Plectin-RACK1 (Receptor for Activated C Kinase 1) Scaffolding

A NOVEL MECHANISM TO REGULATE PROTEIN KINASE C ACTIVITY*

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Agonist-induced translocation of protein kinase C (PKC) isozymes is mediated by receptors for the activated form of the kinase, shuffling it from one intracellular site to another and enhancing its catalytic activity. It is however unknown whether the receptors themselves are anchored to certain intracellular structures prior to their engagement with PKC. We show here sequestration of receptor for activated C kinase 1 (RACK1) to the cytoskeleton through the cytoskeletal linker protein plectin during the initial stages of cell adhesion. We found that upon PKC activation, RACK1 was released from the cytoskeleton and transferred to the detergent-soluble cell compartment, where it formed an inducible triple complex with one of the PKC isozymes, PKCδ, and with plectin. In plectin-deficient cells the cytoskeleton-associated RACK1 fraction was reduced, and the protein was found predominantly at sites to which it normally translocated upon PKC activation. Concomitantly, dislocation of PKCδ and elevated enzymatic activity were observed in these cells. PKCδ was also more rapidly degraded, likely due to its overactivation. We propose a previously unrecognized function of plectin as cytoskeletal regulator of PKC signaling, and possibly other signaling events, through sequestration of the scaffolding protein RACK1.

Plectin is a versatile high molecular weight (>500,000) cytolinker protein. It can bind to all major cytoskeletal filament networks and it plays an important role as mechanical linker and anchoring protein of intermediate filaments (IFs). 1 Several of a series of specific binding partners of plectin identified are constituents of the subplasma membrane protein skeleton or of junctional membrane complexes, such as integrin β4, spectrin/fodrin, and desmoplakin, suggesting that the protein provides a linkage between the cytoskeleton and the cell periphery (1). The crucial role of plectin as a stabilizing element of cells became clearly evident when plectin deficiency due to genetic disorders was found to be the cause for EBS-MD, an autosomal recessive skin blistering disease combined with muscular dystrophy (2), and when a corresponding phenotype was described in plectin knockout mice (3).

A new and at first sight paradoxical aspect of plectin function was revealed when dermal fibroblasts isolated from plectin-deficient mice were examined. After short term adhesion these cells developed increased numbers of focal adhesion contacts (FACs) and of actin stress fibers, and failed to show the characteristic rearrangement of the actin cytoskeleton in response to extracellular stimuli (4). Thus instead of favoring the formation of stable adhesion complexes, as one could have expected based on its stabilizing effect on hemidesmosomal junctions (3), plectin appeared to destabilize actin filaments. These data indicated that plectin does not just serve as a mechanical linker of different structural elements, but plays also a role in their dynamic rearrangements. One possible mechanism how plectin may perform such a diversity of functions could be through the scaffolding of different signaling molecules. Its enormous surface area and multidomain structure would facilitate such a task (5). In fact, we recently found an association of the non-receptor tyrosine kinase Fer with plectin’s N-terminal globular domain and an elevation of Fer kinase activity in the absence of plectin (6). Furthermore, the finding of a high affinity interaction between an N-terminal plectin peptide (residues 95–117) to Siah, an ubiquitin E3 ligase, opened up a potentially new regulatory role of plectin in the selective degradation of proteins (7).

To test more rigorously the idea of plectin functioning as a platform for the assembly of signaling proteins we screened a cDNA library in the yeast two-hybrid system for new interaction partners of plectin and selected for further analysis those with well-established roles in signaling. One of them was RACK1, the receptor for activated C kinase. RACK1, originally described as a 36-kDa homologue of the G protein β-subunit, has been identified as an anchoring protein for protein kinase C (PKC) (8). Defects in the PKC anchoring system due to alterations in RACK1 have been implicated in the process of aging (9), in the development of heart hypertrophy (10), and in the pathophysiology of Alzheimer’s disease (11).

As a plectin-binding protein RACK1 would be an ideal candidate for linking constituents of various signaling pathways to the cytoskeleton, ensuring efficient transmission of signals to cytoskeleton remodeling machineries. Comprising seven WD motif repeats RACK1 has been proposed to form a seven-bladed propeller structure providing multiple protein docking sites (12). Indeed, a variety of interacting proteins has been identified, among them Src kinase (13), and integrins (14). Interestingly, overexpression of RACK1 in fibroblasts led to a phenotype very similar to that of plectin-deficient cells, namely...
enhanced cell spreading, increased numbers of stress fibers and FACS, and decreased cell migration (Ref. 15 and references therein).

We report here that plectin forms an EGF-inducible complex with RACK1 and PKC in the soluble compartment of cells. Examining plectin-null cells, we show that the activity and degradation of PKCδ is increased in the absence of plectin and that there is a significant decrease in the level of cytoskeleton-associated RACK1. On the other hand, we observed a dramatic accumulation of RACK1 at the periphery of plectin-deficient cells, similar to PKCδ phenotype in vivo in wounding cells upon activation with EGF, adhesion on fibronectin, or overexpression of RACK1. Based on this we propose a model where plectin acts as a cytoskeletal platform for PKC and other signaling proteins through sequestration of the scaffold protein RACK1.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The LexA-based yeast two-hybrid system was used according to the user manual (Clontech). A genomic intron-less DNA fragment corresponding to mouse plectin C-terminal amino acids 4235–4580 was generated by restriction enzyme digestion of a lambda clone isolated from a mouse genomic library. This fragment was cloned in frame to the LexA coding sequence to obtain the bait plasmid, pLexA-ple R5. The plasmid was sequenced and correct expression confirmed by immunoblotting analysis of yeast cell lysates using anti-pLexA antiserum (data not shown). pLexA-ple R5, showing no autonomous activation, and a 19-day-old embryo cDNA library in yeast expression vector pJG4–6, were introduced into yeast strain EGY48. Transformants were amplified, and positive clones selected following the protocol recommended in the user manual. Out of 16 positively identified clones, one showed 99% identity to a mouse cDNA sequence corresponding to its C-terminal part (amino acids 173–317) of RACK1.

Cell Cultures and Antibodies—Human adenocarcinoma SW13(1–/−) cells, a cell line lacking vimentin filaments, were a kind gift of Dr. Robert Evans (University of Colorado, Boulder). Mouse dermal fibroblasts were derived from plectin wild type (+/+)/ple(1–/−) and plectin knockout(1–/−)/ple(1–/−) mice, as previously described (16), and used at passage numbers 10–15. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells were seeded at 8 × 10^4 cells/cm^2 except for low density cultures (3 × 10^4 cells/cm^2).

For immunoblotting the following primary and secondary antibodies were used: mouse mAb to RACK1 (Transduction Laboratories; 1:2500), mouse mAb to PKCs (Sigma; 1:500), mouse mAb to PKC8 (Transduction Laboratories; 1:500), phospho-PKC kit 9921 (Cell Signaling Technology Inc.; all 1:1000), anti-plectin antiserum 9 (1:6000) (16). A similar protocol was followed for immunoprecipitation of plectin with RACK1 and PKC in the soluble compartment of cells.

Immunoprecipitation and Immunoblotting—Confluent cultures of SW13 (+/−) cells were treated with 1 ng/ml EGF for 30 min or left untreated. After washing with phosphate-buffered saline, cells were scraped into 50 mM HEPES/HCl, pH 7.0, 5 mM MgCl2, 1 mM EGTA, 100 mM NaCl, 0.5% Triton X-100, 0.1 mM DTT, 0.5 mM MgCl2, or protein G-Sepharose beads (Sigma). Prior to addition of antibodies, protein concentrations were measured in all supernatants (Bradford) and set to similar values. Either 5 μg of mAb to PKCs, 150 μg of hybridoma supernatant containing mAbs to plectin 10F6, 3 μl of anti-plectin antiserum 9, or the corresponding preimmune serum were cleaved by incubation (1 h) with 60 μl of 10% protein A (Amersham Biosciences), or protein G-Sepharose beads (Sigma). After further incubation with 60 μg of 10% protein A or protein G-Sepharose beads for 3 h, extensively washed, and dissolved in sample buffer. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by immunoblotting as described (16). A similar protocol was followed for immunoprecipitation of plectin with total and membrane fractions of mouse p53(−/−)/ple(+/+); or p53(−/−)/ple(−/−) fibroblast cells.

RESULTS

Identification of RACK1 as a Plectin-interacting Protein—The yeast two-hybrid screening of a 19-day-old embryo cDNA library, using a C-terminal domain of plectin (Ple-R5, see Fig. 1) as bait, revealed 16 positive clones. All of them were sequenced and compared with GenBank™ sequences using a BLAST search. One of the cDNA clones showed 99% sequence homology at the nucleotide level to RACK1, the receptor for activated C kinase. RACK1 has been shown to consist of seven WD repeats, which are structurally similar to the G protein β-subunit. The isolated clone encoded WD repeats 3–7 of...
RACK1. Another plectin-binding partner identified in the yeast two hybrid screening was extra-embryonic endodermal cytokeratin type II (19), which was not unexpected considering that the bait protein used included plectin’s C-terminal IF-binding site (1) (see Fig. 1).

EGF-dependent Association of Endogenous RACK1 and PKCβ with Plectin—To confirm plectin-RACK1 binding using an alternative assay and to assess the functional significance of this interaction in mammalian cells, co-immunoprecipitation (co-IP) experiments were performed from SW13 vim(-/-) cell lysates. This type of cells was chosen, because due to its lack of IP expression plectin was expected to be relatively soluble. Furthermore, since the association of RACK1 with some of its binding partners has been reported to be inducible by activators of PKC, the experiments were performed using cells untreated or treated with the (indirect) PKC activator EGF.

As shown by immunoblotting, plectin could be pulled down from cell lysates in a specific manner using either an antiserum or anti-plectin mAb 10F6 in combination with A or G Sepharose beads (Fig. 2A, lanes 1, 2, and 5). RACK1 was found associated with plectin immunocomplexes when cells had been pretreated with EGF, whereas with untreated cells no co-precipitation was observed (Fig. 2B, compare lanes 2 and 6 with 1 and 5). Since RACK1 had previously been shown to bind activated C kinase, we also analyzed co-sedimentation of PKC using a mAb immunoreactive with all three of the classical (Ca2+-dependent) PKC isozymes, α, β, and γ. Similar to RACK1, PKC was found associated with the plectin-RACK1 complex upon EGF treatment of cells, but not in untreated cells (Fig. 2C, compare lanes 2 and 6 with 1 and 5). The PKC band detected in plectin immunoprecipitates was of similar size as the one observed in anti-PKC immunoprecipitates (Fig. 2C, compare lanes 2 and 6 with lane 9). No detectable PKC and negligible amounts of RACK1 were precipitated in controls with corresponding preimmune serum in combination with Sepharose A or G beads alone (Fig. 2, B and C, lanes 3, 4, 7, and 8). Thus EGF caused the selective association of a RACK1/PKC complex with plectin. This scenario resembled RACK1 interactions with different integrin β-chains (14) and insulin-like growth factor I receptor (IGF-IR) (20), both of which were initially discovered in yeast and then shown to be inducible in mammalian cells by PKC activation (with growth factors or PMA).

Plectin Deficiency Leads to Increased Degradation of PKCβ in Mouse Fibroblasts—RACK1 has been shown to enhance the activity of PKC presumably by stabilizing its active conformation (8, 21). Having found that plectin associates with the PKC/RACK1 complex upon activation, next we examined whether plectin deficiency had any effects on PKC activity. For this, we used plectin (ple)-deficient (-/-) mouse fibroblasts, derived from plectin-null mice, and plectin-positive (+/+) control cells (3, 4). Autophosphorylation of PKC isozymes β (22) and δ (23) has been shown to be a prerequisite for their full catalytic activity or, in the case of PKCδ, to be important for the duration of the activation (24). Therefore, we first compared the autophosphorylation levels of PKCα and PKCδ, two of the major PKC isozymes expressed in fibroblasts (25), that both have been reported to be associated with RACK1 (12, 20).
min-treatment with 2 μM of the more specific PKC activator PMA caused a similarly dramatic reduction of PKCβ in both ple(+/+) and (−/−) cells (Fig. 3, A and B, lanes 9, 10). At a lower dosage (0.1 μM), PMA acted like EGF, leading to higher degradation of PKC in ple(−/−) compared with (+/+)) cells (Fig. 3, A and B, lanes 13, 14). On the other hand, as revealed by the ratio of autophosphorylated PKCβ to total PKCβ, the increases in specific autophosphorylation of PKCβ upon activation with either EGF or PMA was more prominent in lysates from ple−/− cells than in those from (+/+ ) cells (Fig. 3C, lanes 7, 8, 11–14). Although PKCβ levels revealed by PKCβ-specific antibodies were very low after chronic PKCα stimulation (signals becoming detectable only after prolonged exposure times) (Fig. 3B, lanes 9, 10), strong bands were detected with anti-phospho-Ser643 antibodies, indicating a much higher level of specific autophosphorylation under these conditions with other conditions (Fig. 3C, lanes 9, 10). Comparable results were obtained when in a similar series of experiments antibodies specific to phospho-Thr505 (another phosphoepitope indicative of the PKCα activated state) were used instead of anti-Ser643 antibodies (data not shown). Both, ple(+/+) and (−/−) cells exhibited similar RACK1 protein levels, which were not significantly altered upon treatments of cells with different stimuli, or at distinct states of cell confluence (Fig. 3A, RACK1). Together, these results suggested increased degradation of PKCβ in ple(−/−) cells, especially in subconfluent cultures and upon short time of adhesion. The notion that reduced PKCβ protein levels in untreated ple(−/−) cells were a consequence of increased degradation of the enzyme was supported by the observation that the degradation process, which normally occurs upon exposure to activating stimuli like EGF or PMA (26), was speeded up in these cells in an uncontrolled way, leading to dramatically reduced levels of the enzyme after prolonged exposure. The observed increase in specific autophosphorylation of the kinase correlated well with its increased degradation after EGF and PMA activation. Interestingly, PKCε, another major isozyme found in fibroblasts, and, like PKCβ, a known interaction partner of RACK1 (25, 27), did not show increased degradation after EGF or PMA activation, nor were its protein levels decreased in ple−/− compared with (+/+ ) cells (data not shown).

Enhanced PKCβ Activity in Plectin(−/−) Mouse Fibroblasts—Previous studies have demonstrated that activation of PKC triggers its ubiquitination and degradation (28). It was therefore conceivable that increased degradation of PKC observed in ple(−/−) cells was a consequence of elevated enzymatic activity. To examine this we performed in vitro kinase assays. PKCβ was immunoprecipitated from ple(+/+) and

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**Fig. 3.** Plectin deficiency leads to increased degradation of PKCβ in mouse fibroblasts. A, plectin-positive (+) and plectin-deficient (−) fibroblasts were seeded at low or high density for different times as indicated. Cell lysates, prepared from cultures without further treatment (lanes 1–6), or after 10, or 30 min incubations with EGF (lanes 7, 8 and 11, 12, respectively), or 10-min incubations with different concentrations of PMA (lanes 9, 10, 13, 14), were subjected to immunoblotting using immunoreagents with specificities as indicated. (Note that incubations of blots with phosphoepitope-specific antibodies routinely were done overnight, those with other antibodies for 60 min). Numbers at right hand margin indicate positions of molecular weight markers (×10−3). Note, double bands in PMA-treated samples (lanes 9, 10, and 13, 14); the upper bands of these duplets may have been derived from p-Ser643PKCβ, which is an alternative phosphoepitope recognized by anti-p-Ser643PKCβ antibodies. B and C, signal intensities of PKCβ and p-Ser643PKCβ bands, densitometrically determined in three independent experiments (including the one shown in A), were normalized to tubulin (B), or to PKCβ (C), respectively. Bars represent average values for all experiments with standard deviations shown. Note, highest values within each of the bar diagrams were set arbitrarily to 100%, allowing comparison within, but not between different diagrams.
immunoblotting using mAb to PKC (autoradiography). The upper part of the membrane was subjected to assessed by exposure of the lower part of the membrane to x-ray film nitrocellulose membrane and phosphorylation of H1 and MyBP was (3) or MyBP (4) as substrates. Lysates used for IP were prepared dioactive ATP and histone 1 (H1), or MyBP, as exogenous solubilized immunoprecipitates were then incubated with ra-

ple(-/-) fibroblast cells, using isozyme-specific antibodies and solubilized immunoprecipitates were then incubated with radioactive ATP and histone 1 (H1), or MyBP, as exogenous PKC-specific substrates. Lysates used for IP were prepared from low-density cultures, because at that stage the difference in PKC levels between ple(+/+) and ple(-/-) cells was relatively high (see Fig. 3B). As monitored by autoradiography, PKCδ derived from ple(-/-) cells, showed significantly higher activity toward both substrates (~60 and ~35% for MyBP and H1, respectively) compared with ple(+/+) cell PKCδ (Fig. 4, A and B, lanes 2–5). The amount of background H1-phosphorylation observed in the absence of anti-PKCδ antibody was negligible (Fig. 4, A and B, lane 1), confirming the specificity of the assay.

Regulation of RACK1 and PKCδ Compartmentalization by Plectin—To investigate the cytoplasmic localization of RACK1 in relation to plectin, mixed 24 h-cultures of ple(+/+) and (-/-) cells were subjected to double immunofluorescence microscopy using mouse mAb to PCKδ and rabbit antisera to plectin. As shown in Fig. 5, A–C, both RACK1 and plectin were found spread all over the cytoplasm under these conditions. However, while plectin showed its typical filamentous staining pattern (in ple(+/+) cells only), RACK1 staining was confined to structures of granular appearance without any significant difference detectable between ple(+/+) and ple(-/-) cells. Upon EGF activation, plectin partially translocated to discrete areas at the cell periphery, most noticeably cellular extensions resembling filopodia (Fig. 5, E and F, white arrows). At the same time, EGF stimulation caused a dispersion of RACK1 granules, resulting in a more intense and diffuse cytoplasmic staining in both ple(+/+) and ple(-/-) cells (Fig. 5, D and F). Similar to plectin, a partial redistribution of RACK1 to the cell periphery occurred. Interestingly, the accumulation of RACK1 at the cell edge was more massive and spatially less restricted in ple(-/-) compared with ple(+/+) cells (Fig. 5, D and F, compare areas marked by yellow versus white arrows). The relocation of RACK1 in ple(+/+) cells appeared to take place concomitantly with that of plectin since both proteins accumulated in the same, filopodia-like extensions (Fig. 5F, white arrows; see also magnification in inset).

Next we investigated the subcellular distribution of the plecin/RACK1 complex to subcellular fractions of fibroblast cells. As shown in Fig. 6, RACK1 was co-immunoprecipitated with plectin from both the membrane and the cytosolic compartments of EGF-stimulated cells. As expected, in similar subfractions of untreated cells and in control experiments performed with ple(-/-) cells no, or very faint, RACK1 signals were detected (Fig. 6). In the cytosolic fraction of EGF-stimulated cells we also found PKCδ to be specifically associated with the RACK1/plectin complex (Fig. 6). The fact that in the membrane fraction no such signal was observed may have been due to the more stringent conditions under which IP from this fraction was carried out. Overall, these data confirmed in fibroblasts the results obtained with epithelial SW13(-/-) cells (see Fig. 2), suggesting that EGF-inducible triple complex formation between plectin, RACK1, and PKCδ, was a general phenomenon.

Co-IP experiments from the cytoskeleton fraction of fibroblasts (where plectin is predominantly localized) would have required even more stringent conditions and could therefore not be performed. To investigate whether plectin associated either constitutively or inducibly with RACK1/PKCδ also in this fraction we chose instead an indirect approach by quantitatively analyzing the subcellular distribution of both proteins in ple(+/+) and ple(-/-) cells. This analysis was performed with cells adhering to culture dishes for either 2 or 24 h, and in addition to untreated and EGF-treated cells, cells treated with PMA (a direct activator of PKC) were analyzed. Compared with ple(+/+) cells, ple(-/-) cells adhering for 2 h without treatment showed dramatically reduced (~50%) RACK1 levels in the cytoskeletal fraction, with a corresponding increase in the cytosolic fraction (Fig. 7, -2 h). After EGF treatment (15 min) of similar cultures, ~25% of cytoskeletal RACK1 translocated to the cytosolic fraction in ple(+/+) cells, whereas in ple(-/-) cells the cytoskeletal RACK1 fraction increased slightly, resulting in a more or less equal distribution to the three fractions tested (Fig. 7, EGF/2h). However, in both ple(+/+) and ple(-/-) cells a dramatic reduction in cytoskeleton-associated RACK1 was observed upon treatment with PMA, leading to ~70 and ~60% cytosolic (soluble) RACK1 in ple(-/-) and ple(+/+) cells, respectively (Fig. 7, PMA/2h). In ple(-/-) cells cultivated for ~24 h a compensatory mechanism seemed to become effective, since RACK1 was found associated with the cytoskeleton at this point to a similar degree as in ple(+/+) cells. Still, in ple(-/-) cells a slight increase in cytosolic RACK1 at the expense of the membrane fraction was observed under these conditions (Fig. 7, -24h). EGF stimulation after 24 h of adhesion increased the cytosolic levels of RACK1 in ple(+/+), but not in ple(-/-) cells, resulting in an almost equal distribution of RACK1 in both cell types (Fig. 7, EGF/24h).

Similar to RACK1, the cytosolic fraction of PKCδ was found increased in ple(-/-) compared with ple(+/+) cells, however with a corresponding reduction in the membrane, not the cytoskeleton fraction (Fig. 7, -2h). Upon stimulation with PMA, in both cell types nearly the entire cytosolic pool of PKCδ translocated to the membrane fraction (Fig. 7, PMA/2h), while translocation upon EGF stimulation was less dramatic (EGF/ 2h). Untreated ple(-/-) cells adhering for 24 h, no longer exhibited decreased levels of the kinase associated with the
**RACK1**

**plectin**

**merge**

**untreated**

**EGF**

**Fig. 5.** EGF-induced accumulation of RACK1 at the cellular periphery of ple(+/−) fibroblasts. Mixed cultures of ple(+/+) and (−/−) cells adhering for 24 h, were left untreated (A–C), or were treated with EGF for 15 min (D–F), fixed with methanol, and processed for double-immunolabeling using mAb to RACK1 (A and D, Texas red optics) and anti-plectin antisera (B and E, FITC optics). Ple(−/−) cells can be distinguished from ple(+/+) cells by the lack of green fluorescence. Note, that the localization of RACK1 was similar in ple(+/+) and ple(−/−) cells in the absence of stimuli (A–C), whereas EGF-induced relocation of RACK1 to the cell periphery is more prominent in ple(−/−) cells (D–F, yellow arrows) compared with ple(+/+) cells (D–F, white arrows). In ple(+/+) cells, peripheral RACK1 is restricted to cell protrusions where it colocalizes with plectin (see boxed area shown as ×2.5 magnified inset in D–F). Bars, 10 μm.

**Fig. 6.** EGF-dependent Co-IP of a RACK1/PKCδ complex with plectin from detergent-soluble cell fractions. Plectin(+/+) and (−/−) fibroblasts were either untreated (−) or treated (+) with EGF (1 ng/ml) for 15 min and then extracted sequentially with different solutions (see text) to prepare membrane and cytosolic fractions. Fractions, obtained from plet(+/+) and plet(−/−) cells (control), containing equivalent amounts of total protein, were subjected to IP using anti-plectin mAb 10F6. Immunocomplexes were separated by SDS-10% PAGE and immunoblottings (IB) performed using anti-plectin antiserum 9, mAb to RACK1, and mAb to PKCδ. Note, EGF-induced plection/RACK1/PKCδ triple complex formation in the cytosolic fraction of plet(+/-) cells.

membrane fraction and thus, a more or less equal distribution of PKCδ to the subcellular fractions was observed (Fig. 7, −/24h). EGF activation after 24 h of cell cultivation did not significantly affect the distribution of PKCδ between the various cell fractions of either cell type (Fig. 7, EGF/24h).

To illustrate on the microscopic level the changes in RACK1 localization caused by plectin deficiency upon short-time (2 h) adhesion, we performed immunofluorescence microscopy of untreated, EGF-, or PMA-treated plet(+/+) and plet(−/−) cells.}

Anti-RACK1/plectin double labeling of untreated wild-type fibroblasts revealed RACK1-positive granular structures mainly confined to areas where filamentous plectin-positive structures were visualized (Fig. 8, B–D). In contrast, corresponding cultures of plectin-deficient cells exhibited reduced RACK1 granularity and a dramatic accumulation of the protein at the cell periphery (Fig. 8A, arrows). A similar pattern was seen in overnight cultures of plet(−/−) cells spread at very low density (data not shown). A noticeable, but far less prominent accumulation of RACK1 (and plectin) at the cell periphery could be observed also in plet(+/-) cells upon EGF treatment (Fig. 8, F–H, arrows), while the distribution of RACK1 in plet(−/-) cells under these conditions was more or less unaltered (Fig. 8E). In contrast to EGF, which caused some accumulation of RACK1 at the cell periphery of plet(+/+) cells, PMA effected in both cell lines a uniform distribution of RACK1 throughout the cytoplasm without any peripheral accumulation (Fig. 8, I and J). However, granularity was still more pronounced in plet(+/+) compared with (−/-) cells. The less filamentous staining pattern of plectin observed in many cells under these conditions (Fig. 8K, cell marked by asterisk) indicated enhanced dissociation of the protein from IFs (29).

Collectively these data suggested that plectin was indispensable for cytoskeleton docking of RACK1 during the initial stages of cell adhesion. Either directly or indirectly through RACK1 anchoring, it also controlled the levels of PKC distributed to cytosolic and membrane fractions. Translocation itself, however, of cytoskeletal and cytosolic RACK1/PKCδ to cytosolic and membrane fractions, respectively, did not seem to require plectin, at least not in the case of PMA.
DISCUSSION

The targeting of enzymes or signaling complexes to certain intracellular sites is an effective way of achieving precise biological responses by modulating enzyme specificity and providing cross-talk between different signaling pathways (30). Signaling scaffolds through which this spatial and temporal regulation occurs have therefore gained more and more attention over the past years. One protein emerging as an important player in this scenario is RACK1. Originally identified as an anchoring protein for PKC, shuttling the active enzyme to different cellular sites, RACK1 has been shown to bind to numerous signaling molecules (12). Several studies have

![Table I. RACK1 and PKCδ distribution](image)

FIG. 7. Association of RACK1 with the cytoskeleton and of PKCδ with the membrane fraction is reduced in ple(−/−) cells after short-time adhesion. A, plectin-positive (+) and ple-deficient (−) fibroblasts, adhering for 2 h or 24 h as indicated, were treated with EGF (1 ng/ml; 15 min), or with PMA (100 nM; 30 min), or were left untreated (−). Cytosolic (1), membrane (2), and cytoskeleton (3) fractions (see text) were subjected to immunoblotting (IB) using mAbs to RACK1 or PKCδ. Note, cytoskeleton fractions analyzed were twice as concentrated as the other fractions to optimize detection. B, bar diagrams showing proportional distribution (%) of RACK1 and PKCδ to subcellular fractions. Values shown are based on densitometrically determined signal intensities of bands, measured in three independent experiments. C, table showing mean values ± S.D. of data shown in B.
shown an inducible association of RACK1 with proteins at the cell periphery/plasma membrane, but little is known about mechanisms triggering its translocation, or how RACK1 is kept away from these sites in the non-stimulated state. In this report we provide evidence for the binding of RACK1 to the cytoskeleton via the cytolinker protein plectin and we propose a role for plectin in regulating the targeting and release of RACK1 to and from the cytoskeleton.

Using the yeast two-hybrid system we found RACK1 to interact with a C-terminal region of plectin that comprises also the major IF binding site of the protein. However, in Co-IP experiments association of plectin with RACK1 was observed only upon EGF treatment of cells, indicating that binding was inducible, and possibly required some additional proteins or cofactors. In fact, the presence of PKC in plectin immunoprecipitates suggested that EGF induced the formation of a plectin/RACK1/PKC triple complex. Two mechanisms of complex formation upon EGF stimulation seemed conceivable. Plectin could either act as a cytoskeletal docking station to which the RACK1/PKC complex would be translocated or it could dissociate from IFs and move together with the RACK1/PKC complex to different cellular sites. We favor the latter mechanism for the following reasons. (i) In co-IP experiments triple-complex formation of plectin, RACK, and PKCβ occurred in the cytosolic (detergent soluble) cell compartment. (ii) After EGF stimulation, colocalization of plectin and RACK1 became very pronounced at the cell periphery, but not at the cytoskeleton, as clearly revealed by immunofluorescence microscopy. The very modest decrease in RACK1’s association with the cytoskeleton and the slight increase of PKCβ observed in cell fractionation experiments might have been due to the previously reported inducible association of both proteins with insoluble components of peripheral focal adhesion contacts (15, 31). (iii) Previous finding have already shown increased solubility of plectin in detergents and its dissociation from IFs upon PMA-induced PKC activation (29). The indirect PKC activator EGF might have similar, but not as strong effects.

What could then be the functional significance of this coordinated plectin/RACK1/PKC translocation? In the absence of plectin we observed that RACK1 was distributed all over the cell periphery of EGF-stimulated cells, whereas in wild-type cells it seemed to be restricted to cellular protrusions, where it colocalized with plectin. Taking into account that PIP2 has been shown to be concentrated in membrane protrusions, such as ruffles and microvillar-like projections (32), and that it binds to plectin (4), an exciting possibility would be that plectin directs RACK1 to PIP2-rich regions of the plasma membrane.

The most intriguing finding of our study was that in the initial stages of adhesion RACK1 massively accumulated at the cell periphery of plectin-deficient cells. In contrast, in control cells RACK1-containing granules were confined to areas where filamentous plectin structures were found, implying an inter-
action of RACK1 with cytoskeleton-bound plectin. In favor of this notion was a dramatic reduction in RACK1’s association with the cytoskeleton in the absence of plectin, as shown by cell fractionation experiments. It should be noted that an accumulation of RACK1 at the plasma membrane and colocalization with integrins β1/β5 were observed also in plectin-positive human U251N glioma cells upon PMA treatment (33) and in CHO cells upon plating on fibronectin (15). However, in both cases the effects were less pronounced compared with ple(−/−) cells. Hence, this suggested that in the absence of plectin, RACK1 accumulates at sites to which it normally moves only upon stimulation with PMA/EGF or signaling via integrins. In line with this, EGF treatment of our control cells after short-time plating resulted in relocation of RACK1 to the cell periphery. Furthermore, cell fractionation experiments revealed dissociation of RACK1 from the cytoskeleton upon treatment with EGF and, especially prominent, PMA. Thus according to our model (Fig. 9), plectin is a RACK1-sequestering protein that keeps RACK1 in a cytoskeleton-associated state in the absence of a stimulus, and contributes to the controlled shuttling of RACK1 upon induction with EGF. Plectin deficiency causes a shift of balance toward soluble cytosolic and cell peripheral states of RACK1, which normally occurs upon EGF/PMA-induced PKC activation. As expected, this phenotype was no longer observed upon prolonged (24 h) adhesion, indicating that RACK1 reached its subcellular destination, but required more time than in the presence of the scaffold. On the other hand, 24-h adhesion of cultures at very low density resulted in a phenotype similar to 2-h adhesion. Thus, plectin-mediated targeting of RACK1 to the cytoskeleton might be conditioned by the extent of cell-cell contacts and modulated by cell motility.

Which consequences could dislocation of RACK1 have for PKC function? RACK1 shuttles the active form of PKC to distinct subcellular sites and enhances the activity of the enzyme. Thereby, both proteins accumulate at sites, which are different from the ones they occupy before PKC activation (21). In line with this, disruption of PKC-RACK1 interaction has been reported to inhibit agonist-induced PKC translocation and function (34). It is therefore conceivable that alone the accumulation of RACK1 at subcellular sites, to which it normally translocated upon PKC activation, may increase the amounts of PKC at these sites and may affect its enzymatic activity. In fact, in ple(−/−) cells we observed dislocation of the PKC isozyme δ, which together with RACK1 accumulated, in the cytosolic fraction at the expense of the membrane fraction. Most importantly, the activity of PKCδ was found to be strongly elevated in ple(−/−) cells, probably due to increased RACK1 levels in the same cellular compartment where PKC was active.

It has been shown that the activation of PKC triggers its degradation and down-regulation (35). Therefore, we suggest that the reduced protein level of PKCδ in ple(−/−) cells was a consequence of the increased activity of the enzyme in these cells. Due to its binding to the activated form of PKC, RACK1 would be expected to exert its effects on the kinase activity under the conditions where the levels of activated PKC are increased, such as after drug-induced (PMA) or physiological (EGF) stimulation. In fact, down-regulation of PKC through degradation, naturally occurring in response to PKC activation, was significantly speeded up in the absence of plectin. This data suggested an uncontrolled increase in the activity of PKC in ple(−/−) cells after PMA/EGF activation, probably due to the pre-enrichment of RACK1 at the sites where PKC became active.

At this point it should be noted that, after EGF- and especially PMA-induced activation, PKCδ accumulated in the membrane fraction, whereas RACK1, its anchoring and activating protein, was found predominantly in the cytosolic fraction. We consider this not to be a discrepancy, however, as the binding of RACK1 to PKC stabilizes the active (open) conformation of the enzyme promoting its association with the membrane (9). Thus, upon its release from the cytoskeleton, RACK1 may bind and activate PKC in the cytosolic fraction without being itself anchored to the membrane. Whether this is in fact the actual mechanism remains to be investigated.

RACK1 was originally connected to PKCβ1-mediated functions, but recent findings have indicated its involvement in the regulation of other PKC isoforms as well (20, 27). It was therefore surprising that in fibroblasts only PKCδ, and not PKCα or PKCe, the other PKC isoforms tested (both of which are known to interact with RACK1), were influenced by the dislocation of RACK1. A possible explanation could be that the effects of RACK1 on PKC activity at least in part are mediated
by other proteins that associate with RACK1 upon PKC activation and selectively influence PKC isozyme δ. A good candidate for such a protein would be tyrosine kinase Src, to which RACK1 binds upon PKC activation, inhibiting its enzymatic activity (13). Uniquely among other PKC isozymes, PKCδ associates with Src, which in turn phosphorylates it, leading to alterations of its activity (36). Hence, one possible sequence of events is that the accumulation of RACK1 at the sites where PKC becomes active leads to a stronger inhibition of Src, causing further up-regulation of PKCδ activity.

The functional importance of assembling RACK1 signaling complexes at certain subcellular sites upon stimulation of cells was clearly revealed in recent studies (20), showing insulin-like growth factor I (IGF-I)- and PMA-inducible association of the RACK1/PKCδ complex with both IGF-I receptor (IGF-IR) and integrin β1. Upon overexpression, RACK1 was found to constitutively associate with IGF-IR, to suppress an unknown integrin β1-associated kinase activity, and to alter binding/phosphorylation of FAC constituents. At the same time, increased numbers of FACs and stress fibers were observed, and RACK1 association with the cytoskeleton was found significantly reduced, highly reminiscent of ple(−/−) cells (4) (and this study).

Based on their observations, Hermoto et al. (20) postulated the existence of an unknown linker protein required for cytoskeleton-docking of RACK1. To our knowledge, our report is the first where with plectin such a linker protein has been identified. We suggest that, similar to RACK1 overexpression, incomplete sequestering of RACK1 at the cytoskeleton in the absence of plectin impairs integrin β1 signaling and leads to increased number of FACs and actin stress fibers.

How does altered activity and localization of PKCδ in ple(−/−) cells fit into this scenario? In fibroblasts PKCδ has been shown to be localized at newly formed FACs in the presence of serum (31) and to exert a negative effect on the formation of FACs (37). However, in epithelial cells it positively associates with FACs (37). How does altered activity and localization of PKCδ in ple(−/−) cells fit into this scenario? In fibroblasts PKCδ has been shown to be localized at newly formed FACs in the presence of serum (31) and to exert a negative effect on the formation of FACs (37). However, in epithelial cells it positively associates with FACs (37). How does altered activity and localization of PKCδ in ple(−/−) cells fit into this scenario? In fibroblasts PKCδ has been shown to be localized at newly formed FACs in the presence of serum (31) and to exert a negative effect on the formation of FACs (37). However, in epithelial cells it positively associates with FACs (37).

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