Regulation of SPIN90 Phosphorylation and Interaction with Nck by ERK and Cell Adhesion*

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SPIN90 is a widely expressed Nck-binding protein that contains one Src homology 3 (SH3) domain, three Pro-rich motifs, and a serine/threonine-rich region, and is known to participate in sarcomere assembly during cardiac myocyte differentiation. We used in vitro binding assays and yeast two-hybrid screening analysis to identify Nck, βPIX, Wiscott-Aldrich syndrome protein (WASP), and ERK1 as SPIN90-binding proteins. It appears that βPIX, WASP, and SPIN90 form a complex that interacts with Nck in a manner dependent upon cell adhesion to extracellular matrix. The βPIX/WASP-SPIN90/Nck interaction was abolished in suspended and cytochalasin D-treated cells, but was recovered when cells were replated on fibronectin-coated dishes. The SPIN90/βPIX/WASP complex was stable, even in suspended cells, suggesting SPIN90 serves as an adaptor molecule to recruit other proteins to Nck at focal adhesions. In addition, we found that overexpression of the SPIN90 SH3 domain or Pro-rich region, respectively, abolished SPIN90/Nck and SPIN90/βPIX interactions, resulting in detachment of cells from extracellular matrix. SPIN90 was phosphorylated by ERK1, which was, itself, activated by cell adhesion and platelet-derived growth factor. Such phosphorylation of SPIN90 likely promotes the interaction of the SPIN90/βPIX/WASP complex and Nck. It thus appears that the interaction of the βPIX/WASP-SPIN90 complex with Nck is crucial for stable cell adhesion and can be dynamically modulated by SPIN90 phosphorylation that is dependent on cell adhesion and ERK activation.

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The abbreviations used are: ECM, extracellular matrix; SPIN90, SH3 protein interacting with Nck, 90 kDa; WASP, Wiscott-Aldrich syndrome protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK1, extra-cellular signal-regulated kinase 1; FBS, fetal bovine serum; FN, fibronectin; GST, glutathione-S-transferase; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PIX, PAK-interacting exchange factor; MFS, phenylmethylsulfonyl fluoride; SH3/H2 domain, Src homology 32 domain; MBP, myelin basic protein; HA, hemagglutinin.

With the aim of understanding better the function of SPIN90, we set out to identify proteins that interact with SPIN90 using in vitro binding assays and yeast two-hybrid screening. We found that SPIN90 has the capacity to mediate formation of protein complexes containing βPIX, WASP, and Nck and that this effect is highly dependent on cell adhesion.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic/antimycotics, complete Freund's adjuvant, incomplete Freund's adjuvant, and trypsin-EDTA were all obtained from Invitrogen. Anti-ERK1 monoclonal antibody was from Zymed Lab-
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orsities Inc. and BD Transduction Laboratories; anti-phospho-ERK1 monoclonal antibody was from Cell Signaling Technology; and anti-phospho-Ser antibody was from Upstate Biotechnology. Horseradish 

peroxidase-labeled anti-mouse immunoglobulin G (IgG) and fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG were from Jackson ImmunoResearch Laboratory. Protein A-Sepharose and glutathione-agarose 4B fast flow were from Amersham Biosciences. Spectra/Por® dialysis membranes (4,000-Dalton cutoff) was from Spectrum Medical Industries, Inc. Primers for PCR and sequencing were synthesized at Genetex. Tn5 T7-coupled recetilcute lysate system was from Promega. [35S]Met and H3[32P]O4 were from PerkinElmer Life Sciences. Rat interferon γ antibody was from Cell Signaling Technology; and anti-

PIX Antibodies

- Phospho-ERK1 (Thr202) and phospho-ERK2 (Thr183) monoclonal antibody was from Cell Signaling Technology; and anti-

GST Pull-down Assays—To assay ERK1 kinase activity (26), HeLa cells were washed with cold PBS and extracted by 

sonication in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM vanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, 1 mM PMSF), after which the cell lysates were collected by centrifugation for 10 min at 4 °C, and the protein content was determined using BCA. Samples containing 1 mg of total protein were then taken for subsequent immunoprecipitation. The supernatants were subjected to SDS-PAGE and immunoblot analysis. 

Immunokinase Assay for Active ERK1—To assay ERK1 kinase activity (26), HeLa cells were washed with cold PBS and extracted by 

sonication in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM vanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, 1 mM PMSF), after which the cell lysates were collected by centrifugation for 10 min at 4 °C, and the protein content was determined using BCA. Samples containing 1 mg of total protein were then taken for subsequent immunoprecipitation. The supernatants were subjected to SDS-PAGE and immunoblot analysis. 

Immunoblot Analysis—Cells were lysed by boiling in a lysis buffer (1% Triton X-100, 10 mM orthovanadate, 10 mM NaF, 10 mM Tris-HCl (pH 7.4), 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin, and 1 mM aprotinin), after which detergent-insoluble materials were removed by 

centrifugation at 12,000 × g for 10 min. Protein concentrations in the soluble fraction were measured using a bicinchoninic acid (BCA) Protein Assay Reagent kit (Fierce). Constant amounts of protein were then separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were then blocked for 1 h with 5% nonfat dry milk or 3% BSA (for phospho-Ser or phospho-ERK1 antibodies) in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20, after which they were incubated first with the respective primary antibodies and then with horseradish peroxidase-conjugated anti-

bit or anti-mouse IgG. The anti-phospho antibodies were used with the Enhanced Chemiluminescence (ECL) reagents (Amersham Biosciences). In some cases, blots were stripped by heating to 60 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) and reprobed.

Co-immunoprecipitation—Cells 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 mM sodium orthovanadate, 10 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM CaCl2, 1 mM MgCl2) supplemented with protease inhibitors. The extracts were then clarified by centrifugation for 10 min at 12,000 × g, and the protein concentrations in the supernatants were determined using BCA. Samples containing 1 mg of total protein were then taken for subsequent immunoprecipitation. The supernatants were subjected to SDS-PAGE and immunoblot analysis. 

Identification of Proteins Associating with SPIN90 in Vitro and in Vivo—Using in vitro and in vivo binding assays, we have identified several proteins that interact with SPIN90. GST pull-down analysis carried out with in vitro-translated proteins showed that SPIN90 co-purified with GST-Nck, βPIX, and -WASP, but not GST-β1 integrin (Fig. 1, A–D). Subsequent co-immunoprecipitation analysis confirmed that SPIN90 does indeed associate with Nck, β1 integrin, βPIX, and WASP (Fig. 1, E–H), suggesting that SPIN90 participates in β1 integrin-mediated signaling via protein–protein interactions. SPIN90 is unlikely to bind to β1 integrin directly, however, because amino
Acid sequence analysis revealed these proteins each to contain multiple Pro-rich motifs and SH3 domains (Fig. 1).

Interaction between SPIN90 and Nck Is Adhesion-dependent—To test the dependence of the SPIN90-Nck interaction on cell adhesion, lysates from adherent or detached cells were subjected to co-immunoprecipitation assays. SPIN90 immunoprecipitates from stably adherent cells contained readily detectable levels of Nck, but no Nck associated with SPIN90 was detected in suspended or cytochalasin D-treated cells (Fig. 2A). Moreover, when lysates from cells placed in suspension for various times were subjected to co-immunoprecipitation, dissociation of SPIN90 from Nck was found to be more than 90% complete within 30 min after cell detachment, and their association was undetectable within 60 min. Notably, this effect was rapidly reversed by replating the cells on FN-coated dishes (Fig. 2B).

By contrast, SPIN90, βPIX, and WASP remained associated in suspended cells. Likewise, the interaction between βPIX and WASP was apparent in both suspended and replated cells. The interaction between Nck and WASP was not detected in suspended cells and only weakly detected in replated cells (Fig. 2C); the interaction between βPIX and Nck was also absent in suspended cells, but was strongly detected in replated cells (Fig. 2D). Taken together, these results suggest that SPIN90, βPIX, and WASP make up a molecular complex that interacts with Nck in a manner dependent on cell adhesion.

SPIN90-Nck Interaction Is Dynamically Regulated by ERK1 Activation—Among the proteins isolated by yeast two-hybrid screening using a rat brain cDNA library and full-length SPIN90 as bait was ERK1, which was identified as binding to SPIN90 (Fig. 3A). GST pull-down assays and co-immunoprecipitation analysis confirmed that ERK1 does indeed bind to SPIN90 both in vitro and in vivo (Fig. 3, B and C). Furthermore, consistent with earlier reports that ERK activity is regulated by cell adhesion (27), we found that phospho-ERK1/2 was undetectable in suspended cells but that levels of phospho-ERK1/2 increased for up to 120 min after replating the cells on FN (Fig. 4A). Pre-treating the cells with PD98059, an ERK inhibitor, before replating blocked the increases in ERK1/2 activation (Fig. 4B).

We next examined SPIN90 phosphorylation in adherent, detached, and replated cells in the absence and presence of 30 μM PD98059. Using in vivo labeling, we detected SPIN90 phosphorylation in both adherent and replated cells, but weak phosphorylation of SPIN90 was detected in detached or PD98059-treated cells. In addition, Ser-phosphorylation of SPIN90 was routinely detected in SPIN90 precipitants from adherent cells, but was abolished by cell detachment or PD98059 treatment (Fig. 4C). We were not able to test for the presence of Thr-phosphorylation because of the absence of available antibodies. In parallel experiments, a strong interaction between SPIN90 and Nck was detected in adherent and replated cells, whereas their interaction almost disappeared in detached and PD98059-treated cells (Fig. 4D).

That ERK1 phosphorylated SPIN90 was confirmed by in vitro kinase assays showing that immunoprecipitates obtained
using anti-phospho-ERK1 antibody in adherent or replated cells were able to phosphorylate purified GST-SPIN90 protein and MBP, and this effect was blocked by detaching the cells or pretreating them with PD98059 (Fig. 5, A and B). Thus, the interaction of SPIN90 and Nck apparently coincides with an ERK1-catalyzed change in the phosphorylation state of SPIN90 during cell adhesion.

Relationship between SPIN90-Nck Interaction and ERK1 Activation by PDGF—It is known that ERK1 can be activated by a variety of growth factors, including PDGF (28). We found that activation of ERK1 by 20 ng/ml PDGF reached to the maximum within 10 min and decreased thereafter (Fig. 6A) and that the interaction of SPIN90 and Nck followed the same time course (Fig. 6B). AG1295 is a specific PDGF receptor antagonist that reversibly inhibits PDGF by competitively binding it its receptor. We found that AG1295 inhibited ERK1 activation by PDGF, as well as the interaction of SPIN90 and Nck (Fig. 6C). Similarly, 30 μM PD98059 specifically inhibited both PDGF-induced ERK1 activation and SPIN90-Nck interaction (Fig. 6D). By contrast, AG1478, an epidermal growth factor (EGF) receptor antagonist, was less able to inhibit the interaction of SPIN90 and Nck (Fig. 6C). These findings clearly indicate that SPIN90 can be a substrate for ERK1 and that its phosphorylation by ERK1 likely modulates SPIN90-Nck interaction.

Interestingly, phosphorylation/activation of PDGF receptors in suspended cells led to recruitment of Nck to the receptor, but not SPIN90. And in the presence of PDGF, non-adherent cells exhibited the induced interaction of SPIN90 and Nck (Fig. 6E), suggesting PDGF can induce SPIN90-Nck interaction and likely potentiates their interaction during cell adhesion.

Modulation of Cell Adhesion by Specific Domains of SPIN90—SPIN90 contains three functional domains or motifs known to mediate protein-protein interaction. To further analyze these interacting motifs, its SH3 domain (HA-SPIN90 SH3), Pro-rich motifs (HA-SPIN90 PR), and C-terminal region (HA-SPIN90 C-term) were, respectively, cloned into an HA-tagged mammalian expression vector (Fig. 7A) and expressed in HeLa cells (Fig. 7B). Fig. 7C shows that overexpression of SPIN90 Pro-rich motifs dramatically suppressed the interaction between SPIN90 and Nck, suggesting that Nck binds to Pro-rich motifs of SPIN90 protein via SH3 domains.
coated dishes suggests their interaction plays a key role in the process of cell adhesion. To test this idea, cell adhesion assays were carried out using cells overexpressing the HA-SPIN90 SH3 domain, Pro-rich motifs, or C-terminal region. Overexpression of the HA-SPIN90 SH3 domain or Pro-rich motifs diminished cell adhesion by up to 60–70%, whereas cell adhesion of cells transfected with HA-SPIN90 C-term was indistinguishable from that in HA vector-transfected cells (Fig. 7D). Apparently, the interactions between SPIN90 and other cellular proteins are essential for stable cell adhesion.

**DISCUSSION**

We previously showed SPIN90 to be a Nck-binding protein widely expressed in a variety of tissues and cell types, and to participate in sarcomere assembly during cardiac myocyte differentiation (22). This protein has a well conserved SH3 domain and three separated Pro-rich motifs capable of mediating protein-protein interactions. Moreover, between two of the Pro-rich motifs, there is a serine/threonine-rich region, indicating the potential for phosphorylation by serine/threonine kinases. Still, the function of SPIN90 remains largely unknown, and few in vivo ligands have been identified.

Particular interactions of a protein with other protein or specific phosphorylation are crucial in a variety of biological processes, including signal transduction. In the present study, therefore, we carried out a series of in vitro binding assays and co-immunoprecipitation analyses with the aim of identifying proteins that bind to SPIN90 and used a yeast two-hybrid screening system to identify kinases capable of phosphorylating it. We found that SPIN90 associates with Nck, βPIX, and WASP. Each of these proteins contains more than one Pro-rich motif or SH3 domain through which they are able to participate in the formation of a protein complex. Indeed, the Nck/SPIN90 interaction is likely mediated through the first and third SH3 domains of Nck (22), which bind to a Pro-rich region of SPIN90. On the other hand, the SH3 domain of SPIN90 likely mediates its interaction with βPIX.

Nck contains three SH3 domains and one SH2 domain that mediate protein-protein interactions. The former are able to bind to a variety of downstream proteins, including SOS, WASP, dynamin, and Cbl, which are all involved in reorganization of actin cytoskeleton (16, 21, 29, 30). βPIX is a Rho family guanine nucleotide exchange factor that interacts with PAK through its SH3 domain and has a role in nuclear signal-
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Fig. 6. Modulation of SPIN90-Nck interaction by ERK1 activated by PDGF. A, serum-starved HeLa cells were incubated with 20 ng/ml PDGF for the indicated times, after which the lysates were immunoblotted with anti-ERK1, anti-phospho-ERK1/2, or anti-SPIN90 antibody. B, lysates from cells treated with PDGF for the indicated times were immunoprecipitated with anti-Nck antibody and immunoblotted with anti-Nck or SPIN90 antibody. C, PDGF-treated cells were incubated with AG1478, an EGF receptor inhibitor, or AG1295, a PDGF receptor inhibitor, after which the lysates were immunoblotted with anti-ERK1 or phospho-ERK1 antibody. To test the effect of PD98059 on SPIN90-Nck interaction, the same cell lysates were immunoprecipitated with anti-Nck antibody and immunoblotted with anti-Nck or SPIN90 antibody. D, cells were treated with 10 or 30 μM PD98059 in the presence or absence of PDGF, after which the lysates were immunoblotted with anti-ERK1 or phospho-ERK1 antibody. E, to identify ERK1 activation by PDGF, adherent and suspended cells, pretreated with or without 20 ng/ml PDGF, were lysed, immunoprecipitated with anti-PDGF receptor antibody, and immunoblotted with anti-p-Tyr, anti-PDGF receptor, anti-Nck, or anti-SPIN90 antibody. Then the same cell lysates were immunoprecipitated with anti-Nck antibody and immunoblotted with anti-Nck or SPIN90 antibody.

by MAPK activation as well as in actin cytoskeleton regulation (31, 32). Members of the PAK family of serinethreonine kinases serve as targets for the small GTP-binding proteins Cdc42 and Rac and have been implicated in a wide range of biological activities related to the modulation of actin cytoskeletal organization (33, 34), including neurite formation, axonal guidance, development of cell polarity, and motile responses (35, 36). The interaction of PAK and Nck is reported to be dynamically regulated by cell adhesion (37). WASP has been shown to regulate the cortical actin cytoskeleton as a downstream effector of Rac and Cdc42 and to integrate signaling cascades leading to actin polymerization (38). In addition, SPIN90 has a SH3 domain very similar to that of WISH, a WASP-binding protein that can enhance N-WASP-induced Arp2/3 complex activation, resulting in rapid actin polymerization (24). Based on these observations and the results of preliminary experiments, we suggest that SPIN90 also plays a role in the actin cytoskeleton rearrangement, including formation of filopodia or lamellipodia, through its interactions with Nck, βPIX, and WASP. Indeed, overexpression of SPIN90 in HeLa cells readily promoted the filopodia formation, and this effect was potentiated by PDGF.²

Ectopic expression of SPIN90 SH3 domain or Pro-rich motifs abolished the interaction of SPIN90 with Nck and βPIX, thereby promoting cell detachment from the ECM. The interaction of SPIN90 and Nck appears to be cell adhesion-dependent and to be reversibly diminished by placing cells in suspension or cytochalasin D treatment, in which cytochalasin D induced actin cytoskeleton disruption and promoted cell detachment and rounding. By contrast, the association between SPIN90 and βPIX or WASP remains intact in suspended cells. These findings suggest that SPIN90 first mediates the formation of the SPIN90βPIX-WASP complex; the interaction between the complex and Nck is subsequently mediated by SPIN90 in a manner dynamically regulated by cell adhesion.

In the correlation with the regulation of SPIN90-Nck interaction, the reverse mechanism of SPIN90-Nck interaction and dissociation reflects that the interaction may be regulated by a post-translational modification, such as phosphorylation. Herein, we also found that ERK1 is able to bind to and efficiently phosphorylate SPIN90. Activation of ERK1 is regulated by cell adhesion as well as a variety of growth factors, including PDGF, insulin, EGF, and fibroblast growth factor (27, 28, 39–41); once activated, ERK is able to phosphorylate numerous proteins, including RSK, c-Fos, c-Jun, c-Myc, c-raf, MAP2, and Mek (42–44). SPIN90 also appears to be phosphorylated by ERK1 in adherent cells, and its phosphorylation may, in turn, facilitate assembly of signaling complexes containing integrin β1, SPIN90, and ERK1, which likely transmit a cell adhesion signal to the cytoskeletal network. It is noteworthy that ERK1/MAPK is targeted to newly forming cell-matrix adhesion by integrin engagement (45), which suggests that the interaction of ERK1 and SPIN90 is closely related in the spatial formation of focal adhesions and that

²C. S. Lim, S. H. Kim, J. G. Jung, J.-K. Kim, and W. K. Song, unpublished data.
phosphorylation of SPIN90 by ERK1 is a crucial step via which the SPIN90 complex is recruited to Nck for stable cell adhesion.

The function of adhesion receptors is regulated by growth factors and other agonists (4). Because the signaling pathways activated by integrins and growth factors share many common elements (46), there are many opportunities for integrin signaling to modulate growth factor signals and vice versa. For example, adhesion to ECM proteins induces transient tyrosine phosphorylation of EGFR and PDGF receptors, which in turn enhances ERK1 activity. In addition to cell adhesion, adhesion receptors may also activate Ras-dependent signaling cascades, which has broad implications for the regulation of protein-protein interactions and anchorage-dependent protein localization.

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