Identification of a Novel Interaction of 14-3-3 with p190RhoGEF*

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Activation of Rho GTPases by guanine nucleotide exchange factors (GEFs) mediates a broad range of cytoskeletal alterations that determine cell shape. In the nervous system, Rho GTPases are essential for establishing highly asymmetrical neuronal forms and may fine-tune the shape of dendrites in differentiated neurons. p190RhoGEF is a brain-enriched, RhoA-specific GEF whose highly interactive C-terminal domain provides potential linkage to multiple pathways in the cell. In the present study, a yeast two-hybrid screen was used to identify 14-3-3η and 14-3-3ε as additional binding partners of p190RhoGEF. Interactions between p190RhoGEF and 14-3-3η were confirmed biochemically and by colocalization of the respective proteins when fused to fluorescent markers and transfected in neuronal cells. We also mapped a unique phosphorylation-independent binding site (I1370QAIQNLI) in p190RhoGEF. Deletion of the binding site abolished interactions in vitro as well as the ability of 14-3-3η to alter the cytoplasmic aggregation of p190RhoGEF in cotransfected cells. The findings suggest a potential role for 14-3-3 in modulating p190RhoGEF activity or in linking p190RhoGEF to the activities of other pathways in the neuron.

Rho GTPases are a subgroup of Ras GTPases and include at least 14 different members (1). They are best characterized for their role in regulating the actin cytoskeleton but also partake in other activities, such as cell proliferation, gene expression, and apoptosis (2, 3). Like other small GTPases, they exhibit both GDP/GTP binding and GTPase activities, and their active states are mediated by cycling between GTP-bound active and GDP-bound inactive forms. Regulation of the cycle is achieved through the opposing effects of three different classes of protein: 1) GTPase-activating proteins, 2) guanine nucleotide exchange factors (GEFs), and 3) guanine nucleotide dissociation inhibitors. Determination of GDP-bound or GTP-bound forms of Rho proteins is linked to other pathways by interactions of regulatory proteins with other components in the cell.

RhoGEFs activate Rho GTPases by catalyzing the exchange of GDP with GTP at the nucleotide binding site. More than 35 RhoGEFs have been identified (1), all containing the signature tandem array of Dbl homology and pleckstrin homology domains responsible for nucleotide exchange activity. RhoGEFs are large proteins with additional functional domains that modulate nucleotide exchange activities or link the RhoGEFs to other pathways in the cell (4–8). Diverse domains on RhoGEFs have evolved in support of varying Rho GTPase activities in different tissues and cells.

p190RhoGEF is a neuron-enriched Rho-specific GDP/GTP exchange factor that activates RhoA in vitro as well as in neuronal cells (9, 10). Upstream of its Dbl homology/pleckstrin homology domain, p190RhoGEF contains a leucine-rich domain and a cysteine-rich zinc finger-like motif, which are dispensable for the catalytic activity of RhoGEF in vivo (9). In addition, p190RhoGEF contains a predicted α-helical coiled-coil motif in the C-terminal region of the molecule. This C-terminal region was recently shown to interact directly with microtubules (10) and c-Jun N-terminal kinase-interacting protein-1 (11) as well as with a destabilizing element in the 3′-untranslated region of the light neurofilament (NF-L) mRNA (12). The highly interactive properties of the C-terminal region of p190RhoGEF are of particular interest in light of their potential involvement in different regulatory processes in the cell.

In this study, we have further explored the interactive properties of C-terminal domain of p190RhoGEF using a two-hybrid screen. We now report the specific binding of 14-3-3 to the C-terminal region of p190RhoGEF. We further show that binding of 14-3-3 is isofrom-specific and that the binding site in p190RhoGEF does not conform to previously reported 14-3-3 binding motifs. We also demonstrate interaction between p190RhoGEF and 14-3-3 when fused to fluorescent proteins and transfected in neuronal cells. Interactions with 14-3-3 link p190RhoGEF with multiple pathways in the neuron and may be instrumental in modulating signal transduction phenomena in differentiated neurons.

EXPERIMENTAL PROCEDURES

DNA Constructs—A cDNA encoding amino acids (aa) 1276–1582 of p190RhoGEF was cloned into pBD-GAL4Cam (Stratagene) as pBD-GAL4Cam/p190RhoGEF-C. Full-length 14-3-3 isoform cDNAs were amplified by polymerase chain reaction from a mouse spinal cord library (Stratagene) and cloned into pGEX-6P-1 (Amersham Pharmacia Biotech), pcDNA3.1/Myc-His (Invitrogen), and pDsRed1-C1 (CLONTECH). HA-tagged p190RhoGEF and p190RhoGEF-C were obtained by inserting cDNAs into pHM6 (Roche Molecular Biochemicals). Point and deletion mutations of p190RhoGEF-C were constructed by polymerase chain reaction and the QuikChange Site-directed mutagenesis kit (Stratagene). All DNA constructs were verified by DNA sequencing.

Yeast Two-hybrid Analysis—Yeast two-hybrid screens were performed according to the manufacturer’s instructions (Stratagene). YRG-2 yeast strain expressing pBD-GAL4Cam/p190RhoGEF-C was transformed with pAD-GAL4Cam containing a murine embryonic day 12.5 spinal cord cDNA library (Stratagene). Transformants (1.4 × 10^5) were selected on synthetic dropout media minus leucine, tryptophan, and histidine and analyzed for β-galactosidase activity. cDNAs were rescued from positive yeast clones using XL1-Blue MRF strain of Escherichia coli. Specificity of protein-protein interactions was assessed by co-transforming selected clones with a pBD-GAL4Cam vector lacking the cDNA insert or pBD-GAL4Cam containing a lamin C cDNA.

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§ The abbreviations used are: GEF, guanine nucleotide exchange factor; aa, amino acids; HA, hemagglutinin; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein.
Biochemical interactions between p190RhoGEF and 14-3-3 binding sites of p190RhoGEF-C, we conducted a series of GST pull-down assays using immobilized 14-3-3, which showed that both GST and GST/14-3-3 fusion proteins interacted with p190RhoGEF-C. These results were consistent with the in vivo binding data obtained in yeast two-hybrid experiments.

To map the 14-3-3 binding site in the C-terminal Domain of p190RhoGEF, we conducted a series of GST pull-down assays using immobilized 14-3-3, which showed that both GST and GST/14-3-3 fusion proteins interacted with p190RhoGEF-C. These results were consistent with the in vivo binding data obtained in yeast two-hybrid experiments.

Localization of the Binding Site of 14-3-3 in the C-terminal Domain of p190RhoGEF—To map the 14-3-3 binding site in yeast, we used a series of GST pull-down assays using immobilized 14-3-3, which showed that both GST and GST/14-3-3 fusion proteins interacted with p190RhoGEF-C. These results were consistent with the in vivo binding data obtained in yeast two-hybrid experiments.

Fig. 1. Interaction between p190RhoGEF and 14-3-3 in yeast two-hybrid screen. A, schematic representation of full-length p190RhoGEF protein and the region (aa 1276–1582) used in the yeast two-hybrid screen (p190RhoGEF-C). B, growth in –Leu/-Trp/-His media (upper panel) and -galactosidase activity (lower panel graph) of yeast cotransformed with pBD-GAL4Cam/14-3-3 and empty pBD-GAL4Cam vector (lane 1), pBD-GAL4Cam/LaminC (lane 2), or pBD-GAL4Cam/p190RhoGEF-C (lane 3) in yeast two-hybrid screens. The results are representative of three separate transformation experiments.

Results

Binding of 14-3-3 to the C-terminal Domain of p190RhoGEF—The C-terminal region of p190RhoGEF was fused in-frame to pBD-GAL4Cam (pBD-GAL4Cam/p190RhoGEF-C) and used as bait to screen a mouse embryonic spinal cord cDNA library (Fig. 1A). Transformants were selected for growth on media lacking leucine, tryptophan, and histidine. Specific protein-protein interactions were confirmed by co-transforming positive clones with empty two-hybrid bait vector or bait vector containing lamin C or p190RhoGEF-C. Six 14-3-3-3p clones and one 14-3-3-3clone were identified by screening 1.4 x 10^6 clones. Yeast co-expressing 14-3-3 and p190RhoGEF-C grew on media minus leucine, tryptophan, and histidine and selected expression vector containing pBD-GAL4Cam/p190RhoGEF-C (Fig. 1B, lane 3) but not when yeast were cotransformed with 14-3-3 and an empty bait vector (Fig. 1B, lane 1) or bait vector containing lamin C (Fig. 1B, lane 2). The findings indicated specific interaction of p190RhoGEF-C with 14-3-3.

Biochemical interactions between p190RhoGEF and 14-3-3 were then tested by GST pull-down assay. Lysates of Neuro 2a cells transfected with HA-tagged p190RhoGEF-C were incubated with GST or GST/14-3-3 fusion proteins. p190RhoGEF-C bound to immobilized GST/14-3-3 and GST/14-3-3 fusion proteins but not to GST protein when lysate proteins were eluted from immobilized GST proteins and immunoblotted with anti-14-3-3 (Fig. 2A). The same GST pull-down assay showed that p190RhoGEF-C also bound to immobilized β and γ but not to ζ or τ isoforms of 14-3-3 (Fig. 2B).

To assess the interaction of p190RhoGEF protein with endogenous 14-3-3, lysates were obtained from cells transfected with HA-tagged p190RhoGEF-C and immunoblotted with anti-14-3-3. The immunoprecipitates were washed extensively and immunoblotted with anti-14-3-3. HA-tagged p190RhoGEF-C was readily detected in immunoprecipitates from Neuro 2a and P19 cells (Fig. 2C), indicating the presence of sufficient amount and form of 14-3-3 in both neuronal cell lines for interaction with p190RhoGEF-C.

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alter the binding of 14-3-3 to p190RhoGEF-C (Fig. 3D). We therefore concluded that binding of 14-3-3 to p190RhoGEF, localized to the sequence between aa 1353 and 1376, was not a phosphorylation-dependent interaction.

To further localize the 14-3-3 binding site, a series of internal deletions between aa 1353 and 1376 of p190RhoGEF-C were constructed (Fig. 3A). The GST pull-down assay showed that deleting aa 1366–1372 diminished the binding of p190RhoGEF-C to 14-3-3, whereas deleting aa 1370–1376 completely abolished the ability of p190RhoGEF-C to interact with 14-3-3 (Fig. 3E). Point mutations in the binding site showed that N1375A and L1376A replacements decreased the binding of 14-3-3 (Fig. 3F); however, no single amino acid mutation within this region eliminated binding of p190RhoGEF-C to 14-3-3. Our findings establish a novel 14-3-3 binding site comprised of uncharged polar and nonpolar residues (I1370QAIQNL) between aa 1353 and 1376.

The immunoprecipitates (IP) were immunoblotted (IB) with anti-HA. Interactions of p190RhoGEF-C were tested by fusing the respective proteins to green (EGFP) and red (DsRed1) fluorescent proteins. HA-tagged p190RhoGEF-C was readily detected in lysates from Neuro 2a cells transfected with mutant HA-tagged p190RhoGEF-C/C2 (S1362A/S1365A); however, deletion of aa 1353–1376 completely abolished the ability of p190RhoGEF-C to interact with 14-3-3. Point mutations in the binding site showed that N1375A and L1376A replacements decreased the binding of 14-3-3 (Fig. 3F); however, no single amino acid mutation within this region eliminated binding of p190RhoGEF-C to 14-3-3. Our findings establish a novel 14-3-3 binding site comprised of uncharged polar and nonpolar residues (I1370QAIQNL) between aa 1353 and 1376.

Co-localization of p190RhoGEF and 14-3-3 proteins in transfected neuronal cells was investigated. In vitro interactions between p190RhoGEF and 14-3-3 were tested by fusing the respective proteins to green (EGFP) and red (DsRed1) fluorescent protein markers and coexpressing the fusion proteins in transfected cells. Confocal microscopy showed that DsRed1/14-3-3 has a fine punctate distribution in cytoplasm and nucleus when transfected alone (data not shown) but becomes redistributed when cotransfected with EGFP/p190RhoGEF-C (Fig. 4A–C). p190RhoGEF and 14-3-3 proteins led to a yellow fluorescence upon merging of the green and red fluorescent images (Fig. 4C) due to their co-localization in punctate aggregates in the cytoplasm of Neuro 2a cells. Fusion proteins were excluded from the nucleus.

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The cytoplasmic aggregation of EGFP/p190RhoGEF-C is due to the context of the full-length protein. The colocalization of DsRed1/14-3-3-η with both EGFP/p190RhoGEF-C and EGFP/p190RhoGEF indicates strong interactive properties of the respective fusion proteins. Further information on the nature of the interactions in vivo were probed by examining the effects on aggregation of fusion proteins by mutating the 14-3-3 binding site in the C-terminal domain of p190RhoGEF (see below).

Coexpression of 14-3-3 and p190RhoGEF Reduces but Does Not Eliminate the Cytoplasmic Aggregation of p190RhoGEF—The cytoplasmic aggregation of EGFP/p190RhoGEF-C is due to the highly interactive properties of the C-terminal domain of p190RhoGEF, and the reduced aggregations of EGFP/p190RhoGEF reflect the modulating effects of the full-length sequence. We used these attributes of the fusion proteins to assess the interactions between 14-3-3-η and p190RhoGEF in vivo and to determine whether the interactions could be altered by mutating the 14-3-3 binding site of p190RhoGEF. Parallel transfections of Neuro 2a cells were conducted in which EGFP/p190RhoGEF-C fusion protein with and without deletion of aa 1370–1376 were transfected alone or cotransfected with DsRed1/14-3-3-η. The extent to which cotransfection of DsRed1/14-3-3-η reduced the aggregation of EGFP/p190RhoGEF-C fusion proteins was quantitated over time. When transfected alone, both wild-type (EGFP/p190RhoGEF-C) and mutant (EGFP/p190RhoGEF-C(Δ1370–1376)) fusion proteins were localized in cytoplasmic aggregates (Fig. 5, A and C). The extent of cytoplasmic aggregation was more frequently observed with the mutant fusion protein. Cotransfection with DsRed1/14-3-3-η reduced the aggregation of wild-type EGFP/p190RhoGEF-C and increased the numbers of cells with diffuse cytoplasmic localization of fusion protein (compare Figs. 5, B and A). Cotransfection with DsRed1/14-3-3-η did not reduce the aggregation of the mutant EGFP/p190RhoGEF-C(Δ1370–1376) lacking the 14-3-3 binding site or increase the numbers of cells with diffusely localized fusion protein (compare Figs. 5, C and D). The ability of DsRed1/14-3-3-η to reduce the aggregation of wild-type EGFP/p190RhoGEF-C fusion protein, but not mutant EGFP/p190RhoGEF-C(Δ1370–1376) fusion protein, was a time-dependent phenomenon and became more apparent at later time points after cotransfection (Fig. 5, E and F).

A similar comparative cotransfection study was conducted using the EGFP marker protein fused to the full-length p190RhoGEF with and without deletion of aa 1370–1376. Low levels of cytoplasmic aggregation of wild-type EGFP/p190RhoGEF (Fig. 6A) was further reduced by cotransfecting DsRed1/14-3-3-η (Fig. 6B). Also, the number of cells with diffusely distributed fusion protein was slightly increased (compare Figs. 6, A and B). The extent of cytoplasmic aggregation of mutant EGFP/p190RhoGEF lacking the 14-3-3 binding site (EGFP/p190RhoGEF(Δ1370–1376)) was diminished by cotransfecting DsRed1/14-3-3-η (compare Figs. 6, C and D). Moreover, the cytoplasmic aggregates of full-length fusion protein were more apparent in cells transfected with the mutant fusion protein (compare Figs. 6, A/B and C/D). The effects of coexpression of DsRed1/14-3-3-η on aggregation of wild-type and mutant EGFP/p190RhoGEF fusion protein have been quantitated in Figs. 6, E and F.

We interpret the results of cotransfection experiments as indicating direct interactions between 14-3-3 and the C terminus of p190RhoGEF as well as some overlap in the 14-3-3 binding site with the self-interactive site(s) in p190RhoGEF. The ability of cotransfections to alter the aggregation of wild-type but not the mutant p190RhoGEF fusion proteins indicates a direct interaction mediated by the 14-3-3 binding site. Furthermore, the enhanced aggregation of mutant fusion protein is consistent the view that these changes may reflect interactions with endogenous 14-3-3 with the same binding site. Evidence of indirect interactions of 14-3-3 and p190RhoGEF was also observed, as suggested by the persistence of DsRed1/14-3-3-η fluorescence in cytoplasmic aggregates of mutant p190RhoGEF fusion proteins (data not shown). The nature of the indirect interaction was not further pursued.

**DISCUSSION**

This study has identified novel interactions between the 14-3-3 adapter protein and the C-terminal domain of p190RhoGEF. The interactions were initially observed using the yeast two-hybrid system and subsequently confirmed by in vitro studies using biochemical methods and by in vivo studies using fluorescent protein-tagged fusion proteins. Biochemical studies also characterized the specificity of p190RhoGEF-C binding to different 14-3-3 isoforms, including η, ε, β, and γ but not ζ or ζ' isoforms. Isoform binding specificities could modify binding affinities to a particular substrate upon dimerization of isoforms or facilitate interactions between diverse substrates upon heterodimerization of isoforms with differing binding properties (15). Such interactions may be particularly relevant in the nervous system where almost all 14-3-3 isoforms are expressed. In most cases, isoform specificities of the different

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2. R. Cañete-Soler, unpublished data.
ligands have not been examined. In the few reported instances, the patterns of binding and non-binding isoforms differ from those reported here (16–18). The unique pattern of 14-3-3 isoform binding to p190RhoGEF may relate to the novel 14-3-3 binding site in the C-terminal domain of p190RhoGEF. Whereas most 14-3-3 binding sites are dependent upon a specific phosphoserine residue within a serine-rich consensus binding motif (13, 14), the binding site in p190RhoGEF (I1370QAIQNL) does not contain a target residue for phosphorylation, and its binding properties are not affected by treatment with nonspecific phosphatase. Both phosphorylation-dependent and -independent binding of ligands to 14-3-3 are believed to occur at a conserved amphipathic groove, with apposing hydrophobic and charged surfaces in all 14-3-3 isoforms (19, 20). It seems likely that the polar and nonpolar residues in the p190RhoGEF binding site may be instrumental in binding to the charged and uncharged surfaces in the amphipathic groove, as disposed in the different isoforms.

Whereas deletion of the binding site in p190RhoGEF was able to abolish interaction with 14-3-3 in a GST pull-down assay and abrogated 14-3-3 effects on self-aggregation of EGFP/p190RhoGEF fusion proteins, deletion of the binding site did not prevent colocalization of DsRed1/14-3-3/EGFP/p190RhoGEF(C1370–1376) fusion protein in the cytoplasmic aggregates. The latter findings could be interpreted as indicating additional interactive sites on p190RhoGEF in vivo or the presence of wild-type endogenous p190RhoGEF in the cytoplasmic aggregates. Alternatively, the colocalization of DsRed1/14-3-3/EGFP/p190RhoGEF(C1370–1376) could reflect the highly interactive properties of the respective fusion protein and their binding to common components in the cell. The latter interpretation would favor the presence of 14-3-3 and p190RhoGEF in a multimolecular complex and could potentiate their respective interactions with the multiple binding partners already defined.

The ability of DsRed1/14-3-3 to reduce the aggregation of EGFP/p190RhoGEF-C indicates that binding of 14-3-3 is able to interfere with self-aggregation sites in the C-terminal domain of p190RhoGEF. The presence of endogenous 14-3-3 may therefore serve to maintain the exchange factor in a non-aggregative state. Indeed, the self-aggregative properties of C-terminal domain of p190RhoGEF are reduced in the context of the full-length protein. In cotransfected cells, the full-length EGFP/p190RhoGEF has a diffuse cytoplasmic distribution with focal submembranous accumulations, not unlike the immunofluorescence localization of endogenous p190RhoGEF (10).

It is noteworthy that the interactions between 14-3-3 and
p190RhoGEF caused a redistribution of fusion proteins in the cytoplasm but did not alter the exclusion of EGFP/p190RhoGEF-C and EGFP/p190RhoGEF from the nucleus. The findings indicate that binding to 14-3-3 does not affect sites in p190RhoGEF governing its lack of nuclear transport, as noted with other 14-3-3 ligands (21). This may be relevant not only in view of the likely considerable excess of endogenous 14-3-3, but also apparent when visualized by red DsRed1/14-3-3 fluorescence (not shown). E–F, quantitation of aggregation of EGFP/p190RhoGEF (E) or EGFP/p190RhoGEF/Δ1370–1376 (F) fusion proteins when transfected alone or cotransfected with DsRed1/14-3-3 at 45 h after transfection. Reduction of cytoplasmic aggregation was also apparent when visualized by red DsRed1/14-3-3 fluorescence (not shown).

FIG. 6. Effects of deleting the 14-3-3 binding site in full-length p190RhoGEF (p190RhoGEF(Δ1370–1376)) on the ability of 14-3-3 to alter cytoplasmic aggregations of p190RhoGEF. A–D, Neuro 2a cells were transfected with wild-type (p190RhoGEF) or mutant (p190RhoGEF(Δ1370–1376)) EGFP fusion proteins alone (A and C) or cotransfected with DsRed1/14-3-3 (B and D). Cotransfection with DsRed1/14-3-3 reduced the cytoplasmic aggregation (arrowheads) of EGFP/p190RhoGEF (compare A and B) but not the aggregation of EGFP/p190RhoGEF(Δ1370–1376) (compare C and D) at 45 h after transfection. Scale bar, 50 μm.

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