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Interactions of the NPXY microdomains of the LDL Receptor-Related Protein 1

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Abstract

The LDL receptor-related protein 1 (LRP1) mediates internalization of a large number of proteins and protein-lipid complexes and is widely implicated in Alzheimer's disease. The cytoplasmic domain of LRP1 (LRP1-CT) can be phosphorylated by activated protein-tyrosine kinases at two NPXY motifs in LRP1-CT; Tyr 4507 is readily phosphorylated and must be phosphorylated before phosphorylation of Tyr 4473 occurs. Pull-down experiments from brain lysate revealed numerous proteins binding to LRP1-CT, but the results were highly variable. To separate which proteins bind to each NPXY motif and their phosphorylation dependence, each NPXY motif microdomain was prepared in both phosphorylated and non-phosphorylated forms and used to probe rodent brain extracts for binding proteins. Proteins that bound specifically to the microdomains were identified by LC-MS/MS, and confirmed by western blot. Recombinant proteins were then tested for binding to each NPXY motif. The NPXY₄₅₀₇ (membrane distal) was found to interact with a large number of proteins, many of which only bound the tyrosine-phosphorylated form. This microdomain also bound a significant number of other proteins in the unphosphorylated state. Many of the interactions were later confirmed to be direct with recombinant proteins. The NPXY₄₄₇₃ (membrane proximal) bound many fewer proteins and only to the phosphorylated form.

Keywords

receptor tyrosine kinase; phosphotyrosine; lipoprotein receptor; signal transduction; protein interactions

The LDL receptor-related protein 1 (LRP1) is an integral membrane protein that carries out the internalization of a large number of proteins, protein complexes and lipoproteins including very low-density lipoproteins, chylomicron remnants, activated α_2 macroglobulin, and the amyloid precursor protein [1,2]. LRP1 plays a role in lipid transport, the uptake of protease-inhibitor complexes and has been implicated in Alzheimer's disease [3].

LRP1 is composed of an entirely extracellular α -chain of 3924 amino acids that is non-covalently linked to a β -chain of 601 amino acids that contains an extracellular region, a single transmembrane region, and a highly conserved cytoplasmic region composed of 100 amino acids including four tyrosine residues. Two of these, Tyr 4473 and Tyr 4507, are present in the

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context of Asn-Pro-X-Tyr (NPXY) motifs. The NPXY motif was first identified in the LDL receptor where it is essential for clathrin-mediated internalization [4]. The CED-6/GULP protein involved in receptor trafficking has also been shown to bind LRP1 and the membrane-distal NPXY₄₅₀₇ motif was implicated in binding [5]. The NPXY₄₅₀₇ motif in LRP1 has been implicated in binding other PTB domain-containing intracellular signaling proteins such as ShcA, Fe65 and Disabled (Dab1) [6–8]. Yeast two hybrid analysis of the LRP1-CT found a number of proteins including several with PTB domains [9]. This study also found that LRP1-CT interacted with a homolog of PIP 4, 5 kinase hinting of a broader role for LRP1, perhaps in assembling signaling complexes. Indeed, other members of the LRP family also play important roles as mediators of signal transduction [10].

LRP1 is tyrosine phosphorylated in v-Src transformed mouse fibroblasts, and Tyr 4507, one of two NPXY tyrosines, was identified as the principle v-Src phosphorylation site in LRP1 [6]. Others have found that LRP1 is tyrosine phosphorylated in response to platelet-derived growth factor [11]. The ShcA PTB domain is known to bind to phosphorylated tyrosine residues present in the context of NPXY motifs, and has been shown to bind phosphorylated LRP1 [6,12]. We recently showed that phosphorylation of the two NPXY motifs in LRP1-CT is sequential. Phosphorylation of the membrane-distal NPXY₄₅₀₇ motif microdomain causes exposure of the membrane-proximal NPXY₄₄₇₃ motif microdomain which is then subsequently phosphorylated [13]. In addition, tyrosine phosphorylation at NPXY₄₄₇₃ prevented binding of Snx17 and enhanced binding of Shp2, which has two SH2 domains that can each bind an NPXY motif [13]. These observations led us to investigate whether other proteins might interact with the NPXY-containing microdomains of LRP1-CT and whether their binding might depend on tyrosine phosphorylation. Since experiments in which LRP1 is isolated from cells could not reveal which NPXY motif microdomain was the site of binding nor the phosphorylation dependence, we decided to prepare each NPXY motif microdomain separately in both phosphorylated and unphosphorylated forms to discover specific protein-protein interactions that were occurring at each site.

Experimental Procedures

Immobilization of LRP1-CT microdomains

The microdomains containing Tyr 4507 (Cys-amino aminohexanoic acid-TNFTNPVY₄₅₀₇ATLY) and Tyr 4473 (Cys-amino aminohexanoic acid-VEIGNPTY₄₄₇₃KMYEGGE) in both phosphorylated and unphosphorylated forms were synthesized and purified from AnaSpec, San Jose, CA. The peptides were immobilized using Sulfolink coupling gel according to the manufacturer's directions (Pierce, Rockford, IL). Due to poor solubility of the unphosphorylated peptides all immobilization reactions were carried out in 3.5M Guanidine HCl, TBS pH 8.0. The amount of peptide immobilized was quantified by UV absorbance. Efficiency of immobilization was found to be 1.5–2.5mg peptide per milliliter of beads.

To verify that peptides were correctly immobilized and accessible, 10μL of peptide beads were added to a 25μg/mL chymotrypsin solution in TBS pH 7.4 and agitated at room temperature for 2 hours. Resulting peptides were mixed 1:1 with alpha-cyano-cinnamic acid (Agilent). Analysis by MALDI-TOF on an Applied Biosystems DE-STR yielded m/z 813.3 for NPXY₄₄₇₃ [(Y)KMYGEEG], 1063.5 for NPXY₄₅₀₇ [(F)TNPVYATLY], and 1143.4 for NPXpY₄₅₀₇ [(F)TNPVpYATLY], suggesting that peptides were susceptible to proteases, thus exposed on the bead surface.

As a further control for specificity of binding, the immobilized NPXpY₄₅₀₇ microdomain (20μL of beads) was treated with 10U shrimp alkaline phosphatase (SAP) (Promega) at 37°C for 12 hours in TBS pH 9.0, 1mM MgCl₂. Non-phosphorylated NPXY₄₅₀₇ as well as NC beads

were also treated with SAP the same way to rule out any other effects the phosphatase treatment may have on the beads.

Cell culture

Control and v-Src-transformed 14.30 and 14.30S3 mouse fibroblasts, and HEK293 cells were grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 25 mM glucose and 10% calf serum at 37°C in 10% CO₂ until confluent and then starved for 12 hours in serum free medium. MEF cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 25 mM glucose and 10% fetal bovine serum at 37°C in 10% CO₂. The 11H4 hybridoma was obtained from ATCC (Manassas, VA) and grown in Iscoves-modified Dulbecco's medium containing 25 mM L-glutamine and 10% FBS at 37°C in 10% CO₂.

Preparation of rat brain lysates

Brains of 3 month old Long Evans rats were resuspended in PLC lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X100, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM Sodium Pyrophosphate, 500 μ M Sodium Vanadate, 1 mM PMSF, 2 mM DTT, with protease inhibitor cocktail (P8340, Sigma-Aldrich) (1 brain in 10 ml) by Dounce homogenization. Lysates were cleared by high-speed centrifugation and incubated batch-wise with immobilized peptides (20 μ L) at 4°C on a rocker for 2 hrs. The beads were washed four times with PLC-lysis buffer, and bound proteins were analyzed by SDS-PAGE.

In-gel digestion

The proteins that bound to the NPXpY₄₅₀₇ microdomain were separated by 1-D SDS-PAGE using 10% Bis-Tris NuPAGE gels (Invitrogen). Gels were stained overnight with Gel Code Blue (Pierce) according to manufacturer's suggested protocol. Bands unique to the NPXpY₄₅₀₇-bound sample were excised, washed twice with 200 μ L of 50% acetonitrile (ACN) and 50% 5 mM DTT / 25 mM NH₄HCO₃ (pH 7.4), reduced in 100mM DTT for 30 minutes at 50°C, washed again, and subsequently alkylated with 100mM iodoacetimide. The gel pieces were dehydrated in pure ACN, rehydrated by addition of 20 μ L of ice-cold 10 ng/ μ L trypsin (Promega) in 25 mM NH₄HCO₃ (pH 7.4), incubated on ice for 30 min and the remaining trypsin solution was removed and replaced with fresh 25 mM NH₄HCO₃ (pH 7.4). The digestion was allowed to continue at 37°C overnight. The peptide mixture was then acidified with 2 μ L of 2% trifluoroacetic acid (TFA) (Fluka) and 2 μ L of acetonitrile, vortexed for 30 min, and the supernatant extracted. Finally, 20 μ L of 20% acetonitrile/ 0.1% TFA was added followed by vortexing to extract the remaining peptides and combined with the previous fraction. The combined extractions were concentrated in a speedvac prior to mass spectrometry.

In solution digestion

Rat brain lysates (3 mls) were cleared by high-speed centrifugation and incubated batch-wise with immobilized peptides (35 μ L) at 4°C on a rocker for 2 hrs. After washing as before, the beads were washed twice with TBS 2mM EDTA, pH 8.0, then resuspended in the same buffer with 0.5% Rapigest® (Waters), and boiled for 10 min to elute bound proteins. The mixture was treated with 3mM TCEP (Sigma) for 30 min, then 6mM iodoacetamide (Sigma) for 30 min, and finally quenched with another 3mM TCEP. Freshly dissolved proteomics grade trypsin (10 μ g, Roche), was added to each sample, and digestion was carried out overnight at 37°C. The digestion was stopped with the addition of 100mM HCl, and incubated at 37°C for 1 hr to breakdown the Rapigest®. Samples were then centrifuged for 30 min at 4°C to remove beads and insoluble degraded lipid. Resulting peptides were extracted via C18 solid phase extraction (Varian, A57203) according to manufacturer's suggested protocol, and concentrated by speedvac prior to MS analysis.

Mass spectrometry

Digested samples were analyzed by electrospray ionization using a QSTAR-Elite hybrid mass spectrometer (Applied Biosystems) interfaced to a capillary column (180 ID, 365 OD) packed with C18 and capped with 0.2 mm filters on either end connected via 4 cm 25 ID (365 OD) capillary to the nanospray source. The spray needle was a pulled capillary 180~15 tip packed with C18. One dimensional chromatography was used for the samples from in-gel digests, whereas 2-dimensional "MUDPIT" chromatography was used for the in solution digests. For 2D chromatography, a strong cation exchange column was inserted in between the autosampler and injector valve. To accomplish the 2D chromatographic separation, the TEMPO LC high flow rate Channel 1 (10 μ l/min) was used to load and separate on the SCX column with buffers A: 2.5% ACN, 0.2 % TFA 0.005% TFA; and Channel 1 B: 500mM ammonium acetate, 5% ACN, 0.2 % TFA. To separate on the C18 reverse phase, the low flow rate Channel 2 (400 nL/min flow) with buffer A: 2.5% ACN, 0.2 %FA 0.005%TFA and Channel 2 B: 100% ACN, 0.2 %FA 0.005% TFA was used. MUDPIT runs were carried out as described previously [14] with an 8 step ion exchange elution. Each was then separated on a 35-min linear gradient from 5 to 40% buffer B at a flow rate of 400 nL/min. LCMSMS data were acquired in a data-dependent fashion by selecting the 4 most intense peaks with charge state 2–4 that exceeded 10 counts with exclusion of former target ions set to always and the mass tolerance for exclusion set to 100 ppm. TOF MS were acquired at m/z 400–1600 Da for 1 sec with 20 time bins to sum. MSMS spectra were acquired from m/z 65 – 2000 Da using "enhance all" and 20 time bins to sum, Dynamic Background Subtract, Automatic Collision Energy, and Automatic MS/MS Accumulation with the Fragment Intensity Multiplier set to 4 and Maximum Accumulation set to 2 sec before exiting the scan.

Database search

Data were analyzed by Analyst 2.0 (Applied Biosystems) and subjected to two different database search protocols. First, spectra were searched with Protein Pilot using the paragon algorithm [15] against both the rat database alone (*Rattus norvegicus*, ncbi 07/06/2006, ftp://ftp.ncbi.nih.gov/genomes/R_norvegicus/protein/) as well as the non-redundant swissprot database (swissprot 10/29/2008, <ftp://ftp.ncbi.nih.gov/blast/db/FASTA/>). Search parameters included fixed carboxymethyl modification of cysteines and included common forms of variable modifications. Only protein hits with at least 2 strong unique peptide matches (>95) were kept. Any hits in the negative control were subtracted from the other samples. The spectra were also searched using Mascot 2.2.1 (Matrix Science) with Mascot Daemon 2.2 (Matrix Science) data import filter parameters set as follows: default precursor charge state 2–4; precursor and MSMS data centroiding using 50% height and 0.05 amu merge distances. MSMS peaks with intensity less than 1% of the base peak were discarded, as were MSMS spectra with less than 22 peaks remaining. Data were searched against the Swissprot database obtained at <ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/> containing 237,168 sequences. The search identified tryptic peptides with up to 2 missed cleavages and used mass tolerances of 100 ppm (MS) and 0.10 Da (MSMS), with variable modifications as follows: deamidation (NQ), oxidation (M), pyro-Glu (N-term E). The search results indicated that individual ion scores > 42 indicate identity or extensive homology ($P < 0.05$). Numbers of peptides reported are for non-redundant peptides. MASCOT and Protein Pilot both identified the same proteins, so the data from Protein Pilot are presented.

Common contaminants including keratin, trypsin, and tubulin were omitted from final table. Results for each microdomain were compared to the results without immobilized peptide and only those proteins that were not observed in the negative control were considered "real". This criteria resulted in 14/39 "real" hits for the NPXpY₄₅₀₇ microdomain, 31/80 "real" hits for the NPXY₄₅₀₇ microdomain, 3/23 "real" hits for the NPXpY₄₄₇₃ microdomain, and no "real" hits for the NPXY₄₄₇₃ microdomain.

Antisera, immunoprecipitation, and immunoblotting

A polyclonal anti-Shp-2 serum was raised against a GST fusion protein containing both SH2 domains. The same technique was used to generate polyclonal anti-Grb2, anti-ShcA, and anti-CSK. a polyclonal anti-LRP1 serum was raised against a GST-fusion protein containing the cytoplasmic domain. Anti-phosphotyrosine monoclonal antibody 4G10, polyclonal anti-p85 PI3K serum, anti-HA (12CA5) and anti-Fe65 (3H6) were purchased from Upstate (Charlottesville, VA). Anti-PLC γ and anti-GAPDH (14C10) were purchased from Cell Signaling Technologies (Danvers, MA). Anti-CrkI (SC-31694) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Src monoclonal antibody 327 was a gift from Dr. T. Hunter (La Jolla, CA). Anti-14-3-3 (57-0700), anti-Ubiquitin (13-1600) and anti-CamK2B (13-9800) were purchased from Zymed (Carlsbad, CA). Anti-GST antibody (27457701) was from GE Healthcare. Immunoprecipitation and immunoblotting were carried out exactly as described previously [6], with the exception of Ubiquitin blots which were autoclaved for 20 minutes after transfer for increased sensitivity [16].

Recombinant protein expression and purification

GST tagged SH2 domains from human Grb2 (58–159), PLC γ -1 (“N” 544–659 & “C” 663–759), and PI3K (“N” 314–446 & “C” 614–724) and full length 14-3-3 γ (genbank ID 21464100) were cloned into pGEX2T (GE Healthcare) using BamHI and EcoRI restriction sites. BL21-DE3 cells were grown to OD₆₀₀ 0.5 at 37°C and protein expression induced with 0.3mM IPTG at 18°C overnight. Cells were harvested by centrifugation, resuspended in 50mM Tris pH 8.0, 500mM NaCl, 1mM DTT, and 1mM PMSF (Sigma) and lysed by sonication. After clearing debris by centrifugation at 12krpm for 40 min, cell extract was loaded over Glutathione-sepharose (GE Biosciences) according to manufacturer’s instructions. Eluted protein was buffer exchanged and concentrated over Y10K centricon (Millipore). 14-3-3 γ was also cloned into pET15 with an N-terminal His tag, and purified by Nickel affinity chromatography followed by gel filtration. Recombinant purified His tagged CSK was a gift from Patricia Jennings. His-tagged CamKII β was purchased from Sigma. Purified calmodulin was purchased from Millipore. SH2 domains of Shp-2 and HA tagged Snx17 were expressed and purified as previously described [13]. For recombinant pulldown reactions 10 μ L of NPXY beads were incubated in a 100nM solution of protein in the same PLC-lysis buffer used for previous pulldowns except for CamKII β which contained 50nM CamKII β and 200nM Calmodulin. Reactions were washed and blotted as described above.

Results

LRP1 co-immunoprecipitates with tyrosine phosphorylated proteins in v-Src transformed fibroblasts

Several groups have shown that LRP1 can become phosphorylated on Tyr in the distal NPXY₄₅₀₇ motif by activated protein-tyrosine kinases [6, Barnes, 2003 #17, 17]. Association of LRP1-CT with “signaling” proteins such as Shp2 and Src has also been demonstrated [6]. These observations led us to hypothesize that other proteins may also bind to LRP1 in a phosphorylation dependent manner.

To find out whether LRP1 is involved in multiple phosphotyrosine-dependent protein-protein interactions, LRP1 was isolated by immunoprecipitation from control and v-Src transformed fibroblasts and the immunoprecipitate was analyzed by anti-P.Tyr immunoblotting. The results show LRP1 is associated with several P.Tyr-containing proteins in v-Src transformed cells (Figure 1A). A significant increase in these interactions was observed when cells were treated with protein tyrosine phosphatase inhibitors orthovanadate and hydrogen peroxide (Figure 1A).

Because tyrosine phosphorylation sites can act as docking sites for other proteins we asked whether any of the proteins that coimmunoprecipitate with LRP1 require Tyr 4507 phosphorylation for binding. The NPXpY₄₅₀₇ microdomain was prepared and immobilized on agarose beads (TNFTNPV(pY₄₅₀₇)ATLY). This immobilized microdomain was first used to probe for binding proteins v-Src transformed fibroblasts (Figure 1B). The results again showed a number of tyrosine-phosphorylated proteins were specifically binding to the microdomain.

LRP1(NPXY)-interacting proteins in the brain

Because LRP1 is highly expressed in the mammalian brain, we sought to find out whether these interactions were only found in v-Src transformed fibroblasts, or whether they were also present in brain tissue. Figure 2 shows that a similarly large number of proteins were found to bind specifically to the phosphorylated microdomain when rat brain lysates were probed. The bands corresponding to proteins binding specifically to the NPXpY₄₅₀₇ microdomain were excised and the proteins were identified by LC-MSMS sequencing (Table 1). A large number of signaling proteins were found to bind specifically to this microdomain in its phosphorylated form.

To better characterize the phosphorylation dependence of the NPXY microdomains, the four microdomains were prepared and analyzed simultaneously; the NPXY₄₄₇₃ microdomain, NPXpY₄₄₇₃ microdomain, NPXY₄₅₀₇ microdomain, and NPXpY₄₅₀₇ microdomain. Anti-P.Tyr blotting of the bound proteins showed a smear of tyrosine phosphorylated proteins binding to the phosphorylated NPXY₄₅₀₇ and NPXY₄₄₇₃, but not to the unphosphorylated forms (Figure 3A). To detect all proteins interacting with these peptides the bound proteins were analyzed by silver staining (Figure 3B). It was evident that while the NPXY₄₄₇₃ microdomain did not seem to have many specifically bound proteins, the NPXY₄₅₀₇ microdomain had a large number of specific interactions. To ensure the differences observed between NPXpY₄₅₀₇ and NPXY₄₅₀₇ were not the result of anything other than phosphorylation, NPXpY₄₅₀₇ beads were treated with shrimp alkaline phosphatase. The resulting silver stained gels showed that removal of the phosphate from NPXY₄₅₀₇ yields a binding profile identical to unphosphorylated NPXY₄₅₀₇ (Figure 4).

Proteins bound to each NPXY motif were eluted and analyzed by in solution trypsin digest followed by 2D LC-MSMS. Proteins with at least two strong (>95 confidence) unique peptide hits are listed in Table 2–Table 4. The phosphorylated membrane distal NPXY₄₅₀₇ microdomain showed dozens of strong protein interactions (Table 2) whereas the phosphorylated membrane proximal NPXY₄₄₇₃ microdomain had very few (Table 3). Besides Grb-2 and Shc-3, both of which were also found in the NPXpY₄₅₀₇ sample, the only protein identified as interacting specifically with NPXpY₄₄₇₃ was peptidyl-prolyl cis-trans isomerase A (Table 3). The unphosphorylated NPXY₄₅₀₇ microdomain also bound many more proteins (Table 4) than the unphosphorylated NPXY₄₄₇₃, which had no protein hits that were not also found in the negative control. A few of the proteins that were observed in the in gel digest analysis of the phosphorylated NPXY₄₅₀₇ interacted more strongly with the unphosphorylated NPXY₄₅₀₇ microdomain. The most likely explanation of these results is that some dephosphorylation of the microdomain occurred during the experiment prior to in gel digestion.

Western blotting confirmation of binding interactions

To confirm the binding of several of the identified proteins to the microdomains of LRP1, bound proteins were resolved by SDS-PAGE, transferred to PVDF membranes and analyzed by immunoblotting. This experiment confirmed that PI3-kinase, Shp-2, PLC- γ , Src, CSK, Shc-3, and Grb-2, present in lysates of rat brains are able to bind to the NPXpY₄₅₀₇ microdomain (Figure 5). As expected from the mass spectrometry data, Shc-3 and Grb2 were found to interact with the NPXpY₄₄₇₃ microdomain also. GAPDH and 14-3-3 γ can bind to the

non phosphorylated NPXY₄₅₀₇. In agreement with our mass spectrometry data, CamKII seems to bind preferentially to unphosphorylated NPXY₄₅₀₇ microdomain with a weaker signal in the NPXpY₄₅₀₇ microdomain lane.

A few expected binding interactions were not observed in our mass spectrometry experiments, and these were also probed by Western blotting. Fe65 had previously been reported to interact with LRP at the NPXY motifs [18], but was not identified in our study. Blotting of the NPXY-bound proteins for Fe65 revealed that indeed, it could bind to both the phosphorylated and unphosphorylated NPXY₄₅₀₇ microdomains. We and others had previously shown that Sorting nexin 17 (Snx17) bound LRP1-CT and that this binding depended on the presence of the NPXY₄₄₇₃ motif [13,19]. Figure 6 shows that recombinant HA tagged Snx17 binds both the unphosphorylated NPXY₄₄₇₃ and the unphosphorylated NPXY₄₅₀₇ motif. These results recapitulate our previous finding in v-Src transformed fibroblasts in which phosphorylation inhibited Snx17 binding, but add an additional finding that Snx17 binds to both of the NPXY motifs in LRP1-CT. It is not too surprising that Snx17 and FE65 were not observed in the proteomics analysis as we have observed low abundance of these proteins in our rodent lysates [13].

Assessment of which proteins bind directly to LRP1-CT

Given the large number of proteins that were observed to bind to the NPXY₄₅₀₇ microdomain, it was possible that most of the interactions were indirect and that the proteins were bound in some sort of large signaling complex. To test for direct interactions, recombinant proteins were prepared and probed for binding to the NPXY microdomains. These experiments revealed that 14-3-3 γ reproducibly interacts with only the NPXY₄₅₀₇ microdomain in either a GST tagged or a His-tagged construct (Figure 6). All SH2 domains from PLC γ , Grb-2, PI3K, Shp-2, and full length CSK were also tested either with a GST or His₈-Ubiquitin tag. These experiments revealed that certain SH2 domains can bind both phosphorylated NPXY₄₄₇₃ and NPXY₄₅₀₇ motifs, and others only bind avidly to NPXpY₄₅₀₇ (Figure 6). Recombinant CamKII β was able to bind to both phosphorylated and non-phosphorylated NPXY₄₅₀₇.

One of the most abundant proteins that bound to the NPXpY₄₅₀₇ motif microdomain was identified as Shp-2. Since previous studies have shown that Shp-2 can interact with both phosphorylated NPXY₄₄₇₃ and NPXY₄₅₀₇ of LRP [13], we used recombinant SH2(N) or SH2(C) domains of Shp-2 fused to ubiquitin to test for direct binding to each NPXY motif. Consistent with our previous results, the NPXpY₄₅₀₇ is the high-affinity binding site (Figure 6). Only on much longer exposures could a band for SH2(C) be detected in the NPXpY₄₄₇₃ lane (data not shown).

Discussion

The LRP1-CT NPXY₄₅₀₇ microdomain may be a signaling “hub”

The LRP1 cytoplasmic domain has been implicated by association in cell signaling. We originally identified LRP1 as a Shc-binding protein [6], and later it was also shown to bind several PTB domain-containing proteins including disabled (Dab1) [7], and by yeast-two-hybrid studies JIP-1 and -2, CAPON, and a PIP4 kinase homolog among others [9]. In addition, it interacts with the PDGF receptor- β [20], is phosphorylated in response to PDGFR ligand binding [11], and is at least sometimes localized to lipid rafts [21]. It also binds Shp2 only in the phosphorylated form, and Snx17 only in the unphosphorylated form [13]. These results were the impetus for the studies presented here, in which the two NPXY microdomains and their phosphorylated counterparts were separately analyzed for protein binding interactions in rodent brain lysates.

The only way to separately isolate such microdomains and to obtain them in pure form (either phosphorylated or unphosphorylated) is to prepare them synthetically. Previous work has shown that this approach is effective for identifying proteins that bind to NPXY motifs and in particular to probe the dependence of binding on tyrosine phosphorylation [22,23]. This approach led to the identification of a variety of proteins that bind specifically to the phosphorylated or unphosphorylated NPXY microdomains of LRP1-CT. In some cases the binding depended strongly on phosphorylation state, as previously seen with peptide array studies with NPXY motifs [23]. Based on our findings the NPXY₄₄₇₃ has very few detectable binding partners, while the NPXY₄₅₀₇ had many direct interactions both in the phosphorylated and unphosphorylated states. It should be noted that the NPXpY₄₄₇₃ microdomain serves as an important specificity control. The proteins observed to bind to the NPXpY₄₅₀₇ microdomain must be recognizing more than just the NPXpY sequence, or they would have also been observed to bind to the NPXpY₄₄₇₃ microdomain. Of all the signaling proteins that bound to the distal NPXpY₄₅₀₇ microdomain, only Shc3 and Grb2 bound also to the NPXpY₄₄₇₃ microdomain.

Many of the signaling proteins bound to the phosphorylated form of the LRP1 NPXY₄₅₀₇ microdomain including PLC γ , PI 3-kinase, Shp-1, Shp-2, Src, Fyn, ShcC and CrkL, are proteins that are recruited to the plasma membrane and are tyrosine phosphorylated in response to extracellular signals [24–31]. Aside from Shp-2, these are all previously unreported interactions, and identification of these proteins as potential LRP1 binding proteins further substantiates the model in which LRP1 functions as a signaling receptor. The finding that CamKII binds both NPXY₄₅₀₇ and NPXpY₄₅₀₇ microdomains gives yet more support for the role of LRP1 in signaling. Meaningful co-localization experiments will require introduction of mutant forms of the LRP1-CT at endogenous levels under controlled phosphorylation conditions. These are quite challenging experiments and were outside the scope of the current project.

Several independent studies have found that the adaptor protein ShcA binds to tyrosine phosphorylated LRP1. In the current study we have identified Shc-3 (ShcC) as an LRP1 binding protein in rat brain lysates. ShcC is a Shc family member that is specifically expressed in the brain [30,32,33]. It is well established that all Shc family members contain an amino-terminal PTB domain that binds to phosphorylated NPXY motifs. In the case of LRP1 it seems that both NPXY motifs in their phosphorylated forms can interact with ShcC.

Shp-2 is a protein-tyrosine phosphatase that contains two SH2 domains followed by a phosphatase domain. We have found that Shp-2 strongly binds to the NPXpY₄₅₀₇ motif and have confirmed that it also binds the phosphorylated LRP1 cytoplasmic domain [13]. This is consistent with the fact that the amino-terminal SH2 domain of Shp-2 binds to phosphorylated tyrosine residues followed by non-polar residues at +1, +3 and +5 [34]. This consensus is matched exactly by the sequence carboxy-terminal to NPXY₄₅₀₇, but not in NPXY₄₄₇₃ which has a much weaker apparent affinity for Shp2. The result that phosphorylated NPXY₄₄₇₃ can bind Shp-2 with weaker affinity agrees with previous results [13]. The observation that Shp2 bound so strongly to the immobilized NPXpY₄₅₀₇ microdomain is most likely due to the fact that Shp2 has two SH2 domains, which can simultaneously engage the multiple copies of the microdomain that are available when it is immobilized. This phenomenon could also be occurring for the other proteins with multiple binding sites.

Both LRP1-CT NPXY microdomains may be involved in receptor sorting

The unphosphorylated NPXY₄₅₀₇ microdomain specifically bound AP-2 adaptors and clathrin as well as many “house-keeping” and metabolic proteins (Table 3). These interactions were specific for the unphosphorylated microdomain and were not observed with the phosphorylated microdomain. These results show how phosphorylation is likely to affect receptor

internalization and sorting. Indeed, such an effect has been previously observed in cells in which stimulation of PDGFR causes changes in LRP1 phosphorylation and subsequent internalization of both proteins [35].

In the course of this study nothing was found specifically interacting with NPXY₄₄₇₃ which was surprising. The peptide was clearly immobilized as the others based on UV follow up of the immobilization and chymotryptic digestion of the beads. Since Snx17 was previously shown to interact with this region of the LRP1-CT [13,19], recombinant Snx17 was tested for binding with all four of the microdomains. In these experiments, specific binding to both NPXY₄₄₇₃ and NXPY₄₅₀₇ was observed. It is possible that in previous studies the NPXY₄₅₀₇ region was in a phosphorylated form thus unable to interact with Snx17, as we reported previously and observed again here [13]. At the very least this demonstrates that NPXY₄₄₇₃ could have specific interactions in the brain, just below our detection limit in this study.

Connection between LRP1 and APP

Several lines of evidence have implicated LRP1 in the development of Alzheimer's disease. APP, the amyloid precursor protein, binds directly to LRP1 [36]. In addition, it has been shown that A β constituent of amyloid plaques, can bind to α_2 M and ApoE; both of which are also ligands for LRP1 [37]. Genetic evidence shows increased presence of certain alleles of LRP1, α_2 M and ApoE in Alzheimer's disease patients [38–40]. Thus it is generally thought that LRP1's involvement in Alzheimer's disease is related to its ability to interact directly or indirectly with APP or A β . The proteins newly identified to bind to LRP1-CT microdomains in this study are peculiarly interesting when combined with recent findings that residues 4504–4510 of LRP affect APP processing and A β generation [41,42]. Therefore these interactions at the NPXY₄₅₀₇ site could play a role in A β generation and thus the onset of Alzheimer's disease. Recent RNAi experiments provide a functional link between Shc and APP processing [43]. It has also been shown that APP can become phosphorylated on Y682 which can then interact with ShcA and ShcC. [44] although it was later found that phosphorylation of APP was not the underlying cause of its processing [45]. The same study also showed that APP processing is affected by several tyrosine kinases including Src, and that the effects are dependent on the presence of LRP [45]. Fluorescence lifetime imaging microscopy showed that APP and LRP are co-localized regardless of tyrosine phosphorylation of LRP [46]. Thus, LRP's affect on APP trafficking is probably best explained by LRP recruiting these signaling molecules to APP.

The specific interaction of NXPY₄₅₀₇ with 14-3-3 proteins provides another link between LRP1 and APP in the light of recent evidence that APP interacts with 14-3-3 γ [47]. Fe65 is another adaptor protein that has been shown to interact with both APP and LRP, and modulate A β generation [18]. Fe65 from rat brains interacted only at the NPXY₄₅₀₇ in both phosphorylation states. This is in clear agreement that only the distal NPXY motif was shown to have effects on APP trafficking and A β generation [41].

The results described here identify several kinases that can bind to LRP1 including; CamKII, Src, Fyn, and CSK. Interestingly, CaM kinase II [48,49] and Src family kinases [50–52] have been implicated previously in Tau phosphorylation. These observations suggest that LRP1 could also be involved in linking APP and A β to Tau hyperphosphorylation, although this conjecture will need to be studied in much more detail.

Abbreviations

LRP1 low density lipoprotein receptor-related protein 1

LRP1-CT	LRP1 cytoplasmic tail (amino acids 4745–4807)
SCX	strong cation exchange
TCEP	triscarboxyethylphosphine
CAMKII	calmodulin-dependent kinase II
CSK	c-src kinase
PI3	phosphoinositide-3
GST	glutathione S-transferase.

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Figure 1. LRP1 associates with multiple tyrosine phosphorylated proteins

A) LRP1 was isolated by immunoprecipitation from control (–) or vanadate/hydrogen peroxide treated (+) 14.30 and v-Src transformed (14.30S3) mouse fibroblasts. Immunoprecipitates were analyzed by anti-P.Tyr immunoblotting. B) Lysates from control or vanadate/hydrogen peroxide treated fibroblasts were incubated with agarose beads containing phosphorylated or unphosphorylated NPXY₄₅₀₇ microdomains. Bound proteins were analyzed by anti-P.Tyr immunoblotting. Anti-LRP1 immunoprecipitates were analyzed in parallel.

Figure 2. A large number of proteins appear to bind to the phosphorylated NPXY₄₅₀₇ microdomain
Rat brain extracts were incubated with either unreacted agarose beads or the phosphorylated NPXY₄₅₀₇ microdomain linked to agarose beads. Bound proteins were analyzed by Gelcode blue staining. Individual protein bands were isolated and identified by mass spectrometry and data-base searching.

Figure 3. Identification of proteins that bound to each NPXY microdomain in either the phosphorylated or unphosphorylated forms

Rat brain extracts were incubated with the each microdomain linked to agarose beads. The sequences were: NC uncoupled beads, Y₄₄₇₃ VEIGNPTYKMYEGGE, pY₄₄₇₃ VEIGNPTpYKMYEGGE, Y₄₅₀₇ TNFTNPVYATLY, and pY₄₅₀₇ TNFTNPVpYATLY. Bound proteins were analyzed by (A) anti-phosphotyrosine blotting or (B) silver staining.

Figure 4. Tyrosine phosphorylation on Y₄₅₀₇ accounts for the differences in binding partners

To ascertain whether the differences between the phosphorylated and unphosphorylated NPXY₄₅₀₇ microdomains was due purely to the tyrosine phosphorylation, and NPXpY₄₅₀₇ and NPXY₄₅₀₇ beads were treated extensively with Shrimp alkaline phosphatase, and subsequently used to probe rat brain lysates, along with untreated pY₄₅₀₇. Samples were visualized by silver staining.

Figure 5. Validation of the specificities of each binding protein assessed by western blot
 Rat brain extracts were incubated with each immobilized peptide. Bound proteins were analyzed by immunoblotting with antisera raised against 14-3-3γ, CamKII, Shp-2, PI3K p85, CSK, Src, N-Shc, PLCγ, Grb-2, GAPDH, Crkl, and Fe65.

Figure 6. Confirmation that some of the interactions are direct

Purified recombinant proteins were incubated with each immobilized peptide. Bound proteins were analyzed by immunoblotting with anti-HA (Snx17), anti-GST (SH2's from Grb2, PLCγ, PI3K p85), anti-14-3-3γ, anti-Ubiquitin (Shp2 SH2s), anti-CSK, and anti CamKIIβ.

Table 1LRP1 NPXpY₄₅₀₇ interacting proteins identified by in-gel digest/LC-MSMS

Protein Accession number	Sequence Coverage	# unique of Peps (>95)
Shp-2	41%	27
gi 84028250		
PLC-gamma 1	15%	20
gi 6981370		
Son of sevenless homolog 1	14%	16
gi 162287023		
pyrroline-5-carboxylate reductase family 2	51%	17
gi 58865992		
growth factor receptor bound protein 2 (GRB2)	58%	13
gi 77539774		
CAM kinase II beta subunit	27%	12
gi 108796657		
Shc-3 (neuronal Shc)	23%	11
gi 48474721		
Tnf receptor-associated factor 3	21%	11
gi 157823541		
PI3-kinase, regulatory subunit, (p85 alpha)	16%	9
gi 6981358		
v-crk sarcoma virus CT10 oncogene homolog	25%	7
gi 56605658		
megakaryocyte-associated tyrosine kinase	20%	7
gi 11177904		
PI3-kinase p110 subunit alpha (isoform 1-3)	8%	7*
gi 109466432, gi 109466430, gi 109466434		
casein kinase 1, delta	20%	7
gi 158631200		
LanC lantibiotic synthetase component C-like 2	18%	6
gi 62079109		
Shp-1	12%	5
gi 16758788		
inositol polyphosphate-5-phosphatase D	5%	5
gi 9506813		
casein kinase 1, alpha 1	18%	5
gi 16758950		
2,4-dienoyl CoA reductase 1, mitochondrial	23%	5
gi 17105350		
pyrroline-5-carboxylate reductase 1	33%	5
gi 157786894		
PREDICTED: similar to RIKEN cDNA 2810457I06	6%	4*

Protein Accession number	Sequence Coverage	# unique of Peps (>95)
(Cbl-interacting protein p70)		
gi 109484556, gi 109483356		
CAM kinase II, alpha	15%	4
gi 6978593		
c-src tyrosine kinase	12%	4
gi 71795633		
Rous sarcoma oncogene (Src)	5%	3
gi 14010835		
CAM kinase II, gamma	23%	3
gi 19424316		
high mobility group box 1, (many isoforms)	8%	3
gi 6981026, gi 157820087, gi 109512438 ...		
Shc-2	7%	3
gi 157820915		
similar to son of sevenless homolog 2	4%	3
gi 109479476, gi 157820915*		
family with sequence similarity 120B	4%	3
gi 157818809		
fyn proto-oncogene	5%	3
gi 6978863		
RAS p21 protein activator 1	2%	2
gi 6981460		
CAM kinase II, delta	12%	2
gi 6978595		
PI3-kinase, catalytic, beta polypeptide	2%	2
gi 16758236		
non-catalytic region of tyrosine kinase adaptor protein 2	6%	2
gi 157817418		
v-crk sarcoma virus CT10 oncogene homolog	4%	1***
gi 9506515		
zeta-chain (TCR) associated protein kinase	2%	1***
gi 58865580		
Tyrosine-protein kinase SYK	2%	1***
gi 3219819		
Serine/threonine-protein kinase DCLK1	2%	1***
gi 20137987		
AP-2 complex subunit mu-1-A	2%	1***
gi 18034787		
hydroxysteroid (17-beta) dehydrogenase 10	5%	1***
gi 13994225		

Protein Accession number	Sequence Coverage	# unique of Peps (>95)
protein kinase N3	1%	1 ***
gi 114145515		
14-3-3 protein zeta/delta	5%	1 ***
gi 62990183		
profilin 2	9%	1 ***
gi 13540707		
glycogen synthase kinase 3 beta	3%	1 ***
gi 14091770		
adaptor protein complex AP-1, beta 1 subunit	1.5%	1 ***
gi 8392872		
Ankyrin repeat and sterile alpha motif domain-containing protein 1B (AIDA-1)	1%	1 ***
gi 182627625		
PREDICTED: similar to Tripartite motif protein 7	2%	1 ***
gi 109490605		

* Peptides can't unambiguously assign an isoform, so all possibilities are listed.

1 strong peptide match MSMS provided in supplementary figures.

Table 2LRP1 NPXpY₄₅₀₇ interacting proteins identified from solution digest/2DLC-MSMS

Protein (14/39 hits kept) Accession number	Sequence Coverage	# unique of Peps (>95)
Shp-2 gi 84028249	33%	15
Shc-3 gi 48474721	21%	7
growth factor receptor bound protein 2 gi 51702260	35%	5
Membrane-associated guanylate kinase inverted 1 gi 123782179	5%	4
CAM kinase II, alpha gi 125285	9%	3
CAM kinase II gamma or beta subunit gi 19424316, gi 108796657, gi 108796659, gi 108796663	3%	2*
c-src tyrosine kinase gi 417209	7%	2
Shp-1 gi 34922906	5%	2
v-crk sarcoma virus CT10 oncogene homolog gi 1169094	10%	2
PI3-kinase, regulatory subunit, (p85 alpha) gi 3914252	3%	2
glutaryl-Coenzyme A dehydrogenase gi 2492632	18%	3
glutamine synthetase 1 gi 55976526	10%	2
pyrroline-5-carboxylate reductase family 2 gi 60390248	18%	4

* Peptides can't unambiguously assign one isoform, so all possibilities are listed.

** Only 1 strong peptide match, MSMS spectrum in supplementary materials.

Table 3LRP1 NPXpY₄₄₇₃ interacting proteins from solution digests identified by mass spectrometry with protein pilot

Protein (3/23 hits kept) Accession number	Sequence Coverage	# unique of Peps (>95)
Shc-3 gi 48474721	16%	6
growth factor receptor bound protein 2 gi 82236546	12%	2
Peptidyl-prolyl cis-trans isomerase A gi 118107	17%	2

Table 4LRP1 NPXY₄₅₀₇ interacting proteins from solution digests identified by mass spectrometry with protein pilot

Protein Accession number	Sequence Coverage	# unique of Peps (>95)
SEC23 homolog A	24%	13
gi 157786714		
clathrin, heavy polypeptide	10%	11
gi 9506497		
brain glycogen phosphorylase	21%	10
gi 158187544		
similar to SEC24 related gene family, member C	14%	10
gi 109502099, gi 109502097, gi 109502091, gi 109502093, gi 109502089, gi 109502095		
adaptor-related protein complex 2, beta 1 subunit	11%	9
gi 18034787		
adaptor-related protein complex 2, mu 1 subunit	29%	10
gi 16758938		
glutamine synthetase 1	27%	7
gi 142349612		
pyrroline-5-carboxylate reductase family 2	32%	8
gi 58865992		
glutaryl-Coenzyme A dehydrogenase	33%	7
gi 157820807		
hydroxysteroid (17-beta) dehydrogenase 10	46%	7
gi 13994225		
CAM kinase II, alpha	23%	7
gi 6978593		
CAM kinase II beta subunit	9%	4*
gi 108796657, gi 108796659, gi 108796663		
14-3-3 protein epsilon	13%	3
gi 13928824		
14-3-3 protein gamma	10%	3
gi 9507245		
14-3-3 protein theta	29%	5
gi 6981712		
14-3-3 protein zeta	20%	4
gi 62990183		
granulin	6%	2
gi 8393493		
Membrane-associated guanylate kinase inverted 1	11%	9
gi 71795664		
Solute carrier family 25 member 4	15%	4
gi 32189355		
Solute carrier family 25 member 12	9%	2

Protein Accession number	Sequence Coverage	# unique of Peps (>95)
gi 34854800		
Solute carrier family 25 member 22	12%	2
gi 62078785		
Solute carrier family 25 member 5	8%	2
gi 32189350		
3-hydroxyacyl-CoA dehydrogenase type-2	33%	5
gi 7387724		
USO1 homolog, vesicle docking protein	8%	5
gi 9507177		
similar glyceraldehyde-3-phosphate dehydrogenase	11%	2*
gi 109504062; gi 109503241		
gephyrin	6%	3
gi 88853561		
phosphofructokinase, platelet	4%	7
gi 57977273		
phosphofructokinase, muscle	3%	2
gi 13929002		
sideroflexin 3	14%	2
gi 12621120		
Ankyrin repeat and sterile alpha motif domain-containing protein 1B (AIDA-1)	3%	2
gi 182627625		
adaptor protein complex AP-2, alpha 1 subunit	6%	3
gi 157823677		
adaptor protein complex AP-2, alpha 2 subunit	2%	2
gi 162138932		

* Peptides can't unambiguously assign an isoform, so all possibilities are listed.