SPIN90 (SH3 Protein Interacting with Nck, 90 kDa), an Adaptor Protein That Is Developmentally Regulated during Cardiac Myocyte Differentiation

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Integrins are a large family of heterodimeric transmembrane receptors that play key roles in mediating interactions between cells and ECM proteins and between intercellular proteins. As such, they are crucially involved in such biological processes as embryonic development, cell migration, and cell growth and differentiation.

The development of cultured cardiac myocytes is characterized by myofibrillogenesis, during which myofibrils are organized into sarcomeric structures. Interactions between integrins and ECM take place continuously throughout sarcomere assembly and underlie the progressive assembly of thick and thin filaments into sarcomeres with appropriately spaced Z-discs. $\beta_1$ integrin is expressed in the costameres of cardiac myocytes, where the cells attach to the collagen network; it forms the strong attachment sites required by highly contractile cells (3, 4). The organization of sarcomere structure within cardiac myocytes is thus crucially dependent on the presence of $\beta_1$ integrin (5). We previously reported that $\alpha_3$ integrin is also localized at costameres and is associated with $\beta_1$ integrin during myofibrillogenesis (6). Moreover, Nck appears to be distributed in a sarcomeric banding pattern in cardiac myocytes, colocalizing at the Z-discs with $\alpha$-actinin, and participating in a $\beta_{1A}$ integrin-mediated signaling pathway (7).

Nck, which contains three Src homology 3 (SH3) domains and one SH2 domain, is ubiquitously expressed in a variety of tissues and cells (8). As it lacks catalytic activity, Nck is referred to as an adaptor molecule, a group of proteins that also includes Grb2 and Crk. SH2 and SH3 domains are peptide motifs found in a wide variety of molecules implicated in the regulation of cell growth (9). SH2 domain-containing proteins capable of tightly associating with catalytic molecules function as adaptors linking the latter to phosphorysosine-containing proteins (10). In addition to recruiting such signaling enzymes as phosphoinositide 3-kinase, phospholipase C-$\gamma$, and p125GAP, adaptor molecules are able to bind to receptor tyrosine kinases via their SH2 domains (11). SH3 domains are mostly found in kinase-mediated signal transduction molecules and in cytoskeletal components (12); the latter possess SH3 motifs that bind to proline-rich motifs (minimal consensus motif: P-X-X-P) in other signaling molecules (13). For example, they bind to SOS, dynamin, Cbl, and Wiskott-Aldrich syndrome protein (WASP), which are responsible for mediating the effects of CDC42 on reorganization of actin cytoskeleton (14–17). Over the past couple of years, several effector molecules that interact with the SH3 domains of Nck have been identified. Nck binds to PRK2, a serine/threonine kinase, closely related to the putative Rho effector, and also to NIK, which activates the mitogen-activated protein kinase kinase kinase/stress-activated protein kinase pathway (18, 19). However, the biological significance of these interactions is not yet known. Furthermore, although we have previously reported on the involvement of Nck in a $\beta_{1A}$ integrin-mediated signaling pathway, the functions of Nck and the
signal molecules with which it interacts in cardiac myocytes are also not yet known.

To better understand the $\beta_2$ integrin-mediated signal pathway, we have used yeast two-hybrid screening to find proteins that interact with Nck. By screening a human heart cDNA library using Nck SH3 domains as bait, we isolated a positive CDNA clone, spin90, which encodes a VIP54-related protein containing an SH3 domain, proline-rich motifs and serine/threonine-rich sequences. Here, we describe its association with Nck and provide evidence of its function in the heart.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, antibiotics/antimycotics, complete Freund’s adjuvant, incomplete Freund’s adjuvant, and trypsin were obtained from Life Technologies, Inc. Tissue culture dishes were from Falcon, Monoclonal anti-actinin antibody (clone EA-53) was purchased from Sigma. Monoclonal anti-Nck antibody was purchased from Transduction Laboratories. Horseradish peroxidase-labeled anti-mouse immunoglobulin (IG) and fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG were from Jackson ImmunoResearch Laboratory. Protein A-Sepharose and glutathione-Sephrose 4B fast flow were purchased from Amersham Pharmacia Biotech. Human heart Matchmaker cDNA library and $\lambda$TriplEX human heart cDNA library were obtained from CLONTECH. The primers used for polymerase chain reaction (PCR) and DNA sequencing were synthesized by Genotech. TNT T7-coupled reticulocyte lysate system was from Promega. $^{32}$PdCTP and $[^{35}]$S)methionine ($[^{35}]$SMet) were from PerkinElmer Life Sciences.

**Cardiac Myocyte Cultures**

Neonatal rat ventricular myocytes were isolated according to the method of Borg et al. (4) and Simpson et al. (20) with major modifications. Briefly, hearts from 12–25 neonatal rats (2–3 days old) were excised and placed in Ads buffer (116 mM NaCl, 20 mM HEPES, 10 mM Na$_2$PO$_4$, 5.5 mM glucose, 5 mM KCl, 0.8 mM MgSO$_4$, pH 7.4), after which they were trimmed of their atria and transferred to fresh Ads buffer. The retained ventricles were then minced, placed in a 50-ml flask containing enzyme digestion solution (Ads buffer containing 65 units/ml collagenase type II and 0.6 mg/ml pancreatin) and incubated at 37 °C for a series of 20-min intervals. After each interval, the solution was discarded, the cell-containing supernatant was collected, and the pellet was resuspended in 10% fetal bovine serum. To enrich the myocyte suspension, the supernatant from the first digestion interval was discarded. The cells from all of the digestions were pooled, washed, and then subjected to centrifugation through a discontinuous Percoll gradient of 1.050, 1.062, and 1.082 g/ml. The interface band between the first and second Percoll layers was discarded. The cells from all of the digestions were pooled, washed, and then subjected to centrifugation through a discontinuous Percoll gradient of 1.050, 1.062, and 1.082 g/ml. The interface band between the first and second Percoll layers was discarded. The cells from all of the digestions were pooled, washed, and then subjected to centrifugation through a discontinuous Percoll gradient of 1.050, 1.062, and 1.082 g/ml. The interface band between the first and second Percoll layers was discarded.

**Cloning the Full-length spin90 CDNA: Phage Library Screening**

The clone identified in the yeast two-hybrid screening did not contain the 3’ end of the gene. To obtain a full-length cDNA, the $\lambda$TriplEX human heart CDNA library was plated and transferred to a Protran BA 85 nitrocellulose membrane (Schleicher & Schuell) according to the manufacturer’s protocol. The library was screened by hybridization using a $^{32}$P-labeled insert as a probe (obtained from clone spin90). After an additional two rounds of purification, three positive $\lambda$TriplEX clones were obtained. The cDNA inserts were sequenced using an ABI PRISM 377 DNA sequencer and PRISM $^{32}$ Ready Reaction Dideoxy Terminator cycle sequencing kit (PerkinElmer Life Sciences). The sequences were then confirmed manually using a T7 Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.).

**Northern Analysis**

The human multiple tissue poly(A)$^+$ RNA blot (2 μg/lane) was purchased from CLONTECH. The cDNA inserts from clone spin90 and $\beta$-actin, which served as a control, were labeled with $^{32}$PdCTP by random-primer extension using the oligonucleotide labeling kit (Amer sham Pharmacia Biotech). These probes were then used for hybridization according to the manufacturer’s protocol, after which the blot was exposed to x-ray film (Fuji Co.) for 12–24 h at –70 °C.

**Construction of Expression Vectors and in Vitro Binding of spin90 with Nck**

**Expression of GST Fusion Proteins for in Vitro Binding Assay—**CDNA’s encoding the full-length of Nck (full Nck; amino acids 1–337), the three SH3 domains (total SH3; amino acids 1–251), the SH2 domain (SH2; amino acids 268–377), and the first SH3 domain (SH3–1; amino acids 1–96), the second SH3 domain (SH3–2; amino acids 68–183), and the third SH3 domain (SH3–3; amino acids 170–251) were amplified by PCR and cloned, in frame, into pGEX4T-1 vector (Amer sham Pharmacia Biotech). GST fusion proteins were overexpressed in bacteria and purified on glutathione-Sepharose columns according to the manufacturer’s protocol.

**Full-down Assay—**To assess in vitro binding, the clone spin90 insert was subcloned into pRSET, a bacterial expression vector, and in vitro translated using a TNT T7-coupled reticulocyte lysate system (Promega). The radiolabeled products were incubated with purified GST or GST fusion proteins bound to glutathione beads. All incubations were performed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin at 4 °C for 6 h. The glutathione beads were then washed four times in PBS containing 1% Triton X-100, and the radiolabeled proteins bound to the beads were solubilized by addition of sodium dodecyl sulfate (SDS) sample buffer in the presence of a reducing agent and subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE). Radioactivity was detected by autoradiography of the dried gels.

**Purification of GST Fusion Proteins and Generation of Antibodies against spin90**

To generate anti-Spin90 antibodies, the cDNA corresponding to full-length Spin90 was amplified by PCR and subcloned, in frame, into pGEX4T-1 vector for GST fusion protein expression. GST-SPIN90 fusion proteins were overexpressed in bacteria and purified according to the method of Merilainen et al. with some modification (22). The cells were suspended in a lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$PO$_4$, 1.8 mM KH$_2$PO$_4$, 1% Triton X-100) and lysed by sonication. SDS sample buffer was added to the lysates, which were then resolved by 8% SDS-PAGE. Thereafter, the GST-SPIN90 fusion protein was electroeluted from the gel using a Bio-Rad model 422 electroeluter, and the purified GST fusion protein was dialyzed and used for immunizations. After the fifth injection, the specificity of the serum was tested by immunoblot analysis, and then further purified by affinity chromatography.

**Western Blotting**

For immunoblot analysis, selected tissues from rat were minced, rinsed once with extraction buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA), and homogenized in extraction buffer containing 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1.5 μM pepstatin, 1 mM aprotinin, and 50 mM sodium fluoride (NaF) using a Dounce homogenizer. The homogenates were incubated for 1 h at 4 °C with gentle agitation and then centrifuged at 12,000 rpm for 15 min at 4 °C. The dissociated cells obtained were lysed by boiling in a lysis buffer (1% SDS, 1 mM sodium orthovanadate, 10 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 1.5 mM pepstatin, and
1 mM aprotinin), and then centrifuged for 10 min to remove insoluble material. Protein concentrations in the soluble fraction were measured using a BCA protein assay reagent kit (Fierce), after which equal amounts of protein were separated by 8% SDS-PAGE. The electro- phoretically separated polypeptides were transferred onto a polyvinylidene difluoride membrane (Bio-Rad), which was then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated first with anti-GST-SPIN90 antibodies and then with horseradish peroxidase-conjugated anti-rabbit IgG. The antigen-antibody complexes were detected using enhanced chemilumi- nescence (ECL) (Amersham Pharmacia Biotech.). In some cases, blasts were stripped by heating them to 55 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) and reprobed with anti-α-tubulin antibody, which served as a control.

**Immunoprecipitation**

Cardiac myocytes grown on collagen-coated culture dishes were washed three times with cold PBS and extracted for 1 h at 4 °C in extraction buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM CaCl2, 1 mM MgCl2) supplemented with protease inhibitors. The extracts were clarified by centrifugation at 10,000 × g, after which the supernatants were determined using the BCA method, and samples containing 1 mg of total protein were taken for subsequent immuno- precipitation. Cell lysates were immunoprecipitated using preimmune serum, anti-GST-Spin90 antibody as a negative control, or anti-GST-SPIN90 antibody as a positive control and then incubated for an additional 4 h at 4 °C with protein A-Sepharose beads. Immunocomplexes were extensively washed with the same extraction buffer, after which the immunoprecipitates were boiled and subjected to 8% SDS-PAGE, and the proteins transferred to a polyvi- dene difluoride membrane (Bio-Rad), which was then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated first with anti-GST-Nck or anti-GST-SPIN90 antibody and then with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted in TBST. After washing three times with TBST, the blots were developed using ECL reagents.

**Detection of SPIN90 within Cardiac Myocytes by Immunofluorescence**

Cardiac myocytes grown on collagen-coated (50 μg/ml) glass coverslips were briefly washed with PBS and then fixed with 5% paraformaldehyde in PBS for 10 min at room temperature. After washing in PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and then incubated for 45 min at room tempera- ture, first with the appropriate primary antibodies diluted with PBS containing 1% bovine serum albumin and then with TRITC-conjugated goat anti-rabbit IgG. For double labeling, these cells were incubated again with the appropriate antibodies and FITC-conjugated goat anti-IgG for 45 min. Thereafter, the coverslips were washed with PBS and mounted with 90% glycerol and 0.1% O-phenylenediamine in PBS. The cells were viewed using a Leica DMRB2 fluorescence microscope equipped with a 63× objective lens and appropriate filters. Fluorescence micrographs were taken on T-max P3200 film (Eastman Kodak Co.).

**Cell Permeabilization and Antibody Internalization**

Cardiac myocytes grown on collagen-coated dishes for 8 days were washed twice with DMEM and exposed to streptolysin O (80 μg/ml) for 30 min at 37 °C (7, 23). The cells were then washed three times with DMEM, and incubated with growth medium containing normal rabbit IgG (NRIgG), or with affinity-purified anti-Nck or anti-SPIN90 antibo- dy (200 μg/ml). After incubation for 1, 12, 24, or 48 h at 37 °C, the cells were extensively washed with DMEM and subjected to immuno- fluorescence assays using anti-α-actinin antibodies to show the sar- comme structure. Cells with disrupted sarcomeres were counted; data from three independent experiments are presented graphically.

**RESULTS**

**Isolation of a cDNA Clone Encoding SPIN90**—In a search to identify the proteins with which the Nck SH3 domains interact, yeast two-hybrid screening analysis was performed. The three SH3 domains of Nck (amino acids residues 1–231) was fused to the GAL4 DNA-binding domain, and used as a screen on a human heart Matchmaker cDNA library. Of 3 × 106 independent clones plated, 15 positive clones were identified that specifically interacted with Nck. Sequence analysis showed that two clones encoded the same protein, which contained a proline-rich motif that matched consensus motifs previously shown to be critical for interaction of SH3 domains with target proteins (13). Of these, clone 12 contained a 2.1-kb gene insert and retained its β-galactosidase activity in secondary screening. This clone, however, seemed to contain only a partial cDNA insert, as a stop codon was found within the 3′ region of the insert. To obtain a full-length cDNA clone, a TriplEx human heart cDNA library was screened using the 2.1-kb cDNA gene insert from clone 12; three clones were isolated, with the size of the largest gene insert being 1.2 kb (clone 1–1). The full-length cDNA was then completed with the contribution of sequences from two clones (12 and 1–1). A schematic diagram of the full-length cDNA shows the overlapping region between clone 12 and the newly obtained 3′ region from clone 1–1 (Fig. 1A). This clone contained a single open reading frame from an ATG start codon to a TAG stop codon followed by a stretch of 762 nucleotides comprising the 3′-untranslated region that contains the polyadenylation signal (AAATAA, 2940–2945) and the poly(A) tail (Fig. 1B).

Analysis of the full-length cDNA showed the clone to encode 722 amino acids, including several regions homologous with other genes in the data base. The N terminus contains an SH3 domain that had the highest homology (35% identity and 50% similarity) with the SH3 domains of Fyn, Yes, and c-Src (Fig. 2A) (24–26). The middle portion of the cDNA contained three proline-rich motifs: a type I motif (RXXPxXP) at amino acids 176–182 and two type II motifs (PXpXpX/R/K) at amino acids 170–176 and 242–249, which are matched with consensus sequences important in mediating SH3 domain binding to protein targets. These motifs seem to be responsible for the interaction between SPIN90 and Nck in the yeast two-hybrid system. The C terminus is very hydrophobic and lacks any obvious sequence motifs. An amino acid sequence comparison revealed that the C terminus of SPIN90 is, in part, identical with VIP54, a VacA-interacting protein colociliated with vimentin- and desmin-contain- ing intermediate filaments in human parietal cells (Fig. 2B) (27). To verify whether these proteins are alternative spliced-variants, RT-PCR was performed using the specific primers for SPIN90 or VIP54. The SPIN90 was detected in all the samples including heart and HeLa cells and heart and HeLa cDNA libraries but VIP54 was not (Fig. 2C). However, to address whether these proteins are the alternative spliced vari- ants, detailed biochemical study will be necessary.

Taken together, these results strongly suggest that the cDNA we have isolated encodes a VIP54-related protein expressed in human heart; therefore, we have designated it SPIN90 (SH3 Protein Interacting with Nck, 90 kDa).

**In Vitro Association between SPIN90 and the SH3 Domains of Nck**—To further investigate the specificity of the interaction between SPIN90 protein and Nck SH3 domains, spin90 was translated in vitro using TNT T7-coupled reticulocyte lysate. In addition, cDNAs encoding Nck protein fragments were cloned into pGEX4T-1 vector and expressed as GST fusion proteins in Escherichia coli. [35S]Met-translated spin90 products were mainly detected at ~90 kDa following SDS-PAGE (Fig. 3A) and were coprecipitated with GST-full Nck, GST-Nck total SH3 (containing three SH3 domains), GST-Nck SH3–1, and GST- Nck SH3–3 (containing the first and third SH3 domains, re- spectively), but not with purified GST protein (Fig. 3B). The translated spin90 product also did not coprecipitate with GST- Nck SH2 (containing the SH2 domain) or with GST-Nck SH3–2 (containing the second SH3 domain). Thus, SPIN90 appears to bind exclusively to the first and third SH3 domains of Nck, in vitro.

**Tissue Distribution of SPIN90**—Northern blot analysis of various human tissues revealed spin90 expression to be fairly ubiquitous. A single transcript about 3.4 kb in length was
expressed in all of the adult tissues examined, except intestine (Fig. 4). The highest levels of expression were found in brain, heart, skeletal muscle, kidney, and liver. Intermediate levels of expressions were detectable in placenta, lung, and leukocyte, and low levels were found in colon, thymus, and spleen.

**SPIN90 Is Ubiquitously Expressed in Rat Tissues—**

To obtain a GST fusion protein using clone spin90, the gene insert from clone spin90 was subcloned into pGEX 4T-1 vector, and the encoded GST-SPIN90 fusion protein was expressed in bacterial cells. After SDS-PAGE, the resultant protein band (115 kDa) was purified by electro-elution. Rabbits were then immunized with the eluted fusion protein to generate a polyclonal antiserum, which recognized species of approximately 115 kDa in bacterial lysates and of about 90 kDa in whole cell extracts from various tissues (Fig. 5A). In particular, a protein highly expressed in heart, skeletal muscle, and two skeletal muscle cell lines (C2C12 and L8E63 cells) was recognized, which is consistent with the Northern blots shown in Fig. 4. SPIN90 was found to be the same size in adult rat heart and in cultured neonatal rat cardiac myocytes, and expression of both SPIN90 and Nck was up-regulated during cardiac myocyte differentiation (Fig. 5B).

**Cellular Localization of SPIN90 during Cardiac Myocyte Differentiation—**

To better understand the function of SPIN90 in cultured cardiac myocytes, immunohistochemical analysis was used to determine its location within the cells. Double immunofluorescent labeling using antibodies against SPIN90 and Nck revealed that, in 9-day cultured cardiac myocytes, Nck and SPIN90 were both distributed in a striated pattern characteristic of the Z-discs (Fig. 6A, c and d); the same pattern of distribution was also observed using a-actinin as a Z-disc marker (Fig. 6A, a). In contrast, anti-GST antibody, serving as a control, showed no specific labeling pattern (Fig. 6A, b). Nck and SPIN90 are thus apparently colocalized at Z-discs in mature cardiac myocytes. To assess whether SPIN90 binds to Nck at the Z-discs, lysates obtained from 9-day cultured cardiac myocytes were immunoprecipitated with anti-Nck antiserum and immunoblotted with anti-SPIN90 antiserum. As shown in Fig. 6B, SPIN90 was indeed immunoprecipitated with Nck, and may thus participate in the β1A integrin-mediated signaling pathway via interaction with Nck.

**Specific Antibodies against Nck or SPIN90 Disrupt Sarcomere Structure in Cardiac Myocytes—**

The localization of SPIN90 in sarcolemmal regions associated with Z-discs suggested a potential role for SPIN90 in the organization of sarcomere structure. To further clarify its function, anti-SPIN90 antiserum was introduced into 8-day cultured cardiac myocytes that had been previously permeabilized with streptolysin O, and its effect on sarcomere structure was examined by analyzing the distribution of anti-a-actinin antibody. Normal 8-day
cultured myocytes exhibited typical sarcomere structure (Fig. 7A, a); streptolysin O itself had no effect on cell viability, which is consistent with our earlier reports (7). Similarly, myocytes exposed to normal rabbit IgG (NRIgG) had little effect on sarcomere structure; in the absence of antibodies, ~6% of cells exhibited disrupted sarcomeres. On the other hand, cells treated with anti-Nck or anti-SPIN90 antibodies exhibited substantially greater numbers of disrupted sarcomeres within 1 h of exposure (Fig. 7B, white bar), and within 24 h, ~40–50% of cells showed disrupted sarcomeres (Fig. 7B, hatched bar). Thus, Nck and SPIN90 may be crucial for the maintenance of sarcomere structure and/or the assembly of myofibrils into sarcomeres.

**DISCUSSION**

Specific protein-protein interactions are essential in many biological processes, including gene replication, transcription, metabolism, and signal transduction. Over the past several years, it has become apparent that, although different signaling molecules may contain distinct catalytic activities, most share several conserved protein domains (10, 11). Among such signaling molecules, Nck is a ubiquitously expressed protein containing one SH2 and three SH3 domains. As Nck lacks any known catalytic domains, it was classified as an adaptor molecule (28). We previously reported that Nck is localized at sarcomeres within cardiac myocytes and takes part in integrin-mediated signal transduction (7); however, Nck’s function during differentiation of cardiac myocytes, as well as the functions of the downstream signaling molecules regulated by Nck, remain unknown.

In the present study, we used the yeast two-hybrid screening system and phage library screening to identify a VIP54-related protein that interacts with two of Nck’s SH3 domains. As determined by SDS-PAGE and amino acid sequence analysis, the positive clone encodes for a 90-kDa protein containing an SH3 domain, which was designated SPIN90 (SH3 Protein Interacting with Nck, 90 kDa).

When the amino acid sequences were compared, the N-terminal SH3 domain of SPIN90 was found to have the highest homology with those of Fyn, Yes, and c-Src. Like most other SH3 domains, that of SPIN90 contains a well conserved two-tryptophan (WW) sequence within the domain. It is interesting that the amino acid sequences within the ligand-binding site are completely conserved among the SH3 domains of Fyn, Yes, c-Src, and SPIN90, suggesting that SPIN90 may interact with a variety of proteins containing proline-rich motifs, including signaling molecules, enzymes, and structural proteins.

SPIN90 also contains three proline-rich motifs: a type I motif (RXXPXXP) at amino acids 162–182 and two type II motifs (PXPPX (IVK)) at amino acids 170–176 and 242–249. These are important in mediating SH3-domain binding to protein targets and are likely to be responsible for the interaction between SPIN90 and Nck. At the SH3-binding motif, two conserved proline residues would protrude from the proline-rich motif and intercalate between the aromatic residues in the ligand-binding site, perhaps forming the long, shallow groove of the SH3 ligand-binding site. Although we do not provide specific evidence that the proline-rich motifs of SPIN90 interact with the SH3 domains of Nck, we clearly confirmed that SPIN90 binds to two of the three SH3 domains of Nck, both in vitro and in vivo.

In addition, serine/threonine-rich sequences within the proline-rich motifs would seem to be candidate sites for phosphorylation by serine/threonine kinases. However, although SPIN90 is phosphorylated at ~90 kDa by Western blot analysis of several tissues and cell lines, the predicted molecular mass of the protein is ~80 kDa. It therefore seems likely that SPIN90 is phosphorylated by serine/threonine kinases.

The C-terminal region of SPIN90 is very hydrophobic and lacks any known sequence motifs. To understand the function...

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**Fig. 2. Alignment and sequence comparison of SPIN90. A, alignment and sequence comparison of the SH3 domains of SPIN90 and other related proteins. The SH3 domain of SPIN90 shows high homology with those of Fyn, Yes, and c-Src. Black boxes indicate the conserved amino acids. B, alignment of the amino acid sequences of SPIN90 and VIP54. Black boxes indicate the conserved amino acids. C, RNAs (5 μg) from heart and HeLa cells and DNAs (4 μg) from human heart tissue and HeLa cell libraries were subjected to RT-PCR using the specific primers for SPIN90 or VIP54. The 5′ primer (5'-TGCAGGACGTGTT-3′) at amino acids 176–182 and two type II motifs XX(XP)X at amino acids 247–252 and 249–254 of SPIN90 also contain three proline-rich motifs: a type I motif (RXXPXXP) at amino acids 162–182 and two type II motifs (PXPPX (IVK)) at amino acids 170–176 and 242–249. These are important in mediating SH3-domain binding to protein targets and are likely to be responsible for the interaction between SPIN90 and Nck. At the SH3-binding motif, two conserved proline residues would protrude from the proline-rich motif and intercalate between the aromatic residues in the ligand-binding site, perhaps forming the long, shallow groove of the SH3 ligand-binding site. Although we do not provide specific evidence that the proline-rich motifs of SPIN90 interact with the SH3 domains of Nck, we clearly confirmed that SPIN90 binds to two of the three SH3 domains of Nck, both in vitro and in vivo.**
Recently, Sano et al. (29) identified the gene AF3p21 (ALL-1 fused gene from chromosome 3p21) as a mixed lineage leukemia (MLL) fusion partner gene whose product is expressed as a fusion protein with MLL. This protein has never been observed as an individual molecule, even though its sequence is completely identical with SPIN90. The SH3 domain-deleted form of AF3p21 can be fused with MLL, and the resultant fusion protein (MLL-AF3p21) seems to be essential for leukemogenesis.

of this region, additional detailed studies will be required. Recently, Sano et al. (29) identified the gene AF3p21 (ALL-1 fused gene from chromosome 3p21) as a mixed lineage leukemia (MLL) fusion partner gene whose product is expressed as a fusion protein with MLL. This protein has never been observed as an individual molecule, even though its sequence is completely identical with SPIN90. The SH3 domain-deleted form of AF3p21 can be fused with MLL, and the resultant fusion protein (MLL-AF3p21) seems to be essential for leukemogenesis.
Unlike most known MLL fusion partner genes (e.g. EEN and ABI-1), which have an SH3 domain in their C terminus, AF3p21 contains an SH3 domain in the N terminus, and MLL-AF3p21 contains no SH3 domains. For this reason, we would expect the downstream region, which includes the proline-rich, serine/threonine-rich, and C-terminal regions of SPIN90, to play an essential role in leukemogenesis.

Comparison of the amino acid sequences revealed that the SPIN90 has a high degree of similarity to VIP54 (VacA-interacting protein), which colocalizes with vimentin- and desmin-containing intermediate filaments in human parietal cells (27). Although both SPIN90 and VIP54 appeared to have the same molecular size of protein. In RT-PCR, SPIN90 was detected in both heart and HeLa cells, respectively, but VIP54 was not. To address whether these two proteins are alternatively spliced variants, further biochemical studies will be conducted.

In conclusion, we have described the sequence of SPIN90 and the binding of it to Nck, and have provided evidence of its function in cardiac myocytes. Together, these findings suggest that SPIN90, which contains an SH3 domain, three proline-rich motifs, and serine/threonine-rich sequences, may be an adaptor protein that acts in concert with Nck to mediate sarcomere development during cardiac myocyte differentiation.
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