The Ras/p120 GTPase-activating Protein (GAP) Interaction Is Regulated by the p120 GAP Pleckstrin Homology Domain*

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Pleckstrin homology domains are structurally conserved functional domains that can undergo both protein/protein and protein/lipid interactions. Pleckstrin homology domains can mediate intra- and inter-molecular binding events to regulate enzyme activity. They occur in numerous proteins including many that interact with Ras superfamily members, such as p120 GAP. The pleckstrin homology domain of p120 GAP is located in the NH₂-terminal, noncatalytic region of p120 GAP. Overexpression of the noncatalytic domains of p120 GAP may modulate Ras signal transduction pathways. Here, we demonstrate that expression of the isolated pleckstrin homology domain of p120 GAP specifically inhibits Ras-mediated signaling and transformation but not normal cellular growth. Furthermore, we show that the pleckstrin homology domain binds the catalytic domain of p120 GAP and interferes with the Ras/GAP interaction. Thus, we suggest that the pleckstrin homology domain of p120 GAP may specifically regulate the interaction of Ras with p120 GAP via competitive intramolecular binding.

PH domains are short (approximately 100 amino acid) motifs found in many proteins involved in signal transduction (1, 2). They may mediate protein/protein as well as protein/lipid interactions (3–7). Perhaps the best characterized role of these domains is the mediation of membrane localization (8–10). PH domains also may assist in regulating enzyme activity by modulating intramolecular interactions (11). They are commonly found in molecules that associate with GTP-binding proteins (12). Thus, PH domains may influence the regulation of both heterotrimeric G proteins and small GTPases of the Ras superfamily.

p120 GAP is a negative regulator of Ras, catalyzing the formation of inactive Ras-GDP from active Ras-GTP by stimulating the intrinsic GTPase activity of Ras (13). However, p120 GAP may play a role in mediating Ras effector function (14, 15). This hypothesis is based largely on the observation that overexpressing sequences from the NH₂-terminal region of p120 GAP (N-GAP), excluding the Ras-interacting catalytic domain, may modulate Ras signaling (16–21). Such dual roles as a negative regulator/positive effector have been previously observed for GAPs acting on heterotrimeric G proteins (22) and the Rho family GAP, IQGAP (23).

N-GAP contains a number of domains that are able to mediate protein/protein interactions, including two SH2 domains, an SH3 domain, and a PH domain (13). We hypothesized that the inhibition of Ras signaling and transformation by N-GAP might be due to a competition between the protein-interaction modules in N-GAP and those same domains in the NH₂-terminal region of full-length p120 GAP for proteins that mediate Ras effector function. If this hypothesis is correct, fragments of N-GAP containing individual domains may also inhibit Ras transformation and signaling.

To determine whether the PH domain of p120 GAP is involved in modulating Ras signal transduction, we expressed the isolated p120 GAP-PH domain and examined its effects upon Ras transformation. We found that overexpressing this PH domain inhibits the ability of Ras to promote transformation in NIH 3T3 cells but does not inhibit the normal growth of the cells. The inhibition of transformation is specific to Ras, because the PH domain did not affect transformation induced by activated Raf, which functions downstream of Ras. The inhibition is specific to the PH domain of p120 GAP, because a heterologous PH domain from RasGRF/CDC25 had no effect. Furthermore, when we evaluated the effect of the PH domain expression on individual Ras signaling pathways, we found that the activation of the Raf/MAPK pathway by Ras was not inhibited but that activation of the JNK pathway was suppressed.

To determine a mechanism for these effects, we examined the binding properties of the p120 GAP PH domain. We found that although the PH domain would not bind Raf-1, it would bind to p120 GAP itself. The binding mapped to the COOH-terminal catalytic domain of p120 GAP, the region that binds Ras. Moreover, the binding of the PH domain and Ras to the catalytic domain of p120 GAP was found to be mutually exclusive.

Thus, the PH domain of p120 GAP can mediate an intramolecular interaction and serve to regulate the binding of Ras and.
p120 GAP. By preventing the interaction of p120 GAP and activated Ras, the isolated PH domain may inhibit Ras-mediated transformation by blocking p120 GAP-dependent signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Generation of PH Domain Expression Constructs—**PH domains of p120 GAP and RasGRF/CDC25 were polymerase chain reaction amplified from the full-length cDNA (p120 GAP residues 471–591; RasGRF/CDC25 residues, 1–144 (generous gift from L. Quilliam, Indiana University)), sequenced, and cloned into the mammalian expression vector pZip-Neo SV(X)1 (24). A fragment containing the COOH-terminal subregion of the PH domain of p120 GAP was cloned in a similar manner (cPH; p120 GAP residues 523–591). PH domain fragments were cloned in the pCGN-Hyg (25) for transient expression in COS-7 cells.

**Cell Culture Assays—**NIH 3T3 cells were grown in 10% calf serum at 10% CO2. To generate cell lines, the cells were transfected with 100 ng of pZip-Neo construct using the calcium phosphate method as described previously (26). Cells were selected in 0.5 mg/ml G418 (Life Technologies, Inc.). For focus assay co-transfections, cells were transfected in a similar manner using 50 ng/dish of activated Ha-Ras(12V) or 100 ng of activated Raf (27) with 2 μg/dish of the appropriate PH domain construct. Foci were scored after 16 days using an inverted microscope. Ras transfections generated 50–100 foci/dish, Raf transfections generated 20–40 foci/dish. MAPK and JNK activation assays were performed in COS-7 cells as described previously (18). Essentially, HA-MAPK or FLAG-JNK were co-transfected transiently into COS-7 cells with Ras and the PH domain constructs using LipofectAMINE. After 48 h the cells were transferred to 0.5% serum and incubated overnight. The cells were lysed, and the inhibitor kinase was immunoprecipitated with the appropriate epitope tag antibody. The levels of Ras, JNK, and MAPK proteins were examined by Western blot to ensure uniformity before assaying the kinases’ activities upon GST-Jun or myelin basic protein. Expression of the HA epitope-tagged PH domain fragment in COS cells was confirmed by Western analysis using anti-HA antiseraum (BAbCo).

**Recombinant Protein Production—**The GAP-PH domain sequences described above were cloned into the GST fusion vector pGEX2T. The RasGRF/CDC25 PH domain was generously supplied as a GST fusion construct by R. Letkowitz (28). These constructs were grown in XL1 Blue bacteria to an optical density (600 nm) of 1, and induced with 0.5 mM isopropyl-β-thiogalactopyranoside for 1 h at 20 °C. The bacteria were pelleted and lysed by treatment with lysozyme followed by three cycles of freezing/thawing in phosphate-buffered saline plus 20 mM EDTA. The lysate was then made up to 1% Triton X-100, 500 mM NaCl, and 10 mM diethiotheritol before shearing by repeated passage through a 21-gauge needle. The lysate was then clarified by centrifugation and mixed with GSH-agarose beads for 4 h at 4 °C. The beads were then washed with phosphate-buffered saline. Recombinant Raf-1 protein was generated by co-infecting SF-9 cells with Raf-1 recombinant baculovirus (generous gift, J. Strom, Glaxo-Wellcome) and isolated by preparing a crude lysate as described previously (29).

Full-length and cGAP protein was supplied as a generous gift by G. Bollag (Onyx Pharmaceuticals). GST-Rap1a was prepared as described previously (30). F. Tamanoi provided the GST fusion construct of the catalytic domain of NFI, NFI-GRD. The protein was expressed and purified as described elsewhere (31), with the exception that the induc- tion period lysate was then made up to 1% Triton X-100, 500 mM NaCl, and 10 mM diethioetheritol before shearing by repeated passage through a 21-gauge needle. The lysate was then clarified by centrifugation and mixed with GSH-agarose beads for 4 h at 4 °C. The beads were then washed with phosphate-buffered saline. Recombinant Raf-1 protein was generated by co-infecting SF-9 cells with Raf-1 recombinant baculovirus (generous gift, J. Strom, Glaxo-Wellcome) and isolated by preparing a crude lysate as described previously (29).

**Protein Binding Assays—**GST and PH domain expression constructs were co-transfected with both activated Ras and p120 GAP-PH domain expression constructs. A heterologous PH domain from RasGRF/CDC25 was used as a negative control. Foci were scored after 16 days. Both the PH domain of p120 GAP and a COOH-terminal fragment (cPH) that lacks the main lipid binding regions (1) inhibited Ras transformation. The PH domain of RasGRF/CDC25 had no such inhibitory effect; thus, the inhibition is specific to the PH domain of p120 GAP. We also performed similar co-transfection experiments with a Raf-1 oncogene activated by truncation (Raf(22W)) (27), because Raf functions downstream of Ras. The PH domain of p120 GAP did not inhibit Raf transformation (Fig. 1). Thus, the inhibition of transformation was specific to Ras and specific to the p120 GAP-PH domain. As a further control, we examined the effects of the isolated 3’ SH2 domain of p120 GAP. We found that although this protein fragment inhibits Ras-mediated transformation, it also blocks Raf transformation, which the PH domain does not (Fig. 1).

**RESULTS**

**Overexpression of the GAP-PH Domain Specifically Inhibits Ras but not Raf Transformation in NIH 3T3 Cells—**To examine the role of the p120 GAP-PH domain in Ras-mediated signal transduction, NIH 3T3 cells were co-transfected with both activated Ras and p120 GAP-PH domain expression constructs. A heterologous PH domain from RasGRF/CDC25 was used as a negative control. Foci were scored after 16 days. Both the PH domain of p120 GAP and a COOH-terminal fragment (cPH) that lacks the main lipid binding regions (1) inhibited Ras transformation. The PH domain of RasGRF/CDC25 had no such inhibitory effect; thus, the inhibition is specific to the PH domain of p120 GAP. We also performed similar co-transfection experiments with a Raf-1 oncogene activated by truncation (Raf(22W)) (27), because Raf functions downstream of Ras. The PH domain of p120 GAP did not inhibit Raf transformation (Fig. 1). Thus, the inhibition of transformation was specific to Ras and specific to the p120 GAP-PH domain. As a further control, we examined the effects of the isolated 3’ SH2 domain of p120 GAP. We found that although this protein fragment inhibits Ras-mediated transformation, it also blocks Raf transformation, which the PH domain does not (Fig. 1).

**Overexpression of the PH Domain of p120 GAP Does Not Alter the Growth Properties of NIH 3T3 Cells—**To ensure that the Ras inhibitory effects were not merely because of cell death induced by the overexpressed PH domain, the effects of the PH domain constructs on cell growth were examined. NIH 3T3 cells were transfected with the full-length and truncated PH domain expression constructs. No difference in the total number of G418-resistant colonies was observed upon selection.
The PH Domain of GAP Regulates Ras/GAP Binding

The PH Domain of p120 GAP Binds p120 GAP—A model for the mode of action of p120 GAP is that in the non-Ras bound state, p120 GAP is folded upon itself and is unfolded when Ras binds to the COOH-terminal catalytic region (21). In the unfolded state, the protein/protein interaction domains in the NH₂-terminal (SH2/SH3/PH) are exposed, allowing them to interact with the appropriate target protein(s). Such a model implies that intramolecular contacts between the NH₂-terminal and COOH-terminal regions of p120 GAP may play a role in regulating p120 GAP function. To determine whether such an interaction could be mediated by the PH domain, we prepared and purified a GST fusion protein of the PH domain of p120 GAP, immobilized it on GSH-coated agarose beads, and incubated the GST fusion protein (or GST alone) with recombinant, baculovirus-derived p120 GAP. After washing, we resolved the precipitated protein by SDS-polyacrylamide gel electrophoresis. Western blotting with a cGAP-specific polyclonal antibody (generous gift from J. Gibbs) was used to assay for p120 GAP binding. Fig. 4 shows that the GST-PH domain fusion protein precipitated the full-length p120 GAP protein. Thus, we have demonstrated that the PH domain of p120 GAP interacts with full-length p120 GAP.

Although the PH domain of p120 GAP inhibits Ras transformation, it does not inhibit Ras-mediated MAPK activation. This suggests that the interaction of the PH domain with Ras effectors is specific and does not involve Raf-1. To confirm this, we performed precipitation experiments as described above using the GST-PH fusion protein and recombinant Raf-1 protein. To visualize Raf-1, we used polyclonal anti-Raf-1 antiserum (C-12, Santa Cruz). The GST-PH domain did not bind Raf-1, although a GST fusion protein of Rap1a (loaded with the GTP analog GMPPCP) previously shown to be a binding partner for Raf-1 (36) did (Fig. 5).

The COOH Terminus of the PH Domain Binds to the COOH Terminus of p120 GAP—To localize the binding determinants, the experiment was repeated with cPH and cGAP. Fig. 6 shows that the cPH domain fusion protein could precipitate cGAP, demonstrating that the COOH-terminal region of the PH domain of p120 GAP binds the COOH terminus of p120 GAP.

Excess Ras Protein Inhibits the Ability of the PH Domain to Bind to cGAP—The binding of the PH domain to cGAP may represent a regulatory mechanism controlling the interaction between p120 GAP and Ras. If so, the binding of the PH domain and Ras to cGAP may be mutually exclusive. To examine this possibility, the GST fusion protein of the PH domain was incubated with cGAP in the presence of excess Ha-Ras (loaded with GMPPCP). Fig. 6 shows that in the presence of excess active Ha-Ras, the binding of the PH domain to cGAP is greatly reduced.

The PH Domain of p120 GAP Inhibits the Ability of cGAP, But Not the Catalytic Domain of NF1, to Stimulate Ras GTPase Activity—If Ras prevents the binding of the PH domain to cGAP, the presence of excess PH domain may inhibit the interaction of Ras with cGAP. The most sensitive assay for Ras/GAP interaction is to measure GAP activity on Ras. Thus, GAP assays were performed in the presence or absence of the GST-PH domain to determine whether excess PH domain protein would inhibit the ability of cGAP to catalyze Ras GTPase activity. Fig. 7A shows that the PH domain of p120 GAP is an effective antagonist of cGAP action on Ha-Ras. Included was a GST-N-Raf control protein, coding for Raf-1 residues 1–140. This protein fragment contains the NH₂-terminal Ras-binding domain of Raf-1, which binds the Ras effector domain but has no catalytic activity. Because N-Raf and cGAP both bind the Ras effector domain, N-Raf should bind Ha-Ras in competition with cGAP, hence inhibiting cGAP activity. We found the PH domain fusion protein to be as efficient as the N-Raf fusion protein at inhibiting the catalysis of Ha-Ras by cGAP.

To further examine the specificity of the PH domain/cGAP interaction, we performed a similar experiment with the catalytic domain of another Ras GAP, NF1. We did not detect any inhibition of the activity of NF1-GRD toward Ha-Ras, demonstrating that the PH domain of p120 GAP interacts only with p120 GAP catalytic domain and not that of NF1.

Inositol Lipids Inhibit the Association of the PH Domain with Full-length GAP—A variety of mitogenic lipids bind PH domains and regulate their interactions with target proteins (37, 38). To determine whether the PH/p120 GAP association might be influenced in this manner, we assayed the GST-PH fusion protein for binding to full-length p120 GAP in the presence and absence of inositol trisphosphate (3.2 μM) or inositol 4,5-bisphosphate (14.7 μM). The p120 GAP/GST-PH binding exper-
The PH Domain of GAP Regulates Ras/GAP Binding

PH domains are present in a diverse array of proteins, many of which are involved in mediating signaling and transformation (1). PH domains mediate protein/protein as well as protein/lipid interactions (37–40). Indeed, one role may be to allow phospholipids to control protein/protein interactions by regulating the protein-binding activity of the PH domain (11).

p120 GAP appears to mediate certain Ras functions that are essential for transformation (14). p120 GAP contains several protein-binding motifs, including an SH3 domain, two SH2 domains and a PH domain. Although binding partners have been identified for the SH2/SH3 domains, the role of the PH domain in p120 GAP function is not known.

We found that expression of the PH domain of p120 GAP could mimic the Ras antagonizing effects seen by expressing the complete NH₂-terminal domain of p120 GAP (19). Thus, the PH domain specifically inhibited Ras-mediated transformation of NIH 3T3 cells but not Raf-mediated transformation. This property is specific to the PH domain of p120 GAP, because the PH domain of RasGRF/CDC25 had no such effect. Interestingly, the expression of the PH domain had no effect on normal cell growth. This suggests that p120 GAP-mediated interactions may be important for transformation but not normal growth of NIH 3T3 cells.

The failure to inhibit cell growth or oncogenic Raf transformation suggests that the action of the isolated PH domain is to...
inhibit some non-Raf/MAPK pathway(s) activated by Ras. We used the JNK pathway as a measure of non-Raf/MAPK pathways. Comparison of the activation of the MAPK pathway and the JNK pathway by Ras in the presence of the PH domain of p120 GAP showed that the PH domain inhibited activation of JNK but not of MAPK. This indicates that p120 GAP plays a specific role in the Ras-mediated activation of the JNK pathway and possibly other non-MAPK pathways regulated by Ras.

To examine the mechanism behind the PH domain-mediated inhibition of Ras transforming activity, we examined the binding properties of a GST-PH domain fusion protein. We found that this protein would not bind to Raf-1 but it did demonstrate stable binding to p120 GAP. Thus, the PH domain of p120 GAP appears to be capable of mediating an intramolecular binding event. We found that the residues responsible for this interaction lie in the COOH-terminal half of the PH domain and the COOH-terminal catalytic region of p120 GAP. Again, this interaction was specific to the PH domain of p120 GAP, because the PH domain from GRF/CDC25 did not interact with p120 GAP sequences (data not shown).

The binding of Ras and the PH domain to the catalytic region of p120 GAP was found to be mutually antagonistic. This suggests that the isolated PH domain, expressed in vivo may antagonize the interaction of Ras with p120 GAP. Furthermore, the binding sites for Ras and the PH domain on the catalytic domain of p120 GAP may overlap.

Bearing this in mind, it is interesting to note that the binding of Gα and the PH domain of βARK to the β subunit of heterotrimeric G proteins is also mutually exclusive. Moreover, the structure of the COOH-terminal region of PH domains and the
switch II region of Go, which is responsible for much of the binding between the α and β subunits, are structurally similar helices supported on a flexible loop (41). Thus, it is possible that isolated PH domains may antagonize signaling through heterotrimeric G proteins by binding to the same site as the Ga subunit, directly preventing the formation of the heterotrimeric complex (28, 42). This suggests that the competition between Ga and the βARK-PH domain for binding to the β subunit may represent a normal, regulatory process. Therefore, it may be possible to draw a parallel to the competition between Ras and the PH domain of p120 GAP for binding to the COOH terminus of p120 GAP. Moreover, this may be a general property of PH domains of proteins involved in G protein function.

In this model, the PH domain of p120 GAP binds to the catalytic domain of p120 GAP, keeping the protein folded upon itself. The binding of activated Ras to p120 GAP displaces the p120 GAP-PH domain from the catalytic region, exposing the NH₂ terminus of p120 GAP and allowing some signaling function. The binding of the isolated PH domain is insufficient to promote the full allosteric changes induced by Ras binding, and therefore the interaction is inhibitory rather than stimulatory. Because the binding of the PH domain to p120 GAP only requires the COOH-terminal half of the PH domain, the NH₂-terminal portion would be available for binding to lipids. These protein/lipid interactions might influence the intramolecular interactions of the COOH-terminal portion of the PH domain.

The ability of the isolated PH domain to inhibit the stimulation of Ras GTPase activity by p120 GAP might suggest that overexpressing the PH domain in cells would result in transformation. This would assume that in the absence of p120 GAP activity, the levels of Ras GTP would increase to the point of provoking transformation. However, as in p120 GAP knock-out cells, this does not occur (10). Presumably, this is due to the presence of other GAP molecules such as NFP1.

The inhibition of Ras transformation by the PH domain of p120 GAP is specific, because a heterologous PH domain from Ras GRF/CDC25 has no such effect. Thus, the Ras/p120 GAP interaction may play a key role in mediating the transforming activity of Ras. This is consistent with a variety of data suggesting that p120 GAP may play a subtle role in modulating Ras signaling through non-Raf/MAPK pathways (19, 43) and supports a dual role for p120 GAP as both a regulator and effector of Ras.

The ability of inositol lipids to inhibit the association of p120 GAP and its isolated PH domain adds a further level to the complexity of Ras regulation. One might envision a scheme where the activation of phosphatidylinositol 3-kinase by Ras results in the elevation of mitogenic lipids that then serve to inhibit intramolecular interactions between the PH and catalytic domains of p120 GAP. This would enhance the interaction of Ras and GAP, facilitating both a p120 GAP-activated signal and the down-regulation of Ras. PH domain-dependent inositol 4,5-bisphosphate-mediated increases in GAP activity have previously been reported for dynamin (6).

Thus, we now demonstrate a role for the PH domain of p120 GAP in directly regulating the interaction between Ras and the catalytic domain of p120 GAP. We suggest that this may be a more general mechanism whereby PH domain containing proteins modulate their interactions with small GTPases. Moreover, we present additional evidence for a role for p120 GAP in Ras-mediated signaling via non-Raf/MAPK effector pathways.

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