The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration

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SUMMARY

The scaffolding protein, Rack1, is a seven-WD-domain-containing protein that has been implicated in binding to integrin β subunit cytoplasmic domains and to members of two kinase families (src and protein kinase C, PKC) that mediate integrin bidirectional signaling. To explore the role of Rack1 in integrin function we have transfected this protein in Chinese hamster ovary (CHO) cells. We have observed no effect of Rack1 overexpression on inside-out signaling as the ligand binding properties of CHO cells also expressing constitutively active or inactive integrins were not affected. In contrast, we observed that cells stably or transiently overexpressing Rack1 had decreased migration compared to mock transfected cells. Stable Rack1 transfectants also demonstrated an increased number of actin stress fibers and focal contacts. These effects on motility and cytoskeletal organization did not appear to result from Rack1 inhibition of src function as downstream substrates of this kinase were phosphorylated normally. In addition, expression of an active src construct did not reverse the migratory deficit induced by Rack1 overexpression. On the other hand when we overexpressed a Rack1 variant with alanine substitutions in the putative PKC binding site in its third WD domain, we observed no deficit in migration. Thus the ability of Rack1 to bind, localize and stabilize PKC isoforms is likely to be involved in aspects of integrin outside-in signaling.

Key words: Rack1, Migration, Integrin, Protein kinase C

INTRODUCTION

The orderly and controlled migration of cells is crucial in many aspects of physiology including embryonic development, immune function, wound repair and angiogenesis. Unregulated or abnormal cell motility also underscores certain pathological situations such as the invasion and metastasis of cancer cells. Cell migration is a complex response to external stimuli involving an interplay between cellular adhesive events and cytoskeletal organization. Signal transduction events involving the regulation of integrin affinity (Huttenlocher et al., 1996), small GTPases (Nobes and Hall, 1995; Nobes and Hall, 1999; Takaishi et al., 1993), the ras/MAP kinase pathway (Klemke et al., 1997; Nguyen et al., 1999), Cas/Crk coupling (Klemke et al., 1998), focal adhesion kinase (Cary et al., 1996; Ilic et al., 1995), phosphatidylinositol 3-kinase (Rodriguez-Vicana et al., 1997), PLCγ (Chen et al., 1994), protein kinase C (Laudanna et al., 1998; Ng et al., 1999; Rigot et al., 1998) and calpain (Huttenlocher et al., 1997), have all been implicated in regulating integrin-mediated adhesion, integrin downstream signaling or actin polymerization leading to motile behavior. Understanding the molecular events that regulate cell migration will be important in designing ways to control this event.

Rack1 is a 36 kDa cytosolic protein composed of seven WD motifs and thus is structurally similar to a G protein β subunit. It was originally identified as a receptor for activated protein kinase C (Ron et al., 1994). In this mode of action, Rack1 acts as neither a substrate nor an inhibitor, but rather enables the translocation of PKC isoforms and stabilizes their active forms (Mochly-Rosen and Gordon, 1998). Indeed, reduced Rack1 levels are correlated with defective PKC translocation in the aging rat brain (Battaini et al., 1997) and disruption of PKC-Rack1 interactions impaired insulin-induced kinase translocation, Xenopus oocyte maturation (Ron et al., 1995) and regulation of calcium channels in cardiomyocytes (Zhang et al., 1997). Rack1 has also been isolated in a yeast two-hybrid screen using an src SH2 domain as bait (Chang et al., 1998). Additional studies suggested that the interaction of Rack1 with src family members inhibited the activity of these kinases in vitro and that Rack1 overexpression in NIH 3T3 and 293T cells decreased growth rates and levels of tyrosine-phosphorylated proteins, respectively (Chang et al., 1998). Thus, Rack1 appeared to have opposing effects on the kinases to which it binds: stabilizing the active conformation of PKC but inhibiting members of the src family. Additional binding partners of Rack1 include integrin β subunit cytoplasmic domains (Liliental and Chang, 1998; Zhang and Hemler, 1999), a phosphodiesterase isoform (Yearwood et al., 1999), certain pleckstrin homology (PH) domains (Rodriguez et al., 1999) and the common β chain of the IL-5/IL-3/GM-CSF receptor (Geijsen et al., 1999). While the functional significance of these latter associations remains undefined, a role for Rack1 as a scaffolding protein is suggested.

Since PKC and src family kinases have been implicated in integrin bidirectional signaling, we wished to explore the functional consequences of Rack1-integrin association. When transiently or stably overexpressed in CHO cell lines, Rack1...
did not appear to affect ligand binding properties. However these transfectants did demonstrate decreased integrin-dependent cell migration and an increased number of actin stress fibers and focal contacts compared to wild-type cells. These effects did not appear to be due to inhibition of src kinase activity and were not reversed with an active src construct. In addition, these migratory defects were not observed when we utilized a Rack1 construct with a mutated PKC binding site. These results suggest that Rack1 is involved in integrin outside-in signaling in a manner that involves its interaction with PKCs but is independent of its reported effects on src.

**MATERIALS AND METHODS**

**Reagents**

A monoclonal antibody against Rack1 was obtained from Transduction Labs while one against talin was from Sigma. An α4β1 antibody (D-57) and the ligand mimetic monoclonal, Pac1, were obtained from Mark Ginsberg and Sandy Shattil, Scripps Research Institute, respectively. All other antibodies (HA epitope, erk, cas, ERK1 and ERK2, phospho-ERK and phosphotyrosine) were from Santa Cruz Biotechnologies. The conjugated reagents, phycocerythrin-streptavidin and FITC-IgG and IgM were from Molecular Probes and Biosource, respectively. The calcein AM dye and rhodamine-phalloidin were obtained from Molecular Probes while matrix streptavidin and FITC-IgG and IgM were from Molecular Probes and Boehringer Mannheim. All synthetic oligonucleotides were from Genosys.

**cDNA cloning**

A partial cDNA for Rack1 encoding residues 81-317 was isolated in a yeast two-hybrid screen using a Hela cell library and the β1 tail as a bait. Two-hybrid screening, yeast manipulations and liquid β-galactosidase assays were done as per the manufacturer’s (Clontech) recommendations. The remaining coding and 5’ non-coding sequences of Rack1 were isolated by RACE-PCR with Hela Marathon-Ready cDNA (Clontech), subcloned into the TA cloning vector (Invitrogen) and confirmed by DNA sequencing. A full-length clone containing a C-terminal HA tag was then generated in the pCDNA3.1 vector (Invitrogen) by first amplifying the partial yeast clone with a 3’ oligonucleotide encoding an HA-tag and an Xhol site and a 5’ oligonucleotide complementary to the yeast plasmid pB42AD, followed by digestion of the product with StyI and XhoI. Next, remaining Rack1 sequences in the TA clone were isolated by digestion with NotI and StyI and finally the two Rack1-encoding pieces ligated into NotI and XhoI-digested pCDNA3.1. An inducible and myc-tagged expression clone for Rack1 was established by subcloning in the pMDHygro vector (Invitrogen). To generate alanine substitutions in the third and sixth WD domains of Rack1 we used appropriately designed oligonucleotides in a site-directed mutagenesis strategy (Quick Change Mutagenesis; Stratagene). All constructs were verified by sequencing before use.

**Cell culture, transfections and analysis of signaling properties**

All CHO cells were maintained in DMEM medium plus 10% fetal calf serum (FCS) and 1% glutamine, nonessential amino acids and penicillin/streptomycin. Stable Rack1 transfectants were generated in wild-type or α4β1-expressing (A5) CHO cells (O’Toole et al., 1990) by cotransfection with HA-tagged Rack1 in pcDNA3.1 and pZeo (Invitrogen) using the lipofectamine reagent (Life Technologies). The medium was changed after 24 hours and after 48 hours the cells were grown in medium containing 250 μg/ml zeocin (Invitrogen). After 2 weeks, colonies were picked, scaled up and analyzed for HA-Rack1 expression by western blotting with anti-Rack1 or anti-HA antibodies. The ligand-binding properties of these transfectants were determined by flow cytometric analysis of Pac1 binding (O’Toole et al., 1990).

Transient expression of Rack1 was achieved using a ponasterone inducible system (Invitrogen). Briefly, the appropriate cell types were cotransfected with the pHOOK vector (Invitrogen) to select for transfectants, pVgRxR and the pND vector containing full-length Rack1. 24 hours after transfection the medium was changed to one containing 0.5% FCS and 5 μM ponasterone (Invitrogen) and the cells were allowed to grow for another 20-24 hours. The transfectants were then isolated by magnetic sorting and induced Rack1 expression was determined by western blotting with an anti-HA-antibody. Expression levels were determined by densitometry with an image analyzer (Alpha Imager 2000, Alpha Innotech Corporation).

To determine the effects of Rack1 overexpression on adhesion-stimulated signaling, we first incubated the appropriate cell types overnight in medium containing 0.5% FCS. The cells were then harvested with trypsin, washed in medium containing 0.5 mg/ml soybean trypsin inhibitor, resuspended in medium containing 0.2% BSA and incubated in suspension culture dishes for 2 hours at 37°C. At this time, some of the cells were collected and washed in phosphate-buffered saline (PBS), while the remainder were allowed to adhere to fibrinogen (fg)-coated dishes (15 μg/ml) for various times. These adherent cells were then likewise collected and washed and all cells lysed on ice in a buffer consisting of 20 mM Tris- HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 3 mM EDTA, 20 mM NaPi, 3 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM PMSF, 10 mM NaF and 1× complete protease inhibitor (Boehringer Mannheim). The lysates were clarified by centrifugation and protein concentration determined by BCA assay (Pierce). For immunoprecipitations, 60 μg of crude lysate were directly used in western blotting analysis with phospho-ERK and ERK1/2-specific antibodies.

**Migration assays**

Transwells were prepared by coating the bottom surface of the membrane with matrix proteins (50 μg/ml in carbonate buffer (0.2 M Na2CO3/NaHCO3, pH 9.3)) for a minimum of 1 hour, and then blocked with 5% milk in PBS plus 0.2% Tween 20. The cells to be examined were incubated in medium containing 0.5% serum overnight and then harvested and resuspended in serum-free medium at approximately 10^4/ml. 0.5 ml of serum-free medium was applied to the bottom chamber while 100 μl of the resuspended cells were then applied to the top of the Transwell, and migration was allowed to proceed for 8-16 hours. At this time medium in the bottom well was replaced with some of the same containing 5 μM calcein AM and migration allowed to proceed for a final 30 minutes. The Transwells were then washed 2× in PBS, the remaining cells on the top wiped off with a Q-Tip, and fluorescence read (excitation 485 nm, emission 530 nm) in a CytoFluor II. These values were normalized relative to the fluorescence of total cells initially seeded to the top of the Transwell and expressed as a percentage of control samples. The control samples were untransfected cells (in experiments where stable transfectants were used) or vector-transfected cells (in experiments where transient transfectants were used).

**Immunofluorescence**

Glass coverslips were coated with 10 μg/ml fibronectin in carbonate buffer for 1 hour at room temperature and then blocked in 1% BSA. 5×10^3 cells were then added and allowed to adhere for 2 hours at 37°C. At this time the cells were fixed with 1% paraformaldehyde, permeabilized
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Rack1, a receptor for activated protein kinase C, has been implicated in binding to the integrin β1, β2, β3 and β5 cytoplasmic domains using the yeast two-hybrid approach (Liliental and Chang, 1998; Zhang and Hemler, 1999). Our two-hybrid studies confirm these results and also suggested an interaction with the β3 cytoplasmic domain (Fig. 1). In addition, Rack1 has been implicated in binding to both protein kinase C and SRC family members, two mediators of integrin bidirectional signaling (Chang et al., 1998; Ron et al., 1994). To begin to examine the role of Rack1 in integrin-mediated functions we stably overexpressed an HA-tagged form of this protein in CHO cells, which also express recombinant αIIbβ3 (A5 cells). After G418 selection and western blotting analysis of individual clones, we identified and maintained two clonal lines for further study. These two lines (R8 and R47) demonstrate both the slower migrating HA-tagged Rack1 (upper bands) as well as the endogenous Rack1 (lower bands) (Fig. 2A). Scanning the western blots suggested a 1.5- to twofold increase in Rack1 expression over levels in untransfected cells. Expression of HA-Rack1 in the A5 cells did not alter integrin expression levels (not shown) or gross morphological features. In all of the subsequent functional analyses described below, the behavior of the two clonal lines appeared identical.

As activators of protein kinase C have been widely used to stimulate integrin binding function, we first sought to determine whether Rack1 overexpression might affect integrin affinity state. Using the monoclonal antibody Pac1 as a ligand mimetic, however, we demonstrated that the clonal lines R8 and R47 remain in the same low-affinity state as their parental line, A5 (Fig. 2B). Ligand binding in these cells could only be stimulated with an activating antibody. Furthermore, Rack1 does not suppress ligand binding when overexpressed in cells with constitutively active, high-affinity integrins (data not shown). Thus Rack1 overexpression does not appear to affect inside-out signaling.

RESULTS

Inside-out signaling is not affected by Rack1 overexpression

Rack1 overexpression alters cytoskeletal structure, focal contact formation and cell migration

To begin to explore the effects of Rack1 on outside-in signaling, we first examined the cytoskeletal organization of the Rack1 overexpressing clones upon adhesion to fG. Immunofluorescence staining of wild-type CHO cells and the clonal line R8 with an anti-talin antibody and phalloidin demonstrated distinct differences between the two cell types. Specifically, the Rack1 overexpressers demonstrated a

Fig. 1. Rack1 interacts with several integrin β subunit cytoplasmic domains. The interaction of full-length Rack1 with a non-specific clone (lamin) and with the cytoplasmic sequences of the β1, β2, β3 and β5 integrins was determined by yeast two-hybrid methodology and quantitated in a β-galactosidase assay (arbitrary units). In addition to previously reported interactions with β1, β2 and β5, Rack1 also interacts with β3.

Fig. 2. The overexpression of Rack1 does not affect integrin inside-out signaling. (A) To explore its effects on integrin binding affinity, we stably expressed Rack1 in a CHO line also expressing αIIbβ3 (A5 cells). Two clonal lines (R8 and R47) expressing both wild-type Rack1 (lower band) and the HA-tagged and transfected Rack1 (upper band) were identified and carried on for further study. (B) The binding of the ligand mimetic Pac1 to A5 cells and the two clonal lines was determined in a flow cytometry assay. Rack1 overexpression did not stimulate high levels of Pac1 binding (solid line) and this could only be accomplished after stimulation of the cell types with an activating antibody, LIBS 6 (shaded histogram). Excess RGD-containing peptide was used in these assays to block binding and demonstrate specificity (dotted line).
A noticeable increase in both the number of stress fibers and focal contacts relative to wild-type cells (Fig. 3). Thus Rack1 overexpression affected the cytoskeletal organization of adherent cells.

The cytoskeletal organization of the adherent, Rack1-overexpressing cells was similar to the observed phenotype of FAK knockout cells (Ilic et al., 1995). Since these cells also demonstrate migration defects, we next sought to determine what effect Rack1 might have on integrin-mediated migration. Using a Transwell migration assay, we have consistently observed that the clonal line R8 demonstrated a 30-60% decrease in migration on fg-coated wells when compared to the parental cell line, A5 (Fig. 4A). As these studies were done with clonal lines, we also wished to see if transient overexpression of Rack1 would have a similar effect on migration. To do this, we subcloned HA-tagged Rack1 into the pINDHGYGRO vector (Invitrogen) and induced its expression with the edcsyne analog, ponasterone (Fig. 4A). These transient transfectants reproducibly demonstrated an approximately twofold increase in Rack1 expression. Similar to those results with the stable lines, transient overexpression of Rack1 in A5 cells also deterred migration on fg (Fig. 4A). Next, we wished to determine if these Rack1 effects were specific to these CHO, αIIbβ3-expressing cells. We therefore stably overexpressed Rack1 in wild-type CHO cells (clonal line CR22) and examined their migratory behavior on fn, which was mediated by endogenous α5β1 (Fig. 4B). As with the A5 cells, overexpression of Rack1 in CHO cells inhibited cell migration. Finally, we observed similar effects on migration when we performed these CHO cells studies in a transient manner (Fig. 4B). Thus migration mediated by different integrins was inhibited.

Fig. 3. Rack1 overexpression alters cytoskeletal organization. Parental CHO cells (left) and the Rack1 overexpressing clone R8 (right) were allowed to adhere to fg and then subjected to immunofluorescence staining as described in Materials and Methods. Analysis was with rhodamine-phalloidin (red) to visualize actin stress fibers and a talin antibody (green) to visualize focal contacts. Rack1 overexpressing cells demonstrate an increase in stress fibers and focal contacts when compared to parental cells.

Fig. 4. Rack1 overexpression inhibits cell migration. (A) A5 cells and the clonal line R8 were allowed to migrate on fg-coated Transwells and the number of migrated cells quantitated by calcein AM staining as described in Materials and Methods (left bars). The stable Rack1 overexpressing line demonstrated an approximate 60% decrease in cell migration relative to the parental line. A5 cells were also transiently transfected with pHOOK and variably with pVgRxR and pINDRack1 as indicated (right bars). Protein expression was induced after 24 hours with 5 μM ponasterone and transfectants isolated after 48 hours by magnetic sorting. Expression of Rack1 was determined by western blotting with an anti-Rack1 antibody (bottom) while migratory properties were analyzed as above in a Transwell assay. (B) Stable expression of Rack1 was also accomplished in wild-type CHO cells. Migration of the parental cells and a Rack1 overexpressing clone (CR22) on fn-coated Transwells were examined as above (left bars). Finally, transient Rack1 expression and migration was accomplished in CHO cells as described above (right bars and bottom). In all cases overexpression of Rack1 inhibited cell migration relative to parental cells.
Rack1 overexpression does not inhibit downstream signaling from src

As noted above, it has been reported that Rack1 can bind to and inactivate src family kinases. To explore whether this might account for the observed phenotype in the Rack1 overexpressing lines, we determined the effects on some of the downstream targets of src signaling. Two substrates of src, FAK and p130cas, have been implicated in migratory function (Cary et al., 1996; Gilmore and Romer, 1996; Klemke et al., 1998) and we initially examined if these substrates were differentially phosphorylated in our Rack1 overexpressing cell lines. To do this A5 and R8 cells were serum starved overnight, put into suspension for 2 hours, and then allowed to adhere to fg for 10 minutes. Lysates from these samples were then immunoprecipitated with anti-FAK and anti-cas antibodies and isolated proteins analyzed by western blotting. Both the Rack1 overexpressing line and wild-type cells demonstrated increases in FAK and cas phosphorylation upon adhesion (Fig. 5A,B). Src activity has also been implicated in the upregulation of the MAP kinases. Therefore, we examined whether ERK1/2 were differentially phosphorylated and activated in Rack1 overexpressing cells. However western blotting with a phospho-ERK specific antibody suggested comparable upregulation of these kinases upon adhesion (Fig. 5C). Thus signaling events downstream of src do not appear to be disrupted in the Rack1 overexpressing cells.

As a final approach to determine if altered src function could account for the decreased motility of Rack1 overexpressing cells, we asked whether we could reverse this phenotype by transfection with an activated src construct. However, transfection of CR22 cells with srcE378G did not enhance migration to wild-type levels (Fig. 6A), despite ample expression of this src variant (Fig. 6B). Thus it appears that Rack1 overexpression did not diminish src activity in our system and this is not a mechanism for the observed migratory defects.

**Inhibition of cell migration is reversed by a mutant Rack1 construct**

Two sequence motifs in Rack1, one in its third WD domain and one in its sixth WD domain, are homologous to areas in other PKC-binding proteins (Ron et al., 1994). Furthermore,
in its WD3 domain, we observed no inhibition of migration (Fig. 7B). All Rack1 variants were expressed to similar extents. Thus PKC interactions with a motif in the third WD domain of Rack1 appear to be important in integrin outside-in signaling, especially with regard to cell migration.

**DISCUSSION**

Racks have been classically defined as saturable, specific receptors that stabilize PKC isoforms in the active state and anchor them to membranes or other functional sites (Mochly-Rosen and Gordon, 1998). The data presented in this paper suggest that Rack1, a receptor for the PKC β isoform that also associates with integrins and src kinases as well (Chang et al., 1998; Liliental and Chang, 1998) is involved in regulating cytoskeletal organization and downstream events such as integrin-mediated cell migration. Rack1 thus represents another player in a cell’s migratory machinery and a novel therapeutic target for regulating this process.

Unlike other proteins which interact with specific integrins (eg. β3-endonexin, Shattil et al., 1995; CIB, Naik et al., 1997), we and others have shown that Rack1 appears to interact with several integrin β subunit tails. The functional studies described here examine Rack1 effects on downstream signaling mediated by the β1 and β3 integrins. We do not yet know if Rack1 overexpression also affects migration mediated by other integrins (β2 and β5). Thus it is not clear if Rack1 broadly affects integrin outside-in signaling or if some measure of specificity, which includes the β1 and β3 integrins, does exist. It is also of interest that we could not overexpress Rack1 to greater than twofold above endogenous levels in either the stable or transient systems. One potential explanation for this is that, like structurally homologous G-protein β subunits, Rack1 may exist as part of a greater protein complex. These additional components may be limiting and therefore affecting Rack1 expression levels.

The multiple binding partners of Rack1 suggest several possibilities whereby this protein might affect cytoskeletal structure and cell motility. First, this protein has been reported to bind to and inactivate src family kinases. Src in turn can be linked to migratory behavior via distinct pathways involving the phosphorylation of FAK and p130cas. Phosphorylation of FAK at residue 925 creates a binding site for the grb2-sos complex and induction of ERK kinase activity through the classical ras pathway. The migration of FG carcinoma cells has been shown to be dependent upon ERK activity and the subsequent phosphorylation of myosin light chain kinase and myosin light chain (Klemke et al., 1997). On the other hand, phosphorylation of p130cas creates a binding site for crk and this association has been demonstrated to be important in the migratory and invasive properties of carcinoma cells in a manner dependent upon the G protein, rac (Klemke et al., 1998). Thus Rack1 inhibition of src kinases seems an attractive hypothesis for the migratory defects we have observed. However, our Rack1 overexpressing cell lines did not demonstrate deficiencies in FAK or cas phosphorylation and MAP kinase activity (Fig. 5), and we do not believe this is a mechanism for reduced migration of our cell lines. In support of this, transfection with a constitutively active src construct did not overcome the migratory defect in Rack1 overexpressing.
cells. In this regard, our results appear to differ from those of Chang et al., where reduced src activity upon Rack1 overexpression could explain their observed phenotypes such as altered growth rates of NIH 3T3 cells (Chang et al., 1998). Cell type differences might be an explanation for these discrepancies. Nevertheless we have not measured src activity directly in our cell lines and cannot discount the possibility that Rack1-mediated inhibition of src activity may limit migration by another means. In this regard, it has been demonstrated that focal adhesion turnover is reduced in cells expressing kinase inactive or myristylation-defective src constructs (Fincham and Frame, 1998). Cells with decreased focal contact turnover would be predicted to have increased adhesive strength, and consequently, decreased motility. Consistent with this, we have observed striking differences in the number of stress fibers and focal contacts between wild-type and Rack1 overexpressing cells (Fig. 3).

On the other hand, Rack1 interaction with PKC isoforms may be important in our observed phenotypes. The involvement of PKCs in several integrin-mediated functions such as spreading (Haller et al., 1998; Vuori and Ruoslahti, 1993), cytoskeletal assembly and adhesion (Haller et al., 1998; Lewis et al., 1996), migration (Derman et al., 1997; Rigot et al., 1998; Volkov et al., 1998), receptor endocytosis (Panetti et al., 1995), and FAK and MAP kinase activation (Lewis et al., 1996; Miranti et al., 1999; Rigot et al., 1998) has been well documented. Interestingly, it has been reported that a specific isoform, PKCζ, is involved in the adhesion and chemotaxis of neutrophils (Laudanna et al., 1998). Thus, the association of Rack1 with integrin tails might enable or otherwise affect PKC isoform-specific localization. Overexpression of Rack1 might disrupt this balance, thereby contributing to the observed cytoskeletal and migratory effects. Although we have not directly analyzed its PKC binding properties, the absence of a migratory deficit in cells overexpressing the WD3 domain variant appears to support this idea. It will be interesting to identify specific residues within this targeted area that mediate these effects. It is presently not clear why cells expressing the WD6 variant did not also have this property. Peptides corresponding to this motif have also been implicated in PKC binding and the downstream functional consequences thereof (Ron et al., 1994; Ron and Mochly-Rosen, 1994). Finally, as Rack1 is composed of WD domains and these motifs have been implicated in protein-protein interactions, it is also conceivable that another unrecognized molecule may mediate our observed effects. In this scenario, Rack1 overexpression and the WD3 domain mutations might affect things such as PKC activity, substrate availability, or protein folding.

In addition to Rack1, two other Racks have been cloned and identified. These include β’-COP, an ePKC receptor (Csukai et al., 1997), and Pick1, an αPKC receptor (Staudinger et al., 1995). While little is known about the structure of Pick1, Rack1 and β’-COP consist of WD repeats, a structural motif held in common with G protein β subunits. Each WD repeat consists of approximately 40 residues with a common core typically bracketed by GH (glycine, histidine) and WD (tryptophan, aspartic acid) (Neer et al., 1994). Structure determinations of WD-containing proteins suggest a propeller motif where each propeller blade consists of four antiparallel β sheets (Garcia-Higuera et al., 1998). WD repeat proteins are involved in diverse cellular functions including cytoskeletal assembly, intracellular trafficking, mRNA splicing, transcriptional regulation and, as implicated in our studies, cell migration. It is not known if the other identified Racks, β’-COP and Pick1, can associate with integrins or are involved in integrin-mediated function. On the contrary, β’-COP has been implicated in vesicular trafficking and Golgi function (Csukai et al., 1997). Nevertheless, it is tempting to speculate that these or other Racks might function to translocate PKCs to integrins or focal contacts and assist in downstream signaling. In light of this idea, it is interesting to note that another WD-containing protein, WAIT-1, has been isolated in a yeast two-hybrid screen using the integrin β7 tail as a bait (Rietzler et al., 1998). It is presently unknown whether WAIT-1 functions as a Rack or what the functional consequences of WAIT-1-integrin β7 interactions might be.

In summary, we have shown that overexpression of the scaffolding protein Rack1 in CHO cells increases the number of actin stress fibers and focal contacts upon adhesion and decreases cell motility in a manner that likely involves its interaction with PKC. Identification of those signaling pathways involving Rack1 and PKC or disrupting their association with integrins might define a novel means of regulating migration in pathological situations.

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