

Phosphorylation of adducin by Rho-kinase,  
an effector of the small GTPase Rho,  
plays a crucial role in cell motility.

(低分子量G蛋白質Rhoの標的蛋白質Rho-kinase  
によるadducinのリン酸化の生理的意義の解析)

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## **Summary**

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  $\alpha$ -adducin and thereby enhances the F-actin-binding activity of  $\alpha$ -adducin *in vitro*. Here I identified the sites of phosphorylation of  $\alpha$ -adducin by Rho-kinase as Thr445 and Thr480. I prepared antibody that specifically recognized  $\alpha$ -adducin phosphorylated at Thr445, and found by use of this antibody that Rho-kinase phosphorylated  $\alpha$ -adducin at Thr445 in COS7 cells in a Rho-dependent manner. Phosphorylated  $\alpha$ -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 tetradecanoylphorbol-13-acetate (TPA) or hepatocyte growth factor (HGF). The microinjection of *botulinum* C3 ADP-ribosyltransferase, dominant negative Rho-kinase or  $\alpha$ -adducin<sup>T445A, T480A</sup> (substitution of Thr445 and Thr480 by Ala) inhibited the TPA-induced membrane ruffling in MDCK cells and wound-induced migration in NRK49F cells.  $\alpha$ -Adducin<sup>T445D, T480D</sup> (substitution of Thr445 and Thr480 by Asp), but not  $\alpha$ -adducin<sup>T445A, T480A</sup>, 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  $\alpha$ -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.

## ***Introduction***

Membrane ruffling, a dynamically fluctuating movement of protrusion consisting of filopodia and lamellipodia, is rapidly induced in response to certain extracellular signals and also induced in the leading edges of motile cells (Stossel, 1993; Lauffenburger 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; Takaishi 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 (Gong et al., 1996; Hirata et al., 1992), neurite retraction in neuronal cells (Nishiki et al., 1990; Jalink 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 its specific targets (Van Aelst and D'Souza-Schorey, 1997). A number of targets of Rho have been identified, including protein kinase N (PKN) (Watanabe et al., 1996; Amano et al., 1996b), Rho-kinase/ROK/ROCK (Leung et al., 1995; Matsui et al., 1996; Ishizaki et al., 1996), the myosin-binding subunit (MBS) of myosin phosphatase (Kimura et al., 1996), p140mDia (Watanabe et al., 1997), citron (Madaule et al., 1995), citron-kinase (Madaule et al., 1998), rhotekin, rhotekin (Reid et al., 1996), and kv1.2 (Cachero et al.,

1998). Rho-kinase, which is activated by Rho, regulates the phosphorylation of myosin light chain (MLC) by the direct phosphorylation of MLC (Amano et al., 1996a) and by the inactivation of myosin phosphatase through the phosphorylation of MBS (Kimura et al., 1996). Rho-kinase regulates the formation of stress fibers and focal adhesions (Leung et al., 1996; Amano et al., 1997; Ishizaki et al., 1997), smooth muscle contraction (Kureishi et al., 1997), and neurite retraction (Kato et al., 1998; Amano et al., 1998; Hirose et al., 1998) through the phosphorylation of MLC. Rho-kinase also phosphorylates the ERM (ezrin/radixin/moesin) family proteins (Matsui et al., 1998) and then regulates the formation of microvilli (Oshiro et al., 1998). However, it remains to be clarified how Rho regulates membrane ruffling and cell motility.

We recently identified adducin as an MBS-binding protein and a substrate of Rho-kinase *in vitro* (Kimura et al., 1998). Adducin is a membrane skeletal protein that binds to the fast growing ends (Kuhlman et al., 1996; Li et al., 1998) and the sides (Mische et al., 1987) of actin filaments (F-actin), and thereby promotes the association of spectrin with F-actin to form a spectrin-actin meshwork beneath plasma membranes (Gardner and Bennett, 1987; Bennett et al., 1988). Adducin is composed of either  $\alpha$  and  $\beta$ , or  $\alpha$  and  $\gamma$  subunits which are closely related in amino acid sequence and domain organization (Gardner and Bennett, 1986; Bennett et al., 1988; Dong et al., 1995). Each adducin subunit has three distinct domains: an amino-terminal head domain, a neck domain, and a carboxy-terminal tail domain

(Joshi and Bennett, 1990; Joshi et al., 1991; Dong et al., 1995). The tail domain has a domain called the myristoylated alanine-rich C kinase-substrate (MARCKS)-related domain, which contains a highly basic stretch and binds to calmodulin (Joshi, 1991; Dong et al., 1995; Matsuoka et al., 1996). Adducin is also a substrate for protein kinase C (PKC) and protein kinase A (PKA) (Palfrey and Waseem, 1985; Kaiser et al., 1989; Dong et al., 1995). The phosphorylation of adducin by PKA reduces the activity of adducin to associate with F-actin and spectrin-F-actin complexes and to recruit spectrin to F-actin (Matsuoka et al., 1996). The phosphorylation of adducin by PKC also inhibits activity of adducin in binding to F-actin and recruiting spectrin to F-actin (Matsuoka et al., 1998). On the other hand, the phosphorylation of adducin by Rho-kinase results in the interaction of adducin with F-actin *in vitro* (Kimura et al., 1998).

In the present study, I identified the sites of phosphorylation of  $\alpha$ -adducin by Rho-kinase as Thr445 and Thr480, and prepared antibody that specifically recognized  $\alpha$ -adducin phosphorylated at Thr445 to elucidate the functions of  $\alpha$ -adducin phosphorylated by Rho-kinase. I found that  $\alpha$ -adducin was phosphorylated at Thr445 via the Rho/Rho-kinase pathway in intact cells, and that the phosphorylation of  $\alpha$ -adducin by Rho-kinase plays a crucial role in membrane ruffling and cell motility.

## **Materials and Methods**

### **Materials and chemicals**

MDCK cells was kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). NRK49F cells was purchased from American Type Culture Collection (Rockville, MD). pEF-BOS-HA-RhoA<sup>V14</sup>, RhoA<sup>N19</sup>, Rac1<sup>V12</sup>, Cdc42<sup>V12</sup>, pEF-BOS-myc-Rho-kinase (full-length, 6-1388 aa), RB (941-1075 aa), RB/PH (TT) (941-1388 aa), and CAT [the catalytic domain of Rho-kinase (6-553 aa)] were constructed as described (Matsui et al., 1996; Amano et al., 1997, 1998; Kuroda et al., 1996). pcDSR $\alpha$ -PKC $\beta$  lacking C1 domain at positions 6-159 aa was constructed as described (Kaibuchi et al., 1989). pQE-rat  $\gamma$ -adducin fragment (319-671 aa; 35H fragment-4D) was kindly provided by Dr. S. Jaken (University of Vermont, VT), and 6 X His-tagged  $\gamma$ -adducin fragment was produced and purified as described (Dong et al., 1995). The expression plasmid of C3 transferase (pGEX-C3) was kindly provided by Dr. A. Hall (University College London, UK), and C3 was produced and purified as described (Fukata et al., 1998). Glutathione-S-transferase (GST)-RB was produced and purified from *Escherichia (E) coli* as described (Amano et al., 1997). GST-CAT was produced and purified from Sf9 cells as described (Matsuura et al., 1987; Amano et al., 1996a). Rac1<sup>N17</sup> was produced and purified from *E. coli* as described (Kuroda et al., 1996). Anti-HA (hemagglutinin) monoclonal antibody (Ab) (12CA5) was purchased from Boehringer Mannheim. [ $\gamma$ -<sup>32</sup>P]ATP was purchased from Amersham Corp. (Buckinghamshire, UK). All materials used in the nucleic acid study were purchased from Takara Shuzo Corp. (Kyoto,

Japan). Other materials and chemicals were obtained from commercial sources.

### **Plasmid Constructs**

The cDNA encoding human  $\alpha$ -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  $\alpha$ -adducin by the use of a baculovirus system, or into the *BamHI* site of pGEX-2T. The cDNA encoding human  $\beta$ -adducin (1-726 aa) (Joshi et al., 1991) was also inserted into the *KpnI* site of pAcYM1-HA. The cDNAs of  $\alpha$ -adducin<sup>T445A</sup>, T480A (AA) and  $\alpha$ -adducin<sup>T445D</sup>, T480D (DD), which were substituted for Thr at both 445 and 480 amino acid residues by Ala or Asp, were generated with the use of a site-directed mutagenesis kit (Stratagene, La Jolla, CA), and subcloned into pEF-BOS-HA, pAcYM1-HA, and pGEX-2T. The cDNAs of  $\alpha$ -adducin<sup>T445A</sup> and  $\alpha$ -adducin<sup>T480A</sup> were also generated as above and subcloned into pGEX-2T.

### **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- $\alpha$ -adducin, GST- $\alpha$ -adducin-AA, GST- $\alpha$ -adducin<sup>T445A</sup> and GST- $\alpha$ -adducin<sup>T480A</sup> were produced and purified from *E. coli*. HA- $\alpha$ -adducin, HA- $\beta$ -adducin, HA- $\alpha$ -adducin-AA and HA- $\alpha$ -adducin-DD were produced in Sf9 cells. The cells expressing HA- $\alpha$ -adducin were suspended in homogenizing buffer (20 mM Tris/HCl at pH 8.0, 1 mM EDTA, 1 mM DTT, 10  $\mu$ M A-PMSF, 10  $\mu$ g/ml leupeptin). The suspension was sonicated and centrifuged at 100,000 X *g* for 1 h at 4°C. The supernatant was applied onto a Mono Q column (Pharmacia

LKB Biotechnology, Uppsala, Sweden) which had been equilibrated with Buffer A (20 mM Tris/HCl at 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- $\alpha$ -adducin was eluted with about 200 mM NaCl and purified to near homogeneity. HA- $\beta$ -adducin, HA- $\alpha$ -adducin-AA, and HA- $\alpha$ -adducin-DD were prepared as HA- $\alpha$ -adducin.

***Determination of phosphorylation sites of  $\alpha$ -adducin by Rho-kinase***

HA- $\alpha$ -adducin (70  $\mu$ g of protein) was phosphorylated with GST-CAT (120 ng of protein) in 2 ml of kinase buffer (50 mM Tris/HCl at pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP for 1 h at 30°C, and the reaction product was digested with *Achromobacter* protease-1 (AP-1) at 37°C for 20 h. The obtained peptides were applied onto a C18 reverse phase column (4.6 X 250 mm, Shiseido Japan) and eluted with a linear gradient of 0-48% 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 (PPSQ-10, Shimadzu, Japan) as described (Bodwell et al., 1991). The fractions obtained from each Edman degradation cycle were measured for <sup>32</sup>P in a Beckman liquid scintillation counter.

***Production of site- and phosphorylation state-specific antibody for  $\alpha$ -adducin***

The phosphopeptide Cys-Gln<sup>440</sup>-Gln-Arg-Glu-Lys-phosphoThr-Arg-Trp-Leu-Asn-Ser<sup>450</sup> was chemically synthesized as an

antigen by Peptide Institute Inc. (Osaka, Japan). Rabbit polyclonal antibody (anti-pT445) was raised against the phosphopeptide and affinity purified as described (Inagaki et al., 1997). To examine the specificity of anti-pT445, equal amounts of HA- $\alpha$ -adducin (40 fmol) with various ratios between phosphorylated and non-phosphorylated proteins were subjected to SDS-PAGE. HA- $\alpha$ -adducin-AA (40 fmol) was incubated in kinase buffer containing GST-CAT and ATP, and subjected to SDS-PAGE. The amount of phosphates incorporated into HA- $\alpha$ -adducin was simultaneously determined by [ $\gamma$ - $^{32}$ P] ATP. Immunoblot analysis was then performed with anti-pT445, anti-pT445 which was preincubated with a 100-fold amount of antigen-phosphopeptide, and anti-HA Ab. For some experiments, HA- $\alpha$ -adducin, HA- $\beta$ -adducin, or 6 X His- $\gamma$ -adducin fragment was separately phosphorylated with GST-CAT, and 40 fmol (as  $^{32}$ P-incorporated amount) of the phosphorylated proteins were 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. NRK49F cells were maintained in DMEM containing 5% calf serum and 1% non-essential amino acids. To obtain MDCK cells stably expressing HA- $\alpha$ -adducin, MDCK cells were transfected with pEF-BOS-HA- $\alpha$ -adducin along with pSVIISR $\alpha$  vector containing the neomycin resistance gene using Lipofectamine (GIBCO BRL, Rockville, MD) as described

(Kuroda et al., 1998), and neomycin-resistant clones were selected.

***Detection of Thr445-phosphorylated  $\alpha$ -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- $\alpha$ -adducin and pEF-BOS-vector, pEF-BOS encoding-HA-Rac1<sup>V12</sup>, HA-Cdc42<sup>V12</sup>, HA-RhoA<sup>V14</sup>, HA-RhoA<sup>N19</sup>, myc-Rho-kinase (full-length), HA-RhoA<sup>V14</sup> and myc-Rho-kinase (full-length), HA-RhoA<sup>V14</sup> and myc-RB, HA-RhoA<sup>V14</sup> and myc-RB/PH (TT), myc-CAT, or pcDSR $\alpha$ -PKC $\beta$ . Separately, COS7 cells were cotransfected with pEF-BOS-HA- $\alpha$ -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% (w/v) trichloroacetic acid. The resulting precipitates were subjected to immunoblot analysis using anti-HA Ab and anti-pT445. MDCK cells stably expressing HA- $\alpha$ -adducin were incubated in the absence or the presence of tetradecanoylphorbol-13-acetate (TPA) for 15 min followed by the incubation in the presence of 0.1  $\mu$ M 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 pM recombinant human hepatocyte growth factor (HGF) (Calbiochem, La Jolla, CA) 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 Ig Ab. For a double immunofluorescence study, the clone stably expressing HA- $\alpha$ -adducin was stimulated as described above.

Phosphorylated adducin and HA- $\alpha$ -adducin were doubly stained with anti-pT445 and anti-HA Ab. The cells were then labeled with FITC-conjugated anti-rabbit Ig Ab and Texas red-conjugated anti-mouse Ig Ab. The cells were examined using a Zeiss axiophoto microscope, or a confocal microscope (Carl Zeiss, Oberkochen, Germany).

### ***Microinjection***

MDCK cells were seeded at a density of  $2.5 \times 10^3$  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 concentrated and during the concentration the buffer was replaced by microinjection buffer (20 mM Tris/HCl at pH 7.4, 20 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 5 mM 2-mercaptoethanol). The indicated proteins were microinjected along with a marker protein (rabbit or mouse IgG at 0.5 mg/ml) into the cytoplasm of cells. Using micro capillaries (tip diameter is 0.5  $\mu$ m), about  $3 \times 10^{-14}$  liter of each protein was microinjected by one injection. When C3 was microinjected at 0.1 mg/ml, the injected amount per cell was about  $3 \times 10^{-15}$  g (the intracellular concentration was estimated to be about 0.15  $\mu$ M). When HA- $\alpha$ -adducin mutants were

microinjected at 5.0 mg/ml, the injected amount per cell was about  $150 \times 10^{-15}$  g (the intracellular concentration was estimated to be about 2.0  $\mu$ M, and it seemed to be much higher than the level of endogenous  $\alpha$ -adducin). Thirty min after the microinjection, the cells were incubated in DMEM containing 200 nM TPA for 15 min at 37°C. The cells were then fixed and stained with anti-pT445 or TRITC-phalloidin as described above.

#### ***Wound healing assay***

Cell migration assay using a wound healing model was performed as described previously (Santos et al., 1997). The confluent monolayer of the cells was scraped with a white chip. Soon after wounding, the medium was changed and indicated recombinant proteins were microinjected along with a marker protein (rabbit IgG, 1.0 mg/ml) into the cytoplasm of only the cells along the wound edge as described above. The original wound edge was determined by taking photographs soon after the cells were wounded and microinjected. After 6 hours the cells were then stained with TRITC-phalloidin, and the cells that moved ( $> 20 \mu$ m) from the original wound edge were counted by taking photographs of the same fields as the original photograph.

#### ***Other Procedures***

SDS-PAGE was performed as described (Laemmli, 1970). Protein concentrations were determined with BSA as the reference protein as described (Bradford, 1976).

## **Results**

### ***Determination of the sites of phosphorylation of $\alpha$ -adducin by Rho-kinase***

The sites of phosphorylation of human  $\alpha$ -adducin by Rho-kinase were first examined as follows. HA-tagged  $\alpha$ -adducin, which was phosphorylated by Rho-kinase in the presence of [ $\gamma$ - $^{32}$ P]ATP, was digested with AP-I and subjected to reverse-phase HPLC as described (Shirataki et al., 1993). Two major radioactive peaks (named peak 1 and 2, respectively) were obtained (Fig. 1A) and their amino acid sequences were determined. The sequence obtained from peak 1 was TRWLNSGRGDEASEEGQNGSSPK, corresponding to residues 445-467 of human  $\alpha$ -adducin, and Thr445 turned out to be the phosphorylation site for Rho-kinase (Fig. 1B-a). The sequence obtained from peak 2 was EDGHRTSTSAVPNLFVPLNTNPK, corresponding to residues 475-497 of human  $\alpha$ -adducin, and Thr480 turned out to be the phosphorylation site for Rho-kinase (Fig. 1B-b). Both phosphorylation sites were in the neck domain of  $\alpha$ -adducin, and were different from previously identified sites of phosphorylation by protein kinase A (PKA) (Ser408, Ser436, Ser481 and Ser726) and by protein kinase C (PKC) (Ser726) (Matsuoka et al., 1996) (Fig. 2). When GST- $\alpha$ -adducin<sup>T445A</sup> or GST- $\alpha$ -adducin<sup>T480A</sup> (substitution of Thr445 or Thr480 by Ala) was phosphorylated by Rho-kinase, GST- $\alpha$ -adducin<sup>T480A</sup> was more efficiently phosphorylated by Rho-kinase than was GST- $\alpha$ -adducin<sup>T445A</sup> (Fig. 3). This result is apparently inconsistent with the observation that the radioactivity of peak 1 containing Thr 445 is lower than that of peak 2 containing Thr480 in the

HPLC analysis (Fig. 1A). 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  $\alpha$ -adducin***

An 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). I prepared and affinity purified the polyclonal antibody that recognized the Thr445-phosphorylated  $\alpha$ -adducin (anti-pT445), against the synthetic phosphopeptide; Cys-Gln-Gln-Arg-Glu-Lys-phosphoThr<sup>445</sup>-Arg-Trp-Leu-Asn-Ser. An immunoblot analysis revealed that anti-pT445 specifically bound to HA- $\alpha$ -adducin phosphorylated by Rho-kinase in a dose-dependent manner, but not to non-phosphorylated HA- $\alpha$ -adducin or HA- $\alpha$ -adducin<sup>T445A</sup>, T480A (substitution of Thr445 and Thr480 by Ala; HA- $\alpha$ -adducin-AA) incubated with Rho-kinase and ATP (Fig. 4A). HA- $\alpha$ -adducin, which was phosphorylated by PKC or PKN at levels similar to that by Rho-kinase, was not recognized by anti-pT445 (data not shown). The binding of anti-pT445 to  $\alpha$ -adducin phosphorylated by Rho-kinase was neutralized by preincubation of the antibody with the antigen phosphopeptide. Rho-kinase phosphorylated  $\beta$  and  $\gamma$ -adducin *in vitro* as well (Fig. 4B). Neither  $\beta$  nor  $\gamma$ -adducin

phosphorylated by Rho-kinase was recognized by anti-pT445 (Fig. 4B). These results indicate that anti-pT445 specifically recognizes only  $\alpha$ -adducin phosphorylated at Thr445.

***Rho/Rho-kinase dependent phosphorylation of  $\alpha$ -adducin in COS7 cells***

I examined whether  $\alpha$ -adducin was phosphorylated via the Rho/Rho-kinase pathway in intact cells by immunoblot analysis with anti-pT445. cDNA of HA- $\alpha$ -adducin was cotransfected into COS7 cells along with plasmids carrying the cDNA of dominant active Rho family members, or dominant active or negative Rho-kinase (Fig. 5). HA- $\alpha$ -adducin was almost equally expressed in all the HA- $\alpha$ -adducin-transfected cells.  $\alpha$ -Adducin phosphorylated at Thr445 was undetectable in serum-starved COS7 cells expressing HA- $\alpha$ -adducin alone. The expression of dominant active RhoA (RhoA<sup>V14</sup>), 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  $\alpha$ -adducin phosphorylation at Thr445, whereas the expression of dominant negative RhoA (RhoA<sup>N19</sup>), 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  $\alpha$ -adducin in COS7 cells expressing RhoA<sup>N19</sup> is unknown, it is likely that the overexpression of GDP-bound RhoA<sup>N19</sup> induced the phosphorylation of  $\alpha$ -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<sup>V12</sup>) or dominant active Cdc42 (Cdc42<sup>V12</sup>) had no effects. Under the conditions, RhoA<sup>V14</sup>, RhoA<sup>N19</sup>, Rac1<sup>V12</sup> and Cdc42<sup>V12</sup> were almost equally expressed (data not shown). The expression of Rho-kinase induced the  $\alpha$ -adducin phosphorylation to some extent. By the coexpression of RhoA<sup>V14</sup> and Rho-kinase, the  $\alpha$ -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). RB (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 C-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 (Leung et al., 1996; Fujisawa 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 et al., 1999). 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., 1999). RB/PH (TT) inhibited the Rho-

kinase-induced stress fiber, but not MRCK-induced stress fiber (Leung et al., 1998) in fibroblasts (Amano et al., submitted for publication). These observations indicate that RB/PH (TT) more specifically inhibits the Rho-kinase activity in intact cells.  $\alpha$ -Adducin phosphorylation at Thr445 in the presence of RhoA<sup>V14</sup> was suppressed by RB and RB/PH (TT) (Fig. 5). Constitutively active Rho-kinase (catalytic domain; CAT) greatly induced the  $\alpha$ -adducin phosphorylation (Fig. 5), whereas constitutively active MRCK did not (data not shown). When the dominant active form of PKC, which activates the TPA-responsive element linked to the chloramphenicol acetyltransferase reporter gene in the absence of phorbol ester (Kaibuchi et al., 1989), was expressed, the phosphorylation of  $\alpha$ -adducin was not detected by anti-pT445 (Fig. 5). HA- $\alpha$ -adducin-AA coexpressed with CAT was not recognized by anti-pT445 (Fig. 5). These results indicate that  $\alpha$ -adducin was specifically phosphorylated at Thr445 via the Rho/Rho-kinase pathway in intact cells.

***Specific phosphorylation of  $\alpha$ -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<sup>V14</sup> nor Rac1<sup>V12</sup> by itself induces membrane ruffling or cell motility (Nishiyama et al., 1994; Takaishi et al., 1993, 1994, 1995; Ridley et al., 1995). I examined the distribution of Thr445-phosphorylated  $\alpha$ -adducin in the TPA-

or HGF-stimulated MDCK cells. I have found by immunoblotting with isoform-specific antibodies that 120 kDa  $\alpha$ -adducin and 90 kDa  $\gamma$ -adducin (alternatively spliced form of 80 kDa  $\gamma$ -adducin) were expressed almost equally in MDCK cells. I have also found that 110 kDa  $\beta$ -adducin was expressed to a lesser extent in MDCK cells. It is likely that anti-pT445 recognizes only phospho-Thr445 in  $\alpha$ -adducin in MDCK cells. The addition of TPA induced membrane ruffling at the outer edge of cell colonies (Fig. 6) as described (Nishiyama et al., 1994; Takaishi et al., 1995). The immunoreactivity of anti-pT445 accumulated in the membrane ruffling areas, where F-actin (Fig. 6) and spectrin (data not shown) also accumulated. Phosphorylated  $\alpha$ -adducin was also detected in the perinuclear region. Similar results were obtained when the cells were stimulated with HGF instead of TPA (Fig. 6). The immunoreactivity with anti-pT445 was abolished by preincubation of the antibody with the antigen phosphopeptide (data not shown). In the non-stimulated MDCK cells, phosphorylated  $\alpha$ -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  $\alpha$ -adducin also accumulated in the leading edge of TPA-induced scattering cells (Fig. 6). To compare the localization of phosphorylated  $\alpha$ -adducin and total  $\alpha$ -adducin, a double-label immunofluorescence study was performed in MDCK cells stably

expressing HA- $\alpha$ -adducin. Immunoreactivity showing the distribution of HA- $\alpha$ -adducin (red) was detected strongly in the cell-cell contact sites (Fig 7, asterisk), weakly in the membrane ruffling areas (Fig 7, arrow), and diffusely in the cytoplasm, as described (Kaiser et al., 1989; Marrs et al., 1993). Phosphorylated  $\alpha$ -adducin (green) was enriched in the membrane ruffling areas in the TPA-stimulated MDCK cells (Fig. 7, arrow). The merged image of red and green immunofluorescence revealed the enrichment of phosphorylated  $\alpha$ -adducin in the membrane ruffling areas and a small increment of phosphorylated  $\alpha$ -adducin in the cytoplasm in the TPA-stimulated cells (Fig. 7-f, greenish image). Phosphorylated  $\alpha$ -adducin did not accumulate at the cell-cell contact sites (Fig. 7-c and f, reddish images indicated by the asterisks). Similar results were obtained when the cells were stimulated with HGF. To confirm that  $\alpha$ -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- $\alpha$ -adducin increased the phosphorylation level of HA- $\alpha$ -adducin at Thr445 (Fig. 8).

***The inhibition of the TPA-induced membrane ruffling by the dominant negative Rho-kinase and  $\alpha$ -adducin mutant***

I 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. 9A 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 (Rac1<sup>N17</sup>) also inhibited the TPA-induced membrane ruffling to a similar extent (Ridley et al., 1995). These results indicate that the Rho-kinase function is required for the TPA-induced membrane ruffling in MDCK cells.

To determine whether the phosphorylation of adducin by Rho-kinase is necessary or sufficient for the membrane ruffling, I produced and utilized cDNAs encoding  $\alpha$ -adducin mutants; HA- $\alpha$ -adducin<sup>T445A, T480A</sup> (HA- $\alpha$ -adducin-AA, substitution of Thr residues by Ala), which was not phosphorylated by Rho-kinase and was expected to serve as the dominant negative form, and HA- $\alpha$ -adducin<sup>T445D, T480D</sup> (HA- $\alpha$ -adducin-DD, substitution of Thr residues by Asp), which was expected to mimic phosphorylated  $\alpha$ -adducin and to serve as the constitutively active form (Sweeney et al., 1994). The microinjection of HA- $\alpha$ -adducin, HA- $\alpha$ -adducin-AA or HA- $\alpha$ -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<sup>V14</sup> alone does not induce the membrane ruffling, and that a lot of processes are required for the induction of membrane ruffling. HA- $\alpha$ -adducin or HA- $\alpha$ -adducin-DD was translocated to the membrane ruffling area (data not shown) and did not inhibit the TPA-

induced membrane ruffling, whereas HA- $\alpha$ -adducin-AA inhibited the ruffling; membrane ruffling was induced in about 60% of TPA-stimulated cells injected with HA- $\alpha$ -adducin or HA- $\alpha$ -adducin-DD, but in only 24% of cells injected with HA- $\alpha$ -adducin-AA (Fig. 9A and B). Although the inhibitory mechanism of HA- $\alpha$ -adducin-AA is not known, HA- $\alpha$ -adducin-AA may substitute for endogenous  $\alpha$ -adducin to oligomerize with endogenous  $\beta$  or  $\gamma$ -adducin, and thereby stay as an inactive complex to inhibit functions of endogenous adducins. Essentially similar results as to the effects of RB/PH (TT) or HA-adducin-AA on membrane ruffling were obtained when MDCK cells were stimulated with HGF instead of TPA (data not shown). To confirm that HA- $\alpha$ -adducin-AA specifically inhibits the function of endogenous  $\alpha$ -adducin but not those of other substrates of Rho-kinase such as MLC or the ERM family proteins, I examined whether the inhibitory effect of HA- $\alpha$ -adducin-AA on the membrane ruffling in MDCK cells was reversed by coinjection of HA- $\alpha$ -adducin or HA- $\alpha$ -adducin-DD. As shown in Fig. 10, HA- $\alpha$ -adducin reversed the inhibitory effect of HA- $\alpha$ -adducin-AA in a dose dependent manner. HA- $\alpha$ -adducin-DD more efficiently reversed the inhibitory effect. These results indicate that HA- $\alpha$ -adducin-AA specifically inhibits the function of endogenous  $\alpha$ -adducin but not those of other substrates. When HA- $\alpha$ -adducin-AA was microinjected into NIH3T3 cells, HA- $\alpha$ -adducin-AA did not affect the LPA-induced formation of stress fibers, which is mediated by the MLC phosphorylation through the Rho/Rho-kinase pathway (Chihara et al., 1997; Amano et al., 1998), whereas RB/PH (TT) inhibited the LPA-induced formation of stress fibers as

described (Amano et al., 1998) (Fig. 11). HA- $\alpha$ -adducin-AA did not affect the LPA-induced formation of microvilli, which is mediated by the ERM phosphorylation through the Rho/Rho-kinase pathway, under the conditions in which RB/PH (TT) inhibited the LPA-induced formation of microvilli (Oshiro et al., 1998) (data not shown). Taken together, these results suggest that HA- $\alpha$ -adducin-AA specifically interferes with the pathway mediated through the  $\alpha$ -adducin phosphorylated by Rho-kinase in intact cells.

When HA- $\alpha$ -adducin-DD, but not HA- $\alpha$ -adducin-AA, was comicroinjected with RB or RB/PH (TT), the inhibitory effect of RB or RB/PH (TT) on the TPA-induced membrane ruffling was counteracted (Fig. 12A and B). Under these conditions, HA- $\alpha$ -adducin also counteracted the inhibitory effect of RB/PH (TT), but not that of RB (Fig. 12B). This difference is likely due to the distinct mode of action between RB and RB/PH (TT). RB/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 RRRLSSLRA without titrating out Rho (Amano et al., 1999). The excess of HA- $\alpha$ -adducin over RB/PH (TT) in the cells may neutralize a competitive inhibition of Rho-kinase by RB/PH (TT), and then HA- $\alpha$ -adducin can be phosphorylated by the activated Rho-kinase. Consistently, when RB/PH (TT) was microinjected at higher concentrations, HA- $\alpha$ -adducin could not counteract the inhibitory effect of RB/PH (TT), whereas HA- $\alpha$ -adducin-DD was still capable of counteracting (data not shown). In contrast to RB/PH (TT), RB 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 (Amano et al., 1997). Thus, HA- $\alpha$ -adducin may not overcome the inhibitory effect of RB in MDCK cells. On the other hand, because HA- $\alpha$ -adducin-DD can mimic  $\alpha$ -adducin phosphorylated at Thr445 and Thr480 *in vivo*, HA- $\alpha$ -adducin-DD may bypass Rho-kinase to induce membrane ruffling during the action of TPA irrespective of the Rho-kinase activity. HA- $\alpha$ -adducin-DD counteracted neither the inhibitory effect of C3 nor that of Rac1<sup>N17</sup> (Fig. 12A and B). GTP $\gamma$ S-bound RhoA<sup>I41</sup>, 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- $\alpha$ -adducin-DD serves as the constitutively active form, and that  $\alpha$ -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). I then investigated whether the  $\alpha$ -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). I performed a wound healing assay by the use of NRK49F 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 NRK49F cells were

more suitable for a wound healing assay than were MDCK cells. Linear wounds were made in a confluent monolayer of NRK49F cells and about 200 cells along the wound edge were microinjected with C3, RB/PH (TT), or the  $\alpha$ -adducin mutants. Six hours after wounding, the cells that moved ( $> 20 \mu\text{m}$ ) from the original wound edge were counted. I also evaluated the wound-induced migration by a timelapse recording. When maltose-binding protein (MBP) was injected as a control protein, 63% of the total injected cells showed wound-oriented migration, and about 60% of the total injected cells had membrane rufflings (Fig. 14A and B). About 90% of the migrating injected cells had membrane rufflings in their leading edges (Fig. 14A). I found that  $\alpha$ -adducin phosphorylated at Thr445 accumulated at the membrane ruffling areas in the leading edge and the perinuclear region in migrating NRK49F cells (Fig. 13), 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. 14A and B). The microinjection of HA- $\alpha$ -adducin-AA also inhibited the migration; only 21% of cells injected with HA- $\alpha$ -adducin-AA migrated to the wound (Fig. 14A and B). When C3, RB/PH (TT) or  $\alpha$ -adducin-AA 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 rufflings (Fig. 14A). Whatever proteins were injected, the majority (about 90%) of migrating injected cells had membrane

ruffling in the leading edge. These results suggest that the inhibition of cell migration by HA- $\alpha$ -adducin-AA, C3 or RB/PH (TT) correlates with the inhibition of membrane ruffling. In the HA- $\alpha$ -adducin-injected cells, the wound-oriented migration and membrane ruffling were not inhibited (Fig. 14A and B). HA- $\alpha$ -adducin-DD also inhibited the migration; 32% of cells injected with HA- $\alpha$ -adducin-DD migrated to the wound (Fig. 14A and B). However, the percentage of the cells with membrane ruffling in the total injected cells (about 60%) was much bigger than that of the migrating cells in the total injected cells (32%). The majority of migrating cells injected with  $\alpha$ -adducin-DD had membrane rufflings in the leading edge, and about one half of the stationary cells injected with  $\alpha$ -adducin-DD had membrane rufflings (Fig. 14A-k). These results indicate that  $\alpha$ -adducin-DD did not inhibit the membrane ruffling but did inhibit cell migration, suggesting that cycling between the phosphorylated and dephosphorylated states of  $\alpha$ -adducin appears to be necessary for cell migration, but not for membrane ruffling. Consistently, RhoA<sup>V14</sup> or CAT as well as Rac1<sup>N17</sup> inhibited the wound-induced migration (data not shown).

## **Discussion**

### ***Phosphorylation sites of $\alpha$ -adducin by Rho-kinase and site- and phosphorylation-state specific antibody***

In the present study, I identified the sites of phosphorylation of  $\alpha$ -adducin by Rho-kinase as Thr445 and Thr480 in the neck domain.  $\alpha$ -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  $\alpha$ -adducin, Thr445 and Thr480 are unique for Rho-kinase. Anti-pT445, a site- and phosphorylation-state specific antibody for  $\alpha$ -adducin, recognized HA- $\alpha$ -adducin phosphorylated by Rho-kinase, but not by PKC or PKN. Anti-pT445 recognized neither non-phosphorylated HA- $\alpha$ -adducin nor HA- $\alpha$ -adducin<sup>T445A, T480A</sup> incubated with Rho-kinase and ATP (Fig. 4A). These results indicate that anti-pT445 is a useful tool with which to detect the specific phosphorylation of  $\alpha$ -adducin by Rho-kinase both *in vitro* and *in vivo*, distinguishing from the phosphorylation of  $\alpha$ -adducin by PKC. Although Rho-kinase phosphorylated  $\beta$ - and  $\gamma$ -adducin *in vitro* (Fig. 4B), the phosphorylation sites have not been determined. Since the surrounding amino acid sequences of Thr437 in rat  $\gamma$ -adducin (QQREKTRWLNS) are completely identical to those of Thr445 in  $\alpha$ -adducin (selected as antigen phosphopeptide for anti-pT445), the result that anti-pT445 did not cross-react with rat  $\gamma$ -adducin phosphorylated by Rho-kinase (Fig. 4B) revealed that Rho-kinase does not primarily phosphorylate  $\gamma$ -adducin at Thr437. The surrounding amino acid sequences of Thr431 in  $\beta$ -

adducin (QQKEKTRWLNT) are a little bit different from those of Thr445 in  $\alpha$ -adducin. At this stage, I do not know whether Rho-kinase phosphorylates  $\beta$ -adducin at Thr431. Adducin is thought to be comprised of  $\alpha$ - and  $\beta$ -, or  $\alpha$ - and  $\gamma$ -adducin *in vivo* (Bennett et al., 1988; Dong et al., 1995). I found that in MDCK and NRK49F cells a heterodimer of adducin which consists of  $\alpha$ - and  $\gamma$ -adducin was mainly expressed (data not shown). Therefore, it is likely that anti-pT445 recognizes only phospho-Thr445 in  $\alpha$ -adducin but not in  $\beta$ - and  $\gamma$ -adducin in both cells (see below). Further analysis is necessary to understand how the phosphorylation of  $\beta$ - or  $\gamma$ -adducin is regulated through Rho-kinase *in vitro* and *in vivo*.

***In vivo phosphorylation of  $\alpha$ -adducin via the Rho/Rho-kinase pathway***

I here found using anti-pT445 that Thr445-phosphorylation of  $\alpha$ -adducin occurred in a Rho/Rho-kinase dependent manner in COS7 cells (Fig. 5). I further found that  $\alpha$ -adducin was phosphorylated at Thr445 in the TPA-stimulated MDCK cells (Fig. 8). TPA also drives the activation of PKC. Indeed, TPA induces the phosphorylation of  $\alpha$ -adducin at Ser726 in renal proximal tubule epithelial cells and MDCK cells (Fowler et al., 1998; Matsuoka et al., 1998). However, it is unlikely that PKC directly phosphorylates  $\alpha$ -adducin at Thr445 in the TPA-stimulated cells, because anti-pT445 did not recognize  $\alpha$ -adducin which was phosphorylated by PKC *in vitro* and was coexpressed with the dominant active PKC in COS7 cells. Rac and Cdc42 are

also involved in TPA-induced phenomena such as membrane ruffling (Ridley et al., 1995). It is possible that the protein kinases activated by Rac or Cdc42 phosphorylate  $\alpha$ -adducin. However, I found that neither Rac1<sup>V12</sup> nor Cdc42<sup>V12</sup> induced the Thr445-phosphorylation of  $\alpha$ -adducin in COS7 cells, and that the dominant active MRCK, which is identified as the target of Cdc42 (Leung et al., 1998) and Rac1 (Nakamura et al., manuscript in preparation), induced the phosphorylation of MLC but not of  $\alpha$ -adducin at Thr445. Taken together, these results indicate that in intact cells  $\alpha$ -adducin was specifically phosphorylated at Thr445 via the Rho/Rho-kinase pathway during the action of TPA. Although *in vitro* experiment using GST- $\alpha$ -adducin<sup>T445A</sup> and T480A (Fig. 3) suggests that Thr445 is a preferential site of phosphorylation by Rho-kinase *in vitro*, at this stage it is unknown which of Thr445 or Thr480 is a preferential site in intact cells.

***Involvement of the phosphorylation of  $\alpha$ -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 are thought to regulate the membrane ruffling downstream of extracellular signals in certain types of cells, because C3 inhibits the TPA or HGF-induced membrane ruffling in

epithelial cells such as MDCK and KB cells (Nishiyama et al., 1994; Takaishi et al., 1995). Here, I found that Thr445-phosphorylated  $\alpha$ -adducin accumulated at the TPA-induced membrane ruffling areas and the leading edges of migrating cells (Fig. 6 and 13). I furthermore found that RB and RB/PH (TT) as well as C3 inhibited the TPA-induced membrane ruffling, that  $\alpha$ -adducin-AA also inhibited the membrane ruffling as the dominant negative  $\alpha$ -adducin (Fig. 9A and B), and that  $\alpha$ -adducin-DD specifically counteracted the inhibitory effect of RB and RB/PH (TT) on the membrane ruffling (Fig. 12). These results indicate that  $\alpha$ -adducin is a crucial substrate of Rho-kinase downstream of Rho for membrane ruffling. The fact that  $\alpha$ -adducin-DD counteracted the inhibitory effect of RB but not that of C3 (Fig. 12B) is probably due to the difference between the inhibitory mechanisms by RB and C3. RB is the fragment of Rho-kinase which consists of the Rho-binding domain of Rho-kinase. RB 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<sup>V14</sup>, 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<sup>Y42C</sup> abolishes interaction with PKN but maintains interaction with ROCK-I (an isoform of Rho-kinase) and RhoA<sup>F39L,C20R</sup> maintains interaction with PKN but abolishes interaction with ROCK-I (Sahai, Albert, and Treisman, 1998). RhoA<sup>Y42C</sup> induces stress fiber formation, whereas RhoA<sup>F39L,C20R</sup> does not induce stress fiber

formation, suggesting that ROCK-I but not PKN is required for stress fiber formation. These observations indicate that there are distinct interfaces in the effector domain of Rho for the different classes of effector proteins.

Although at this time I 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 Swiss3T3 cells (Amano et al., 1996), and the Rho-dependent recruitment of p140mDia to the dynamic membrane structures in Swiss3T3 cells (Watanabe, et al., 1997). C3 may inhibit the membrane ruffling by impairing the whole functions of Rho, whereas RB may inhibit the membrane ruffling by mainly (or specifically) impairing the function of Rho-kinase.

I also found that not only C3 and RB/PH (TT) but also  $\alpha$ -adducin-AA inhibited cell motility as well as membrane ruffling in NRK fibroblasts (Fig. 14). This suggests that  $\alpha$ -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 to 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

(Matsumura 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 Swiss3T3 or NIH3T3 fibroblasts, in which the dominant active Rac by itself is sufficient to induce the membrane ruffling (Hall, 1998),  $\alpha$ -adducin phosphorylated at Thr445 was not observed in the dominant active Rac-induced ruffling areas, and neither C3 , RB/PH (TT) nor HA- $\alpha$ -adducin-AA inhibited the dominant active Rac-induced ruffling (data not shown). On the other hand, I found that in KB epithelial cells as well as MDCK epithelial cells,  $\alpha$ -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  $\alpha$ -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 that in other types of cells such as Swiss3T3 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  $\alpha$ -adducin function through phosphorylation by Rho-kinase and PKC**

The present results indicate that the phosphorylation of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -adducin by Rho-kinase is known to promote the binding of  $\alpha$ -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. 15). 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  $\alpha$ -adducin by PKC inhibits the activity of

adducin in promoting the formation of a spectrin-actin complex *in vitro* (Matsuoka et al., 1998). TPA induces the phosphorylation of  $\alpha$ -adducin at Ser726 and the redistribution of  $\alpha$ -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; Matsuoka et al., 1998). Interestingly,  $\alpha$ -adducin phosphorylated at Ser726 was also observed in the membrane ruffling areas (Matsuoka et al., manuscript in preparation). It is conceivable that  $\alpha$ -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  $\alpha$ -adducin-DD inhibits the wound-induced migration of NRK49F cells but not their membrane ruffling. This suggests that the "on" and "off" states of  $\alpha$ -adducin are necessary for cell migration (Fig. 15).

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## **Footnotes**

Abbreviations used in this paper: PKN, protein kinase N; MBS, myosin-binding subunit; MLC, myosin light chain; ERM, ezrin/radixin/moesin; PKC, protein kinase C; PKA, protein kinase A; RB, Rho-binding domain; PH, pleckstrin homology domain; CAT, catalytic domain; GST, glutathione-S-transferase; HA, hemagglutinin; Ab, antibody;  $\alpha$ -adducin-AA,  $\alpha$ -adducin with substitution of Thr445 and Thr480 by Ala;  $\alpha$ -adducin-DD,  $\alpha$ -adducin with substitution of Thr445 and Thr480 by Asp; MBP, maltose-binding protein; TPA, tetradecanoylphorbol-13-acetate; HGF, hepatocyte growth factor; LPA, lysophosphatidic acid.

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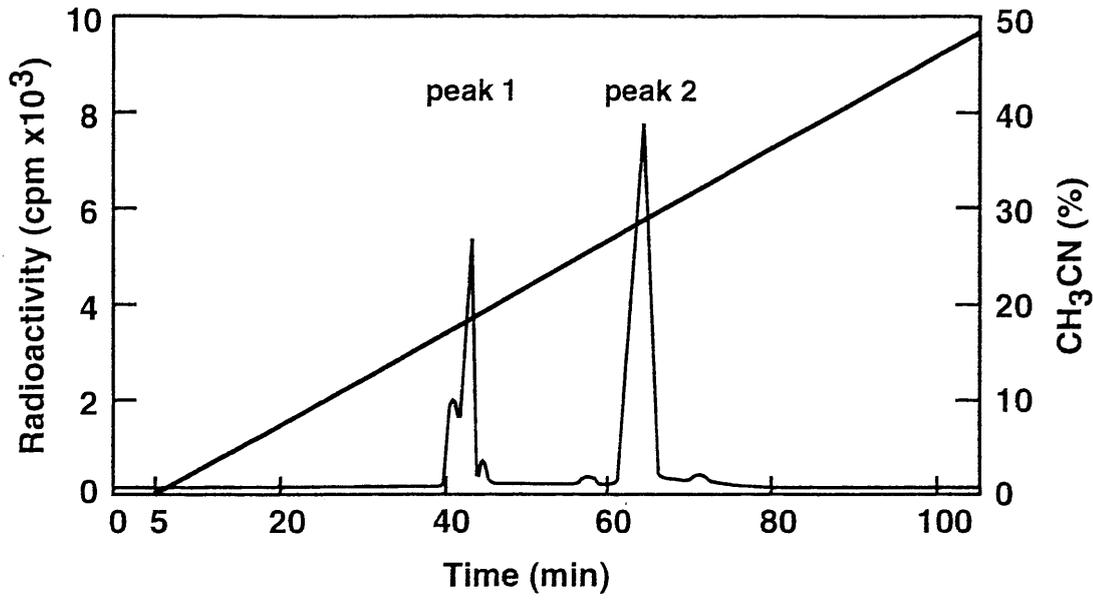
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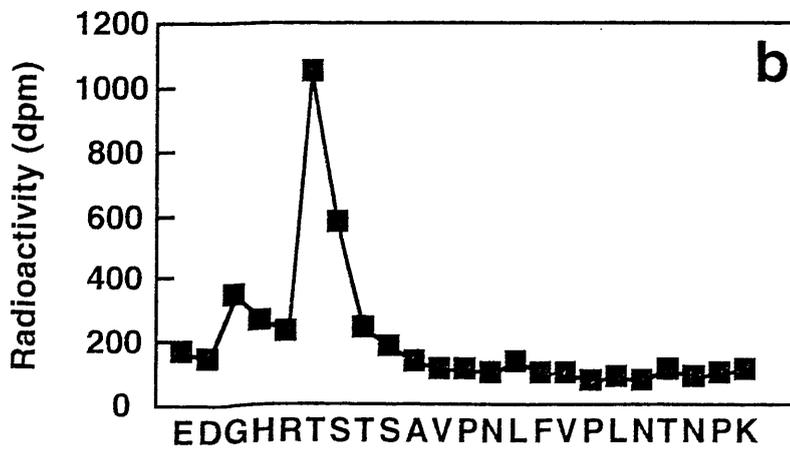
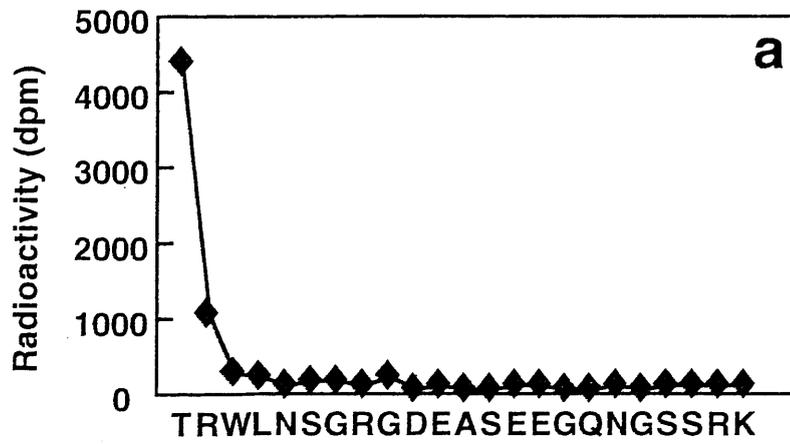
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**Fig. 1**

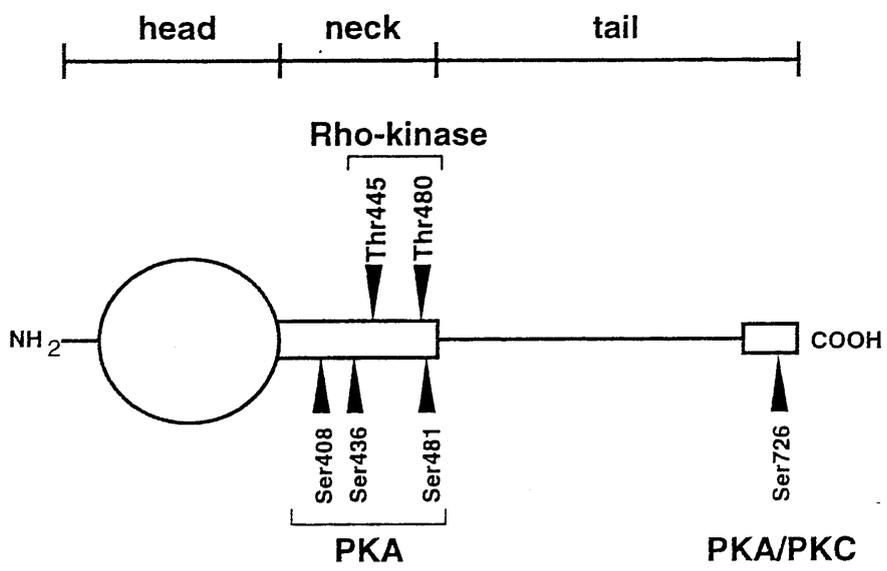
**A**



**B**



**Fig. 2**



**Fig. 3**

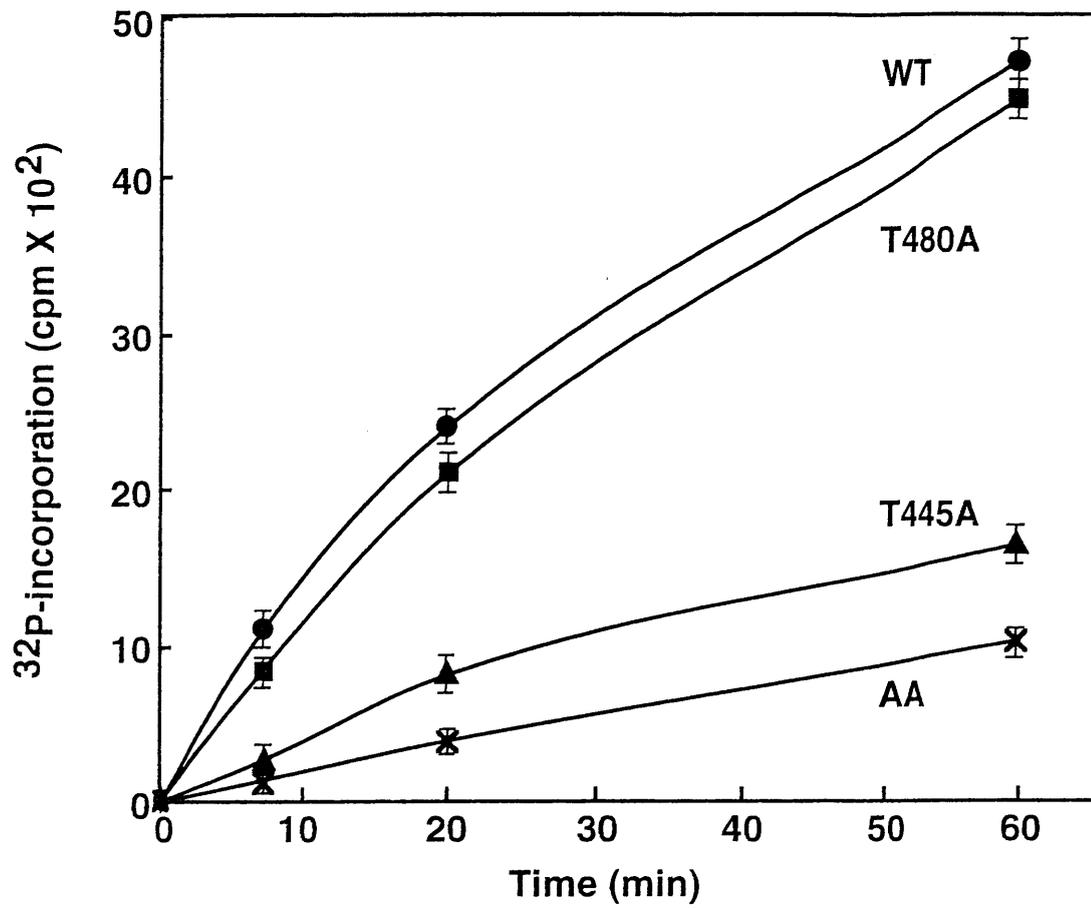
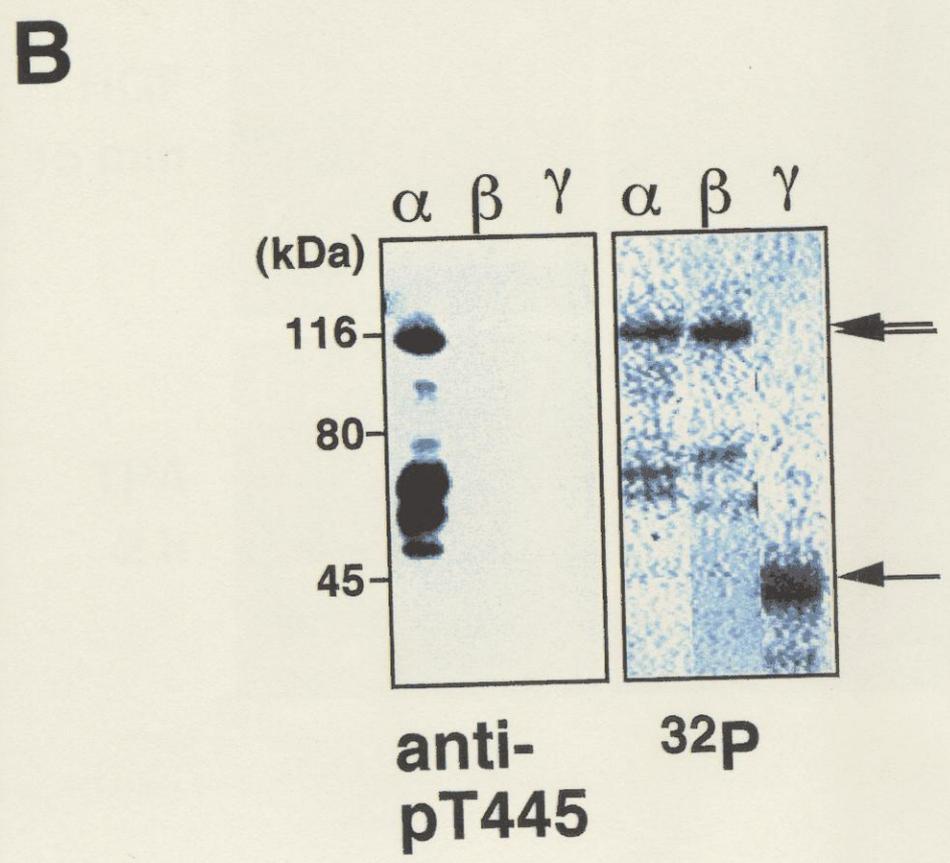
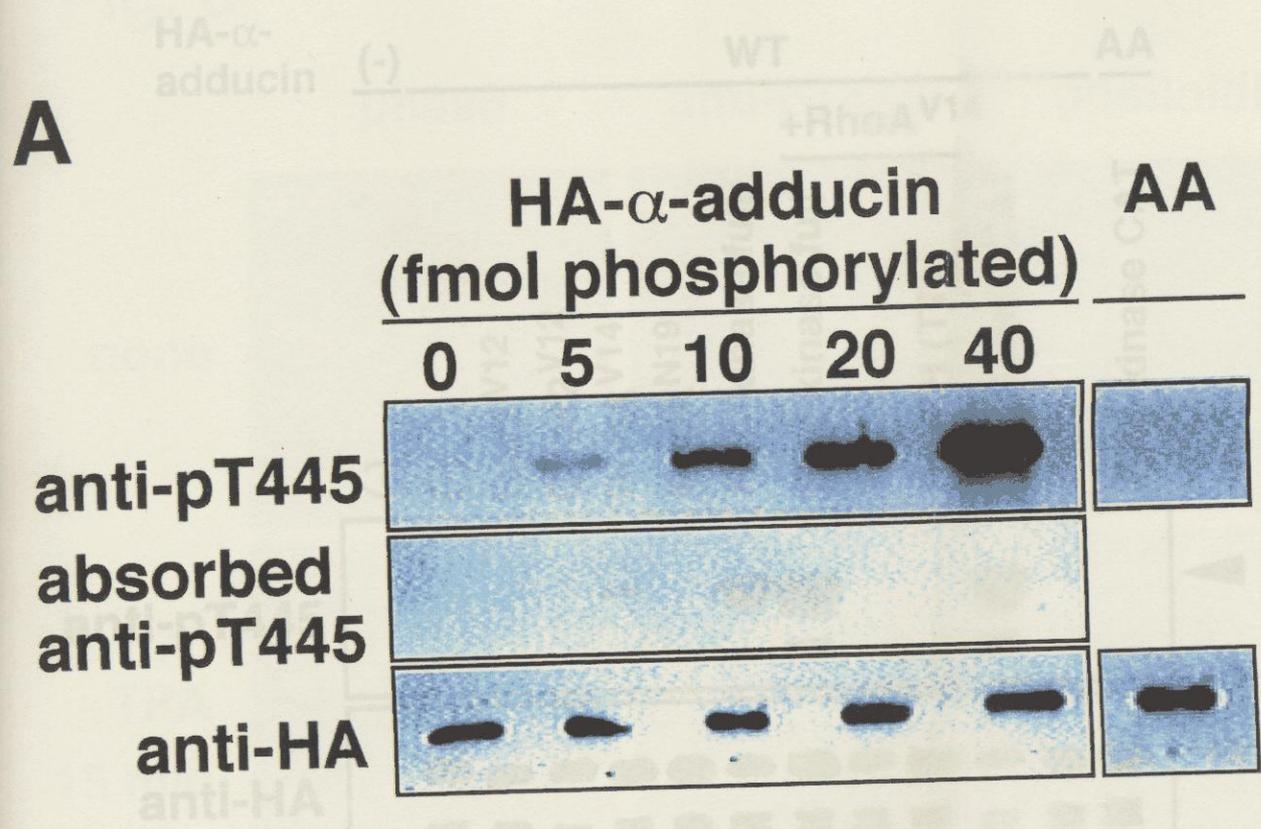
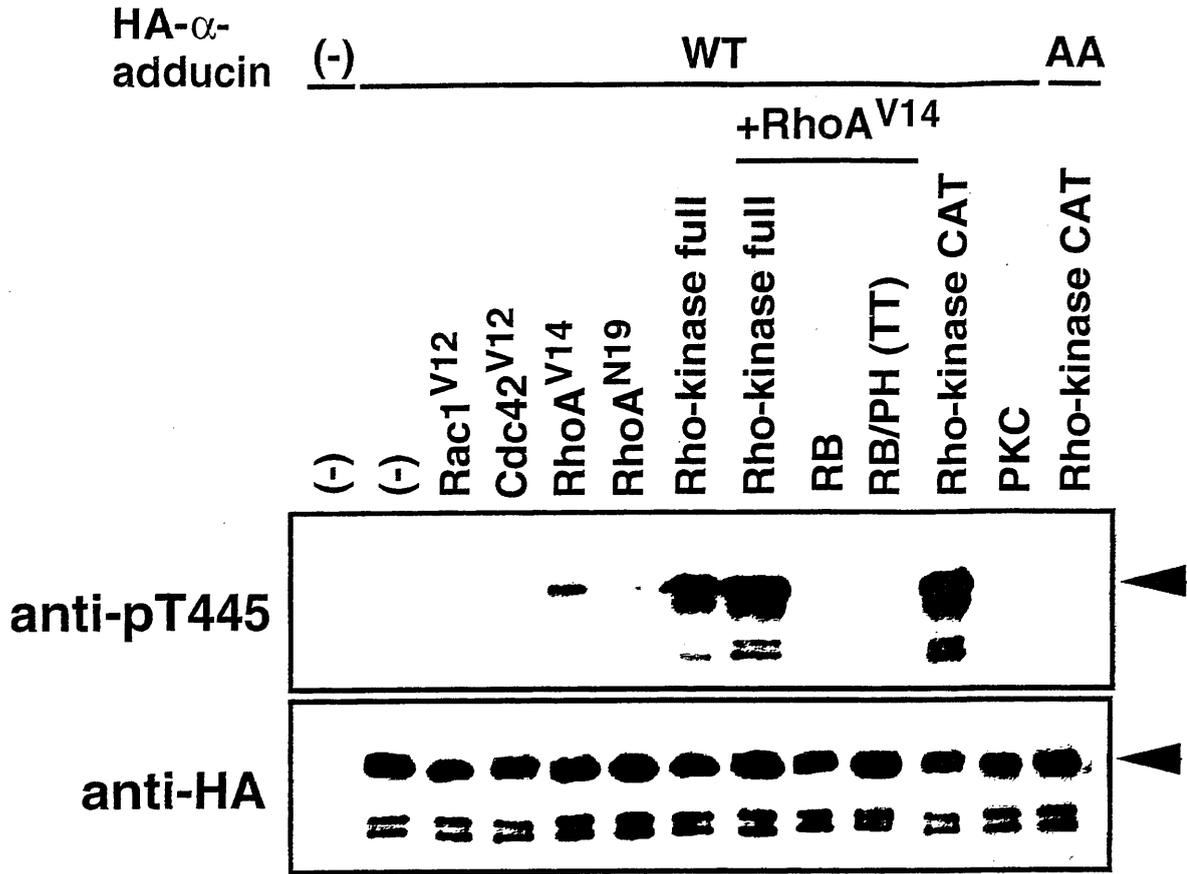


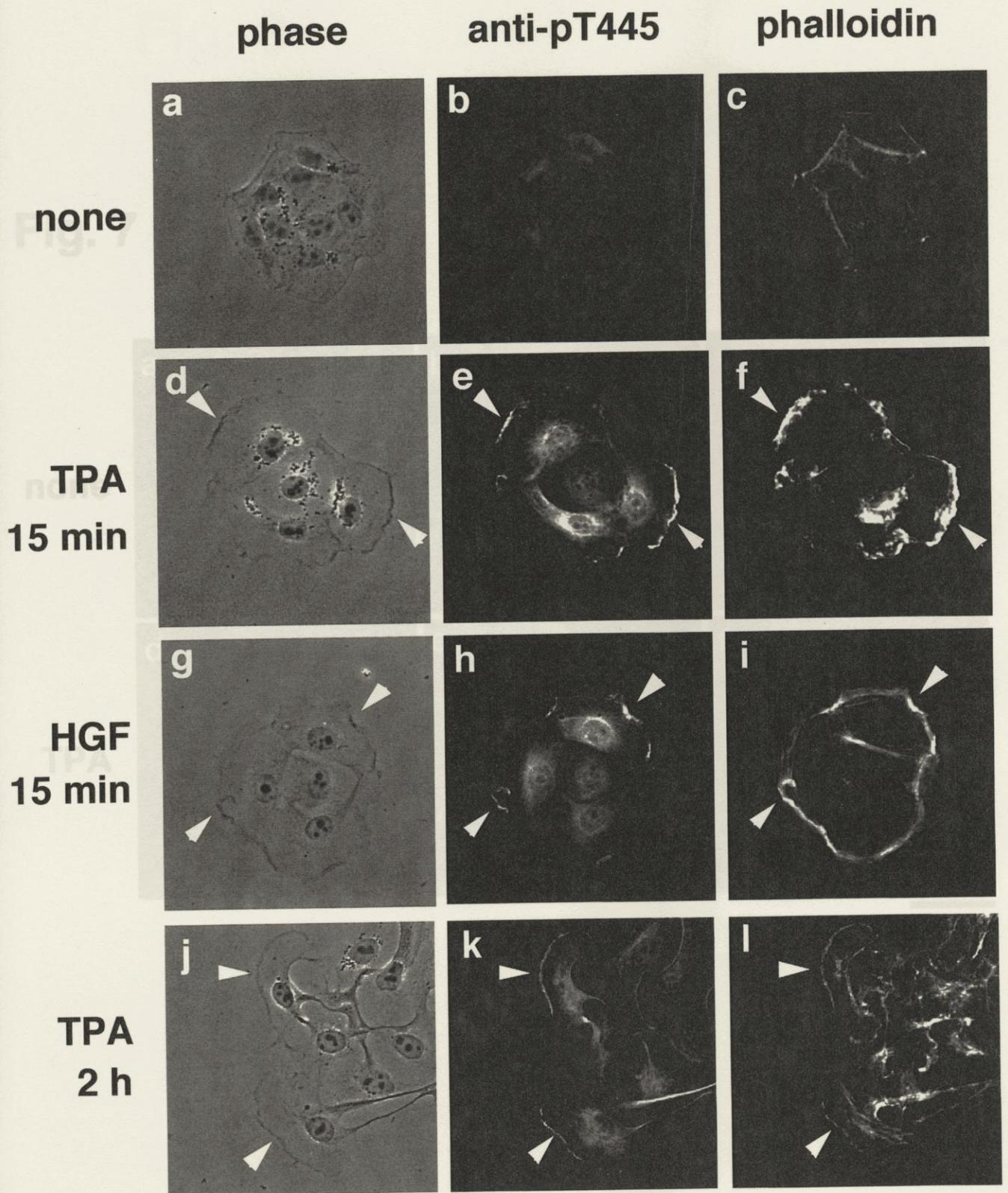
Fig. 5  
**Fig. 4**



**Fig. 5**



**Fig. 6**



**Fig. 7**

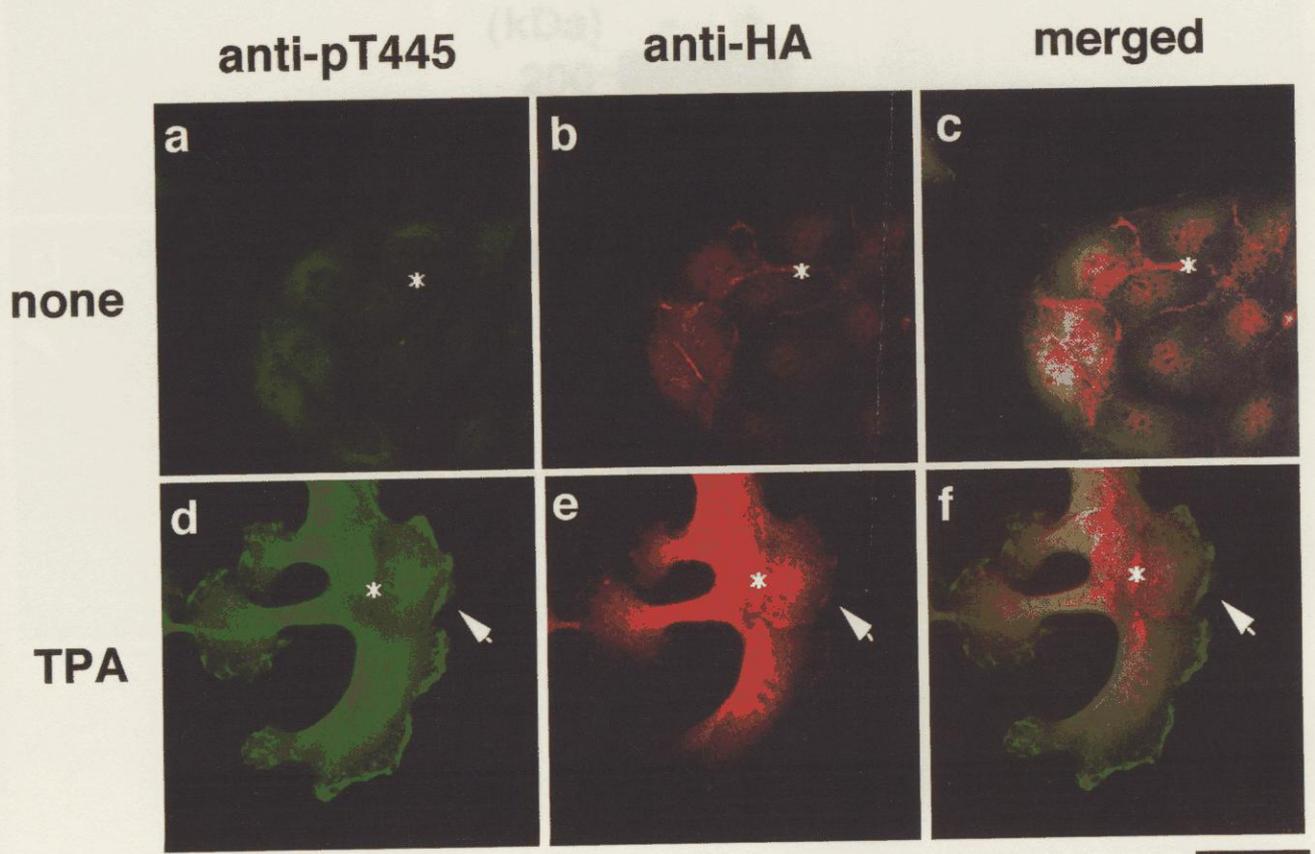


Fig. 9

A

Fig. 8

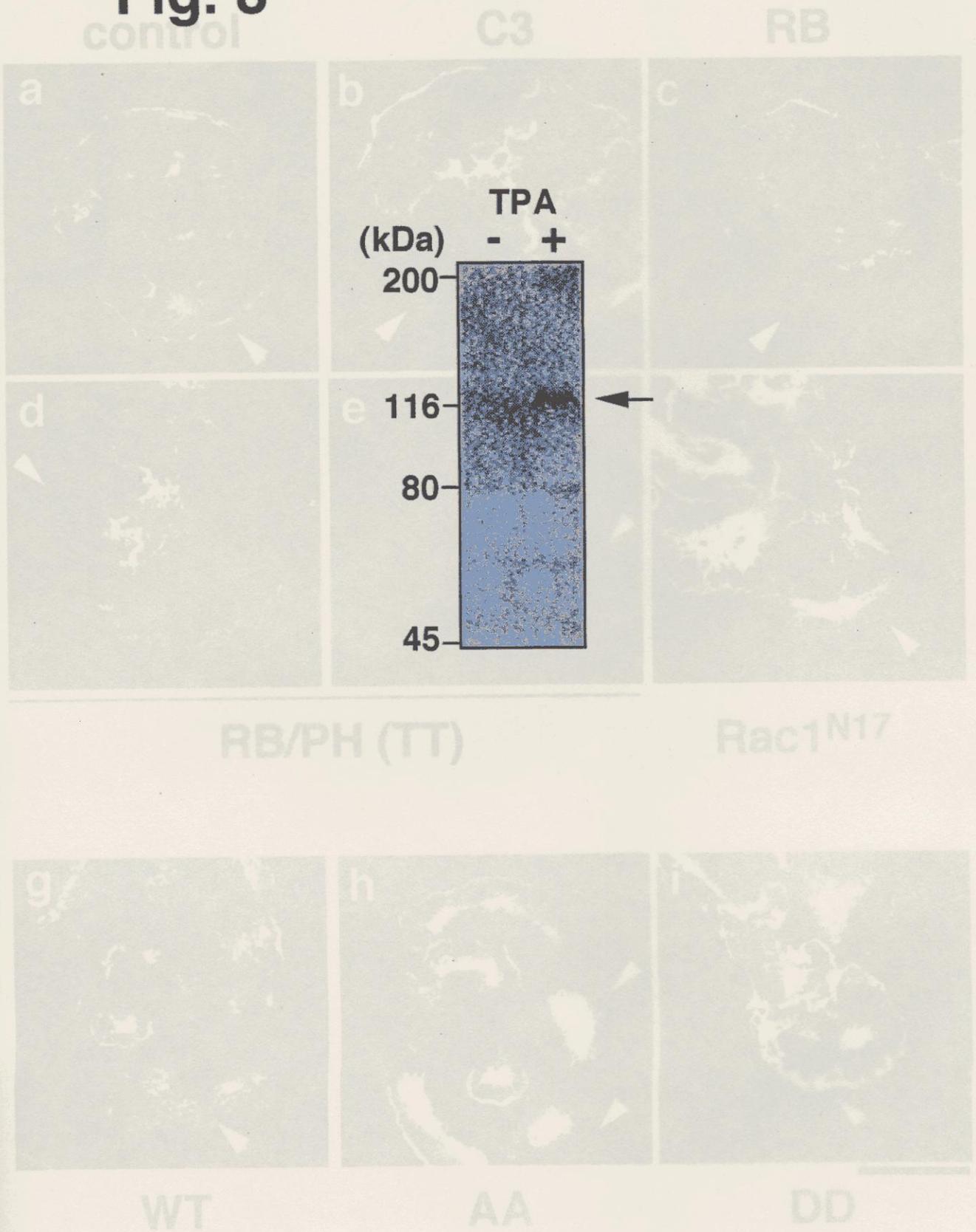
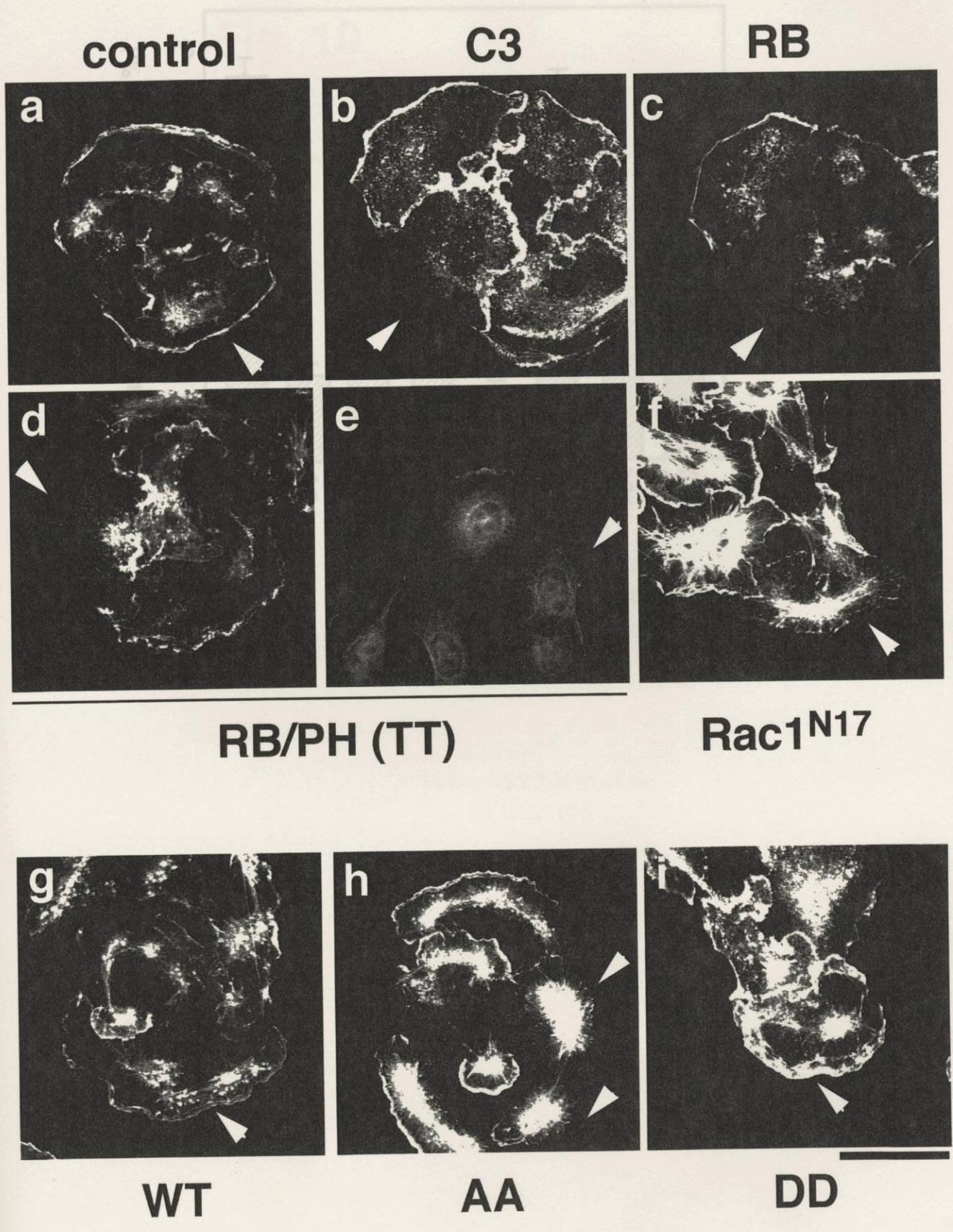


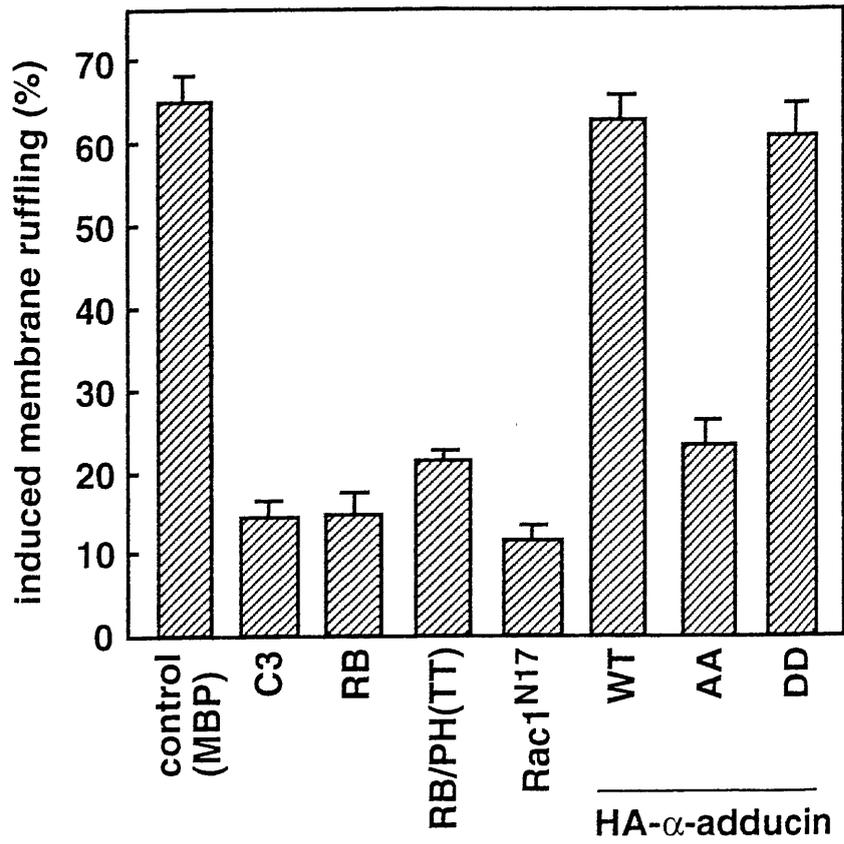
Fig. 9

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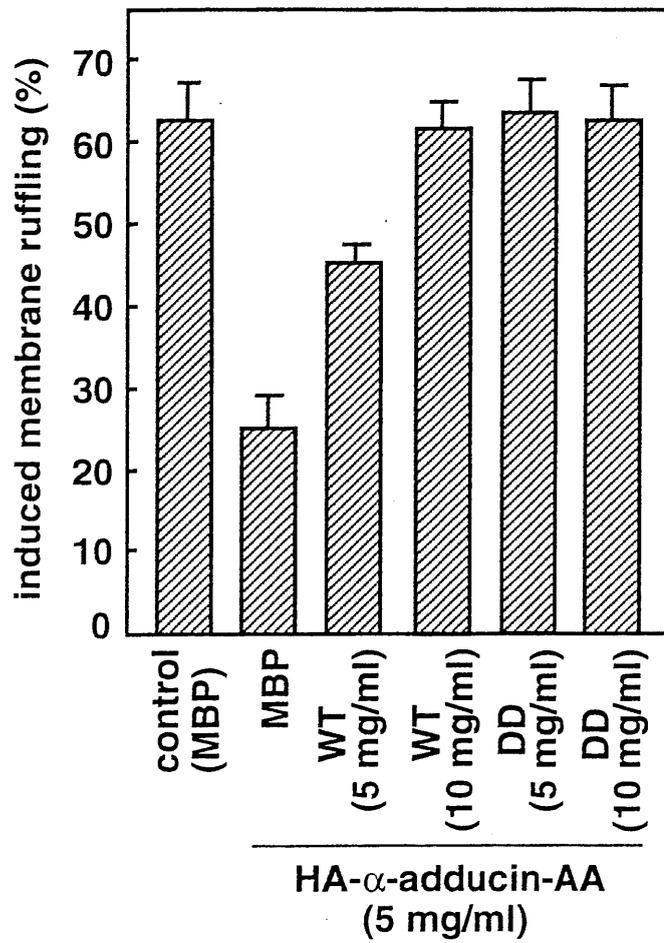


**Fig. 9**

**B**



**Fig. 10**



**Fig. 11**

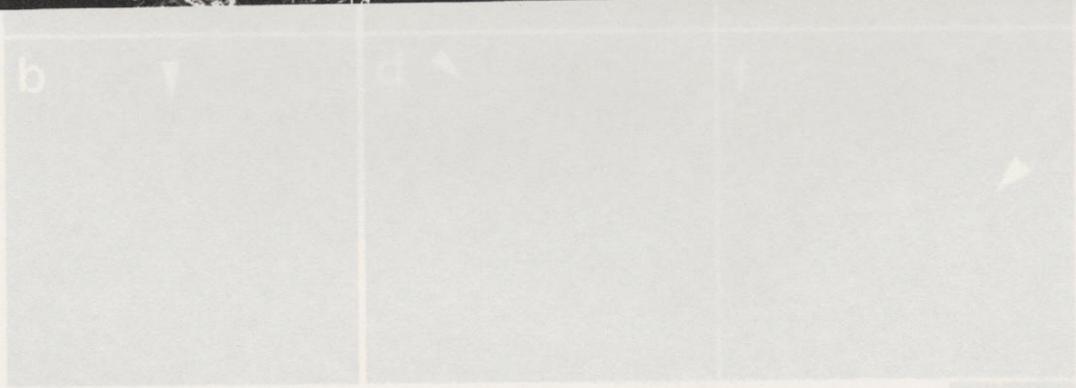
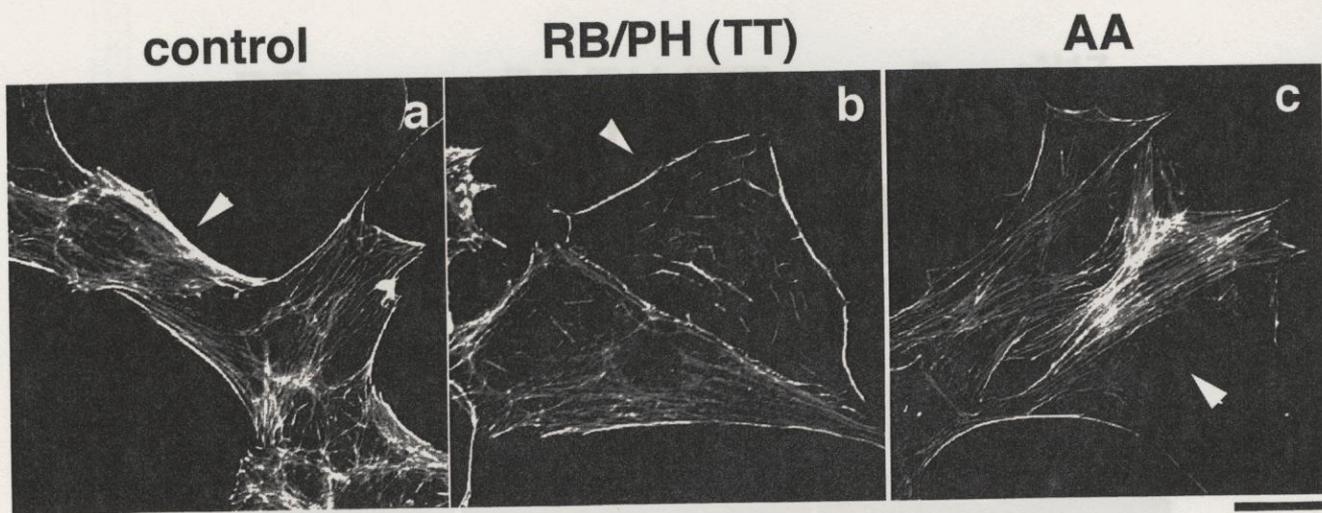


Fig.12

B

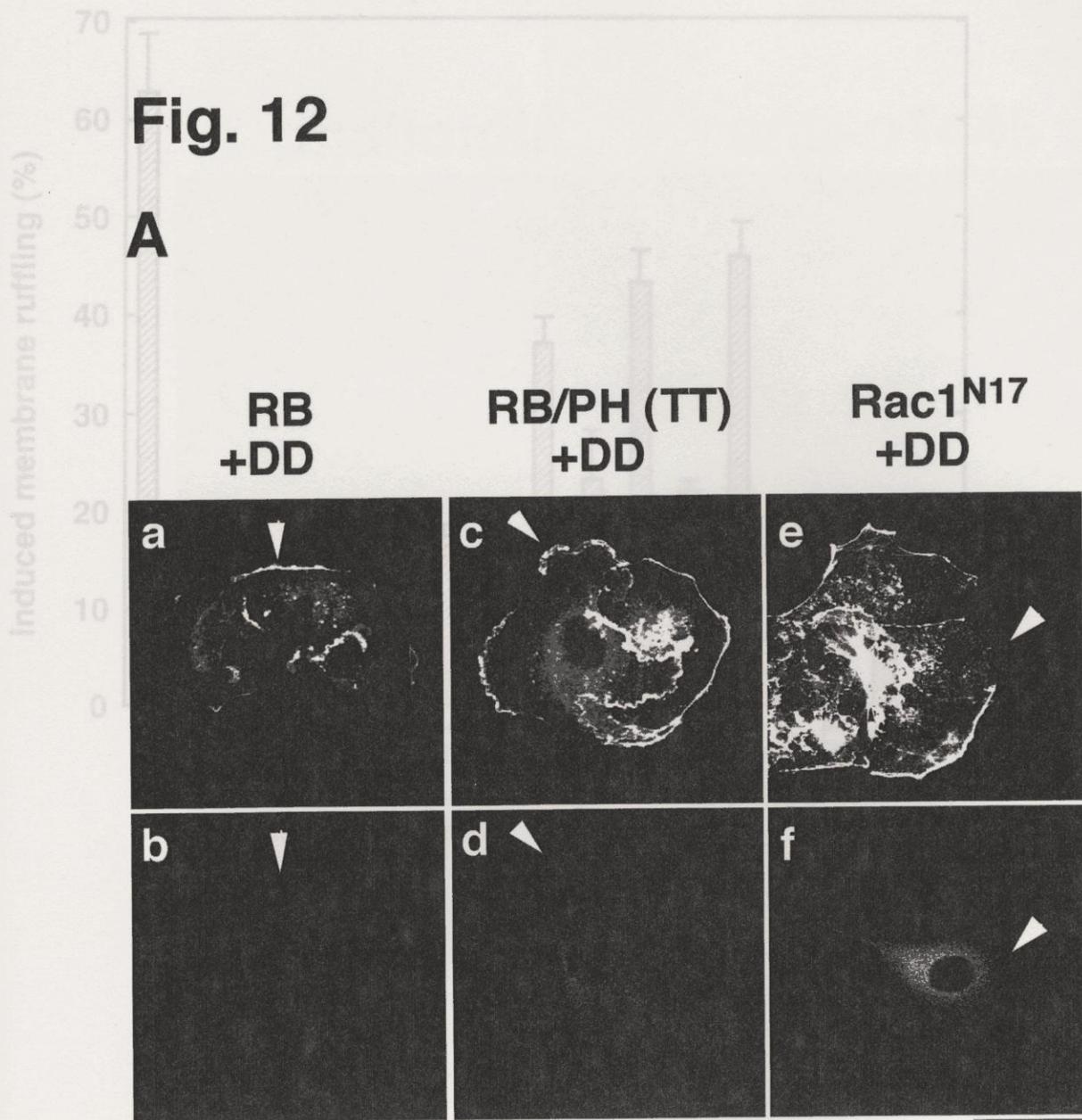


Fig.12

B

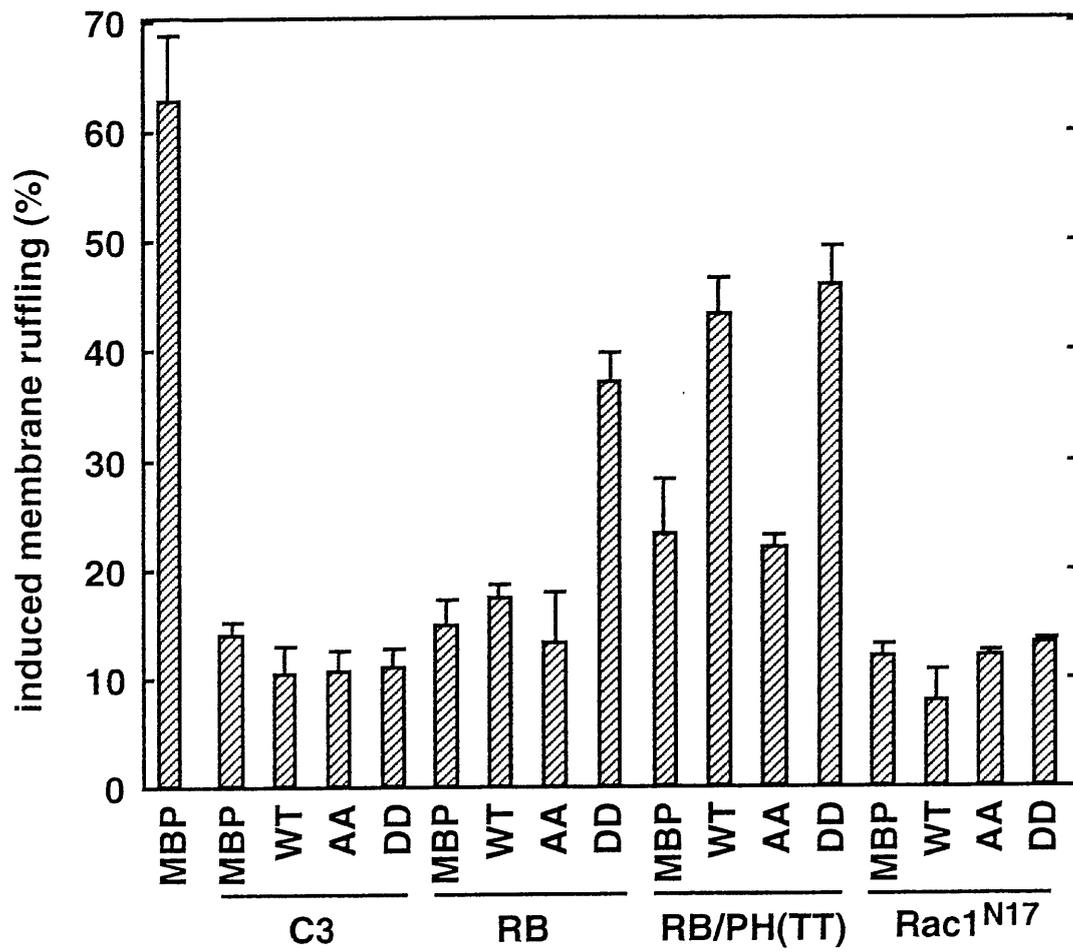


Fig. 13

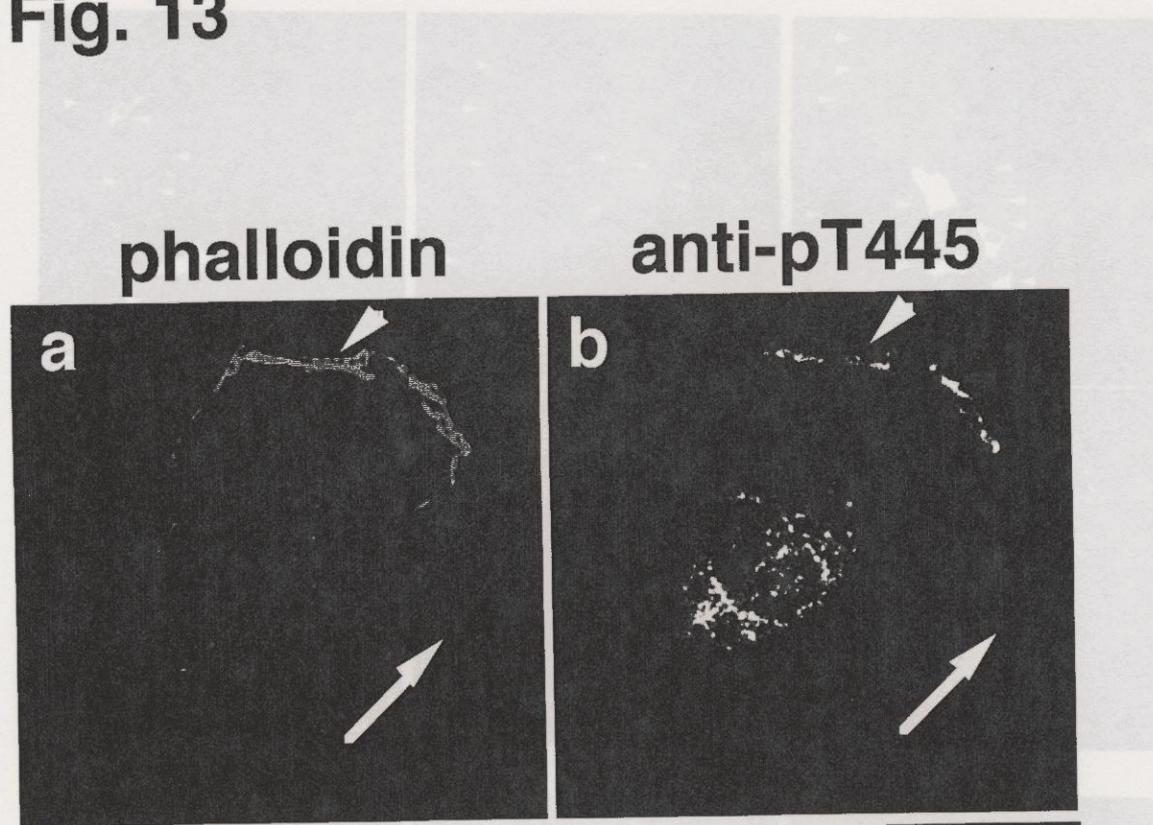
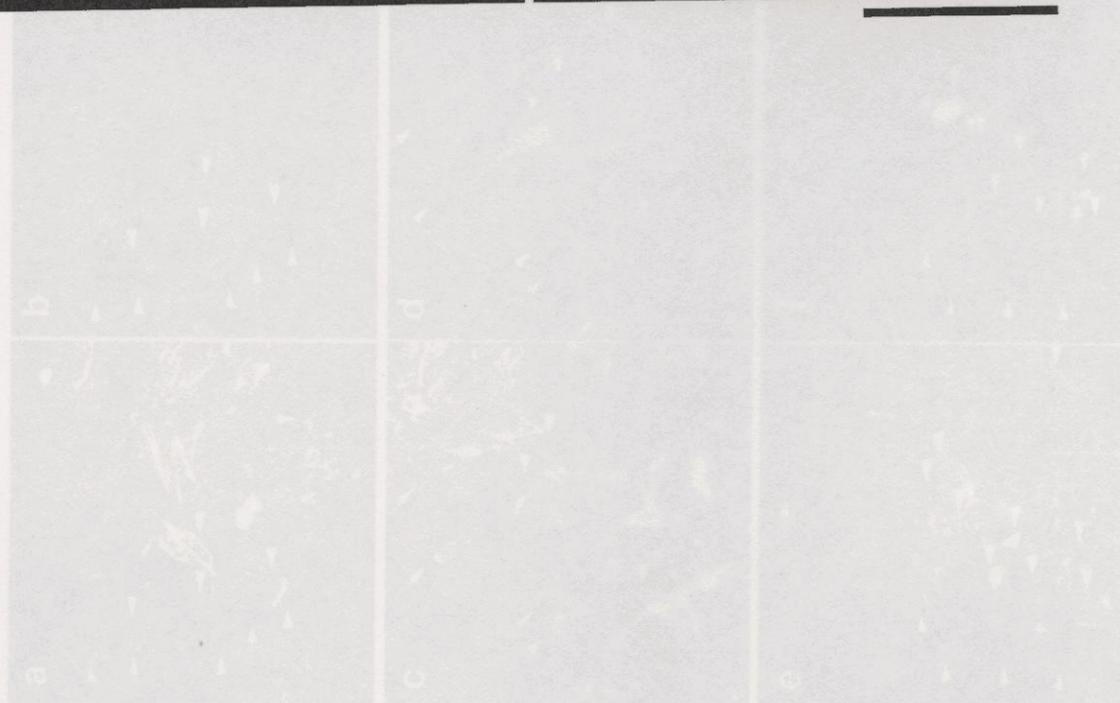


Fig. 14

A



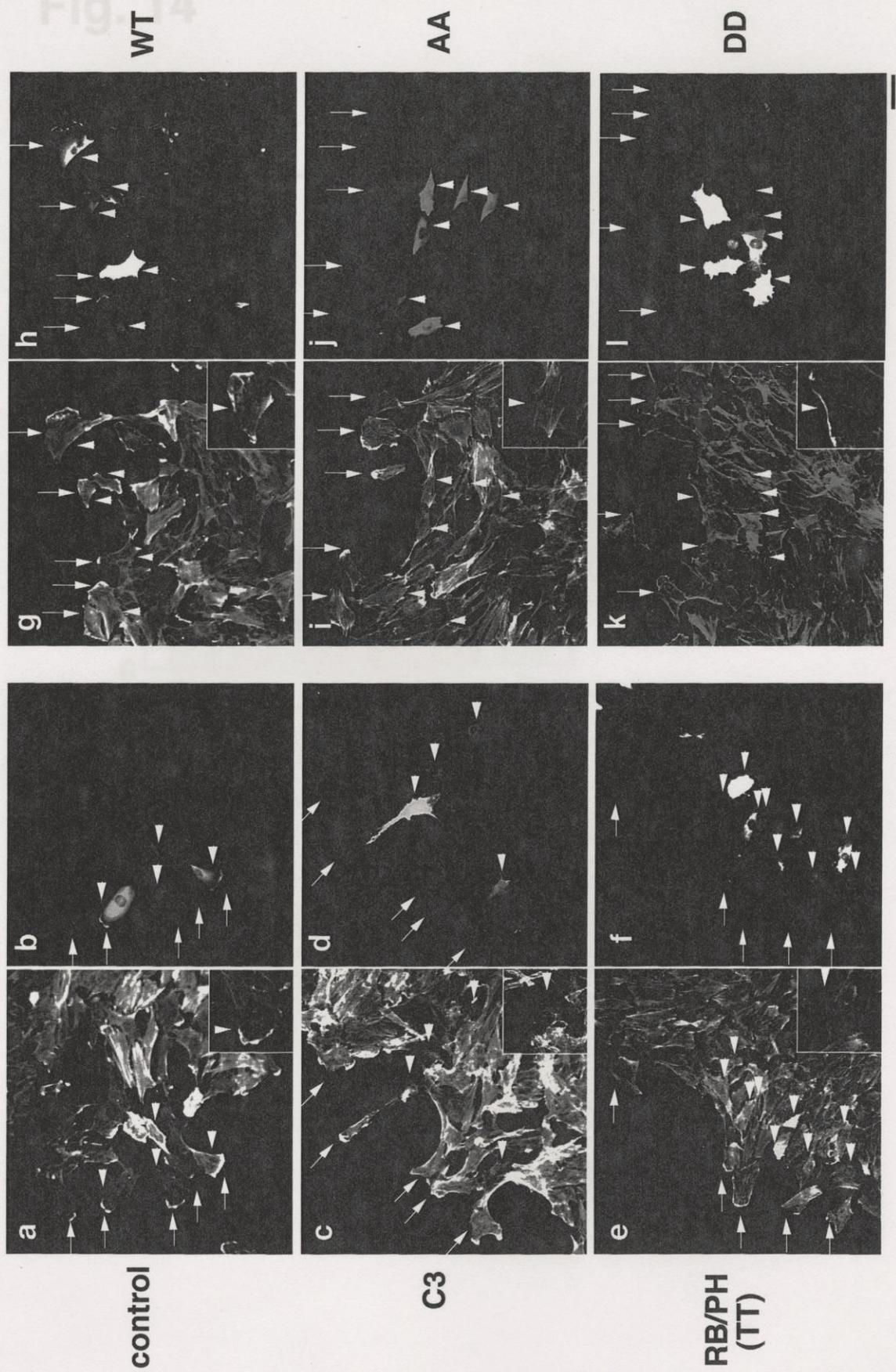
control

33

RB/PH  
(TT)

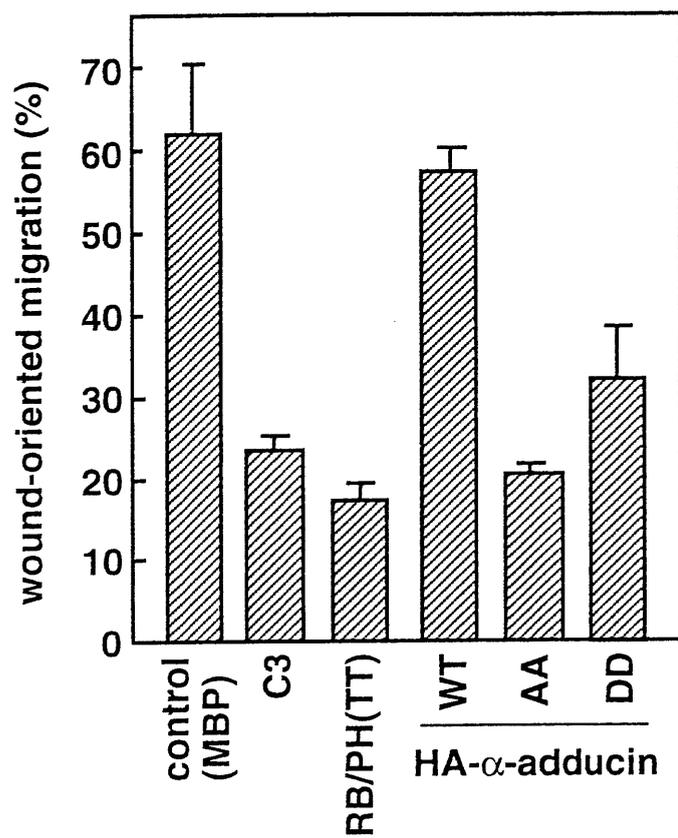
Fig. 14

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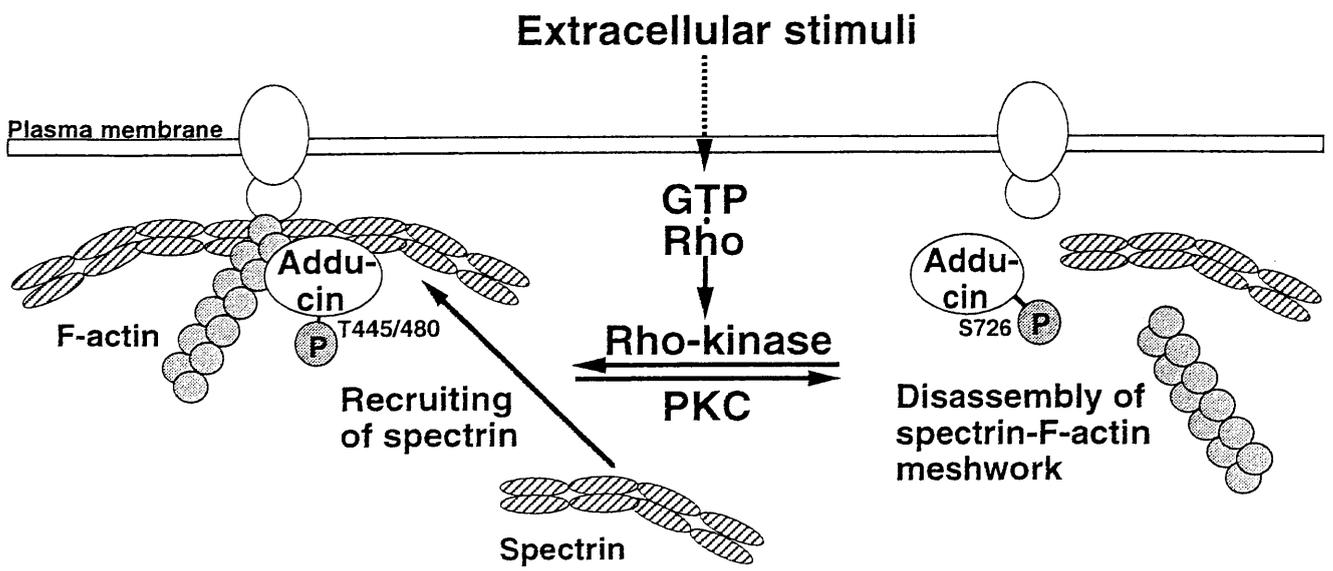


**Fig. 14**

**B**



**Fig. 15**



### **Figure Legends**

**Fig. 1.** Determination of the sites of phosphorylation of  $\alpha$ -adducin by Rho-kinase. (A) Reverse phase HPLC for AP-1 fragments of  $\alpha$ -adducin phosphorylated by Rho-kinase was carried out under the conditions described in "Materials and Methods". Two major radioactive peaks (named peak 1 and peak 2) were obtained. CH<sub>3</sub>CN, acetonitrile. (B) Amino acid sequences of phosphopeptides derived from  $\alpha$ -adducin phosphorylated by Rho-kinase. The radioactive peak 1 (a) and 2 (b) were separated. Phosphoamino acid sequencing of each peak was carried out with a peptide sequencer, and the fractions obtained from each Edman degradation cycle were measured for <sup>32</sup>P in a liquid scintillation counter.

**Fig. 2.** Structure and phosphorylation sites of  $\alpha$ -adducin. A model for  $\alpha$ -adducin based upon Ref. (Matsuoka et al., 1996) is presented. The phosphorylation sites of  $\alpha$ -adducin for PKA, PKC and Rho-kinase are presented.

**Fig. 3.** Preferential phosphorylation of  $\alpha$ -adducin at Thr445 by Rho-kinase *in vitro*. GST- $\alpha$ -adducin (WT) (●), GST- $\alpha$ -adducin-AA (X), GST- $\alpha$ -adducin<sup>T445A</sup> (▲), and GST- $\alpha$ -adducin<sup>T480A</sup> (■) were phosphorylated by GST-CAT for various periods. Data are means  $\pm$  SEM of triplicate determinations.

**Fig. 4.** Specificity of anti-pT445. (A) HA- $\alpha$ -adducin (40 fmol) containing the indicated amounts of HA- $\alpha$ -adducin phosphorylated by Rho-kinase, and HA- $\alpha$ -adducin-AA (40 fmol) phosphorylated by Rho-kinase were subjected to immunoblot

analysis with anti-pT445, anti-pT445 preincubated with a 100-fold amount of antigen-phosphopeptide, and anti-HA antibody. The amount of phosphates incorporated into HA- $\alpha$ -adducin was simultaneously determined by [ $\gamma$ - $^{32}$ P] ATP. (B) Anti-pT445 reacted with only  $\alpha$ -adducin phosphorylated by Rho-kinase. HA- $\alpha$ -adducin, HA- $\beta$ -adducin, or 6 X His- $\gamma$ -adducin fragment (319-671 aa) was separately phosphorylated with GST-CAT, resolved by SDS-PAGE, and visualized by an image analyzer (*right panel*). Forty fmol (as  $^{32}$ P-incorporated amount) of phosphorylated proteins were subjected to immunoblot analysis with anti-pT445 (*left panel*). Arrows from the *top* indicate the positions of the intact HA- $\alpha$ -adducin, HA- $\beta$ -adducin, or 6 X His- $\gamma$ -adducin, respectively. Anti-pT445 also bound to the degradation products of HA- $\alpha$ -adducin. The results are representative of three independent experiments.

**Fig. 5.** The phosphorylation of  $\alpha$ -adducin via the Rho/Rho-kinase pathway *in vivo*. pEF-BOS-HA- $\alpha$ -adducin (WT) (3  $\mu$ g) was cotransfected into COS7 cells with pEF-BOS vector (6  $\mu$ g), pEF-BOS encoding HA-Rac1<sup>V12</sup> (3  $\mu$ g), HA-Cdc42<sup>V12</sup> (3  $\mu$ g), HA-RhoA<sup>V14</sup> (3  $\mu$ g), HA-RhoA<sup>N19</sup> (3  $\mu$ g), myc-Rho-kinase (full-length) (3  $\mu$ g), HA-RhoA<sup>V14</sup> (3  $\mu$ g) and myc-Rho-kinase (full-length) (3  $\mu$ g), HA-RhoA<sup>V14</sup> (3  $\mu$ g) and myc-RB (6  $\mu$ g), HA-RhoA<sup>V14</sup> (3  $\mu$ g) and myc-RB/PH (TT) (6  $\mu$ g), myc-CAT (3  $\mu$ g) or pcDSR $\alpha$ -PKC (6  $\mu$ g). pEF-BOS-HA- $\alpha$ -adducin-AA (3  $\mu$ g) was also cotransfected with myc-CAT (3  $\mu$ g). As a negative control, pEF-BOS vector (12  $\mu$ g) alone was transfected. After a 24 h-incubation, the transfected cells were incubated in serum-

free medium for 24 h. The lysates of the cells were subjected to immunoblot analysis with indicated antibodies. The amounts of the sample in lanes of HA- $\alpha$ -adducin or HA- $\alpha$ -adducin-AA cotransfected with CAT of the upper panel were 30% of the others. Arrowheads indicate the positions of the intact band of HA- $\alpha$ -adducin. The anti-pT445-immunoreactive bands just below the intact HA- $\alpha$ -adducin correspond to endogenous  $\alpha$ -adducin. The additional bands which anti-pT445 recognized were the degradation products, because these proteins were also detected by anti-HA antibody. The ratios of phosphorylation at Thr445 to expressed HA- $\alpha$ -adducin were quantitated, and the proportions relative to the ratio of CAT-induced phosphorylation of HA- $\alpha$ -adducin were indicated in brackets as follows: HA- $\alpha$ -adducin alone, or with Rac1<sup>V12</sup>, or Cdc42<sup>V12</sup> (0%); with RhoA<sup>V14</sup> (4%); with RhoA<sup>N19</sup> (0.3%); with Rho-kinase (20%); with RhoA<sup>V14</sup> and Rho-kinase (32%); with RhoA<sup>V14</sup> and RB (0.1%); with RhoA<sup>V14</sup> and RB/PH (TT) (0%); with CAT (100%); with PKC (0%); HA- $\alpha$ -adducin-AA with CAT (0%). The results are representative of three independent experiments.

**Fig. 6.** The localization of Thr445-phosphorylated  $\alpha$ -adducin in the TPA- or HGF-induced membrane ruffling. The serum-deprived MDCK cells were stimulated with 200 nM TPA (d-f) or 50 pM HGF (g-i) for 15 min or with 200 nM TPA for 2 h (j-l). Non-stimulated (a-c) and stimulated cells were doubly labeled with anti-pT445 (b, e, h and k) and TRITC-phalloidin (c, f, i and l). Phase contrast images are shown

(a, d, g and j). Arrowheads indicate the induced-membrane ruffling areas or the leading edges. Bar, 25  $\mu$ m.

**Fig. 7.** The enrichment of phosphorylated  $\alpha$ -adducin in the membrane ruffling areas. MDCK cells stably expressing HA- $\alpha$ -adducin were established. Non-stimulated (a, b, and c) or TPA-stimulated (d, e, and f) MDCK cells stably expressing HA- $\alpha$ -adducin were doubly stained with anti-pT445 (green; a and d) and anti-HA (red; 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  $\mu$ m.

**Fig. 8.** The TPA-induced Thr445-phosphorylation in  $\alpha$ -adducin. MDCK cells stably expressing HA- $\alpha$ -adducin were stimulated by vehicle (indicated by -) or TPA (indicated by +) for 15 min followed by the incubation in the presence of calyculin A, and then lysed and subjected to immunoblot analysis with anti-pT445. An arrow indicates the phosphorylated HA- $\alpha$ -adducin. These results are representative of three independent experiments.

**Fig. 9.** The inhibition of the TPA-induced membrane ruffling by the mutants of Rho-kinase and  $\alpha$ -adducin. (A) The serum-deprived MDCK cells were microinjected with MBP (2 mg/ml) (a), C3 (0.1 mg/ml) (b); GST-RB (2 mg/ml) (c), MBP-RB/PH (TT) (2 mg/ml) (d and e), or Rac1<sup>N17</sup> (0.2 mg/ml) (f), HA- $\alpha$ -adducin (WT) (5 mg/ml) (g), HA- $\alpha$ -adducin-AA (5 mg/ml) (h), or HA- $\alpha$ -adducin-DD (5 mg/ml) (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 (a-d and f-i) and Thr445-phosphorylated  $\alpha$ -adducin (e) were visualized. Arrowheads indicate the microinjected cells. Bar, 25  $\mu$ m. (B) The ratios of the membrane ruffling-induced cells to the cells injected with proteins described in (A) are indicated. Data are means  $\pm$  SEM of triplicate determinations.

**Fig. 10.** Specificity of the effect of HA- $\alpha$ -adducin-AA on the TPA-induced membrane ruffling. HA- $\alpha$ -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  $\pm$  SEM of triplicate determinations.

**Fig. 11.** HA- $\alpha$ -adducin-AA had no effect on stress fiber formation. The serum-deprived NIH3T3 cells were microinjected with MBP (2 mg/ml) (a), MBP-RB/PH (TT) (2 mg/ml) (b), or HA- $\alpha$ -adducin-AA (5 mg/ml) (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  $\mu$ m. These results are representative of three independent experiments.

**Fig. 12.** Effects of  $\alpha$ -adducin mutants on the membrane ruffling in the cells introduced with the dominant negative Rho-kinase. MBP, HA- $\alpha$ -adducin (WT), HA- $\alpha$ -adducin-AA, or HA- $\alpha$ -adducin-DD (10 mg/ml) was comicroinjected with C3 (0.1

mg/ml), GST-RB (2 mg/ml), MBP-RB/PH (TT) (2 mg/ml), or Rac1<sup>N17</sup> (0.2 mg/ml) along with a marker protein (rabbit IgG, 0.5 mg/ml) into the serum-deprived MDCK cells. After a 30 min-incubation, the cells were stimulated with 200 nM TPA for 15 min. (A) The cells into which HA- $\alpha$ -adducin-DD was comicroinjected with GST-RB (a and b), MBP-RB/PH (TT) (c and d), and Rac1<sup>N17</sup> (e and f) are shown. The cells were doubly labeled with TRITC-phalloidin (a, c and e) and FITC-anti-rabbit Ig Ab (b, d and f). Arrowheads indicate the microinjected cells. Bar, 25  $\mu$ m. These results are representative of three independent experiments.

(B) The ratios of the membrane ruffling-induced cells to injected cells are indicated. Data are means  $\pm$  SEM of triplicate determinations.

**Fig. 13.** The localization of Thr445-phosphorylated  $\alpha$ -adducin in the migrating NRK49F cell. NRK49F cells which migrated in response to the wounding were stained with TRITC-phalloidin (a) and anti-pT445 (b). Arrows indicate the direction of migration. Arrowheads indicate the membrane ruffling in the leading edge. Bar, 20  $\mu$ m.

**Fig. 14.** Effects of the mutants of Rho-kinase and  $\alpha$ -adducin on cell migration in a wound healing assay. (A) A confluent monolayer of NRK49F cells was linearly wounded with a white chip. Soon after wounding, MBP (5 mg/ml) (a and b), C3 (0.1 mg/ml) (c and d), MBP-RB/PH (TT) (5 mg/ml) (e and f), HA- $\alpha$ -adducin (WT) (5 mg/ml) (g and h), HA- $\alpha$ -adducin-AA (5 mg/ml) (i and j), or HA- $\alpha$ -adducin-DD (5 mg/ml) (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 ( $> 20 \mu\text{m}$ ) from the original wound edge were counted by taking photographs of the same fields after 6 hours. The cells were doubly labeled by staining with TRITC-phalloidin (a, c, e, g, i, and k) and FITC-anti-rabbit Ig Ab (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,  $20 \mu\text{m}$ . These results are representative of three independent experiments.

(B) The ratios of migrating cells to the total injected cells are indicated. Data are means  $\pm$  SEM of triplicate determinations.

**Fig. 15.** Model for the role of adducin phosphorylated by the Rho/Rho-kinase pathway in cell motility. A spectrin-F-actin meshwork is linked to the plasma membrane by association with membrane accessory proteins such as band 4.1. Upon the activation of Rho-kinase by Rho during the action of extracellular stimuli,  $\alpha$ -adducin is phosphorylated at Thr445 and Thr480. Phosphorylated  $\alpha$ -adducin binds to F-actin, and then facilitates the recruitment of spectrin to F-actin. This may promote the formation of a spectrin-F-actin meshwork beneath plasma membrane for membrane ruffling. On the other hand, PKC phosphorylates  $\alpha$ -adducin

at Ser726, and then inhibits the activity of adducin in recruiting spectrin to F-actin. This may in turn induce the disassembly of a spectrin-F-actin meshwork. Transition between the phosphorylation states of  $\alpha$ -adducin by Rho-kinase and PKC may regulate the remodeling of membrane ruffling, which is important for cell migration.

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