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Protein-protein interactions of the LRP1 cytoplasmic domain modulated by tyrosine phosphorylation

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Nubile, Gina Faye

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Protein-Protein Interactions of the LRP1 Cytoplasmic Domain Modulated by Tyrosine Phosphorylation

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Chemistry by Gina Faye Nubile

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Professor Peter van der Geer, Co-chair
Professor Joe Noel
Professor Emmanuel Theodorakis
Professor Jean Wang

2007
The dissertation of Gina Faye Nubile is approved, and it is acceptable in quality and form for publication on microfilm:

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Co-chair

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University of California, San Diego

2007
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LIST OF SYMBOLS AND ABBREVIATIONS

LRP: Low Density Lipoprotein Receptor Related Protein
LDL-R: Low Density Lipoprotein Receptor
ATP: adenosine 5'-triphosphate
GST: Glutathione S-transferase
HA: Hemagglutinin
MALDI: Matrix-Assisted Laser Desorption Ionization
MS: Mass spectrometry
WT: wild type
PTB: Phosphotyrosine binding
PID: Phosphotyrosine interaction domain
PTP: phosphatase
Aβ: Amyloid beta
AD: Alzheimer’s Disease
ApoE: Apolipoprotein E
SH2: Src-homology 2
APP: Amyloid precursor protein
NF-M: neurofilament protein – medium
H/D: hydrogen deuterium
CT: cytoplasmic tail
ER: endoplasmic reticulum
IP: immunoprecipitation
WB: western blot
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
EGF: epidermal growth factor
Csk: C-terminal Src kinase
bFGF: basic fibroblast growth factor
Ubq: ubiquitin

Amino Acids
Y: tyrosine
F: phenylalanine
E: glutamine
C: cysteine
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Chapter 2 is, in part, a reprint of a submitted manuscript in review with the title: Structural and Functional Consequences of LRP1 Tyrosine Phosphorylation. Co-authors aside from the dissertation author and the principle investigator include Peter van der Geer. The dissertation author was the primary researcher and author of this manuscript.
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FIELDS OF STUDY

Major Field: Biochemistry

Biochemistry: Studies in Structural Affects of Tyrosine Phosphorylation and Functional Consequences
The low-density lipoprotein receptor-related protein (LRP1) is a member of the low-density lipoprotein (LDL) receptor family. This family of receptors is classically viewed as endocytic receptors. New, emerging evidence though points to the fact that some of these receptors are functionally dynamic and can fulfill different roles within the cell. One of these multipurpose LDL-R type receptors is LRP, which can function in both an endocytic role or in a signaling role.

While the bulk of the receptor’s endocytic machinery is located outside the cell, the signaling component of LRP is a small, 100 amino acid, cytoplasmic domain that contains two NPXY sequence motifs. NPXY motifs are potential sites of phosphorylation and are binding sites for PTB or SH2
domains. Much work to date has demonstrated an important role of the distal NPXY motif in LRP signaling, and it is the only site of known tyrosine phosphorylation in the receptor tail. The lack of observable phosphorylation to the proximal NPXY motif could be due to structural constraints.

To probe the accessibility of these sites to tyrosine phosphorylation, the GST-fusion proteins of wild-type and phosphorylated LRP cytoplasmic tail (LRP-CT) were studied by H/D exchange mass spectrometry. We discovered that some regions of the protein do in fact show reduced amide exchange, including the first NPXY motif containing Y4473. Experiments on the phosphorylated form (pY4507) showed changes in amide exchange, such that the first NPXY motif became exposed. This result suggested that the site at 4507 was always exposed, but the site at 4473 may be sequestered in a folded part of the protein.

*In vitro* kinase reactions using $^{32}$P-[ATP] and wild-type or mutant GST-LRP-CT, Tyr to Phe mutations at each NPXY motif (or Glu at distal Y4507) residue, and phosphopeptide mapping proved that both NPXY motifs can be phosphorylated. Further experiments showed that the Y4473 site can become phosphorylated *in vivo*. Overall, our data suggests a model in which the NPXY motifs of the LRP-CT are phosphorylated in a sequential manner. The distal motif is always accessible to potential kinases and is phosphorylated first (Y4507). This event effects distant residues within the protein causing the proximal NPXY motif to become more accessible. This allows phosphorylation to occur at Y4473.
This method of phosphorylation of the LRP-CT has implications for its interactions with other proteins. We studied the effect of LRP-CT tyrosine phosphorylation on its interactions with several proteins, one of which is a novel binding partner that we identified using an affinity peptide search based on the LRP-CT NPXY<sub>4507</sub> region and MS/MS. This protein, Shp2, a protein tyrosine phosphatase, requires LRP tyrosine phosphorylation to bind. Another known LRP-CT binding protein, Fe65, exhibited a slight preference for a phosphorylated binding site. However, this preference may be due more to the “opening up” affect of phosphorylation, as distant residues from the NPXY motif appear to be important for binding. Finally, sorting nexin (Snx17) binding was regulated by LRP-CT phosphorylation. Interestingly, Snx17 binds to the proximal NPXY motif, but this binding is blocked by phosphorylation at Y4473. However, phosphorylation at Y4507 increases binding, presumably by making the Y4473 region more accessible.
CHAPTER ONE

Introduction
Background of the LDL-R Family

Cells are separated from their environment by the plasma membrane. However, for cells to function properly they need to exchange both information and materials with the outside world. A variety of proteins are expressed on the cell surface to mediate these exchanges. One such example are proteins which are cell surface receptors. A diversity of receptors, ubiquitous and cell type dependent, have been studied.

Genes encoding cell receptors compromise about 5% of the human genome, which relates to over 1000 different proteins (Ben-Shlomo, et al., 2003). The largest receptor family is the GPCRs which encompass greater than 860 different members. Other main receptor families include receptor tyrosine kinases, receptor tyrosine phosphatases, integrins, guanylate cyclase, Notch, and LDL (Strachan, et al., 2006). Receptors are responsible for binding a diverse range of ligands such as hormones, ions, neurotransmitters, amino acids, natural products, lipids, and more (Armbruster and Roth, 2005). These regulated binding events help to maintain the physiological environment of the cell.

Two categories of receptors include signaling receptors or endocytic receptors. Signaling receptors function by binding ligand on the extracellular surface and transmitting signals inside the cell. Endocytic receptors work mainly to deliver cargo from the extracellular space to the intracellular space. My dissertation work has focused on the low-density-lipoprotein receptor related protein (LRP). LRP is a member of the LDL receptor family. This family of receptors is classically viewed as endocytic. An interesting characteristic of LRP
is that it displays both types of behaviors, signaling and endocytosis. Initial research on LRP detailed its role as an endocytic receptor, but later work, including mine, has demonstrated a role of LRP in signaling.

**LDL-R family members**

The low density lipoprotein (LDL) receptor (LDL-R) is the founding member of the LDL-R family of endocytic type I receptors (Brown and Goldstein, 1986). The main role of these receptors originally was the endocytosis of bound ligands, such that they are delivered to the lysosome for degradation. This internalization is constitutive; the receptors will recycle all the time whether ligand is bound on the extracellular portion of the receptor or not. The receptors exhibit tight binding to their respective ligands, displaying both unique and overlapping specificity across the family. All the receptors in the family are composed of similar structural units; ligand binding class A repeats, EGF precursor homology domains with YWTD repeats, an O-linked sugar domain enriched in serine and threonine residues, a single pass transmembrane domain, and a short cytoplasmic domain (Figure 1.1). The ligand binding domain(s) is arranged in a different number of repeats depending on the specific receptor. Each ligand binding repeat is composed of approximately 40 amino acids, with six cysteine residues in three disulfide bonds which help form the structure of the repeat. The disulfide bonds are arranged as C1-C3, C2-C5, and C4-C6. The repeats present a negatively charged surface which aids the interaction between the receptor and the positively charged ligands like ApoE/ApoB bound lipoprotein.
Figure 1.1: Schematic diagram of LDL-R family members. The two largest members are LRP1 and LRP2 (megalin). LRP1 is sometimes viewed as repeating units of the LDL receptor, as can be observed in the above depiction. Adapted from (Sugiyama, et al., 2000).
Most of the receptors contain at least one NPXY sequence in the cytoplasmic domain. For some of the receptors this sequence can act as an internalization signal or a docking site as part of a signaling event.

**LDL-R**

LDL-R is the prototype of the family. It contains one ligand binding domain composed of seven complement-type repeats (Brown and Goldstein, 1986). It is highly expressed in the liver. LDL-R is best known for its role in the regulation of cholesterol levels in the circulation. In this manner, many of the ligands that it binds are lipid and lipid products. Lipid bound particles bind to the ligand binding repeats on the extracellular portion of the receptor and are internalized into the cell when the receptor clusters into clathrin-coated pits for endocytosis.

The LDL-R is responsible for maintaining cholesterol levels between extracellular and intracellular sources. Mutations in the LDL-R gene can result in the disease familial hypercholesterolemia (FH), the most common single allele human disease. The disease is autosomal dominant, and heterozygotes occur 1 in every 500 persons. There are over 150 mutant alleles of the LDL-R that have been discovered to be associated with FH (Hobbs, et al., 1992). Many of these mutations occur within a ligand binding domain and disrupt the structure of the repeat (Blacklow and Kim, 1996). This severely decreases the ability of the receptor to take up lipid particles. Without functional LDL-R to maintain
homeostasis of cholesterol levels, patients with these mutations quickly develop atherosclerosis.

**Megalin**

Megalin is also known as LRP-2, and is more similar to LRP than the LDL-R based on its larger size and presence of two NPXY motifs (Saito, et al., 1994). It also contains several different domains like src-homology binding regions, and protein kinase/casein kinase II sites. These regions would suggest that megalin could play more of a role in signaling than in endocytosis. Also like LRP, it has four ligand binding domains, each made up of 7, 8, 10, and 11 complement repeats respectively. Megalin is expressed in adsorptive epithelial cells such as in the lung and kidney (Saito, et al., 1994). Due to its tissue distribution and endocytic roles, megalin is implicated in the reabsorption of calcium and vitamins to cells (Lundgren, et al., 1994, Moestrup, et al., 1996).

**VLDL-R**

The very-low-density lipoprotein receptor (VLDL-R) is very similar to the LDL-R except that the ligand binding domain of the VLDL-R contains eight repeats instead of seven (Takahashi, et al., 1992). The VLDL-R gene is highly conserved across different species (95% identity across mammals), hence it is believed that have a significant function for the cell (Webb, et al., 1994). VLDL-R can also bind apoE lipoproteins, and thus plays some role in the uptake of triglyceride-rich particles. However, its exact role in this function is not clear due
to its tissue localization. Contrary to the LDL-R, the VLDL-R is highly expressed in the brain as opposed to the liver. This has implications for its different functions. For example, VLDL-R, in conjunction with the ApoER2 receptor, is essential for proper neuronal development. Knockout mice for both receptors displayed inverted cortical layering and a general phenotype indistinguishable from a reeler mouse (Trommsdorff, et al., 1999).

_ApoER2_

ApoER2 is very similar to both LDL-R and VLDL-R; however there are several splice variants of the apoER2 gene that can result in slightly different products (Kim, et al., 1996, Kim, et al., 1997, Novak, et al., 1996). For example, one splice variant results in a furin cleavage site near the membrane of the cell which can yield a soluble form of the receptor from the extracellular domain. The apoER2 receptor also contains a unique proline rich sequence in its cytoplasmic tail. As compared to its closest family members, apoER2 has the slowest endocytosis rate. Some theorized that this is an indication that the receptor plays a larger role in signaling than in endocytosis. This model is supported by the recent discovery of the role of VLDL-R and apoER2 with the extracellular protein Reelin and the intracellular protein Dab in neuronal development. Reelin is secreted from cells and binds to the extracellular ligand binding domain of VLDL-R and ApoER2. This leads to the interaction of the adaptor protein Dab1 with the NPXY motif the receptors. This interaction is needed to mediate the signal
between the secreted ligand and intracellular action thru Dab1 for proper cortical plate layering (Trommsdorff, et al., 1999).

LR11

LR11 is also known as SORLA-1 (Jacobsen, et al., 1996, Yamazaki, et al., 1996). It is quite different from the other receptors in this family as it does not contain any EGF precursor repeats. It contains a single ligand binding domain made up of 11 complement repeats, and its cytoplasmic domain has one internalization sequence. The extracellular domain of this receptor can be cleaved to a soluble form that is capable of binding ligand in the extracellular space (Hampe, et al., 1999, Zhu, et al., 2004).

LRP5 and LRP6

LRP5 and LRP6 are different from other members of the LDL-R family in that LRP5/LRP6 are composed of four EGF repeats and three complement type repeats in a unique arrangement (Brown, et al., 1998, Hey, et al., 1998). They also have a longer cytoplasmic domain, about twice that of LRP (~ 200 amino acids) and both lack an NPXY motif. These receptors have been studied more for their signaling functions than for endocytosis. For example, LRP5/LRP6 appear to function as co-receptors for Wnt-signaling (Pinson, et al., 2000, Tamai, et al., 2000, Wehrli, et al., 2000). Wnts are secreted proteins that are expressed during vertebrate embryonic development and signal through their specific receptor, Frizzled (Wodarz and Nusse, 1998). LRP6 knockouts in mice caused
developmental defects similar to those observed with Wnt mutations (Pinson, et al., 2000). It was later found that LRP6 mutants with deletion of the cytoplasmic domain blocked signaling by Wnt and that the extracellular ligand binding domain of LRP6 could bind Wnt-1 (Tamai, et al., 2000).

Work on LRP5 revealed that mutation of this gene caused osteoporosis-pseudoglioma syndrome (Gong, et al., 2001). LRP5 may also have some association with type 1 diabetes, as the LRP5 gene was mapped within a region on chromosome 11q13 that is linked to the disease (Hey, et al., 1998). Along these lines, studies have found that LRP5 is essential for proper glucose-induced insulin secretion (Fujino, et al., 2003).

**LRP1**

The low density lipoprotein receptor-related protein (LRP) is one of the largest members of the low density lipoprotein receptor gene family. LRP is composed of 2 chains, a 515 kD α-chain and a 85 kD β-chain, which are created by a furin cleavage of the single LRP chain in the trans-Golgi. (Herz, et al., 1988). The α-chain is entirely extracellular and composed of four ligand binding domains, only three of which are complete. These are class A type complement repeats, organized in increasing numbers of repeats, 2, 8, 10, 11. These are accompanied by EGF-like class B repeats. The complement, or ligand binding domains, have been shown to bind to over 30 different ligands from proteases and protease inhibitor complexes, lipoproteins, growth factors, transport proteins, and more (Table 1.1) (Hussain, et al., 1999). It has been reported that N-linked
glycosylation of LRP is required for its proper folding. When cells were treated with tunicamycin, a nucleoside antibiotic that inhibits N-linked glycosylation, improperly folded LRP aggregates were formed (McCormick, et al., 2005). Receptor Associated Protein (RAP) is a 39 kD protein that acts as a folding chaperone for LRP (Willnow, et al., 1996) (Croy, et al., 2003). RAP also binds to the LRP ligand binding domains and inhibits the binding of other ligands to LRP prematurely in the Golgi. RAP is normally localized to the ER based on its ER localization signal (Willnow, et al., 1996). However extracellularly added RAP can act as a general antagonist for LRP and the LDL-R ligand binding interactions (Croy, et al., 2003, Fisher, et al., 2006, Lee, et al., 2006). The ligand binding repeats require calcium for proper folding. The beta chain contains an extracellular portion with O-linked glycosylation sites, and YWTD repeats (which form a beta propeller in the LDL receptor (Rudenko, et al., 2002)). The beta chain passes through the membrane in a single pass and ends with a short 100 amino acid cytoplasmic tail. The cytoplasmic tail contains 2 NPXY motifs, a signature of the LDL-R family. The distal NPXY motif was originally believed to be the endocytosis signal for the receptor based on homology with the LDL-R (Paccaud, et al., 1993). Later it was discovered that the YXXL sequence was the dominant internalization signal (Li, et al., 2000).
Table 1.1: Proteins that bind to the extracellular ligand binding domains of LRP. Adapted from (Strickland and Ranganathan, 2003)

<table>
<thead>
<tr>
<th>Proteases and Cofactors</th>
<th>Protease</th>
<th>Lipoprotein and lipid metabolism</th>
<th>Matrix proteins</th>
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**LRP in Endocytosis**

LRP is a very large receptor and so it requires a lot of help for proper folding and transport to the cell surface to function. The YXXL sequence and di-leucine motif is needed for LRP function as an endocytosis receptor (Li, et al., 2000). LRP is highly expressed in the liver and brain, and so many of its ligands are relevant to maintaining cholesterol homeostasis or clearance of proteins in the brain. A diverse range of ligands have been discovered to interact with the LRP extracellular ligand binding domains (Table 1.1). LRP is even believed to help regulate the composition of receptors on the cell surface by forming heterocomplexes with certain receptors (APP, uPAR, TF, PDGF-R) and aiding in their endocytosis (Gonias, et al., 2004).

Endocytosis of a ligand by LRP proceeds in several steps (Figure 1.2). First, ligand must be recognized and bound by the receptor. This is important since certain ligands like alpha-2-macroglobulin or ApoE can only bind to LRP in their activated or lipid-bound form. Next, the receptor clusters into clathrin-coated pits to be endocytosed. After this step, the ligand dissociates from the receptor (presumably aided by the drop in pH of the endosome as seen in the LDL-R (Rudenko, et al., 2002)). The ligand is degraded in the lysosome, while the receptor is then recycled back out to the cell surface.
Intracellular domain – Structure, Binding

LRP is unique in the low density lipoprotein receptor family because it is one of three receptors that contain two NPXY motifs (the others are megalin and LRP1B). For the rest of this work the NPXY motifs will be referred to as NPXY$_{4473}$ and NPXY$_{4507}$. Much work has been done to demonstrate the importance the NPXY$_{4507}$ motif for LRP function. For example, it was discovered that Y4507 can be phosphorylated in v-src transformed cells (Barnes, et al., 2003). Phosphorylation at this site can act as a binding site for the adaptor protein Shc. This interaction brings Shc close to the plasma membrane and can lead to downstream signaling events through the Ras/MAPK pathway (Figure 1.3).

This is just one example demonstrating the partnership between LRP and adaptor proteins (Gotthardt, et al., 2000, May, et al., 2004, Trommsdorff, et al., 1998). Adaptor proteins contain protein-protein interaction domains such as SH2, PTB, WW, and SH3 domains and help to transmit signals by assembling multimeric protein complexes. Phospho-tyrosine binding domains (PTB) and Src-Homology 2 (SH2) domains both interact with NPXY motifs but in slightly different contexts. PTB domains can bind NPXY motifs in a phosphorylation dependent or independent manner depending on the specific protein and recognize the following consensus, $\Psi$XNPXY, where $\Psi$ is hydrophobic residue and X is any amino acid (Forman-Kay and Pawson, 1999, Margolis, et al., 1999). SH2 domains also recognize phosphorylated tyrosine residues within NPXY motifs, but their binding depends on residues C-terminal to the NPXY.
Figure 1.2: Examples of LRP-mediated endocytosis of ligand complexes. A) Proteinase-Inhibitor complexes. C) Apoprotein enriched lipid particles. In each case, the complex binds to the receptor on the surface of the cell, and becomes internalized. In the lysosome, the ligand is degraded and processed while the receptor exits the lysosome and is shuttled back out to the cell surface. Adapted from (Willnow, et al., 1999)
Figure 1.3: Diagram of NGF signaling through the TrkA receptor in nerve cells. The arrow is pointing to the role of Shc as an adaptor protein involved in the signaling cascade, ultimately leading to MAPK activation and gene transcription. LRP1 can act in a similar role as the TrkA receptor as a scaffold site for Shc and the same downstream components. (Adapted from Basic Neurochemistry)
sequence (Songyang, et al., 1994). NPXY motifs are present in a wide variety of proteins such as receptor tyrosine kinases, integrins, G-protein-coupled receptors, lipoprotein receptors, and phosphatases (Smith, et al., 2006). They were originally identified as a sequence responsible for the internalization of receptors into clathrin-coated pits, and later recognized for their ability to act as binding sites for protein interaction domains (Paccaud, et al., 1993). Many proteins have been discovered to interact with these motifs in the LRP cytoplasmic domain (Table 1.2). Although the purpose of all of these intracellular interactions has yet been determined, they are believed to be important for the signaling function of the receptor.

LRP contains many potential phosphorylation sites as it contains four tyrosines, six threonines, and three serines. Previous work has shown that LRP is phosphorylated on all of these residues in cells (Barnes, et al., 2003, Li, et al., 2001, Loukinova, et al., 2002, Ranganathan, et al., 2004), although the levels of tyrosine phosphorylation are low in comparison to serine and threonine phosphorylation. The significance of all the phosphorylation sites has yet to be determined, but some sites have been studied.

Work on tyrosine phosphorylation showed that phosphorylation of Y4507 occurs in cells in response to PDGF-BB and by v-src (Barnes, et al., 2003, Loukinova, et al., 2002). Phosphorylation of a receptor tail in response to growth factor binding would again suggest that LRP could play a role in signaling. For example, phosphorylation of Y4507 promotes binding of Shc (Barnes, et al., 2003).
<table>
<thead>
<tr>
<th>Intracellular Protein</th>
<th>Method of Identification</th>
<th>Purpose of Interaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe65, Dab1</td>
<td>Yeast Two-Hybrid Adaptor</td>
<td>Adaptor</td>
<td>(Trommsdorff, et al., 1998)</td>
</tr>
<tr>
<td>Shc</td>
<td>GST pulldown, Affinity peptide</td>
<td>Adaptor</td>
<td>(Barnes, et al., 2003)</td>
</tr>
<tr>
<td>SEMCAP-1, JIP1/2, PSD-95, OMP25, ICAP-1</td>
<td>Yeast Two-Hybrid Adaptors, Unknown</td>
<td>Adaptors, Unknown</td>
<td>(Gotthardt, et al., 2000)</td>
</tr>
<tr>
<td>Snx17</td>
<td>GST-pulldown</td>
<td>Receptor recycling?</td>
<td>(Stockinger, et al., 2002)</td>
</tr>
<tr>
<td>GULP</td>
<td>GST-pulldown</td>
<td>Engulfment?</td>
<td>(Su, et al., 2002)</td>
</tr>
</tbody>
</table>
This event recruits Shc close to the plasma membrane where it can interact with other proteins and be phosphorylated as well. This series of event leads to activation of the Ras/MAPK pathway. Work on other potential phosphorylation sites indicates that PKA and PKCa are responsible for the phosphorylation of S4520 (S76), and possibly other sites as well (Li, et al., 2001, Ranganathan, et al., 2004). Phosphorylation of S76 helps mediate endocytosis of the receptor, as a minireceptor with S76A mutation displayed slower rates of endocytosis (Li, et al., 2001).

**LRP in Signaling**

LRP has been implicated in many signaling events. LRP is believed to be a pro-survival receptor in cells. LRP was selectively up regulated in Schwann cells after sciatic nerve injury (Campana, et al., 2006). This up regulation was associated with increased cell survival, and a decrease in activated caspase-3, a signaling protein leading to apoptosis. LRP has been shown to mediate the inhibition of smooth muscle cell migration in response to growth factors via binding of extracellular ApoE (Swertfeger, et al., 2002). LRP also appears to regulate several proteins involve in the makeup of the extracellular matrix. Other researchers determined in a proteomics-type experiment (Gaultier, et al., 2006) that LRP expression in cells lead to the decrease in mRNA of several proteins.

Even more interesting was the fact that this regulation was not dependent on the endocytic activity of the receptor. Another signaling cascade that LRP is involved in is the JNK/cJun/Elk-1 pathway (Lutz, et al., 2002). Again, this event
was proven to be independent of the extracellular domain of LRP, as chimeric minireceptors containing IgG domains instead of the LRP extracellular domain were used for experiments. In this case, LRP expression inhibited the activity of the transcription factors Elk-1 and c-Jun. This event was associated with an increase in JNK retention at the plasma membrane.

There has also been recent work in the field on proteolytic processing of the LRP receptor and its potential signaling effects. Proteolysis of LRP by matrix metalloproteases results in the release of a soluble extracellular fragment of LRP (Rozanov, et al., 2004). May et al. discovered that the proteolytic processing of the cytoplasmic tail can result in release of a soluble intracellular fragment. This process involved at least two steps, the first of which was shedding of the LRP ectodomain (May, et al., 2002). Later it was discovered that β-secretase (BACE, β site of APP-cleaving enzyme) can induce this ectodomain shedding (von Arnim, et al., 2005) and that LRP can interact with presenilin-1 (PS1) (Lleo, et al., 2005).

Presenilin is part of the gamma-secretase complex. Taken together, these two studies would suggest that β- and γ-secretase can act in concert to process LRP; the first step being cleavage and loss of the extracellular domain, followed by a second cleavage and release of a cytoplasmic fragment to the intracellular space. This pathway is similar to what occurs in Notch and APP processing. Proteolysis of Notch has been shown to lead to a free cytoplasmic fragment that translocates to the nucleus and activates transcription of certain
genes (Artavanis-Tsakonas, et al., 1999). It is possible that LRP could participate in a similar mechanism and also signal through the nucleus.

LRP is an important protein for both its endocytic and signaling roles. Deletion of the LRP gene is embryonic lethal in mice (Herz, et al., 1992). It has been implicated in the development of Alzheimer’s disease (AD)(Arelin, et al., 2002), familial Hypercholesterolemia (Van Leuven, et al., 2001), atherosclerosis (Boucher, et al., 2003) and coronary artery disease (Pocathikorn, et al., 2003). LRP functions in conjunction with the LDL-R to clear chylomicron remnants from the circulation into the liver (Rohlmann, et al., 1998).

**LRP in Alzheimer’s Disease**

Alzheimer’s Disease (AD) is the leading cause of dementia in the elderly. AD is a disorder of the central nervous system which can present itself in two types, early and late-onset. Late or age-dependent onset is most common. Early onset AD is sporadic and caused by multiple factors. The causes of AD are not clear, but the two main hypotheses involve the proteins amyloid-precursor protein (APP) and the microtubule associated protein tau (Figure 1.4). The plaques often found in the brains of AD patients are composed of extracellular Aβ (Kang, et al., 1987). Also present in the brain of AD patients are tangles, which are composed of intracellular paired helical filaments of hyperphosphorylated tau (Selkoe, 2001). The APP theory is interesting in that it involves LRP in many levels. In fact, there are several genetic links, direct and indirect, between AD and LRP.
There are 2 pathways for the processing of APP: amyloidogenic and non-amyloidogenic (Figure 1.5). The amyloidogenic pathway results when APP is cleaved first by beta-secretase on the extracellular surface, and then by gamma secretase within the membrane. These cleavage events generate Aβ peptides (Aβ40 or Aβ42), which can aggregate in the extracellular space in senile plaques of AD patients (King and Scott Turner, 2004). The majority of APP is processed through the non-amyloidogenic pathway by alpha secretase (Ikezu, et al., 1998). Aβ peptide generation is important because it has many links to LRP. As an endocytic receptor, LRP can internalize Aβ when bound by apoE or activated α2-macroglobin (Haas, et al., 1997, LaDu, et al., 1997, Narita, et al., 1997, Pillot, et al., 1999, Qiu, et al., 1999). Many other ligands of LRP are also found in senile plaques such as apoE, activated α2-macroglobin, tissue and urokinase-type plasminogen activators, plasminogen activator inhibitor-1, lipoprotein lipase, and lactoferrin (Rebeck, et al., 1995). One theory of AD is that LRP function or expression is impaired. This is supported by the fact that many of the above LRP ligands are found in increased amount in the serum of probable AD patients (Aoyagi, et al., 1992, Licastro, et al., 1995, Taddei, et al., 1997).

LRP also plays a role in the processing of the APP receptor itself. Much of this work is still ongoing and the clear meaning of the APP-LRP processing link is an evolving process. It is unclear if LRP plays a protective role against Aβ toxicity or not. The internalization of APP was found to be impaired in CHO cells lacking LRP (Pietrzik, et al., 2002). The level of Aβ peptide was also decreased
Figure 1.4: Schematic of tau and Aβ hypothesis of Alzheimer's Disease. Insoluble amyloid plaques composed of Aβ peptide and other LRP1 ligands form in the extracellular space of neurons. Inside the cell, hyperphosphorylated tau forms neurofibrillary tangles. Adapted from (Selkoe, 2004)
in these same cells. The investigators discovered that both of these defects in APP processing could be restored by expression of the c-terminal tail of LRP. Other studies showed that LRP could have a protective role when cells were challenged with both Aβ and an LRP ligand such as alpha-2-macroglobulin (Van Uden, et al., 2000).

In these two cases, it is hard to reconcile the opposing effects of LRP. In the first case, the LRP cytoplasmic domain appears to have a negative role in the development of AD, while the second example shows a positive role through the LRP extracellular domain. Some of these contrary results are likely due to different expression levels of LRP and other important involved proteins in different cell types. There are many genetic links to AD, some which are direct proven mutations and others that are indirect, and the validity of which is unclear.

Familial AD is directly inherited by a gene mutation, of which there are three known for AD, presenilin-1, presenilin-2, and APP (Kwok, et al., 1997, Tanzi, et al., 1996). These cases are rare, accounting for less than 5% of all AD patients. Most AD cases are a result of multiple genetic and environmental factors known as “sporadic” AD. For example, the apolipoprotein E (APOE) has a strong and well studied connection to AD. The ApoE4 allele increases the risk of developing AD (Corder, et al., 1993, Saunders, et al., 1993, Strittmatter, et al., 1993). The ApoE2 allele is associated with protective affects against AD (Benjamin, et al., 1994, Beyer, et al., 2002). Most controversial is the C766T polymorphism of LRP. Some studies claim an association of T allele with a protective effect, some with the opposite effect, and other studies concluded no
Figure 1.5: Schematic of APP processing. The top pathway of $\alpha$-secretase followed by $\gamma$-secretase cleavage is the non-amyloidogenic pathway. The lower route of $\beta$-secretase followed by $\gamma$-secretase cleavage represents the amyloidogenic pathway. $\alpha$-secretase and $\beta$-secretase cleave APP in the extracellular domain, while the $\gamma$-secretase complex cuts APP within the transmembrane region. Taken from (King and Scott Turner, 2004)

Goals of Dissertation

This project started with the very simple and common question about the structure and function relationship of the LRP cytoplasmic tail and how they might be connected by a small unpublished result. In studying Src phosphorylation of the LRP cytoplasmic tail it was noted that LRP was phosphorylated to a higher extent when the protein was denatured before the kinase reaction. As discussed above, the LRP cytoplasmic tail has two NPXY motifs, in addition to two other tyrosine residues, that could be phosphorylated. To date, only Y4507 has been shown to be phosphorylated in cells. However, this denaturing result hinted that the LRP cytoplasmic tail (LRP-CT) could have structure to it that under normal conditions prevented full phosphorylation of the cytoplasmic domain. When the protein was denatured by heat any inherent structure of the cytoplasmic domain was lost and thus more tyrosine residues were available to Src.

Work described in this dissertation will clearly explain how this phenomenon is possible. The next chapter will discuss the sequential phosphorylation of LRP and how it can affect the receptor’s interaction with other proteins in the cell. Using both in vitro and in vivo experiments, we have shown that in fact both tyrosine residues within the LRP NXPY motifs are phosphorylated.
The following chapters will extend this knowledge in other directions, first looking for proteins that can interact with the LRP-CT using affinity peptides and GST fusion proteins with in-gel digests and MS/MS. Then we will look at two specific LRP interactions, the first with PTB/PID-domain containing protein Fe65 and the second with the SH2 domain containing protein Shp2. Both of these proteins contain domains that interact with NPXY motifs; however, we will show that these interactions are very different from each other.

The interaction between LRP and Fe65 was found to be phosphorylation independent and potentially requires residues outside of the NPXY motif for optimal binding. We also observed regions distant from the PID1 domain, which is responsible for binding to LRP, can interfere with binding to PID1.

The interaction between LRP and Shp2 was phosphorylation dependent and the NPXY motifs were the only important regions. The only regions required for binding to LRP were the SH2 domains of Shp2. It appears that each SH2 domain binds to a specific NPXY motif within LRP cytoplasmic tail.

Shp2 is a novel LRP binding protein. Studies here clearly show that the interaction is direct and mediated by the SH2 domains of Shp2 and the NPXY motifs of LRP. This appears to be the first LRP interaction that requires both NPXY motifs for optimal binding. Fe65 is an established LRP binding protein, but with unconfirmed binding parameters. Hopefully the work put forth here will help better define this interaction and aid in the understanding of cellular processes involving these protein complexes.
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CHAPTER TWO

Structural and Functional Consequences of LRP1 Tyrosine Phosphorylation
Introduction

The low density lipoprotein receptor-related protein (LRP1) is an endocytic receptor involved in the uptake of diverse range of ligands including lipoproteins, proteases and protease/inhibitor complexes, matrix proteins, growth factors and intracellular proteins (Strickland and Ranganathan, 2003). LRP is present at the cell surface as a heterodimer, which consists of a 515 kDa alpha chain and a 85 kDa beta chain that are non-covalently associated (Herz, et al., 1988). The alpha chain is completely extracellular and is made up of a series of complement-type (class A) ligand binding repeats organized in four domains, and EGF-like (class B) repeats. The ligand binding repeats have been shown to bind up to 30 different extracellular ligands, many of which are found in the proteinaceous plaques in the brains of Alzheimer's Disease patients (Rebeck, et al., 1995). Other hints that LRP may be involved in Alzheimer's Disease include the fact that the LRP cytoplasmic tail interacts with PSD95 which may link LRP to the NMDA receptor (Gotthardt, et al., 2000, May, et al., 2004) and with Fe65, providing a direct link between LRP and APP (Pietrzik, et al., 2004, Trommsdorff, et al., 1998).

The beta chain is composed of YWTD repeats, a single transmembrane domain, and a 100 amino acid cytoplasmic region with two NPXY motifs (NPXY_{4473} and NPXY_{4507}) (Herz, et al., 1988). LRP was originally identified as an internalization receptor. It was thought that LRP cycled continuously between the cell surface and intracellular compartments. Ligands are bound at the cell surface and delivered to the endosomes followed by recycling of the receptor
back to the cell surface. The two NPXY motifs present within the short cytoplasmic tail were thought to mediate interactions with the internalization machinery. In fact, the membrane proximal NPXY motif has been identified as the binding site for sorting nexin 17 (Snx17) and knock-in experiments in mice showed a strong phenotype upon mutation of this site (Roebroek, et al., 2006, van Kerkhof, et al., 2005). Mutational studies identified the YXXL motif (which overlaps with Y4507) as critical for LRP receptor endocytosis (Li, et al., 2000).

More recently this image of LRP as a revolving door in the plasma membrane has started to change. An exciting new avenue of research was opened up when the cytoplasmic domain of LRP was shown to bind the adapter protein, Shc (Barnes, et al., 2001). Shc has been shown to be involved in the activation of the Ras-MAP kinase pathway upon activation of protein-tyrosine kinases and its binding to LRP required phosphorylation of Y4507 (Barnes, et al., 2003, Barnes, et al., 2001). These observations provided the first hint that LRP may be involved in the assembly of signaling complexes. Recently, LRP was shown to bind JIP, a scaffold protein involved in JNK activation (Gotthardt, et al., 2000, Lutz, et al., 2002). The distal NPXY\textsubscript{4507} motif was also identified as the primary site of binding to the adaptor protein Dab1, involved in neuronal path-finding during the development of the central nervous system (Trommsdorff, et al., 1998). Another adaptor protein involved in engulfment called GULP also required the distal NPXY\textsubscript{4507} motif (Goto and Tanzi, 2002). Deletion of both NPXY motifs was required to abrogate binding of Fe65, a neuronal adaptor protein that was initially identified as a transcription factor, and mutations at the
distal NPXY4507 motif affected processing of APP which also binds Fe65 (Pietrzik, et al., 2002, Pietrzik, et al., 2004).

The distal NPXY4507 motif can be phosphorylated in v-Src transformed cells (Barnes, et al., 2003, Loukinova, et al., 2002) and following stimulation of cells with PDGF-BB (Barnes, et al., 2003, Loukinova, et al., 2002). It is surprising that phosphorylation of only the distal of the two NPXY motifs has been observed because the amino acid sequences amino-terminal to Y4473 and Y4507 are very similar (Barnes, et al., 2001, Loukinova, et al., 2002). This raises the question as to why v-Src does not phosphorylate Y4473. One possible explanation is that the NPXY4473 site is bound by a protein that prevents access to the kinase. If this is the case, in vitro phosphorylation of the purified cytoplasmic tail should result in phosphorylation of this site. A second possibility is that the NPXY4473 site is not exposed at the surface of the protein and therefore cannot act as a substrate for phosphorylation. Alternatively, there could be structural constraints that would prevent the NPXY4473 site from acting as a substrate for phosphorylation. We report here that the proximal NXPY4473 motif of LRP can be tyrosine phosphorylated. However, our observations strongly suggest that the cytoplasmic region of LRP is phosphorylated in a sequential manner, such that Y4473 can only become phosphorylated after Y4507 is phosphorylated. We further show by in vivo and in vitro experiments that phosphorylation of Y4507 increases the solvent accessibility of much of the cytoplasmic tail, providing an explanation as to why Y4473 may only be accessible after Y4507 is
phosphorylated. Finally, using GST pull-down experiments we show Snx17 binding at the proximal NPXY$_{4473}$ is regulated by phosphorylation.

**Materials and Methods**

**Materials**

293 cells were grown in Dulbecco-Vogt’s modified Eagle’s medium containing 10% fetal bovine serum with 50 µg/mL penicillin and streptomycin, 5% CO$_2$. Anti-LRP1 antibody 11H4 was used as a hybridoma supernatant (ATCC, Manassas, VA) grown in Iscove’s modified Dulbecco’s medium containing 10% fetal bovine serum and 25 mM L-glutamine. Anti-phosphotyrosine monoclonal antibody G410 and anti-HA monoclonal antibody 12CA5 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and Babco (Richmond, CA). Anti-myc antibody, α-cyano-4-hydroxycinnamic acid, and Protein A was obtained from Sigma (St. Louis, MO). Goat anti-mouse secondary antibody was purchased from Upstate Biotechnology, Inc (Lake Placid, NY). Glutathione sepharose was purchased from GE Healthcare (Piscataway, NJ). Pepsin beads were obtained from Pierce Biotechnology (Rockford, IL). Recombinant Src kinase domain and YopH phosphatase plasmids were a kind gift from Dr. John Kuriyan (University of California, Berkeley, CA).

**Expression and Purification of Proteins**
The entire cytoplasmic domain of LRP (residues 4444-4544) was cloned into pGEX-4T2. GST fusion proteins were expressed in pLysS cells and induced with 0.1 mM IPTG overnight at room temperature. Briefly, the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS), 1 mM benzamidine, 1 mM DTT, 1 µg/mL leupeptin, aprotinin), sonicated on ice, and centrifuged for 30 min at 10,000 rpm. The supernatant was incubated with 10 mL of glutathione sepharose for 1 hour at 4°C. Resulting slurry was then washed exhaustively with TBS, and fusion proteins were eluted as 1 mL fractions with 10 mM reduced glutathione in TBS, pH 7.4. We found that maintaining the LRP cytoplasmic domain on GST reduced aggregation and improved stability of the protein, so this was how all of the protein was prepared. Eluted protein was dialyzed overnight in TBS, 4°C and the amount of protein obtained was determined by BCA assay (Pierce Biotechnology, Rockford, IL). Mutations in the LRP cytoplasmic tail at Tyr 4473 and Tyr 4507 were introduced using Stratagene Site Directed Mutagenesis Kit and mutant cDNAs were sequenced for fidelity. A double mutant, termed Y2F, was also created combining phenylalanine mutations at both Y4473 and Y4507.

*Phosphopeptide Mapping*

Full-length Src (Upstate, Lake Placid, NY) was used to phosphorylate the tyrosine residues of the LRP NPXY motifs for the H/D exchange and peptide mapping experiments. Higher levels of phosphorylation were achieved in later experiments using bacterially expressed recombinant Src kinase domain
(referred to as recombinant Src). Recombinant Src was expressed and purified according to protocol (Seeliger, et al., 2005).

To map the location of phosphorylation sites in the LRP cytoplasmic domain, 1µg of GST-LRP-CT (and each mutant Y4473F, Y4507F, Y2F, Y4507E, respectively) was incubated with 25 µL of kinase buffer (100 mM Tris, 10 mM MnCl₂, 10 mM MgCl₂, 1 mM DTT, 25 µCi [γ³²P]-ATP, 1 µL full-length Src (Upstate)) for 15 min in a 37°C water bath. The reaction was quenched by addition of 2X SDS-sample buffer on ice. Samples were resolved by SDS-PAGE and phosphorylated bands were localized by autoradiography, removed from the gel, and digested with trypsin (van der Geer and Hunter, 1994). Phosphopeptides were separated by electrophoresis at pH 1.9 for 35 min at 1kV on an HTLE 7000 electrophoresis instrument (C.B.S. Scientific). Subsequently, peptides were resolved in a second dimension by chromatography on a TLC plate in isobutyric acid buffer. Phosphopeptides were then visualized by autoradiography. Amino acid analysis was performed with phospho-serine, phospho-threonine, and phospho-tyrosine standards to confirm that peptide spots represented only phospho-tyrosine peptides.

In vitro kinase reactions

To follow the phosphorylation of the LRP cytoplasmic tail in a quantitative manner, a radiolabeled in vitro kinase reaction was set up in which 6 µg of GST-LRP was incubated in 40 µL of kinase buffer (100 mM Tris, 10 mM MnCl₂, 10 mM MgCl₂, 1 mM DTT, 0.2 mCi γ³²P-[ATP], and recombinant Src (Seeliger, et al.,
2005)) in a 37°C water bath. Reactions with equal amounts of GST-LRP-CT and mutants Y4437F, Y4507F, Y2F, Y4507E, and a GST control were set up in parallel. Individual samples at 5, 10, 20, 30, and 60 min were removed and quenched into chilled tubes containing 2X SDS-sample buffer. Proteins were separated by SDS-PAGE and the gel was stained and dried. Phosphorylated proteins were observed by autoradiography.

**Transient Expression of LRP1β mutants with v-src**

Phosphorylation of LRP in vivo was performed as described previously (Barnes, et al., 2003). An expression construct, termed Myc-LRPβ, was used to transiently express LRP in cells. This construct is the beta chain of LRP with a signal peptide and a Myc tag fused to the amino-terminus. 293 cells were co-transfected at 30% confluency with 1 µg of pcDNA3-Myc-LRPβ (wild-type, Y4473F, Y4507F, Y2F, and Y4507E) and with 1 µg v-Src. Cells were lysed 48 h after transfection in PLC-lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 500 µM Na₃VO₄, 1 mM PMSF, 10 µg/mL aprotinin and leupeptin). Lysates were incubated with 1 µL of anti-myc antibody for 1 h at 4°C, and then with 100 µL of Protein-A slurry (10%). Beads were collected by centrifugation and washed 4 times with PLC-lysis buffer. Immunoprecipitates were analyzed by Western blot for tyrosine phosphorylation.

**Immunoblotting**
Proteins were transferred to a nitrocellulose membrane and blocked for 1 h at room temperature with 1% casein in TBS with 0.1% Tween 20 (TBST) and incubated with a 1:100 dilution of anti-LRP antibody 11H4 or 1:20 dilution of anti-HA antibody 12CA5 in TBST for 1 h at room temperature. Blots were washed twice for 10 min with TBST, and twice with TBS for 10 min, and then incubated with a 1:5000 dilution of goat anti-mouse-HRP (Upstate, Lake Placid, NY) for 30 min at room temperature, and developed with ECL reagents (Thorpe and Kricka, 1986). For immunoblotting with anti-P-Tyr antibody 4G10, membranes were blocked with 2% BSA in TBST and then incubated with a 1:10 dilution of antibody 4G10 in TBST with 2% BSA, for 1 h each.

**Hydrogen/Deuterium Exchange Mass Spectrometry**

Purified GST-LRP-CT or full-length Src (Upstate) phosphorylated GST-LRP-CT-P (400 µg, in TBS, pH 7.4) was bound to glutathione sepharose and cleaved with thrombin in a minimum volume by washing the beads with TBS and then exchanging for thrombin cleavage buffer (TBS, 2.5 mM CaCl₂, pH 7.4, 1:50 thrombin). After rocking for 1 hr at room temperature free LRP-CT was collected by centrifugation and used immediately for hydrogen/deuterium experiments. The cleaved protein retains two extra residues, GS, at the N-terminus as a result of the thrombin cleavage. A sample (2 µL) of free LRP-CT was mixed with 20 µL of ²H₂O for an exchange period of 30 sec – 10 min at room temperature. Non-deuterated controls were also performed substituting water for deuterium and treated in the same manner. Experiments were performed in triplicate. The
samples were quenched by the addition of chilled 0.1% TFA (adjusted with 2% TFA so that final pH of mixed solution was 2.2), and the mixture was added to pre-washed (25 µL beads washed twice with 0.1% TFA) pepsin beads. Beads were kept on ice and vortexed once a minute for 10 min. At the end of the digestion, the supernatant was collected by centrifugation and dispensed into three separate aliquots which were immediately frozen in liquid nitrogen to be analyzed later.

Peptides produced by pepsin digests were identified in the following manner. A sample of GST-LRP-CT in solution was cleaved with thrombin in cleavage buffer and incubated with glutathione sepharose to collect the GST tag. The sample was spun in a microcolumn, and the free LRP-CT was collected for a pepsin digest in the same way as described above. The resulting peptides were separated on an analytical HPLC (Waters, 300 x 19 mm id) column using a gradient of 0-50% B (A: 0.1% TFA, B: 90% acetonitrile, 0.1% TFA) over 60 min at 10 mL/min. Fractions corresponding to chromatographic peaks were collected, lyophilized, and resuspended in 10 µL of 50/50 acetonitrile and 0.5% TFA. Each sample was mixed 1:1 with matrix (5.0 mg/mL α-cyano-4-hydroxycinnamic acid in 0.01% TFA: acetonitrile), spotted individually on a MALDI target plate, and sequenced using MALDI tandem mass spectrometry (MS/MS) on a Q-STAR XL hybrid quadrupole time of flight mass spectrometer equipped with an orthogonal MALDI source (Applied Biosystems). Spectra were analyzed manually with Data Explorer software (Applied Biosystems).
Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used to monitor deuterium incorporation into the amide positions of the LRP-CT. Spectra were acquired on a Voyager DE-STR instrument (Applied Biosystems) as previously described (Mandell, et al., 1998). The matrix used was 5.0 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) dissolved in a solution containing a 1:1:1 mixture of acetonitrile, ethanol, and 0.1% TFA. The pH of the matrix was adjusted to pH 2.2 using 2% TFA. The matrix solution was chilled on ice for at least 60 min prior to use, and the MALDI target plates were chilled overnight at 4°C. Mass spectra of the pepsin digests were analyzed as described previously (Mandell, et al., 1998) to determine the average number of deuterons incorporated into each peptide. The centroids of the individual peptides were calculated using the CAPP software program and the number of deuterium atoms incorporated during the exchange period was determined by subtracting the centroid of the undeuterated peptide from that of the deutrated peptide. Only backbone deuteration was reported for each peptide as side-chain contributions were subtracted from the total number of deuterons incorporated for each peptide and the results were corrected for back-exchange during analysis as previously described (Hughes, et al., 2001). The data from six peptides that gave the best continuous coverage of the protein are presented although more peptides were observed in the spectra.

To directly compare the amount of deuterium incorporation into the phospho- vs. non-phospho-LRP-CT, an experiment was performed in which $^{14}$N-LRP and $^{15}$N-phospho-LRP were compared in the same sample. This
experiment was used to confirm results from previous experiments in which phosphorylated and nonphosphorylated LRP samples were analyzed separately. For $^{15}$N-LRP labeled sample, protein was grown in M9 minimal media using $^{15}$N-NH$_4$Cl. Labeled protein was then phosphorylated with full-length Src (Upstate, Lake Placid, NY) as before. Equal amounts of $^{14}$N-GST-LRP-CT and $^{15}$N-GST-LRP-CT-P were bound to glutathione sepharose beads and mixed together. Beads were washed and incubated with thrombin as before and free LRP-CT was collected by centrifugation. Free protein was then used immediately for H/D experiments as above.

**GST-fusion pulldown assay for Snx17 interaction with GST-LRP-CTs.**

The murine Snx17 cDNA clone was obtained from the ATCC, amplified by PCR, and subcloned into the EcoRI and XbaI sites of the mammalian expression vector pcDNA3.1 modified with an amino terminal HA tag. A full-length construct (residues 1-470) of Snx17 was expressed transiently in 293 cells. Cells were transfected at 30% confluency using 1 µg of pcDNA3-HA-Snx17 with Transfectin (Biorad, Hercules, CA). Cells were lysed 48 h after transfection in HUNT buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 50 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 10 µg/mL aprotinin and leupeptin.

The phosphorylation dependence of the LRP-Snx17 interaction was tested in vitro in pulldown experiments in which mammalian expressed Snx17 (1-470) was probed using GST-LRP-CT fusion proteins as bait. Known amounts of each GST-LRP-CT fusion protein were bound to glutathione sepharose beads as
already described. 25 µg of GST-LRP-CT bound beads were mixed with 1 mL of cell lysate from HA-Snx17 transfected cells in HUNT buffer and incubated with rocking at 4°C for 2 hrs. Beads were collected by centrifugation and washed 3 times with HUNT buffer. Samples were boiled for 10 min in 2X SDS-sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes.

**Co-immunoprecipitation of Snx17 and LRP1**

293 cells were transiently transfected with 1 µg of myc-LRP1β, 1 µg of v-Src, and 2 µg of HA-Snx17 using Transfectin (Biorad, Hercules, CA). Cells were lysed 48 hr after transfection with 1 mL PLC-lysis buffer after washing with ice-cold TBS. Lysates were rocked for 1 hr with anti-HA antibody 12CA5 at 4°C, and then with Protein-G slurry (Upstate, Lake Placid, NY) for an additional hr. Immunoprecipitates were washed four times with PLC lysis buffer and analyzed by anti-HA or anti-LRP1 immunoblotting.

**Circular Dichromism and NMR.**

GST-LRP-CT and GST-LRP-CT-P were tested for structure of the LRP-CT by circular dichroism and nuclear magnetic resonance. CD experiments were performed with an Aviv 202 spectropolarimeter (Aviv Biomedical, Lakewood, NJ). Spectra are reported as mean residual ellipticity (MRE) as follows:

\[
MRE = \text{deg} / (10 \times L \times M \times \text{(# of peptide bonds)})
\]

Where deg is the CD signal, L is the cell pathlength in cm, and M is protein concentration in molarity.
For CD spectra, GST-LRP-CT bound to glutathione sepharose was cleaved with thrombin in a minimum volume, and the free LRP-CT was collected and concentrated to 63 µM in 25 mM Tris, 50 mM NaCl, pH 7.4. GST, GST-LRP-CT, and GST-LRP-CT-P were also tested so that the GST signal could be subtracted from the GST-LRP-CT signal in case the fusion protein would give a better spectrum than the free cleaved LRP-CT.

For NMR, $^{15}$N-GST-LRP-CT labeled sample was prepared as stated above. The sample was concentrated to 0.6 mM in 50 mM Tris, 150 mM NaCl in 90% H$_2$O /10%$^2$H$_2$O. NMR experiments were obtained on a Bruker DRX 600MHz spectrometer.

**Results**

Tyrosine phosphorylation of LRP-CT NPXY motifs occurs in a sequential manner

LRP contains two NPXY motifs in its cytoplasmic domain. It has previously been shown that Y4507 in the distal NPXY$_{4507}$ motif of LRP can be phosphorylated in response to stimulation with PDGF-BB and in v-Src transformed cells (Barnes, et al., 2003, Loukinova, et al., 2002). Barnes and coworkers also mentioned that Y4473 in the proximal NPXY motif could be phosphorylated if the LRP protein was first denatured, but did not follow up on this preliminarily observation (Barnes, et al., 2003). To further characterize the resistance of Y4473 to phosphorylation we carried out *in vitro* kinase reactions with recombinant Src using a GST-fusion protein containing the cytoplasmic
domain of LRP (GST-LRP-CT) and [γ-32P]-ATP as substrates. In a time-course experiment the wild type fusion protein continued to incorporate 32P for at least an hour as did the Y4473F mutant (Figure 2.1). In contrast, the Y4507F and the 2F (Y4473F, Y4507F) mutants fusion proteins proved to be poor substrates for phosphorylation by recombinant Src. To find out whether phosphorylation at Y4507 could affect phosphorylation at Y4473 we generated a mutant fusion protein in which Y4507 was replace by glutamic acid. This fusion protein incorporated substantial amounts of 32P and was shown to be a better substrate for recombinant Src than the Y4473F mutant (Figure 2.1). Thus substitution of Y4507 with glutamic acid to mimic phosphorylation at Y4507 allowed phosphorylation at Y4473 to occur. These data indicate that both NPXY motifs can be phosphorylated, however, the phosphorylation of Y4473 requires prior introduction of phosphate or a negative charge at Y4507.

Phosphopeptide mapping confirms phosphorylation of both of NPXY motifs in LRP

In order to unequivocally show dual tyrosine phosphorylation of both NPXY motifs in LRP we repeated the in vitro kinase reactions and analyzed phosphorylated proteins by phosphopeptide mapping. Wild-type GST-LRP-CT phosphorylated with full-length Src yielded two major 32P-containing peptides (Figure 2.2A). These peptides were identified by comparison of maps obtained from wild type and mutant proteins (Figure 2A-C). This analysis indicates that the main phosphopeptide seen on maps of the wild type protein represents the
Figure 2.1: Phosphorylation time course of GST-LRP and mutant forms. GST and GST-LRP wild-type, Y4473F, Y4507F, Y2F, and Y4507E were incubated in kinase buffer with γ³²P-[ATP] for 1 hr. Individual samples were removed at time points of 5, 10, 20, 30, and 60 min and resolved by SDS-PAGE. The top panel of each group represents the autoradiograph of the samples while the bottom panel represents a coomasie stained gel of the respective protein loading controls. The arrows point to LRP tyrosine phosphorylation and Src autophosphorylation.
Y4507 phosphorylation site, while the minor peptide represents the Y4473 phosphorylation site (Figure 2D). The relative intensities of the phosphopeptides indicated that the majority of phosphorylation was found on Y4507 while the Y4473 had lower intensity corresponding to a lower level of phosphorylation at this site. Possible phosphorylation of the other two tyrosine residues in the LRP cytoplasmic region was ruled out by use of a Y2F mutant control which did not incorporate any label. The results from this mapping experiment confirmed that either phosphorylation of Y4507 or substitution with glutamate results in phosphorylation of Y4473.

**Phosphorylation of NPXY_{4473} in vivo**

We have shown that Y4473 can be phosphorylated *in vitro*. To test whether Y4473 can also be phosphorylated *in vivo*, as had been shown for Y4507, we co-expressed the myc-tagged LRP-β chain (Myc-LRPβ) and v-Src (Barnes, et al., 2003, Loukinova, et al., 2002). Myc-LRPβ was isolated by anti-Myc immunoprecipitation antibodies and probed for tyrosine phosphorylation. In the presence of v-Src, we observed phosphorylation of the wild-type protein as well as the Y4473F, and Y4507E mutants (Figure 2.3). The highest level of phosphorylation was seen in the wild type protein (Figure 2.3). Phosphorylation of the Y4473F mutant was the second most intense, followed by the Y4507E. These results confirm that Y4473 can be phosphorylated *in vivo*. The Y4507E myc-LRPβ appeared to be a better kinase substrate then the Y4507F form, in
agreement with the *in vitro* results. The observation that the wild type protein was phosphorylated to a higher level than the Y4473F mutant again indicates that phosphorylation of the Y4473 residue by v-Src is occurring in these cells. Given that Y4473 can be phosphorylated *in vitro* and *in vivo*, but that it requires prior phosphorylation at Y4507, we surmised that the cytoplasmic domain might have structure constraints preventing phosphorylation by Src, but that phosphorylation at Y4507 might relieve these constraints.

*Structural characterization of the cytoplasmic domain of LRP*

Having confirmed the dual tyrosine phosphorylation of LRP, we sought to determine the mechanism behind the differential phosphorylation of the NPXY motifs. To test whether a negative charge at Y4507 leads to a change in accessibility of Y4473 we first utilized CD and NMR techniques to learn more about the structure of the LRP-CT. The cytoplasmic tail is 100 amino acid residues long and has no significant predicted structure according to homology or sequence predictors. The CD spectrum of the untagged cytoplasmic region suggested that it forms a random coil (Figure 2.4). Additional CD spectra collected on the GST-LRP-CT and GST and NMR spectra of $^{15}$N-labeled GST-LRP-CT substantiated the notion that the LRP-CT is relatively unstructured (Figure 2.5).

*H/D exchange of wild-type and phosphorylated LRP-CT shows increased solvent accessibility in the phosphorylated form*
Figure 2.2: Phosphopeptide mapping of GST-LRP-CT. Protein was incubated in kinase buffer with $\gamma^{32}$P-[ATP] for 15 min at 37ºC and then separated on an SDS-PAGE gel. The phosphorylated proteins were treated as described in “Materials and Methods”. The resulting TLC plate was then exposed to film to create a autoradiograph of the labeled phosphopeptides. A) Wild-type GST-LRP. B) Y4507E GST-LRP. C) Y4473F GST-LRP. D) Schematic of respective LRP phosphopeptides.
Figure 2.3: *In vivo* phosphorylation of LRP Y4473. 293 cells were transfected with different myc-βLRP constructs or control plasmid with (lanes 2, 4, 6, 8, 10, 11) and without (lanes 1, 3, 5, 7, and 9) the presence of v-Src. Myc-βLRP immunoprecipitates were probed for tyrosine phosphorylation by anti-P-Tyr immunoblotting.
In order to discover whether the LRP-CT was at all structured and whether phosphorylation affected the structure, we measured the amide exchange of the LRP peptides. The experiment involves incubating freshly prepared LRP-CT or phospho-LRP-CT in buffered deuterium for a set period of time, quenching the reaction by lowering the pH and temperature, and digesting the protein with pepsin. The resulting peptides were analyzed by mass spectrometry (Figure 2.6). The extent of exchange depends on a number of factors including whether or not the amide is sequestered from solvent because it is in a folded part of the molecule and/or is participating in a hydrogen bond. We identified a set of peptides that would allow us to monitor 82% of the protein, including the two NPXY motifs (Figure 2.7A). Upon phosphorylation, the peptides covering the NPXY$_{4507}$ motif are no longer observed. Surprisingly, much of the phosphorylated LRP-CT became more deuterated than the unphosphorylated protein (Figure 2.7 B-E). The difference was quantitatively significant at all time points measured (Table 2.1 gives the 2 min data). To control for the possibility of a systematic difference between the samples that may lead to such an overall difference in deuteration, we prepared a sample of the phosphorylated protein that was $^{15}$N-labeled and compared it to the unphosphorylated protein that was $^{14}$N-labeled in the same reaction mixture (Figure 2.8). This analysis confirmed the global differences in solvent accessibility between the unphosphorylated and phosphorylated proteins. The changes in solvent accessibility that occurred upon phosphorylation led us to ask if the same changes could be induced simply by the presence of a negative charge at the NPXY$_{4507}$ site.
Figure 2.4: CD spectrum of free LRP-CT. GST-LRP was cleaved with thrombin to generate the free cytoplasmic domain of LRP. The protein was concentrated and exchanged into a low salt buffer for sample preparation.
Figure 2.5: HSQC spectrum of $^{15}$N-GST-LRP. GST-LRP was prepared in minimal media with $^{15}$N-labeled ammonium chloride. Sample conditions were 0.6 mM $^{15}$N-GST-LRP in 50 mM Tris, 150 mM NaCl in 90% H$_2$O /10%D$_2$O. NMR experiments were obtained on a Bruker DRX 600MHz spectrometer.
To this end, we repeated the H/D exchange experiment with the Y4507E mutant. The overall deuterium incorporation of Y4507E mutant was higher than both the wild-type and phosphorylated protein. This was not surprising because at the time these experiments were performed, we did not have high activity Src and the phosho-LRP-CT was only approx. 30% phosphorylated. Due to the low activity of the Src only Y4507 and not Y4473 was phosphorylated. In fact, the peptide spanning residues 4492 – 4507 decreased in intensity in the pepsin digest of phosho-LRP-CT because some of it was phosphorylated, but the peptide spanning 4466 - 4475 did not decrease in intensity.

The effect of LRP tyrosine phosphorylation on interaction with Snx17

Sorting nexin protein Snx17 was recently shown to bind to the NPXY$_{4473}$ motif of LRP (van Kerkhof, et al., 2005). The dual phosphorylation of the LRP-CT lead us to investigate the effect of phosphorylation on the binding of Snx17 to LRP-CT. To determine if the phosphorylation state of the LRP-CT could effect its ability to interact with Snx17, we conducted in vitro binding experiments with unphosphorylated and phosphorylated GST-LRP-CT (and GST-LRP-CT-P) fusion proteins. Lysates of 293 cells expressing HA-tagged Snx17 were incubated with GST-LRP-CT fusion proteins immobilized on glutathione sepharose and bound proteins were analyzed by anti-HA immunoblotting. We observed specific binding of HA-Snx17 to GST-LRP-CT, but not to the GST control (Figure 2.9). Phosphorylation on Y4473 blocked binding. Binding was increased in the Y4473F-P and Y4507E mutants. These results suggest
Figure 2.6: Diagram of H/D exchange experiment. A) The protein is incubated in deuterated buffer for some time during which certain amide hydrogens will exchange with the deuterium. The reaction is then quenched and digested with pepsin. The resulting peptides retain the deuterium and can be observed as mass shifts in the spectrum by MALDI-TOF. B) A representative peptide from a protein region that is buried and not accessible to exchange will take on few if any deuterons. The top spectrum from a sample incubated in water and the middle and bottom panels are the same sample in D$_2$O after 2 or 5 min of exchange. The average mass envelope of a protected peptide does not shift much. C) A representative peptide from a surface loop that is accessible to exchange. The top spectrum is the sample in water and the middle and bottom spectra are the same sample in D$_2$O for 2 or 5 min of exchange. The average mass envelope shifts to higher masses as more deuterons are incorporated.
Figure 2.7: Increased deuterium exchange of LRP-CT upon phosphorylation. A) Pepsin cleavage of LRP-CT resulted in 9 peptides that covered 82% of the protein sequence as shown in the peptide coverage map. Tyrosine residues in context of NPXY motifs are marked in bold. B) The N-terminus of LRP-CT, which was covered by the peptide of mass 1249.72 (residues 4442 – 4452) showed higher deuteration in the phosphorylated form; (i) the peptide before deuteration of LRP-CT, (ii) the peptide after LRP-CT was deuterated for 30 sec, (iii) the peptide after phospho-LRP-CT was deuterated for 30 sec. C) Residues 4453-4465 of LRP-CT (the peptide of mass 1491.66) under the same conditions as in B. D) Residues 4466 – 4475 of LRP-CT which include the NXPY_{4473} (peptide of mass 1151.57) under the same conditions as in B. E) Residues 4492 – 4507 of LRP-CT which include the NXPY_{4507} (peptide of mass 1838.89) under the same conditions as in B except panel (iii) not shown because the intensity of the peptide signal decreased markedly due to phosphorylation. Data for the C-terminal region is not shown.
Table 2.1: Summary of H/D exchange data comparing wild-type LRP-CT and phosphorylated LRP-CT.

<table>
<thead>
<tr>
<th>Peptide (N to C terminal)</th>
<th>Coverage region</th>
<th>Number of Amides</th>
<th>Deuteration in LRP</th>
<th>Deuteration in LRP-P (pY4507)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1249.72</td>
<td>4442-4452</td>
<td>10</td>
<td>7.48 ± 0.05</td>
<td>8.45 ± 0.23</td>
</tr>
<tr>
<td>1377.61</td>
<td>4452-4464</td>
<td>11</td>
<td>7.29 ± 0.02</td>
<td>8.99 ± 0.27</td>
</tr>
<tr>
<td>1491.64</td>
<td>4453-4465</td>
<td>12</td>
<td>7.82 ± 0.21</td>
<td>9.73 ± 0.26</td>
</tr>
<tr>
<td>1151.57</td>
<td>4466-4475</td>
<td>8</td>
<td>4.93 ± 0.15</td>
<td>6.47 ± 0.14</td>
</tr>
<tr>
<td>1800.80</td>
<td>4465-4480</td>
<td>14</td>
<td>8.08 ± na</td>
<td>10.35 ± 0.04</td>
</tr>
<tr>
<td>1691.83</td>
<td>4493-4507</td>
<td>12</td>
<td>8.59 ± 0.04</td>
<td>Na</td>
</tr>
<tr>
<td>1838.89</td>
<td>4492-4507</td>
<td>12</td>
<td>11.33 ± 0.09</td>
<td>Na</td>
</tr>
<tr>
<td>1704.74</td>
<td>4511-4526</td>
<td>15</td>
<td>7.05 ± na</td>
<td>9.57 ± 0.14</td>
</tr>
<tr>
<td>1965.03</td>
<td>4527-4544</td>
<td>15</td>
<td>8.57 ± 0.46</td>
<td>12.19 ± 0.09</td>
</tr>
</tbody>
</table>
Figure 2.8: Wild-type and phosphorylated forms of the LRP1-CT were studied in the same sample by H/D exchange mass spectrometry by MALDI-TOF. An 15N labeled sample of GST-LRP1 was prepared and phosphorylated in vitro with full-length Src. The phosphorylated sample was then mixed 1:1 with the wild-type 14N GST-LRP1-CT sample and the mixture was bound to glutathione sepharose. After washing the slurry with TBS, the proteins were cleaved from the sepharose with thrombin. The free LRP1-CT mixture was then used immediately for H/D experiments. Shown in A is the representative spectrum of a single peptide, 1249.72, from the mixture. The first peak is the 14N-LRP1-CT 1249.72 peak, and the second peak corresponds to the 15N–LRP1-CT-phospho peak. Wild-type and phosphorylated samples, nondeuterated control (i), and wild-type and phosphorylated LRP1, 30 sec deuteration (ii).
strongly that the interaction between LRP and Snx17 is regulated directly or indirectly by tyrosine phosphorylation. The same phenomenon was observed in vivo. 293 cells were transfected with HA-Snx17 and mycLRPβ wild-type or Y4507E with and without the presence of v-Src (Figure 2.10). Using the HA-tagged Snx17 to immunoprecipitate myc-tagged LRP1β we observed similar results as our in vitro experiment. Binding to wild-type LRP was almost too weak to observe and the presence of v-Src wiped out any interaction. However, much stronger binding was seen with the Y4507E mutant, which again was lost upon phosphorylation by v-Src. This proves that Snx17 can not bind to LRP when Y4473 is phosphorylated.
Figure 2.9: Phosphorylation dependence of LRP-Snx17 interaction in vitro. 293 cells were transfected with HA-tagged Snx17 and cell lysates were incubated with various GST or GST-LRP-CT fusion proteins in phosphorylated or unphosphorylated forms bound to glutathione sepharose. Bound proteins were analyzed by anti-HA (top panel) or anti-LRP (bottom panel, loading controls) immunoblotting.
Figure 2.10: LRP-Snx17 interaction in vivo is modulated by phosphorylation. 293 cells were transfected with HA-tagged Snx17 and myc-LRPβ, with or without the presence of v-Src. HA-Snx was immunoprecipitated with anti-HA antibody and protein G slurry and bound proteins were detected by western blot with the appropriate antibodies. (Wild-type myc-LRPβ samples are not shown as binding was too weak to be observed with scanning).
Discussion

*Implications of sequential phosphorylation at the two NPXY motifs in LRP.*

The LRP-CT contains two NPXY motifs, the distal of which has been implicated in many signaling pathways (Barnes, et al., 2003, Loukinova, et al., 2002, Swertfeger, et al., 2002) and the proximal of which has been shown to be involved in receptor recycling and demonstrated a strong phenotype in knock-in mice (8,9). Phosphorylation of the distal NPXY\textsubscript{4507} motif allows binding of Shc (Barnes, et al., 2003, Barnes, et al., 2001) and may modulate binding of other adapter proteins as well. We previously reported that Y4507 is the principle phosphorylation site on LRP because tyrosine phosphorylation *in vitro* and *in vivo* was blocked by mutation of this residue (Barnes, et al., 2003). Phosphopeptide mapping studies reported here show that LRP is phosphorylated on two tyrosines that are represented by two phosphopeptides on the peptide map. Mutation of Y4507 results in disappearance of both phosphopeptides. Mutation of Y4473 results in the disappearance of one phosphopeptide, which most likely represents Y4473. Mutation of Y4507 to glutamate blocks phosphorylation on Y4507 but allows phosphorylation on Y4473. The results published previously and those reported here are consistent with a model in which the LRP-CT is phosphorylated in a sequential manner, in which phosphorylation of Y4507, or mutation to glutamate, is a prerequisite for phosphorylation of Y4473. Here we found that phosphorylation of Y4507 increases the solvent accessibility of Y4473. The increase in solvent accessibility is mirrored by an increase in accessibility to
Src. The increase in phosphorylation and solvent accessibility is also seen in the Y4507E mutant. The observation that phosphorylation of one NPXY motif changes the reactivity of the other is, as far as we know, novel. It hints at an interplay between the two sites that may affect which proteins bind where and when.

The interplay between the two NPXY motifs on LRP may be relevant to our understanding of APP processing. APP is a type I integral membrane protein of unknown function that is expressed on the cell surface (Selkoe, 2001). APP is subject to ectodomain shedding, carried out by α or β secretase, followed by intramembrane cleavage, carried out by γ-secretase (Golde, et al., 1992, Haass, et al., 1992, Sisodia, et al., 1990). This proteolytic processing gives rise to various products including Aβ, a major constituent of the amyloid plaques that play a role in the etiology of Alzheimer’s disease. LRP deficiency has been shown to cause a decrease in APP internalization and APP processing, resulting in a decrease in the levels of Aβ. This defect can be rescued by expression of an LRP β chain deletion mutant that lacks most of its extracellular domain. The ability of this mutant to rescue APP processing depends on the presence of the distal NPXY motif (Pietrzik, et al., 2002). LRP associates with APP indirectly via the adapter protein Fe65, a protein that contains two PTB domains, which are known to bind to NPXY motifs (Bork and Margolis, 1995, Pietrzik, et al., 2004). Over expression of the adaptor protein Fe65 changed cellular levels of the mature form of LRP and/or APP suggesting that processing of LRP and APP to their mature forms is linked by Fe65 binding (Guenette, et al., 2002, Guenette, et
al., 1999). These results would make sense if phosphorylation of the distal NPXY<sub>4507</sub> altered the binding of Fe65 to LRP, but much more work is required to prove this conjecture.

*Phosphorylation at Y4507 opens the structure of LRP-CT near Y4473.*

Amide exchange followed by mass spectrometry revealed different solvent accessibility of the two NPXY motifs in LRP. Residues 4467-4475, including the proximal NPXY<sub>4473</sub> motif, were one of the most solvent inaccessible regions in LRP, but upon phosphorylation this region showed significantly increased accessibility. Thus, the proximal NPXY<sub>4473</sub> motif appears to be sequestered from solvent and unavailable for phosphorylation by the Src in the native state of the protein, but when the distal site becomes phosphorylated, the proximal site becomes more exposed to both solvent and to the Src active site. This result helps to define a mechanism for the observed sequential phosphorylation.

LRP is one of many receptors that have short cytoplasmic domains. Little structural work has been done on these domains and structure prediction programs generally predict no regular secondary structure. The CD and NMR studies presented here indicate that the LRP-CT, like the APP cytoplasmic domain (APP-CT), is mainly random coil (Kroenke, et al., 1997). The APP-CT is approximately half the size of the LRP-CT and contains a single NPXY motif (NPTY<sub>687</sub>) which forms a type I reverse turn (Kroenke, et al., 1997). NMR structural studies revealed that phosphorylation affects the structure of the APP-CT, but not at the NPXY motif. Phosphorylation at T668 in APP-CT was shown
to alter the cis-trans ratio of P669 located in a second type 1 reverse turn comprised of residues TPEE$_{671}$ some 15 amino acids upstream from the NPXY. No long-distance structural changes were seen near the NPXY motif upon phosphorylation of T668 (Kroenke, et al., 1997, Ramelot and Nicholson, 2001). In contrast, phosphorylation of LRP-CT at the distal NPXY$_{4507}$ caused structural changes more than 30 residues away at the proximal NPXY$_{4473}$. Our results are consistent with the possibility that the LRP proximal NPXY$_{4473}$ is present within a type I reverse turn similar to that seen in APP-CT since a tight turn would reduce solvent accessibility and would restrict access to the Src active site. It is interesting that maintenance of the turn structure appears to depend on the sequence some 30 amino acids away. Introduction of a phosphate at Y4507 or replacement of Y4507 with a negatively charged glutamic acid appears to disrupt the turn and open up the Y4473 region.

**Phosphorylation of Y4507 alters binding events at Y4473.**

It is usually thought that phosphorylation alters binding at the site of phosphorylation. For example, binding of Shc and Dab1 localized to the NPXY$_{4507}$ motif and a few surrounding residues using peptides as the pull-down bait (Barnes, et al., 2001, Trommsdorff, et al., 1998) and phosphorylation at Y4507 was required for the binding of Shc in both the peptide and full LRP-CT pull-downs (Barnes, et al., 2003).

Binding of Snx17 aids in the proper recycling of the receptor as mutation of the Y4473 binding site and siRNA knockdown of Snx17 expression in U87
cells lead to a decrease in the distribution of LRP on the cell surface (van Kerkhof, et al., 2005). It has also been reported that Y4473 is necessary for correct basolateral sorting of LRP in polarized cells (Marzolo, et al., 2003). Snx17 interacts with the LRP-CT at the NPXY<sub>4473</sub>, but it was unclear what effect phosphorylation of LRP would have on the interaction (van Kerkhof, et al., 2005). Knowing that both tyrosine residues in the NPXY motifs could be phosphorylated and that phosphorylation of Y4507 changed the accessibility of NPXY<sub>4473</sub>, we sought to carry-out binding experiments with Snx17 under different phosphorylation conditions. By using a series of GST-LRP-CT mutants and their phospho forms, we determined that Snx17 prefers to bind to the unphosphorylated form of LRP. Phosphorylation of the LRP binding site at Y4507 in the Y4473F mutant, or replacement with a glutamate residue, enhanced binding of Snx17, but phosphorylation of the wild type protein abolished binding. Similarly, phosphorylation of the Y4507E mutant, which is only phosphorylated at Y4473, inhibited the interaction with Snx17 (Figure 2.7). These results conclusively show that Snx17 binding is modulated by phosphorylation of the LRP-CT, and recapitulate the sequential phosphorylation effects described earlier. Since it is known that Snx17 is involved in LRP recycling and vesicle sorting, these results suggest an interplay between control of the phosphorylation state of the LRP-CT and receptor sorting/recycling. Others have proposed that alteration of LRP recycling and/or sorting may affect APP processing and Aβ peptide production (Pietrzik, et al., 2002) and our results
suggest a mechanism by which phosphorylation of the LRP-CT may be important in this control.
References


CHAPTER THREE

Probing the Fe65-LRP Interaction
Introduction

Alzheimer’s disease is the leading neurological disorder affecting the elderly. The causes of the disease are still not clear but there are many hypotheses. LRP is implicated in the development of Alzheimer’s disease (AD) in several ways (Kang, et al., 1997). There are some genetic polymorphisms that seem to be tied to AD (Carter, 2007, Hollenbach, et al., 1998, Panza, et al., 2006). Also, several LRP ligands are found in the plaques that are hallmarks of AD (Rebeck, et al., 1995). One particular ligand, ApoE, has a strong effect. ApoE (Apolipoprotein E) has three variants, ApoE2, E3, and E4. One variant (E2) is protective towards AD, while another (E4) increases the chances one has of developing AD (Benjamin, et al., 1994, Beyer, et al., 2002, Corder, et al., 1993, Saunders, et al., 1993, Strittmatter, et al., 1993).

Another ligand with strong ties to LRP is the amyloid precursor protein (APP). APP is a transmembrane receptor of unknown function. APP can be cleaved by 3 different secretases (Ikezu, et al., 1998, Selkoe, 2001). If the receptor is cleaved by beta secretase followed by gamma secretase, the Aβ peptide is released into the exoplasm (Hardy, 1992). This Aβ peptide is the hallmark component of the plaques that mar the brains of AD patients. LRP can bind this peptide, in complex with a carrier like ApoE, and internalize the ligand into the cell (Haas, et al., 1997, Kounnas, et al., 1995, LaDu, et al., 1997, Narita, et al., 1997, Pillot, et al., 1999, Qiu, et al., 1999). It is also possible that the two receptors can dimerize at the cell surface (Ulery, et al., 2000). Finally the
cytoplasmic tails of the two receptors both bind simultaneously the adaptor protein Fe65 (Pietrzik, et al., 2004).

Fe65 was first discovered as a mRNA highly expressed in rat brain. The Fe65 family actually contains three proteins; Fe65, Fe65L1, and Fe65L2 (Duilio, et al., 1998, Guenette, et al., 1996). The major difference in these proteins is the size of the region N-terminal of the WW domain and their distribution (Figure 3.1). Fe65 mRNA is mostly expressed in the brain, whereas human Fe65L1 mRNA is ubiquitously expressed and rat Fe65L2 mRNA is found in the brain and the testis (Russo, et al., 1998). All three proteins have all been shown to bind to APP (Guenette, et al., 1999, Tanahashi and Tabira, 2002). Double knockout mice, lacking both Fe65 and Fe65L1, exhibit severe defects of the central nervous system (Guenette, et al., 2006), a result similar to experiments using APP triple knockout mice.

Fe65 contains a WW domain, which can interact with proline rich sequences, and two PID domains (Duilio, et al., 1991). The PID domain is another name for the PTB domains although PID is often used for interactions that can be phosphotyrosine independent. The WW domain has been shown to bind to Mena (mammalian enabled) (Ermekova, et al., 1997), possibly connecting Fe65 to cytoskeleton rearrangement, based on Mena’s high abundance in focal contacts and interactions with actin. The N-terminal PID, or PID1, interacts with the transcription factor CP2/LSF/LBP-1 (Zambrano, et al., 1998) and LRP (Trommsdorff, et al., 1998). This PID can also interact with Tip60, another transcription factor regulator (Cao and Sudhof, 2001).
Figure 3.1: Fe65 family members with their domain structure. Taken from (Russo, et al., 1998). The sequence numbering used for comparison is rat Fe65, human Fe65L, and rat Fe65L2.
This leads to the possibility that Fe65 is involved in the transactivation of genes. In fact, there is evidence that the APP-CT is translocated to the nucleus through its interaction with Fe65/Tip60 complex (Cao and Sudhof, 2001).

The PID2, or C-terminal PID domain interacts with APP, and its family members APLP1 and APLP2 (Bressler, et al., 1996, Fiore, et al., 1995, Guenette, et al., 1996). This interaction, along with the PID1/LRP interaction, connects Fe65 to Alzheimer’s disease (Figure 3.2). Much work has been focused on the effects of Fe65 on APP and LRP in relation to AD. However, the specifics of this interaction and its implications are not yet clear. Results from different investigators vary, especially when different cell lines are used for study. For example, in 293 cells transfected with Fe65, Ando et al. found that levels of Aβ, APP secretion, and APP maturation were all reduced (Ando, et al., 2001). However, Guenette et. al. found that in H4 neuroglioma cells APP maturation and APP secretion were increased under the same conditions (Guenette, et al., 1999). They also discovered later that LRP levels were decreased in these same cells (Guenette, et al., 2002). Finally, another group observed increased APP maturation, secretion, and Aβ production when APP was over-expressed in a stable Fe65-expressing MDCK cell line (Sabo, et al., 1999). Some parts of the story are clear though. For example, it is known that Fe65 interacts with APP using the PID2 domain and the NPXY motif of APP (Duilio, et al., 1998, Fiore, et al., 1995, Guenette, et al., 1996). The PID1 domain of Fe65 interacts with LRP (Trommsdorff, et al., 1998). We wished to further study the interaction between
Figure 3.2: Diagram of LRP-Fe65-APP interaction. Much work has been done detailing the role of LRP and Fe65 in the process of APP. APP cleavage can result in Aβ peptide, a major component of AD plaques. Fe65 is believed to bridge the two receptors via its PTB (or PID) domains. Taken from (King and Scott Turner, 2004)
Fe65 and LRP to see if it was phosphorylation dependent and to better define the regions required for binding. We also wanted to test if the PID2 domain would have any effect on the Fe65-LRP interaction.

**Materials and Methods**

*Expression and Purification of Fe65*

The full-length Fe65 (WW PID1PID2) cDNA (residues 236-708) and a cDNA encoding just the WW PID1 domains (residues 236-514) were cloned into a pHis8 vector. Proteins were expressed in NZY media and induced overnight at 18°C with 0.1mM IPTG. The cell pellet was resuspended in 50 mM Tris, 150 mM NaCl, 1mM PMSF, 1 mM BME, pH 7.4 and sonicated on ice. The resulting supernatant was centrifuged for 30 minutes at 12,000 rpm at 4°C. The cleared lysate was added to 5 mL of prewashed NTA-Ni slurry and incubated at 4°C for at least 1 hr. The slurry was washed extensively with 20 mM imidazole in TBS until the protein absorbance of the wash was about zero. The protein was then eluted with 8mL of 250 mM imidazole in TBS. The protein was then further purified from contaminating proteins by running over a S-200 size exclusion column using TBS. Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL).

*Expression of LRP-CT fusion protein and GST-APP-CT*
For GST-LRP-CT proteins, cDNA encoding the entire cytoplasmic domain of LRP1 (residues 4444-4544) was cloned into pGEX-4T2. GST fusion proteins were expressed in pLysS cells and induced with 0.1mM IPTG overnight at room temperature. Briefly, the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl (TBS), 1mM benzamidine, 1 mM DTT, 1ug/mL leupeptin, aprotinin), sonicated on ice, and centrifuged for 30 min at 10,000 rpm. The supernatant was incubated with 10 mL of glutathione sepharose for 1 hour at 4ºC. Resulting slurry was then washed exhaustively with TBS, and fusion proteins were eluted as 1 mL fractions with 10mM reduced glutathione in TBS, pH 7.4.

We found that maintaining the LRP1 cytoplasmic domain on GST reduced aggregation and improved stability of the protein, so this was how all of the protein was prepared. Eluted protein was dialyzed overnight in TBS, 4ºC and the amount of protein obtained was determined by BCA assay (Pierce Biotechnology, Rockford, IL). Mutations in the LRP1 cytoplasmic tail at Tyr 4473 and Tyr 4507 were introduced using Stratagene Site Directed Mutagenesis Kit and confirmed by DNA sequencing. A double mutant, termed Y2F, was also created combining phenylalanine mutations at both Y4473 and Y4507. The entire cytoplasmic domain of APP was cloned in pGEX-4T2 and expressed and purified as stated above for GST-LRP-CT.

Ubq-LRP-CT – The cDNA encoding the entire cytoplasmic domain of LRP was cloned into a pHis8 vector which had been modified with an N-terminal ubiquitin tag (including a P->G mutation at the fusion site to reduce proteolytic
cleavage). Ubiquitin fusion proteins were expressed in BL21 DE3 cells in 1L of LB and induced overnight with 0.1 mM IPTG. The cell pellet was resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, 1mM BME, 1 µg/mL leupeptin and aprotinin, and 1mM PMSF, pH 7.4), sonicated on ice (30 sec pulses over 2 minutes) and centrifuged for 30 minutes at 12,000 rpm. The supernatant was incubated with 5 mL of Ni-NTA resin (Qiagen) for 1 hour at 4°C. The slurry was washed until elute A280 was near zero with 20 mM imidazole, 1mM BME, 50 mM Tris, 150 mM NaCl, pH 7.4. Proteins were eluted as one fraction with 250 mM Imidazole in TBS, pH 7.4 and separated from contaminating proteins by size exclusion chromatography (S-75 column with 50 mM Tris, 150 mM NaCl, pH 7.4). Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL).

**GST Pulldowns**

Equal amounts of GST-LRP-CT in phosphorylated and non-phosphorylated forms were bound to glutathione sepharose beads at 1mg/mL (as 50% slurry). The amount of protein on the beads was measured by BCA assay (Pierce). To an eppendorf tube was added 10 µg of Fe65 (WW PID1 or WW PID1PID2), 50 µL of GST-LRP-CT slurry (for 25 µg of bound LRP1 protein), and 800 µL of TBS-X buffer (50 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% Triton-X-100, pH 7.4). Reactions were allowed to incubate at 4°C for 2 hours. Samples were spun down and washed 4 times with TBS-X buffer, aspirated to dryness, and resuspended in 40 µL of 2X reducing sample buffer.
Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed for either bound Fe65 with anti-His antibody (1:3000, Qiagen, Penta-His Ab) or loading controls with anti-LRP1 antibody 11H4 (1:500).

**Peptide Pulldowns**

Purified recombinant Fe65 (WW PID1) was incubated with 10 µL of NPXY_{4507} peptide in PLC-lysis buffer for 4ºC for 2 hours. Samples were spun down and washed 4 times with PLC buffer, aspirated to dryness, and resuspended in 40 µL of 2X reducing sample buffer. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed for bound Fe65 with anti-His antibody (1:3000, Qiagen, PentaHis Ab).

**Trypsin Digestion of Ubq-LRP-CT or Ubq-LRP-CT/Fe65 complex**

The his-tag of Ubq-LRP-CT was cleaved with thrombin by incubating 250 µg of Ubq-LRP-CT in a total volume 500 µL of TBS with 2.5 mM CaCl₂ and 1:100 dilution of thrombin. The reaction was rocked at room temperature for 1 hr and then 100 µL of NTA-Ni beads was added to the tube, rocking, for another hr. This step was repeated once to ensure that any uncleaved HisUbq-LRP-CT was removed from the solution. The beads were pelleted by centrifugation and the supernatant with Ubq-LRP-CT (no his tag) was collected. His-tagged WW PID1 Fe65 (25 µg) was bound to 25 µL of NTA-Ni beads by rocking in solution for 1 hr at room temperature. The beads were spun and washed three times with TBS and left in a small volume (100 µL). To this sample was added 50 µg of Ubq-
LRP (100 µL) and 400 µL TBS-X, rocking at room temperature for 1 hr. The sample was then centrifuged, washed with TBS-X three times to remove nonspecific binding, and aspirated to dryness. The sample was resuspended in 100 µL TBS and 2 µL of trypsin was added. A sample of Ubq-LRP-CT 100 µL (50 µg) was set up in parallel with 2 µL of trypsin. The reactions were incubated in a 37°C heat block for 1 hr with mixing every 10 min. The samples were centrifuged and the supernatant collected for MS/MS on a QSTAR Elite hybrid QqTOF mass spectrometer and analysis with Analyst software (Applied Biosystems) and Mascot (Matrix Science).

Generation of Fe65 Antibody

Full-length (residues 236-708) his-tagged Fe65 was prepared as detailed above. The his tag was removed by thrombin cleavage (50 mM Tris, 2.5 mM CaCl₂, 1:100 thrombin, rocking, room temp, 1 hr) after the protein had been eluted from the nickel resin with 8 mL of 250 mM imidazole in TBS and dialyzed into TBS. The cleaved product was then further purified by size-exclusion chromatography (S-200) in TBS, pH 7.4. To generate Fe65 polyclonal antibody, two New Zealand white rabbits were injected with a mixture of Fe65 and adjuvant. For the first immunization, 400 µg of Fe65 in 1 mL of buffer was mixed with 1 mL of Freund’s Complete Adjuvant (Sigma, St. Louis, MI) in two glass syringes with a luer-lock connector. The resulting 2 mL was split between the two rabbits for injections each time. Every two weeks, approximately 40 mL of blood was collected from each rabbit, and they received an immunization boost.
consisting of 200 µg Fe65 in 1 mL and 1 mL of Freud’s Incomplete Adjuvant (Sigma, St. Louis, MI) prepared just as before. The bleeds were prepared by incubating in a 37ºC water bath for 45 min with a wooden stick in the center of the corning tube. Next the clot was separated from the sides of the corning tube by scraping the edges with another wooden stick. The tube was covered with parafilm and placed at 4ºC overnight. The serum was then decanted from the blood clot into a centrifuge tube and spun at 10,000 rpm for 15 min. The serum supernatant was then collected and stored at 4ºC for immediate use or at 20ºC for long-term storage.

Results

*Fe65 binds to LRP in vitro with a weak dependence on phosphorylation*

As mentioned above, Fe65-LRP interactions have clear implications for Alzheimer’s disease, but the sites of interaction of the two proteins were unknown. To test for phosphorylation dependence the GST-LRP-CT proteins unphosphorylated and phosphorylated forms were used for pulldown assays with various Fe65 constructs. We used two different Fe65 constructs, one containing the WW domain and both PIDs, and one containing just the WW domain and PID1 (Figure 3.3). The WW PID1 (MW = 32,715) and GST-LRP-CT (MW =37,000) were overlapping on an SDS-PAGE gel so the interactions were probed by western blot. Furthermore, the Fe65 antibody reacted non-specifically with the GST-LRP-CT proteins on the membrane, and so we chose to use the His-tag
of the Fe65 protein as our probe. Also note that we chose to work with the shorter Fe65 construct and not the “true” full-length 97kD form. Fe65 is proteolytically cleaved near the N-terminus of the WW domain to a 65kD form in most cells, although the ratio of 97kD to 65 kD Fe65 is species and cell dependent (Hu, et al., 2005).

We discovered that the Fe65-LRP interaction was buffer dependent. When high detergent buffer was used such as PLC (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM MgCl2, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 500 µM Na3VO4, 1 mM PMSF, 10 µg/mL aprotinin and leupeptin), no interaction between Fe65 and LRP was observed. However if we used a less stringent buffer such as 50 mM Tris, 150 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, and 1% Triton-X-100, termed TBS-X for the rest of this work. Pulldowns with GST, GST-LRP-CT, and GST-LRP-CT-phospho and Fe65 showed a small, approximately 2-fold increase in Fe65 binding to LRP in the presence of phosphorylation (Figure 3.4).

*Fe65 binding is enhanced by upstream LRP residues*

We previously designed an additional LRP construct, termed 5up, which included 5 residues from the LRP transmembrane domain in the GST-LRP-CT sequence. This protein, when incubated with recombinant Fe65, exhibited stronger binding than the wild-type GST-LRP-CT protein (Figure 3.5). Again, interaction was slightly enhanced by LRP phosphorylation. We performed a proteolytic cleavage protection assay with the Fe65-LRP complex. A complex of ubiquitin fusion LRP-CT (Ubq-LRP-CT) and Ni-bound WW PID1 Fe65
Figure 3.3: Fe65 constructs used for *in vitro* experiments. Proteins were expressed and purified in *E.coli* with an N-terminal His8 tag. Shown on the bottom is the GST-LRP-CT construct with the respective NPXY motifs. GST-LRP-CT could be phosphorylated *in vitro* with recombinant expressed Src in kinase buffer. Wild-type phosphorylated LRP would be phosphorylated on both Y4473 and Y4507.
Figure 3.4: GST-LRP-CT pulldown of Fe65 WW PID1. Purified Fe65 was mixed in TBS-X buffer with various GST, GST-APP-CT controls, or GST-LRP-CT mutants and their phosphorylated forms bound to glutathione sepharose. Complexes were spun, washed, and bound proteins were analyzed by western blot with anti-His antibody.
Figure 3.5: GST-LRP-CT pulldowns with Fe65 (WW PID1). Top panel: Schematic of 5up LRP construct. Bottom panel: GST proteins bound to glutathione sepharose beads were incubated with His-tagged Fe65. Beads were spun and washed, and bound proteins were analyzed by anti-His immunoblotting. No binding was observed with GST or GST-APP-CT controls. There was a small, 2 fold difference in binding between phosphorylated and nonphosphorylated forms of GST-LRP-CT (as determined by ImageJ quantification of bands). Notice that the inclusion of the transmembrane residues LRP in the 5up construct increases the binding between LRP and Fe65. The extra band in phosphorylated lanes corresponds to recombinant His-tag Src used to phosphorylate LRP that was still retained on GST-LRP-CT.
was formed, washed, and digested with trypsin at 37ºC for 1 hr. A reaction with just Ubq-LRP-CT was set up in parallel. The ubiquitin fusion construct of LRP-CT was used instead of GST-LRP-CT because the ubiquitin fusion was not digested as readily and thus reduced the complexity of the samples. The reactions were spun down and the resulting tryptic peptides were removed from the supernatant and subjected to MS/MS analysis on a QSTAR Elite hybrid QqTOF mass spectrometer. We found the N-terminal region of LRP was protected in the Fe65-LRP complex sample. Analysis of the chromatographic ion peaks showed that an N-terminal peptide (RVQGAKGFQHQR) present in the Ubq-LRP-CT was absent from the Ubq-LRP-CT/WW PID1 complex sample (Figure 3.6B, D). A control peptide present in both spectra (MTNGAMNVEIGNPTYK) however displayed similar intensity, indicating that the absence of the N-terminal peptide from the complex sample was not the result of weaker signal (Figure 3.6A, C).

*Fe65 binding requires contacts outside of the NPXY$_{4507}$ motif*

We have access to a small agarose linked peptide of the LRP1 sequence surrounding the NPXY$_{4507}$ motif (Sequence = TNFTNPVY/pATLY). Binding of endogenous brain lysate Fe65 in pulldowns using these peptides was never observed. This was puzzling as many reports have shown that binding of Fe65 to LRP is dependent on the NPXY$_{4507}$ region. For example, Pietrzik et. al. found that only deletion of the NPXY$_{4507}$ resulted in abrogated APP processing, presumably through Fe65 binding. These data suggested the NPXY$_{4507}$ peptide,
as representative of the Fe65 binding site, should be able to pull out Fe65. One explanation for never observing this binding with rat brain lysate was that the endogenous form of rat Fe65 is a longer 97kD version. To test the ability of the peptides to interact with our Fe65 construct we incubated the NPXY_{4507} and NPXY_{4507}-P peptide with purified recombinant Fe65 WW PID1 in TBS-X buffer and probed the samples for Fe65 binding. No interaction was observed between the peptide and Fe65 under conditions where binding was observed with GST-LRP-CT (Figure 3.7).

**Fe65 PID2 domain blocks binding of LRP to Fe65**

We compared the ability of LRP to interact with two different forms of Fe65, a full-length construct of the WWPID1PID2 domains and a shorter construct which lacks the C-terminal PID2 domain. There is some evidence in the field that there are intramolecular interactions between the different domains of Fe65, such that the PID1 domain may be inaccessible under some conditions. We performed GST-LRP-CT pulldown experiments with His-tagged constructs of Fe65. Fe65 WW PID1PID2 was incubated for 2 hours at 4°C in TBS-X buffer with glutathione sepharose bound GST-LRP-CT or GST-LRP-CT-phospho or GST control (Figure 3.8). We observed little to no binding of FL Fe65 to either GST-LRP-CT protein, but binding was increased by transmembrane residues located in 5up LRP. This result would support the idea that the PID2 domain of Fe65 blocks binding interactions at the PID1 domain, and that Fe65 binding specificity also relies on contacts outside of the NXPY motif. We also observed
Figure 3.6: Proteolytic cleavage protection of Ubq-LRP-CT amino terminus in LRP/Fe65 complex. Ubq-LRP-CT or Ubq-LRP-CT complexed with WW PID1 Fe65 was subjected to trypsin digestion. The tryptic peptides were separated and analyzed by MS/MS on a QSTAR Elite hybrid QqTOF mass spectrometer. Shown above is the extracted ion chromatogram for the monoisotopic peak of a control peptide (MTNGAMNVEIGNPTYK) present in equal intensity in both the C) Ubq-LRP-CT spectrum and in the A) complex spectrum. In comparison, the extracted ion chromatogram for the monoisotopic peak representing an N-terminal peptide (RVQGAKGFQHQR) in D) Ubq-LRP-CT spectrum and in the B) complex spectrum. The arrow points to the chromatographic peak for the RVQGAKGFQHQR peptide, which is absent in the complex spectrum B) above.
Figure 3.7: The LRP NPXY\textsubscript{4507} motif is not sufficient for Fe65 binding. The LRP NPXY\textsubscript{4507} peptide (pep) and the phosphorylated form (pep-P) were incubated with purified recombinant Fe65, either WW PID1 or WW PID2 PID2. Samples were probed for Fe65 binding by anti-His immunoblotting.
Figure 3.8: GST-LRP-CT pulldowns with Fe65 FL (WW PID1 PID2). GST proteins bound to glutathione sepharose beads were incubated with His-tagged Fe65. Beads were spun and washed, and bound proteins were analyzed by anti-His immunoblotting. No binding was observed with GST and little to no binding was seen with wild-type LRP. There was a small enhancement of binding to the phosphorylated form of GST-LRP-CT. Notice that the inclusion of the transmembrane residues LRP in the 5up construct increases the binding between LRP and Fe65.
a small enhancement of Fe65 binding to the phosphorylated LRP form, similar to what was observed with WW PID1 binding.

Discussion

Protein-protein interactions are complex and modulated by many factors, including phosphorylation, conformational changes, inhibitory molecules, and other post-translational modifications. When studying interactions such as LRP1 and Fe65 it is important to remember that these components inside the cell are in a constant state of change. For example, LRP recycles between the cell surface and the endosome. The amount of LRP on the cell surface therefore is always changing. These events are then further perturbed by external stimuli, such as PDGF-BB stimulation, that can result in LRP1 phosphorylation (Loukinova, et al., 2002).

Using purified, recombinant protein in pulldown assays several important factors about the Fe65-LRP interaction were determined. First, the binding of Fe65 to LRP1 was slightly dependent on phosphorylation. The number of phosphorylation sites did not matter, as wild-type phosphorylated LRP (Y4473p/Y4507p) bound just as well as Y4473F LRP (Y4507p). Using this same method we also observed that Fe65 binding to LRP was enhanced by the inclusion of upstream transmembrane residues of the LRP sequence. Finally, we found that the NPYX\textsubscript{4507} motif alone (Sequence TNFTNPVY/pATLY) was not
sufficient for Fe65 binding, as a peptide based on this sequence was not able to
bind to Fe65.

These data suggests two possibilities. One, it is possible that Fe65 does not bind to the NPXY$_{4507}$ region of LRP, but to a different part of the sequence. The second possibility is that Fe65 does in fact bind to this region, but the binding is weak and thus also depends on contacts outside of the NPXY$_{4507}$ motif. Our studies alone demonstrating tighter binding with the 5up LRP construct and the inability of the NPXY$_{4507}$ peptide to bind Fe65 support these two theories, but do not favor one over the other. The small increase in Fe65 binding observed to phospho-LRP correlates with the stronger binding to the 5up LRP construct. As discussed in Chapter 2, phosphorylation at Y4507 opens up distant regions of the protein such that the N-terminus of LRP is more accessible in the phosphorylated form of LRP. If Fe65 does indeed bind to the juxtamembrane region of LRP, then phosphorylation of the protein would make those regions more accessible for Fe65 binding.

The second theory is supported by work by other investigators such as the finding that the Fe65-LRP complex in cells was only blocked when a double NPXY deletion LRP construct was co-expressed with Fe65 in HEK 293T cells transiently expressing Flag-Fe65 and a LRP-CT construct (Pietrzik, et al., 2004). Fe65-LRP complexes could be recovered in the presence of both LRP-CTΔ1 (lacking residues 4469-4484) and LRP-CTΔ2 (lacking residues 4486-4507), respectively. This would suggest that Fe65 can bind to either NPXY motif. In the
context of our peptide experiment we would propose that Fe65 binding requires at least one LRP NPXY motif and residues outside of this sequence.

It is difficult to reconcile results of these and other binding experiments with biological tests that sought to link LRP to APP processing. For example, in LRP-deficient CHO 13-5-1 cells transiently co-expressing an APP695 construct and an LRP-CT construct, any deletion to the NPXY$_{4507}$ motif resulted in abrogated APP processing (Pietrzik, et al., 2002), while loss of the NPXY$_{4473}$ motif had no effect. If APP processing by LRP is in fact mediated by Fe65 acting as a bridge between the two proteins then this experiment would suggest that Fe65 can discriminate between the two NPXY motifs.

There are several explanations for these contrary results. One, the results could be cell type dependent. One experiment was performed using HEK 293T cells and saw no dependence on the NPXY motif, while the second experiment in CHO 13-5-1 cells saw the opposite. Second, there could be other factors involved in the APP processing experiment then just formation of an LRP-Fe65-APP complex. It is possible the while LRP-Fe65 binding does not solely depend on the NPXY$_{4507}$ motif, the signaling and processing machinery for LRP-mediated APP processing could depend on that sequence. It is likely that both of these factors are involved, which makes our *in vitro* studies very interesting as we can examine binding interactions without the variables of changing cellular components or cell types.

A final variable in this complex interaction is the nature of the Fe65 itself. Fe65 is a 97kD protein with three defined protein-protein interaction domains; a
WW domain, and an N-terminal and C-terminal PID domain. Often overlooked, but still important, is the region N-terminal to the WW domain, which is highly negatively charged and contains potential serine phosphorylation sites within conserved (S/T)PXX or (S/T)(D/E)_3 motifs. In fact, this region has been shown to be phosphorylated, and may play some role in targeting of the molecule between the cytoplasm and the nucleus (Sabo, et al., 1999, Zambrano, et al., 1998). Phosphorylation of the amino-terminus of Fe65 may either inhibit its interaction with certain proteins such as LRP1, or could target Fe65 to different regions of the cell and thus change the pool of interacting proteins.

Another important characteristic of Fe65 is the evidence that the two PID domains may interact with each other (Cao and Sudhof, 2004, Hu, et al., 2005). This theory proposes that the PID2 domain swings back onto the PID1 domain and blocks any interactions at this region. It is then necessary for APP (or another PID2 binding protein) to first bind to the PID2 region to open up the protein and allow access to the PID1 domain. Our data supports this theory of PID2 inhibition. GST-LRP-CT was unable to pulldown full-length Fe65, but could interact with the protein if the PID2 domain was lost.

One question that could arise from these cell-based experiments with APP processing is the phosphorylation state of LRP. Our in vitro studies indicate that this particular question does not matter since we found that phosphorylation had only a minimal affect on the Fe65-LRP interaction. There is also the question of what form of Fe65 is present in the cell. There is evidence that both the amino-terminus with its many potential phosphorylation sites and the carboxy terminal
PID domain can regulate Fe65 interactions with other proteins (Cao and Sudhof, 2004, Hu, et al., 2005, Zambrano, et al., 1998). Our results agree with these studies suggesting that the presence of the PID2 domain of Fe65 limits the interactions at the PID1 domain. It may be important to more greatly consider this factor when dealing with processes involving Fe65.

The next step for this project will be to study structural data on the Fe65-LRP interaction and PID intramolecular interaction. H\(^{2}H\) exchange mass spectrometry is an excellent tool to address these questions. If the two PID domains do interact with each other then we will be able to observe protection of those regions by decreased deuterium incorporation of the respective peptides. In this same manner we should also be able to map the Fe65 binding site to LRP. This may prove more difficult however if the binding is weak or promiscuous, as our data indicates. If Fe65 does not discriminate between the two NPXY motifs, then we may observe protection all across the LRP molecule. However, even that result would be informative, as it would tell us that Fe65 does not have a definitive binding site on LRP.
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CHAPTER FOUR

Directed Proteomics
**Introduction**

Proteomics is a powerful and growing field of biochemistry in the last 10 years due to commercialization of techniques and reagents. Proteomics is the complete dissection of the protein world through mass spectrometry. Common methods for identifying proteins from mixtures include 2D gel electrophoresis, followed by staining, protease cleavage of protein bands, MS/MS, and analysis. Analysis software includes web-based programs such as Mascot (www.matrixscience.com), ProteinProspector (http://prospector.ucsf.edu), or Sequest. In general these programs attempt to identify the protein in question by a database search against the input MS/MS or MS peptide data. The results are scored based on how well the calculated versus experimental data match up. The more peptides one can match to a protein, the higher the confidence that the identification is correct.

Constant improvements to these techniques over time have lead to more automation and more powerful instruments. The low analyte concentration requirement is one of the great advantages of mass spectrometry. Techniques now developed with mass spectrometry have been utilized to examine protein-protein interactions on large scale samples, map sites of post-translational modifications, test changing protein levels in response to specific stimuli, and much more.

One method for investigating protein interactions is the use of affinity peptides or proteins (Figeys, et al., 2001, Wilhelmsen, et al., 2004). In this case,
the researcher is interested in studying the interaction of a particular protein, or sequence within a protein, with the native cellular components. The protein or sequence is covalently linked to a bead support by various chemistries (amide, sulfo, biotin linkage, etc). The resulting slurry is then incubated in tissue or cellular lysate of choice for a period of time and then washed extensively to remove non-specific binding proteins. Interacting proteins still left on the bait are eluted and separated by gel electrophoresis and again identified by MS/MS sequencing (Figure 4.1).

In collaboration between the Komives and van der Geer groups, several proteins that interacted with the second NPXY motif of LRP1 were identified. A 12 amino acid long peptide of the sequence TNFTNPVY/pATLY (NPXY$_{4507}$ or NPXY$_{4507}$-phospho) was used as a bait in both a non- and phosphorylated version of NPXY tyrosine residue. Testing several different protein sources (liver and brain lysates, A431 cells, S7A cells) we noticed many protein bands that interacted specifically with the phosphorylated peptide (Figure 4.2). These bands were then excised from the gel, digested with trypsin, and prepared for MS/MS identification of the proteins. Several kinases (such as casein kinase I (CKI), calcium-calmodulin-kinase II (CaMKII), Src, and Csk) were shown to interact with LRP. Many of these were confirmed by western blotting the same pulldowns with specific antibodies to the kinases. Given the discovery that the first NPXY motif was shown to bind Snx17, (van Kerkhof, et al., 2005) further directed proteomics experiments using the full GST-LRP-CT fusion protein phosphorylated by Src
Figure 4.1: General schematic for the MS/MS identification of protein bands from a stained gel through in-gel digestion.
Figure 4.2: MS/MS identification of proteins that associated with the LRP NPXY_{4507}-P motif. LRP affinity peptide beads for NPXY_{4507} and NPXY_{4507}-P sequences were incubated with rat brain lysate. Bound proteins were visualized by Gel Code Blue staining, desired bands were excised from the gel, and prepared for in gel digestion with trypsin and subsequent MS/MS and analysis for identification.
and a NPXY\textsubscript{4473} based affinity peptide (Sequence VEIGNPTYKMYEGGE, with version of Y and Yp) were undertaken.

**Materials and Methods**

*Preparation of cell or tissue lysate*

A 50 mL volume homogenizer was prechilled and kept in an ice bucket at all times. Frozen rats brains were ground into a fine powder with a mortar and pestle while immersed in liquid nitrogen. The powder was immediately transferred to the chilled homogenizer with 10 mL of PLC buffer. Another 10 mL of PLC buffer was used to rinse the homogenizer. The rat brain powder was then homogenized on ice with 10 strokes of pestle A and 10 strokes of pestle B. The rat brain supernatant was then centrifuged for 15 min at 12,000 rpm. The supernatant was transferred to a new tube and centrifuged again. This process was repeated until a clear lysate was obtained. The lysate was diluted with PLC buffer so that the final volume was approximately 1 brain in 20 mL. This resulted in an average protein concentration of 25 mg/mL.

*GST-LRP-CT or affinity peptide pulldown*

GST-LRP-CT proteins were prepared as stated in Chapter 2, “Materials and Methods”. Phosphorylated forms were generated by either *in vitro* kinase reaction with v-src (commercial or expressed) or from expression in TK cells. 50 µg of bound GST-LRP-CT was incubated with 1 mL of diluted rat brain lysate (5
µg/µL by BCA assay) at 4ºC for 2 hours. Samples were washed five times with PLC buffer and once with TBS, aspirated to dryness, and resuspended in 2X reducing sample buffer. For peptides, 25 µL beads (Blocked, NPXY_{4473}, or NPXY_{4473}-P) were incubated with 1 mL of prepared rat brain lysate at 4ºC for 2 hours. Samples were washed and prepared as above.

*In-gel digest and MS/MS*

Prepared pulldown samples in 2X reducing sample buffer were run on NuPage 12% Bis-Tris gels in MOPS running buffer in an X-cel electrophoresis chamber (Invitrogen, Carlsbad, CA). Gels were fixed for 30 min in MOPS fixing buffer and then stained overnight with silver stain (Morrissey, 1981) or Gel Code Blue (Pierce, Rockford, IL). Bands of interest were cut from the gel and placed into prewashed eppendorf tubes for digest. For the digest, proteins were first reduced and alkylated, and then subjected to trypsin digestion. Tryptic peptides were analyzed by MS/MS on a Q-STAR XL.

*Affinity peptide preparation*

Hook Agarose (G-Biosciences, St. Louis, MO) was washed with PBS and aspirated to almost dryness (200 µL beads). Peptides (phosphorylated and nonphosphorylated) were obtained from AnaSpec (San Jose, CA). The peptide (1 mg) was dissolved in 500 µL of PBS. The heterolinker MBS (Pierce, Rockford, IL) was dissolved in 1 part DMSO to 1 part PBS to a final volume of 500 µL for 5 mg of MBS. The peptide was mixed with the MBS solution and the pH carefully
adjusted as close to 7.2 as possible. The peptide/MBS solution was then added to the Hook Agarose and rocked at room temperature for 2 hours. The slurry was spun and washed 4X with PBS and left as a 50% slurry, which was approximately 1 mg/mL peptide. A set of blocked beads was prepared in a similar manner except that no peptide was added, and a 30 min incubation in 50 mM Cysteine was added before the final washes to cap activated MBS molecules.

**Phosphorylation of LRP**

Phosphorylation of LRP was achieved in kinase reactions by three different sources of kinase: commercial Src, a phosphorylating cell line, or expressed Src. GST-LRP-CT phosphorylated by commercial src (Upstate, Lake Placid, NY) was as follows. GST-LRP-CT (100 µg) was mixed in solution with kinase buffer (100 mM Tris, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM DTT, 1 µL Src (Upstate), and 1 mM ATP) at room temp for 4 hours. Glutathione agarose was then added to solution and rocked at room temp for 1 hr. Beads were spun and washed three times with PLC buffer.

GST-LRP-CT was phosphorylated by the TKX1 cell line as follows. LRP plasmid pGEX-2T was transformed into TKX1 cells and grown in 1L of LB. After cells reached the required density, expression of LRP protein was induced with 0.1 mM IPTG for 4 hrs at 37°C. Cells were then collected by centrifugation, resuspended in TK induction media (0.5L), and grown for 2 more hours at 37°C.
At this point, the protein was purified as described in “Materials and Methods” of Chapter 2.

GST-LRP-CT was phosphorylated by expressed Src as follows. Src was prepared according to protocol (Seeliger, et al., 2005). Briefly, BL21 DE3 cells were transformed with Src kinase domain plasmid and Yop phosphatase plasmid and grown in 1L of LB kanamycin/streptomycin. Cells were induced overnight at 18°C with 0.2 mM IPTG after reaching an OD_{600nm} of 1.2. The pellet was collected by centrifugation, resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, pH 8.0, 1 mM PMSF), sonicated on ice, and the lysate cleared by centrifugation at 10,000 rpm for 30 min. The cleared supernatant was incubated with 5 mL of NTA-Ni slurry for 2 hr at 4°C. The resin was then washed with 50 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0 until the OD_{280nm} was approximately zero. The protein was eluted from the Ni column with 8 mL of 250 mM imidazole in 50 mM Tris, 500 mM NaCl and dialyzed into storage buffer (50 mM Tris, 100 mM NaCl, pH 8.0, 5% glycerol, and 1 mM DTT). GST-LRP-CT was rocked in solution with a 1:100 dilution of expressed Src in kinase buffer (100 mM Tris, 10 mM MgCl\textsubscript{2}, 10 mM MnCl\textsubscript{2}, 1 mM DTT, and 1 mM ATP) for 1 hr at room temperature. Glutathione agarose was then added to the solution and rocked at room temp for 1 hr. Beads were spun and washed three times with PLC buffer. The extent of phosphorylation could be observed by checking the band shifts of phosphorylation versus non-phosphorylated LRP in silver-stained gels.

*Phosphorylation of GST-LRP-CT by CKI and CaMKII*
CKI and CaMKII were immunoprecipitated from rat brain lysate using 2 µL of CKI antibody (Santa Cruz) and 2 µL of CaMKII antibody (Zymed). Antibodies were added to 1 mL of rat brain lysate and rocked at 4ºC for 1 hr and then with 100 µL of Protein-G slurry (10%) for another hr. Beads were spun down, washed four times with PLC buffer and twice with kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT), and resuspended in 100 µL of kinase buffer. Immunoprecipitates were split between two samples such that each reaction was composed of 50 µL of CKI or CaMKII immunoprecipitate, 25 µL of GST-LRP-CT or GST-LRP-CT-P, and 24 µL of 4X kinase buffer, and 1 µL of γ-³²P-[ATP]. Samples were incubated at 37ºC for 1 hr with occasional mixing. Samples were spun down and 40 µL of supernatant was removed and mixed with 2X reducing sample buffer. Samples were resolved by SDS-PAGE and the gel was dried and exposed to film.

Phosphorylation of tau by LRP associated kinases

Rat brain lysate was incubated with 10 µL of NPXY₄₅₀₇ or NPXY₄₅₀₇-P peptide beads for 2 hrs at 4ºC. Beads were washed four times with PLC buffer and twice with kinase buffer. Beads were aspirated to dryness and then resuspended in 25 µL of kinase buffer containing γ-³²P-[ATP] and 25 µL of tau (1 mg/mL). Samples were incubated at 37ºC for 15 min, spun briefly, and 25 µL of supernatant was removed for sample. Samples were resolved by SDS-PAGE and the gel was dried and exposed to film. Recombinant tau 2N4R was a kind gift from Dr. John Lew.
Immunoblot confirmation of LRP interacting proteins

Rat brain lysate was incubated with 10 μL of NPXY<sub>4507</sub> beads, 10 μL of NPXY<sub>4507</sub>-P beads, or 50 μg of GST, GST-LRP-CT, or GST-LRP-CT-P bound to glutathione sepharose for 2 hrs at 4ºC. Beads were washed four times with PLC buffer, aspirated to dryness, and resuspended in 2X reducing sample buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked for 1 hr with 1% casein in TBS-T, and probed for CaMKII or CKI (from NPXY<sub>4507</sub> beads) or NF-M (from GST-LRP-CT beads). For CaMKII (Invitrogen, Carlsbad, CA) the antibody was diluted 1:500 in 1% casein in TBS-T. For CKI (Santa Cruz Biotechnology, Santa Cruz, CA) the antibody was diluted 1:1000 in 1% casein in TBS-T. For NF-M (Sigma, St. Louis, MO), the antibody was diluted 1:1000 in 1% casein in TBS-T. All antibodies were incubated at room temperature for 1 hr, followed by washes with TBS-T, and probing with appropriate HRP specific secondary antibodies. Blots were then washed again, twice with TBS-T for 10 min each, and then twice with TBS for 10 min each. Blots were developed with ECL reagents.

Optimization of Experimental Conditions

In general, our search for LRP1 binding partners started with the fusion protein bound to glutathione sepharose at 1 mg/mL and incubated with cell or tissue lysate. The protein could also be phosphorylated in vitro with v-src before being bound by the support. We discovered that there were many conditions to
optimize with this experiment such as the amount of lysate, phosphorylation system, and preparation of lysate.

Optimization of the amount of protein bait to use in each incubation was neccessary. The NPXY peptide was linked at a very high concentration, which presented a large number of ligand binding sites. We could recover a cleaner pulldown by first incubating the lysate with GST-slurry. After one hour incubation with just GST, the sample was spun down and the lysate removed. The lysate was then added to GST-LRP-CT slurry and incubated again. In this way we were able to remove some of the GST-only binding proteins from our experiment. Higher amounts of protein were required to pulldown interactions of the same strength as the NPXY peptides. Part of this problem was due to the fact that the GST-LRP-CT bait was somewhat unstable and there were many breakdown products in the bait that could not be removed.

Different buffers for lysate preparation and washing were also tested. The PLC buffer contained several detergents including Triton-X-100 and glycerol. The HUNT buffer was milder, with only 0.5% NP-40. We also tried an SDS based buffer, but this was harder to work with, as too high an SDS content would cause precipitation in the buffer at the lower temperatures used for the incubation. Lysate from cell sources gave cleaner results than those from tissue sources. It was necessary to subject brain tissue lysate to many rounds of centrifugation in order to remove all contaminating lipid materials. Without these added spins the resulting pulldowns had a high level of background binding.
Trials of in vitro phosphorylation methods

One of the goals of this experiment was to find proteins that differentially interacted with the non or phosphorylated versions of LRP. Our initial experiments had trouble meeting this goal. The reason for this problem was that the in vitro kinase reaction was very inefficient. By observing the molecular weight shifts of the full length GST-LRP-CT protein, it was possible to estimate how much phosphorylation had occurred (Figure 4.3). The kinase reaction using commercially purchased v-Src resulted in about 15% of the protein becoming phosphorylated. This meant that in our phosphorylated pulldowns there was very little phosphorylated protein in the bait to make a difference. We also determined by mass spectrometry we were not phosphorylating the Y4473 residue as a result of the low efficiency phosphorylation. This resulted in our phosphorylated versus nonphosphorylated samples appearing virtually identical.

To get around this problem other methods were explored. Our first alternative was to use a phosphorylating E. coli strain. This was a commercial strain of E. coli that had been transformed to express the tyrosine kinase elk (Stratagene, San Diego, CA). The protein of interest was first induced in the cells, followed by spinning down the pellet and changing the media. The new media would lead to the induction of the tyrosine kinase and thus phosphorylation of the target protein. In this system we found that all of the GST-LRP-CT present was phosphorylated.

However, there were problems with this method as well. The elk tyrosine kinase has a broad specificity, and appeared to phosphorylate all the tyrosine
residues in LRP. We can observe this phenomenon by the molecular weight shifts of all the mutant LRP forms, even the 2F (Figure 4.4). LRP contains 4 tyrosine residues, 2 of which are in the context of NPXY motifs, and could become phosphorylated. We were also able to show that when cleaved with thrombin the free LRP domain of all forms, even the Y2F mutant, were phosphorylated.

Fortunately, our next alternative proved much better. Work by Seelinger et al. demonstrated the expression and purification of the tyrosine kinases Src and Abl (Seeliger, et al., 2005). The plasmid for the expression of Src and Yop phosphatase was obtained from Dr. Kuriyan and used to produce phosphorylated GST-LRP-CT fusion proteins. Achievement of efficient, fast, and specific phosphorylation of our target proteins was now possible (Figure 4.5). With this system we now had a better bait system in place to test with our lysates.

Results

Functional analysis of kinase interactions with LRP

As mentioned above, the LRP NPXY\textsubscript{4507} based affinity peptide lead to the identification of several kinases that associated with LRP. These interactions were confirmed by Western blot (Figure 4.6). CKI and CaMKII are serine/threonine kinases. LRP has been shown to be phosphorylated on serine and threonine residues in the cell. In particular, S76 of LRP is phosphorylated by PKA, and other sites may be targets of PKC as well.
Figure 4.3: GST-LRP-CT phosphorylated \textit{in vitro} by commercial Src. GST-LRP-CT was incubated in kinase buffer with or without Src; the samples were separated by SDS-PAGE and visualized by silver stain. The incomplete shift of the GST-LRP-CT + Src sample shows that only a small percent of the protein is actually phosphorylated.
Figure 4.4: Phosphorylation of GST-LRP-CT in TKX1 cells. GST-LRP-CT and mutants were expressed in TKX1 cells as described in “Materials and Methods” to generate the phosphorylated form of the protein. However, the broad specificity of the Elk kinase caused it to phosphorylate all tyrosine residues in the GST-LRP-CT protein. A) Silver stain gel of GST-LRP-CT, GST-LRP-CT Y2F, and their phosphorylated forms grown in TKX1 cells. The phosphorylated protein shifts to a higher molecular weight in both the wild-type and Y2F mutant. B) GST-LRP-CT Y2F TK phosphorylated protein was probed by western blot with anti-phospho-tyrosine antibody. Both the full-length protein and the free Y2F cytoplasmic domain (No GST) are still recognized by the antibody. This proves that the extra phosphorylation is also occurring at the 2 tyrosine residues outside of the LRP NPXY motifs, and not just the GST domain. This proves that the GST-LRP-CT was becoming phosphorylated on more sites than we desired. still phosphorylated.
Figure 4.5: GST-LRP-CT phosphorylated in vitro with expressed recombinant Src. GST-LRP-CT wild-type or Y2F mutant (Y4473F/Y4507F) was incubated for 1 hr in kinase buffer, with or without expressed Src. Proteins were separated on an SDS-PAGE gel and visualized by silver stain. The wild-type protein completely shifts to the higher molecular weight, whereas there is no unwanted shift in the Y2F protein.
Figure 4.6: Western blot analysis confirms LRP interaction with CaMKII and CKI. Agarose bead bound affinity peptides were incubated with rat brain lysate, spun, washed, and bound proteins were separated on an SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with the appropriate antibodies. Lanes 1 and 3 represent samples from the NPXY$_{4507}$ peptide, while lanes 2 and 4 represent samples from NPXY$_{4507}$-phospho peptide. The CKI antibody did not yield a good signal and thus the arrows help to point out bound CKI. Different isoforms were recognized by the antibody and interact with the LRP affinity peptide.
(Li, et al., 2001, Ranganathan, et al., 2004). To determine if LRP might be a substrate for either of these peptide associated kinases, CKI or CaMKII immunoprecipitated from rat brain lysates was incubated with GST-LRP-CT or GST-LRP-CT-P in kinase buffer with γ-\(^{32}\)P-[ATP]. Proteins were separated by SDS-PAGE and phosphorylation was determined by bands in the autoradiograph (Figure 4.7). Preliminary results from this experiment indicate that both kinases do indeed phosphorylate LRP, at least \textit{in vitro}.

\textbf{LRP associated kinases can phosphorylate Tau}

LRP has implications in the development of Alzheimer’s disease. Several of the kinases identified in our screen with the NPXY\textsubscript{4507}-P peptide are known to phosphorylate tau, the microtubule associated protein also implicated in AD (Grundke-Iqbal, et al., 1986, Iqbal, et al., 2005, Singh, et al., 1995, Singh, et al., 1997, Singh, et al., 1996). To test whether our LRP associated kinases would also phosphorylate tau affinity peptide beads were incubated in rat brain lysate and washed as before. The beads were then incubated in kinase buffer with γ-\(^{32}\)P-[ATP] and recombinant tau. The samples were separated on an SDS-PAGE gel and bands visualized by autoradiography (Figure 4.8). We found that tau was indeed phosphorylated in the presence of LRP associated kinases.

\textit{Discovery of a new LRP interacting partner}

From our screens using GST-LRP-CT fusion protein we identified a neurofilament protein, NF-M (Genbank
Figure 4.7: LRP is a substrate for CaMKII and CKI in vitro. CaMKII and CKI were immunoprecipitated from rat brain lysate and incubated with GST-LRP-CT or GST-LRP-CT-P in kinase buffer with γ-32P-[ATP] in solution. A sample of the supernatant was removed and separated on a SDS-PAGE gel, dried on filter paper, and exposed to film. The autoradiograph reveals γ-32P-phosphorylated LRP by CaMKII and CKI. Lanes 1 and 3 contained GST-LRP-CT while lanes 2 and 4 contained GST-LRP-CT-P. Shown above is the LRP-CT sequence with the potential serine/threonine sites numbered.
Figure 4.8: Autoradiograph shows that LRP associated kinases can phosphorylate tau. LRP affinity peptides beads NPXY$_{4507}$ and NPXY$_{4507}$$^\text{P}$ were incubated with rat brain lysate for 2 hrs at 4°C. The beads were spun, washed, resuspended in kinase buffer with recombinant tau (2N4R, MW ~ 46 kD) and γ-$^32\text{P}$-[ATP], and incubated at 37°C for 15 min. The beads were spun and the supernatant collected for SDS-PAGE analysis. The gel was dried on filter paper and exposed to film.
Figure 4.9: Identification of NF-M by MS/MS. A) Tryptic peptides produced from MS spectrum were fed into the Mascot web-based software program and searched against a database for matches. The probability Mowse score is shown in A) 4 total hits with various scores were found for this set of peptides. The highest score, for NF-M, is significant. B) A partial list of the peptides that matched to the NF-M sequence. A total of 15 peptides were matched. C) MS spectrum of a single peptide from NF-M band. D) MS/MS of peptide from C). Some of the b and y ions are labeled.
It bound to both the non and phosphorylated forms of LRP. This protein was found in our screens using rat brain tissue lysate. NF-M is an intermediate filament of 90 kD and one of 3 family members (also NF-H and NF-L). It contains 2 coiled-coiled domains and a central conserved \( \alpha \)-helix domain that helps mediate interactions between the filament proteins. The LRP-NF-M interaction was also confirmed by Western blots of the same samples with anti-NF-M antibody (Figure 4.10).

**Early work with a NPXY\(_{4473} \) based affinity peptide**

The observation of dual tyrosine phosphorylation in LRP and the discovery of a novel NPXY\(_{4473} \) interacting protein prompted us to probe interactions via the NPXY\(_{4473} \) with an affinity peptide as well. The peptide to represent the first NPXY motif was 15 amino acids long, contained a N-terminal cysteine, and a caproic acid linker (Figure 4.11A) (Sequence VEIGNPTYKMYEGGE, with version of Y and Yp). The presence of a lysine residue in the sequence prevented us from using required thiol chemistry and covalently linked the peptide to a commercial resin via the added non-native N-terminal cysteine. This system was not as clean as the amide peptide linkage; however we were still able to observe some differences between the phosphorylated peptides and nonphosphorylated peptides (Figure 4.11B). Over 30 bands from these samples are being analyzed by in gel digestion and MS/MS to identify interacting proteins. This work is still in progress.
Figure 4.10: The neurofilament protein NF-M binds to LRP \textit{in vitro}. GST, GST-LRP-CT or GST-LRP-CT-P bound to glutathione sepharose was incubated with rat brain lysate for 2 hr at 4°C. Samples were then spun, washed, separated on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed for NF-M.
Figure 4.11: Affinity peptide based on NPYX_{4473} motif of the LRP cytoplasmic tail.
A) Design of the NPYX_{4473} based affinity peptide in a phosphorylated and nonphosphorylated version. The ‘X’ denotes an amino caproic acid linker.
B) Affinity peptide beads were incubated with cell or tissue lysate for 2 hr at 4°C, spun, washed, separated on a commercial 10% gel, and stained with Gel Code Blue (Pierce). Lanes 1, 4, and 7 were incubated with blocked beads. Lanes 2, 5, and 8 were incubated with the NPYX_{4473} based peptide beads. Lanes 3, 6, and 9 were incubated with the NPYX_{4473}-P based peptide beads. Lanes 1-3 used U87 cell lysate, lanes 4-6 used 293 cell lysate, and lanes 7-9 used rat brain lysate. It is hard to resolve the gel bands from the included scan, but over 30 bands were able to be removed for identification.
Discussion

Mass spectrometry is a very powerful tool in the study of proteomics. MS has the ability to accurately determine protein mass and unambiguously identify proteins by peptide fragmentation sequencing or MS/MS. This approach was used to identify proteins that interact with the LRP cytoplasmic tail. The use of LRP affinity peptides, combined with in gel digestion and MS/MS, lead to the identification of over 15 novel binding proteins to the LRP-CT.

It was important to demonstrate that these interactions are significant and not just the result of domain-domain interactions without specificity. To this end we focused on several of the kinases that were discovered to bind to LRP-CT. We chose to focus on CaMKII and CKI. Neither of these kinases contain any domains (PTB or SH2) that one would expect to find binding to a NPXY motif (Hsu, et al., 1998, Tapia, et al., 1994). We hypothesized that if the kinase interacted with LRP at the NPXY\textsubscript{4507} motif, then perhaps the other regions of LRP could be a substrate for the kinase. We could easily test if this was possible.

We were pleasantly surprised therefore to discover that both kinases were able to phosphorylate GST-LRP-CT-P in an \textit{in vitro} kinase reaction. CaMKII was a better kinase towards LRP than CKI based on the intensity of the respective bands from the autoradiograph. The phosphorylated samples appeared as a ladder of different molecular weight proteins. Some of the phosphorylated bands were shifted to a much higher molecular weight than one might expect. This could be the result of multiple serine/theronine residues becoming
phosphorylated. In fact the multiple bands in the lane are likely the result of additional residues being phosphorylated in the protein, thus affecting its mobility in the gel.

There have been other reports of serine/theronine phosphorylation of LRP, but none involving CaMKII or CKI. In fact, serine and threonine phosphorylation is much more abundant than tyrosine phosphorylation in the LRP-CT (Ranganathan, et al., 2004). So far only PKC\textsubscript{\textalpha} and PKA have been identified to phosphorylate the LRP-CT on serine and threonine residues, with PKC\textsubscript{\textalpha} on S76, and PKA potentially on T16/S73/S76/S79 (Li, et al., 2001, Ranganathan, et al., 2004). Phosphorylation on these sites is implicated with affecting the endocytosis rate of LRP and modulating interactions with the adaptor proteins Dab-1 and CED-6/GULP (Li, et al., 2001, Ranganathan, et al., 2004). Both CaMKII and CKI are ubiquitously expressed, although CaMKII is enriched in the brain, as is LRP. This fact, along with the strong phosphorylated bands present in the autoradiograph, suggests that CaMKII could be a relevant kinase for LRP \textit{in vivo}.

The tau of neurofibrillary tangles in brains of Alzheimer’s disease patient’s is hyperphosphorylated on serine and threonine residues (Grundke-Iqbal, et al., 1986, Hanger, et al., 1998). CaMKII and CKI were recently shown to both target tau protein as well (Iqbal, et al., 2005, Singh, et al., 1995, Singh, et al., 1996). Many other kinases, such as Cdk5, p38-MAPK, and GSK3-\textbeta, are also involved in the hyperphosphorylation of tau at more than 30 serine/threonine residues (Grundke-Iqbal, et al., 1986, Singh, et al., 1997). Of these kinases, three were
found to also bind to LRP in our NPXY$_{4507}$-P affinity peptide screen. We found that these LRP associated kinases could phosphorylate tau when the kinases were pulled out of rat brain lysate with the NPXY$_{4507}$-P peptide beads.

Most AD therapies in current development focus on Aβ, and not tau which makes up the neurofibrillary tangles inside of neurons. Tau neurofibrillary tangles are often viewed as a downstream affect of Aβ buildup and dysfunction and thus not looked upon as a therapeutic target. However, new studies have demonstrated that tau may indeed be a useful drug target (Marx, 2007). Transgenic mice with tau reduction (Tau +/- or -/-) in the AD mouse model suffered fewer behavioral deficits associated with the hAPP background than the Tau +/- controls (Roberson, et al., 2007).

Our tau result is very interesting in that it provides a link of communication between amyloid and tau, the two main hallmarks of AD. LRP is involved in the processing of APP and thus Aβ peptide of amyloid plaques. There is no clear connection between the development of amyloid plaques and neurofibrillary tangles in AD. Our result indicates that the two may be connected through LRP. The LRP-CT has been shown to affect the ratio of Aβ produced (Pietrzik, et al., 2002). This same region is responsible for binding to the tau phosphorylating kinases CaMKII, CKI, and Src. LRP could be a scaffold that concentrates these kinases such that they are available to phosphorylate substrates such as tau. Much more work needs to done in this area however, starting with whether this event occurs in vivo as it does in vitro. We also plan to determine which LRP
associated kinase is responsible for the tau phosphorylating activity by treating our samples with kinase specific inhibitors.

We have added one novel binding protein to our list using a full-length fusion protein bait system. LRP-CT interacts with the neurofilament protein NF-M in a phosphorylation independent manner. NF-M is involved in shaping the intracellular scaffold and maintaining stability of axons (Lariviere and Julien, 2004). NF-M is one of three neurofilament proteins in adult neurons. Neurofilaments form 10 nm filaments as heteropolymers of NF-L with either NF-M or NF-H (Ching and Liem, 1993, Lee, et al., 1993). NF-M does not contain any known NPXY binding domains, but it does contain several KSP repeats (Hoffman and Lasek, 1975, Liem, et al., 1978, Napolitano, et al., 1987).

There is some evidence in the literature for NF-M binding to other proteins such as the dopamine receptor. This binding appears to be mediated by the C-terminal tail of NF-M and helps to translocate the receptor to target areas within the cell (Kim, et al., 2002). It is possible that NF-M could play a similar role for LRP. It is interesting that NF-M also was found to be hyperphosphorylated and in the presence of AD plaques (Wang, et al., 2001). NF-M could be yet another LRP related protein found to be associated with AD. It is not clear yet what is the significance of the LRP/NF-M interaction. More work in the future is needed to study this interaction in vivo.

Future work using this “fishing” method should consider advantages and problems inherent with this technique. The full-length fusion protein system proved to be quite troublesome and not as fruitful as our peptide based systems.
Reasons for this include the instability of the fusion protein, difficulties in efficient phosphorylation, and high background. It is important to remember the limitations of this type of “fishing” system as well. For example, the search may be limited to high affinity interactions. The stringency of the interactions can be modulated by the type of buffer used as well. Some low affinity interactions, or interactions involving a low abundance protein, may be overlooked by the pulldown system.

However, our efforts to expand this affinity based search are still ongoing. We are encouraged by the high number of bands found to bind to the NPXY$_{4473}$/NPXY$_{4473}$-$P$ based affinity peptides. To date, only Snx17 has been identified to bind to this site (van Kerkhof, et al., 2005). Yet mutation of this site in knock-in mice had a deleterious phenotype (Roebroek, et al., 2006), which would suggest that the site is important and that more is going on here than previously observed. Hopefully we will identify several novel interactions that can be confirmed in vivo as well.
References


CHAPTER FIVE

Characterizing the novel LRP-Shp2 Interaction
Introduction

As mentioned in the previous chapter, affinity peptide experiments based on the LRP NPXY_{4507} motif yielded a diverse list of interacting proteins, one of which was Shp2. This band was the strongest LRP interacting protein, by both affinity peptide and GST-LRP pulldown experiments. Given the high affinity of this interaction it was probed in more detail.

Shp2 Introduction: Structure and Function

Shp2 is a protein tyrosine phosphatase (PTP). These proteins function to remove phosphate residues from protein tyrosine kinases (PTK). Over 107 PTPs have been identified in the human genome (Alonso, et al., 2004). PTPs can be either receptor-like or non-receptor forms, and classical (dephosphorylate tyrosine only) or dual-specificity (tyrosine and serine/threonine) (Feng, 1999, Neel, et al., 2003, Ostman, et al., 2006). Enzyme activity is located in a 208 residue long phosphatase domain. The highly conserved active site contains a critical cysteine residue. The PTP functions by forming a covalent phosphate intermediate between the PTP and substrate, followed by hydrolysis of the phosphate bond (Figure 5.1). In the first step, the active site cysteine acts as a nucleophile to accept the substrate phosphate. In the second step, the Asp residue in the WPD loop acts as general base which accepts a proton from an attacking water molecule. The water molecule in was activated by a glutamate residue from another loop. This final step leads to the hydrolysis of the phosphocysteine complex.
Figure 5.1: Mechanism of PTP dephosphorylation of substrate. Taken from (Roskoski, 2005) In the first step, the conserved cysteine of the PTP active site acts as a nucleophile to attack the phosphate of the substrate protein and creates a phosphoenzyme intermediate. The active PTP is regenerated by the loss of the phosphate.
PTPs can function as negative or positive regulators depending on their substrate. There are several levels of regulation on PTPs, including phosphorylation, proteolytic cleavage, degradation, and oxidation of the catalytic cysteine residue (Ostman, et al., 2006). Many PTPs are tumor suppressors such as DEP1, Shp1, and PTP1B (Ostman, et al., 2006); however, Shp2 (PTPN11) was the first PTP to be confirmed as a proto-oncogene. Shp2 is composed of two tandem SH2 domains and a catalytic PTP domain. Shp2 is a classical, non-receptor PTP. It was recently determined to be a cause of Noonan syndrome, a disease characterized by hypertelorism, short stature, cardiac anomalies, and motor delay. Approximately 50% of disease cases are caused by autosomal dominant mutation of Shp2 (Tartaglia, et al., 2001). It is not yet clear how the missense mutations in Shp2 lead to disease; however they appear to be less severe gain-of-function mutations than those that lead to Shp2-related cancer. Many questions about how Shp2 functions were answered when the structure was solved.

The crystal structure of Shp2 clearly revealed the autoinhibition mechanism of Shp2 (Figure 5.2). In the inactive state, the N-terminal SH2 domain interacts with the PTP domain, thus preventing access to potential substrates (Hof, et al., 1998). There are two varying models on the activation of Shp2. In the first case, a bi-phosphorylated protein (or two singly phosphorylated proteins) are presented to the inactive PTP and allow activation by opening up the molecule. The Shp2 tandem SH2 domains bind the phosphorylated proteins and allow the PTP domain to access potential
Figure 5.2: Shp2 crystal structure taken from (Hof, et al., 1998). Shp2 residues 1-527 were crystallized in the closed, autoinhibited conformation. Only the last 66 residues of the c-terminal tail were missing. A) Ribbon diagram of Shp2 structure. The N-SH2 domain is colored in yellow, the C-SH2 domain in green, and the PTP domain in blue. B) All nonhydrogen atom structure with same coloring as in A). Also shown in magenta are the PTP catalytic site residues, and in red are the peptide contact residues when the SH2 domains are bound by phosphopeptides.
substrates. In the second case, tyrosine residues in the C-terminal tail of Shp2 can be phosphorylated and interact with the SH2 domains. This intramolecular interaction again frees up the PTP domain for activation towards substrates (Figure 5.3).

Shp2 has been implicated as a positive regulator in the Ras-ERK pathway by at least two different mechanisms (Ren, et al., 2004, Zhang, et al., 2004). In one system, Shp2 dephosphorylates a specific tyrosine residue on PAG, a Csk regulating enzyme. This prevents Csk from phosphorylating Src on an autoinhibitory residue in the c-terminal tail of Src (Y529) (Zhang, et al., 2004). The now active Src can go on activate Ras, which leads to ERK activation. In a second pathway, Shp2 is recruited near the plasma membrane by the bis-phosphorylated ligand Gab1 in response to EGF stimulation. This association activates Shp2 and leads it to dephosphorylate another Gab1 bound protein called paxillin. The dephosphorylation event results in the dissociation of Csk from the complex, and thus an increase in Src activity since Csk is no longer around to phosphorylate Src at Y530, an autoinhibitory site for Src activation (Ren, et al., 2004). Activation of Src can then lead to ERK activation (Figure 5.4). The role of Shp2 in cells is still unclear, as in some cases the PTP appears to be a positive regulator, and in other cases it is a negative regulator (Meng, et al., 2002, Neel, et al., 2003). As mentioned in Chapter 4, an interaction between LRP1 and Shp2 was observed. We desired to determine the role of LRP1 in Shp2 function. The initial observation was made by using an affinity peptide based on the LRP1 amino acid sequence surrounding the 2nd NPXY motif.
Figure 5.3: Proposed mechanisms of Shp2 activation. In the top pathway a bi-phosphorylated ligand binds to the N- and C-terminal SH2 domains, relieving the N-SH2 domain inhibition of the PTP domain. In the bottom pathway, the c-terminal tail of Shp2 is phosphorylated on two tyrosine residues which then swing back to interact with the SH2 domains, again opening up the PTP domain to substrates and activating the enzyme. Taken from (Neel, et al., 2003).
Figure 5.4: Mechanism of Shp2 activation in the Src signaling pathway induced by EGF stimulation. Taken from (Ren, et al., 2004).
Shp2 was found to specifically interact with the phosphorylated version of this peptide. Later this interaction was confirmed in cells by co-immunoprecipitation, and then *in vitro* using purified proteins (GST-LRP-CT fusion protein could pulldown Shp2 from cells).

Although it was likely that the SH2 domains of Shp2 were mediating the binding to LRP it was still possible that other regions also contributed to the specificity. It was also not clear if both SH2 domains were involved in binding or just one. LRP be a substrate for Shp2, or merely a binding site. LRP could also act as an activator for Shp2, in a manner similar to Grb2-associated binder-1 (Gab1) (Cunnick, et al., 2001). Gab1 is an adaptor protein which contains an N-terminal pleckstrin homology (PH) domain and proline rich regions which can bind to SH3 domains (Holgado-Madruga, et al., 1996, Weidner, et al., 1996). Upon EGF stimulation of cells, Gab1 is phosphorylated on several tyrosine residues, which leads to its association with Shp2 and other proteins (Cunnick, et al., 2001, Holgado-Madruga, et al., 1996, Shi, et al., 2000). Cunnick et. al. found that Gab1 was phosphorylated on Y627 and Y659, and that both of these sites were essential for EGF-stimulated Erk activation. Shp2 bound to both of these residues, but activation alone was not enough for EGF stimulated and Shp2 mediated Erk activation, as a constitutively active Shp2 mutant did not lead to Erk activation. The physical association of Shp2 with Gab1 was necessary for Erk activation, indicating that Shp2 needs to be recruited to the Gab1 to act on other localized proteins as well.
In parallel, if both NPXY tyrosine residues were phosphorylated, they could act as a bisphosphate binding ligand, interacting with the tandem SH2 domains of Shp2. This interaction would free the catalytic site from inhibition by the N-terminal SH2 domain and thus lead to activation of Shp2. LRP can also be a scaffold to recruit Shp2 to the membrane and thus associate with other localized proteins.

**Materials and Methods**

*Cloning of Shp2 constructs*

Shp2 cDNA was a kind gift from Dr. Gen-Sheng Feng. Shp2 constructs were cloned into pcDNA3-HA vector for expression in mammalian cells. This vector is amp/G418 resistant and contains an N-terminal HA tag. Primers were obtained from Integrated DNA Technologies (Coralville, IA) and designed as follows to insert with a 3' EcoRI site and 5' XbaI site (all primer are listed in 5' to 3' direction):

**Shp2 Full-length (1-598)**

Forward CAT GCA TGC GAA TTC GCA TGA CAT CGC GGA GA
Reverse CTG ACG TCT AGA TCA TCT GAA ACT CCT CTG

**Shp2 (1-531)**

Reverse AGT CAG TCT AGA TCA GCG CTG CAG AGT CTC
SH2 domain constructs were cloned into the bacterial expression vector pHis8, containing an N-terminal His-tag followed by ubiquitin and a multiple cloning site. Primers were obtained from Integrated DNA Technologies (Coralville, IA) and designed as follows to insert with a 5’ BamHI site and 3’ NotI site (all primers are listed in 5’ direction). The forward BamHI primer contains a four glycine repeat
sequence to act as a linker between the ubiquitin fusion site and Shp2 protein fragments.

Shp2 N-SH2
Forward CAT GGA TGG GAT CCG GAG GAG GAG GAA TGA CAT CGC GGA GAT GG
Reverse GGC ACA TGC GGC CGC TTA AGA GGT AGG GTC TGC ACA GTT CAG

Shp2 C-SH2
Forward CAT GGA TGG GAT CCG GAG GAG GAG GAT TCC ATG GTC ACT TGT CT

Shp2 (1-222, tandem SH2 domains)
Reverse CAT GCA TGG CGG CCG CTC AAC GAG TTG TGT TGA GGG GCT G

Clones were created by PCR of native Shp2 sequence followed by digestion of PCR product with restriction enzymes and subsequent cloning. All constructs were confirmed by DNA sequencing.

*Cell culture*
293 cells were grown in Dulbecco-Vogt’s modified Eagle’s medium containing 10% fetal bovine serum, with 50 µg/mL penicillin and streptomycin, 5% CO₂.

Expression and purification of proteins

GST-LRP proteins were expressed and purified as stated in “Materials and Methods of Chapter 2.

Shp2 SH2 domains – Shp2 protein domains were cloned into a pHis8 vector from the J. Noel lab. The vector was modified by cloning the ubiquitin coding sequence in frame between the his tag and the multiple cloning site. N-terminal ubiquitin fusion proteins of each Shp2 fragment were expressed in BL21 DE3 cells in 1L of LB and induced overnight with 0.1 mM IPTG. The cell pellet was resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, 1mM BME, 1 µg/mL leupeptin and aprotinin, and 1 mM PMSF, pH 7.4), sonicated on ice (30 sec pulses over 2 minutes) and centrifuged for 30 minutes at 12,000 rpm. The supernatant was incubated with 5 mL of Ni-NTA resin (Qiagen) for 1 hour at 4°C. The slurry was washed until elute the absorbance at 280 nm was near zero with 20 mM imidazole, 1 mM BME, 50 mM Tris, 150 mM NaCl, pH 7.4. Proteins were eluted as one fraction with 250 mM imidazole in TBS, pH 7.4 and separated from contaminating proteins by size exclusion chromatography (S-75 column with 50 mM Tris, 150 mM NaCl, pH 7.4). Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL).
Transient transfection of Shp2 constructs and mycLRPβ

Cells were split in 60 mm dishes the day before transfection. Cells were transfected with 1 µg of pcDNA3.1-HA Shp2, 1 µg mycLRPβ, and 1 µg v-Src at 40% confluency using 2 µL of Transfectin reagent (Biorad, Hercules, CA). After 48 hrs cells were rinsed twice with ice cold TBS, and then lysed in 1 mL PLC lysis buffer (with 1 mM DTT and fresh proteinase inhibitors). Cells were scraped a total of 3 times in PLC, with resting on ice for 5 minutes in between each. Collected lysates were allowed to rest on ice in an eppendorf tube for 10 minutes and then centrifuged at 13,000 rpm for 20 minutes to pellet nuclei and cell debris. Cleared lysates were then incubated for 1 hour with 40 µL of 12CA5 anti-HA antibody (Babco, Richmond, CA) and then with 50 µL of Protein-G 10% slurry, rocking at 4ºC. Samples were washed four times with PLC lysis buffer, aspirated to dryness, and resuspended in 35 µL of 2X reducing sample buffer. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-HA antibody 12CA5 at a 1:25 dilution and anti-LRP antibody 11H4 at a 1:10 dilution (ATTC, Manassas, VA).

GST-LRP pulldown of Shp2 truncated forms

GST-LRP proteins were purified as detailed above. Proteins were phosphorylated in vitro by mixing each fusion protein with v-Src in kinase buffer (100 mM Tris, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 1 mM ATP) for 30 min at room temperature. Proteins were then bound to glutathione sepharose and washed 3X PLC buffer. Bound proteins (25 µg) were then added to 293 cell
lysates containing full-length HA-tagged Shp2 and allowed to mix at 4°C for 1 hour. Samples were spun down and washed 4X with PLC buffer, aspirated to dryness, and resuspended in 35 μL of 2X reducing sample buffer. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed for anti-HA with 12CA5 antibody to detect Shp2 binding.

**GST pulldown of Shp2 SH2 domains with LRP1 mutants**

Proteins were expressed and purified as detailed above. Equal amounts of GST-LRP (Y4507E and phosphorylated form) or Ubq-LRP (wild-type and phosphorylated form) and Shp2 His-tagged SH2 domain (N-SH2, C-SH2, or 222) were mixed in TBS-X buffer at 4°C for 1 h and then 25 μL of NTA-Ni (50% slurry) was added, mixing for an additional hr. Beads were spun down by centrifugation, washed 3 times with TBS-X, aspirated to dryness, and resuspend in 2X reducing sample buffer. Samples were resolved by SDS-PAGE and visualized by silver stain.

**Results**

**Shp2 interactions with LRP in vivo**

The Shp2/LRP interaction was confirmed *in vivo* by immunoprecipitation of co-transfected 293 cells. Cells transiently expressing full-length HA-tagged Shp2, mycLRPβ, and v-src were lysed in PLC buffer, incubated with anti-HA antibody, and the resulting immunoprecipitates probed with anti-LRP antibody to detect LRP1 binding. Shp2 could interact with wild-type and Y4473F mycLRPβ,
but not with Y4507F, Y4507E, or Y2F mycLRP1β, and only in the presence of v-Src. These results indicate that Shp2 only interacted when the mycLRPβ was phosphorylated (Figure 5.5).

**Shp2 needs its N-terminal SH2 domain to bind to LRP-P**

The LRP binding site for Shp2 appeared rather clearly to be the NPXY\textsubscript{4507} motif. To determine the region of Shp2 responsible for binding a series of Shp2 constructs (Figure 5.6) was designed. These constructs were cloned into a mammalian vector to be expressed in cells and then tested for their ability to interact with LRP. Lysates from 293 cells transfected with a specific Shp2 construct were incubated with GST-LRP-CT-phospho bound to glutathione sepharose. Interactions with Shp2 were determined by western blot by probing for the HA tag of the Shp2 construct. The GST-LRP-P could interact with all forms of Shp2 except the PTP and 109. The 109 construct was missing the N-terminal SH2 domain, and bound very weakly (Figure 5.7). The PTP construct was missing both SH2 domains, and thus was not expected to bind to LRP.

**Shp2 can bind to both NPXY motifs of LRP**

The initial discovery of the LRP-Shp2 interaction was limited to the NPXY\textsubscript{4507} region because that was believed to be the only site of phosphorylation. The discovery that both NPXY motifs of LRP can be tyrosine phosphorylated suggested the idea that both motifs could interact with Shp2. To test this idea, phosphorylation of Y4473 in the context of the glutamate mutant was used. Pulldown experiments using bound fusion protein of GST-LRP-CT
Figure 5.5: LRP interacts with Shp2 in vivo. 293 cells were transfected with different myc-tagged LRPβ constructs, v-Src, and HA-tagged Shp2. HA-tagged Shp2 was immunoprecipitated from cell lysates with the anti-HA antibody and protein-G agarose. Immunoprecipitates were spun, washed, separated on a SDS-PAGE gel, transferred to a nitrocellulose membrane, and bound proteins were probed with the appropriate antibodies. Even though the expression of the Y4473F LRP mutant is much higher, the amount bound to Shp2 is still lower than that of wild-type LRP.
Figure 5.6: Shp2 truncation and mutant constructs (murine). Shp2 constructs were created by PCR and ligated at EcoRI and XbaI sites into the pcDNA3 mammalian vector which includes an N-terminal HA tag.
Figure 5.7: Shp2 binding to LRP is mediated by the SH2 domains. 293 cells were transfected with various HA-tagged Shp2 constructs, v-Src, and myc-tagged LRPβ. HA-Shp2 was immunoprecipitated from cell lysates with anti-HA antibody and protein-G agarose. Immunoprecipitates were spun, washed, and bound proteins were detected by western blot with the appropriate antibodies.
Figure 5.8: Shp2 can interact with Y4473p in the LRP-CT. GST-LRP proteins were bound to glutathione sepharose and incubated with lysates of 293 cells that had been transfected with HA-Shp2. Beads were spun, washed, and bound proteins were detected by western blot with anti-HA antibody.
GST-LRP-CT-P, and GST-LRP-CT-P (Y4507E) were mixed with 293 cell lysates and bound proteins were probed by western blot for Shp2. Shp2 did in fact bind to the glutamate mutant, suggesting that either NPXY motif can act as a Shp2 binding site as long as it is phosphorylated (Figure 5.8).

**Determination of Shp2 SH2 domain specificity for LRP**

To determine if the two SH2 domains of Shp2 had specificity for the LRP NPXY motifs the N and C-terminal SH2 domains were expressed as his-tagged ubiquitin fusion proteins and incubated with Ubq-LRP-CT, Ubq-LRP-CT-P, GST-LRP-CT Y63E, or GST-LRP-CT -Y63E-P. The proteins were mixed with NTA-Ni slurry, spun, washed, separated by SDS-PAGE, and bound proteins were visualized by silver stain (Figure 5.9). Both SH2 domains could bind to either phosphorylated NPXY motif.
Figure 5.9: Shp2 SH2 domains can bind to both NPXYp motifs. Recombinant proteins were mixed in solution and pulled out with glutathione sepharose by the GST-tag of the LRP constructs. (Top panel) Western blot of pulldowns with tandem SH2 domains 1-222 or (middle panel) the individual SH2 domains N-SH2 or (bottom panel) the individual C-SH2 domain of Shp2. Lanes 1 was the negative control showing no binding when GST-LRP was not phosphorylated. Shp2 SH2 domain binding was probed by anti-His immunoblotting.
Discussion

Shp2 is a protein tyrosine phosphatase that functions in many cases to activate signaling cascades mediated by growth factor simulation, such as ERK. For example, it has been shown that Shp2 is recruited to the PDGF receptor upon receptor stimulation by PDGF-BB (Klinghoffer and Kazlauskas, 1995). Some studies have shown that Shp2 inactivation is necessary for PDGF function, while others indicate Shp2 activity has a positive role on PDGF function (Meng, et al., 2002, Neel, et al., 2003). PDGF stimulation of cells also results in tyrosine phosphorylation of LRP1, and possibly formation of a LRP1-PDGF-R heterodimer (Loukinova, et al., 2002, Newton, et al., 2005).

We have shown by both in vivo and in vitro experiments that Shp2 also binds to LRP1. It is possible that the positive and negative effects of Shp2 on PDGF-R activation could be modulated by the interaction between LRP1 and Shp2 as they are both affected by PDGF stimulation. For example, Shp2 could promote PDGF-R activity by directly interacting in the Src signaling pathway, but in some cases under certain conditions Shp2 could be localized to LRP1 and thus not exert any activity towards PDGF-R activation. Shp2 targeting could be directed by any of its domains and regulated by phosphorylation.

Different deletion mutants of Shp2 expressed in mammalian cells were tested for which domains were necessary bind to LRP1. As expected, the SH2 domains of Shp2 were vital for binding to LRP1. A construct lacking the tandem SH2 domains did not bind to LRP1 at all, indicating that the PTP domain does not contribute any binding specificity towards LRP1. This is contrary to what was
found in bFGF induced *Xenopus* mesoderm induction in which some substrate specificity was directed by the PTP domain of Shp2 (O'Reilly and Neel, 1998). Another construct that lacked only the N-terminal SH2 domain (Shp2 109) was able to bind weakly to LRP1. The stronger influence of the N-SH2 domain is in agreement with what was observed in the *Xenopus* experiment using Shp2 mutants of the SH2 domains (O'Reilly and Neel, 1998). These data suggest that both SH2 domains can bind to LRP1, raising the possibility that each SH2 domain interacts with a specific NPXYp motif in LRP1.

This idea is supported by the *in vitro* experiment in which binding of Shp2 to GST-LRP-CT (4507E-P) was observed. This binding was weaker than that observed with GST-LRP-CT (Wild-type-P). Wild-type GST-LRP-CT-P has two phosphorylated NPXY motifs as opposed to Y4507E-P which has only one. These results demonstrated weaker binding to the Y4507E protein (and in the 109 *in vivo* case above) agreed with the theory that both NPXYp motifs are required for optimal binding to Shp2 via its tandem SH2 domains.

Unfortunately, this experiment repeated *in vivo* to demonstrate Shp2 binding to mycLRPβ-Y4507E failed, as no binding was observed. This could be due to a lower amount of phosphorylation at Y4473 in cells. However a difference in binding between wild-type LRP1 and Y4473F LRP1 was observed. Binding of Shp2 to LRP1 was significantly lower in the Y4473F mutant. This result made sense because both NPXY motifs are phosphorylated in the wild-type protein. Since in the Y4473F mutant only half of the potential sites are phosphorylated only half the binding is observed. This provides more evidence
that the two SH2 domains work in tandem to create a high affinity binding interaction.

To prove this theory a matrix of LRP1 fusion proteins in phosphorylated and nonphosphorylated forms with the SH2 domains of Shp2, N-SH2, C-SH2, and 1-222 was designed to test the interactions of the individual Shp2 domains with LRP. The individual SH2 domains did not discriminate between the two phosphorylated NPXY motifs. In contrast, each SH2 domain was able to bind to each phosphorylated NPXY motif. This was shown by the use of wild-type phosphorylated protein and the Y4507E-P protein. It has been demonstrated that the SH2 domains can both bind to the phosphorylated Y4507 residue. The Y4507E-P protein demonstrated binding at phosphorylated Y4473. These data indicated that fully phosphorylated LRP (Y4473p/Y4507p) could act as a bis-phosphorylated ligand for Shp2 and lead to its activation in cells.

Future work in cells is needed to confirm this theory. It would be interesting to see if the activation of Src family kinases is affected by LRP knockdown in cells. If LRP does help activate Shp2, then one would expect that loss of LRP would result in a decrease in Src activation due to the retention of autoinhibitory tyrosine phosphorylation on its c-terminal tail. Secondly, LRP may be acting as a scaffold that helps target Shp2 to the membrane. Previous studies have shown that Shp2 recruitment to the plasma membrane is vital for its function (Allard, et al., 1996, Fujioka, et al., 1996). Binding of Shp2 to LRP may change the subset of intracellular protein substrates for Shp2.
Finally, LRP may also affect Shp2 activity by protecting it from oxidation during PDGF stimulation. PDGF stimulation results in tyrosine phosphorylation of LRP in cells, but it also initiates a reactive oxygen intermediates (ROI) cascade that acts on Shp2 (Meng, et al., 2002). Under these conditions Shp2 undergoes oxidation and becomes inactive. This is why cells are sometimes treated with hydrogen peroxide prior to lysing when protein phosphorylation is being studied. The hydrogen peroxide oxides the catalytic cysteine in the active site of phosphatases, such as Shp2, and inhibits their activity towards phosphorylated substrates. PDGF stimulation induced oxidation of Shp2 is limited to the pool of Shp2 associated with the PDGF-R (Meng, et al., 2002). If the intracellular pool of Shp2 is associated with LRP instead it would be protected from oxidation and thus remain active. It would be interesting to discover if the amount of activated Shp2 changes with PDGF stimulation between LRP +/+ and LRP -/- cells.
References


