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The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase

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The small GTP-binding protein Rho functions as a molecular switch in the formation of focal adhesions and stress fibers, cytokinesis and transcriptional activation. The biochemical mechanism underlying these actions remains unknown. Using a ligand overlay assay, we purified a 160 kDa platelet protein that bound specifically to GTP-bound Rho. This protein, p160, underwent autophosphorylation at its serine and threonine residues and showed the kinase activity to exogenous substrates. Both activities were enhanced by the addition of GTP-bound Rho. A cDNA encoding p160 coded for a 1354 amino acid protein. This protein has a Ser/Thr kinase domain in its N-terminus, followed by a coiled-coil structure ~600 amino acids long, and a cysteine-rich zinc finger-like motif and a pleckstrin homology region in the C-terminus. The N-terminus region including a kinase domain and a part of coiled-coil structure showed strong homology to myotonic dystrophy kinase over 500 residues. When co-expressed with RhoA in COS cells, p160 was co-precipitated with the expressed Rho and its kinase activity was activated, indicating that p160 can associate physically and functionally with Rho both *in vitro* and *in vivo*.

Keywords: coiled-coil structure/myotonic dystrophy kinase/protein kinase/Rho/small GTP-binding protein

Introduction

The Ras-related small GTP-binding proteins control a diverse array of cellular processes by shuttling between the inactive GDP-bound form and the active GTP-bound form. Among them, the Rho subfamily of proteins including Rho, Rac and Cdc42 regulate various forms of focal complexes and actin cytoskeletons. In fibroblasts, Rho regulates the formation of focal adhesions and stress fibers, Rac regulates growth factor-induced membrane ruffling and Cdc42 regulates filopodia formation (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kozma *et al.*, 1995; Nobes *et al.*, 1995). In this fashion, Rho controls the adhesion of fibroblasts to the substratum. Rho appears to act in a similar way in platelets and lymphocytes to regulate the avidity of cell surface integrins and to control their adhesion (Morii *et al.*, 1992; Tominaga *et al.*, 1993). Rho also plays a critical role in cytokinesis by inducing and maintaining the contractile ring, another important actin-based cytoskeletal structure (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993). In addition to the organization of these specific actin cytoskeletons, recent studies have shown that Rho is involved in the regulation of transcriptional activation by serum response factor (Hill *et al.*, 1995), in cell-cycle progression (Yamamoto *et al.*, 1993) and in cell transformation (Khosravi-Far *et al.*, 1995).

Although these actions of Rho are well known, the biochemical mechanism by which it evokes these effects remains unknown. We have previously demonstrated that lysophosphatidic acid (LPA)- or GTPγS-induced tyrosine phosphorylation of focal adhesion kinase and paxillin in cultured Swiss 3T3 fibroblasts was blocked by the inactivation of Rho by the treatment of the cells with botulinum C3 exoenzyme, and suggested that Rho is upstream of some tyrosine kinase(s) (Kumagai *et al.*, 1993; Seckl *et al.*, 1995). We also showed that several Ser/Thr kinases were activated in a Rho-dependent manner in Swiss 3T3 cells and rat 3Y1 fibroblasts (Kumagai *et al.*, 1995). These results indicate the involvement of protein kinases in Rho-mediated signal transduction. Consistent with this idea, Rho-induced formation of focal adhesions and stress fibers can be blocked by the kinase inhibitor genistein (Ridley and Hall, 1994). On the other hand, the activation of lipid kinases such as phosphatidylinositol-3-kinase (Zhang *et al.*, 1993) and phosphatidylinositol-4-phosphate 5-kinase (Chong *et al.*, 1994) by Rho has been reported in platelets and fibroblasts respectively. The latter activation has been suggested to control actin polymerization through localized increases in phosphatidylinositol-(4,5)bisphosphate (PIP_2) level (for review see Stossel, 1993). However, unlike Rac and Cdc42 (Zheng *et al.*, 1994; Tolias *et al.*, 1995), neither has been shown to associate directly with Rho in a GTP-dependent manner.

Using an *in vitro* binding assay, Manser *et al.* (1994) identified a Ser/Thr protein kinase designated as $p65^{PAK}$ that binds directly to the GTP-bound form of Rac and Cdc42 and is activated by this binding. We have employed a similar approach and identified a 160 kDa cytosolic protein that binds specifically to the GTP-bound form of Rho. Here we report that this protein is a novel Ser/Thr protein kinase containing a long coiled-coil structure, with strong homology to myotonic dystrophy kinase. Our study shows that this protein designated as $p160^{ROCK}$ (a Rho-associated, coiled-coil containing protein kinase) not only physically but also functionally associates with GTP-bound Rho both *in vitro* and *in vivo*.

Results

Identification and purification of p160 Rho-binding protein

In order to identify proteins that bound to the GTP-bound form of Rho, we performed a ligand overlay assay with

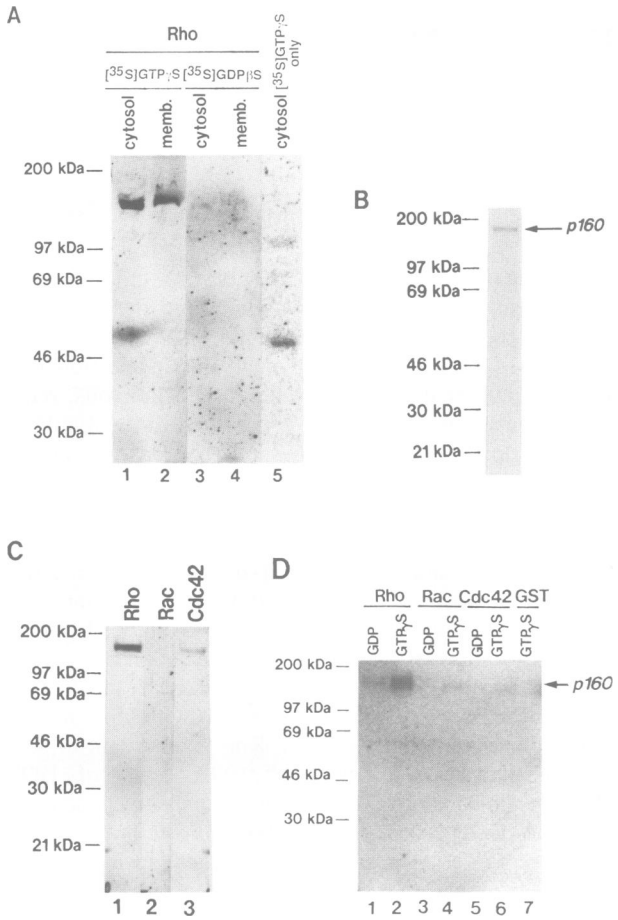

Fig. 1. (**A**) Identification of a Rho-binding protein in human blood platelets by the ligand overlay assay. Human blood platelets were homogenized, and the cytosolic and membrane fractions were separated by centrifugation. These fractions were subjected to a ligand overlay assay and were probed with either [^{35}S]GTPγS–Rho (lanes 1 and 2), [^{35}S]GDPβS–Rho (lanes 3 and 4) or [^{35}S]GTPγS alone (lane 5). Purification of p160 Rho-binding protein (**B**) and the specificity of the binding to the Rho subfamily of proteins (**C**). p160 was purified as described in Materials and methods. The final preparation was subjected to SDS–PAGE for silver staining (B), and for ligand overlay analysis (C) with [^{35}S]GTPγS-bound recombinant RhoA (lane 1), Rac1 (lane 2) and Cdc42 (lane 3). (**D**) Precipitation of the purified p160 by recombinant Rho-immobilized beads. Purified p160 was incubated with GTPγS- (lane 1) or GDP- (lane 2) bound GST–Rho, GTPγS- (lane 3) or GDP- (lane4) bound GST–Rac1, GTPγS- (lane 5) or GDP- (lane 6) bound GST–Cdc42, or with GST and GTPγS alone (lane 7), and precipitated by the addition of glutathione–Sepharose. The precipitated complex was then washed and subjected to the immunoblot analysis using anti-peptide antibody to p160 as described in Materials and methods.

recombinant Rho loaded with [^{35}S]GTPγS as a probe. Using this procedure, we identified a 160 kDa protein in human platelet homogenates that was labeled with this probe, and was distributed mainly in the cytosol (Figure 1A). No appreciable binding was seen with [^{35}S]GDPβS–Rho or [^{35}S]GTPγS alone (Figure 1). This protein also bound to the [^{35}S]GTPγS-bound form of native Rho purified from bovine adrenal glands (Morii *et al.*, 1988), and to Rho loaded with [α-^{32}P]GTP. The latter binding decreased with the hydrolysis of the bound nucleotide (data not shown). These results suggest that this protein, p160, binds specifically to the GTP-bound form of Rho.

p160 was purified from the platelet cytosol by sequential chromatography using DEAE–Sepharose, Red A–Sepharose, hydroxyapatite and Mono Q columns. As shown in Figure 1B, the final preparation showed a single protein band at a molecular weight of 160K on SDS–PAGE. About 10 μg of the purified p160 protein was obtained from a typical purification procedure of ~800 mg protein of the platelet cytosol. To determine its binding specificity among the Rho subfamily of proteins, the purified protein was renatured on a membrane and then overlaid with the same amount of [^{35}S]GTPγS-loaded recombinant RhoA, Rac1 or Cdc42. As shown in Figure 1C, p160 showed strong binding to RhoA and only weak binding to Cdc42, and almost no binding was observed for Rac, indicating that the binding of p160 was specific to Rho. We next examined if Rho and p160 could interact in solution. Purified p160 was incubated with either the GTPγS- or the GDP-bound form of GST–Rho, GST–Rac or GST–Cdc42, which was then precipitated with glutathione–Sepharose. As shown in Figure 1D, p160 was precipitated quantitatively by incubation with GTP-bound Rho, whereas no precipitation occurred with GDP-bound Rho. Little precipitation was observed with either Rac1 or Cdc42.

Identification of p160 as a protein serine/threonine kinase

Since our previous results indicated that some protein kinases lie downstream of Rho in the LPA signaling pathway (Kumagai *et al.*, 1993, 1995), we suspected that p160 might be a kinase. We examined this possibility by incubating purified p160 with [γ-^{32}P]ATP. As shown in Figure 2A, the ^{32}P radioactivity was incorporated into p160 in this incubation, and phosphoamino acid analysis revealed that [^{32}P]phosphate was incorporated into both serine and threonine but not to tyrosine residues (Figure 2B). The kinase activity of p160 was then examined by incubating purified p160 with several substrate proteins. As shown in Figure 2C, p160 phosphorylated both myelin basic protein (MBP) and histone. This kinase activity co-eluted precisely with the Rho-binding activity from the Mono Q column, suggesting that the kinase activity was associated with p160 itself (data not shown). We next examined the effects of GTP–Rho on the kinase activity of p160. Purified p160 was incubated with [^{32}P]ATP plus either histone, MBP or vehicle, in the presence of either GTPγS- or GDP-bound Rho. As shown in Figure 2D, p160 phosphorylated histone in a time dependent manner. This reaction was stimulated by ~2-fold in the presence of GTPγS–Rho. A similar magnitude of stimulation was also found for both autophosphorylation and MBP phosphorylation (data not shown). No stimulation was found with either Rac1 or Cdc42 (Figure 2D).

cDNA isolation and the predicted structure of p160

Purified p160 was subjected to peptide sequencing analysis, and seven partial amino acid sequences were obtained. Using a degenerate oligonucleotide corresponding to one of the sequences, AP23, as a probe in the initial screening and then using the obtained cDNA inserts sequentially as probes, four overlapping clones (designated as P2, N, 4N and C) were obtained from cDNA libraries prepared from human megakaryocytic leukemia MEG-01 cells (Figure 3A). The complete p160 cDNA sequence that was determined from these clones contained an open

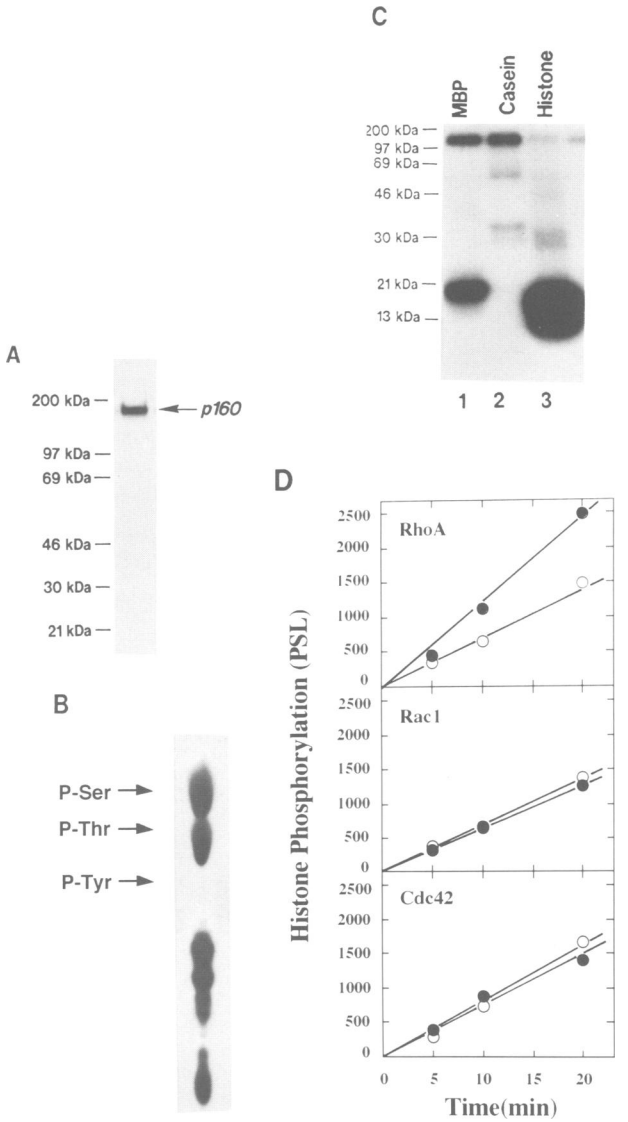

Fig. 2. (**A**) Autophosphorylation of p160. Purified p160 was incubated with [γ-^{32}P]ATP and then subjected to SDS–PAGE. An autoradiogram is shown. (**B**) Phosphoamino acid analysis. The [^{32}P]phosphorylated band of p160 was excised from the PVDF membrane and subjected to the analysis. P-Ser, P-Thr and P-Tyr denote the electrophoretic positions of phosphoserine, phosphothreonine and phosphotyrosine respectively. (**C**) Phosphorylation of MBP and histone by p160. Purified p160 was incubated with either MBP, casein or histone in the presence of [^{32}P]ATP, and the phosphorylation reaction was analyzed as described in Materials and methods. (**D**) Activation of histone phosphorylation by GTPγS-bound Rho. The reaction was carried out in the presence of GTPγS-bound (●) or GDP-bound (○) GST–Rho (upper panel), GTPγS-bound (●) or GDP-bound (○) GST–Rac1 (middle panel), GTPγS-bound (●) or GDP-bound (○) GST–Cdc42 (lower panel), and the radioactivity incorporated into histone was measured by a Bioimage Analyzer BAS2000, and expressed as PSL (photo-stimulated luminescence) values. Histone phosphorylation by reaction without recombinant Rho was not significantly different from that with GDP-bound Rho (data not shown).

reading frame of 4062 bp, and encoded a protein of 1354 amino acids with a calculated molecular weight of 158 166 Da (Figure 3B). This deduced amino acid sequence contained all seven partial amino acid sequences determined from the purified p160, suggesting that the cDNA encoded the purified protein. To further prove its identity, we expressed the p160 cDNA as a myc-tagged protein in cultured COS-7 cells, and used the cell lysates in the overlay assay. As shown in Figure 3C, [^{35}S]GTPγS–Rho binding was observed at M_r 160K in the lysates from the transfected cells but not mock-transfected cells, and this binding completely overlapped with the tag staining. Northern blot analysis revealed a 7.5 kb transcript that was highly expressed in the heart, lung, skeletal muscle, kidney and pancreas, and to a lesser extent in placenta and liver, and in only a trace amount in the brain. An additional 6.0 kb transcript was found in skeletal muscle and pancreas mRNA (Figure 3D).

A comparison of the predicted p160 protein sequence with other sequences in databases identified several domains within the molecule (Figure 4). A conserved protein kinase sequence was identified in the N-terminal 262 amino acids (amino acids 72–334), which is characteristic of protein serine/threonine kinase of the protein kinase A family (Hanks *et al.*, 1988). The sequence spanning this kinase domain (amino acids 1–420) showed strong homology to myotonic dystrophy kinase (MD-PK) (Brook *et al.*, 1992; Mahadevan *et al.*, 1993); 44% identity over 420 amino acids (Figure 4A). Following the kinase domain is a sequence of ~600 amino acids (amino acids 423–1096) that has homology to various coiled-coil proteins such as myosin heavy chain tails. An analysis using the algorithm developed by Lupas *et al.* (1991) revealed a high probability of forming a coiled-coil structure in this region (Figure 4B). MD-PK also has a coiled-coil structure (~100 amino acids long) following the kinase domain and this region showed significant homology to the corresponding region of p160; ~34% homology between amino acids 421–522 of p160 and amino acids 421–525 of MD-PK. The C-terminus part of p160 contains a pleckstrin homology (PH) domain (Musacchio *et al.*, 1993) and a cysteine-rich zinc finger motif (Figure 4C and D). The PH domain was split into two regions between subdomains 5 and 6 by the insertion of the cysteine-rich region. The Cys-rich region was homologous to the cysteine-rich domains of protein kinase C (Quest *et al.*, 1994; Zhang *et al.*, 1995), with a 50 amino acid stretch consisting of H-X_{12}-C-X_2-C-X_{15}-C-X_2-C-X_4-H-X_2-H-X_8-C (C5H3) (single letter codes, X denoting variable residues).

Association of p160 and Rho and the activation of kinase activity in vivo

To examine if p160 interacts with Rho *in vivo* in the cell, we expressed myc-tagged p160 with either Val^{14}- or wild type rhoA tagged with hemaglutinin epitope in cultured COS cells. Cell lysates were then prepared, and subjected to immunoprecipitation with a 9E10 anti-myc antibody. As shown in Figure 5A, this procedure recovered p160 quantitatively in the precipitates, and co-precipitated both the Val14 mutant and the wild type of expressed Rho with p160. The precipitation of Rho depended on both the expression of p160 and the use of the 9E10 antibody. The kinase activity of these precipitates was then examined using histone as a substrate. As shown in Figure 5B, the p160 precipitated from the lysates of the cells co-expressing either Val14 or wild type Rho showed ~2-fold higher phosphorylation than that from the control cells expressing p160 alone. These results indicate that p160 was stably associated with Rho *in vivo* in the cells and was activated by this association.

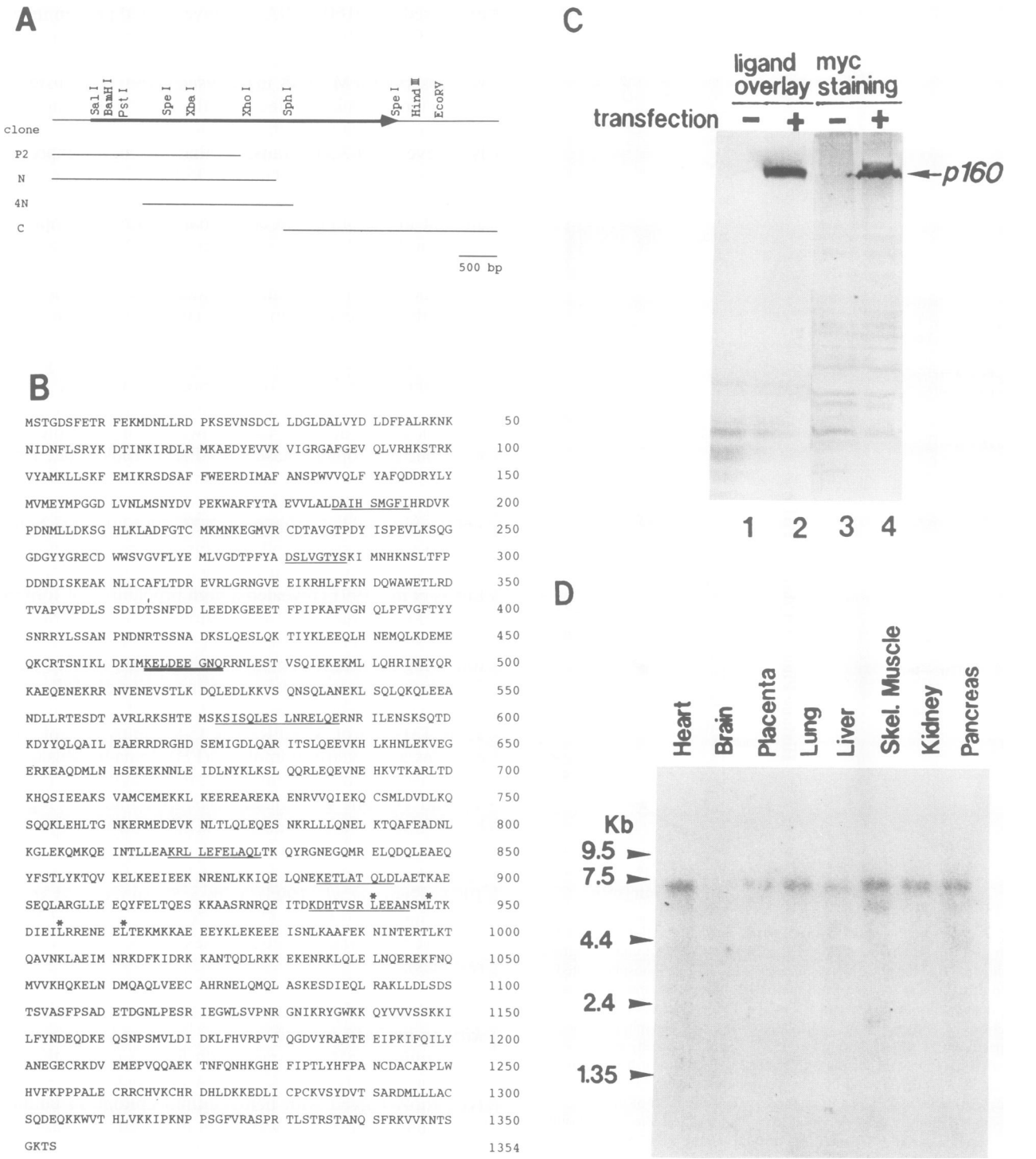

Fig. 3. (**A**) Schematic representation of the isolated p160 clones. Restriction enzyme sites on a composite cDNA are shown. The open reading frame is shown by a thick arrow. (**B**) Deduced amino acid sequence of p160. The sequence for the peptide AP-23 is double underlined, and other partial amino acid sequences are underlined. Leucine residues in a leucine zipper-like sequence are indicated by asterisks. The accession number of the nucleotide sequence in the GenBank™/EMBL Data Bank is U43195. (**C**) Rho-binding to the expressed protein. Lysates from the transfected (lanes 2 and 4) and mock-transfected (lanes 1 and 3) cells were used for the ligand overlay assay (lanes 1 and 2), and for immunostaining with the anti-myc antibody (lanes 3 and 4). (**D**) Northern blot analysis of p160 expression in various human tissues.

Discussion

p160^{ROCK} as a putative Rho target

In this study, a ligand overlay assay was used to identify a novel protein kinase, p160, as a Rho-binding protein. p160 is a protein Ser/Thr kinase that has multiple domains including a coiled-coil structure. On the basis of these features, we have designated p160 as a Rho-associated, coiled-coil containing protein kinase (ROCK). The findings that only the GTP-bound form of Rho binds to this

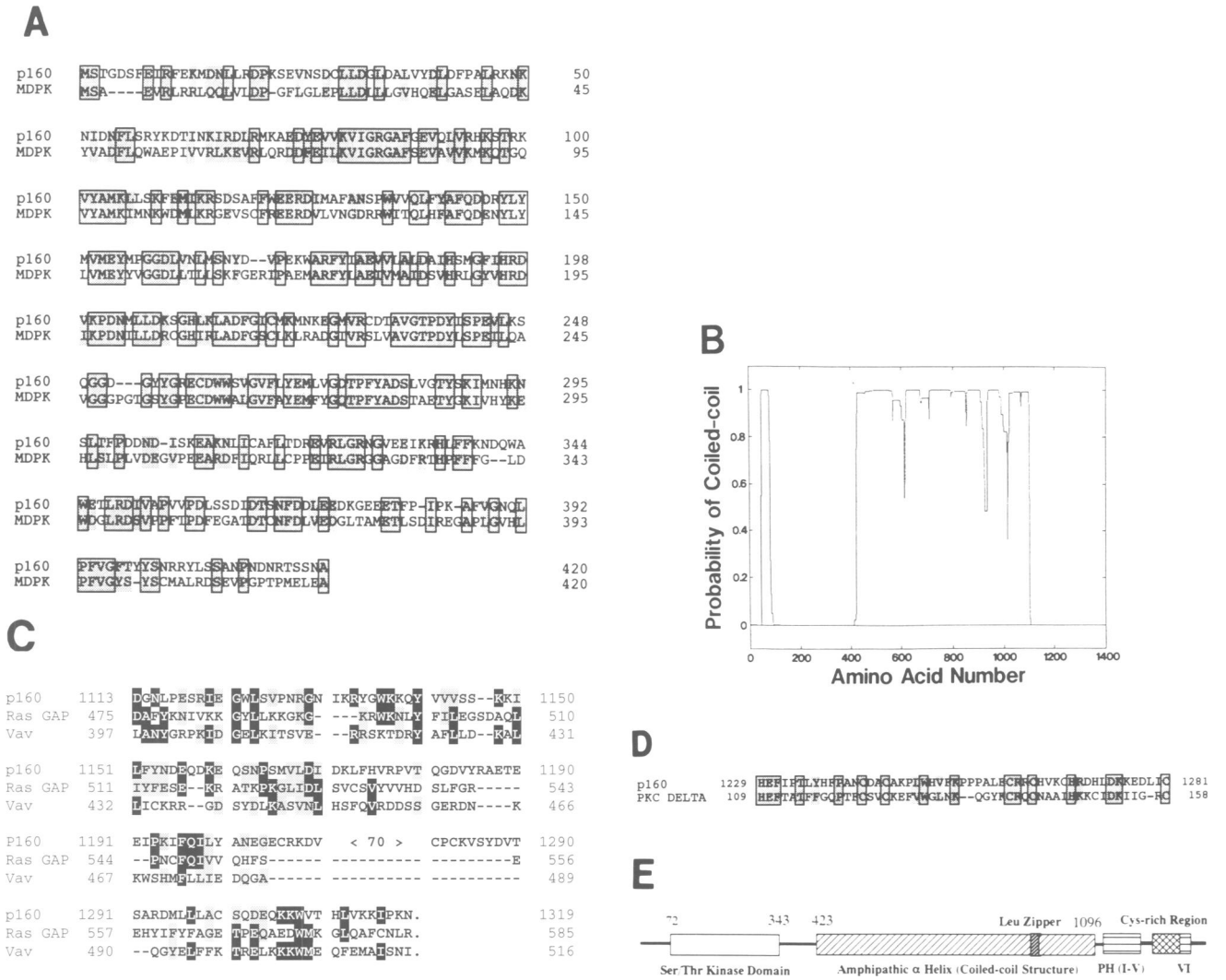

Fig. 4. Structure of p160. (**A**) Alignment of the kinase domain to myotonic dystrophy kinase (MD-PK, accession number L08835). Identical amino acids are boxed, and conservative replacement is shadowed. (**B**) Analysis of the coiled-coil region. The probability of forming a coiled-coil structure was calculated for each residue with a window of 28 amino acids by the method of Lupas *et al.* (1991). (**C**) Alignment of the PH domain. The PH domain of p160 was compared with Ras GTPase activating protein and Vav, according to Musacchio *et al.* (1993). Identical amino acids are shown by white letters in black, and conservative changes are shadowed. (**D**) Alignment of the Cys-rich region. The Cys-rich region of p160 was compared with the Cys-1 region of PKCδ (accession number Z22521). (**E**) A schematic representation of the structure of p160.

protein and activates its kinase activity *in vitro*, and that both association with and activation by Rho also occur *in vivo*, suggest that p160^{ROCK} is a good candidate for a downstream effector in the Rho signaling pathway. The comparable *in vivo* actions on p160 of wild type Rho and the Val14 mutant may indicate that the p160 binding to Rho suppressed its GTPase activity, as was observed for Cdc42 by p65^{PAK} (Manser *et al.*, 1994).

Possible role of protein kinase in Rho-mediated cellular processes

The involvement of protein kinases and protein phosphorylation in Rho-mediated cellular responses has been previously suggested. For example, LPA, which induces the formation of focal adhesions and the assembly of stress fibers in a Rho-dependent manner (Ridley and Hall, 1992), also activates several Ser/Thr kinases as well as a tyrosine kinase, p125^{FAK}, in a Rho-dependent manner (Kumagai *et al.*, 1993, 1995). Furthermore, Rho-induced formation of focal adhesions and stress fibers is inhibited by a kinase inhibitor genistein (Ridley and Hall, 1994). Involvement of a protein kinase(s) is also suggested for cytokinesis (Mabuchi and Takano-Ohmuro, 1990), for the increase in the calcium sensitivity of smooth muscle (Trinkle-Mulcahy *et al.*, 1995) and for the stimulus-evoked neurite retraction of cultured neuroblastoma cells (Jalink and Moolenaar, 1992), all of which are Rho dependent (Hirata *et al.*, 1992; Mabuchi *et al.*, 1993; Jalink *et al.*, 1994). More recently, Rho has been shown to regulate transcriptional activation by serum response factor (Hill *et al.*, 1995). This activation does not correlate with the activation of the MAP-kinases ERK, SAPK/JNK or MPK2/p38; hence, the existence of a novel Rho-mediated kinase cascade leading to SRF activation was postulated, analogous to the Ras/Raf/MAPK pathway leading to TCF activation (Hill and Treisman, 1995), or the Rac or Cdc42/PAK/JNK pathway leading to c-Jun activation (Coso *et al.*, 1995; Minden *et al.*, 1995). p160^{ROCK} may be involved in one or more of these cellular processes.

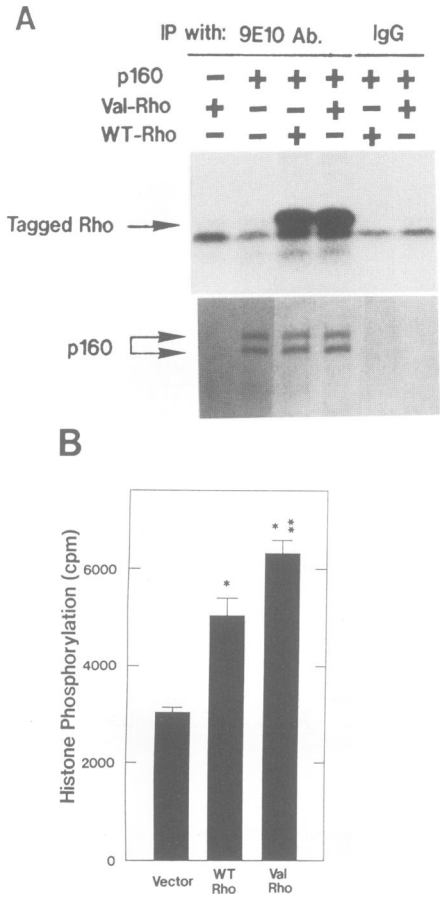

Fig. 5. (**A**) Co-precipitation of p160 and Rho from COS-7 cells. The expression of p160 and Rho in COS cells and the immunoprecipitation with the anti-myc antibody were carried out as described in Materials and methods. Each immunoprecipitate was subjected to the ADP-ribosylation reaction (above) and to immunoblotting with the anti-myc antibody (below). The positions of the HA-tagged expressed Rho and myc-tagged expressed p160 (a doublet) are shown by the arrows. The lowest ADP-ribosylation band seen clearly in the third and fourth lanes from the left and weakly in the second lane represents endogenous Rho. (**B**) Activation of p160 kinase activity *in vivo*. p160 was co-expressed in COS cells with either Val14-rhoA, wild type (WT) rhoA or vector alone, and then collected from the cell lysates by precipitation with the anti-myc antibody. The kinase activity in the precipitates was assayed as described in Materials and methods. The control experiment was carried out in the mock-transfected cells in each series of experiment, and the obtained values (400–700 c.p.m.) were subtracted. The results of five experiments are shown as mean ± SEM. $*P<0.01$ compared with the p160 plus vector. The kinase activity in the precipitates from Val14-rhoA-cotransfected cells was also significantly higher than that from the wild type rhoA-cotransfected cells ($**P<0.01$).

Possible functions of other domains of p160^{ROCK}

The activation of p160^{ROCK} is quite modest when compared with the marked stimulation of p65^{PAK} by Cdc42 or Rac (Manser *et al.*, 1994; Martin *et al.*, 1995). In this respect, it is more similar to the Raf kinase which is only marginally activated by Ras (Zhang *et al.*, 1993; Yamamori *et al.*, 1995). Recent studies indicate that Raf requires membrane recruitment for its activation, and that this recruitment is executed by Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994; Marais *et al.*, 1995). Like the Ras and Raf kinase, p160^{ROCK} may be recruited by Rho to a specific site on the membrane for its activation. Its high homology to MD-PK over the kinase and coiled-coil regions supports this notion, because a recent histochemical study (van der Ven *et al.*, 1993) showed that the latter protein was localized to specific sites in muscle cells, such as the myotendinous junction of skeletal muscle, the intercalated disc of cardiac muscle and the dense plaques of smooth muscle. These are all areas where muscle fibers fuse to the membrane, and many focal adhesion proteins also accumulate in these regions. p160^{ROCK} may also accumulate in sites where the cytoskeleton anchors to the membrane, and this accumulation may be regulated in a Rho-dependent manner. The coiled-coil structure of p160 is indicative of its interaction with itself or other coiled-coil proteins, many of which are cytoskeletal proteins. It is intriguing to hypothesize that such an association may be regulated by Rho. In addition, the PH domain and the Cys-rich region in the C-terminus of p160 may indicate its interaction with cell membranes via the binding to PIP_2 and to diacylglycerol respectively (Harlan *et al.*, 1994; Zhang *et al.*, 1995). This interaction may be evoked by the formation of these lipids upon cell activation.

Multiplicity of Rho-mediated pathways and target molecules

As discussed above, it now appears that Rho regulates multiple pathways within the cell, i.e. the induction of focal adhesions and stress fibers, the regulation of the contractile ring in cytokinesis, the increase in the calcium sensitivity in smooth muscle, nuclear transcription and cell transformation. It is an interesting question to ask how these pathways are regulated and where they diverge. The most plausible possibility is that Rho has multiple effectors, and each effector drives a separate pathway. Multiple target molecules have already been found for Ras (e.g. see Rodoriguez-Viciana *et al.*, 1994) and for Rac/Cdc42 (Manser *et al.*, 1993, 1994; Diekmann *et al.*, 1994; Zheng *et al.*, 1994). It is, therefore, likely that Rho also has more than one effector.

Using a yeast two hybrid system and a ligand overlay assay, we have already found several other molecules that interact specifically with the GTP-bound form of Rho. One of these is a molecule having a coiled-coil structure, PH domain and a Cys-rich region similar to p160 (Madaule *et al.*, 1995). Others include another protein Ser/Thr kinase, protein kinase N and a novel molecule containing a domain homologous to the regulatory domain of PKN which we have designated rhophilin (Watanabe *et al.*, 1996). In addition to these molecules, two lipid kinases and one phospholipase have been reported to be activated and regulated by Rho in the cell: PI-3-kinase (Zhang *et al.*, 1993), PI-4-P-5-kinase (Chong *et al.*, 1994) and phospholipase D (PLD) (Malcom *et al.*, 1994). Although the direct binding of Rho to these lipid kinases and lipase has not yet been demonstrated, recent studies have reported that Rho can activate a highly purified preparation of PLD (Siddiqi *et al.*, 1995; Singer *et al.*, 1995). It is therefore likely that several molecules serve as Rho effectors in the cell. They may be differentially expressed in different cell types or during different phases of growth, and thereby mediate different actions of Rho. It is also conceivable that these molecules interact with different regions of the Rho molecule, form a multi-protein signaling complex and then work cooperatively within a single cell. Sup-

porting this idea, an earlier study (Self and Hall, 1993) has demonstrated the presence of multiple transduction domains within the Rho molecule. Identification of $p160^{ROCK}$ will facilitate studies over these issues to clarify the mechanism by which extracellular stimuli evoke a variety of cell responses via Rho GTPase.

Materials and methods

Materials

[^{35}S]GTPγS (1000 Ci/mmol), [^{35}S]GDPβS (1000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]NAD (800 Ci/mmol) were purchased from Dupont-New England Nuclear. [^{125}I]protein A (100 μCi/μg) was obtained by ICN Biomedicals Inc. Recombinant human RhoA, Rac1 (a plasmid provided by Y.Takai) and Cdc42Hs (a plasmid provided by P.Polakis) were expressed as glutathione-*S*-transferase fusion proteins and then purified as previously described (Morii *et al.*, 1993). DEAE–Sepharose CL-6B and a Mono Q HR5/5 column were obtained from Pharmacia Biotech. Red A–Sepharose and Macro-Prep Ceramic hydroxyapatite (40 μm particles) columns were purchased from Amicon and Bio-Rad respectively.

Anti-peptide antibody to the N-terminal portion of p160 was raised by Research Genetics in rabbits immunized with a synthetic peptide, acetylated Ser-Thr-Gly-Asp-Ser-Phe-Glu-Thr-Arg-Phe-Glu-Lys-Met-Asp-Cys conjugated keyhole limpet hemocyanin, and antiserum 20 490 was used for immunoblot analysis.

Ligand overlay assay

Cell homogenates (100 μg protein each) or purified proteins were subjected to SDS–PAGE on 8% gels, and the separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The proteins on the membranes were denatured and renatured as previously described (Manser *et al.*, 1992, 1994), except that the renaturation was performed overnight at 4°C in Dulbecco's phosphate buffered saline (PBS) containing 0.1% bovine serum albumin, 0.5 mM $MgCl_2$, 50 μM $ZnCl_2$, 0.1% Triton X-100 and 5 mM dithiothreitol. The recombinant GTP-binding proteins were loaded with radiolabeled guanine nucleotides by incubating 40 μM of protein with 100 μM of each nucleotide (1000 Ci/mmol) in 25 mM Tris–HCl, pH 7.5, containing 1 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl, 0.05% Tween 20 and 5 mM dithiothreitol at 30°C for 30 min. The radioactivity bound to the recombinant proteins was determined by filter assay as previously described (Morii *et al.*, 1988). The radionucleotide-bound recombinant protein was then added to the renatured proteins at 5 nM, and the incubation was performed as previously described (Manser *et al.*, 1994). The membrane was subsequently washed, dried and exposed to X-ray film for autoradiography.

Purification of p160 Rho-binding protein

Human blood platelets were collected from the buffy coat fraction as described previously (Morii *et al.*, 1992). All of the subsequent procedures were performed at 4°C. Washed platelets from 100 units of blood were homogenized by 10 strokes in a Potter–Elvehjem homogenizer in 100 ml of buffer A (10 mM Tris–HCl, pH 7.4, containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine hydrochloride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 100 μM PMSF), and then centrifuged at 100 000 *g* for 60 min. The supernatant containing 970 mg of protein was applied to a DEAE–Sepharose CL-6B column (bed volume, 70 ml) equilibrated with buffer A. The elution was performed with a linear gradient of 0–0.5 M NaCl in buffer A (total volume, 1200 ml). p160 Rho-binding protein was recovered as a broad peak at 0.25 M NaCl. The active fractions, containing 170 mg protein, were pooled and applied to a Red A column (bed volume, 7 ml) equilibrated with buffer A. The column was washed successively with 150 ml of buffer A containing 1 M NaCl, and then with 50 ml of buffer A containing 1.5 M NaCl. The elution was performed with 50 ml of the same buffer containing 1.5 M NaCl and 50% ethylene glycol. The recovered fraction (17.5 mg of protein) was dialyzed for 48 h against two changes of 1 l each of buffer A. The dialyzates were appplied to 2 ml of a Macro-Prep Hydroxyapatite column equilibrated with 10 mM potassium phosphate, pH 7.0, and the elution was performed with a linear gradient of 10–350 mM potassium phosphate, pH 7.0 (total volume, 90 ml). The active fraction (0.75 mg of protein) was then applied to a Mono Q HR 5/5 column, and the proteins were eluted with a linear gradient of 150–500 mM NaCl in a total volume of 35 ml. p160 was eluted in a broad peak at 350 mM NaCl , and this was designated as the final preparation.

Affinity precipitation of p160 with GST–RhoA

A hydroxyapatite fraction containing ~0.2 pmol of p160 was dialyzed against the overlay buffer. To this fraction was added 80 pmol of recombinant GST–RhoA, GST–Rac1 or GST–Cdc42 loaded with either GTPγS or GDP, or the equivalent amounts of GST and GTPγS, and the total volume was adjusted to 200 μl with the final nucleotide concentrations of 20 μM. Incubation was carried out at 4°C for 60 min with gentle shaking. A 30 μl aliquot of glutathione–Sepharose pre-equilibrated with the overlay buffer containing either nucleotide was then added, and the incubation continued for another 30 min at 4°C. The mixtures were spun down at 1000 *g* for 5 min. The pellets were then washed twice with a washing buffer (25 mM MES–NaOH, pH 6.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% Triton X-100, and 20 μM GTPγS or GDP). The Sepharose beads were then suspended in an equal volume of 2× Laemmli sample buffer. The suspensions were boiled and the extracts were subjected to immunoblot analysis using anti-peptide antibody 20 490 to p160 as decribed previously (Kumagai *et al.*, 1993).

Autophosphorylation of p160 and phosphoamino acid analysis

The Mono Q fraction of p160 (8 ng of protein) was incubated with 4 μM [γ-^{32}P] ATP (25 Ci/mmol) at 30°C for 30 min in 50 mM HEPES–NaOH, pH 7.3, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.03% Briji 35 and 2 mM dithiothreitol. After the incubation, the solution was mixed with an equal volume of 2× Laemmli sample buffer, boiled for 5 min and then applied to SDS–PAGE. The gel was stained with Commassie Blue, dried and subjected to autoradiography. For the phosphoamino acid analysis, the radiolabeled protein was transferred to a PVDF membrane. The radioactive band was then excised and subjected to phosphoamino acid analysis as previously described (Kumagai *et al.*, 1995).

Phosphorylation reactions

The Mono Q fraction of p160 (30 ng of protein) was incubated with 40 μM [γ-^{32}P]ATP (3.3 Ci/mmol) and with 3 μg of either histone (HF2A, Worthington), dephosphorylated casein (Sigma) or MBP (Gibco) in the presence of 0.4 μM GDP- or GTPγS-loaded GST–RhoA, GST–Rac1 or GST–Cdc42 at 30°C in a total volume of 31 μl. A 7 μl aliquot was taken at 0, 5, 10 and 20 min, mixed with an equal volume of 2× Laemmli sample buffer, and applied to SDS–PAGE. The gel was stained with Commassie Blue, dried and subjected to analysis by a Bioimage Analyzer BAS2000 (Fuji Photo Film Co., Tokyo).

Peptide sequencing

The Mono Q fraction containing ~30 pmol of p160 was subjected to SDS–PAGE and then transferred to a PVDF membrane. The proteins were stained with Ponceau S, and the p160 band was cut out. The immobilized p160 was digested with *Achromobacter* lysyl endopeptidase or endoproteinase Asp-N, and the peptides released were separated and sequenced as previously described (Iwamatsu, 1992; Maekawa *et al.*, 1994).

cDNA cloning and sequence analysis

Poly(A)$^+$ RNA was prepared from cultured MEG-01S human megakaryocytic leukemia cells (Hirata *et al.*, 1991) and the λgt10 and λZAP-II cDNA libraries were constructed as previously described (Kakizuka *et al.*, 1993). The λgt10 library was first screened with a 20mer degenerate oligonucleotide probe corresponding to the 7mer partial amino acid sequence AP-23. One positive clone containing a 2.6 kbp insert (clone P2) was isolated from 3×10^5 plaques. This clone contained a nucleotide sequence corresponding to the probe and encoded two other peptide sequences derived from p160 in-frame. Using the 450 bp 5′-part of this cDNA as a probe, the λZAP-II cDNA library was then screened, and one clone, clone N, was isolated. This clone contained a cDNA which overlapped P2 and extended 450 bp into the 3′-end. Using this 3′-extended part as a probe, clone 4N was obtained from the λgt10 library, which had a 3′-extension of 350 bp. Clone C was then isolated using this 3′-extension as a probe from the same library, and extended the 3′-end by another 2.5 kbp. Nucleotide sequence was determined on both strands using the dideoxy chain termination method. Sequence comparisons were made using BLAST (Altschul *et al.*, 1990) against a non-redundant PDB + SwissProt + Spupdate + PIR + GenPept + GPupdate database. The coiled-coil structure probability was analyzed by the algorithm developed by Lupas *et al.* (1991).

Northern blot analysis

Northern blot analysis was performed on Human Multiple Tissue Blots I (CLONTECH) with a ^{32}P-labeled 5′-fragment of *NotI*/*Bam*HI digests of P2 as a probe. Hybridization was carried out in 5× SSPE containing 50 mM sodium phosphate, pH 6.5, 50% formamide, 5× Denhardt's, 0.1% SDS and 0.2 mg/ml yeast tRNA at 42°C for 16 h. The filter was washed three times in 2× SSC–0.1% SDS at 42°C for 15 min, and then exposed to an X-ray film for 4 days.

Construction of expression vectors and transfection experiments

The full size cDNA used for expression was constructed as follows. A plasmid DNA pBluescript SK$^+$ carrying the clone 4N cDNA was digested with *Sph*I and *Sac*I, and then ligated with a *Sph*I–*Spe*I fragment of clone C cDNA and a *Spe*I–*Sma*I–*Eco*RV–*Sac*I linker to produce plasmid4N-C. PCR was then performed with clone N cDNA as a template with the forward primer, 5′-GGGGAGCTCAAGGTACCTCG-AGTGGGGACAGTTTTGAG-3′ and with the reverse primer 5′-CGCC-TGCAGGCTTTCATTCGTAAATCTCTG. The PCR fragment was subcloned into pBluescript SK$^+$ (Stratagene) and sequenced. The plasmid carrying this product was digested with *Bam*HI and *Eco*RV, and then ligated with a *Xba*I–*Eco*RV fragment from plasmid 4N-C and a *Bam*HI–*Xba*I fragment from clone N. An *Asp*718–*Eco*RV fragment was excised from the resultant plasmid and then inserted into a pCMX vector carrying a myc epitope sequence (Dyck *et al.*, 1994). COS-7 cells were plated at 10^5 cells per 3.5 cm dish and cultured overnight, and then transfected with 1.5 μg of the pCMX vector with lipofectamine. The cells were incubated in Opti-MEM for 6 h, and then cultured in DMEM containing 10% fetal calf serum for 18 h. The medium was removed and the cells were washed twice with PBS, and lyzed in 100 μl of 2× Laemmli sample buffer. Thirty μl aliquots of the lysates were subjected to SDS–PAGE, and the separated proteins were transferred to either a PVDF membrane or a nitrocellulose membrane. Immunoblotting using a 9E10 anti-myc epitope antibody and the ligand overlay analysis were carried out as described.

Cotransfection of p160 cDNA with Val14- and wild type rhoA

COS-7 cells were plated at a density of 1.2×10^5 cells per 6 cm dish. After culturing for 1 day, the medium was removed, and the cells were transfected with 2.25 μg of pCMX-myc-p160 or pCMX-myc, together with 0.75 μg of either pEF-BOS-HA (*Hemophilus influenza* hemagglutinin)-tagged-Val14-rhoA, pEF-BOS-HA-rhoA or vector alone, using lipofectamine in 2 ml of Opti-MEM. At 6 h, 3 ml of Opti-MEM was added and the cells were cultured for another 30 h. The cells were washed once with ice-cold PBS and lyzed with a lysis buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 25 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.2 mM PMSF, 2 mM dithiotheritol, 0.2 mM sodium vanadate, 0.05% Triton X-100 and 0.1 μM calyculin A) on ice for 20 min. The lysates were centrifuged at 10 000 *g* for 10 min, and the supernatant was collected. Either a 9E10 antibody or control IgG coupled to protein G–Sepharose was then added to the supernatant, and the mixture was shaken at 4°C for 3 h. The suspension was centrifuged at 1000 *g* for 5 min, and the resultant pellets were washed three times with 0.5 ml of the lysis buffer. For the ADP-ribosylation reaction, the pellets were resuspended in 500 μl of the ADP-ribosylation buffer without dithiothreitol (Morii *et al.*, 1988). One hundred μl aliquots were then taken and used for immunoblotting with anti-myc antibody after precipitation. The remaining 400 μl of suspension was spun down, and the pellet was resuspended in 34 μl of ADP-ribosylation buffer. [^{32}P]NAD (10^6 c.p.m./pmol) was added to a concentration of 1 μM, and the reaction was allowed to proceed with 400 ng of botulinum C3 exoenzyme at 30°C for 12 h. The analysis of the ADP-ribosylation reaction was performed as previously described (Morii *et al.*, 1988). For the kinase assay, the pellets were washed once with 20 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$ and 0.1 μM calyculin A, and were then resuspended in the phosphorylation buffer. The phosphorylation reaction was carried out as described above with histone as the substrate.

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