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Tyrosine Phosphorylation Regulates Alpha II Spectrin Cleavage by Calpain

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Spectrins, components of the membrane skeleton, are implicated in various cellular functions. Understanding the diversity of these functions requires better characterization of the interacting domains of spectrins, such as the SH3 domain. Yeast two-hybrid screening of a kidney cDNA library revealed that the SH3 domain of αII-spectrin binds specifically isoform A of low-molecular-weight phosphotyrosine phosphatase (LMW-PTP). The αII-spectrin SH3 domain does not interact with LMW-PTP B or C nor does LMW-PTP A interact with the αI-spectrin SH3 domain. The interaction of spectrin with LMW-PTP A led us to look for a tyrosine-phosphorylated residue in αII-spectrin. Western blotting showed that αII-spectrin is tyrosine phosphorylated in vivo. Using mutagenesis on recombinant peptides, we identified the residue Y1176 located in the calpain cleavage site of αII-spectrin, near the SH3 domain, as an in vitro substrate for Src kinase and LMW-PTP A. This Y1176 residue is also an in vivo target for kinases and phosphatases in COS cells. Phosphorylation of this residue decreases spectrin sensitivity to calpain in vitro. Similarly, the presence of phosphatase inhibitors in cell culture is associated with the absence of spectrin cleavage products. This suggests that the Y1176 phosphorylation state could modulate spectrin cleavage by calpain and may play an important role during membrane skeleton remodeling.

First identified at the intracellular surface of the erythrocyte plasma membrane, spectrins (Sp) are now known to be the central components of the membrane skeleton, a ubiquitous and complex spectrin-actin scaffold located under the lipid bilayer of metazoan animal cells (for review, see references 4 and 21). Numerous studies on red cells, particularly those in hereditary hemolytic anemia, have clearly established the organization of the erythrocyte skeleton and its importance in maintaining erythrocyte shape, stability, and deformability. Spectrins are giant extended flexible molecules composed of two subunits (αI and βI in red cells) which intertwine to form αβ heterodimers. Spectrin exists as elongated tetramers resulting from self-association of αβ heterodimers. Sp tetramers constitute the filaments of the lattice, the nodes of which are crossed-linked by short actin filaments. This spectrin-based skeleton is bound to various transmembrane proteins through two connecting proteins, ankyrin and protein 4.1.

In nonerythroid mammal cells, α (αI and αII) and β (βI to βV) chains are encoded by two and five genes, respectively, each of these genes producing several isoforms by alternative splicing. Despite this diversity, all Sp chains present the same structural organization mainly made up of a succession of triple-helical repeat units, 22 for α chains and 17 for β chains except βV, which has 30 repeats. These units are characteristic of spectrin family members. They are about 106 amino acids long and folded in a coiled-coil structure made up of three helices (A, B, and C). Beside these repeat units, spectrin isoforms can also contain several interacting domains, such as SH3 domain, EF hands, PH domains, and binding domains for ankyrin, actin, protein 4.1, and calmodulin.

In nonerythroid cells, spectrin isoforms are not evenly distributed at the plasma membrane. Spectrins are also present in the Golgi apparatus, in cytoplasmic vesicles (16, 41), and in the nucleus (31). Several mechanisms appear to control spectrin dynamic distribution at the protein level, such as serine phosphorylation (19) and proteolysis by calpain and caspase. Spectrin binds Ca^{2+} and calmodulin which regulate spectrin binding to the membrane (43).

The multiple physiological functions attributed to spectrins are related to both their cellular locations and the nature of proteins that interact with them. Spectrins and the spectrin-based skeleton are considered to participate in the formation and maintenance of specialized plasma membrane domains in epithelial cells (17), in neurons (5), and in striated muscle cells (7, 22, 33). They are considered to stabilize integral membrane proteins, to reduce their endocytic rate, and to confer resiliency and durability on the membrane itself. Recent studies also suggest that spectrin may play a role in membrane protein sorting, vesicle trafficking (3), endocytosis (27), and neurite outgrowth (22, 38, 46). The recent description of spectrin mutations in quivering mice that manifest auditory and motor neuropathies (35) confirms their important functions in the maintenance of specialized subcellular domains.

The involvement of spectrins in many diverse physiological processes can be explained by their modular structure that combines numerous protein-interacting domains in a number of different isoforms. One approach for obtaining insight into

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the function(s) of spectrins is to define the proteins interacting with its domains in specific cell types. We have focused our study on a particular domain of 350 residues located within the middle part of the αII-spectrin. This area contains two repeat units (α9 and α10) together with several additional sequences. These additional sequences include (i) an SH3 domain, (ii) a calmodulin binding site, and (iii) two cleavage sites for proteases, such as calpains and caspase 3.

SH3 domains are 60-amino-acid-long sequences that are present in many signaling and cytoskeletal proteins. Despite the modest sequence homology, the three-dimensional structure is well conserved. They mediate protein interactions by binding short proline-rich sequences bearing the consensus motif PXXP, where X is any amino acid. The αII-Sp SH3 domain is highly conserved between species, with 100% identity between birds and mammals, suggesting important and conserved functions.

The protein E3B1, a substrate for tyrosine kinase, has been identified as a ligand for the αI-Sp SH3 domain (51) but no partner has been clearly defined for the αII-Sp SH3 domain. Using the yeast two-hybrid system, we identified isoform A of low-molecular-weight phosphotyrosine phosphatase (LMW-PTP) as a specific partner for the αII-Sp SH3 domain. As not reported previously to our knowledge, we demonstrated that αII-Sp was tyrosine phosphorylated in cells. We identified one tyrosine residue (Y1176) that is phosphorylated and dephosphorylated in vivo. This residue is located in the specific calpain cleavage site, near the SH3 domain, and is an in vitro substrate for two tyrosine kinases of the Src family, Src and Lck, and for the isoform A of LMW-PTP. Phosphorylation of this residue antagonizes calpain proteolytic activity. LMW-PTP A can dephosphorylate phosphotyrosine 1176 and so modulate spectrin susceptibility to calpain.

MATERIALS AND METHODS

Construction of rat kidney cDNA libraries for yeast two-hybrid screening. RNAs were isolated from adult male rat kidney (Sprague Dawley) by using the guanidium thiocyanate-phenol-chloroform extraction method (12). First-strand cDNA synthesis was performed on 4.5-μg polyadenylated RNAs (twice purified on an oligo(dT) column [Pharmacia, Uppsala, Sweden]) using avian myeloblastosis virus reverse transcriptase in the presence of 50-base oligonucleotide primers [including the *Xho*I restriction site and an 18-base poly(dT) sequence] and a nucleotide mixture containing 5-methyl dCTP. After second-strand synthesis by DNA polymerase I (Pol I) in the presence of RNase H, double-stranded cDNA uneven termini were filled with *Pfu* DNA polymerase. *Eco*RI adapters (phosphorylated 9-mer and dephosphorylated 13-mer nucleotides, cDNA synthesis kit; Stratagene) were bound to cDNAs by T4 DNA ligase (Gibco-BRL, Life Technologies). After phosphorylation and *Xho*I digestion, cDNAs were size fractionated on a Sepharose CL2B column. The cDNAs (above 600 bp) were introduced directionally as *Eco*RI-*Xho*I fragments into a modified version of plasmid pGAD GH, which includes a longer multiple cloning site. After electrotransformation of the DH10B strain (Gibco BRL, Life Technologies) with recombinant plasmids, 2.7×10^6 independent clones were obtained (with insert sizes ranging from 600 to 4,200 bp, with a mean value of 1,250 bp) and 2% of the clones were empty clones.

Screening of spectrin SH3 domain-binding proteins by yeast two-hybrid system. The sequence encoding the αII-Sp SH3 domain (E970 to P1026) was amplified with primers GGGGGATCCGGGAGCTGGTCTTG and GGAATTCTAGGGGTCCAATTTCTTCAC containing the *Bam*HI and *Eco*RI sites (underlined), respectively, and cloned to the C terminus of the LEX A DNA-binding domain into pLEX10 (generous gift from J. H. Camonis). Overnight culture of the yeast strain L40 established with the αII-Sp SH3-pLEX plasmid on selective medium lacking tryptophan (DO-W) was diluted to 5×10^6 cells/ml and grown for 3 h to 10^7 cells/ml before transfection with 100 μg of cDNA library plasmids. Transformation efficiency was estimated by dilution spreading on medium lacking tryptophan and leucine (DO-WL). As the bait did not induce background, His^+ clones were selected on medium lacking tryptophan, leucine, and histidine (DO-WLH) after 3 to 4 days of growth at 30°C. Expression of β-galactosidase was estimated on filter and liquid assays using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrates, respectively. Recombinant pGAD was recovered from His^+LacZ^+ yeasts and selected after transformation of an HB101 *Escherichia coli* strain grown on a selective medium lacking leucine.

Sequences were obtained from PCR-amplified products (forward primer, CGATGATGAAGATACCCCACC, and reverse primer, promT7-TAATAC GACTCACTATAGGGCGA) and were subsequently submitted to BLAST search (http://www.ncbi.NLM.NIH.gov/BLAST).

Analysis of interaction specificity using yeast mating strategy. The L40 yeast strain was transfected with pGAD plasmids containing different LMW-PTP inserts (full-length sequence, residues 1 to 158, and partial sequences, residues M1 to T78, Q33 to T78, and Q33 to H158) obtained with the following primers (cloning sites are underlined): forward M1, GGGGGGAATTCAGATGGCG GAACAGGCTACC; forward Q33, GGGGGAATTCAAAACATCTCAGAGA ATTGGAG; reverse H158, GGGGGCTCGAGCCTCAGTGGGCCTTCTCCA AG; and reverse T78, GGGGCTCGAGTCAGGTAATCTGCCGGGCAAC.

Transfected L40 cells were mated with the complementary yeast strains AMR70 established with different SH3 baits cloned in pLEX: mutant αII-Sp SH3, αI-Sp SH3 (residues 980 to 1036), and full-length SCD2 (11). Mutations of αII-Sp SH3 were introduced as previously described (34) by using the forward primer pLex-*Bam*HI-*Kpn*I, which alters the unique *Bam*HI site into a *Kpn*I site (underlined): selection primer, CGACTGGCTGGAATTCGGT ACCGAGCTGGTCTTGGCT, and reverse primers containing mutations (in bold), GAGAAGAGTCCCCGAGCGGTCACCATGAAGAAG, ACTCAAC AGCACCAACGAGGATTGGTGGAAAGT, and CGTCAGGGTTTTGTG CTGGCTGCGTACGTGAAG, for introduction of the E985A, K1002E, and P1017L mutations, respectively.

Expression and purification of recombinant peptides. Sequences corresponding to αII-Sp SH3 (E970 to P1026), αI-Sp SH3 (A970 to Q1055 cDNA amplified with primers containing cloning sites (underlined) GGGGATCCGCTGCAC CAGTGGAGGGAGT and GGGAATTCCTGGGTGATGTTTCCTGGCT), αII-Sp SH3-α9-α10 (D885 to L1229, GGGCCATGGACCTGGAGGACTCTC TGCAG and GGGGGATCCCAAGAGCTGGCTCCGTTC), and LMW-PTP (1 to 157) were PCR amplified with *Pfu* polymerase from different sources (rat kidney cells, human lymphocytes, and reticulocytes). After digestion with appropriate restriction enzymes, amplification products were cloned either into *Bam*HI/*Eco*RI-linearized pGEX-2T plasmid (Pharmacia) for glutathione *S*-transferase (GST) fusion peptides or into *Nco*I/*Bam*HI-linearized pQE-60 plasmid (Qiagen) for His_6-tagged peptides. The Y1176E mutation was introduced as described above with the reverse primer containing mutations (in bold), CTGTGCAAC AACAGGAAGTGGAGGGCATGATGCCCAGGGATGA. GST-fusion peptides were expressed in *E. coli* strains JM109 (Promega) or $ompT^-$ (a protease deficient strain) while His_6-tagged peptides were expressed in strain M15. After induction with 0.5 to 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C, cells were lysed by sonication in Triton X-100–phosphate-buffered saline (PBS) buffer containing antiproteases (10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 10 μg of pepstatin/ml, and 1 mM PMSF). His_6-tagged peptides were purified on nickel-agarose beads according to the instructions of Qiagen. GST-fusion proteins were purified on a glutathione-Sepharose column according to the instructions of Amersham Pharmacia Biotech. Proteins were further purified by anion-exchange chromatography on a Resource Q column (Amersham Pharmacia Biotech) in 20 mM Tris-HCl (pH 7.4)–1 mM β-ME–1 mM EDTA with a linear gradient of NaCl.

Transfection and cell culture. Sequences corresponding to the wild type and Y1176E mutant SH3-α9-α10 were amplified (forward primer, CCCGGATC CACCATGGACCTGGAGGACTCTCTGCAGGC containing the *Bam*HI site [underlined]; reverse primer containing the FLAG sequence [bold] and an *Eco*RI site [underlined], CCCGAATTC**ATTTGTCATCGTCATCTTTGTAGTC** CAAGAGCTGGCTCCGTTCCTC from pQE60 constructs and introduced into the pcDNA 3 plasmid). COS cells were grown in Dulbecco minimal essential medium containing 10% serum and transfected using Lipofectamine as recommended by the manufacturer (Invitrogen). RCCD1 cells were grown on collagen-coated plastic dishes as previously described (8). When indicated, pervanadate solution was added at 0.1 mM to culture medium 30 min before cell scraping.

Immunoprecipitation and immunoblottings. Polyclonal antibodies were obtained after the immunization of rabbits (Eurogentec) with GST fusion protein (GST–LMW-PTP A and GST-Sp αII SH3 domain) and purification by affinity chromatography (HiTrap; Amersham Pharmacia Biotech) using the central regions (residues 33 to 78) of LMW-PTP A and SH3-α9-α10-His_6 peptides, re-

spectively, as the affinity matrix. After washing, cells were lysed as previously described (14) for 20 min on ice in radioimmunoprecipitation (RIPA) lysis buffer containing antiproteases and 1 mM orthovanadate when cells were preincubated with pervanadate. Immunoprecipitations with anti-FLAG M2-Agarose Affinity gel (Sigma) were performed on RIPA supernatants (1 ml). Immunoprecipitations with immune serum directed against Sp αII/βII (generous gift from B. Geny, Paris, France) were performed on RIPA supernatants precleared with normal rabbit serum (50 μl) and protein-G Agarose (Sigma).

Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose. Immunoblots were blocked in PBS buffer containing 1.5% bovine serum albumin. After washing with PBS buffer containing 0.1% Tween 20–0.1% bovine serum albumin, immunoblots were probed either with a horseradish peroxidase-conjugated phosphotyrosine antibody (PY99) (Santa Cruz Biotechnology) or with affinity-purified immunoglobulin G (IgG) directed against the αIISp-SH3 domain or the LMW-PTP A and then with secondary antibodies conjugated with horseradish peroxidase. Blots were developed with the SuperSignal West Pico chemiluminescence's substrate (Pierce). If necessary, blots were stripped with Restore buffer (Pierce) and then blocked and probed again.

In vitro interactions and cross-linking experiments. In vitro interactions were performed at 4°C with 10 μg of immobilized recombinant peptides (either on Sepharose 4B gluthatione beads or on nickel beads) and recombinant peptides free of GST (purified after thrombin cleavage) in 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.01 to 0.2% Nonidet NP-40, and anti-proteases. After extensive washings, bound proteins were analyzed by SDS-PAGE. Cross-linking between LMW-PTP (1 to 10 μM) and different SH3 peptides (1 to 10 μM) was performed with bifunctional reagent disuccinimidyl suberate (DSS) (Pierce) at 1 mM and analyzed by SDS-PAGE.

Kinase, phosphatase, and calpain assays. Phosphorylation of spectrin recombinant peptides was performed using Src and Lck kinases (Upstate Biotechnology) in the presence of [γ-^{32}P]ATP as recommended by the manufacturer, but without O vanadate. Samples were resolved by SDS-PAGE. The amount of radioactivity incorporated was determined by Instant Imager (Packard). Recombinant LMW-PTP activity was estimated in acetate buffer at pH 5.5 on *para*-nitrophenylphosphate (pNPP) by colorimetric assay (at 410 nm) and on phosphorecombinant peptides by Instant Imager after separation on SDS-PAGE. μ-Calpain activity (Chemicon) was studied with SH3 α9-α10-His_6 recombinant peptides in 100 to 150 mM NaCl–25 mM Tris-HCl buffer, pH 7.5, in the presence of 500 μM Ca^{2+} and 50 μM calmodulin. Native and cleaved products were evaluated from Coomassie blue-stained gels by densitometry scanning.

Nucleotide sequence accession number. The nucleotide sequence of rat LMW-PTP A has been submitted to the DDBJ/EMBL/GenBank databases under accession number AF171072.

RESULTS

Looking for partners: the αII-Sp SH3 domain can recruit low-molecular-weight phosphotyrosine phosphatase. We looked for putative partners of the αII-Sp SH3 domain from a rat kidney cDNA library by using the yeast two-hybrid system with the αII-Sp SH3 domain (residues E970 to P1026) fused to the DNA binding domain of LexA. Then, 309 clones having the $Leu^+/Trp^+/His^+/LacZ^+$ phenotype were selected from 1.3×10^7 transformants. Seventy-eight out of 309 clones were found to encode isoform A of LMW-PTP. These 78 clones exhibited a weak β-galactosidase activity on both filter and liquid assays. These clones were derived from numerous independent RNA molecules as their sequence displayed different 5′ and 3′ ends; however, all of them contained at least the full-length coding sequence with insert sizes larger than 1,500 nucleotides (nt).

αII-Sp SH3 domain interacted specifically with isoform A of LMW-PTP. In mammals, the single LMW-PTP gene codes for three distinct isoforms, A, B, and C (10, 45). Isoforms A and B result from splicing of two alternative exons coding for residues 41 to 74 out of 158 residues, while isoform C does not contain this sequence (Fig. 1A). As LMW-PTP B and C had not been selected by the αII-Sp SH3 bait, their presence was checked in the library by analysis of the size of PCR products for isoform C and of the restriction pattern obtained after *Bgl*II digestion, which enabled isoforms A and B to be distinguished. Results showed that the library contains the three isoforms, indicating that the restricted selection of isoform A was not due to the absence of isoforms B and C in the library. In addition, cDNA of each of the three human isoforms was obtained by reverse transcription-PCR of lymphocyte mRNA and their interaction with spectrin SH3 was tested in the two hybrid systems. As shown in Fig. 2, the αII-Sp SH3 domain interacts with human isoform A but not with human isoforms B or C. This confirmed that the αII-Sp SH3 domain interacted specifically with isoform A of human and rat LMW-PTP.

Using a yeast mating strategy, we tested the hypothesis that residues 41 to 74, specific to isoform A, were responsible and sufficient for binding to the SH3 domain. Sequences encompassing the N-terminal (residues 1 to 78), the C-terminal (residues 33 to 158), and the central (residues 33 to 78) regions of LMW-PTP A were fused to the Gal4 activating domain. However, no interaction was detected with these three partial sequences of LMW-PTP A, suggesting that the interaction required the entire LMW-PTP A molecule (Fig. 2).

To better characterize the specificity of the interaction, we studied the behavior of LMW-PTP A versus various SH3 domains. We used the αI-Sp SH3 domain and two other unrelated SH3 domains from the yeast SCD2 protein (11) that share 68, 28, and 18% identity with the αII-Sp SH3 domain, respectively (Fig. 1B). As shown in Fig. 2, LMW-PTP A did not interact with any of the other tested SH3 domains in the yeast two-hybrid assay.

Mutations affecting two highly conserved residues in all SH3 domains (corresponding to E985 and P1017 in the αII-Sp SH3 sequence; Fig. 1B) have been associated with a complete loss of interactions in vitro as well as with a loss of function in vivo (15, 18, 37, 47). Structural studies of several SH3 domains have revealed that these two conserved residues are involved in the formation of the ligand-binding surface. Interaction of mutant P1017L and E985A αII-Sp SH3 peptides with LMW-PTP A was analyzed using the yeast two-hybrid system. The presence of either mutation totally abolished the interaction as revealed by the absence of growth on triple-selective medium DO-WLH (Fig. 2) and of β-galactosidase activity (data not shown). In contrast, mutation of a nonconserved Lys (K1002E) did not affect the interaction.

In vitro experiments confirmed weak but specific interaction. In order to confirm the results obtained with the two-hybrid system, LMW-PTP A and B as well as several peptides encompassing the αII-Sp SH3 domain were expressed as either GST- or His_6-tagged proteins. GST–αII-Sp SH3 peptide bound to glutathione beads did not retain purified recombinant LMW-PTP A (devoid of GST) (data not shown). Similarly, GST–LMW-PTP A bound to glutathione beads did not retain αII-Sp SH3-α9-α10-His_6 peptides. These negative results could be explained by a weak interaction between the two partners, in keeping with the observation of a weak β-galactosidase activity observed in the two-hybrid system. To verify this hypothesis, cross-linking agent (DDS) was added to the binding reaction solution containing both partners (at 1 to 10 μM). Subsequent analysis of the proteins by SDS-PAGE (Fig. 3A) revealed the presence of an additional band in the sample containing the αII-Sp SH3/LMW-PTP A mixture. This new

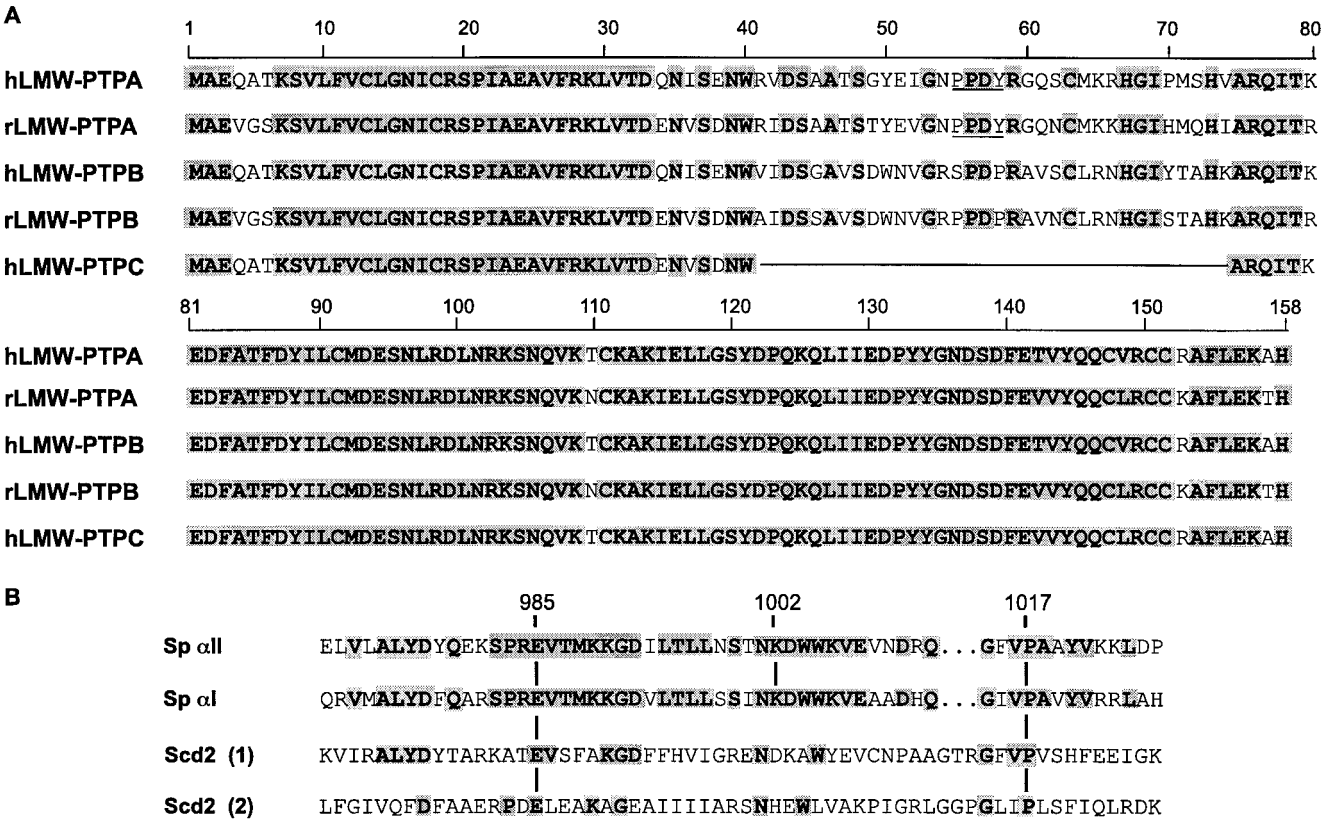

FIG. 1. Comparative analysis of amino acid sequence of LMW-PTP isoforms and SH3 domains used in this study. Identical residues are highlighted in grey. (A) Sequence alignment of human and rat LMW-PTP isoforms (accession numbers P24666, P24667, P41498, and H33956). Alternative splicing leads to different sequences for residues 41 to 74 between isoforms A and B. Isoform C is characterized by the absence of residues 41 to 74. The PPXY motif is underlined. (B) Sequence alignment of SH3 domains from mammal αII-spectrin (accession number A35715), human αI-spectrin (accession number P02549), and yeast SCD2 protein (11). The numbered residues have been mutated.

band showed an apparent molecular weight compatible with the formation of a complex between both species in a 1:1 ratio. No such additional band was detected on Coomassie blue-stained gels in the mixture containing either αI-Sp SH3 instead of αII-Sp SH3 or LMW-PTP B instead of LMW-PTP A (Fig. 3A). The identity of this new band was confirmed by Western blottings (Fig. 3B). This new band corresponds to the complex consisting of αII-Sp SH3 and LMW-PTP A since it was recognized by both antibodies directed against the SH3 domain and LMW-PTP A. This band was absent when the P1017L mutant αII-Sp SH3 peptide was used instead of the wild-type SH3 (Fig. 3B). These results confirm that the interaction between αII-Sp SH3 and LMW-PTP A occurs with high specificity but weak affinity.

LMW-PTP A was present with spectrin-based skeleton in detergent-insoluble fraction. LMW-PTPs are essentially considered to be cytosolic enzymes in a number of tissues; however, a fraction has also been observed in the detergent-insoluble pellet of NIH 3T3 cells (14). Using Western blotting, we analyzed the distribution of isoform A between the detergent-soluble and -insoluble fractions obtained from a rat kidney cell line (RCCD1). Immunopurified polyclonal antibodies raised against LMW-PTP A showed the presence of this phosphatase not only in the detergent-soluble fraction but also in the de-

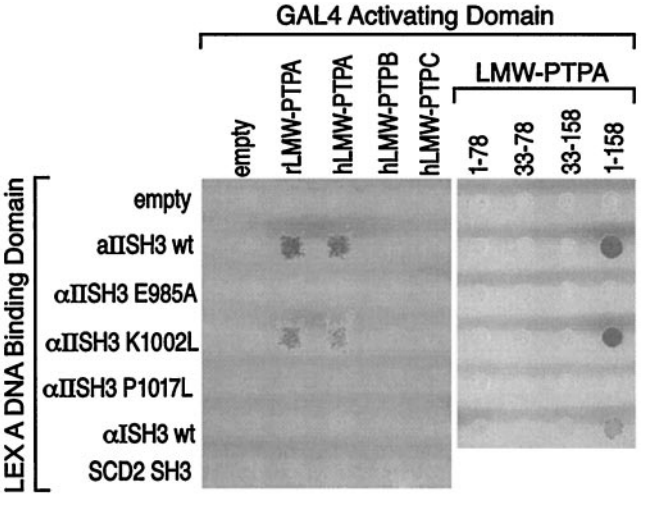

FIG. 2. Analysis of SH3 domains/LMW-PTP interactions using yeast mating. AMR70 yeast cells transformed with different pLexA-SH3 plasmids were mated with L40 yeast cells transformed with pGAD plasmids containing different sequences of rat and human LMW-PTP. As revealed by yeast growth on selective medium DO-WLH, interactions occurred only between either wild-type or K1002L αII-Sp SH3 peptides and full-length LMW-PTP A.

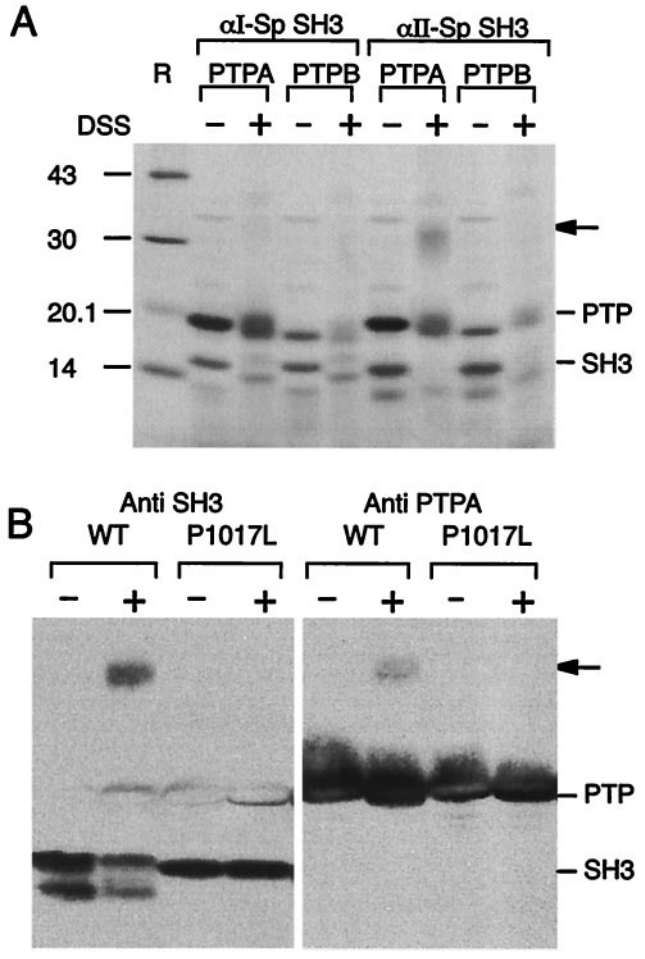

FIG. 3. In vitro interactions between α-spectrin SH3 domains and LMW-PTP isoforms. (A) Coomassie blue-stained gel. LMW-PTP A (19.5 kDa) and LMW-PTP B (19 kDa) (at 10 μM) were incubated with either αI-Sp SH3 (10.6 kDa) or αII-Sp SH3 (9.7 kDa) in the presence of cross-linking reagent (DSS). Interaction occurred only between αII-Sp SH3 and LMW-PTP A, as revealed by the presence of a new band (indicated by an arrow) with an apparent molecular mass of 32 kDa. Lane R, molecular mass markers. (B) Western blots. Wild-type (WT) and mutant (P1017L) αII-Sp SH3 peptides (at 1 μM) were incubated with LMW-PTP A in the presence of DSS. Western blots were revealed by antibodies directed against either the αII-Sp SH3 domain or LMW-PTP A. In the presence of DSS, both antibodies revealed the same additional band (indicated by an arrow) in the mixture containing wild-type αII-Sp SH3 peptide. In contrast, no such a band was detected in the presence of mutant αII-Sp SH3.

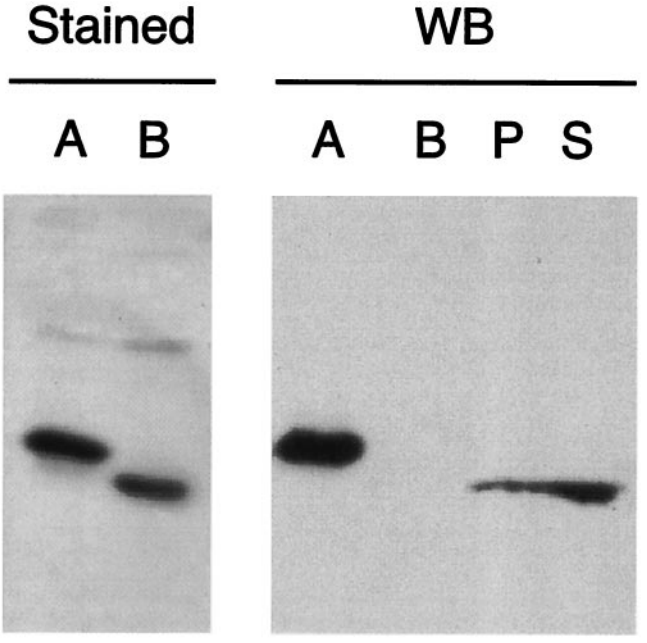

FIG. 4. Distribution of LMW-PTP A in cell detergent extracts. Shown are a Coomassie blue-stained gel and a Western blots (WB). The immunopurified antibodies directed against LMW-PTP A specifically recognized LMW-PTP A (lane A) and did not label LMW-PTP B (lane B). These antibodies revealed the presence of LMW-PTP A not only in the detergent-soluble fraction (lane S) but also in the detergent-insoluble fraction (lane P) obtained from the rat kidney cell line RCCD1.

tergent-insoluble fraction (Fig. 4), which contains the spectrin-based skeleton.

αII-spectrin was tyrosine phosphorylated in vivo. The interaction between the αII-Sp SH3 domain and LMW-PTP A could have two biological implications. On the one hand, the interaction with spectrin could target this enzyme to the vicinity of its known substrates, which are transmembrane proteins (42). On the other hand, spectrin itself could be a substrate: the αII-Sp from mammals has been reported to be tyrosine phosphorylated in vitro by Src and spleen tyrosine kinases (1, 48). We studied endogenous αII-Sp tyrosine phosphorylation in a rat kidney cell line (RCCD1) by Western blotting using anti-phosphotyrosine antibodies. Spectrin was immunoprecipitated by anti-αII/βII-Sp antibodies and then probed using Western blotting with anti-phosphotyrosine antibodies, and after stripping, with anti-αII-Sp SH3 antibodies (Fig. 5). When cells were treated with a phosphatase inhibitor (pervanadate) 30 min before lysis, anti-phosphotyrosine antibodies strongly labeled a high-molecular-mass peptide displaying an apparent molecular mass in agreement with that of the full-length αII-spectrin (280 kDa). This band was recognized by anti-αII-Sp SH3 antibodies. In the absence of phosphatase inhibitors in cell culture, the αII-spectrin was faintly labeled by anti-phosphotyrosine antibodies. Moreover, apart from the 280-kDa peptide, the αII-Sp SH3 antibodies revealed a 150-kDa peptide that could correspond to the well-determined spectrin cleavage product (23). This 150-kDa peptide has been demonstrated to result from either calpain or caspase 3 cleavage at two close residues, Y1176 and D1185, respectively, which are located in the α10 repeat unit, near the SH3 domain (residues 971 to 1025). In pervanadate-treated cells, the 150-kDa peptide was not detected, suggesting a protective effect of phosphatase inhibitor on spectrin cleavage.

Phosphorylation occurred on Tyr residue 1176 located in calpain cleavage site. Our results showed an interaction between the αII-Sp SH3 domain and LMW-PTP A and suggested a relationship between the phosphorylation of spectrin and its proteolytic cleavage at residues located near the SH3 domain. We therefore looked for tyrosine phosphorylation sites close to the SH3 domain. Computer analysis (using the prediction programs PROSITE and NETPHOS) of the amino acid sequence

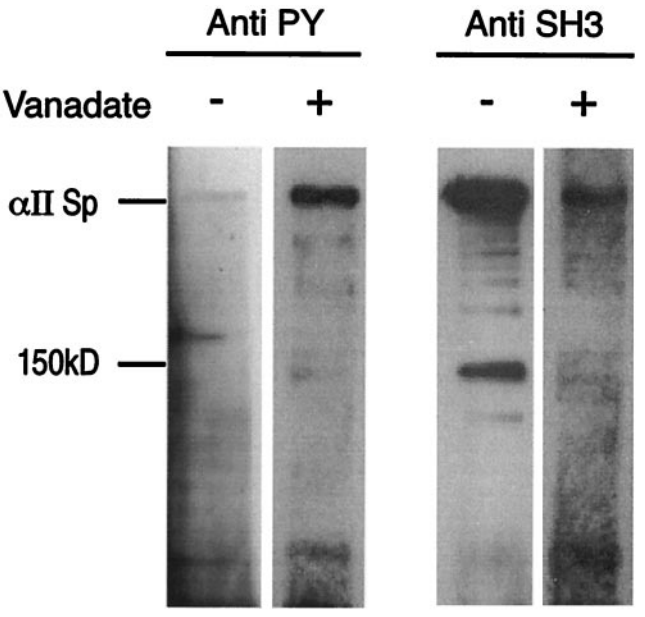

FIG. 5. αII-spectrin phosphorylation in cells and effects of phosphatase inhibitor. Sp αII/βII was immunoprecipitated with anti-Sp αII/βII antibodies from detergent-soluble extracts of RCCD1 cells. Prior to SDS lysis, cells were incubated for 30 min in the presence of phosphatase inhibitor (pervanadate) when indicated. The immunoprecipitate was probed with anti-phosphotyrosine (PY) and then stripped and probed with anti-αII-Sp SH3 antibodies.

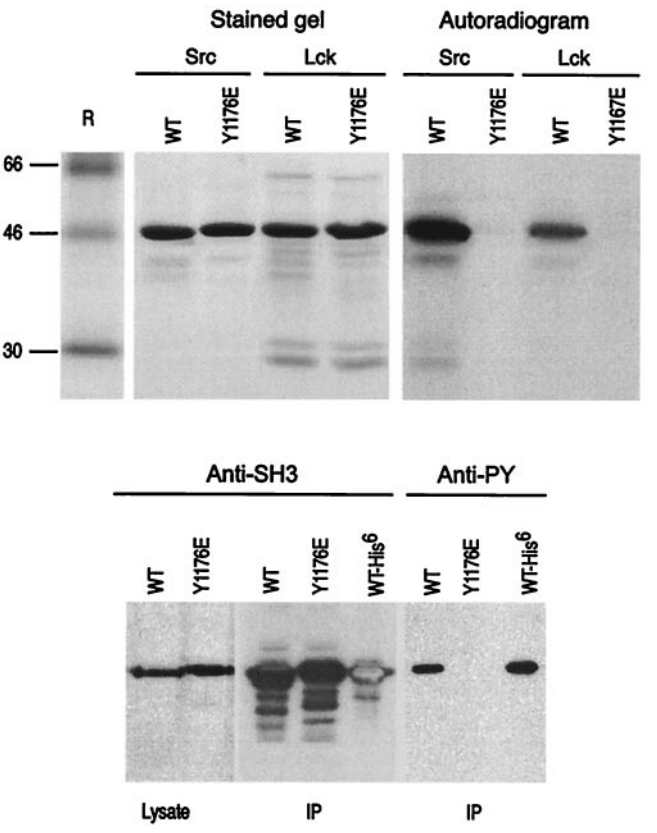

FIG. 6. Identification of a phosphorylation site in αII-spectrin, located near the SH3 domain. (Top) His-tagged recombinant peptides (wild-type [WT] and Y1176E mutant SH3-α9-α10) were submitted to in vitro Src and Lck phosphorylation in the presence of [^{32}P]ATP. Wild-type peptide was phosphorylated by both enzymes, whereas the Y1176E mutant SH3-α9-α10-His$_6$ was not. Lane R, molecular mass markers. (Bottom) Expression and phosphorylation of wild-type (WT) and Y1176E mutant SH3-α9-α10 peptides in COS cells (preincubated in presence of pervanadate) were analyzed by Western blotting on cell lysates and after immunoprecipitation (IP) by anti-Flag antibodies. Peptides were revealed by antibodies directed against αII-Sp SH3 and then by anti-phosphotyrosine (PY). WT-His$_6$ corresponds to the recombinant SH3-α9-α10-His$_6$ peptide phosphorylated in vitro by Src.

encompassing the SH3 domain and its two adjacent repeat units (α9 and α10) predicted one or two potential tyrosine phosphorylation sites (Y1073 and Y1176). We studied the in vitro activity of two tyrosine kinases, Src and Lck, on a set of either GST- or His$_6$-tagged peptides encompassing the human sequences corresponding to the SH3 domain alone or with one (α9) or two (α9 and α10) adjacent repeat units in the presence of [γ-^{32}P]ATP. These peptides have a high Tyr residue content (14 for GST and 3, 7, and 8 for Sp αII SH3, SH3-α9, and SH3-α9-α10, respectively). Only the Sp recombinant peptide containing the α10 repeat (SH3-α9-α10 peptide) was phosphorylated. The peptide was highly phosphorylated by the two kinases (Fig. 6, upper panel), up to 90% by Src. The α10 repeat bears the calmodulin binding site and the cleavage sites for calpain and caspase (at residues Y1176 and D1185, respectively). This peptide differs from the other tested peptides only by the presence of one additional Tyr residue (Y1176) located in the calpain cleavage site (40). Site-directed mutagenesis resulting in Y1176E amino acid substitution totally abolished in vitro spectrin phosphorylation by Src and Lck (Fig. 6, upper panel). This site was predicted by NETPHOS with a probability of 0.76.

Whether or not the Y1176 residue is phosphorylated in vivo was further tested by transient expression of FLAG-tagged SH3-α9-α10 peptides (wild-type and Y1176E mutant) in COS cells. Western blottings of cell lysate (obtained from transfected cells incubated for 30 min in the presence of pervanadate prior to lysis) were probed with anti-αII-Sp SH3 antibodies. The results indicated that both wild-type and mutant peptides were well expressed and highly stable as indicated by the presence of a unique band with an apparent molecular weight similar to that of the His-tagged peptide (Fig. 6, lower panel). After immunoprecipitation by anti-FLAG antibodies, both wild-type and mutant peptides were labeled by anti-αII-Sp SH3 antibody, but only the wild-type SH3-α9-α10 peptide was recognized by anti-phosphotyrosine antibodies. The anti-phosphotyrosine antibodies did not react with the mutant Y1176E peptide. These data confirm the residue Y1176 as a phosphorylation site recognized in vivo by cellular kinases. In the absence of cell treatment with phosphatase inhibitors, phosphorylation of this residue was very weak, indicating that this residue is also an in vivo substrate for tyrosine phosphatase (data not shown). So, the phosphorylation state of Y1176 is determined by a dynamic equilibrium between kinases and phosphatases.

Spectrin phosphorylation affected calpain cleavage. After the identification of residue Y1176 located in the calpain cleavage site as a phosphorylation site, we analyzed in vitro the effects of this phosphorylation on αII-Sp cleavage by μ-calpain.

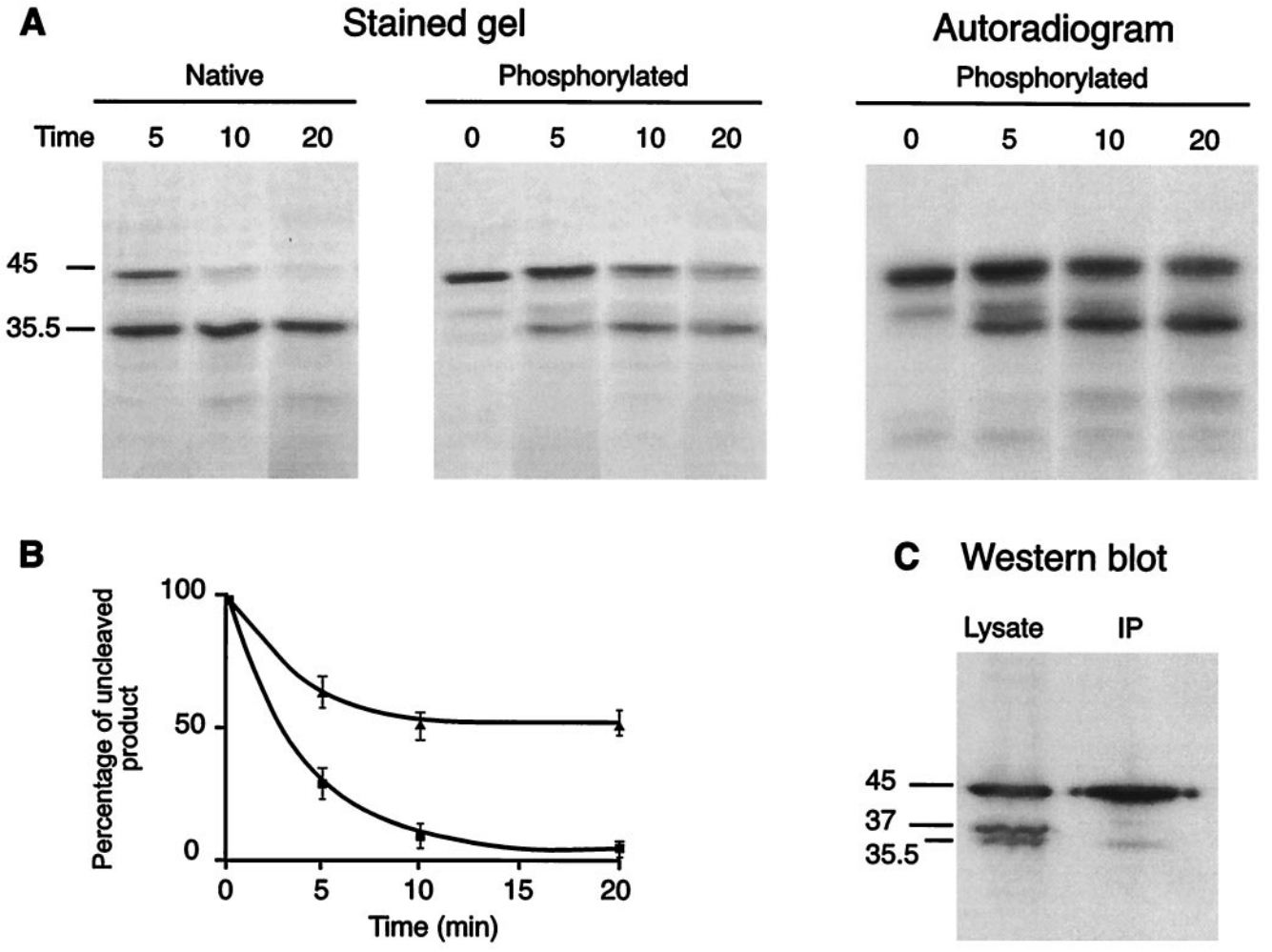

FIG. 7. αII-spectrin phosphorylation and protease cleavage. (A) In vitro kinetic studies of μ-calpain activity on ^{32}P-phosphorylated and native SH3-α9-α10-His_6 peptides. The 35.5-kDa peptide corresponds to the cleavage product. (B) Graph showing μ-calpain activity on native (■) and phosphorylated (▲) peptides calculated from Coomassie blue-stained gels and autoradiograms. (C) Expression and cleavage of Flag-tagged SH3-α9-α10 peptide expressed in COS cells. In the absence of phosphatase inhibitor, anti-αII-Sp SH3 antibody revealed the full-length peptide (45 kDa) and two proteolytic products (37 and 35.5 kDa). The 45-kDa peptide immunoprecipitated (IP) only anti-Flag antibodies.

In the presence of calmodulin (50 μM) and Ca^{2+} (500 μM), phosphorylated peptides (His_6-tagged SH3-α9-α10) were less sensitive to μ-calpain than nonphosphorylated peptides (Fig. 7A and B): after 10 min of incubation, the nonphosphorylated SH3-α9-α10 peptide was almost completely cleaved, whereas only 40% of the phosphorylated form was cleaved by μ-calpain. Mass spectrometry of peptide products after calpain cleavage confirmed that spectrin cleavage occurred after residue Y1176 (data not shown).

As phosphorylation of Y1176 residue appears to protect spectrin against in vitro calpain degradation, we further investigated in vivo proteolysis of FLAG-tagged SH3-α9-α10 peptide transiently expressed in COS cells. In the absence of phosphatase inhibitors (Fig. 7C), Western blottings of cell lysate exhibited three bands (with apparent molecular masses of 45, 37, and 35.5 kDa) labeled by anti-αII-Sp SH3 antibodies, whereas in the presence of phosphatase inhibitor (Fig. 6, lower panel), only the 45-kDa peptide (corresponding to the full-length peptide) was detected. Anti-FLAG antibodies were able to pull down the 45-kDa peptide but not the 37- or 35.5-kDa peptides, suggesting that both these peptides have lost the FLAG located at the COOH end. This was confirmed by Western blottings on cell lysate using anti-FLAG antibodies that recognized only the 45-kDa band (data not shown). These two 37- and 35.5-kDa peptides probably arise from proteolytic cleavage that must occur between the SH3 domain and the FLAG. The apparent molecular masses of these peptides (37 and 35.5 kDa) are compatible with a cleavage at residues D1185 and Y1176 by caspase 3 and calpain, respectively.

Spectrin is substrate for LMW-PTP A. We checked whether this Src-phosphorylated residue could be an in vitro substrate of LMW-PTP A. Since the linkage of GST to the N terminus of LMW-PTP does not affect the kinetic properties of the enzymes (13), the activity of both LMW-PTP A and B expressed as GST-fusion proteins was tested on *para*-nitrophenylphosphate (pNPP) and on the Src-phosphorylated peptide (SH3-α9-α10 peptide). Recombinant LMW-PTP B displayed a higher activity on pNPP than on LMW-PTP A (100 times as

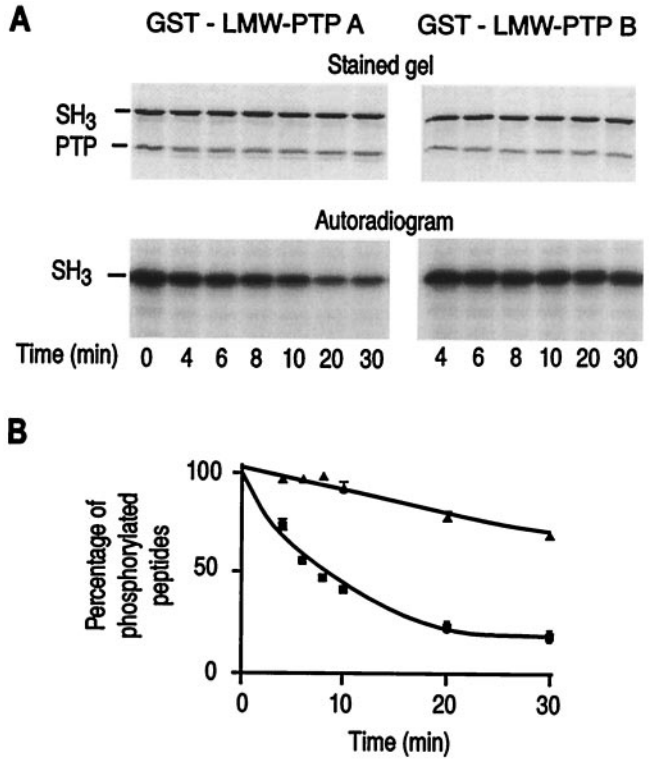

FIG. 8. Kinetic studies of LMW-PTP A and B activity on phosphorylated spectrin peptides. After phosphorylation by Src, recombinant peptide SH3-α9-α10-His_6 (indicated by SH3) was submitted to dephosphorylation by LMW-PTP A and B (indicated by PTP). (A) Coomassie blue-stained gel and autoradiogram. (B) Activity of LMW-PTP A (■) and LMW-PTP B (▲).

estimated by the number of pNPP molecules hydrolyzed per microgram of recombinant peptides), but LMW-PTP A was considerably more active on spectrin peptides than isoform B (Fig. 8). After 10 min of incubation at the same enzyme-substrate ratio, more than 50% of the spectrin was dephosphorylated by isoform A. In contrast, no detectable loss of phosphate was observed in the presence of isoform B after 10 min of incubation, and only a 25% loss of phosphate label was observed after 30 min of reaction.

DISCUSSION

Spectrin was tyrosine phosphorylated in vivo on residue Y1176. There is very little information available on the phosphorylation of spectrins, but in vivo ^{32}P labeling of spectrin has revealed phosphorylation of β-spectrins, mainly on serine but also threonine residues (19, 24, 39). In erythrocytes, βI-spectrin was reported to contain at least six phosphorylatable sites in vivo (36). The locations of these phosphorylated residues, the kinases, and phosphatases involved in this process as well as the functional significance of βI-spectrin phosphorylation are not clear. Manno et al. (30) demonstrated that an increase in the phosphorylation of βI-spectrin causes a decrease in the mechanical stability of red blood cell membrane. Serine phosphorylation of βII-spectrin has been associated with spectrin redistribution in epidermal growth factor-stimulated A 431 cells (9) and during mitosis (19). These finding support the view that βI-spectrin phosphorylation may regulate physiological functions in vivo. Concerning α-spectrins, mammalian αII-spectrin is an in vitro substrate for tyrosine kinases such as Src (1). The α-spectrin from *Torpedo* has been shown to be tyrosine phosphorylated (2). In this study, we demonstrate for the first time by immunoblotting using anti-phosphotyrosine antibodies that the mammalian endogenous αII-spectrin is tyrosine phosphorylated in vivo. This tyrosine phosphorylation of αII-spectrin depends greatly on kinase/phosphatase equilibrium since this phosphorylation cannot be detected in the absence of phosphatase inhibitors, which could explain why it was not previously detected.

Expression of mutant peptides in COS cells has allowed us to identify residue Y1176, located in the hypersensitive site for calpains (23, 40), as an in vivo site for cellular kinases and phosphatases.

In vitro experiments showed that this tyrosine can be a substrate for nonreceptor tyrosine kinases, such as Src and Lck. Some lines of evidence suggest that Src may be the physiological kinase: (i) Src is more active on residue Y1176 than Lck, (ii) in our in vitro studies (data not shown), Src preferentially phosphorylated Sp peptides rather than LMW-PTP A, which has been demonstrated to be a physiological target for Src (14), and (iii) Src and spectrin have been colocalized (20). The Src involvement in Sp phosphorylation on residue Y1176 in cells is likely but remains to be confirmed.

LMW-PTP A interacted with αII-Sp SH3 domain and dephosphorylated Y1176. Our in vitro studies have shown that residue Y1176 is a substrate for LMW-PTP A. We suspected spectrin might be a substrate for LMW-PTP A since we detected an interaction between LMW-PTP and the αII-Sp SH3 domain by two-hybrid screening of a rat kidney cDNA library. LMW-PTP A appears to be a highly specific partner for the αII-Sp SH3 domain as indicated by the following observations: (i) this interaction is conserved in humans, although LMW-PTP A sequences differ slightly between the two species (20 different residues out of 158; Fig. 1), (ii) LMW-PTP B (with 21 different residues) and LMW-PTP C (with these 21 residues deleted) did not interact with the αII-Sp SH3 domain, (iii) LMW-PTP A did not interact with either the αI-Sp SH3 domain (68% identity with αII-Sp SH3) or two other unrelated SH3 domains, and (iv) mutations of αII-Sp SH3 on residues involved in SH3 domain functions led to a loss of interaction with LMW-PTP A.

Surprisingly, the LMW-PTP A sequence did not contain the PXXP core motif usually flanked by positively charged residues, mainly Arg [RXX(X)PXXP or PXXP(X)R], whereas rat LMW-PTP B contains this motif (PPXPR). However, recent data have extended the repertoire of SH3 domain binding motifs to Tyr-based motifs, such as RKXXYXXY and PXXDY (28, 32), indicating that SH3 domain binding motifs are not so restrictive. Rat and human LMW-PTP A do not contain such a motif, but they contain a similar sequence (PPDYR) that is not present in isoforms B or C. Mutagenesis experiments are in progress to test whether this motif is the SH3-binding site. However, this short sequence is probably not sufficient since any interaction can be detected with partially truncated proteins that contain this sequence, suggesting that the whole molecule is required in the interaction. Selection of

clones with only the full-length sequence in the two-hybrid screens reinforces such a hypothesis.

The interaction of αII-Sp SH3 with LMW-PTP A was weak since we detected it only in the two-hybrid assay and in vitro, in the presence of a cross-linking agent. During evolution it seems that SH3 domains have been selected to recognize their ligands with a high specificity but a relatively low affinity. Such interactions are ideal for signaling domains that must recognize selective ligands to transduce information accurately, but with low affinity to allow sensitive and dynamic modulation in response to changing signals. Typically, the K_d values of ligands binding SH3 domains are low (between 1 and 100 μM for synthetic peptides). Recent data have suggested that SH3 domain interactions may be regulated in the cell, for example, by phosphorylation. The interaction between Sos and Grb2 and the interaction between WASP and PSTPIP can be regulated by phosphorylation of either the ligand or the SH3 domain (49, 50). So, the interaction between spectrin and LMW-PTP A can involved both the Sp SH3 domain and the phosphorylated Y1176 residue. Interaction with the αII-Sp SH3 domain could participate either in the recruitment or in the regulation of the enzyme activity, and it could also make the enzyme-substrate interaction more specific. Such effects have been observed between PTP 1B and its substrate, the protein p130cas: the interaction between the PTP 1B proline-rich region and the p130cas SH3 domain is required for substantial tyrosine dephosphorylation of p130cas and for the function of PTP 1B as a negative regulator of integrin signaling (29).

Moreover, LMW-PTPs are 18-kDa enzymes that are distantly related to other PTPases: they contain only the catalytic domain whereas other PTPases also possess regulatory or targeting domains. Because LMW-PTPs apparently lack regulatory elements, their regulation and targeting are not fully understood, although Y131 and Y132 phosphorylation by kinases of the Src family up-regulate their catalytic activity (44). The Sp αII SH3 domain ability to bind LMW-PTP A correlates with the presence of a tyrosine phosphorylation site near the SH3 domain. It is noticeable that the αI-spectrin chain, which does not contain this tyrosine or the calpain cleavage site, has an SH3 domain unable to recruit LMW-PTP. Similarly, our in vitro studies have shown that spectrin is a better substrate for LMW-PTP A than for isoform B. The isoforms A and B are quite similar; they arise from alternative splicing of two homologous exons of the same gene. However, the two enzymes differ in substrate specificity (towards synthetic substrates as well as more physiological protein targets) and in response to inhibitors or activators, indicating that isoforms have acquired different physiological functions (13). Several proteins have been identified as LMW-PTP B substrates (PDGF-R, EGF-R, insulin-R, and MCSF-R), whereas ephrin receptor tyrosine kinases are the only physiological targets of LMW-PTP A known so far (42). Spectrin is a new candidate for a physiological substrate for LMW-PTP A, since a fraction of LMW-PTP A that is mainly cytosolic is associated with the skeleton in a detergent-insoluble fraction.

Spectrin cleavage by calpain and membrane skeleton remodeling. As we have shown, αII-spectrin phosphorylation on residue 1176 modifies its susceptibility to calpains, ubiquitous Ca^{2+}-dependent proteases. Modification of sensitivity to proteolysis by phosphorylation has already been observed: the glutamate ionotropic AMPA receptors are more resistant to calpain when phosphorylated (6). In contrast, tyrosine phosphorylation of cortactin increases its sensitivity to calpain (26).

Proteolysis of the αII-spectrin by calpain is well known and has been documented as a consequence of cell injury in several tissues. It also correlates with platelet activation, neutrophil degranulation, neuronal long-term potentiation, NMDA receptor activation, stimulation of exocytosis in adrenal chromaffin cells, and endocytosis (27). The action of calpain on spectrin is of significant interest because of its dramatic effect on the stability of the spectrin-based skeleton: spectrin cleavage by calpain leads up to a loss of spectrin ability to cross-link actin filaments and to bind to the membrane (25). Thus, modulation of spectrin proteolysis by phosphorylation must be important for stability and reorganization of the spectrin-based skeleton and consequently for spectrin-based skeleton functions. These data raise the question of the events that can modify the balance of kinase/phosphatase activities.

Taken together, our results demonstrate that calpain-mediated truncation of spectrin can be regulated not only by Ca^{2+}-calmodulin level but also by phosphorylation.

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