		S149	Phospho	69.09	100%	novel

6.4.3 Identification of ShcC Phosphorylation Sites in Human Brain Frozen Tissue Samples

Eight phosphorylation sites in endogenous expressed ShcC were identified in high risk neuroblastoma cells. Because phosphorylations are a reversible modification that can be modified by several biological or artificial factors including cell culture methods or specific media growth factor, we decided to verify the presence of the previously identified phosphorylation sites in human frozen tissue samples(31). The ShcC protein is reported to be expressed in only a small subset of tissues, from which brain tissue is the

one showing the most prominent expression. After obtaining all the required IRB permissions, our collaborators from Phoenix Children's Hospital provided us ten samples of brain human tissue from epilepsy biopsies to conduct our analysis in ShcC. We purified the protein from ~230 mg of tissue and conducted the analysis of ShcC as detailed in material and methods. The ten samples were analyzed using LC-MS/MS, and the phosphorylation sites were identified. Figure 6.3 shows the protein expression levels of ShcC and the overall tyrosine phosphorylation levels of all the samples provided. It was noticeable that sample one in the 2nd batch contained low detectable levels of tyrosine phosphorylation and ShcC protein. This sample had the longest tissue processing time (TPT>3 hours). TPT accounts for the time lapse that passed since the tissue was removed from the patient until it was stored in liquid nitrogen for long time storage. Because phosphorylations are labile modifications that can be removed by intrinsic tissue phosphatases that remain active while the sample is not frozen, it is likely that this sample lost many of its phosphorylations during the long tissue processing time. This loss was also reflected on the MS analysis, which shows less phosphopeptides in this sample (Figure 6.4, top right).

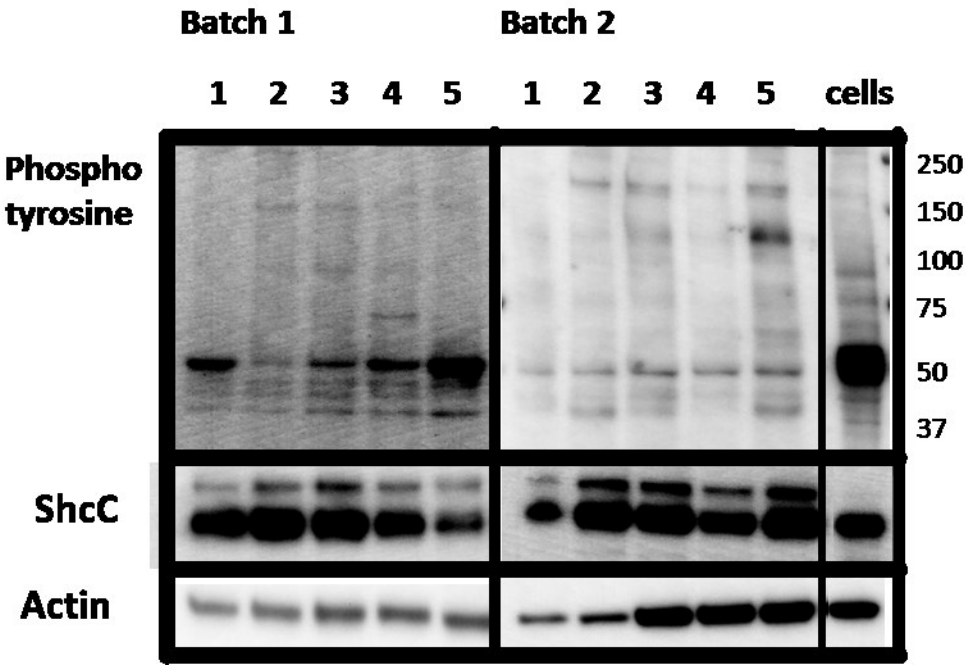

Figure 6.3. Western blots showing the protein levels of ShcC and overall phosphotyrosine phosphorylation in 10 human brain tissue samples and high risk neuroblastoma cells as controls. The samples were processed in two batches of five samples each.

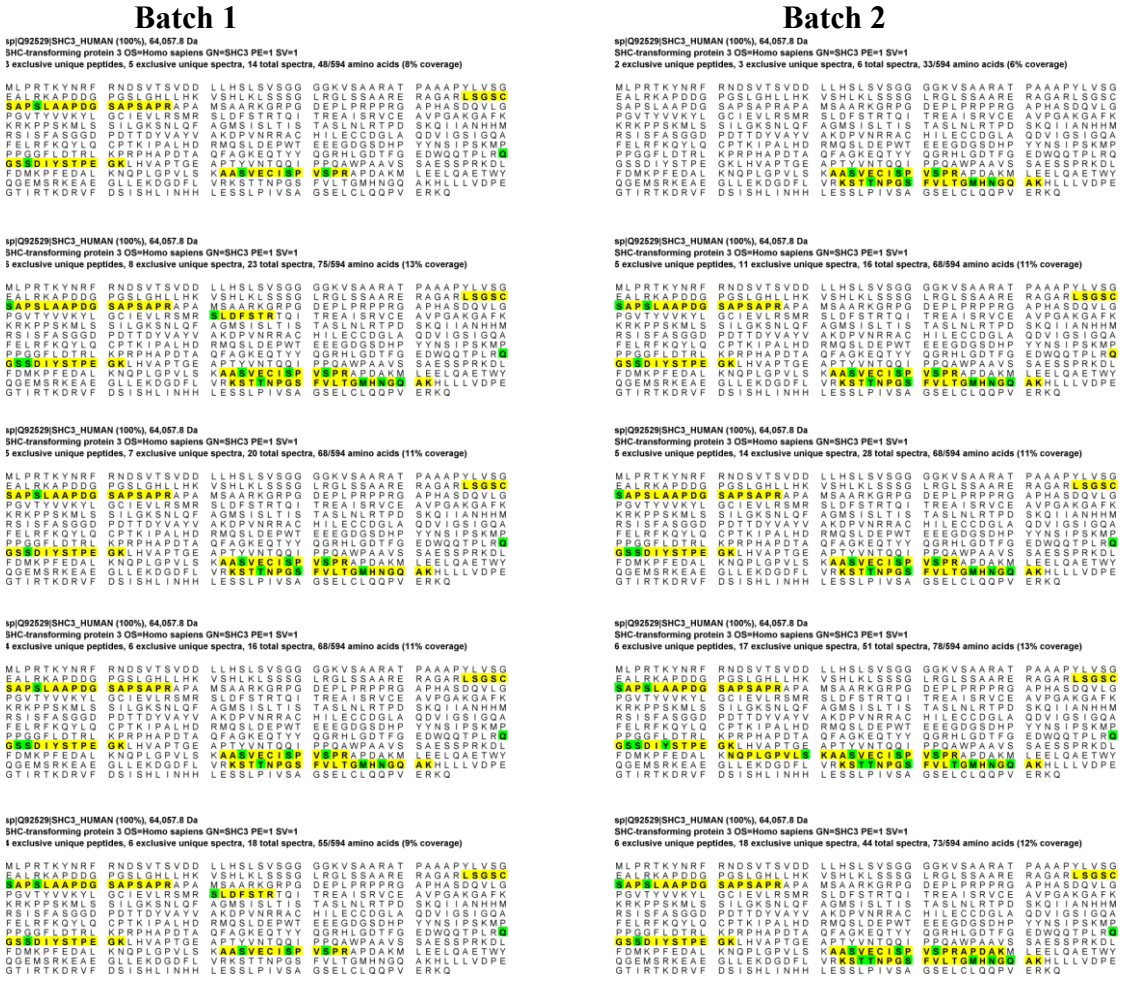

Figure 6.4. Sequence coverage and phosphorylation sites identified in ShcC protein extracted from 10 human brain tissue samples.

The protein coverage and the schemes of the entire putative phosphorylation sites for all the samples are presented in Figure 6.4. All the phosphorylation sites were then evaluated using Ascore and manual validation before assigning the phosphorylation to the site with certainty. Table 6.3shows the compilation of all the Ascore verified phosphorylation sites in human tissue samples. A total of nine phosphorylation sites were identified in ShcC in brain tissues, and from those Y406, S474, S479 and S482 have been

previously seen in MS shotgun analyses. S171, S402, S470, T526 and S530 have never been reported before. Two phosphorylations, T526 and S530, were present in the C-terminal region of ShcC, which is especially interesting because they were present only in the tissue samples but not in the cell lysates. This may indicate that those modifications may be triggered by biological conditions present in the tissue but absent in the cells, such as microenvironment elements or extracellular matrix interactions.

Table 6.3. Summary of the Ascore validated ShcC phosphorylation sites identified in human brain tissues. The table indicates the position of the validated phosphorylation sites, the Ascore of the best peptide, the localization probability and the novelty of the identified site.

Protein Accession	Protein Name	Site	Modif	Best Ascore	Localization Probability	Novelty
sp|Q92529| SHC3_ HUMAN	SHC-transforming protein 3 (ShcC) OS=Homo sapiens GN=SHC3 PE=1 SV=1	S171	Phospho	122.47	100%	novel
		S402	Phospho	32.22	100%	novel
		Y406	Phospho	29.21	100%	known
		S470	Phospho	1,000.00	100%	novel
		S474	Phospho	1,000.00	100%	known
		S479	Phospho	1,000.00	100%	known
		S482	Phospho	1,000.00	100%	known
		T526	Phospho	30.46	100%	novel
		S530	Phospho	21.44	100%	novel

6.4.4 Attempt to Identify Phosphorylations in High-Risk Neuroblastoma Human Frozen Tissue Samples

In an attempt to follow up the experiments conducted in brain tissue, we performed a series of experiments in a second set of tissues containing seven human frozen tissues of pathology confirmed high risk neuroblastoma tumors. These tissues hypothetically contain high levels of NMYC and ShcC proteins, or at least NMYC which, by definition, is over-expressed in high risk neuroblastoma. After conducting the proteins extraction using our optimized protocol for tissue samples we obtained a good protein yield ranging from 50 to 93 ug protein/mg of tissue; however, the western blot analysis revealed that neither proteins were present in detectable levels in any of the eight tissue samples (Figure 6.5). This observation was extremely disappointing because the lack of the endogenous target proteins did not allow us to complete our phosphorylation analysis in neuroblastoma tissue. Rather than being caused by a failure in our protocol, the lack of target proteins is most likely related to difficulties inherent to the samples analyzed, including high homogeneity of the tissue samples and long processing times. It is well known that neuroblastoma produces very heterogeneous type of tumors(32-36); therefore, it is possible that the analyzed samples contained a larger amount of stroma or other connecting tissue that decreased the net amount of tumor tissue in the samples. It was also noticeable that some of the tissue containing low levels of phosphorylations which may have been caused by sample temperature variations. This incidence, although not present in all of the samples, may have decreased the net phosphorylations in the affected tissues.

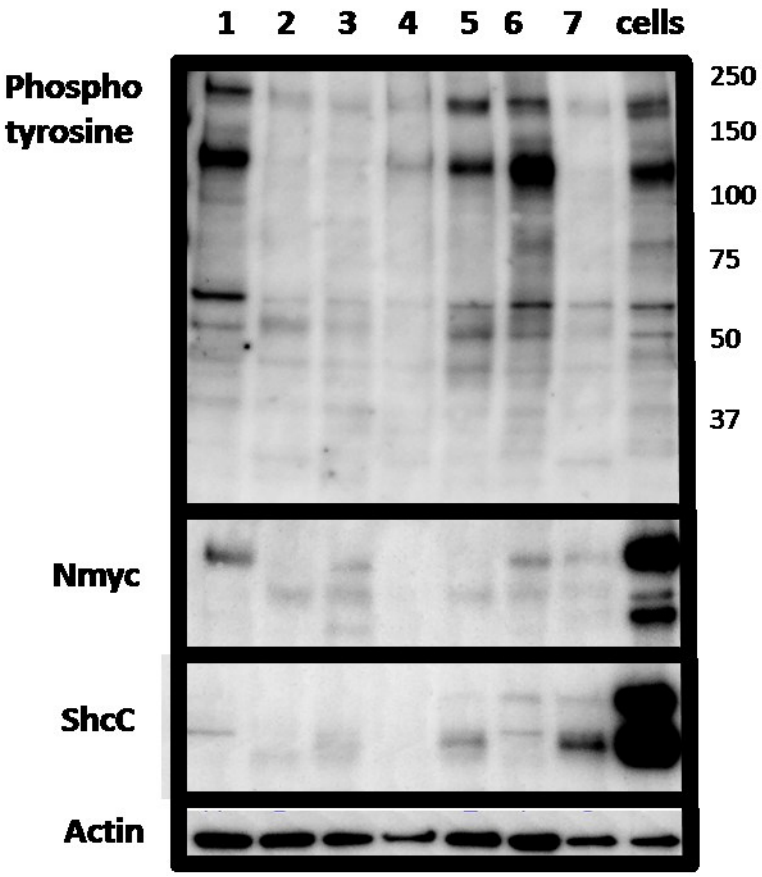

Figure 6.5. Western blots showing the protein levels of ShcC, NMYC and overall phosphotyrosine phosphorylations in 7 human high risk neuroblastoma tissues and high risk neuroblastoma cells as controls.

6.5 Discussion

6.5.1 Biological Role of the Novel ShcC and NMYC Phosphorylation Sites

The method described above was utilized to identify several novel phosphorylation is two key high risk neuroblastoma oncoproteins. Both targets belong to well defined protein families: ShcC is part of the 'SHC' family of scaffold proteins, composed of 4 members (ShcA, ShcB, ShcC and ShcD). NMYC is part of the "MYC"

family of transcription factors constituted by the 3 members c-MYC, NMYC and MYCL1. It is remarkable that for both proteins, ShcC and NMYC are the predominant member of their families being expressed in the central nervous system (CNS) while other members are expressed in other tissues. It is also noteworthy that the two proteins are homologues to well described oncoproteins also within their families. ShcC is 47% identical to ShcA (aka Shc), a well-defined protein involved in the cell signaling of breast(37, 38), prostate cancers(22, 28), B-cell lymphoma(39). NMYC also shares 47% exact homology to c-MYC (aka MYC), one of the first described proto-oncogenes that is a key participant in the cell signaling of breast, prostate, liver and other types of cancer (40-44). To hypothesize the role of the novel phosphorylation sites identified in ShcC and NMYC we used homology studies to compare the amino acid sequence of the novel sites with their well-defined homologues ShcA and c-MYC.

6.5.2 Key Functional Domains of Shc Family of Proteins

The Shc family is a group of adaptor proteins that mediate the transduction of cell signals by providing a scaffold to recruit multiple signaling components. ShcA and ShcC share a 47% exact sequence homology and contain the same functional domains; however, they are both expressed in a different subset of tissues. ShcA is found ubiquitously with the exception of the CNS where ShcC is primary found. ShcA has been identified as a central component in the transduction of several cell signals, from which RAS/MAPK, PI3K/ALK and RTKs are the most relevant. ShcA holds four protein domains which are shared with ShcC: an N-terminal Collagen rich domain (CH2), a Phospho Tyrosine Binding domain (PTB), another Collagen rich domain (CH1) and a Src Homology2 (SH2) domain. This unique structure (CH2-PTB-CH1-SH2) allows these

proteins to interact simultaneously with a large subset of proteins and act as a convergence point in several cell signals by facilitating the contact of other way-independent proteins.

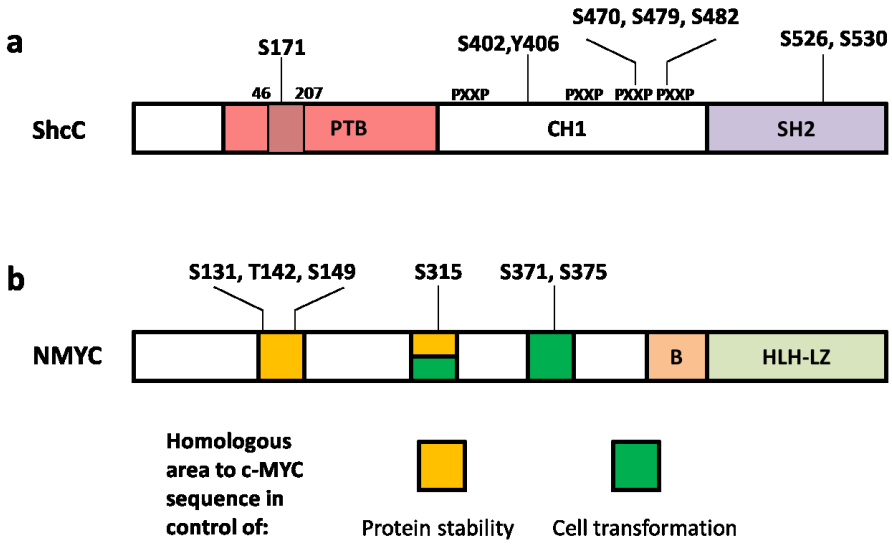

Figure 6.6. (a) Phosphorylation sites identified in ShcC neuroblastoma cells and brain tissue samples are localized in functional domains of the SHC family of proteins. S171 is located in the46-207 active pocked of PTB domain. S470, S479 and S482 are located inside two PXXP sequences responsible for SH3 binding partner recognition. S526 and S530 are located in the middle of SH2 domain responsible for binding to several Tyrosine Receptor Kinases. (b) Phosphorylation sites identified in NMYC neuroblastoma cells homologous to known active phosphorylation sites in the canonical c-MYC proto-oncogene. The sites regulate protein stabilization and/or cell transformation.

As shown in Figure 6.6a, the novel phosphorylation at Serine 171 is located inside the PTB domain, which is the most important domain in regulating its binding events.

This serine is also located in the pocket comprising the amino acids 46-207, which has been identified as critical for the regulation of Shc-PTB binding to partners(45). Serines 402, 470, 474, 479, 482 and Tyrosine 406 are located inside the CH1 domain. This area, rich in glycine and proline, moderates the Shc binding to multiple proteins through its interaction with their Src Homology 3 domain (SH3). The interaction of Shc-Ch1 and SH3 is in turn regulated by the Pro-X-X-Pro motifs that bind to the SH3 domains of multiple kinases such as Src, Fyn, Lyn and others(45). The phosphorylation sites S470, S474, S479, and S482 that we identified in neuroblastoma cells are located inside of two out of the four P-X-X-P domains of the CH1 domain of ShcC. Therefore, it is possible that those sites may regulate the interaction of ShcC with other kinases. Also within CH1 domain, tyrosine 406 was identified. This phosphorylation site is part of a canonical try-X-Asp-X binding pocket for the RAS/MAPK signal member Grb2. Finally, the two novel T526 and S530 phosphorylation sites were located inside the SH2 domain, a phosphotyrosine recognition domain that is known to interact with the receptor tyrosine kinases family (RTKs).

All the phosphorylation sites identified in ShcC using cells and tissues were located in functional domains of the Shc family, and some of them are surrounded by amino acids sequences linked to specific protein interactions. It is thus possible that the identified sites may also play a role in the regulation of ShcC-mediated cell signals.

6.5.3 Key Functional Phosphorylation Sites of c-MYC Are Homologous to Novel NMYC Phosphorylations

NMYC is the neuronal counterpart of c-MYC, one of the most well-known proto-oncogenes in the cancer field. Both proteins have 47% exact homology and perform

similar functions in distinctive tissue sub-types. C-MYC is ubiquitously expressed while NMYC is mainly expressed in the tissues of the CNS. C-MYC has been studied in great detail: to-date, 27 phosphorylation sites have been described in c-MYC. In striking contrast, only 6 sites for NMYC are currently known. Using our methodology, we increased the NMYC phosphorylation coverage by detecting 8 novel phosphorylation sites. Several of the novel identified sites share a close homology to previously active identified sites in c-MYC.

c-Myc Serines 344 and 348 have been reported as two sites that regulate the cell transformation of SH-EP and MCF10A cells in soft agar(46). As shown in Figure 6.6b, the two sites are homologous to the novel NMYC sites S371 and S375 we identified in NMYC. The c-MYC site S329 has been shown to increase c-MYC protein stability and through that an increase in the tumorigenicity of B-cell lymphoma cells.(39) This position is homologous to the NMYC sequence where the novel sites S352 and S355 were located. Lastly, the S131, T142 and S149 of NMYC share a close homology to a regulatory sequence in c-MYC that undergoes ubiquitination and as a result regulates proteosomal c-myc degradation and protein stability. This sequence was found acetylated in c-MYC mediated cancers at positions K143, K147 and K148 as a mechanism to avoid ubiquitination and resulting protein degradation(47-49). In our analysis, we found that NMYC was heavily phosphorylated in the same sequence, which may indicate an alternate mechanism to avoid ubiquitination via phosphorylation in neuroblastoma cells.

6.6 Conclusions

In this work a series of purification steps were utilized to increase the phosphorylation detection of two key neuroblastoma proteins. This protocol directly addressed the problem of an in-depth analysis of the phosphorylation sites of key proteins in complex biological mixtures. The method was proved to be efficient for analyzing target proteins in cell and human tissue lysates. Immunoprecipitation and SDS gel were first utilized to separate the target proteins from the more abundant components of the cell. Then, the proteins were in-gel digested with trypsin, and phosphorylated peptides were further concentrated using TiO2 beads. This methodology was tested in endogenous proteins extracted from cells and human tissue samples and showed good results when target proteins are expressed in samples. Results showed a considerable increase in phosphorylation site detection for both proteins. For NMYC, 11 sites were identified and unambiguously assigned. 8 of these sites have not been previously reported. For ShcC a total of 9 sites were identified, 4 of which were novel. All of the novel sites were present in either functional protein domains or known homologous active sequences in both proteins. The above system suggests that a combination of two protein purification methods followed by a specific phospho peptide enrichment step is an attractive way to identify novel phosphorylation sites in low abundance proteins concealed in complex mixtures of cells or tissue lysates.

6.7 References

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CHAPTER 7

CONCLUDING REMARKS

7.1 Abstract

The work presented in the preceding chapters describes the study of two post translational modifications. Chapters 2, 3 and 4 focus in general on the study of glycosylation and specifically on the development of a method to label bacterial glycans with fluorescent nano-particles. These chapters also address the application of the fluorescent probes to study the interaction of glycans and peptides in a microarray format.

Chapters 5 and 6 focus on the study of phosphorylation with a specific focus on the identification of novel phosphorylations sites in two key neuroblastoma proteins.

The conclusions for each investigation are summarized here.

7.2 Conclusions

The research described in Chapters 2 and 4 sought to create a novel fluorescent conjugate of bacterial lipopolysaccharide (LPS) and Quantum Dots (QDots). This construction was achieved by means of a ternary solvent system of Water – Chloroform – Methanol. The mixture of the three solvents allowed the hydrophobic QDots and the hydrophilic LPS to co-exist in a single phase. The novel conjugates readily dissolved in water, which allowed us to use them in physiological conditions. This research was the first to synthesize a QDot-LPS conjugate and to use them to label live cells. After publication, different groups utilized the method successfully. One interesting example was the use of QDot-LPS conjugates to follow the TLR2 response in live mice using biophotonic imaging.(1)

Chapter 3 described the use of the QDots-LPS conjugates to interrogate a 10,000 peptide microarray. The objective of the experiment was to explore the capabilities of the

array to bind a series of LPS of divergent glycan composition. Using this technique we identified a list of peptides that bound to LPS. The interaction between the peptides and the LPS was then validated using three orthogonal methods: SPR, ITC and flow cytometry. These analyses showed that the peptides bind to the LPS not only on the surface of the array but also in solution with a Kd of 1.75uM. A value comparable to natural occurring LPS binding peptides such as human lactoferrin (Kd=1.5uM) and polymyxin (Kd=1.1-5.8uM). Using flow cytometry, it was also noticed that the peptides had the capability to bind to the natural occurring LPS directly on the surface of bacteria. Something interesting and unexpected that it's worth nothing is that some of the selected LPS binding peptides presented antimicrobial activity. We conducted a series of growth inhibitory assays to show that 8 of the 16 LPS binding peptides inhibited bacterial growth. This effect was traced down to the amino acid composition of these peptides rich in amino acids of positive change that resembled the sequence of some natural occurring antimicrobial peptides. These observations were noted in the publication resulted of this work. (2)

The research in Chapter 6 sought to create a MS methodology to identify the phosphorylation sites of two selected neuroblastoma oncoproteins. In a first approach, we digested the entire proteome of a selected neuroblastoma cell lines into peptides. Then, we repeated the approach using titanium dioxide enriched for the phosphorylated species. From the 578 phospho-proteins identified, none of the key neuroblastoma proteins was found. This result supported the notion of utilizing a target-specific approach to achieve our goal. Using immune affinity purification before the MS, we were able to identify our target proteins; however, the phosphorylation identification was still poor. The problem

was approached by combining two protein purification steps, followed by titanium dioxide enrichment. This methodology resulted in a massive increase in phosphopeptide detection. A total of 9 and 11 phosphorylation sites were identified in both proteins from which 5 and 8 were novel sites.

Chapter 5 expands respectively on this results for one of the proteins, the transcription factor NMYC. Previously only 6 phosphorylation sites were known in NMYC. With our methodology this number increased to 14. Remarkably, several of the novel sites were homologous to active phosphorylation sites in c-MYC, a member of the same family of proteins that is also amplified in cancer cells. Using homology studies, we showed that the phosphorylation sites of both proteins correlate in position and in amino acid sequence. This supports the possibility that the novel NMYC sites may be also active in cancer.

Additionally, using computational studies we predicted a series of kinases that potentially phosphorylate the novel NMYC sites. Using in vitro kinase assays we showed that Chk1, Ck1 and Ck2 had the capability of phosphorylate NMYC in vitro.

7.3 Future Directions

The results in Chapter 3 showed the capability of the 10,000 peptide microarray to discriminate glycan differences in selected lipopolysaccharides. It was also shown that the identified peptides not only bound to LPS but presented antimicrobial activity. These two observations open a large number of possibilities to explore. The glycan recognition capabilities of the approach can be further used to explore glycan diversity. For example, other glycan conjugates not only from bacteria but from other organism can be tested in

an attempt to identify peptides binding profiles of glycans. These profiles can potentially be utilized in a similar way to the current lectin microarrays technologies but without the protein instability limitations that characterize them. The capability of this method to identify potential antimicrobial peptides is also a very interesting area of exploration. Glycans from other bacterial species can be potentially utilized in a similar fashion to the method described here. Teichoic acid from Gram negative bacteria or trehalose conjugates from mycobacteria are other potential targets for this approach.

The results in Chapter 5 and 6 showed the identification of several novel phosphorylation sites in NMYC and ShcC. It was also shown that those sites are homologous to active phosphorylation sites in proteins of the same family or were locates in the midst of active domains. Further characterization of those sites is required to define their role in protein function and neuroblastoma tumorigenicity. We already initiate this process by utilizing site directed mutagenesis to mutate each of the phosphorylation sites to alanine or glutamic acid. These two amino acids resemble the negative and positive phosphorylation site of the mutated residue. The next step would be to test these mutants in the context of a molecular or cellular model that allow us to quantify the effect of each site in the protein activity. This can be accomplished for examples, using a cellular knock in model, were each mutant is transfected into a mammal cells and phenotypical changes are measured. The results of these experiments would provide detailed information of the mechanism of NMYC and ShcC and have the potential to uncover the underlying mechanism regulating the role of these two molecules in the tumorigenicity of high risk neuroblastoma.

7.4 References

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ASU Knowledge Enterprise Development

Office of Research Integrity and Assurance

To:	Joshua Labaer Center for
From:	Carol Johnston, Chair Biosci IRB
Date:	05/19/2011
Committee Action:	**Expedited Approval**
Approval Date:	05/19/2011
Review Type:	Expedited F5
IRB Protocol #:	1105006461
Study Title:	Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity
Expiration Date:	05/18/2012

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ASU Knowledge Enterprise Development

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To:	Joshua Labaer Center for
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Date:	04/26/2012
Committee Action:	**Renewal**
Renewal Date:	04/26/2012
Review Type:	Expedited F5
IRB Protocol #:	1105006461
Study Title:	Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity
Expiration Date:	05/17/2013

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Date:	05/22/2013
Committee Action:	**Renewal**
Renewal Date:	05/22/2013
Review Type:	Expedited F5
IRB Protocol #:	1105006461
Study Title:	Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity
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It is the Principal Investigator's responsibility to obtain review and continued approval of ongoing research before the expiration noted above. Please allow sufficient time for reapproval. Research activity of any sort may not continue beyond the expiration date without committee approval. Failure to receive approval for continuation before the expiration date will result in the automatic suspension of the approval of this protocol on the expiration date. Information collected following suspension is unapproved research and cannot be reported or published as research data. If you do not wish continued approval, please notify the Committee of the study termination.

This approval by the Biosci IRB does not replace or supersede any departmental or oversight committee review that may be required by institutional policy.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

Please retain a copy of this letter with your approved protocol.

Phoenix Children's Hospital IRB Approval Letter 2011 – 2012

PHOENIX CHILDREN'S Hospital

April 7, 2011

RECEIVED
08 APR 2011 AF

Francis Eshun, MD
Phoenix Children's Hospital
Hematology/Oncology
1919 E. Thomas Road
Phoenix, AZ 85016

RE: PCH IRB #: 10-082: The study title: Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity.

Dear Dr. Eshun:

I have reviewed your request for expedited approval of the new study listed above. Your study is eligible for expedited review under FDA and DHHS (OHRP) regulations category 5, materials collected for non research purposes.

This approval includes:
- IRB Application
- Protocol
- Waiver of or Alteration of Informed Consent/Assent
- Waiver of HIPAA Authorization

You are granted permission to conduct your study as described in your application effective immediately.

Approval Period - April 7, 2011 - April 6, 2012

An expedited review process is used for research activities that present no more than minimal risk to human subjects, and are listed in one or more of the categories authorized by 45 CFR 46.110 and 21 CFR 56.110.

Any changes to the study must be promptly reported and approved. Some changes may be approved by expedited review; others require full board review. No changes may be implemented prior to IRB approval. If any adverse events occur, please report them at once to the IRB.

Please note the IRB number assigned to your study and refer to it on all correspondence.

If you have any questions or require further information contact Linda Balk at 602-546-0209; lbalk@phoenixchildrens.com in the IRB Office.

Sincerely,

Mitchell Shub, MD
Co-Chair, PCH Institutional Review Board

1919 East Thomas Road • Phoenix, AZ 85016 • Phone (602) 546-1000 • www.phoenixchildrens.com

PHOENIX CHILDREN'S Hospital

RECEIVED

April 7, 2011

Francis Eshun, MD
Phoenix Children's Hospital
Center for Cancer and Blood Disorders
1919 E. Thomas Road
Phoenix, AZ 85016

RE: PCH IRB #10-082- The study title: Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity.

You have the permission of Phoenix Children's Hospital (PCH) to conduct the study noted above.

The research will be guided by Good Clinical Practices and conducted in accordance with all applicable Federal, State, and Local Laws and Regulations. In addition, the research will be conducted in compliance with the policies and procedures of the PCH Office of Research and the PCH Institutional Review Board (IRB).

Enclosed is the following documentation for your records:

- Scientific Review Committee approval letter
- IRB approval letter
- Material Transfer Agreement

Please do not hesitate to contact the Office of Research with any questions or concerns. We look forward to the successful completion of this study.

Sincerely,

Ashley Lopez, MS
Director of Research

1919 East Thomas Road • Phoenix, AZ 85016 • Phone (602) 546-1000 • www.phoenixchildrens.com

Phoenix Children's Hospital IRB Approval Letter 2012 - 2013

PHOENIX CHILDREN'S Hospital

RECEIVED 15 MAR 2012

March 15, 2012

Francis Eshun, MD
Phoenix Children's Hospital
Hematology/Oncology
1919 E. Thomas Road
Phoenix, AZ 85016

RE: IRB #10-082: The study title: Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity (Continuing Review Application)

Dear Dr. Eshun:

I have reviewed your request for continuing review of the study listed above. This study qualifies for expedited review under FDA and DHHS (OHRP) regulations category 5, materials collected for non research purposes.

This is to confirm that I have approved your request for continuation. You are granted permission to continue your study as described effective immediately.

This approval includes the following:

- Continuing Review Application (2/21/2012)
- Project Updated (February 2012)

The study is next subject to continuing review on or before 3/14/2013, unless closed before that date. You may not continue the study beyond the expiration date noted above. You must apply for re-approval 45 days in advance of expiration to allow adequate time for IRB review.

As Principal Investigator you are responsible for assuring that:

- The approved protocol is followed and prior IRB approval is obtained for any changes (including changes in recruitment procedures, subject, population, location, protocol); and
- Any problems are reported promptly to the IRB (including adverse events and deviations from the approved protocol).

Approval period: 3/15/2012 - 3/14/2013

If you have any questions, please contact Linda Balk at 602-546-0209; lbalk@phoenixchildrens.com.

Sincerely,

Mitchell Shub, MD
Co-Chair, PCH Institutional Review Board

cc: Garrick Fenton

1919 East Thomas Road • Phoenix, AZ 85016 • Phone (602) 546-1000 • www.phoenixchildrens.com

Phoenix Children's Hospital IRB Approval Letter 2013 - 2014

PHOENIX CHILDREN'S *Medical Group*

May 10, 2013

Francis Eshun, MD
Phoenix Children's Hospital
Hematology/Oncology
1919 E. Thomas Road
Phoenix, AZ 85016

RECEIVED 5/10/13 SRC

RE: IRB #10-082: The study title: Analysis of the phosphorylation sites and kinases implicated in the phosphorylation of ShcC, a scaffold protein over-expressed in high-risk neuroblastoma, and their implication in tumorigenicity

Dear Dr. Eshun:

I have reviewed your request for continuing review of the study listed above. This study qualifies for expedited review under 21 CFR 56.110; 45 CFR 46.110, category 5, materials collected for non-research purposes.

This is to confirm that I have approved your request for continuation. You are granted permission to continue your study as described effective immediately.

This approval includes the following:
- Continuing Review Application (2/22/2013)
- Addition of Sam Chimienti as Research Coordinator
- Conflict of Interest Form for Sam Chimienti
- Removal of Garrick Fenton, Nicole Pawenski, and Chasity Wellnitz as members of the study staff

Since your study expired on 3/14/2013, you are not permitted to use any data collected between 3/14/2013 and 5/9/2013.

The study is next subject to continuing review on or before 5/9/2014, unless closed before that date. You may not continue the study beyond the expiration date noted above. You must apply for re-approval 45 days in advance of expiration to allow adequate time for IRB review.

As Principal Investigator you are responsible for assuring that:
- The approved protocol is followed and prior IRB approval is obtained for any changes (including changes in recruitment procedures, subject, population, location, protocol); and
- Any problems are reported promptly to the IRB (including adverse events and deviations from the approved protocol).

Approval period: **5/10/2013 - 5/9/2014**

If you have any questions, please contact the IRB Office at 602-546-0141.

Sincerely,

Mitchell Shub, MD
Co-Chair, PCH Institutional Review Board

cc: Sam Chimienti