Morphological Changes and Detachment of Adherent Cells
Induced by p122, a GTPase-activating Protein for Rho*

(Received for publication, October 19, 1998, and in revised form, April 7, 1999)

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We recently cloned a novel signaling molecule, p122, that shows a GTPase-activating activity specific for Rho and the ability to enhance the phosphatidylinositol 4,5-bisphosphate-hydrolyzing activity of phospholipase C δ1 in vitro. Here we analyzed the in vivo function of p122. Microinjection of the GTPase-activating domain of p122 suppressed the formation of stress fibers and focal adhesions induced by lyso phosphatic acid, suggesting a GTPase-activating activity for Rho as in vitro. Transfection of p122 also induced the disassembly of stress fibers and the morphological rounding of various adherent cells. Analyses using deletion and point mutants demonstrated that the GTPase-activating domain of p122 is responsible for the morphological changes and detachment and that arginine residues at positions 668 and 710 and a lysine residue at position 706 in the GTPase-activating domain are essential. Using Fluo-3-based Ca2+ microscopy, we found that p122 evoked a rapid elevation of intracellular Ca2+ levels, suggesting that p122 stimulates the phosphatidylinositol 4,5-bisphosphate-hydrolyzing activity of phospholipase C δ1. These results demonstrate that p122 synergistically functions as a GTPase-activating protein specific for Rho and an activator of phospholipase C δ1 in vivo and induces morphological changes and detachment through cytoskeletal reorganization.

Recent studies have indicated a close association between the regulation of cytoskeletal assembly and phosphatidylinositol (PI)3 metabolism. That is, a number of PI 4,5-bisphosphate (PIP2)-binding proteins including gelsolin, cofilin, profilin, and α-actinin are known to bind to actin and regulate cytoskeletal assembly (1–4). In addition, Rho has been shown to enhance the activity of PI 4-phosphate 5-kinase, the PIP2-synthesizing enzyme (5), and the overexpression of PI 4-phosphate 5-kinase shows a GTPase-activating activity specific for Rho (6). Here we analyzed the in vivo function of p122. Microinjection of the GTPase-activating domain of p122 suppressed the formation of stress fibers and focal adhesions induced by lysophosphatic acid, suggesting a GTPase-activating activity for Rho as in vitro. Transfection of p122 also induced the disassembly of stress fibers and the morphological rounding of various adherent cells. Analyses using deletion and point mutants demonstrated that the GTPase-activating domain of p122 is responsible for the morphological changes and detachment and that arginine residues at positions 668 and 710 and a lysine residue at position 706 in the GTPase-activating domain are essential. Using Fluo-3-based Ca2+ microscopy, we found that p122 evoked a rapid elevation of intracellular Ca2+ levels, suggesting that p122 stimulates the phosphatidylinositol 4,5-bisphosphate-hydrolyzing activity of phospholipase C δ1. These results demonstrate that p122 synergistically functions as a GTPase-activating protein specific for Rho and an activator of phospholipase C δ1 in vivo and induces morphological changes and detachment through cytoskeletal reorganization.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Microinjection—Madin-Darby canine kidney (MDCK) cells, baby hamster kidney (BHK21) cells, and mouse Swiss 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and t-glutamine. For microinjection of proteins, 1 × 10^5 MDCK cells were seeded onto glass coverslips as described previously (14). After 4–7 days, the cells were washed extensively and starved in serum-free culture medium for 24–36 h. Recombinant proteins were microinjected at a concentration of 1 mg/ml along with 10 mg/ml FITC-dextran (average M, 20,000 Sigma) as a marker into the cytoplasm of the quiescent, serum-starved cells. For microinjection of plasmids, 1 × 10^8 MDCK cells were seeded onto glass coverslips and cultured for 1 day. Various plasmids were microinjected at a concentration of 0.1 mg/ml into the nuclei. Microinjection was performed using Eppendorf 5170 micromanipulator and 5246 microinjector (Eppendorf, Germany).

Expression and Purification of Recombinant Proteins for Microinjection—For construction of the N- and C-terminal recombinant p122 proteins, p122-N and p122-C, p122 cDNA was mutagenized by the polymerase chain reaction (PCR) to create an Ndel site at the starting ATG and codon 617 using the following primer: 5’TATctagaCATAT- GATCCCTAACCAAAATTTGAGCC-3’ and 5’AGGATGAAAAGGAT- CAAGGTTCCGAG-3’ (the XhoI site is indicated by lowercase letters, and the Ndel site is underlined), respectively. The PCR product encoding amino acid residues 1 to 533 was digested with Xbcl and EcoRV, subcloned into the corresponding sites of pBluescript SK (Stratagene, CA) and then sequenced. The plasmid was digested with Ndel and XhoI (which cuts in the multiple cloning sites of pBluescript) and subcloned into the Ndel-XhoI sites of a bacterial expression vector pET-15b.
p122 Acts as a RhoGAP and a Stimulator of PLC\(\delta\)I in Vivo

(Novagen, WI) to obtain the plasmid p122-N. For construction of the plasmid p122-C, the PCR product encoding amino acid residues 617 to 1083 was digested with NotI and BamHI (which cuts in the 3' untranslated segment of p122 cDNA), subcloned into the NotI-BamHI sites of pET-15b, and then confirmed by sequence analysis. The plasmids were routinely transformed into Escherichia coli BL21(DE3)pLysS to allow for the expression of (His)\(_6\)-tagged recombinant p122-N and p122-C proteins (Fig. 1A). The recombinant proteins were purified with a nickel-bound HiTrap chelating column (Amersham Pharmacia Biotech) and dialyzed against phosphate-buffered saline (PBS) for microinjection. The purified proteins were probed with a 1:1000 dilution of nickel-nitrilotriacetic acid horseradish peroxidase conjugate (Qiagen, CA) and visualized by enhanced chemiluminescence detection using an ECL kit (Amersham Pharmacia Biotech) (Fig. 1B).

Plasmid Construction and Mutagenesis—The various p122 constructs are summarized in Fig. 4A. To express a green fluorescent protein (GFP) fused to p122 in mammalian cells, a 5' XhoI site was introduced into the 5'-flanking sequence of the reading frame by PCR amplification using the primer 5'-AGAGCTCGAGACCATATGATC-3' (the XhoI site is underlined) and verified by sequencing. To construct pGFP-WT, the full-length p122 cDNA was digested with XhoI and KpnI (which cuts in the 3'-untranslated segment of p122 cDNA) and then inserted into the corresponding sites of the mammalian expression vector, pEGFP-C1 (CLONTECH, Palo Alto, CA), resulting in a fusion protein with GFP at the N terminus. Deletion derivatives of p122 were generated using the following restriction sites: XhoI and BamHI at positions 1–117 (pGFP-117AC), XhoI and EcoRI at positions 1–534 (pGFP-534AC), EcoRI and KpnI at positions 798–1083 (pGFP-789AN), and SalI and KpnI at positions 949–1083 (pGFP-949DN). For construction of pGFP-863AC, p122 cDNA was mutagenized by PCR to create a KpnI site at codon 864 using the primer 5'-CCGTTAATTCGTCAAGGGGCCGAGCAG-3' (the KpnI site is underlined). The PCR product encoding residues 1 to 863 was digested with XhoI and KpnI and then subcloned into the corresponding sites of pGFP-C1.

Nucleotide changes in the p122 cDNA were engineered by site-directed mutagenesis using a QuikChang mutagenesis kit (Stratagene). The mutant primers used to generate pGFP-R668E, pGFP-K706E, pGFP-R668E/K706E, pGFP-D997E, and pGFP-D997E/R668E were designed to replace the wild-type residues with corresponding residues in the RhoGAP domains of RhoGAP-1 and RhoGAP-2, respectively, and the mutant sequences were verified by DNA sequencing.

RESULTS

p122-C Inhibits Formation of Stress Fibers and Focal Adhesions—To address the physiological activities of the GAP domain of p122 in signaling pathways specific for Rho \textit{in vivo}, purified recombinant p122-C proteins encompassing the complete GAP domain (Fig. 1) were microinjected into Swiss 3T3 cells. In agreement with the previous results (14), removal of serum from the culture medium of Swiss 3T3 cells resulted in a reduction of both actin stress fibers and focal adhesions containing phosphotyrosine and vinculin, whereas lysophosphatidic acid (LPA) stimulation restored stress fibers and focal adhesions in the serum-starved cells. Using this system, we examined whether p122-C could inhibit LPA-stimulated changes in actin fiber organization and focal adhesions \textit{in vivo}. When cells were microinjected with p122-C and then treated with LPA for 10 min, the LPA-induced formation of stress fibers and focal adhesions was completely abolished as compared with un.injected cells (Fig. 2, A–F). In contrast, p122-C had no effect on platelet-derived growth factor-induced membrane ruffling, which is mediated by Rac proteins (Fig. 2, G and H).

Immunofluorescence Microscopy—Cells growing on glass coverslips or glass-bottomed dishes were rinsed in PBS, fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After washing three times with PBS, actin filaments were stained with tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin (Sigma) for 30 min at room temperature. To visualize focal adhesions, cells were incubated with a 1:500 dilution of antiphosphotyrosine antibody, 4G10 (Upstate Biotechnology), or a 1:100 dilution of anti-vinculin antibody (VIN-11-5, Sigma) for 1 h, washed three times with PBS, and then incubated for 30 min with a 1:100 dilution of rhodamine B-conjugated goat anti-mouse immunoglobulins (BIOSOURCE, Camarillo, CA). For visualization of myc-tagged Val14-RhoA, monoclonal antibody 9E10 was used as a primary antibody.

\(\text{Ca}^{2+}\) Imaging—MDCK cells were loaded with 5 \(\mu\text{M}\) fluo-3/AM (Dojindo, Japan) and 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR). A 250 mM stock solution of 20 mM salt solutions (20 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), and 13.8 mM glucose) for 20 min at 37 °C, incubated with the solution for 1 h at 37 °C, and then washed with PBS. The loaded cells were mounted on the stage of an inverted microscope and microinjected with recombinant p122-N or p122-C protein at a concentration of 1 mg/ml into the cytoplasm. Fluorescence images were obtained by excitation at 480 nm using xenon lamps. The emission signal at 520 nm was collected with a cooled charge-coupled device camera, and the digitized signals were stored and analyzed with Merlin digital imaging system (Olympus, Japan).

To examine the potential functions of whole p122 molecules in mammalian cells \textit{in vivo}, we constructed an expression vector, pGFP-WT, encoding a GFP fused to full-length p122. The plasmid was introduced into a variety of cell types, Swiss 3T3, MDCK, and BHK21, by liposome-mediated transfection. The p122-GFP fusion products were expressed in these cells and easily identified under fluorescence microscopy. After 24 h, all cells expressing p122 fusion proteins became...
p122 Acts as a RhoGAP and a Stimulator of PLC\(\delta1\) in Vivo

Profoundly rounded, refractile in cell shape, and left beaded dendritic process-like structures (i.e. protrusions) attached to the substrate (Fig. 3). In contrast, cells transfected with a plasmid encoding GFP alone displayed diffuse fluorescence throughout the cytoplasm and nucleus and exhibited a regular phenotype on morphologic examinations (data not shown). These results indicate that the p122 protein induces morphological rounding not only in fibroblasts but also in other adherent cell types.

The GAP Domain of p122 Is Responsible for the Morphological Changes and Actin Organization—Next, we constructed expression vectors encoding various p122 deletion mutants (Fig. 4A) and expressed the truncated proteins in BHK21 cells (Fig. 4B). The expression of pGFP-534AC (Fig. 4B, c), encoding the first 534 amino acids of p122, displayed diffuse staining throughout the cytoplasm and did not cause the morphological changes as seen with pGFP-117AC (data not shown). The expression of pGFP-958AN (Fig. 4B, i) and pGFP-949\(\Delta\)N (data not shown), encoding part of the C-terminal region of p122, also did not produce the changes. On the other hand, the expression of pGFP-863\(\Delta\)C and pGFP-617\(\Delta\)N (Fig. 4B, e and g) induced morphological rounding and detachment as shown with pGFP-WT (Fig. 4B, a). These results indicate that the GAP domain of p122 is necessary for the rounding and detachment of these cells.

Because these morphological alterations were likely to be accompanied by changes in actin organization, we visualized F-actin fibers by rhodamine-conjugated phalloidin staining. As shown in Fig. 4B, b, stress fibers were absent from cells expressing the p122-GFP fusion protein after 4 h of transfection. The expression of pGFP-863\(\Delta\)C (Fig. 4B, f) and pGFP-617\(\Delta\)N (Fig. 4B, h) induced the disassembly of stress fibers, whereas the expression of pGFP-534\(\Delta\)C and pGFP-798\(\Delta\)N did not affect actin organization (Fig. 4B, d and j). These observations indicate that the GAP domain of p122 regulates actin organization.

To further confirm the specificity of GAP, we used a constitutively active mutant of Rho, V14-RhoA. This mutant V14-RhoA causes a large decrease in intrinsic GTPase activity and is unresponsive to all GAP proteins. Because BHK21 cells are not suitable for nuclear injection of plasmids, we used MDCK cells and examined the effect of V14-RhoA on the morphological changes caused by p122. Microinjection of both pGFP-WT and pEXV-myc-VAL14-RhoA (kindly provided by A. Hall of University College London via S. Narumiya of Kyoto University) into MDCK cells resulted in a flat morphology (Fig. 5, A–C), whereas cells expressing p122 alone was prospectively rounded (Fig. 5, D–F). These results indicate that active V14-RhoA can neutralize the effect of p122 on the morphological changes, suggesting that p122 is a Rho-specific GAP.

Effect of Substitutions in the GAP Domain of p122 on Cell Morphology and Actin Organization—Crystal structure analyses of p50rhoGAP have suggested that Arg-85 and Asn-194 are important for binding to Rho proteins and enhancing GTPase activity (15). The positions of these amino acids correspond to Arg-668 and Asn-779 of p122 (Fig. 6B). Moreover, sequence alignment of the GAP domains of various GAPs reveals conserved lysine and arginine residues. Based on these findings, we replaced Arg-668 with Glu (R668E), Lys-706 with Glu (K706E), Arg-710 with Glu (R710E), or Asn-779 with Val (N779V) (Fig. 6A) and transfected these mutants independently into BHK21 cells. As shown in Fig. 7, A and B, cells expressing of R668E, K706E, or R710E underwent no morphological alterations, whereas cells expressing N779V showed an altered morphology and detachment. Furthermore, no obvious alterations of F-actin organization in R668E, K706E, or R710E-transfected cells as compared with nontransfected cells were detected (Fig. 7A, b, d, and f). In contrast, overexpression of N779V induced a loss of stress fibers (Fig. 7A, h). These results suggest that
p122 Acts as a RhoGAP and a Stimulator of PLCδ1 in Vivo

**DISCUSSION**

Here we demonstrated that the overexpression of p122 induces in adherent cells morphological changes and detachment from the substrate. The LPA-induced formation of stress fibers and focal adhesions is suppressed by microinjecting the GAP domain of p122 into cells, whereas platelet-derived growth factor-induced ruffle formation is not affected (Fig. 2). Cell rounding is also detected within 24 h after p122 transfection and the formation of stress fibers and focal adhesions is suppressed during the course of these changes. These changes induced by p122 are attenuated by cotransfection with the constitutive active mutant of Rho, V14-RhoA. We previously reported that p122 shows strong GAP activity for RhoA but not for Rac or Cdc42 in vitro (13). Considering that Rho controls the formation of stress fibers and focal adhesions rather than membrane ruffling and filopodia formation, it appears certain that p122 functions as a GAP for Rho in vivo and is involved in the

**Fig. 4.** Effect of p122 and its deletion mutants on morphological alterations and the organization of actin stress fibers. A, schematic representation of GFP fused to p122 and the individual mutants produced by eukaryotic expression vectors. Shaded boxes denote GFP regions, whereas solid boxes denote GAP domains. The numbers refer to the positions of amino acids in p122. Pluses and minuses are arbitrary designations to indicate the relative activity of the GFP fusion proteins in producing the morphological alterations. B, BHK21 cells were transiently transfected with plasmids pGFP-WT (a and b), pGFP-634ΔC (c and d), pGFP-668ΔC (e and f), pGFP-617ΔN (g and h), or pGFP-798ΔN (i and j). After 4 h (a and b) or 24 h (c–j), the cells were examined under fluorescence microscopy and photographed using a FITC-compatible filter (a, c, e, g, and i). In identical cell fields, F-actin was visualized with TRITC-phalloidin (b, d, f, h, and j). The bar represents 20 μm. WT, wild type.

**Fig. 5.** Effects of dominant-active V14-RhoA on p122-induced morphological alterations. MDCK cells were microinjected with plasmids pEXV-myc-VAL14-RhoA and pGFP-WT (A–C) or pGFP-WT (D–F) into the nucleus. After 24 h, cells expressing GFP fusion proteins were examined under fluorescence microscopy (A and D). V14-RhoA was stained with anti-myc antibody 9E10 followed by rhodamine B-conjugated goat anti-mouse antibody (B and E). Identical cell fields are shown under phase-contrast microscopy (C and F). The arrowheads indicate the microinjected cells. The bar represents 20 μm.
regulation of cytoskeletal disassembly.

Although stress fibers and focal adhesions have been widely studied in many types of cells, their regulations in vivo are not yet clear. The formation of focal adhesions is considered to be associated with integrin clustering, which is induced by exogenous stimuli such as formylpeptides and aggregation (16). This clustering is suppressed by a Rho inhibitor, C3 transferase (16). Stress fibers, in concert with focal adhesions, also regulate cell adhesion and motility. In MDCK cells, scatter factor/hepatocyte growth factor stimulates motility and spreading, whereas active Rho inhibits these reactions, presumably by promoting filament assembly and adhesion (17). These results indicate that p122 is involved in the regulation of cell adhesion and motility through reorganization of cytoskeletal components. In this context, the intracellular mechanisms underlying the regulation and localization of p122 are intriguing.

It is thought that GAPs stimulate the GTP hydrolysis of monomeric G proteins by supplying basic amino acid residues to the active center of G proteins, resulting in a stabilization of the increasing negative charge in the center (18, 19). Almost all GAPs contain two conserved arginine residues and one conserved lysine residue (Arg-668, Lys-706, and Arg-710 in p122), and mutations in any of these basic residues significantly reduce GAP activity (20). Moreover, the recently determined crystal structure of RhoA in its GDP-bound form with bound GTP S-domain, which formed a transition-state analogue, suggested that Arg-668 and Asn-194 to valine does not affect the activity of p122. These results indicate that residues Arg-668, Lys-706, and Arg-710 are essential for the function of p122 as a Rho-specific GAP in vivo, possibly by stabilizing the transition state of GTP hydrolysis. The importance of a charged residue at Arg-668 is also supported by Muller et al. (22). They demonstrated that mutation of arginine to methionine in the GAP domain of myr 5 (Arg-1695) corresponding to Arg-668 of p122 or Arg-85 of p50rhoGAP abolished the GAP activity both in vitro and in vivo (22). On the other hand, it is interesting that changing Asn-779 (equivalent to Asn-194 of p50) to valine does not affect the activity of p122, which is inconsistent with above crystal analysis. Asn-779 may not be essential for the biological activity of p122.

In addition to its Rho GAP activity, p122 is able to enhance the PIP2-hydrolyzing activity for PLC61 in vivo (13). Using the Ca2+-sensitive dye fluo-3, we demonstrate in this report that p122-C, but not p122-N, when injected into MDCK cells, leads to an increase in fluo-3 fluorescence. Therefore, the rise in Ca2+ sensitive dye fluo-3, we demonstrate in this report that p122-C, but not p122-N, when injected into MDCK cells, leads to an increase in fluo-3 fluorescence. Therefore, the rise in Ca2+ is consistent with the hypothesis that p122 leads to an increase in PLC61 activity, leading to increased hydrolysis of PIP2 and the generation of inositol 1,4,5-trisphosphate, which induces Ca2+ release (Fig. 8) (23, 24). In addition, it is also possible that the hydrolysis of PIP2 is enhanced by active PLC61, resulting in the release of actin-binding proteins from the membrane or PIP2 micelles. Free actin-binding proteins bind to G-actins, a process that accelerates the disassembly of actin fibers. These sequential reactions are quite similar to those induced by Rho GAPs. It is conceivable that the PLC61-enhancing activity of p122 acts synergistically with its GAP activity in causing the morphological changes and detachment. In fact, Tribioli et al. (25) previously reported that overexpression of p115, which is a protein with characteristics of a RhoGAP predominantly expressed in hematopoietic cells, in fibroblasts inhibited stress...
Fig. 8. Microscopic analysis of the intracellular Ca\textsuperscript{2+} increase in MDCK cells loaded with fluo-3. Cells microinjected with recombinant proteins, p122-N (A) or p122-C (B) are indicated by arrows. The gallery shows selected images from the time series at the indicated time after microinjection. The difference 20 s minus 0 s images (20-0) are shown at the right. The scale bar shows the fluorescence intensity map. The peak intensity in the center of the cell (20-0 in B) reflects the greater thickness in the central region of the cell and possibly the different fluorescent properties of the indicator, fluo-3, in the cytoplasm and the nucleus (30). C, time courses of fluorescence intensity marked with the area in microinjected cells (a and b) were shown. The arrow indicates the time at which recombinant proteins were microinjected. The data were collected in independent experiments (n = 11 for p122-N, n = 6 for p122-C).

Acknowledgments—We thank Dr. A. Hall for providing the eukaryotic expression vector, pEXV-myс-VAL14-RhoA, Dr. K. Matsuoka for providing Swiss 3T3 cells, H. Ohashi and J. Yamaki for technical assistance, and K. Suyama and S. Takano for photographic assistance.

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