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Regulation of SPIN90 by Cell Adhesion and ERK Activation

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Abstract

SPIN90 was identified to form molecular complex with βPIX, WASP and Nck. This complex shows that SPIN90 interacts with Nck in a manner dependent upon cell adhesion to extracellular matrix, but SPIN90·βPIX·WASP complex was stable even in suspended cells. This suggests that SPIN90 serves as an adaptor molecule to recruit other proteins to Nck at focal adhesions. 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·β PIX·WASP complex and Nck. It thus appears that the interaction of the SPIN90·βPIX·WASP 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.

SPIN90 directly binds syndapin I, syndapin isoform II-1 and II-s via its PRD region *in vitro, in vivo* and also associates with endocytosis core components such as clathrin and dynamin. In neuron and fibroblast, SPIN90 colocalizes with syndapins as puntate form, consistent with a role for SPIN90 in clathrin-mediated endocytosis pathway. Overexpression of SPIN90 N-term inhibits receptor-mediated endocytosis. Interestingly, SPIN90 PRD, binding interface of syndapin, significantly blocks internalization of transferrin, demonstrating SPIN90 involvement in endocytosis *in vivo* by interacting syndapin. Depletion of endogenous SPIN90 by introducing α-SPIN90 also blocks receptor-mediated endocytosis.

Actin polymerization could generate force facilitating the pinch-out event in endocytosis, detach newly formed endocytic vesicle from the plasma membrane or push out them via the cytosol on actin tails. Here we found that SPIN90 localizes to high actin turn over cortical area, actin-membrane interface and membrane ruffle in PDGF treated cells. Overexpression of SPIN90 has an effect on cortical actin rearrangement as filopodia induction and it is mediated by the Arp2/3 complex at cell periphery. Consistent with a role in actin organization, GFP-SPIN90 present in actin comet tail generated by PIP5 kinaseγ overexpression. Therefore this study suggests that SPIN90 is functional linker between endocytosis and actin cytoskeleton.

Introduction

Endocytosis internalizes part of plasma membrane and components of the extracellular medium.

Endocytosis has been first event at vesicular trafficking. Endocytic pathways are tightly regulated to control all aspects of intercellular communication in multicelluar organisms. Several accessory proteins assist the formation of a clathrin-coated vesicle and may help in coat assembly and regulate coat dynamics.

Dynamin is a mechanochemical enzyme that pinches vesicles from the plasma membrane in endocytosis. C-terminal PRD of dynamin associates with several SH3 domain containing proteins that is integrated in clathrin-mediated endocytosis. Syndapin is one of dynamin interacting protein via its SH3 domain. SH3 domain of syndapin inhibits receptor-mediated endocytosis in vivo.

The actin cytoskeleton is essential for endocytosis. Actin polymerization could generate force facilitating the pinch-out event. Abp1, cortactin are integrated in endocytosis via SH3 domain that is dynamin binding interface.

In this study, we demonstrate that SPIN90 plays a role in receptor-mediated endocytosis by impairing uptake of transferrin with overexpressing proline rich domain (PRD). SPIN90 associates with F-actin and colocalizes at cortical actin rich region and actin-membrane interface in PDGF treated cell. SPIN90 promotes actin reorganization like filopodia and it shows to localize to the tail of actin comet tail induced by phosphatidylinositol phosphate 5-kinases. These suggest that SPIN90 is supported by the link between endocytosis and actin cytoskeleton.

Materials and Methods

Yeast two-hybrid screening

Bait, a cDNA clone encoding spin90, was constructed into pGBKT7. Rat brain cDNA library in the pACT2, GAL4 AD vector was purchased from Clontech and amplified. Yeast two-hybrid screenings were performed according to the Clontech MATCHMAKER Two-Hybrid System manual, using lithium acetate (LiAc) method for preparing competent yeast cells.

GST pull down assay and co-immunoprecipitation

For GST pull-down assay, GST fused recombinant proteins were purified according to the manufacturer's protocol. Other constructs were translated in vitro using TNT T7-coupled reticulocyte lysate system. The [^{35}S] radiolabeled products were incubated with purified GST or GST fused proteins immobilized to glutathione-Sepharose beads. Bound proteins were subjected to SDS-PAGE, and were visualized by autoradiography. For coimmunoprecipitation assays, Lysates were immunoprecipitated using anti-syndapins or anti-SPIN90 antibodies as a probe and incubated for an additional 4h at 4℃ with protein A-sepharose bead. Immunoprecipitates were subjected to SDS-PAGE, and blots were incubated with anti-SPIN90 or anti-syndapins antibodies and were developed using ECL reagent.

Endocytosis assay (Transferrin uptake assay)

Transferrin uptake assays were performed after transfection. Cells were starved in serum-free medium for 2 h and 25μg/ml Alexa® 488-Transferrin or 25μg/ml Texas red-Transferrin were loaded for 15 min at 37°C. Images were acquired by using a Cool-SNAP^{TM}HQ CCD camera driven by

Metamorph. Fluorescence intensity quantitation was analyzed with IPLab.

Immunofluorescence

For immunofluorescence staining, cells were washed 3 times with PBS in 1mM CaCl2, 1mM MgCl2 and fix in 2% freshly paraformaldehyde for 10min. cells were permeabilized in 0.2% Triton X-100 for 10min. The slides were incubated with primary antibodies for 1h at room temperature and subsequently washed three times with PBS. Fluorophore-conjugated secondary antibodies were applied for 1h at room temperature. After three times washes, cells were mounted. Images were acquired by cooled CCD camera driven by MetaMorph Imaging software.

Results and Discussion

SPIN90 interacts with syndapin family *in vitro* and *in vivo* and endocytic proteins

We confirmed interaction between SPIN90 and YTH screening isolated protein, syndapin *in vitro* and *in vivo* using GST pull down assay or coimmunoprecipitation. Especially this interaction is due to their conserved domains that are proline-rich domain (PRD) of SPIN90 and src homology 3 (SH3) domain of syndapin. SPIN90 also associated with endocytic complex proteins such as clathrin, dynamin and vesicle associated membrane protein2 (Vamp2). These results showed that SPIN90 might be implicated in endocytosis pathway.

SPIN90 colocalizes syndapins in hippocampal neurons and COS-7 cells

To investigate subcellular localization of SPIN90 and syndapin, immunofluorescence assay was performed using affinity-purified anti-SPIN90 and anti-syndapin. SPIN90 immunoreactivity partialy overlapped with syndapin1 distribution at small punctuate structures in cell body, neurite, presynaptic nerve terminals and varicosities of hippocampal neurons. This immunoreactivity also partially colocalized with Vamp2 (synaptobrevin) as a synaptic marker. In COS-7 cells, SPIN90-positive dots were scattered throughout the cytoplasm or periphery, and colocalized syndapin II in cytoplasmic region and periphery region as like vesicular structures. These suggested that SPIN90 associated with syndapins, consistent with role for endocytosis pathway.

Interaction of SPIN90 integrates in receptor-mediated endocytosis via its PRD region

To demonstrate a correlation of interaction of SPIN90 protein in endocytosis, as suggested by sucellular distribution of SPIN90, we performed uptake assay using fluorescent (Texas Red or Alexa® flour-488) labeled transferrin in COS-7 cells transfected with HA-tagged SPN90 constructs. Overexpression of SPIN90 full-length blocked receptor-mediated endocytosis. Interestingly, SPIN90 PRD, binding interface of syndapin, highly impaired transferrin endocytosis. This result showed that the interaction of SPIN90 with syndapin functionally implicated in endocytosis pathway.

Syndapins rescue inhibition of endocytosis in SPIN90 PRD overexpressed COS-7 cells.

In order to examine whether the endocytosis block by overexpressing SPIN90 PRD construct was

caused by interruption of sydapins functions, we performed to rescue assay. While overexpression of SPIN90 PRD strongly blocked uptake of transferrin, endocytosis was restored in COS-7 cells co transfected with SPIN90 PRD and syndapins (syndapin I, syndapin II-l and syndapin II-s). Endocytosis levels were restored approximately to those observed in untransfected cells. These results suggested that interaction between SPIN90 and syndapin was implicated in endocytic function and both molecules were crucial factor for receptor-mediated endocytosis.

Depletion of endogenous SPIN90 lead to endocytosis impairments

We next asked whether it would be possible that interference with SPIN90 function also blocks endocytic uptake. We interrupted SPIN90 functions *in vivo* by introducing anti-SPIN90 antibodies into the cells using microinjection system (eppendorf). While Normal IgG injected cells did not appear any inhibition of endocytosis, introducing anti-SPIN90 antibodies caused a strong inhibition of transferrin uptake. These results represented that SPIN90 in cellular function took part in endocytosis.

SPIN90 over expression induce cortical actin reorganization

To examination of SPIN90 role in actin rearrangements, we transiently overexpressed SPIN90 constructs. Overexpression of SPIN90 in cells provoked dramatic change of cortical actin rearrangement. Cells transfected with SPIN90 full-length dramatically changed their morphology and induced microspike like filopodia. This overexpression phenotype was observed in transiently transfected cells, in contrast, untransfected cells showed a few short filopodia. Two part of SPIN90 domain were transiently transfected. Overexpression of N-terminal domain showed little filopodia induction as untransfected cells and accumulated in nucleus but overexpression of C-terminal domain showed strong induction of filopodia rather than those of untransfected cells. Thus these results suggested that C-terminal region of SPIN90 had a crucial role in actin dynamics.

SPIN90 colocalizes to F-actin at membrane interface and membrane ruffle in response to growth factors

SPIN90 immunoreactivity colocalized F-actin structure in the interface of actin-membrane. Only a partial colocalization was observed in the cortical areas. However GFP-SPIN90 highly accumulated at membrane ruffle area in response to PDGF and colocalized F-actin and localized to tip of filopodia in constant positive Cdc42 (Cdc42G12V) transfected cells. Thus, these results seemed that SPIN90 was localize in highly actin dynamics region and has an effect on actin reorganization.

SPIN90 induce cortical actin reorganization through the Arp2/3 complex

As above conclusion, overexpression of SPIN90 induces cortical actin rearrangement. Next, to demonstrate whether SPIN90 induced actin reorganization involved the Arp2/3 complex, HA-SPIN90 and GFP-Arp3 co transfected cells, visualized with an antibody, showed colocalization at dynamic actin reorganization site at cell cortex. Moreover, we confirm that SPIN90 also associated

with Arp2/3 complex using coimmunoprecipitation. This result suggests that actin rearrangements induced SPIN90 were mediated by the Arp2/3 complex.

SPIN90 is present at actin comet tail by overexpression of PIP5Kγ in living cells

Actin tail appears to begin at plasma-membrane site of pinosome formation and the machinery for propulsive actin polymerization is concentrated in ruffles, actin tails play a pivotal role in the motility of cell organelles. To investigate whether SPIN90 is present in actin comet tail in PIP5K overexpressed cells, we acquired time-laps imaging with living cells that were cotransfected with GFP-SPIN90 and PIP5Kγ. In time-laps imaging with living cells that were cotransfected with GFP-SPIN90 and PIP5K, Motile comet tail structures were observed. This seemed that SPIN90 is a component of actin comet tail, macropinosomes or vesicle traffic complex, consistent with our observation that SPIN90 functions to regulate actin-based vesicle motility.

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-precipitated with GST-Nck, βPIX and WASP, but not GST-β1 integrin. Subsequent co-immunoprecipitation analysis confirmed that SPIN90 does indeed associate with Nck, b1 integrin, βPIX, and WASP, suggesting that SPIN90 participates in β1 integrin mediated signaling via protein-protein interactions.

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. 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, the interaction between βPIX and Nck was also absent in suspended cells, but was strongly detected in replated cell. 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

ERK1was identified as binding to SPIN90 using yeast two-hybrid screening. GST pull-down and co-immunoprecipitation analysis confirmed that ERK1 bind to SPIN90 both *in vitro* and *in vivo*. Furthermore, consistent with earlier reports that ERK activity is regulated by cell adhesion. 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. Using *in vivo* labeling, we detected SPIN90 phosphorylation in both adherent and replated cells, but weak phosphorylation of SPIN90

was detected in detached or ERK inhibitor, PD98059,- treated cells. 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.

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. We found that activation of ERK1 by 20 ng/ml PDGF reached to the maximum within 10 min and decreased thereafter and that the interaction of SPIN90 and Nck followed the same time course. We found that AG1295, PDGFR antagonist, inhibited ERK1 activation by PDGF, as well as the interaction of SPIN90 and Nck. Likewise, 30uM PD98059 specifically inhibited both PDGF induced ERK1 activation and SPIN90-Nck interaction.

These findings clearly indicate that SPIN90 can be a substrate for ERK1 and that its phosphorylation by ERK1 likely modulates SPIN90-Nck interaction.

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