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

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In the yeast two-hybrid screening, we have isolated a cDNA clone from a human heart library using Nck Src homology 3 (SH3) domains as bait. The full-length cDNA, which encoded 722 amino acids, was identified as a VIP54-related gene containing an SH3 domain, proline-rich motifs, a serine/threonine-rich region, and a long C-terminal hydrophobic region. We refer to this protein as SPIN90 (SH3 Protein Interacting with Nck, 90 kDa). The amino acid sequence of the SH3 domain has the highest homology with those of Fyn, Yes, and c-Src. SPIN90 was broadly expressed in human tissues; in particular, it was highly expressed in heart, brain, and skeletal muscle, and its expression was developmentally regulated during cardiac myocyte differentiation. SPIN90 is able to bind to the first and third SH3 domains of Nck, *in vitro*, and is colocalized with Nck at sarcomere Z-discs within cardiac myocytes. Moreover, treatment with antisera raised against SPIN90 disrupted sarcomere structure, suggesting that this protein may play an important role in the maintenance of sarcomere structure and/or in the assembly of myofibrils into sarcomeres.

Integrins are a large family of heterodimeric transmembrane receptors that play key roles in mediating interactions between cells and ECM[1] 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 (1).

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 (2). β_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 β_1 integrin (5). We previously reported that α_3 integrin is also localized at costameres and is associated with β_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 α-actinin, and participating in a β_{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 phosphotyrosine-containing proteins (10). In addition to recruiting such signaling enzymes as phosphoinositide 3-kinase, phospholipase C-γ, 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 domains that bind to proline-rich motifs (minimal consensus motif: P*XX*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/mitogen-activated protein 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 β_{1A} integrin-mediated signal pathway, the functions of Nck and the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF303581.

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[1] The abbreviations used are: ECM, extracellular matrix; SPIN90, SH3 Protein Interacting with Nck, 90 kDa; SH, Src homology; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; GST, glutathione *S*-transferase; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase pair(s); TBST, Tris-buffered saline with Tween 20; MLL, mixed lineage leukemia; NRIgG, normal rabbit IgG.

signal molecules with which it interacts in cardiac myocytes are also not yet known.

To better understand the β_{1A} 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, antibiotic/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-α-actinin antibody (clone EA-53) was purchased from Sigma. Monoclonal anti-Nck antibody was purchased from Transduction Laboratory. Horseradish peroxidase-labeled anti-mouse immunoglobulin (IgG) and fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG were from Jackson Immunoresearch Laboratory. Protein A-Sepharose and glutathione-Sepharose 4B fast flow were purchased from Amersham Pharmacia Biotech. Human heart Matchmaker cDNA library and λTriplEX 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}P]dCTP and [^{35}S]methionine ([^{35}S]Met) 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 modification. 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 NaH_2PO_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 centrifuged, 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 dissociation 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 1.062 and 1.082 was collected and used as the sole source of purified myocytes. These myocytes were resuspended in DMEM supplemented with 10% fetal bovine serum, plated at a density of 200 cells/mm^2 on collagen-coated dishes (50 μg/ml), and maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Yeast Two-hybrid Screening

The yeast two-hybrid screening system was used as described by Fields *et al.* (21). A cDNA encoding the three SH3 domains of Nck (amino acids 1–251) was amplified via PCR and cloned into pGBT9 vector containing the GAL4 DNA-binding domain. The Nck construct was cotransformed into the yeast strain HF7c with human heart Matchmaker cDNAs containing the activation domain of GAL4 in the pGAD10 vector using the lithium acetate (LiAc) method. Approximately 3×10^6 yeast transformants were pooled and subjected to selections as described below. The transformants were selected at 30 °C on synthetic complete medium plates lacking tryptophan, leucine, and histidine and containing 4 mM 3-amino- (1,2,4)-triazole. After 8–10 days, prey plasmids exhibiting β-galactosidase activity were recovered from His$^+$ colonies and were tested in a cotransformation assay with the bait construct, pGBT9/Nck SH3 or with control pGBT9 vector. To assay for β-galactosidase activity, individual yeast transformants were patched onto filter paper and frozen in liquid nitrogen, after which the filters were stained with buffer (consisting of 0.01% 5-bromo-4-chloro-3-indolyl β-galactopyranoside, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 50 mM β-mercaptoethanol) at 30 °C.

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 λTriplEX 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 pTriplEX plasmids were obtained. The cDNA inserts were sequenced using an ABI PRISM 377 DNA sequencer and PRISM™ 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 β-actin, which served as a control, were labeled with [^{32}P]dCTP by random-primer extension using the oligonucleotide labeling kit (Amersham 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—cDNAs 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), 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 (Amersham Pharmacia Biotech). GST fusion proteins were overexpressed in bacteria and purified on glutathione-Sepharose columns according to the manufacturer's protocol.

Pull-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_2PO_4, 1.8 mM KH_2PO_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 mM leupeptin, 1.5 mM 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 (Pierce), after which equal amounts of protein were separated by 8% SDS-PAGE. The electrophoretically 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 chemiluminescence (ECL) (Amersham Pharmacia Biotech.). In some cases, blots 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.

Coimmunoprecipitation

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 $CaCl_2$, 1 mM $MgCl_2$) supplemented with protease inhibitors. The extracts were clarified by centrifugation for 10 min at 10,000 × *g*, after which the protein concentrations of supernatants were determined using the BCA method, and samples containing 1 mg of total protein were taken for subsequent immunoprecipitation. Cell lysates were immunoprecipitated using preimmune serum, anti-GST-Nck, or anti-GST-SPIN90 antibody as a probe and then incubated for an additional 4 h at 4 °C with protein A-Sepharose beads. Immune complexes 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 polyvinylidene difluoride membrane. The resultant blots were blocked 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 temperature, first with the appropriate primary antibodies diluted with PBS containing 1% bovine serum albumin and then with TRITC-conjugated goat anti-IgG. For double staining, 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 under a Leica DMRBE 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 antibody (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 immunofluorescence assays using anti-α-actinin antibodies to show the sarcomere 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 1–251) was fused to the GAL4 DNA-binding domain and used to screen a human heart Matchmaker cDNA library. Of 3 × 10^6 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 not found within the 3′ region of the insert. To obtain a full-length cDNA clone, a λTriplEX 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. 1*A*). 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 (AATAAA, 2940–2945) and the poly(A) tail (Fig. 1*B*).

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. 2*A*) (24–26). The middle portion of the cDNA contained three proline-rich motifs: a type I motif (R*XX*P*XX*P) at amino acids 176–182 and two type II motifs (PP*X*P*X*(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 colocalized with vimentin- and desmin-containing intermediate filaments in human parietal cells (Fig. 2*B*) (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. 2*C*). However, to address whether these proteins are the alternative spliced variants, 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*. [^{35}S]Met-translated *spin90* products were mainly detected at ~90 kDa following SDS-PAGE (Fig. 3*A*) 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, respectively), but not with purified GST protein (Fig. 3*B*). 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

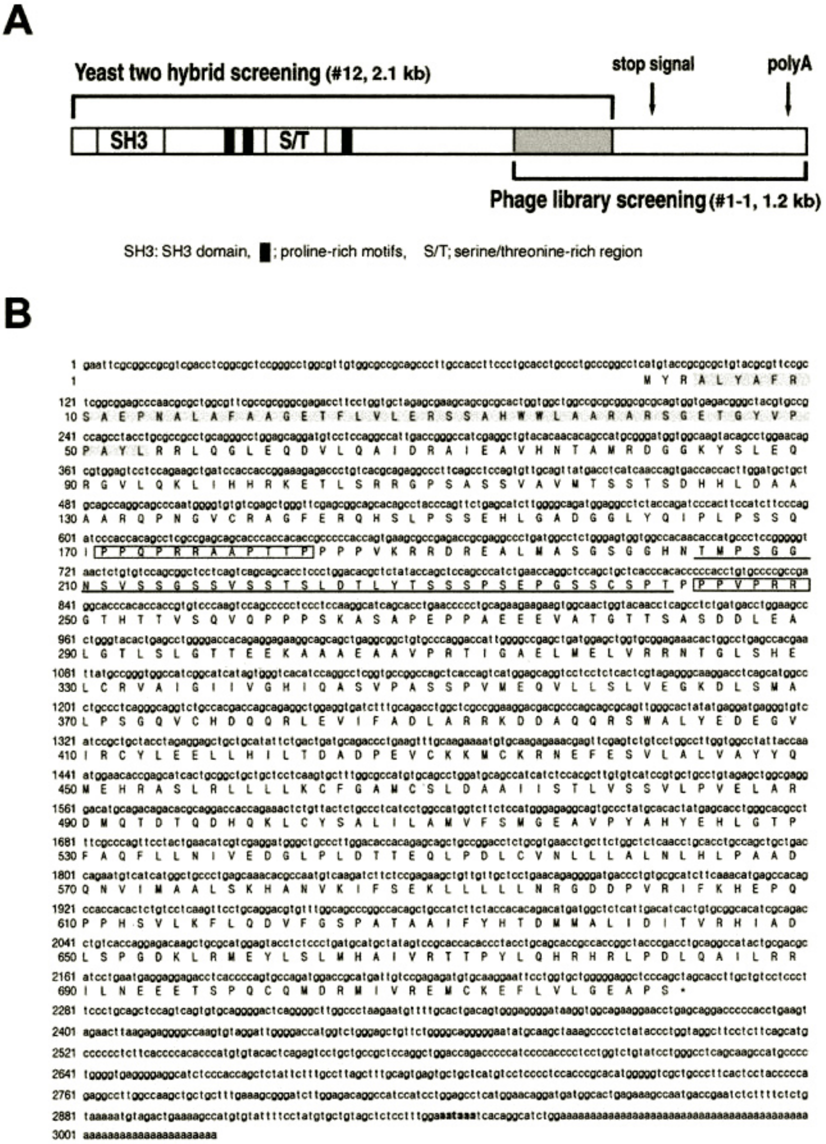


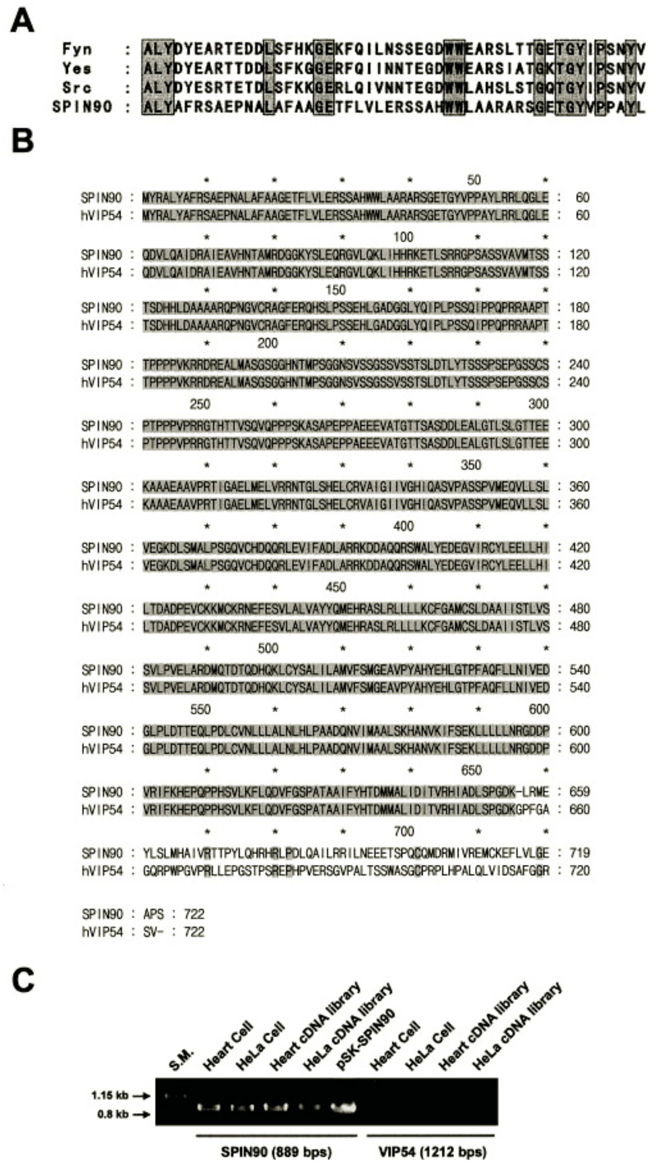
FIG. 1. **Cloning of *spin90*.** *A*, strategy for cloning a full-length *spin90* cDNA. Clone 12 (~2.1 kb) was obtained by two-hybrid screening of a human heart cDNA library using Nck SH3 domains as bait. Clone 1–1 (~1.2 kb) was obtained from human heart phage library by screening with the gene insert of clone 12. The overlapping region is *shaded*. *B*, nucleotide and deduced amino acid sequences of *spin90*. Overlapping clones 12 and 1–1 complete the full-length sequence. The open reading frame begins at position 94, which corresponds to the putative ATG start codon. An *asterisk* at position 2260 indicates the TAG termination codon. The polyadenylation recognition sequence (AATAAA, 2940–2945) is in *bold*. The SH3 domain is *shaded*, serine/threonine-rich sequences are *underlined*, and proline-rich motifs are *boxed*. The accession number of SPIN90 in GenBank™ is AF303581.

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. 5*A*). 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. 5*B*).

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. 6*A*, *c* and *d*); the same pattern of distribution was also observed using α-actinin as a Z-disc marker (Fig. 6*A*, *a*). In contrast, anti-GST antibody, serving as a control, showed no specific labeling pattern (Fig. 6*A*, *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. 6*B*, SPIN90 was indeed immunoprecipitated with Nck, and may thus participate in the β_{1A} 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-α-actinin antibody. Normal 8-day



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Fyn    : ALYDYEARTEDDLSFHKGEKFQILNSSEGDWWEARSLTTGETGYIPSNYV
Yes    : ALYDYEARTTDDLSFKGGERFQIINNTEGDWWEARSIATGKTGYIPSNYV
Src    : ALYDYESRTETDLSFKKGERLQIVNNTEGDWWLAHSLSTGQTGYIPSNYV
SPIN90 : ALYAFRSAEPNALAFAAGETFLVLERSSAHWWLAARARSGETGYVPPAYL
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                *         *         *         *        50         *
SPIN90 : MYRALYAFRSAEPNALAFAAGETFLVLERSSAHWWLAARARSGETGYVPPAYLRRLQGLE :  60
hVIP54 : MYRALYAFRSAEPNALAFAAGETFLVLERSSAHWWLAARARSGETGYVPPAYLRRLQGLE :  60
                *         *         *       100         *         *
SPIN90 : QDVLQAIDRAIEAVHNTAMRDGGKYSLEQRGVLQKLIHHRKETLSRRGPSASSVAVMTSS : 120
hVIP54 : QDVLQAIDRAIEAVHNTAMRDGGKYSLEQRGVLQKLIHHRKETLSRRGPSASSVAVMTSS : 120
                *         *       150         *         *         *
SPIN90 : TSDHHLDAAAARQPNGVCRAGFERQHSLPSSEHLGADGGLYQIPLPSSQIPPQPRRAAPT : 180
hVIP54 : TSDHHLDAAAARQPNGVCRAGFERQHSLPSSEHLGADGGLYQIPLPSSQIPPQPRRAAPT : 180
                *       200         *         *         *         *
SPIN90 : TPPPPVKRRDREALMASGSGGHNTMPSGGNSVSSGSSVSSTSLDTLYTSSSPSEPGSSCS : 240
hVIP54 : TPPPPVKRRDREALMASGSGGHNTMPSGGNSVSSGSSVSSTSLDTLYTSSSPSEPGSSCS : 240
              250         *         *         *         *       300
SPIN90 : PTPPPVPRRGTHTTVSQVQPPPSKASAPEPPAEEEVATGTTSASDDLEALGTLSLGTTEE : 300
hVIP54 : PTPPPVPRRGTHTTVSQVQPPPSKASAPEPPAEEEVATGTTSASDDLEALGTLSLGTTEE : 300
                *         *         *         *       350         *
SPIN90 : KAAAEAAVPRTIGAELMELVRRNTGLSHELCRVAIGIIVGHIQASVPASSPVMEQVLLSL : 360
hVIP54 : KAAAEAAVPRTIGAELMELVRRNTGLSHELCRVAIGIIVGHIQASVPASSPVMEQVLLSL : 360
                *         *         *       400         *         *
SPIN90 : VEGKDLSMALPSGQVCHDQQRLEVIFADLARRKDDAQQRSWALYEDEGVIRCYLEELLHI : 420
hVIP54 : VEGKDLSMALPSGQVCHDQQRLEVIFADLARRKDDAQQRSWALYEDEGVIRCYLEELLHI : 420
                *         *       450         *         *         *
SPIN90 : LTDADPEVCKKMCKRNEFESVLALVAYYQMEHRASLRLLLLKCFGAMCSLDAAIISTLVS : 480
hVIP54 : LTDADPEVCKKMCKRNEFESVLALVAYYQMEHRASLRLLLLKCFGAMCSLDAAIISTLVS : 480
                *       500         *         *         *         *
SPIN90 : SVLPVELARDMQTDTQDHQKLCYSALILAMVFSMGEAVPYAHYEHLGTPFAQFLLNIVED : 540
hVIP54 : SVLPVELARDMQTDTQDHQKLCYSALILAMVFSMGEAVPYAHYEHLGTPFAQFLLNIVED : 540
              550         *         *         *         *       600
SPIN90 : GLPLDTTEQLPDLCVNLLLALNLHLPAADQNVIMAALSKHANVKIFSEKLLLLLNRGDDP : 600
hVIP54 : GLPLDTTEQLPDLCVNLLLALNLHLPAADQNVIMAALSKHANVKIFSEKLLLLLNRGDDP : 600
                *         *         *         *       650         *
SPIN90 : VRIFKHEPQPPHSVLKFLQDVFGSPATAAIFYHTDMMALIDITVRHIADLSPGDK-LRME : 659
hVIP54 : VRIFKHEPQPPHSVLKFLQDVFGSPATAAIFYHTDMMALIDITVRHIADLSPGDKGPFGA : 660
                *         *         *       700         *         *
SPIN90 : YLSLMHAIVRTTPYLQHRHRLPDLQAILRRILNEEETSPQCQMDRMIVREMCKEFLVLGE : 719
hVIP54 : GQRPWPGVPRLLEPGSTPSREPHPVERSGVPALTSSWASGCPRPLHPALQLVIDSAFGGR : 720

SPIN90 : APS : 722
hVIP54 : SV- : 722
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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-TGGCAG-3′, 1947–1966) was picked from the homology region of SPIN90 and VIP54, and the 3′ primers were from 3′-untranslated region of SPIN90 (5′-GCTTTCTCAGTGCCATCATCC-3′, 2817–2836) or VIP54 (5′-CGTTGCTCCTGGTGCTGAAC-3′, 3140–3159). SPIN90 (889 base pairs) was detected in all the samples but VIP54 (1212 base pairs) was not. The accession number of VIP54 in GenBank™ is AJ242655. *S.M.*, size marker.

cultured myocytes exhibited typical sarcomere structure (Fig. 7*A*, *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) and then immunolabeled with anti-α-actinin antibodies also exhibited typical sarcomeric patterns (Fig. 7*A*, *b*). On the other hand, exposing the cells to anti-Nck or anti-SPIN90 antisera and then immunolabeling with anti-α-actinin antibodies revealed severely disrupted sarcomere structures (Fig. 7*A*, *c* and *d*).

The percentage of sarcomeres disrupted by antibody treatment was calculated from the mean number of cardiac myocytes containing disrupted sarcomeres (Fig. 7*B*). Streptolysin O and 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. 7*B*, *white bar*), and within 24 h, ~40–50% of cells showed disrupted sarcomeres (Fig. 7*B*, *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 β_{1A} 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; hence, it 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 (R*XX*P*XX*P) at amino acids 176–182 and two type II motifs (PP*X*P*X*(R/K)) 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. In fact, although SPIN90 is detected 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

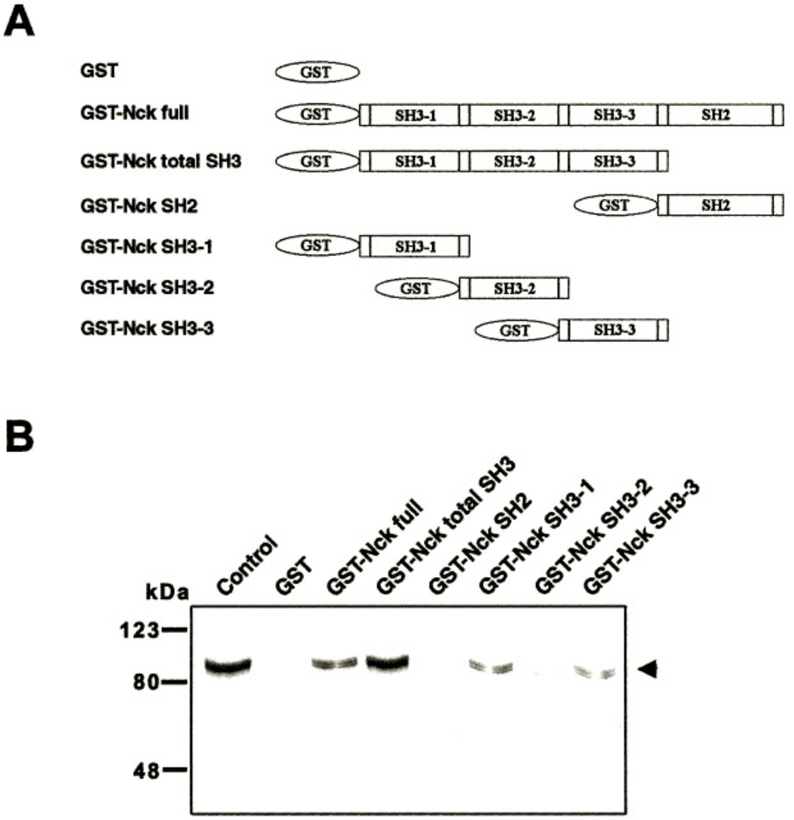

FIG. 3. ***In vitro* association of SPIN90 with Nck.** All recombinant GST-Nck fusion proteins overexpressed in *E. coli* were immobilized on glutathione-Sepharose beads, after which GST pull-down assays were performed by incubating the beads with [^{35}S]Met-labeled, *in vitro* translated SPIN90 for 6 h at 4 °C. Bound proteins were eluted, analyzed by SDS-PAGE, and visualized by autoradiography. SPIN90 binds to GST-full Nck, GST-total SH3, GST-SH3–1, and GST-SH3–3, but not to GST-SH2, GST-SH3–2, or GST alone (control).

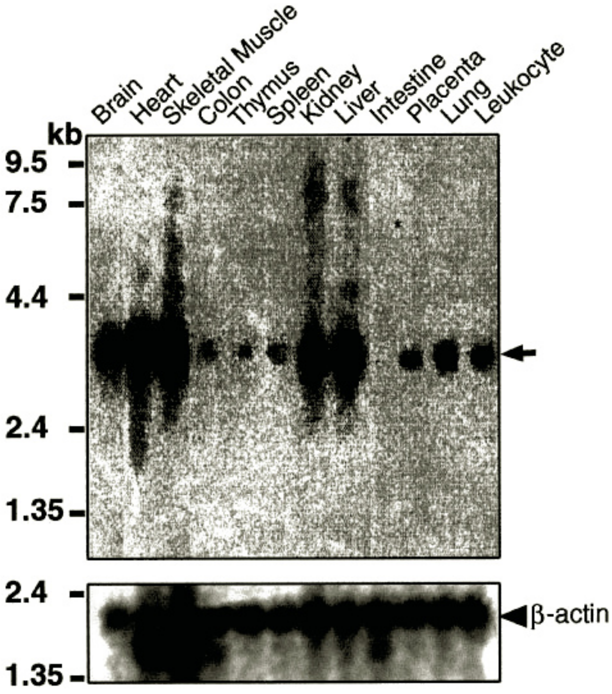

FIG. 4. **Expression of SPIN90 mRNA in various human adult tissues.** Poly(A)$^+$ RNAs isolated from the indicated human tissues (CLONTECH) were hybridized with the ^{32}P-labeled cDNA fragment corresponding to the nucleotides 1–2145 of *spin90*. Molecular size makers are indicated on the *left*. The SPIN90 mRNA (~3.4 kb) appears to be ubiquitously expressed.

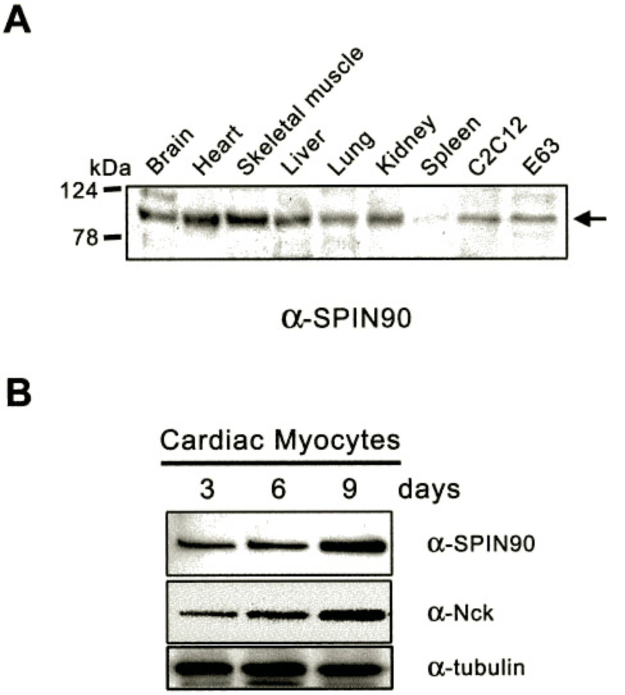

FIG. 5. **Expression of SPIN90 protein in various rat tissues.** *A*, antiserum raised against SPIN90 recognized a 90-kDa protein in various rat tissues and cell lines. Highest levels of SPIN90 expression were found in heart, skeletal muscle, and two skeletal muscle cell lines (C2C12 and L8E63). *B*, expression of SPIN90 in neonatal rat cardiac myocytes. Expression of SPIN90 and Nck are up-regulated during cardiac myocyte differentiation. α-Tubulin was used as a control.

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.

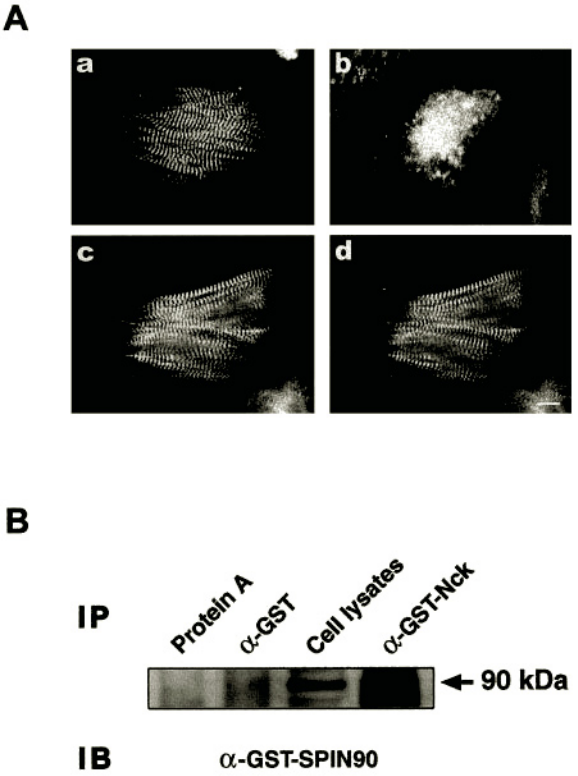

FIG. 6. **Intracellular localization of SPIN90 in cultured cardiac myocytes.** *A*, after 9 days in culture, cardiac myocytes were fixed, permeabilized, and labeled first with anti-α-actinin (*a*) or anti-GST antibodies (*b*) and then with TRITC-conjugated goat anti-IgGs. For double labeling, the fixed cells were first incubated with monoclonal anti-Nck antibodies and FITC-conjugated goat anti-mouse IgG (*c*) and then incubated again with anti-SPIN90 antiserum and TRITC-conjugated goat anti-rabbit IgG (*d*). Nck (*c*) and SPIN90 (*d*) localized at the Z-discs in a pattern characteristic of sarcomeric banding, a pattern not detected when cells were labeled with α-GST antibody (*b*). α-Actinin served as a Z-disc marker (*a*). *B*, *in vivo* association between SPIN90 and Nck SH3 domains in cardiac myocytes. To confirm the interaction between Nck and SPIN90 *in vivo*, lysates of cardiac myocytes (9-day cultures) were immunoprecipitated with anti-GST-Nck antiserum, and the precipitants were immunoblotted with anti-SPIN90 antiserum. Protein A and anti-GST antiserum were used as controls. *Bar* = 10 μm.

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 size of transcripts and show the same tissue distribution pattern, immunoblot analysis revealed that they encode different 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. However, it seems that these two proteins are closely related to each other. VIP54 was found to be associated with vimentin, and SPIN90 also seems to be able to associate with cytoskeletal proteins. For example, immunofluorescent labeling showed SPIN90 to be localized at Z-discs of cardiac myocytes, and introduction of SPIN90 antiserum into cells disrupted sarcomere structure. This suggests that SPIN90 may participate in the maintenance of sarcomere structure and/or the assembly of myofibrils into sarcomeres via association with cytoskeletal proteins. In addition, we reported previously that Nck participates in downstream signaling triggered by β_{1A} but not β_{1D} integrin. Thus, the association of SPIN90 with Nck in sarcomeres of cardiac myocytes supports the notion that SPIN90 is involved in a β_{1A} integrin-mediated signal transduction pathway initiated by the interaction of integrin and ECM during cardiac myocyte differentiation, though additional detailed studies will be required to determine specifically how this protein is involved.

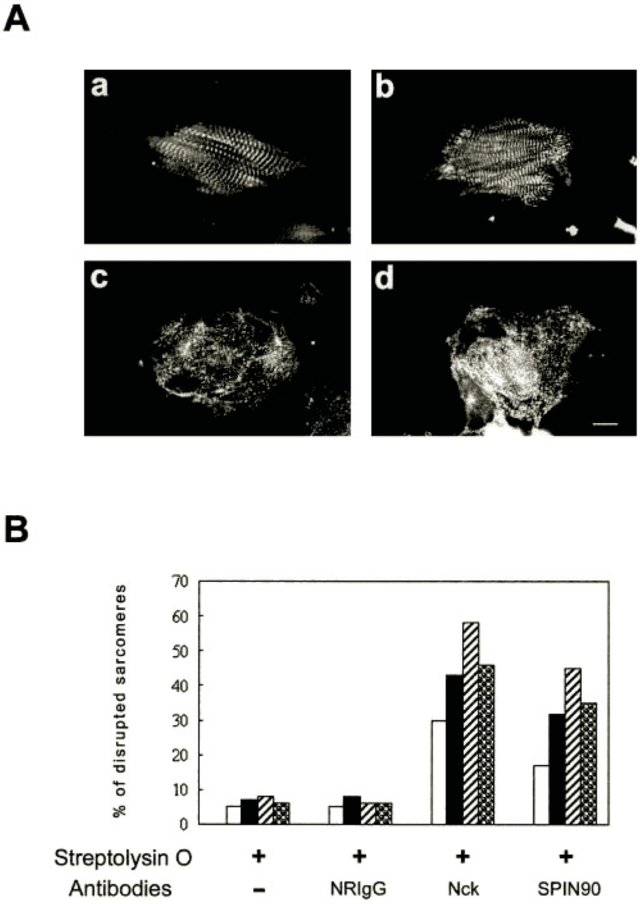

FIG. 7. **Disruption of sarcomere structure by anti-Nck and anti-SPIN90 antibodies.** *A*, myocytes cultured for 8 days were treated with streptolysin O for 30 min to permeabilize their cell membranes. To visualize the changes in sarcomere structure, following antibody treatment for 24 h, the cells were labeled with anti-α-actinin antibodies and FITC-conjugated goat anti-mouse IgG. Untreated cells exhibited typical sarcomeric structures (*a*), as did cells treated with NRIgG, which served as a control (*b*). In contrast, anti-Nck (*c*) and anti-SPIN90 (*d*) antibodies caused clear disruptions of sarcomeric structure. *B*, the percentage of disrupted sarcomeres was counted from the mean number of myocytes in which disrupted sarcomeres were observed. In the presence of streptolysin O and NRIgG, the incidence of sarcomeric disruption remained at basal levels (~6%). In contrast, anti-Nck and anti-SPIN90 antibodies disrupted ~50% of sarcomeres. *White bar*, 1 h; *black bar*, 12 h; *hatched bar*, 24 h; *dotted bar*, 48 h after internalization of NRIgG, anti-Nck, or anti-SPIN90. *Bar* = 10 μ m.

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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