Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo

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Summary

Ezrin/radixin/moesin (ERM) proteins crosslink actin filaments to plasma membranes and are involved in the organization of the cortical cytoskeleton, especially in the formation of microvilli. ERM proteins are reported to be activated as crosslinkers in a Rho-dependent manner and are stabilized when phosphorylated at their C-terminal threonine residue to create C-terminal threonine-phosphorylated ERM proteins (CPERMs). Using a CPERM-specific mAb, we have shown, in vivo, that treatment with C3 transferase (a Rho inactivator) or staurosporine (a protein kinase inhibitor) leads to the dephosphorylation of CPERMs, the translocation of ERM proteins from plasma membranes to the cytoplasm and microvillar breakdown. We further elucidated that ERM protein activation does not require C-terminal phosphorylation in A431 cells stimulated with epidermal growth factor. In certain types of kidney-derived cells such as MDCK cells, however, ERM proteins appear to be activated in the absence of Rho activation and remain active without C-terminal phosphorylation. Interestingly, microinjection of an aminoglycoside antibiotic, neomycin, which binds to polyphosphoinositides, such as phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], affected the activation of ERM proteins regardless of cell type. These findings not only indicate the existence of a Rho-independent activation mechanism of ERM proteins but also suggest that both Rho-dependent and -independent activation of ERM proteins require a local elevation of PtdIns(4,5)P_2 concentration in vivo.

Key words: ERM protein, Microvilli, Rho, PtdIns(4,5)P_2, Neomycin

Introduction

Ezrin/radixin/moesin (ERM) proteins are three closely related proteins from the band 4.1 superfamily and are thought to function as crosslinkers between plasma membranes and actin filaments (for reviews, see Vaheri et al., 1997; Bretscher, 1999; Mangeat et al., 1999; Tsukita and Yonemura, 1999). They are mainly localized just beneath the plasma membranes of cellular protrusions, such as microvilli. Suppression of ERM protein expression with antisense oligonucleotides in cultured cells resulted in complete disappearance of microvilli (Takeuchi et al., 1994), indicating their essential role in microvillar formation. The C-terminal domain of ERM proteins binds to actin filaments (Algrain et al., 1993; Turunen et al., 1994; Henry et al., 1995; Martin et al., 1995; Pestonjamasp et al., 1995), and the N-terminal, called the FERM domain (band 4.1, ezrin, radixin, moesin homology domain; Chishti et al., 1998), associates directly with the cytoplasmic domains of several integral membrane proteins such as CD43, CD44, ICAM-1, -2 and -3 (Algrain et al., 1993; Yonemura et al., 1993; Tsukita et al., 1994; Henry et al., 1995; Helander et al., 1996; Hirao et al., 1996; Serrador et al., 1997; Heiska et al., 1998; Yonemura et al., 1998) or indirectly with the Na^+/H^+ exchanger 3 via a cytoplasmic phosphoprotein called EBP50 or NHE-RF (Reczek et al., 1997; Yun et al., 1997; Murthy et al., 1998). Overexpression of the C-terminal half of ezrin or radixin induced abnormally long cellular protrusions (Henry et al., 1995; Martin et al., 1995), and overexpression of the N-terminal of ezrin prevented formation of microvilli (Crepaldi et al., 1997). However, full-length ERM proteins did not affect the cell morphology.

Soluble ERM proteins in the cytoplasm are ‘dormant’ in terms of their crosslinking activity through intramolecular association between FERM and C-terminal tail domains (Gary and Bretscher, 1995); this interaction was recently confirmed by crystal structure analysis (Pearson et al., 2000). When these dormant ERM proteins are activated, both domains are exposed and allowed to interact with membrane proteins (for the FERM domain) and actin filaments (for the C-termimal tail) (Berryman et al., 1995; Bretscher et al., 1995; Gary and Bretscher, 1995; Hirao et al., 1996; Matsui et al., 1998). These activated ERM proteins together with ERM-binding membrane proteins are directly involved in the organization of microvilli (Yonemura et al., 1999).

Evidence explaining the mechanism of ERM protein activation is accumulating. Polyphosphoinositides such as phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] bind to ERM proteins and appear to open closed ERM proteins, enhancing their binding to membrane proteins and actin

filaments (Niggli et al., 1995; Hirao et al., 1996; Heiska et al., 1998; Huang et al., 1999; Nakamura et al., 1999; Barret et al., 2000; Niggli, 2001). In support of this, recent crystal structure analysis showed that the N-terminal half of radixin has a basic cleft that can bind to the headgroup of PtdIns(4,5)P_2 (Hamada et al., 2000).

Phosphorylated ERM proteins, especially ERM proteins phosphorylated at the C-terminal threonine residue (C-terminal threonine-phosphorylated ERM proteins; CPERMs), are also considered to be closely involved in ERM protein activation. The amount of moesin phosphorylated at the C-terminal threonine residue increases in platelets upon activation by thrombin (Nakamura et al., 1995). At the initial stage of anoxic injury and apoptosis, most microvilli disappear from the cell surface, and in both cases ERM proteins are translocated from the microvilli to the cytoplasm with concomitant dephosphorylation (Chen et al., 1995; Kondo et al., 1997; Hayashi et al., 1999). Moesin phosphorylated in vitro and phosphorylated moesin purified from platelets showed enhanced binding both to actin filaments and to EBP50 (Simons et al., 1998; Nakamura et al., 1999). Hayashi et al. showed that CPERMs represent an active form of ERM proteins in vivo in terms of their exclusive localization at the plasma membrane (Hayashi et al., 1999). This was experimentally confirmed using ERM proteins in which the C-terminal threonine residue was mutated to aspartic acid (Oshiro et al., 1998; Huang et al., 1999; Yonemura et al., 1999). However, this phosphorylation may not be required for activating ERM proteins but for stabilizing activated ERM proteins, as phosphorylation of the C-terminal half of radixin does not enhance its actin-filament-binding ability but rather inhibits its interaction with the FERM domain (Matsui et al., 1998).

A small GTPase, called Rho, which organizes stress fibers (Tapon and Hall, 1997), is also thought to be involved in ERM protein activation. Interestingly, ERM proteins may regulate Rho activity and vice versa (for reviews, see Bretscher, 1999; Mangeat et al., 1999; Tsukita and Yonemura, 1999). The in vitro association of ERM proteins with the plasma membranes of cultured cells was dependent on Rho activation (Hirao et al., 1996). In several lines of cultured cells, activation of RhoA but not Rac1 or Cdc42 produced CPERMs, forming microvilli with a concomitant accumulation of ERM proteins in microvilli (Shaw et al., 1998; Matsui et al., 1998; Matsui et al., 1999). One of the direct effectors of Rho, Rho-kinase, can phosphorylate the C-terminal threonine of ERM proteins in vitro (Matsui et al., 1998) and appears to do so also in vivo (Oshiro et al., 1998). However, Matsui et al. (Matsui et al., 1999) showed that Rho-dependent production of CPERMs was not suppressed by Y-27632, a specific inhibitor of ROCK kinases, including Rho-kinase (Uehata et al., 1997). Overexpression of another direct effector of Rho, phosphatidylinositol 4-phospate 5-kinase type Iα (PtdIns4*P* 5-kinase α), which produces PtdIns(4,5)P_2, increased the level of CPERMs and induced microvillar formation (Matsui et al., 1999). Furthermore, Barret et al. showed that mutagenesis of the PtdIns(4,5)P_2-binding site in the N-terminal domain of ezrin alters its membrane localization (Barret et al., 2000), suggesting that PtdIns(4,5)P_2 is important in ERM protein function at the plasma membrane.

Thus, it was postulated that PtdIns(4,5)P_2, which is produced in a Rho-dependent manner, directly activates ERM proteins and that activated ERM proteins are phosphorylated at their C-terminal threonine residue for stability (Matsui et al., 1999). To evaluate this hypothesis, we further examined the roles of Rho, CPERMs and PtdIns(4,5)P_2 in various types of cultured cells. During the course of these studies we found, unexpectedly, that in certain cell lines that neither the activation of Rho nor the phosphorylation of ERM proteins was required in the activation of ERM proteins or for their stabilization. In this study, we examined the molecular mechanism behind the Rho-independent activation of ERM proteins, paying special attention to the role of PtdIns(4,5)P_2.

Materials and Methods

Cells and antibodies

A431, HeLa, Madin-Darby bovine kidney (MDBK), PtK2, LLC-PK1, NIH3T3, CHO, CV1, P3 and 3Y1 cells were obtained from Health Science Research Resources Bank (Osaka, Japan). L, MTD-1A and MDCK I cells (Richardson et al., 1981) were provided by M. Takeichi (Kyoto University, Kyoto, Japan). MDCK II (Richardson et al., 1981) cells were a gift from K. Murata (National Institute for Physiological Sciences, Okazaki, Japan). Mouse anti-ERM mAb (CR22) with higher affinity to moesin than to ezrin/radixin, rat anti-CPERMs mAb (297S), rat anti-ezrin mAb (M11), rat anti-moesin mAb (M22) and rabbit anti-ERM polyclonal antibody (pAb) (TK89) have been described previously described previously (Sato et al., 1991; Takeuchi et al., 1994; Matsui et al., 1998; Kondo et al., 1997). Mouse anti-hemagglutinin (HA) mAb (12CA5) was purchased from Boehringer Mannheim Biotechnology. Mouse anti-vesicular stomatitis virus glycoprotein G (VSVG) mAb (clone P5D4) was purchased from Upstate Biotechnology. Inc. C3 transferase, a potent inhibitor of Rho (Narumiya et al., 1988), was produced in *Escherichia coli* and purified. Aminoglycosides, neomycin sulfate and spectinomycin dihydrochloride were purchased from Sigma and used after neutralization. Ampicillin sodium salt was from NACALAI TESQUE. INC. Staurosporine, a protein kinase inhibitor, was purchased from Wako Pure Chemical Industries, Ltd.

EGF treatment of A431 cells

Subconfluent A431 cells grown on coverslips were cultured in DMEM without FCS for 6-24 hours. Recombinant human epidermal growth factor (EGF) (GIBCO BRL) was added at a final concentration of 100 ng/ml. Cells were incubated for 30 seconds to 30 minutes and quickly fixed and processed for immunofluorescence microscopy.

Microinjection

Several reagents were introduced into cells by microinjection using a set of manipulators (MN-188 and MO-189; Narishige, Tokyo, Japan) connected to an Eppendorf microinjector 5242 (Eppendorf, Inc., Hamburg, Germany) and a Zeiss Axiovert 135 microscope. As markers for microinjected cells fluorescein-conjugated 70,000 MW anionic, lysine-fixable dextran (F-dextran) or tetramethylrhodamine-conjugated 70,000 MW, lysine-fixable dextran (Rh-dextran) (Molecular Probes, Inc.) was used. C3 transferase (1μg/μl) or antibiotics such as neomycin sulfate (1-10 mM) were microinjected with 1% F- or Rh-dextran in PBS into the cytoplasm of cultured cells on coverslips. For transfection, expression vectors pEF-BOS-HAx3-V14RhoA for constitutively active RhoA, pA/mEz-VSVG for VSVG-tagged ezrin (Yonemura et al., 1999), pA/mEz-T/A-VSVG where the C-terminal threonine residue of ezrin was mutated to alanine (Yonemura et al., 1999) or pEF-BOS-HAx3-PI4P5K for PI4P5Kα (Matsui et al., 1999) at 0.2-0.6 μg/μl in a solution containing 1 mM

EDTA and 10 mM Tris-HCl (pH 8.0) was injected into the nuclei of cells. Cells were examined 6-8 hours after injection.

Immunofluorescence microscopy

In most cases, cells were fixed with ice-cold 10% trichloroacetic acid (TCA) for 15 minutes (Hayashi et al., 1999). When F-dextran or Rh-dextran was microinjected into cells, cells were fixed with ice-cold 10% TCA plus 1% paraformaldehyde to ensure the immobilization of F- or Rh-dextran within cells. The presence of paraformaldehyde in 10% TCA solution did not affect the staining of ERM proteins or CPERMs. To visualize actin filaments with rhodamine phalloidin or FITC phalloidin (Molecular Probes, Inc.), cells were fixed with 4% paraformaldehyde in 0.1 M Hepes buffer (pH 7.5) for 15 minutes. After three washes with PBS containing 30 mM glycine (G-PBS), cells were treated with 0.2% Triton X-100 in G-PBS for 10 minutes and washed with G-PBS. Cells were soaked in blocking solution (G-PBS containing 4% normal donkey serum) for 5 minutes and incubated with primary antibodies diluted with the blocking solution for 30 minutes. Cells were then washed three times with G-PBS and incubated with secondary antibodies for 30 minutes. FITC- or Cy3-conjugated donkey anti-mouse IgG antibody, FITC- and Cy3-conjugated donkey anti-rat IgG antibody and FITC-, Cy3- and Cy5-conjugated donkey anti-rabbit IgG antibody were from Jackson ImmunoResearch Laboratories, Inc. Cy5-conjugated goat anti-mouse IgG antibody was from Amersham International. Cells were washed three times then mounted in 90% glycerol-PBS containing 0.1% para-phenylendiamine and 1% n-propylgalate. Specimens were observed using a Zeiss Axiophot photomicroscope or Olympus IX70 with appropriate combinations of filters and mirrors. Images were recorded with a cooled CCD camera (SenSys 0400, 768×512 pixels; Photometrics) controlled by a Power Macintosh 7600/132 and the software package IPLab Spectrum V3.1 (Scanalytics Inc.).

Electron microscopy

L cells were cultured on CELLocate coverslips (Eppendorf) and microinjected with C3/F-dextran or neomycin/F-dextran. They were kept in a CO_2 incubator for 30 minutes and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 hours at room temperature. L cells treated with staurosporine (10 nM) for 1 hour were also fixed under the same condition. Microinjected cells were identified under a fluorescence microscope, and their phase contrast as well as fluorescence images were recorded together with their location information printed on CELLocate coverslips. Samples were then processed conventionally and examined under a scanning electron microscope (S-3500N, Hitachi Co.). Microinjected cells were identified on the basis of the recorded images.

For transmission electron microscopy, MDCK II cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 hours at room temperature and then post-fixed with 1% OsO_4 in the same buffer for 2 hours on ice. The samples were rinsed with distilled water, stained with 0.5% aqueous uranyl acetate for 2 hours at room temperature, dehydrated with ethanol and embedded in Polybed 812 (Polyscience). Ultra-thin sections were cut, doubly stained with uranyl acetate and lead citrate and viewed with a JEM 1010 transmission electron microscope (JEOL).

Soluble and insoluble ERM proteins

Subconfluent L cells cultured on a 35 mm dish were treated with 10 nM staurosporine for 1 hour then washed twice with ice-cold PBS. After removal of PBS, 500 μl of an ice-cold sonication buffer (150

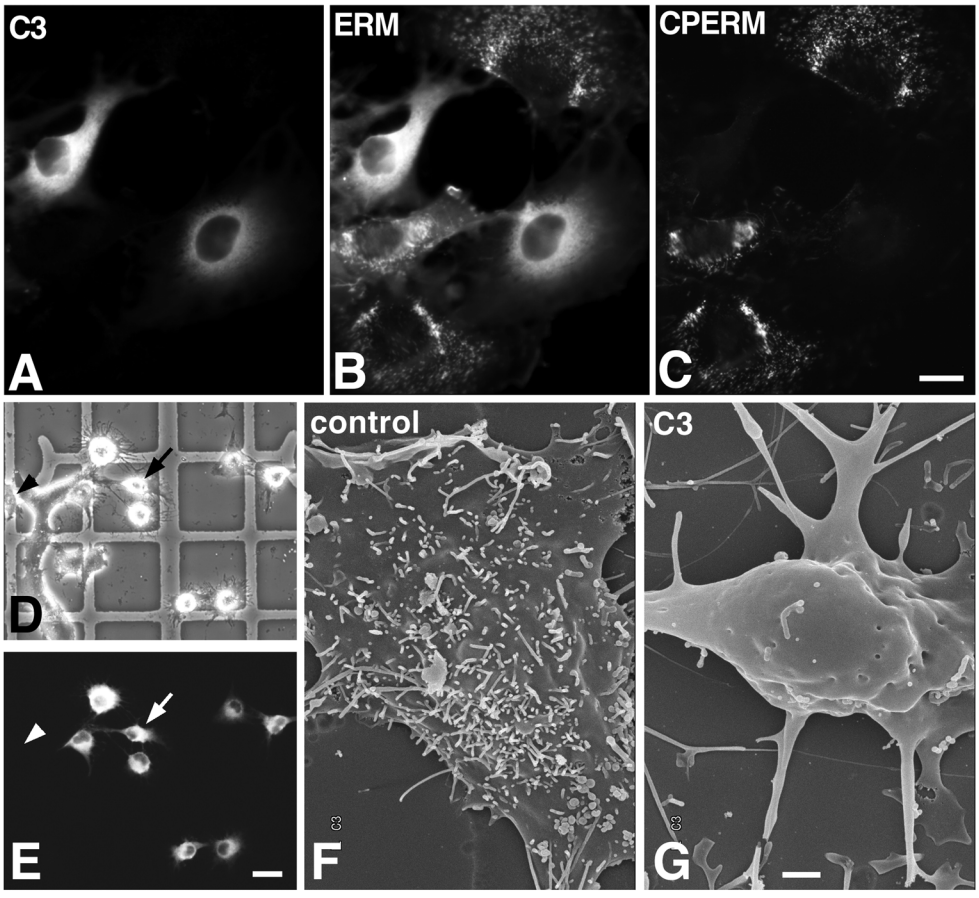

Fig. 1. Inactivation of ERM proteins in L cells by microinjection of C3 transferase. (A-C) Rh-dextran and C3 transferase were co-microinjected into L cells. After a 30 minute incubation, cells were fixed with TCA/formaldehyde, and microinjected cells were identified by the fluorescence of Rh-dextran (A). Cells were then doubly stained with anti-ERM mAb, CR22 (B) and anti-CPERM mAb (C). C3 induced dephosphorylation of CPERMs (C), with concomitant translocation of ERM proteins from microvilli to the cytoplasm (B). (D-G) L cells grown on CELLocate™ coverslips were microinjected with C3/F-dextran followed by glutaraldehyde fixation. Their phase-contrast (D) and fluorescence (E) images were recorded to identify microinjected cells. Scanning electron microscopy showed that a non-microinjected cell (arrowheads) bore numerous microvilli on its surface (F), whereas a microinjected cell (arrow) was characterized by a smooth cell surface (G). Bars, A-C, 10 μm; D, E, 10 μm; F, G, 2 μm.

mM NaCl, 1 mM EGTA, 1 mM DTT, 10 μg/ml leupeptin, 10 mM Hepes buffer, pH 7.5) containing 10 nM staurosporine was added. Cell were scraped off and collected into 1.5 ml tubes. 500 μl of the cell suspension was transferred to a new tube and sonicated. The homogenates were centrifuged at 10,000 ***g*** for 10 minutes at 4°C to recover the soluble and insoluble fractions. Equivalent amounts of supernatant and pellet were applied to SDS-polyacrylamide gels followed by immunoblotting with pAb TK89.

To examine the effects of neomycin on the solubility of ERM proteins, subconfluent L cells or MDCK II cells cultured on a 35 mm dish were processed as described above, except that 1 mM neomycin sulfate was added to the sonication buffer instead of staurosporine. After sonication, the homogenates were kept on ice for 30 minutes. Then soluble and insoluble fractions were recovered and analyzed as described above.

Results

Microvillar formation with concomitant CPERM production and ERM protein translocation in L cells is blocked by C3 transferase

Lysophosphatidic-acid-induced relocalization of epitope-tagged radixin stably expressed in NIH3T3 cells and lysophosphatidic-acid-induced rapid phosphorylation of ERM proteins at the C-terminal threonine in Swiss 3T3 cells are blocked by C3 transferase, a potent inhibitor of Rho (Shaw et al., 1998; Matsui et al., 1998). Therefore, we first addressed whether Rho activity is required for endogenous ERM protein activation, including its phosphorylation, translocation to microvilli and formation of microvilli in cells cultured under conventional conditions. C3 was microinjected into L fibroblasts. Within 30 minutes of microinjection, CPERMs were dephosphorylated almost completely, and ERM proteins were translocated from the microvilli to the cytoplasm (Fig. 1A-C). As a negative control, 1% BSA was microinjected into L cells, which showed no change either in the level of phosphorylation or in the localization of ERM proteins (data not shown). As a positive control, destruction of stress fibers by C3 (Ridley et al., 1992) was confirmed in L cells (data not shown). To confirm whether ERM protein translocation from microvilli with concomitant dephosphorylation of CPERMs represents microvillar breakdown, we examined cell surface morphology using a scanning electronic microscope. Compared with control L cells, which have numerous microvilli, C3-microinjected L cells had a smooth cell surface with only a few microvilli, if any (Fig. 1D-G). Thus, in L cells, Rho activity is required for ERM protein activation under conventional culture conditions. This was confirmed in other types of cells, including epithelial MTD-1A, PtK2 and fibroblastic 3Y1 cells (data not shown).

In serum-starved A431 cells, EGF treatment causes rapid and transient microvillar elongation and membrane ruffling with concomitant ERM protein translocation to these structures and CPERM production (Chinkers et al., 1979; Bretscher, 1989; Yonemura et al., 1999). C3 blocked this EGF-induced ERM protein activation and microvillar elongation in A431 cells (Fig. 2A-C). In serum-starved A431 cells where the amount of CPERM is very low, expression of a constitutively active form of Rho (V14RhoA) resulted in a dramatic increase in the amount of CPERM localized at microvilli (Fig. 2D,E). Thus, Rho activity is indispensable for ERM protein activation in several types of cells.

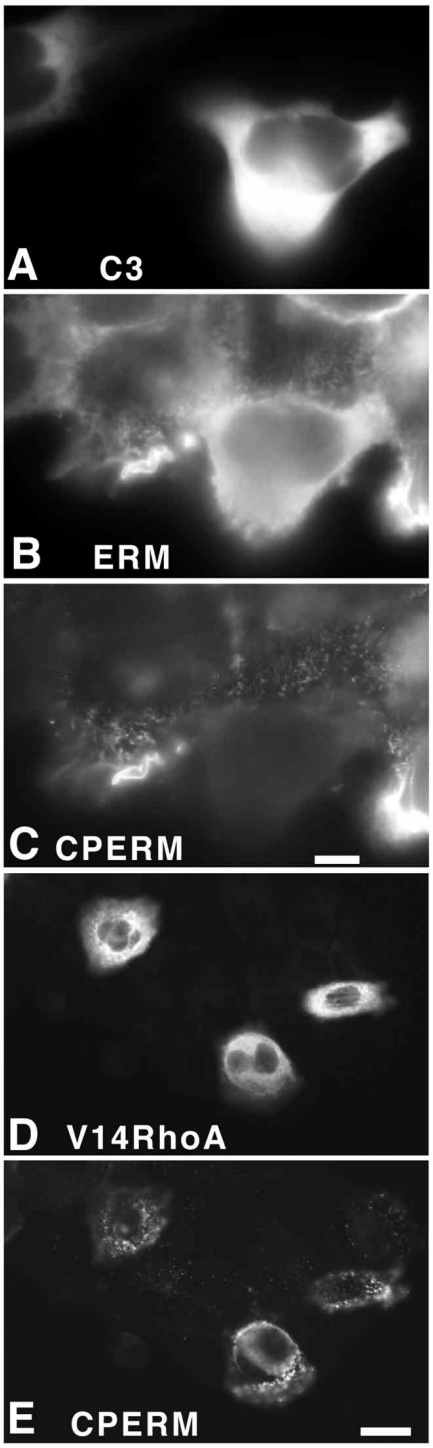

Fig. 2. Rho-dependent ERM protein activation in A431 cells. Serum-starved A431 cells were microinjected with C3/Rh-dextran (A), incubated for 10 minutes, then stimulated with EGF for 5 minutes. Cells were fixed and doubly stained with anti-ERM pAb, TK89 (B) and anti-CPERM mAb (C). C3 completely suppressed both the production of CPERMs and microvillar elongation with concomitant recruitment of ERM proteins. Constitutively active RhoA (V14RhoA) was expressed in serum-starved A431 cells (D,E). Cells were doubly stained with anti-HA mAb for detection of HA-tagged V14RhoA (D) and anti-CPERM mAb (E). CPERMs were dramatically increased in microvilli in cells where Rho activity was increased. Bars, A-C, 10 μm; D,E, 20 μm.

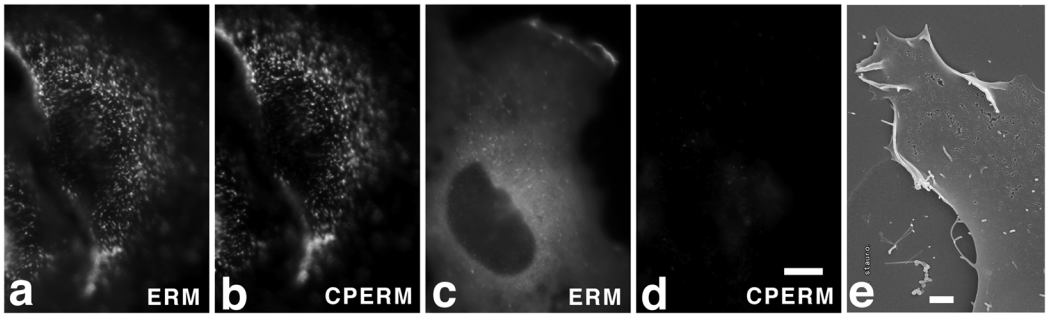

Fig. 3. Dephosphorylation leads to inactivation of ERM proteins in L cells. (A) Control L cells (a,b) and 10 nM staurosporine-treated L cells (c,d) were doubly stained with anti-ERM mAb, CR22 (a,c), and anti-CPERM mAb (b,d). Staurosporine induced a dephosphorylation of CPERMs and translocation of ERM proteins from microvilli to the cytoplasm. Scanning electron microscopy showed that a staurosporine-treated L cell lost microvilli on its cell surface (e). Bars, a-d, 10 μm; e, 2 μm. (B) L cells cultured in the absence (control) or presence (stauro.) of 10 nM staurosporine were homogenized and centrifuged. Equivalent amounts of supernatant (S) and pellet (P) were subjected to immunoblotting with anti-ERM pAb, TK89. Note that staurosporine induced translocation of ERM proteins from the insoluble to soluble cell fraction (S).

Dephosphorylation of CPERMs can result in microvillar breakdown, but CPERM production is not necessary for EGF-induced microvillar formation

We next addressed whether phosphorylation of ERM proteins at the C-terminal threonine is required for ERM protein activation. Since staurosporine, a potent protein kinase inhibitor with a broad specificity, suppresses production of CPERMs in platelets (Nakamura et al., 1995), L cells were cultured in the presence of staurosporine at 10 nM for 1 hour. Almost all CPERMs were dephosphorylated, and ERM proteins were translocated from microvilli to the cytoplasm (Fig. 3Aa-d). Scanning electron microscopy confirmed the disappearance of microvilli from the surface of staurosporine-treated L cells (Fig. 3Ae). Biochemical findings were consistent with these morphological observations (Fig. 3B): in the homogenate obtained from staurosporine-treated L cells, ERM proteins were mostly recovered in the soluble fraction after centrifugation, whereas from control L cells a considerable amount of ERM protein was associated with the insoluble fraction. Similar effects of staurosporine were observed in epithelial MTD-1A, A431, fibroblastic NIH3T3, CV1 and myeloma P3 cells (data not shown). These results indicate that phosphorylation at the C-terminal threonine is required for ERM proteins to function as crosslinkers in these cells. CPERM production may be required for activating ERM proteins, or it may be required only for maintaining their active state. In other words, without phosphorylation, activated ERM proteins may be quickly inactivated by their intramolecular association. To solve this problem, we tried to activate ERM proteins within cells experimentally and examined the role of CPERM production. We chose A431 cells for this purpose because activation of ERM proteins in an inactive state is clearly observed, especially following EGF stimulation after serum-starvation; this was not observed in other cells. Interestingly, when serum-starved staurosporine-treated A431 cells were stimulated with EGF, elongation of microvilli-like structures and recruitment of ERM proteins to these structures occurred without CPERM production (Fig. 4Aa-d). These structures were confirmed to contain actin filaments (such as microvilli and ruffling membranes) (Fig. 4Ae,f), although staurosporine treatment affected the shape of these structures to some extent. These ERM proteins returned to the cytoplasm faster than those in control cells stimulated with EGF (Fig. 4Ba-j). The C-terminally VSVG-tagged ezrin (Ez-VSVG) was previously shown to behave in a similar manner to endogenous ezrin (Algrain et al., 1993; Crepaldi et al., 1997). When the C-terminal threonine residue of ezrin (T567) was mutated to alanine (Ez-T/A-VSVG; non-phosphorylatable ezrin), the expressed mutant ezrin in serum-starved A431 cells was recruited to microvilli and ruffling membrane after EGF stimulation, as Ez-VSVG was (Fig. 5), supporting the theory that ERM proteins can be activated without phosphorylation. We could not confirm this rapid and transient translocation of ERM proteins by the biochemical analysis described above, probably because not only activating but also inactivating pathways are working after EGF stimulation, rendering activated ERM protein unstable during cell fractionation (data not shown). These findings show that production of CPERMs is not required for ERM protein activity itself and confirm the role of phosphorylation in maintaining the active state of ERM proteins in vivo.

Rho-independent activation of ERM proteins

In contrast, in kidney-derived cells such as MDCK II cells, microinjection of C3 transferase did not dephosphorylate CPERMs (Fig. 6Aa) and did not affect their subcellular localization, although stress fibers were apparently disrupted (Fig. 6Ab). Similar findings were obtained with other kidney-derived cells: MDCK I, LLC-PK1 and MDBK cells (data not shown). These findings were consistent with a previous report showing no effects of C3 on the distribution of ERM proteins in MDCK cells (Kotani et al., 1997). Interestingly, staurosporine also induced dephosphorylation of CPERMs in these cells, but microvilli-like structures on apical

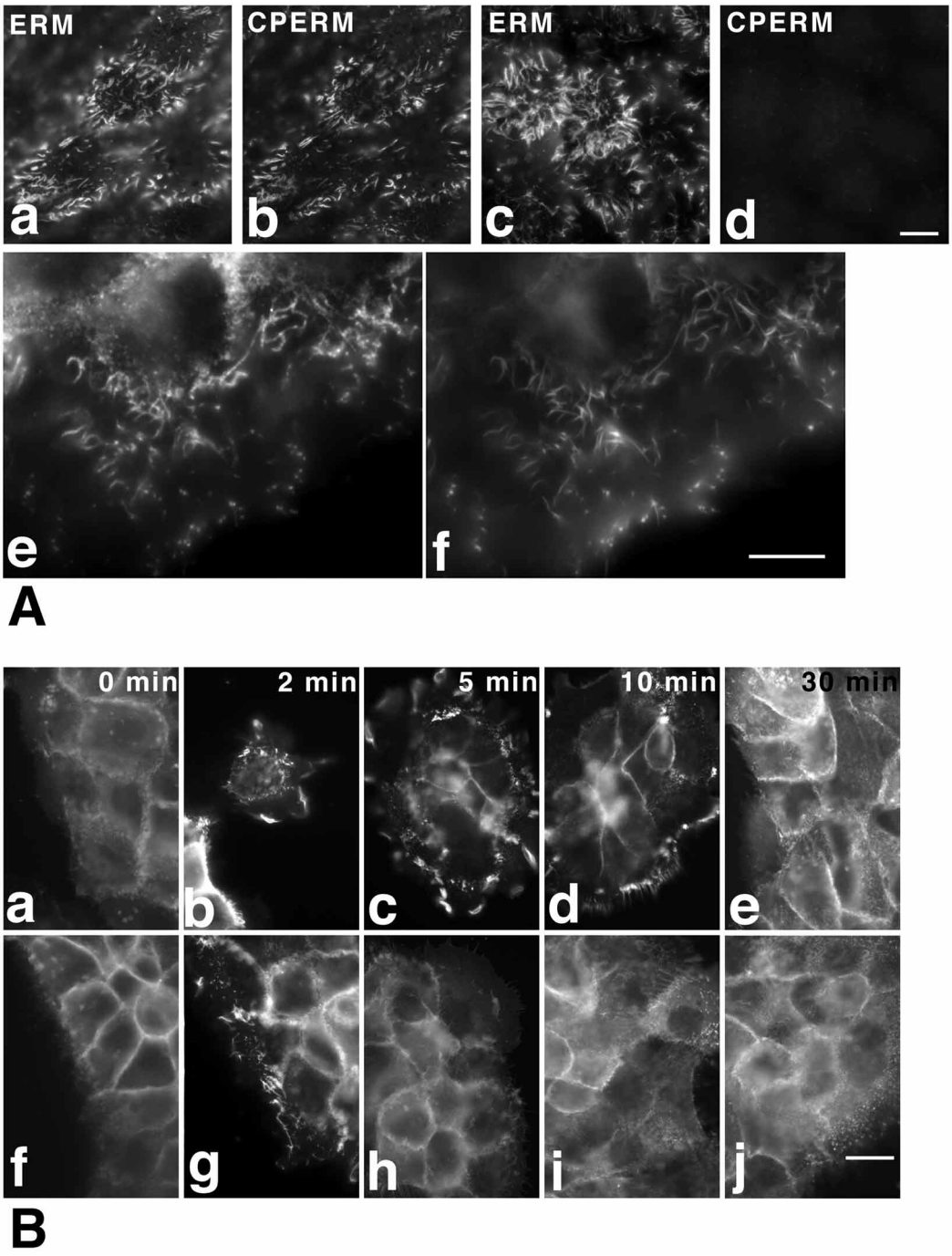

Fig. 4. Phosphorylation is not required for activation of ERM proteins but required for the maintenance of the active state in A431 cells. (A) Serum-starved A431 cells cultured in the absence (a,b) or presence (c-f) of staurosporine (10 minutes at 100 nM) were stimulated with EGF for 30 seconds and stained with anti-ERM mAb, CR22 (a,c) and anti-CPERM mAb (b,d). Although CPERMs were almost completely dephosphorylated by staurosporine treatment, microvillar elongation and recruiting of ERM proteins appeared normal. EGF-induced microvilli-like structures in the presence of staurosporine contained both ERM proteins stained with anti-ERM pAb, TK89 (e) and actin filaments stained with rhodamine phalloidin (f). Bars, a-d,10 μm; e,f, 10 μm. (B) Translocation of ERM proteins in A431 cells after EGF stimulation. Serum-starved cells cultured in the absence (a-e) or presence (f-j) of 25 nM staurosporine for 1 hour were stimulated with EGF. Cells were fixed with TCA and stained with anti-ERM mAb, CR22. Times after EGF stimulation (0 minute: a,f; 2 minutes: b,g; 5 minutes: c,h; 10 minutes: d,i; 30 minutes: e,j) are indicated. Note that ERM proteins recruited to cell surface structures such as microvilli and ruffling membranes after EGF stimulation from the cytoplasm were relocated to the cytoplasm much faster in the presence of staurosporine than in its absence. Bar, 20 μm.

membranes remained intact or occasionally elongated where dephosphorylated ERM proteins were concentrated (Fig. 6Ac,d). Transmission electron microscopy revealed that these structures are normal microvilli (Fig. 6Ae,f). In the homogenate obtained from staurosporine-treated MDCK cells, ERM proteins were recovered both in the soluble and the insoluble fraction after centrifugation; the same result was obtained for control MDCK II cells (Fig. 6B). These findings indicate that in these cells ERM proteins are activated in a Rho-independent manner and that their activated forms are stabilized by mechanisms other than phosphorylation of their C-terminal threonine residue.

Involvement of PtdIns(4,5)P_2 both in Rho-dependent and -independent activation of ERM proteins

PtdIns(4,5)P_2 activates ERM proteins as crosslinkers in vitro, and this activation had nothing to do with phosphorylation (Hirao et al., 1996). Furthermore, PtdIns(4,5)P_2 was suggested to play a crucial role in a Rho-dependent activation of ERM proteins in vivo (Matsui et al., 1999). We thus examined the effects of increasing PtdIns(4,5)P_2 concentration on the ERM activity within cells in more detail. Firstly, PtdIns4*P* 5-kinase α, which produces PtdIns(4,5)P_2 (Ishihara et al., 1996), was overexpressed in serum-starved A431 cells in which Rho was not activated and CPERMs were undetectable. As shown

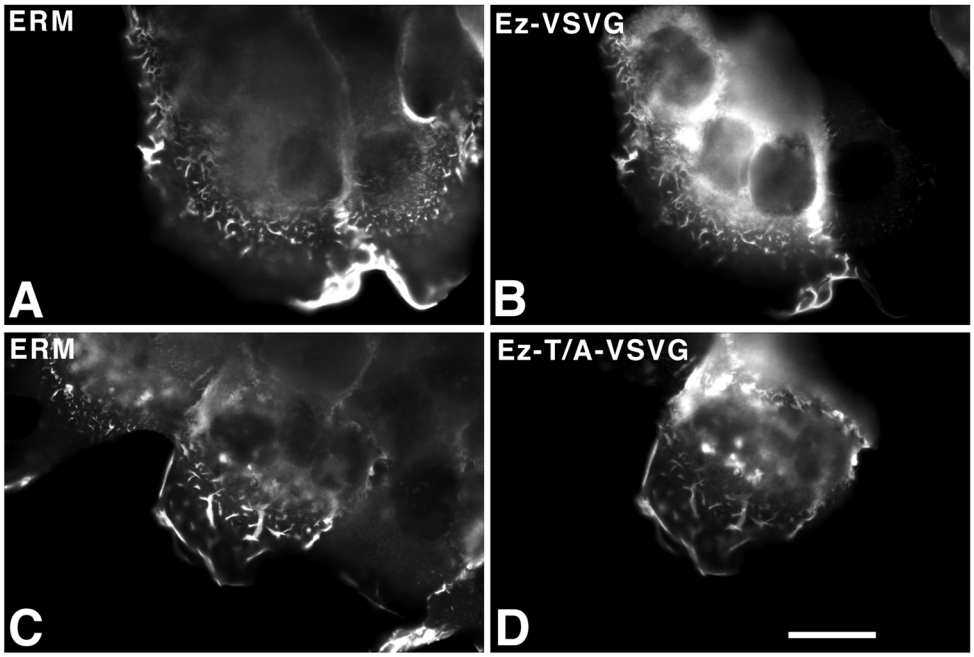

Fig. 5. An ezrin mutant that cannot be phosphorylated can be recruited to microvilli and ruffling membranes. A431 cells expressing VSVG-tagged mouse ezrin (Ez-VSVG) (A,B) and its mutant where the C-terminal threonine was mutated to alanine (Ez-T/A-VSVG) (C,D) were serum starved and stimulated with EGF for 1 minute. Cells were fixed with TCA and stained with anti-ERM pAb, TK89 (A,C) and anti-VSVG mAb, P5D4 (B,D). Both Ez-VSVG and Ez-T/A-VSVG were recruited to microvilli and ruffling membranes. Bar, 20 μm.

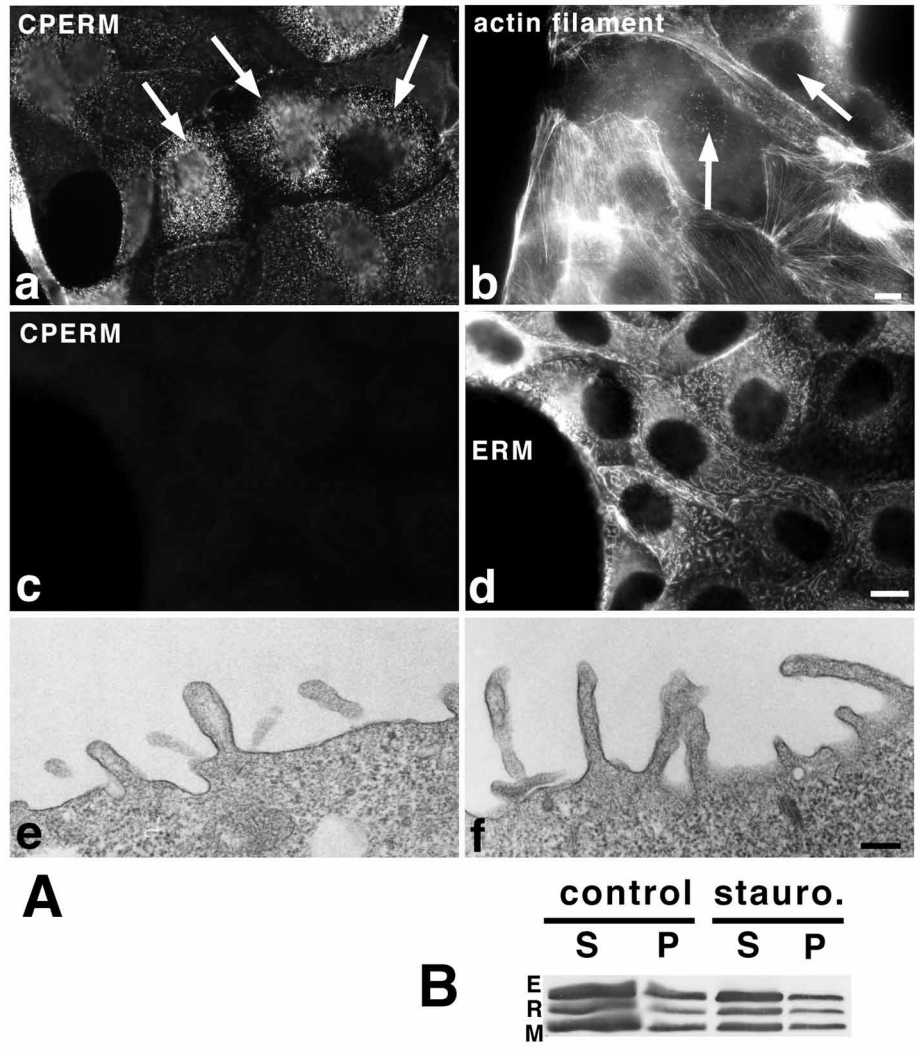

Fig. 6. Rho- and phosphorylation-independent activation of ERM proteins in MDCK II cells. (Aa,Ab) MDCK II cells were microinjected with C3/Rh-dextran (arrows in a,b). After a 30 minute incubation, cells were fixed with TCA and stained with anti-CPERM mAb (a) or fixed with formaldehyde and stained with FITC phalloidin (b). Because phalloidin cannot bind to actin filaments fixed with TCA, double staining with anti-CPERM mAb and phalloidin of the same specimen was impossible. Although C3 suppressed Rho activity enough to affect stress fibers (b), it showed no effects on CPERMs (a). (Ac,Ad) MDCK II cells were treated with 200 nM staurosporine for 10 minutes, then double stained with anti-CPERM mAb (c) and anti-ERM mAb, CR22 (d). Although CPERMs were mostly dephosphorylated (c), microvilli, which were sometimes elongated, remained with accumulated ERM proteins. (Ae,Af) Transmission electron micrographs of control (e) and staurosporine-treated (200 nM for 10 minutes) (f) MDCK II cells. The morphology of microvilli of staurosporine-treated MDCK II cells is normal, although they are often elongated. Bars, a,b, 10 μm; c,d, 10 μm; e,f, 0.2 μm. (B) MDCK II cells cultured in the absence (control) or presence (stauro.) of 200 nM staurosporine were homogenized and centrifuged. Equivalent amounts of supernatant (S) and pellet (P) were subjected to immunoblotting with anti-ERM pAb, TK89. Note that both in the absence and presence of staurosporine, a considerable amount of ERM protein was recovered in the insoluble (P) fraction.

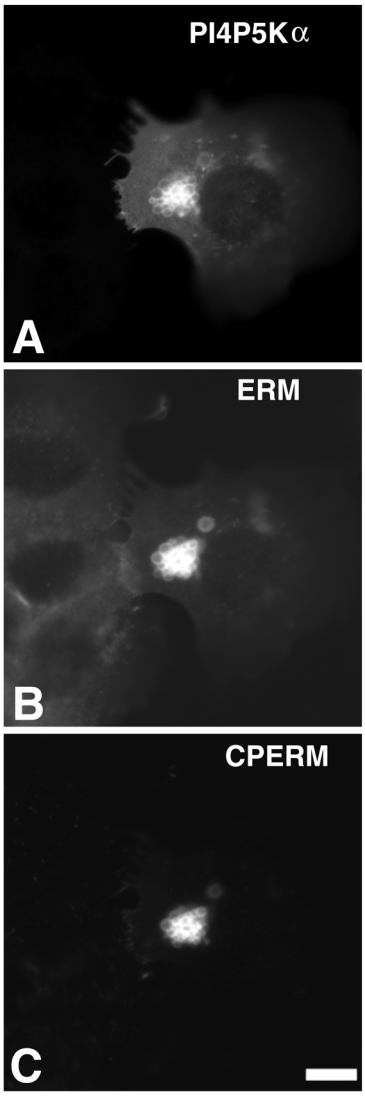

Fig. 7. Local expression of PtdIns4*P* 5-kinase (PI4P5Kα) and local activation of ERM proteins. HA-tagged PtdIns4*P* 5-kinase was overexpressed in serum-starved A431 cells that were triple stained with anti-HA mAb (A), anti-ERM pAb, TK89 (B) and anti-CPERM mAb (C). PtdIns4*P* 5-kinase was occasionally concentrated on the surface of vesicular structures (A) where ERM proteins were recruited (B) and phosphorylated (C). Bar, 10 μm.

in Fig. 7A,B, overexpressed PtdIns4*P* 5-kinase α was occasionally associated with vesicular structures in the cytoplasm to which considerable amounts of ERM proteins were recruited. Immunofluorescence microscopy showed that these recruited ERM proteins were phosphorylated at their C-terminal threonine residue (Fig. 7C). Actin filaments were also concentrated at these vesicles (data not shown). Thus, we concluded that the local production of PtdIns(4,5)P_2 by PtdIns4*P* 5-kinase α on the vesicles recruited and activated the ERM proteins and phosphorylated them without Rho activity.

We next examined the effects of depleting intracellular PtdIns(4,5)P_2. Neomycin, an aminoglycoside antibiotic that binds to polyphosphoinositides, especially PtdIns(4,5)P_2, with high affinity was used (Wang et al., 1984). As neomycin could not cross plasma membranes effectively, we microinjected neomycin into the cytoplasm to interfere with PtdIns(4,5)P_2 and PtdIns(4,5)P_2-binding proteins. As shown in Fig. 8Aa-c, microinjection of 10 mM neomycin induced rapid dephosphorylation of CPERMs and translocation of ERM proteins from microvilli to the cytoplasm within 10 minutes in L cells. Scanning electron microscopy showed that neomycin caused microvillar breakdown (Fig. 8Ad). One of the aminoglycosides, spectinomycin, which has a lower affinity for PtdIns(4,5)P_2 than neomycin (Wang et al., 1984), had no effect on ERM proteins at a concentration of 10 mM (data not shown). Ampicillin, which is from a different group of antibiotics, had no effect at 10 mM (data not shown). Supporting these morphological observations, when L cell homogenate was incubated in the presence of 1 mM neomycin for 30 minutes followed by centrifugation, ERM proteins were mostly recovered in the soluble fraction, whereas in the absence of neomycin, considerable amounts of ERM proteins were associated with the insoluble fraction (Fig. 8B). Similar findings were obtained for other types of cells in which ERM proteins were activated in a Rho-dependent manner as described above.

The question of whether PtdIns(4,5)P_2 is also involved in the Rho-independent activation of ERM proteins naturally arose. Interestingly, when 10 mM neomycin was microinjected into MDCK I, MDCK II, LLC-PK1 or MDBK cells, ERM proteins were translocated from microvilli to the cytoplasm (Fig. 8Cb). As shown above, in these cells the C-terminal threonine is not necessarily phosphorylated for activated ERM proteins to be stabilized, but CPERMs disappeared by microinjection of neomycin (Fig. 8Ca). Neomycin microinjection inhibited EGF-induced ERM protein activation in serum-starved A431 cells (data not shown). This finding indicates the importance of PtdIns(4,5)P_2 in ERM protein activation common in a variety of cells.

We used an ezrin-, moesin-specific mAb or a pan-ERM pAb (M11, M22 and TK89, respectively) for immunofluorescence microscopy to see whether activation mechanisms of ERM proteins differ from each other. In all cases described in this study, ezrin, moesin and total ERM proteins behaved similarly (data not shown). In many cases, the expression level of radixin was too low for detection by immunofluorescence microscopy. Considering that all ERM proteins behaved similarly in cell fractionation experiments and that cellular distributions of ERM proteins are quite similar to each other in many types of cultured cells (Franck et al., 1993; Takeuchi et al., 1994), each ERM protein appears to use the same activation mechanism.

Discussion

This study presented several lines of evidence concerning the relationship between the activated ERM proteins and CPERMs. In L cells, three reagents all with different actions, C3 transferase, staurosporine and neomycin, induced the same phenomena: (i) the amount of CPERM decreased; (ii) ERM proteins were translocated from microvilli to the cytoplasm; (iii) microvilli were deconstructed. It is reasonable to assume that the translocation of ERM proteins to the cyotoplasm is a result of ERM protein inactivation, which prevents ERM proteins from working as membrane-cytoskeleton crosslinker. This translocation of ERM proteins resulted in microvillar breakdown. In L cells, dephosphorylation of CPERMs by staurosporine appeared to cause ERM protein inactivation, and CPERMs appeared to be functionally equivalent to active ERM proteins. However, production of CPERMs was not required for either the microvilli formation in A431 cells stimulated with EGF or microvilli formation of MDCK II cells cultured under normal conditions. Because each ERM protein is highly

conserved among mammals, the characteristics of ERM proteins revealed in A431 and MDCK II cells appear to be common to ERM proteins from a variety of cells. Therefore, we account for these phenomena as follows: ERM protein activation can occur without ERM protein phosphorylation at the C-terminal threonine. However, its phosphorylation generally occurs in vivo and can stabilize the active state of ERM proteins. Thus, CPERMs represent activated ERM proteins. Dephosphorylation of CPERMs can induce intramolecular association between FERM and C-terminal tail domains, resulting in ERM protein inactivation. If the frequency of activation is low, most ERM proteins are kept in an inactive state. Conversely, if the frequency is high enough, ERM proteins can be kept in an active state without phosphorylation. Alternatively, the active state of ERM proteins may also be stabilized through a system different from ERM protein phosphorylation.

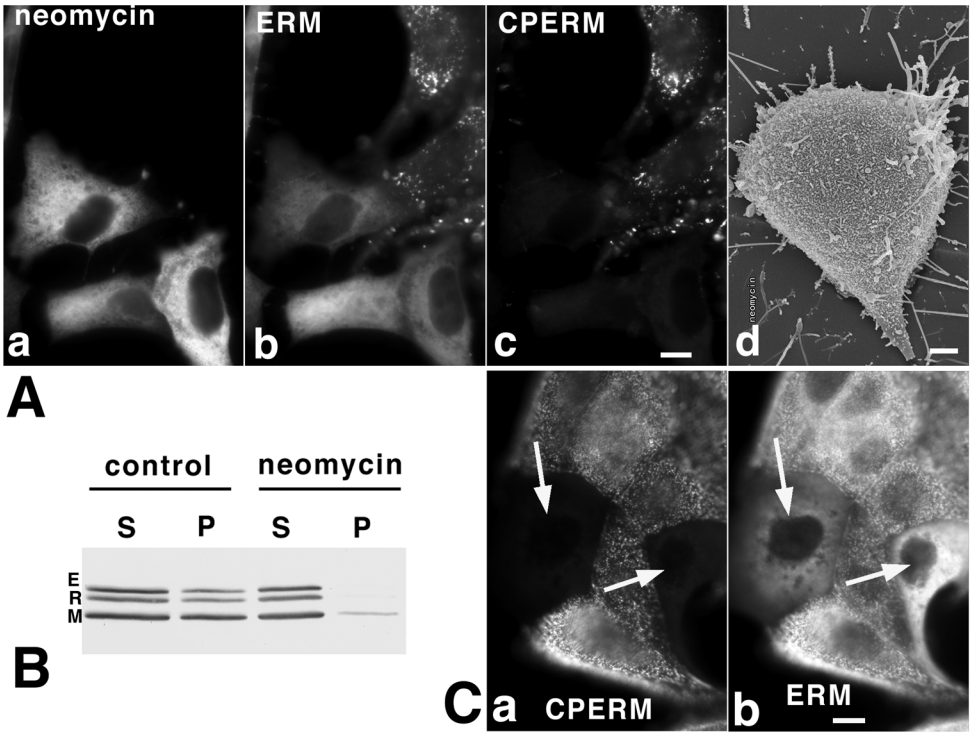

Fig. 8. Inactivation of ERM proteins by neomycin. (A) L cells were microinjected with 10 mM neomycin/F-dextran and incubated for 10 (a-c) or 30 (d) minutes. Cells were double stained with anti-ERM mAb, CR22 (b) and anti-CPERM mAb (c). In neomycin-microinjected cells (a), ERM proteins were dephosphorylated (c) and translocated to the cytoplasm (b). Scanning electron microscopy showed that microvilli disappeared from the cell surface (d). (B) L cells were homogenized in the presence of 1 mM neomycin, incubated for 30 minutes on ice and centrifuged. The supernatant (S) and pellet (P) were resolved by SDS-PAGE followed by immunoblotting with anti-ERM pAb, TK89. Neomycin induced translocation of ERM proteins from the insoluble to soluble cell fraction. (C) MDCK II cells were microinjected with 10 mM neomycin/F-dextran and incubated for 10 minutes. Cells were doubly stained with anti-CPERM mAb (a) and anti-ERM mAb, CR22 (b). In neomycin-microinjected cells (arrows), ERM proteins were dephosphorylated (a) and translocated to the cytoplasm (b). Bars, Aa-c, 10 μm; Ad, 2 μm; C, 10 μm.

One of the CPERMs, moesin, and a moesin mutant, in which aspartate was substituted for threonine 558 to mimic a CPERM, bind to actin filaments more strongly than non-phosphorylated wild-type moesin in vitro (Huang et al., 1999; Nakamura et al., 1999). However, this study indicates that the production of CPERM is not essential for ERM proteins to work as membrane-cytoskeleton crosslinkers in vivo.

Next, we were able to clarify the role of Rho activity in the activation of ERM proteins. We confirmed that ERM protein activation is dependent on Rho activity in a group of cultured cells, including L, MTD-1A, PtK2, 3Y1 and A431 cells. Because the transient activation of ERM proteins in A431 cells via EGF stimulation, which is observed even in the presence of staurosporine, was not observed after microinjection of C3, Rho appears to play an important role in the activation of ERM proteins rather than in the maintenance of the active state, that is, phosphorylation. We clearly visualized here that increasing Rho activity activates ERM proteins in serum-starved A431 cells. In this group of cells, the activity of PtdIns4*P* 5-kinase α may be functionally Rho-dependent, and therefore C3 transferase may have decreased the amount of PtdIns(4,5)P_2 required for ERM protein activation.

We further found that ERM protein activation with concomitant CPERM production is independent of Rho activity in a group of cells including MDCK cells. Since dephosphorylation of CPERMs by staurosporine did not result in ERM protein inactivation in this group of cells, ERM proteins may be stimulated for activation very frequently. Conversely, this group of cells may have systems such as oligomerization or adduct formation of ERM proteins other than the production of CPERMs to maintain activated ERM proteins (Berryman et al., 1995). If PtdIns(4,5)P_2 is important for ERM protein activation generally, then PtdIns4*P* 5-kinase in this group of cells may be functionally Rho independent. Actually, the activity of PtdIns4*P* 5-kinase has been reported to be dependent on Rac (Hartwig et al., 1995) or ARF6 (Honda et al., 1999); however, unknown systems for PtdIns4*P* 5-kinase synthesis may be dominant in these cells.

Lastly, we presented several findings showing that polyphosphoinositides, especially PtdIns(4,5)P_2, are essential for ERM protein activation regardless of Rho activity. In serum-starved, PtdIns4*P* 5-kinase-overexpressed A431 cells, ERM proteins were translocated to the surface of cytoplasmic vesicles where PtdIns4*P* 5-kinase was localized, resulting in the production of CPERMs and in further recruitment of actin filaments. This supports the idea that local accumulation of PtdIns(4,5)P_2 causes local activation of ERM proteins. On the other hand, microinjection of neomycin into L cells to disrupt the interaction between PtdIns(4,5)P_2 and ERM proteins

induced inactivation of ERM proteins, as seen by dephosphorylation of CPERMs, translocation of ERM proteins from microvilli to the cytoplasm and microvillar breakdown. Similar results were obtained with all cultured cells tested. Translocation of ERM proteins from the insoluble fraction to the soluble fraction was demonstrated biochemically by adding neomycin to cell homogenates. These findings show that one polyphosphoinositide, PtdIns(4,5)P_2, is the most likely candidate for a direct activator of ERM proteins in vivo at present.

Barret et al. mapped the PtdIns(4,5)P_2-binding site in ezrin and generated ezrin mutants that were unable to bind to PtdIns(4,5)P_2 in vitro (Barret et al., 2000). Furthermore, one of these ezrin mutants was unable to associate with the plasma membrane. These results suggested that PtdIns(4,5)P_2 has an important role in ERM protein activation and membrane targeting. Our results with PtdIns(4,5)P_2 confirm and extend their data and interpretation.

Neomycin binds to polyphosphoinositides including PtdIns(4,5)P_2 with high affinity (Schacht, 1978; Wang et al., 1984; Gabev et al., 1989) and has been used for inhibition of binding between PtdIns(4,5)P_2 and PtdIns(4,5)P_2-binding proteins in permeabilized cells or in vitro (Cockcroft et al., 1987; Liscovitch et al., 1991). When neomycin was microinjected into sea urchin eggs (Swann et al., 1992) or *Xenopus* oocytes (Carnero and Lacal, 1995), several events at fertilization and oocyte maturation were blocked by neomycin in the cytoplasm at a concentration of 5-10 mM. In this study, 10 mM neomycin was microinjected, and its intracellular concentration could be roughly estimated as 1 mM, a reasonable concentration for inhibiting binding between PtdIns(4,5)P_2 and PtdIns(4,5)P_2-binding proteins in the cytoplasm. Although microinjection of neomycin into cultured cells appears to be rarely performed, this technique can be applied widely to analyze the roles of PtdIns(4,5)P_2 and PtdIns(4,5)P_2-binding proteins.

Another issue that we should discuss here is the relationship between ERM proteins and the toxicity of aminoglycosides. Aminoglycosides were reported to accumulate specifically in renal proximal tubular cells and hair cells of the inner ear, where they cause cell injury (Tulkens, 1989; Lim, 1986). Interestingly, microvillar breakdown was observed in these cells (Wersäll et al., 1973; Jones and Elliott, 1987). Therefore, it is interesting to speculate that the suppression of the Rho-independent activation of ERM proteins by accumulated neomycin is directly involved in the nephrotoxicity (and probably also ototoxicity) of aminoglycosides.

A conformational change in an ERM protein upon activation, which was proposed as a model (Gary and Bretscher, 1995), is being evaluated by crystal structure analysis (Pearson et al., 2000). However, several important points have not been clarified yet. What is the kinase(s) responsible for the production of CPERMs in vivo that maintains the active state of ERM proteins? If PtdIns(4,5)P_2 is the major activator for ERM proteins, what are the spatial and temporal changes in PtdIns(4,5)P_2 concentration within cells? What is the role of ERM proteins in the regulation of Rho activity in vivo? Furthermore, the specific role of each ERM proteins or their synergistic roles needs to be elucidated. Although moesin-null mice appeared normal (Doi et al., 1999), they should be examined in more detail. Radixin- or ezrin-null mice may serve as a model to demonstrate the basic functions of ERM proteins in the body.

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