Phosphorylation of Adducin by Rho-Kinase Plays a Crucial Role in Cell Motility

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Abstract. Adducin is a membrane skeletal protein that binds to actin filaments (F-actin) and thereby promotes the association of spectrin with F-actin to form a spectrin-actin meshwork beneath plasma membranes such as ruffling membranes. Rho-associated kinase (Rho-kinase), which is activated by the small guanosine triphosphatase Rho, phosphorylates α-adducin and thereby enhances the F-actin-binding activity of α-adducin in vitro. Here we identified the sites of phosphorylation of α-adducin by Rho-kinase as Thr445 and Thr480. We prepared antibody that specifically recognized α-adducin phosphorylated at Thr445, and found by use of this antibody that Rho-kinase phosphorylated α-adducin at Thr445 in MDCK cells in a Rho-dependent manner. Phosphorylated α-adducin accumulated in the membrane ruffling area of Madin-Darby canine kidney (MDCK) epithelial cells and the leading edge of scattering cells during the action of tetradeoxyribosylphorbol-13-acetate (TPA) or hepatocyte growth factor (HGF). The microinjection of Botulinum C3 A D P-ribosyl-transferase, dominant negative Rho-kinase, or α-adducinT445A,T480A (substitution of Thr445 and Thr480 by Ala) inhibited the TPA-induced membrane ruffling in MDCK cells and wound-induced migration in NRK49F cells. α-AdducinT445D,T480D (substitution of Thr445 and Thr480 by Asp) counteracted the inhibitory effect of the dominant negative Rho-kinase on the TPA-induced membrane ruffling in MDCK cells. Taken together, these results indicate that Rho-kinase phosphorylates α-adducin downstream of Rho in vivo, and that the phosphorylation of adducin by Rho-kinase plays a crucial role in the regulation of membrane ruffling and cell motility.

Keywords: Rho • Rho-kinase • adducin • membrane ruffling • cell motility

Membrane ruffling, a dynamically fluctuating movement of protrusion consisting of filopodia and lamellipodia, is rapidly induced in response to certain extracellular signals and is also induced in the leading edges of motile cells (Stossel, 1993; Laffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Welch et al., 1997). Membrane ruffling is thought to be important for cell motility (Mitchison and Cramer, 1996). The small GTPase Rho regulates the formation of actin stress fibers and focal adhesions (Ridley and Hall, 1992, 1994; Hall, 1998), membrane ruffling (Nishiyama et al., 1994; Takaiishi et al., 1995) and cell motility (Takaishi et al., 1993, 1994) in certain types of cells, cell morphology (Paterson et al., 1990), cell aggregation (Tominaga et al., 1993), smooth muscle contraction (Hirata et al., 1992; Góng et al., 1996), neuret retraction in neuronal cells (Nishiki et al., 1990; Jalil et al., 1994), the formation of microvilli (Shaw et al., 1998), and cytokinesis (Kishi et al., 1993; Mabuchi et al., 1993). Rho exerts its biological functions through interaction with specific targets (Van Aelst and D’Souza-Schorey, 1997). Several targets of Rho have been identified, including protein kinase N (PKN) 1 (Amano et al., 1996b; Watanabe et al., 1996), Rho-kinase/ROK/ROCK (Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996), the myosin-binding subunit (MBS) of myosin phosphatase (Kimura et al., 1996), α-adducin with substitution of Thr445 and Thr480 by Ala; α-adducin-DD, α-adducin with substitution of Thr445 and Thr480 by Asp; CAT, catalytic domain; ERM, ezrin/radixin/moesin; G5T, glutathione-S-transferase; HAB, hemagglutinin; HGF, hepatocyte growth factor; LPA, lyposphosphatic acid; MBP, maltose-binding protein; MBS, myosin-binding subunit; MLC, myosin light chain; PH, pleckstrin-homology domain; PK, protein kinase; RB, Rho-binding domain; TPA, tetradeoxyribosylphorbol-13-acetate.

1. Abbreviations used in this paper: α-adducin-A A, α-adducin with substitution of Thr445 and Thr480 by Ala; α-adducin-α, α-adducin with substitution of Thr445 and Thr480 by Asp; CAT, catalytic domain; ERM, ezrin/radixin/moesin; G5T, glutathione-S-transferase; HAB, hemagglutinin; HGF, hepatocyte growth factor; LPA, lyposphosphatic acid; MBP, maltose-binding protein; MBS, myosin-binding subunit; MLC, myosin light chain; PH, pleckstrin-homology domain; PK, protein kinase; RB, Rho-binding domain; TPA, tetradeoxyribosylphorbol-13-acetate.
Materials and Methods

Materials and Chemicals

MDCK cells were kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). NR K 49F cells were purchased from American Type Culture Collection. pEF-BOS-HA-RhoA34V, RhoA N17, Rac1V12, Cdc42V12, pEF-BOS-myc-Rho-kinase, full-length, 16–188 amino acids (aa), Rho-kinase binding domain (R B; 941–1075 aa), pRB/PH (TT) (941–1388 aa), and CAL the catalytic domain of Rho-kinase, 6–553 aa) were constructed as described (Kuroda et al., 1996; Matsuoka et al., 1996; Amano et al., 1997, 1998). pCDNA-ΔPK C α lacking C1 domain at positions 6–159 aa was constructed as described (K ibuchi et al., 1989). pQ-E-R-t-α-adducin fragment (319–671 aa; 35H fragment-4D) was kindly provided by Dr. S. Jaken (University of Vermont, Burlington, VT), and 6 X His-tagged γ-adducin fragment was produced and purified as described (Dong et al., 1995). The expression plasmid of C3 transglutaminase (pGEX-C3) was kindly provided by Dr. A. Hall (University of Vermont, Burlington, VT), and C3 was produced and purified as described (Fukata et al., 1998). Glutathione-S-transferase (GST)-BB was produced and purified from Escherichia coli as described (Amano et al., 1997). GST-CAT was produced and purified from Sf9 cells as described previously (Matsura et al., 1987; Amano et al., 1996a). Rac1V12 was produced and purified from E. coli as described (Kuroda et al., 1996). A nN-hemagglutinin (HA) monoclonal A b (12CA5) was purchased from Behringer Mannheim. 32p-AP-1 TPT was purchased from Amersham Corp. A ll materials used in the nuclei acid study were purchased from Takara Shuzo Corp. Other materials and chemicals were obtained from commercial sources.

Plasmid Constructs

The cDNA encoding human α-adducin (1–737 aa; Joshi et al., 1991) was inserted into the KpnI site of pEF-BOS-HA, into the KpnI site of pAcYM1-HA to obtain recombinant α-adducin by the use of a baculovi

Protein Purification

RB/PH (TT) (Amano et al., 1998) was expressed as a maltose-binding protein (MBP) fusion protein in E. coli and purified. GST-α-adducin, GST-β-adducin, and GST-γ-adducin were produced and purified from E. coli. HA-α-adducin, HA-β-adducin, HA-α-adducin-A A, and HA-α-adducin-D D were produced in Sf9 cells. The cells expressing HA-α-adducin were suspended in homogenizing buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 μM A -PMSF, 10 μM leupeptin). The suspension was sonicated and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was applied onto a mono-Q column (Pharmacia LKB Biotechnology) which had been equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT). The column was then washed, and the proteins were eluted with a linear concentration gradient of NaCl (0–600 mM) in buffer A. HA-α-adducin was eluted with ≈200 mM NaCl and purified to near homogeneity. HA-β-adducin, HA-α-adducin A A, and HA-α-adducin-D D were prepared as HA-α-adducin.

Determination of Phosphorylation Sites of α-Adducin by Rho-Kinase

HA-α-adducin (70 μg of protein) was phosphorylated with GST-CA T (120 ng of protein) in 2 ml of kinase buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, 1 mM EGT A, 1 mM DTT) containing 100 μM 32p-AP-1 TPT for 1 h at 30°C, and the reaction product was digested with A chromobacter protease-1 (AP-1) at 37°C for 20 h. The obtained peptides were applied onto a C18 reverse-phase column (4.6 × 250 mm; Shimazu, Japan) and eluted with a linear gradient of 0–4% acetonitrile for 100 min at a flow rate of 1.0 ml/min by HPLC (System Gold, Beckman). The radioactive peaks were separated and phosphoamino acid sequencing was carried out with a peptide sequencer (PPS-10; Shimadzu, Japan) as described (Bodwell et al., 1991). The fractions obtained from each E domain degradation cycle were measured for 32p in a Beckman liquid scintillation counter.

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Production of Site- and Phosphorylation State-specific Antibody for α-Adducin

The phospheptide Cys-Gln-Glu-In-Arg-Glu-Lys-phospho-Thr-Arg-Trp-Leu-Asn-Ser-Glu was chemically synthesized as an antigen by Peptide Institute Inc. Rabbit polyclonal antibody (anti-pT445) was raised against the phospheptide and affinity purified as described (Inagaki et al., 1997). To examine the specificity of anti-pT445, equal amounts of HA-α-adducin (40 fmol) with various ratios between phosphorylated and nonphosphorylated proteins were subjected to SDS-PAGE. HA-α-adducin (40 fmol) was incubated in kinase buffer containing GST-CAT and A TP, and subjected to SDS-PAGE. The amount of phosphates incorporated into HA-α-adducin was simultaneously determined by γ[32P]ATP. Immunoblot analysis was then performed with anti-pT445, anti-pT445 which was preincubated with a 100-fold amount of antigen-phosphate peptide, and anti-HA A b. For some experiments, HA-α-adducin, HA-ji-adducin, or 6X His-α-adducin fragment was separately phosphorylated with GST-CAT, and 40 fmol (as 32P-incorporated amount) of phosphorylated proteins was subjected to immunoblot analysis with anti-pT445.

Cell Culture

MDCK cells and NIH3T3 cells were maintained in DMEM containing 10% calf serum, streptomycin, and penicillin. COS7 cells were maintained in DMEM containing 10% FBS, streptomycin, and penicillin. NRK 49F cells were maintained in DMEM containing 5% calf serum and 1% nonessential amino acids. To obtain MDCK cells stably expressing HA-α-adducin, MDCK cells were transfected with pEF-BOS-HA-α-adducin along with a marker vector containing the neomycin resistance gene using Li-copfection (GIBCO BRL) as described (Kuroda et al., 1998), and neomycin-resistant clones were selected.

Detection of Thr445-phosphorylated α-Adducin in Intact Cells

The transfection of plasmids into COS7 cells was carried out by the standard DEAE-dextran method. COS7 cells were cotransfected with pEF-BOS-HA-α-adducin and pEF-BOS-vector, pEF-BOS encoding HA-Rac V12, HA-Cdc42 V12, HA-RhoA V14, HA-RhoA N34, myc-Rho-kinase (full-length), HA-RhoA V14 and myc-Rho-kinase (full-length), HA-RhoA V14 and myc-RB, HA-RhoA V14 and myc-R-β/PH (TT), myc-CAT, or pcDNA3-α adding PKCB. Separately, COS7 cells were cotransfected with pEF-BOS-HA-α-adducin-AA and myc-CAT. After a 24-h incubation, the transfected cells were incubated in serum-free medium for 24 h. The cells were treated with 10% (vol/vol) trichloroacetic acid. The resulting precipitates were subjected to immunoblot analysis using anti-HA A b and anti-pT445. MDCK cells stably expressing HA-α-adducin were incubated in the absence or the presence of tetradecanoylphorbol-13-acetate (TPA) for 15 min followed by incubation in the presence of 0.1 μM calyculin A for 10 min. The cells were then treated as COS7 cells and subjected to immunoblot analysis.

Immunofluorescence Analysis

MDCK cells were deprived of serum for 24 h and incubated in DMEM containing 200 nM TPA or 50 μM recombinant human hepatocyte growth factor (HGF; Calbiochem) for 15 min at 37°C. For cell scattering, the cells were not deprived of serum and incubated in DMEM containing 5% calf serum and 200 nM TPA for 2 h. The cells were fixed with 3.0% formaldehyde in PBS for 10 min and treated with PBS containing 0.2% Triton X-100 for 10 min. Phosphorylated adducin and F-actin were doubly stained with anti-pT445 and TRITC-phalloidin. For anti-pT445, the cells were then labeled with FITC-conjugated anti-rabbit IgG A b. For a double immunofluorescence study, the clone stably expressing HA-α-adducin was stimulated as described above. Phosphorylated adducin and HA-α-adducin were doubly stained with anti-pT445 and anti-HA A b. The cells were then labeled with FITC-conjugated anti-rabbit IgG A b and Texas red-conjugated anti-mouse IgG A b. The cells were examined using a Zeiss Axiophot microscope or a confocal microscope (Carl Zeiss).

Microinjection

MDCK cells were seeded at a density of 2.5 × 104 cells per 13-mm cover glass in 60-mm tissue culture dishes and incubated for 8 h. Then, the cells were deprived of serum for 24 h. For microinjection, proteins were con-
analysis (Fig. 1 A). This may be due to the different recovery of each peptide during separation by HPLC. Taken together, these results suggest that Thr445 is a preferential site of phosphorylation by Rho-kinase in vitro.

Production and Characterization of the Site- and Phosphorylation State–specific Antibody for α-Adducin

A n antibody that recognizes the phosphorylated state of a substrate at a specific site is a useful tool with which to evaluate site-specific phosphorylation in vivo and to visualize the cellular distribution of the protein phosphorylated at a specific site (Inagaki et al., 1997). We prepared and affinity purified the polyclonal antibody that recognized the Thr445-phosphorylated α-adducin based on Matsuoka et al. (1996) is presented. The phosphorylation sites of α-adducin for PKA, PKC, and Rho-kinase are presented. (D) Preferential phosphorylation of α-adducin at Thr445 by Rho-kinase in vitro. GST-α-adducin (WT, circles), GST-α-adducin-AA (AA, X), GST-α-adducinT445A (T445A, triangles), and GST-α-adducinT480A (T480A, squares) were phosphorylated by GST-CAT for various periods. Data are means ± SEM of triplicate determinations.
We examined whether α-adducin was phosphorylated via the Rho/Rho-kinase pathway in COS7 cells by immunoblot analysis with anti-pT445. cDNA of HA-α-adducin was cotransfected with plasmids carrying the cDNA of dominant active Rho family members, or dominant active or negative Rho-kinase (Fig. 3). HA-α-adducin was almost equally expressed in all the HA-α-adducin-transfected cells. α-Adducin phosphorylated at Thr445 was undetectable in serum-starved COS7 cells expressing HA-α-adducin alone. The expression of dominant active RhoA (RhoA V14), a RhoA mutant that is defective in GTPase activity and thought to be constitutively the GTP-bound form in the cells, induced a small increment of the α-adducin phosphorylation at Thr445, whereas the expression of dominant negative RhoA (RhoAN19), a RhoA mutant that preferentially binds GDP rather than GTP and is thought to be constitutively the GDP-bound form in the cells, had minimal effects. Although the exact reason for the minimal increase in phosphorylated α-adducin in COS7 cells expressing RhoAN19 is unknown, it is likely that the overexpression of GDP-bound RhoA N19 induced the phosphorylation of α-adducin to a minimal extent because GDP • Rho also activates Rho-kinase in vitro to a small extent as described (Matsui et al., 1996). Dominant active Rac1 (Rac1 V12) or dominant active Cdc42 (Cdc42 V12) had no effects. Under the conditions, RhoA V14, RhoAN19, Rac1 V12, and Cdc42 V12 were almost equally expressed (data not shown). The expression of Rho-kinase induced the α-adducin phosphorylation to some extent. By the coexpression of RhoA V14 and Rho-kinase, the α-adducin phosphorylation was further enhanced. Rho-kinase is composed of catalytic (CAT), coiled-coil (COIL), Rho-binding (RB), and pleckstrin-homology (PH) domains (Matsui et al., 1996). Rac1 (941–1075 aa) binds to Rho and inhibits the Rho-dependent Rho-kinase activity in vitro and in vivo (Amano et al., 1997). RB/PH (TT) (941–1388 aa), which is the carboxy-terminal portion of Rho-kinase and encompasses the RB and PH domains, has point mutations in the RB domain and does not bind to Rho (Fujisawa et al., 1996; Leung et al., 1996; Amano et al., 1998). RB/PH (TT) inhibits the lysophosphatidic acid (LPA)-induced stress fiber formation in fibroblasts and neurite retraction in neuroblastoma cells (Amano et al., 1998), suggesting that RB/PH (TT) serves as the dominant negative form of Rho-kinase. Recently, we found that RB/PH (TT) interacted with the catalytic domain of Rho-kinase and thereby inhibited the Rho-kinase activity in vitro without titrating out Rho (Amano, M., K. Chihara, N. Nakamura, T. Kameko, Y. Matsuura, and K. Kaibuchi, manuscript submitted for publication). We further found that RB/PH (TT) inhibited the lysophosphatidic acid (LPA)-induced stress fiber formation in fibroblasts and neurite retraction in neuroblastoma cells (Amano et al., 1998), suggesting that RB/PH (TT) serves as the dominant negative form of Rho-kinase in vitro without titrating out Rho (Amano, M., K. Chihara, N. Nakamura, T. Kameko, Y. Matsuura, and K. Kaibuchi, manuscript submitted for publication). We further found that RB/PH (TT) inhibited the lysophosphatidic acid (LPA)-induced stress fiber formation in fibroblasts and neurite retraction in neuroblastoma cells (Amano et al., 1998), suggesting that RB/PH (TT) serves as the dominant negative form of Rho-kinase. Recently, we found that RB/PH (TT) interacted with the catalytic domain of Rho-kinase and thereby inhibited the Rho-kinase activity in vitro without titrating out Rho (Amano, M., K. Chihara, N. Nakamura, T. Kameko, Y. Matsuura, and K. Kaibuchi, manuscript submitted for publication). We further found that RB/PH (TT) had no effect on catalytic activity of PKN or myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), which is identified as a target of Cdc42 and is homologous to Rho-kinase within the kinase domain (Leung et al., 1998), in vitro (Amano et al., 1997). RB/PH (TT) inhibited the lysophosphatidic acid (LPA)-induced stress fiber formation in fibroblasts and neurite retraction in neuroblastoma cells (Amano et al., 1998), suggesting that RB/PH (TT) serves as the dominant negative form of Rho-kinase. Recently, we found that RB/PH (TT) interacted with the catalytic domain of Rho-kinase and thereby inhibited the Rho-kinase activity in vitro without titrating out Rho (Amano, M., K. Chihara, N. Nakamura, T. Kameko, Y. Matsuura, and K. Kaibuchi, manuscript submitted for publication). We further found that RB/PH (TT) had no effect on catalytic activity of PKN or myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), which is identified as a target of Cdc42 and is homologous to Rho-kinase within the kinase domain (Leung et al., 1998), in vitro (Amano et al., 1997). RB/PH (TT) inhibited the lysophosphatidic acid (LPA)-induced stress fiber formation in fibroblasts and neurite retraction in neuroblastoma cells (Amano et al., 1998).
Specific Phosphorylation of α-Adducin at Thr445 in the TPA-induced Membrane Ruffling Areas

Both Rho and Rac are thought to be necessary for the HGF- and TPA-induced membrane ruffling and HGF-induced cell motility in MDCK epithelial cells, although neither RhoA V14 nor Rac1V12 by itself induces membrane ruffling or cell motility (Takaishi et al., 1993, 1994, 1995; Nishiyama et al., 1994; Ridley et al., 1995). We examined the distribution of Thr445-phosphorylated α-adducin in the TPA- or HGF-stimulated MDCK cells. We have found by immunoblotting with isofrom-specific antibodies that 120-kD α-adducin and 90-kD γ-adducin (alternatively spliced form of 80-kD γ-adducin) were expressed almost equally in MDCK cells. We have also found that 110-kD β-adducin was expressed to a lesser extent in MDCK cells. Therefore, it is likely that anti-pT445 recognizes only phospho-Thr445 in α-adducin in MDCK cells. The addition of TPA induced membrane ruffling at the outer edge of cell colonies (Fig. 4 A) as described (Nishiyama et al., 1994; Takaishi et al., 1993). The immunoreactivity of anti-pT445 accumulated in the membrane ruffling areas, where F-actin (Fig. 4 A) and spectrin (data not shown) also accumulated. Phosphorylated α-adducin was also detected in the perinuclear region. Similar results were obtained when the cells were stimulated with HGF instead of TPA (Fig. 4 A). The immunoreactivity with anti-pT445 was abolished by preincubation of the antibody with the antigen phosphopeptide (data not shown). In the nonstimulated MDCK cells, phosphorylated α-adducin was diffusely present in the cytoplasm, but not in the free ends of the plasma membrane. Between 2 and 16 h after the addition of TPA, the cells dissociated from each other and scattered, with polarized morphology and membrane ruffling in the leading edge as described (Takaishi et al., 1994; Ridley et al., 1995). Phosphorylated α-adducin also accumulated in the leading edge of TPA-induced scattering cells (Fig. 4 A). To compare the localization of phosphorylated α-adducin and total α-adducin, a double-label immunofluorescence study was performed in MDCK cells stably expressing HA-α-adducin. Immunoreactivity showing the distribution of HA-α-adducin (red) was detected strongly in the cell-cell contact sites (Fig. 4 B, asterisk), weakly in the membrane ruffling areas (Fig. 4 B, arrow), and diffusely in the cytoplasm, as described (Kaiser et al., 1989; Marrs et al., 1993). Phosphorylated α-adducin (green) was enriched in the membrane ruffling areas in the TPA-stimulated cells (Fig. 4 B).
ulated MDCK cells (Fig. 4B, arrow). The merged image of red and green immunofluorescence revealed the enrichment of phosphorylated α-adducin in the membrane ruffling areas and a small increment of phosphorylated α-adducin in the cytoplasm in the TPA-stimulated cells (Fig. 4B, panel f, greenish image). Phosphorylated α-adducin did not accumulate at the cell-cell contact sites (Fig. 4B, panels c and f, reddish images indicated by the asterisks). Similar results were obtained when the cells were stimulated with HGF. To confirm that α-adducin is specifically phosphorylated at Thr445 during the action of TPA, immunoblot analysis with anti-pT445 was performed. The addition of TPA to the MDCK cells stably expressing HA-α-adducin increased the phosphorylation level of HA-α-adducin at Thr445 (Fig. 4C).

Figure 4. The localization of Thr445-phosphorylated α-adducin in the TPA- or HGF-induced membrane ruffling. (A) The serum-deprived MDCK cells were stimulated with 200 nM TPA (panels d–f) or 50 pM HGF (panels g–i) for 15 min or with 200 nM TPA for 2 h (panels j–l). Nonstimulated (panels a–c) and stimulated cells were doubly labeled with anti-pT445 (panels b, e, h, and k) and TRITC-phalloidin (panels c, f, i, and l). Phase-contrast images are shown (panels a, d, g, and j). Arrowheads indicate the induced membrane ruffling areas or the leading edges. Bar, 25 μm. (B) The enrichment of phosphorylated α-adducin in the membrane ruffling areas. MDCK cells stably expressing HA-α-adducin were established. Nonstimulated (panels a–c) or TPA-stimulated (panels d–f) MDCK cells stably expressing HA-α-adducin were doubly stained with anti-pT445 (green; panels a and d) and anti-HA (red; panels b and e). c and f show merged images of a and b, and d and e, respectively. Arrows indicate the induced membrane ruffling areas. Asterisks indicate the cell-cell contact sites. Bar, 25 μm. (C) The TPA-induced Thr445 phosphorylation in α-adducin. MDCK cells stably expressing HA-α-adducin were stimulated by vehicle (–) or TPA (+) for 15 min followed by incubation in the presence of calyculin A, and then lysed and subjected to immunoblot analysis with anti-pT445. An arrow indicates the phosphorylated HA-α-adducin. These results are representative of three independent experiments.

The Inhibition of the TPA-induced Membrane Ruffling by the Dominant Negative Rho-Kinase and α-Adducin Mutant

We next examined whether the Rho/Rho-kinase pathway was required for the TPA-induced membrane ruffling. The microinjection of C3, which is thought to interfere with endogenous Rho functions, RB, or RB/PH (TT) inhibited the TPA-induced membrane ruffling and the accumulation of phosphorylated adducin in the free end of the plasma membrane (Fig. 5, A and B); membrane ruffling was induced in 64% of TPA-stimulated cells in the outer edges of cell colonies, but in only 14% of cells injected with C3, 15% of cells injected with RB, and 22% of cells injected with RB/PH (TT). Dominant negative Rac (Rac1N17) also inhibited the TPA-induced membrane ruf-
Figure 5. The inhibition of the TPA-induced membrane ruffling by the mutants of Rho-kinase and α-adducin. (A) The serum-deprived MDCK cells were microinjected with MBP (2 mg/ml; panel a), C3 (0.1 mg/ml; panel b), GST-RB (2 mg/ml; panel c), MBP-RB/PH (TT) (2 mg/ml; panels d and e), or Rac1N17 (0.2 mg/ml; panel f), HA-α-adducin (WT) (5 mg/ml; panel g), HA-α-adducin-AA (5 mg/ml; panel h), or HA-α-adducin-DD (5 mg/ml; panel i) along with a marker protein (rabbit or mouse IgG, 0.5 mg/ml). After a 30-min incubation, the cells were stimulated with 200 nM TPA for 15 min. F-actin (panels a–d and f–i) and Thr445-phosphorylated α-adducin (panel e) were visualized. Arrowheads indicate the microinjected cells. Bar, 25 μm. (B) The ratios of the membrane ruffling–induced cells to the cells injected with proteins described in A are indicated. Data are means ± SEM of triplicate determinations. (C) Specificity of the effect of HA-α-adducin-AA on the TPA-induced membrane ruffling. HA-α-adducin-AA (5 mg/ml) was microinjected along with indicated proteins. The ratios of the membrane ruffling–induced cells to the injected cells are indicated. Data are means ± SEM of triplicate determinations. (D) HA-α-adducin-A A had no effect on stress fiber formation. The serum-deprived NIH3T3 cells were microinjected with MBP (2 mg/ml; panel a), MBP-RB/PH (TT) (2 mg/ml; panel b), or HA-α-adducin-A A (5 mg/ml; panel c). After a 30-min incubation, the cells were stimulated with 50 ng/ml LPA for 20 min. F-actin was visualized. Arrowheads indicate the microinjected cells. Bar, 10 μm. These results are representative of three independent experiments.
To determine whether the phosphorylation of adducin by Rho-kinase is necessary or sufficient for the membrane ruffling, we produced and used cDNA encoding α-adducin mutants; HA-α-adducinT445A,T480A (HA-α-adducin-A A, substitution of Thr residues by Ala), which was not phosphorylated by Rho-kinase and expected to serve as the dominant negative form, and HA-α-adducinT445D,T480D (HA-α-adducin-DD, substitution of Thr residues by Asp), which was expected to mimic phosphorylated adducin and to serve as the constitutively active form (Sweeney et al., 1994). The microinjection of HA-α-adducin, HA-α-adducin-A A, or HA-α-adducin-DD by itself did not induce the membrane ruffling in MDCK cells (data not shown). This observation is consistent with the notion that RhoA\textsuperscript{V14} alone does not induce the membrane ruffling, and that a lot of processes are required for the induction of membrane ruffling. HA-α-adducin or HA-α-adducin-DD was translocated to the membrane ruffling area (data not shown) and did not inhibit the TPA-induced membrane ruffling, whereas HA-α-adducin-A A inhibited the ruffling; membrane ruffling was induced in ~60% of TPA-stimulated cells injected with HA-α-adducin or HA-α-adducin-DD, but in only 24% of cells injected with HA-α-adducin-A A (Fig. 5 A and B). Although the inhibitory mechanism of HA-α-adducin-A A is not known, HA-α-adducin-A A may substitute for endogenous α-adducin to oligomerize with endogenous β- or γ-adducin, and thereby stay as an inactive complex to inhibit functions of endogenous adducins. Essentially similar results as to the effects of TPA/PH (TT) or HA-α-adducin-A A on membrane ruffling were obtained when MDCK cells were stimulated with HGF instead of TPA (data not shown). To confirm that HA-α-adducin-A A specifically inhibits the function of endogenous adducin but not those of other substrates of Rho-kinase such as MLC or the ERM family proteins, we examined whether the inhibitory effect of HA-α-adducin-A A on the membrane ruffling in MDCK cells was reversed by coinjection of HA-α-adducin or HA-α-adducin-DD. As shown in Fig. 5 C, HA-α-adducin reversed the inhibitory effect of HA-α-adducin-A A in a dose-dependent manner. HA-α-adducin-DD more efficiently reversed the inhibitory effect. These results indicate that HA-α-adducin-A A specifically inhibits the function of endogenous adducin but not those of other substrates. When HA-α-adducin-A A was microinjected into NIH 3T3 cells, HA-α-adducin-A A did not affect the LPA-induced formation of stress fibers, which is mediated by MLC phosphorylation through the Rho/Rho-kinase pathway (Chihara et al., 1997; A mano et al., 1998), whereas RBP/PH (TT) inhibited the LPA-induced formation of stress fibers as described (A mano et al., 1998; Fig. 5 D). HA-α-adducin-A A did not affect the LPA-induced formation of microvilli, which is mediated by ERM phosphorylation through the Rho/Rho-kinase pathway, under the conditions in which RBP/PH (TT) inhibited the LPA-induced formation of microvilli (Oshiro et al., 1998; data not shown). Taken together, these results suggest that HA-α-adducin-A A specifically interferes with the pathway mediated through the α-adducin phosphorylated by Rho-kinase in intact cells.

When HA-α-adducin-DD, but not HA-α-adducin-A A, was microinjected with RBP or RBP/PH (TT), the inhibitory effect of RBP or RBP/PH (TT) on the TPA-induced membrane ruffling was counteracted (Fig. 6 A and B). Under these conditions, HA-α-adducin also counteracted the inhibitory effect of RBP/PH (TT), but not that of RBP (Fig. 6 B). This difference is likely due to the distinct mode of action between RBP and RBP/PH (TT). RBP/PH (TT) interacts with the catalytic domain of Rho-kinase and inhibits the activity of Rho-kinase in a manner competitive with peptide substrates of Rho-kinase such as RRLSSLRA without titrating out Rho (A mano, M., K. Chihara, N. Nakamura, T. Kaneko, Y. Matsuura, and K. Kaibuchi, manuscript submitted for publication). The excess of HA-α-adducin over RBP/PH (TT) in the cells may neutralize a competitive inhibition of Rho-kinase by RBP/PH (TT), and then HA-α-adducin can be phosphorylated by the activated Rho-kinase. Consistently, when RBP/PH (TT) was microinjected at higher concentrations, HA-α-adducin could not counteract the inhibitory effect of RBP/PH (TT), whereas HA-α-adducin-DD was still capable of counteracting (data not shown). In contrast to RBP/PH (TT), RBP binds to the effector domain of Rho, and is expected to inhibit the interaction of Rho-kinase with Rho and thereby the activation of Rho-kinase by Rho (A mano et al., 1997). Thus, HA-α-adducin may not overcome the inhibitory effect of RBP in MDCK cells. On the other hand, because HA-α-adducin-DD can mimic α-adducin phosphorylated at Thr445 and Thr480 in vivo, HA-α-adducin-DD may bypass Rho-kinase to induce membrane ruffling during the action of TPA irrespective of the Rho-kinase activity. HA-α-adducin-DD counteracted neither the inhibitory effect of C3 nor that of Rac1\textsuperscript{N17} (Fig. 6, A and B). GTPγS-bound RhoA\textsuperscript{V14}, which is insensitive to C3, could completely overcome the inhibitory effect of C3 as described (Nishiyama et al., 1994; data not shown). Taken together, these results indicate that HA-α-adducin-DD serves as the constitutively active form, and that α-adducin is a crucial substrate of Rho-kinase downstream of Rho for membrane ruffling.

Adducin Phosphorylation via the Rho/Rho-Kinase Pathway and Cell Motility

Membrane ruffling is thought to be an essential event in cell motility (Mitchison and Cramer, 1996). We then investigated whether the α-adducin phosphorylation by Rho-kinase is involved in cell motility. C3 has been shown to inhibit the wound-induced migration of IEC-6 cells (Santos et al., 1997). We performed a wound healing assay by the use of NRK 49F cells to investigate roles of Rho-kinase and adducin in cell motility, since it was difficult to estimate the precise distance moved in MDCK cells and to evaluate cell motility induced by TPA or HGF, and NRK 49F cells were more suitable for a wound healing assay than were MDCK cells. Linear wounds were made in a confluent monolayer of NRK 49F cells and ~200 cells along the wound edge were microinjected with C3. RBP/PH (TT), or the α-adducin mutants. 6 h after wounding, the cells that moved (>20 μm) from the original wound edge were counted. We also evaluated the wound-induced migration by a time-lapse recording. When MBP was in-
jected as a control protein, 63% of the total injected cells showed wound-oriented migration, and ~60% of the total injected cells had membrane ruffling (Fig. 7, B and C). About 90% of the migrating injected cells had membrane ruffling in their leading edges (Fig. 7 B). We found that α-adducin phosphorylated at Thr445 accumulated at the membrane ruffling areas in the leading edge and the perinuclear region in migrating NRK49F cells (Fig. 7 A), as shown in MDCK cells. C3 and RB/PH (TT) inhibited the migration to the wound; only 24% of cells injected with C3 and 17% of cells injected with RB/PH (TT) migrated to the wound (Fig. 7, B and C). The microinjection of HA-α-adducin-DD also inhibited the migration; only 21% of cells injected with HA-α-adducin-DD migrated to the wound (Fig. 7, B and C). When C3, RB/PH (TT), or α-adducin-DD was injected, the percentages of the cells with membrane ruffling in the total injected cells (~20%) were roughly the same as those of the migrating cells in the total injected cells (17–24%), and the stationary injected cells had few membrane ruffles (Fig. 7 B). Whatever proteins were injected, the majority (~90%) of migrating injected cells had membrane ruffling in the leading edge. These results suggest that the inhibition of cell migration by HA-α-adducin-DD, C3, or RB/PH (TT) correlates with the inhibition of membrane ruffling. In the HA-α-adducin-injected cells, the wound-oriented migration and membrane ruffling were not inhibited (Fig. 7, B and C). HA-α-adducin-DD also inhibited the migration; 32% of cells injected with HA-α-adducin-DD migrated to the wound (Fig. 7, B and C). However, the percentage of the cells with membrane ruffling in the total injected cells (~60%) was much bigger than that of the migrating cells in the total injected cells (32%). The majority of migrating cells injected with α-adducin-DD had membrane ruffles in the leading edge, and about one-half of the stationary cells injected with α-adducin-DD had membrane ruffles (Fig. 7 B, panel k). These results indicate that α-adducin-DD did not inhibit the membrane ruffling but did inhibit cell migration, suggesting that cycling between the phosphorylated and dephosphorylated states of α-adducin appears to be necessary for cell migration, but not for membrane ruffling. Consistently, RhoA V14 or CAT as well as Rac1 N17 inhibited the wound-induced migration (data not shown).

Discussion

Phosphorylation Sites of α-Adducin by Rho-Kinase and Site- and Phosphorylation State–specific Antibodies

In this study, we identified the sites of phosphorylation of α-adducin by Rho-kinase as Thr445 and Thr480 in the neck domain. α-Adducin is phosphorylated at Ser408, Ser436, and Ser481 in the neck domain by PKA, and at Ser726 in the MARCKS-related domain by PKC and PKA (Matsuoka et al., 1996). Among the in vitro phosphorylation sites of α-adducin, Thr445 and Thr480 are unique for Rho-kinase. We raised and used a site- and phosphorylation state–specific antibody for α-adducin. A anti-pT445 recognized HA-α-adducin phosphorylated by Rho-kinase, but not by PKC or PKN. A anti-pT445 recog-
Figure 7. Effects of the mutants of Rho-kinase and α-adducin on cell migration in a wound healing assay. (A) The localization of Thr445-phosphorylated α-adducin in the migrating NRK 49F cell. NRK 49F cells which migrated in response to the wounding were stained with TRITC-phalloidin (panel a) and anti-pT445 (panel b). Arrows indicate the direction of migration. Arrowheads indicate the membrane ruffling in the leading edge. Bar, 20 μm. (B) A confluent monolayer of NRK 49F cells was linearly wounded with a white chip. Soon after wounding, MBP (5 mg/ml; panels a and b), C3 (0.1 mg/ml; panels c and d), MBP-RB/PH (TT) (5 mg/ml; panels e and f), HA-α-adducin (WT) (5 mg/ml; panels g and h), HA-α-adducin-AA (5 mg/ml; panels i and j), or HA-α-adducin-DD (5 mg/ml; panels k and l) was microinjected along with a marker protein (rabbit IgG, 1.0 mg/ml) into the cytoplasm of only the cells along the wound edge. The original wound edge was determined by taking photographs soon after the cells were wounded and microinjected, and then the cells that moved (>20 μm) from the original wound edge were counted by taking photographs of the same fields after 6 h. The cells were doubly labeled by staining with TRITC-phalloidin (panels a, c, e, g, i, and k) and FITC anti–rabbit IgG Ab (panels b, d, f, h, j, and l). Arrows indicate the positions of the migrating wound edge cells. Arrowheads indicate the injected cells. Morphologies of the injected cells that showed the typical phenotypes were shown in insets. Bar, 20 μm. These results are representative of three independent experiments. (C) The ratios of migrating cells to the total injected cells are indicated. Data are means ± SEM of triplicate determinations.
nized neither nonphosphorylated HA-α-adducin nor HA-α-adducin\(^{\text{445A}, \text{T480A}}\) incubated with Rho-kinase and ATP (Fig. 2 A). These results indicate that anti-pT445 is a useful tool with which to detect the specific phosphorylation of α-adducin by Rho-kinase both in vitro and in vivo, distinguishing from the phosphorylation of α-adducin by PKC. Although Rho-kinase phosphorylated β- and γ-adducin in vitro (Fig. 2 B), the phosphorylation sites have not been determined. Since the surrounding amino acid sequences of Thr437 in rat γ-adducin (Q Q R E K T R W L N S) are completely identical to those of Thr445 in α-adducin (selected as antigen phosphopeptide for anti-pT445), the result that anti-pT445 did not cross-react with rat γ-adducin phosphorylated by Rho-kinase (Fig. 2 B) revealed that Rho-kinase does not primarily phosphorylate γ-adducin at Thr437. The surrounding amino acid sequences of Thr431 in β-adducin (Q Q K E T R W L N T) are a little bit different from those of Thr445 in α-adducin. At this stage, we do not know whether Rho-kinase phosphorylates β-adducin at Thr431. A ducin is thought to be comprised of α- and β-, or α- and γ-adducin in vivo (Bennett et al., 1988; Dong et al., 1995). We found that in MDCK and NRK49F cells a heterodimer of adducin which consists of α- and γ-adducin was mainly expressed (data not shown). Therefore, it is likely that anti-pT445 recognizes only phospho-Thr445 in α-adducin but not in β- and γ-adducin in both cells (see below). Further analysis is necessary to understand how the phosphorylation of β- or γ-adducin is regulated through Rho-kinase in vitro and in vivo.

**In Vivo Phosphorylation of α-Adducin via the Rho/Rho-Kinase Pathway**

Using anti-pT 445, we found that Thr445 phosphorylation of α-adducin occurred in a Rho/Rho-kinase-dependent manner in COS7 cells (Fig. 3). We further found that α-adducin was phosphorylated at Thr445 in the TPA-stimulated MDCK cells (Fig. 4 C). TPA also drives the activation of PKC. Indeed, TPA induces the phosphorylation of PKC. Although Rho-kinase phosphorylated α-adducin in vitro (Fig. 2 B), the phosphorylation sites have not been determined. Since the surrounding amino acid sequences of Thr437 in rat γ-adducin (Q Q R E K T R W L N S) are completely identical to those of Thr445 in α-adducin (selected as antigen phosphopeptide for anti-pT445), the result that anti-pT445 did not cross-react with rat γ-adducin phosphorylated by Rho-kinase (Fig. 2 B) revealed that Rho-kinase does not primarily phosphorylate γ-adducin at Thr437. The surrounding amino acid sequences of Thr431 in β-adducin (Q Q K E T R W L N T) are a little bit different from those of Thr445 in α-adducin. At this stage, we do not know whether Rho-kinase phosphorylates β-adducin at Thr431. A ducin is thought to be comprised of α- and β-, or α- and γ-adducin in vivo (Bennett et al., 1988; Dong et al., 1995). We found that in MDCK and NRK49F cells a heterodimer of adducin which consists of α- and γ-adducin was mainly expressed (data not shown). Therefore, it is likely that anti-pT445 recognizes only phospho-Thr445 in α-adducin but not in β- and γ-adducin in both cells (see below). Further analysis is necessary to understand how the phosphorylation of β- or γ-adducin is regulated through Rho-kinase in vitro and in vivo.

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**Involvement of the Phosphorylation of α-Adducin via the Rho/Rho-Kinase Pathway in Membrane Ruffling and Cell Motility**

Membrane ruffling, which is induced by several growth factors and is observed in the leading edges of motile cells, is thought to play an essential role in cell motility. The regulatory mechanism of the membrane ruffling is less well characterized. Not only Rac but also Rho is thought to regulate the membrane ruffling downstream of extracellular signals in certain types of cells, because C3 inhibits the TPA- and HGF-induced membrane ruffling in epithelial cells such as MDCK and KB cells (Nishiyama et al., 1994; Takaishi et al., 1995). Here, we found that Thr445-phosphorylated α-adducin accumulated at the TPA-induced membrane ruffling areas and the leading edges of migrating cells (Figs. 4 and 7 A). We furthermore found that Rho and RB/PH (TT) as well as C3 inhibited the TPA-induced membrane ruffling. The fact that α-adducin-DD specifically counteracted the inhibitory effect of Rho but not that of C3 (Fig. 6 B) is probably due to difference between the inhibitory mechanisms by Rho and C3. Rho is the fragment of Rho-kinase which consists of the Rho domain of Rho-kinase. Rho binds to the effector domain of Rho, and then inhibits the interaction of Rho with Rho-kinase (Amano et al., 1997). Recent studies revealed that the various effector mutants of RhoA\(^{\text{V14}}\), in which interaction with some effectors is maintained but with others is lost, make it possible to dissect the diverged pathways downstream of Rho. For example, RhoA\(^{\text{T42C}}\) abolishes interaction with PKN but maintains interaction with ROCK-1 (an isoform of Rho-kinase) and RhoA\(^{\text{F38L,C20R}}\) maintains interaction with PKN but abolishes interaction with ROCK-1 (Sahai et al., 1998). RhoA\(^{\text{T42C}}\) induces stress fiber formation, whereas RhoA\(^{\text{F38L,C20R}}\) does not induce stress fiber formation, suggesting that ROCK-1 but not PKN is required for stress fiber formation. These observations indicate that there are distinct interfaces in the effector domain of Rho for different classes of effector proteins. Although at this time we cannot completely rule out the possibility that RB inhibits interaction of Rho with the other Rho targets such as PKN and p140mDia, RB may inhibit preferentially the interaction of Rho with Rho-kinase rather than with other Rho targets. On the other hand, C3 interferes with the whole functions of Rho. In fact, C3 inhibits the activity of PKN as well as Rho-kinase in Swiss 3T3 cells (Amano et al., 1996) and the Rho-dependent recruitment of p140mDia to the dynamic membrane structures in Swiss 3T3 cells (Watanabe et al., 1997). C3 may inhibit the membrane ruffling by impairing the whole functions of Rho, whereas Rho may inhibit the membrane ruffling by mainly (or specifically) impairing the function of Rho-kinase.
We also found that not only C3 and RB/PH (TT) but also α-adducin-AA inhibited cell motility as well as membrane ruffling in NRK fibroblasts (Fig. 7, B and C). This suggests that α-adducin is one of the substrates for Rho-kinase involved in cell motility, probably regulating the membrane ruffling in the leading edges of motile cells. As for the substrates of Rho-kinase, MLC is another major substrate which is thought to play an important role in cell motility. Indeed, injection of anti-MLC kinase antibody diminishes the cell motility of macrophages (Wilson et al., 1991). Moreover, phosphorylated MLC is enriched in both the leading edges and rear ends of motile fibroblasts and epithelial cells (Matsunaga et al., 1998), suggesting that a force derived from myosin-actin filament driven by the MLC phosphorylation contributes to cell motility. Thus, Rho, acting through Rho-kinase, appears to regulate cell motility through the spatial and temporal regulation of phosphorylation of certain substrates including adducin and MLC.

It should be noted that in Swiss 3T3 or NIH 3T3 fibroblasts, in which the dominant active Rac by itself is sufficient to induce the membrane ruffling (Hall, 1998), α-adducin phosphorylated at Thr445 was not observed in the dominant active Rac-induced ruffling areas, and neither C3, RB/PH (TT), nor HA-α-adducin-AA inhibited the dominant active Rac-induced ruffling (data not shown). On the other hand, we found that in KB epithelial cells as well as MDCK epithelial cells, α-adducin phosphorylated at Thr445 accumulated in the TPA-induced membrane ruffling areas, and that C3 and RB/PH (TT) inhibited the TPA-induced membrane ruffling (data not shown). When RB/PH (TT) or α-adducin-AA was injected to MDCK cells, the HGF-induced membrane ruffling was also inhibited as the TPA-induced membrane ruffling (data not shown). The regulatory system of the membrane ruffling may vary among cell types. It is conceivable that the membrane ruffling in a certain type of cell such as MDCK epithelial cells may require not only the Rac-mediated pathway but also the phosphorylation of adducin by the Rho/Rho-kinase pathway, whereas in other types of cells such as Swiss 3T3 fibroblasts may require only the Rac-mediated pathway. Further studies are necessary to determine what causes the differences between the two types of cell lines.

**Regulation of α-Adducin Function through Phosphorylation by Rho-Kinase and PKC**

These results indicate that the phosphorylation of α-adducin by the Rho/Rho-kinase pathway plays a crucial role in the regulation of membrane ruffling and cell motility. What is the function of the α-adducin phosphorylation in membrane ruffling and cell motility? Membrane ruffles contain complicated cytoskeletal structures composed of F-actin and other F-actin-associated proteins (Mitchison and Cramer, 1996). Spectrin, which binds to F-actin beneath plasma membrane and forms a membrane cortical meshwork, also accumulates at the leading edges of motile cells (Burridge et al., 1982; Sormunen and Lehto, 1995). This lattice-like meshwork is dynamically reconstructed and required for the formation of membrane ruffling (Lauffenburger and Horwitz, 1996). Since the phosphorylation of α-adducin by Rho-kinase is known to promote the binding of α-adducin to F-actin (Kimura et al., 1998) and this in turn induces the recruitment of spectrin to F-actin (Gardner and Bennett, 1987; Bennett et al., 1988), it is possible that the formation of a spectrin-F-actin meshwork is dynamically regulated in the active membrane ruffling by the Rho/Rho-kinase pathway (Fig. 8). It is also possible that Rho-kinase modulates the F-actin-capping activity (Li et al., 1998) or cross-linking activity (Mische et al., 1987) of adducin and then regulates actin dynamics in the membrane ruffling areas. Adducin is also a substrate of PKC, which is activated in response to TPA or HGF, and the phosphorylation of α-adducin by PKC inhibits the activity of adducin in promoting the formation of a spectrin-actin complex in vitro (Matsunaga et al., 1998). TPA induces the phosphorylation of α-adducin at Ser726 and the redistribution of α-adducin phosphorylated at Ser726 from the cell membrane to cytoplasm accompanied by that of spectrin in renal proximal tubule epithelial and MDCK cells (Fowler et al., 1998; Matsunaga et al., 1998). Interest-
ingly, α-adducin phosphorylated at Ser726 was also observed in the membrane ruffling areas (Matsuka, Y., and V. Bennett, manuscript in preparation). It is conceivable that α-adducin phosphorylated by PKC dissociates from a spectrin-F-actin meshwork during membrane ruffling. Thus, it is tempting to speculate that the adducin function may be cyclically regulated through the phosphorylation by Rho-kinase and PKC during the action of TPA or HGF, and the alternative phosphorylation contributes to the turnover of a spectrin-F-actin meshwork for membrane ruffling. In this regard, it is interesting that turnover of a spectrin-F-actin meshwork during membrane ruffling is cyclically regulated through the phosphorylation by Rho-kinase and PKC during the action of TPA or HGF.


Adducin Phosphorylation Downstream of Rho


