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p160^{ROCK}, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions

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Abstract p160^{ROCK} is a serine/threonine protein kinase that binds selectively to GTP-Rho and is activated by this binding. To identify its function, we transfected HeLa cells with wild type and mutants of p160^{ROCK} and examined morphology of the transfected cells. Transfection with wild type and mutants containing the kinase domain and the coiled-coil forming region induced focal adhesions and stress fibers, while no induction was observed with a kinase-defective mutant or a mutant containing only the kinase domain. Furthermore, Rho-induced formation of focal adhesions and stress fibers was inhibited by co-expression of a mutant defective in both kinase and Rho-binding activities. Rho, however, still induced an increase in F-actin content in these cells. These results suggest that p160^{ROCK} works downstream of Rho to induce formation of focal adhesions and that Rho-induced actin polymerization is mediated by other effector(s).

Key words: Rho; p160^{ROCK}; Focal adhesion; Stress fiber; Actin polymerization

1. Introduction

The Rho family of small GTPases regulate cell shape and cell motility through reorganization of actin cytoskeletons [1,2]. In cultured fibroblasts and epithelial cells, Rho regulates the formation of focal adhesions and stress fibers to facilitate cell adhesion to the extracellular matrix via integrins [3,4]. Rho also regulates the avidity of integrins in platelets and lymphocytes to control their adhesion [5,6]. Rac, on the other hand, induces membrane ruffling with dot-like focal complexes [7,8]. While several putative target proteins for Rho and Rac have been isolated, the biochemical mechanisms of the morphological changes induced by these GTPases remain unclarified (see [1,2] for review). One of the putative Rho target proteins is a serine/threonine protein kinase, p160^{ROCK} [9]. This kinase has a kinase domain in its amino-terminus, a long amphipathic α-helix capable of forming a coiled-coil structure in the middle, and a cysteine-rich zinc finger-like motif and a PH region in its carboxy-terminus. A homologue of p160^{ROCK} has been isolated and called either ROKα [10], Rho-kinase [11] or ROCK-II [12]. Recently, Leung et al. [13] reported that expression of ROKα induces focal adhesion- and stress fiber-like structures in cultured cells. Whether p160^{ROCK} elicits the same or a different phenotype is an intriguing question, because they show only 45–55% identity beside the kinase domain [12]. It also remains to be established whether these kinases work downstream of Rho in the cell, because two groups recently reported that both ROKα and p160^{ROCK} can interact with activated Rac in vitro and suggested that they may work downstream of Rac [14,15]. This point has not been clarified by the previous study [13]. We report here that p160^{ROCK} works downstream of Rho and not of Rac to mediate formation of focal adhesions and stress fibers.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, Dulbecco's phosphate buffered saline; PCR, polymerase chain reaction; PH, pleckstrin homology; p160^{ROCK}, a Rho-associated, coiled-coil forming protein kinase

2. Materials and methods

2.1. Materials and cell culture

Anti-vinculin antibody (V-4505) and anti-RhoA polyclonal antibody (SC-179) were purchased from Sigma and Santa Cruz Biotechnology, respectively. Anti-Myc antibody (9E10) [16] was a gift from S. Nishikawa, Kyoto University. Antibody to p160^{ROCK} (C9) was raised in rabbits by immunizing with GST-fused recombinant protein of the C-terminal potion of p160^{ROCK} (aa 1096 to aa 1354) [17] and used. Plasmids of pEXV-Val^{14}-RhoA and pEXV-Val^{12}-Rac1 were provided by A. Hall. HeLa cells were grown in DMEM supplemented with 10% FCS.

2.2. Expression constructs

All constructs of p160^{ROCK} were tagged with a Myc epitope at the amino-terminus and expressed. 5′-*Hin*dIII fragment of pCMX-*myc*-p160^{ROCK} [9] was ligated to a plasmid pBluescript SK+ digested with *Hin*dIII to produce pBluescript-*myc*-N. To construct pBluescript-*myc*-p160, pBluescript-*myc*-N was digested with *Bam*HI-*Not*I, and a *Bam*HI-*Sma*I fragment of pCMX-*myc* p160^{ROCK} was ligated. A *Sal*I-*Bst*XI fragment of pBluescript-*myc*-p160 was inserted into pCAG vector [18] to produce pCAG-*myc*-p160 (WT). A *Xho*I-*Eco*RV-*Xho*I linker was inserted into the 3′-*Xho*I site of pCAG-*myc*-p160 (WT) to construct pCAG-*myc*-p160$^{myc-727}$ with three frame stop codons. *Asp*718-*Msc*I, *Asp*718-*Sph*I, *Asp*718-*Xho*I, *Asp*718-*Xba*I and *Asp*718-*Spe*I fragments were ligated to an *Asp*718-*Eco*RV fragment of pCAG-*myc*-p160$^{myc-727}$ to generate pCAG-*myc*-p160Δ1, 2, 3, 4 and 5, respectively.

For site-directed mutagenesis of Lys^{105} to Ala, oligonucleotides 5′-TGCTGCGGGATCCCAAATCGG-3′, 5′-CAAATTTGCTGAGAAGCGCCATGGCATATACC-3′, 5′-GGTATATGCCATGGCGCTTCTCAGCAAATTTG-3′ and 5′-GAACTACTTCTGCAGTATAGAATCG-3′, designated primers A, B, C and D, corresponding to nucleotide sequences 50–71, 300–331, 300–331 and 526–550 of the coding region of p160^{ROCK} cDNA (underlined, the complementary sequences), respectively, were synthesized. PCR was performed with pCMX-*myc*-p160^{ROCK} as a template using primer pairs A and B, and C and D, separately. These products were combined, and PCR was performed using primers A and D. The product was digested with *Bam*HI and *Pst*I, and was inserted into a *Bam*HI-*Pst*I fragment of pCMX-*myc*-p160^{ROCK} and pCMX-p160^{ROCKI1009A} [17] to make a kinase defective mutant (KD) and a kinase defective, Rho-binding negative mutant (KD-IA), respectively. Fragments of pCMX-p160^{ROCK}(KD) and p160^{ROCK}(KD-IA) were excised and inserted into pCAG vector to produce pCAG-*myc*-KD and -KD-IA, respectively. To generate pCAG-*myc*-C, PCR was performed with pCMX-*myc*-p160^{ROCK} as a template using the forward primer 5′-CG-

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GGGTACCGCCAGCAAAGAGAGTGATATTGAG-3′ and the reverse primer 5′-TTGTCCAATTATGTCACACCACAG-3′. This product was digested with *Asp*718-*Sma*I, and then ligated to an *Asp*718-*Eco*RV fragment of pCAG-*myc*-p160$^{myc-727}$.

2.3. Transfection, immunofluorescence, Western blotting and immune complex kinase assay

HeLa cells were plated on a coverglass at a density of 7.5×10^4 cells per 3.5 cm dish and at a density of 1.5×10^5 cells per 6 cm dish for immunofluorescence and immunoprecipitation studies, respectively. After culture for one day, cells were transfected with plasmid DNA by the application of lipofectamine-DNA coprecipitates. At 4 h, DMEM containing 10% FCS was added and cells were cultured for another 12 h. Cells were then washed twice with PBS. Lysis of washed cells and immunoprecipitation of cell lysates with anti-Myc antibody were carried out as described previously [9].

Fixation, permeabilization and blocking for immunofluorescence were performed as described previously [19] except that cells for anti-vinculin staining were simultaneously treated with 4% formaldehyde and 0.1% Triton X-100 in Ca^{2+} and Mg^{2+}-free PBS. Cells were first incubated with either anti-Myc (9E10) antibody (17 mg/ml) (1:500 in blotto), anti-vinculin antibody (1:100 in blotto), anti-RhoA antibody (1:50 in blotto), anti-p160^{ROCK} C9 (1:100 in blotto) or combinations of anti-vinculin and either anti-Rho or anti-p160 antibodies. Anti-Myc (9E10) and anti-vinculin antibodies were detected with FITC-conjugated anti-mouse IgG. Rhodamine-conjugated or Cy2-conjugated anti-rabbit IgG were used to detect signals of anti-Rho and anti-p160 antibodies. For F-actin staining, rhodamine-conjugated phalloidin (Molecular Probes) was added after incubation with the second antibody. Cells were photographed on a Zeiss Axiophot fluorescence microscope or were analyzed at 0.36 μm optical sections on a Bio-Rad MRC-1024 Confocal Imaging System and built-up images were constructed.

3. Results and discussion

3.1. Expression of p160^{ROCK} and its mutants

p160^{ROCK} is a multi-domain protein containing a kinase domain, a coiled-coil forming α-helix region, the Rho-binding Ydomain, a PH and a cysteine-rich regions. To identify its role in Rho-mediated cellular processes and to identify the function of each domain, we expressed wild type (WT) as well as mutants of this protein. The mutants used in this study were five truncation mutants having the above domains deleted from the C-terminus (Δ1–Δ5), a C-terminal fragment containing the PH and cysteine-rich regions only (C), and full length proteins with a point mutation in the kinase domain (KD) and with point mutations both in the kinase and the Rho-binding domains (KD-IA) (Fig. 1A). Plasmids carrying these constructs were transfected into HeLa cells, and lysates of transfected cells were used for immunoprecipitation and immunoblotting analysis. As shown in Fig. 1B, proteins of the expected sizes were recovered with each transfection. Expression levels of the mutants Δ2 and KD-IA were significantly lower than the others. Immunoprecipitates were then subjected to the kinase assay with histone as a substrate (Fig. 1C). Phosphorylation was observed in WT protein as well as the Δ1–Δ4 mutants. The Δ5 mutant showed little histone phosphorylation, although it gave an autophosphorylation band. Apparently no phosphorylation was observed in the C or the KD-IA mutant. It was neither detected in the KD mutant (data not shown).

3.2. Induction of focal adhesion- and stress fiber-like structures by p160^{ROCK} and its mutants

We next identified cells expressing these proteins by the staining of Myc epitope tagged to the N-terminus of each protein, and characterized the morphology of each transfectant. Transfected cells were then subjected to double staining for vinculin and actin. The characteristic phenotype of each

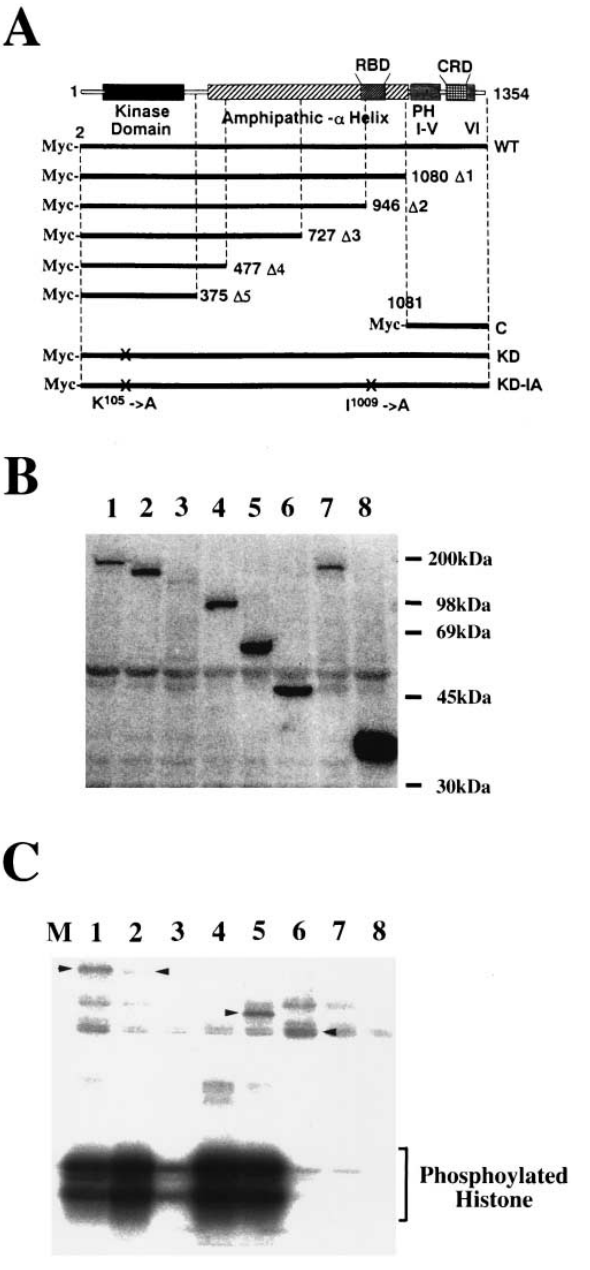
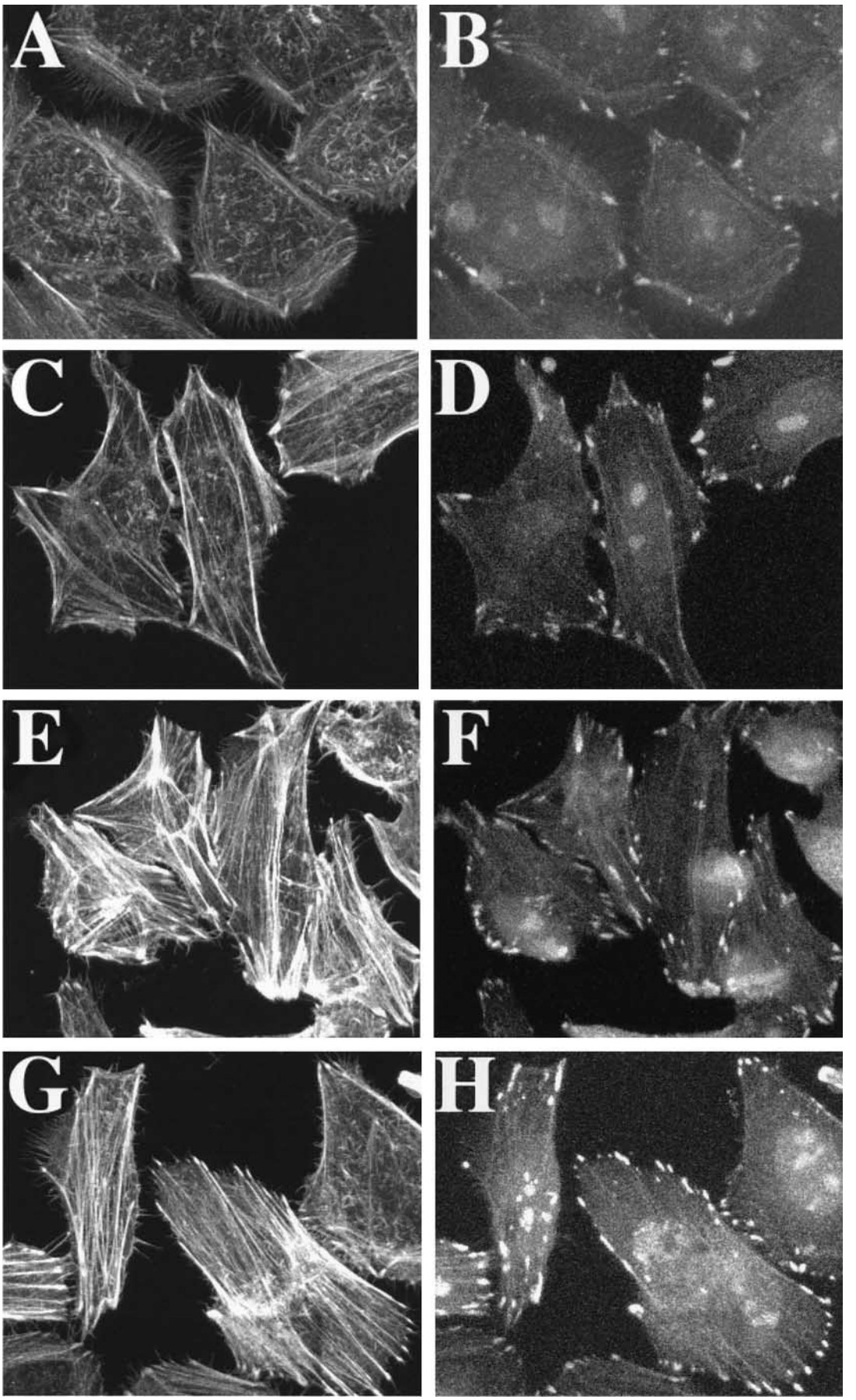


Fig. 1. Diagrammatic representation of p160^{ROCK} mutants (A), and immunoblotting (B) and immune complex kinase assay (C) of expressed proteins. A: Structural domains of p160^{ROCK} are schematically represented at the top and the eight mutants are represented by the thick lines below. Numbers indicate amino acid residues of the amino- and carboxyl-termini of each mutant. The positions of point mutations are indicated by X. B and C: Expression of p160^{ROCK} mutants in HeLa cells. Expressed proteins in HeLa cells were immunoprecipitated with anti-Myc antibody (9E10), and immunoprecipitates were subjected to Western blotting (B) and to a phosphorylation reaction (C) as described [9]. Lane 1, WT; lane 2, Δ1; lane 3, Δ2; lane 4, Δ3; lane 5, Δ4; lane 6, Δ5; lane 7, KD-IA; lane 8, C. In B, cell lysates from one 6 cm dish were used except for analysis of the mutants Δ2 and KD-IA, which used lysates from six dishes. Positions of molecular weight markers are indicated at the right margin. In C, one tenth each of lysates from seven 6 cm dishes were used. Arrowheads indicate autophosphorylated bands of each mutant. M represents cells transfected with a vector alone.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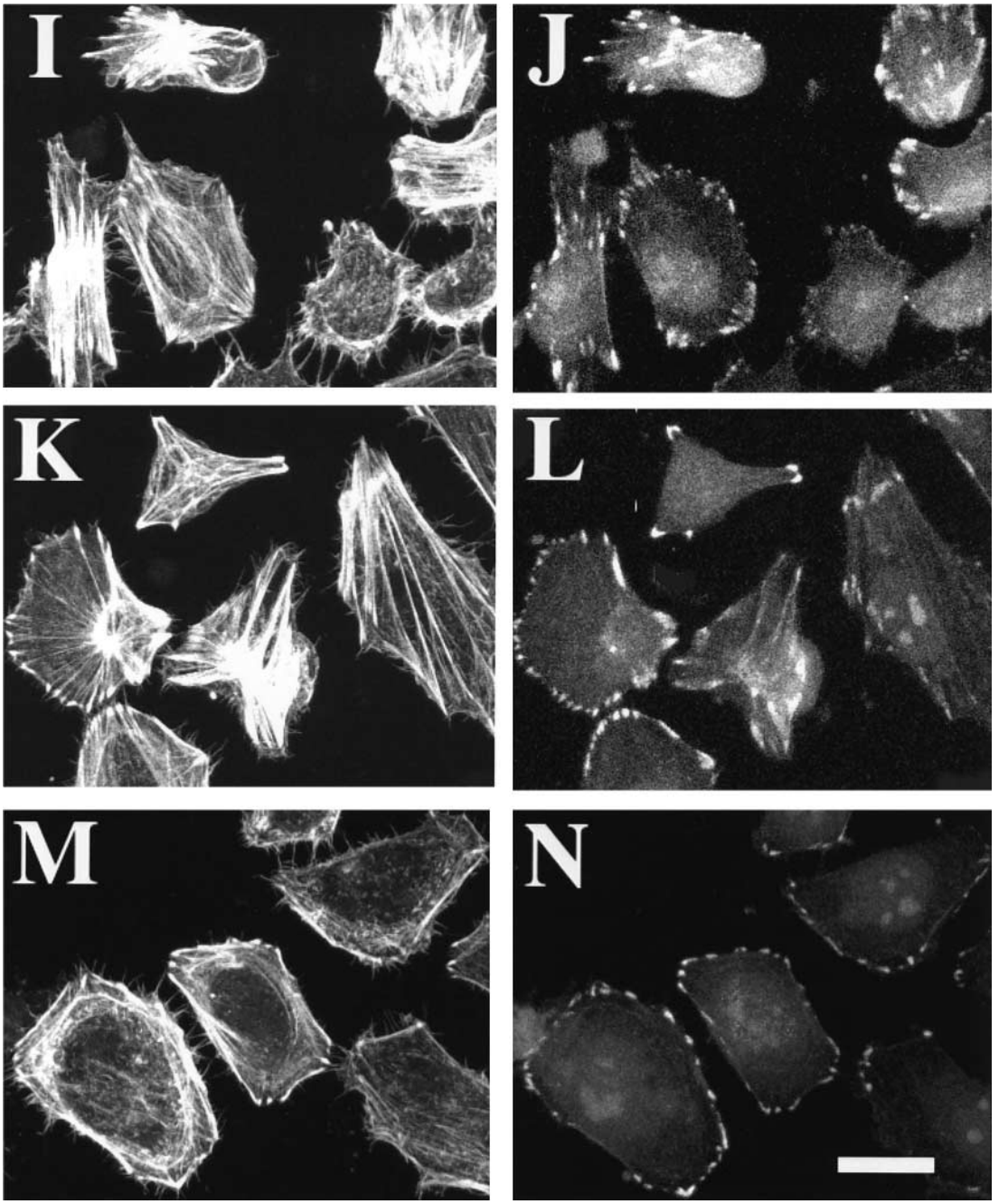

Fig. 2. Morphology of HeLa cells overexpressing p160^{ROCK} mutants. HeLa cells were transfected with 1 μg of vector alone (A and B), or vector carrying WT (C and D), Δ1 (E and F), Δ2 (G and H), Δ3 (I and J), Δ4 (K and L) or Δ5 (M and N) mutant of p160^{ROCK}. After 16 h of incubation, cells were double-stained with rhodamine-phalloidin (left panels; A, C, E, G, I, K and M) and with anti-vinculin antibody (right panels; B, D, F, H, J, L and N). Images built up from optical sections by a confocal imaging system are shown. Scale bar, 20 μm.

transfectant is shown in Fig. 2. Expression of WT resulted in elongated rectangular to polygonal cell shapes with pointed edges (Fig. 2C,D). Thin actin bundles were formed and terminated at the pointed edges. Punctate vinculin staining was observed at the termini of the actin bundles. Upon expression of Δ1 mutant, actin bundles spanning the cells became thick and increased in number. Consistently, vinculin staining increased in size and number and became more dense (Fig. 2E,F). Essentially the same morphology was observed in cells transfected with Δ2 mutant (Fig. 2G,H), indicating that Rho binding was not required for induction of this change. In cells expressing Δ3 mutant, vinculin staining became more dense and larger, and some of them were located within the cells or concentrated at one edge of the cells (Fig. 2I,J). Actin bundles thicker and shorter than those found in the Δ1 and Δ2-transfected cells were seen between the vinculin-containing structures (Fig. 2I,J). Similar changes were found in cells expressing Δ4 (Fig. 2K,L). In contrast to these findings, no significant morphological changes were found in cells expressing Δ5, the kinase domain alone (Fig. 2M,N). Expression of the kinase-defective mutant, KD, also failed to induce the phenotypic change (data not shown).

3.3. *A dominant negative form of p160^{ROCK} blocks Rho-induced formation of focal adhesions and stress fibers but not Rac-induced membrane ruffling*

The above results thus demonstrated that expression of p160^{ROCK} can induce focal adhesion-like structures as evidenced by vinculin accumulation and stress fiber-like actin bundles extending between these structures. They also suggested that both kinase activity and a part of the coiled-coil forming region are required for this induction. The observed phenotype appeared similar to that reported for ROKα in the previous study [13]. However, these phenotypes were not exactly the same as that induced by activated Rho [13] (Figs. 2

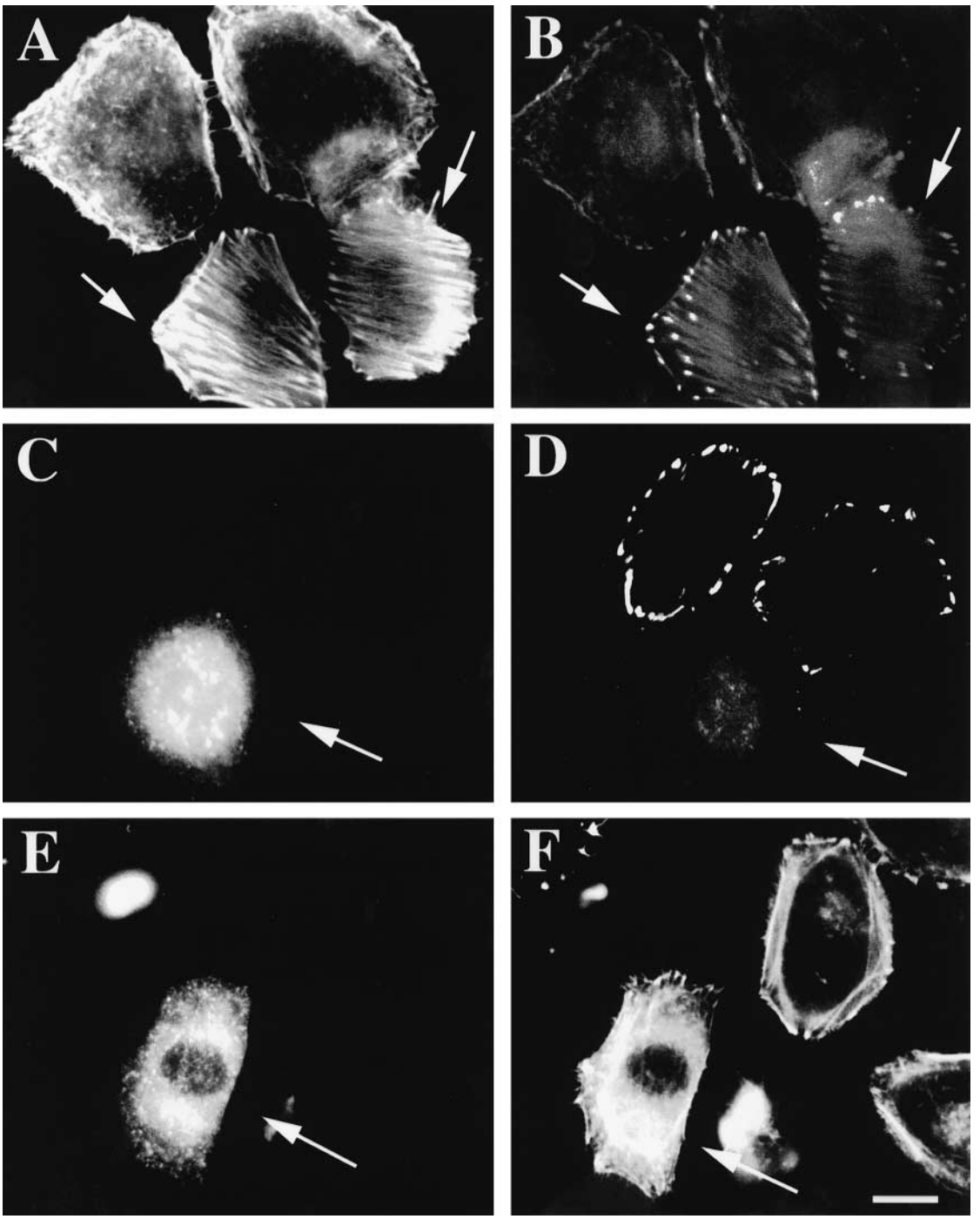

Fig. 3. Inhibition of Rho-induced formation of focal adhesions by co-expression of KD-IA mutant of p160^{ROCK}. HeLa cells were transfected either with 0.2 μg of pEXV-Val^{14}-RhoA or with 0.2 μg of pEXV-Val^{12}-Rac1 alone (A and B for Rho, G and H for Rac) or together with 2 μg of pCAG-KD-IA mutant of p160^{ROCK} (C-F for co-transfection with Rho, I and J for co-transfection with Rac). After 16 h of incubation, cells were double-stained either with rhodamine phalloidin (A and G) and anti-vinculin (B and H), with anti-RhoA antibody (C) and anti-vinculin antibody (D), with anti-RhoA antibody (E) and rhodamine phalloidin (F), with anti-p160 antibody and rhodamine phalloidin (I) or with anti-p160 antibody and anti-vinculin antibody (J). Arrows indicate cells expressing Val^{14}-RhoA (A-F) and cells expressing the KD-IA mutant (I and J). Photographs taken on a fluorescence microscope (A–F) and built-up images by a confocal imaging system (G–J) are shown. Scale bar, 20 μm.

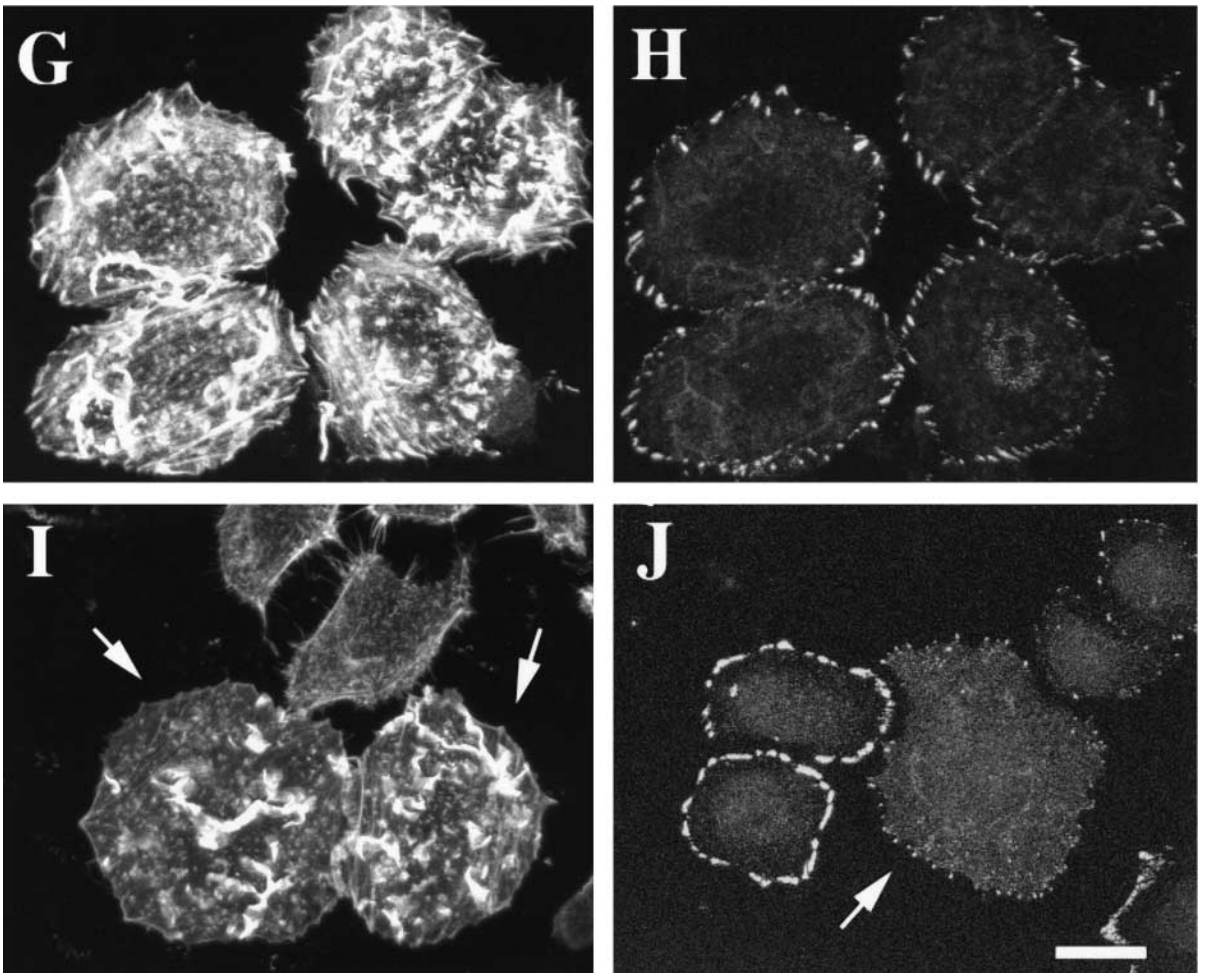

Fig. 3. (*continued*).

and 3). We, therefore, examined the involvement of p160^{ROCK} in Rho-induced focal adhesion and stress fiber formation by co-transfecting HeLa cells with Val^{14}-RhoA and KD-IA, a kinase-defective, Rho-binding negative mutant. Transfection with Val^{14}-RhoA alone strongly induced both stress fibers and focal adhesions (Fig. 3A,B). Co-expression of the KD-IA mutant of p160^{ROCK} suppressed induction of focal adhesions as shown in Fig. 3C,D. Stress fibers were not found, but a generalized increase in the amount of F-actin was observed in these cells (Fig. 3E,F). Co-expression of the C mutant also suppressed Val^{14}-RhoA-induced formation of focal adhesions and stress fibers (data not shown). We next examined if the KD-IA and C mutants inhibit a morphological phenotype evoked by Val^{12}-Rac. Transfection of Val^{12}-Rac alone strongly induced formation of membrane ruffling and dot-like focal complexes with concomitant Rho-dependent formation of stress fibers as described before [7] (Fig. 3G,H). Co-expression with the KD-IA mutant did not inhibit the formation of the former structures induced by activated Rac, but did inhibit the Rho-dependent latter structures (Fig. 3I,J). A similar effect was found with co-expression of the C mutant (data not shown). These results strongly suggest that p160^{ROCK} selectively works downstream of Rho to induce focal adhesions and stress fibers in the cell. Whether ROKα exerts the same function in the cell remains to be tested. Interestingly, Val^{14}-RhoA still increased F-actin content in these cells, suggesting that Rho-induced actin polymerization is mediated by a different effector. This is consistent with previous studies showing that a kinase inhibitor, staurosporine, inhibits focal adhesion formation without affecting the F-actin increase induced by Rho, and that blocking of actin polymer-

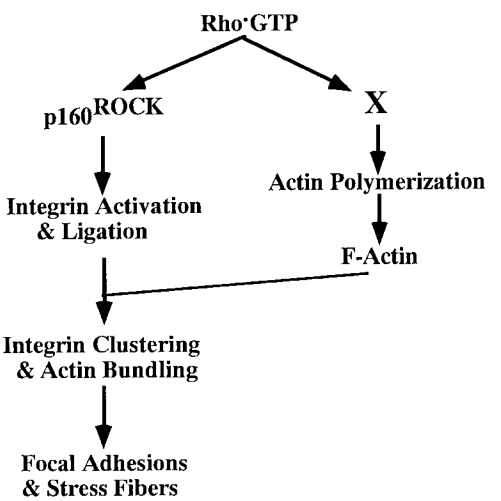

Fig. 4. Proposed action of p160^{ROCK} in Rho-mediated formation of focal adhesions and stress fibers.

ization with cytochalasins could not inhibit Rho-mediated cell adhesion and focal adhesion formation [6,8]. We have recently isolated a novel Rho-binding protein, which induces actin polymerization in transfected cells (Watanabe, N. and Narumiya, S., unpublished observation).

Focal adhesion formation requires integrin activation, its ligation with ligands and clustering of ligated integrins. Bundling of F-actin as stress fibers may accompany integrin clustering [20]. Based on the present findings, we postulate the role of $p160^{ROCK}$ in Rho-mediated actions as depicted in Fig. 4. Clustering of ligated integrins and bundling of F-actin may be induced by a p160-mediated increase in myosin contractility as reported for Rho-kinase by Kimura et al. [21]. It is of interest that expression of the C mutant alone can induce dominant negative effects on Rho, indicating that this truncation product competes with endogenous $p160^{ROCK}$ for the binding at targeting sites. These issues will be addressed in future studies by identifying interacting molecules and substrate(s) of this kinase.

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