TYROSINE 302 IN RACK1 IS ESSENTIAL FOR IGF-I-MEDIATED COMPETITIVE BINDING OF PP2A AND β1 INTEGRIN AND FOR TUMOUR CELL PROLIFERATION AND MIGRATION

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Running title: PP2A and β1 integrin bind to Tyrosine 302 in RACK1

IGF-I regulates a mutually exclusive interaction of PP2A and β1 integrin with the WD repeat scaffolding protein RACK1. This interaction is required for the integration of IGF-I Receptor and adhesion signaling. Here we investigated the nature of the binding site for PP2A and β1 integrin in RACK1. A WD7 deletion mutant of RACK1 did not associate with PP2A but retained some interaction with β1 integrin, whereas a WD6/WD7 mutant lost the ability to bind to both PP2A and β1 integrin. Using immobilized peptide arrays representing the entire RACK1 protein, we identified a common cluster of amino acids (FAGY) at positions 299-302 within WD7 of RACK1 which were essential for binding of both PP2A and β1 integrin to RACK1. PP2A showed a higher level of association with a peptide in which Y302 was phosphorylated compared with an unphosphorylated peptide, whereas β1 integrin binding was not affected by phosphorylation. RACK1 mutants in which either the FAGY cluster or Y302 were mutated to AAAF, or F, respectively, did not interact with either PP2A or β1 integrin. These mutants were unable to rescue the decrease in PP2A activity caused by suppression of RACK1 in MCF-7 cells with siRNA. MCF-7 cells and R+ cells (IGF-IR over-expressing fibroblasts) expressing these mutants exhibited decreased proliferation and migration, whereas R- cells (IGF-IR null fibroblasts) were unaffected. Taken together the data demonstrate that Y302 in RACK1 is required for interaction with PP2A and β1 integrin, for regulation of PP2A activity, and for IGF-I-mediated cell migration and proliferation.

RACK1 is a highly conserved scaffolding protein with 7 WD repeats that functions as a 7 sided propeller protein and displays significant homology to the beta subunit of hetero-trimeric G proteins (1,2). Although RACK1 was originally named for its function in targeting activated C kinases around the cell, it has also been shown to act as a broadly active scaffolding protein in diverse essential cellular functions including transcription and translation (1,3). RACK1 has a well established role in integrin and growth factor signaling (4-6). It can interact with β1 and β3 integrin receptors, and regulate the assembly of protrusions necessary for cell adhesion and migration in a Src-dependent manner (4). RACK1 also scaffolds members of the Erk signalling pathway to promote integrin-mediated activation of MAP kinases (7).

We and others have previously demonstrated that RACK1 can regulate IGF-I Receptor (IGF-IR) and integrin signalling in transformed cells (5,8), which may be essential for IGF-I-mediated signalling via the MAP kinase pathway. Over-expression of RACK1 enhances IGF-I-mediated cell migration and activation of MAP kinases (8). Cells expressing a mutant of the IGF-I Receptor (Y1250/1251F), which does not interact with RACK1 (9) are deficient in
promoting IGF-I-mediated cell survival, migration, and activation of the MAP kinase pathway (10,11) and are also deficient in transforming activity (12) and cytoskeletal organization (13). RACK1 scaffolds β1 integrin to a complex at the IGF-IR and promotes recruitment and dissociation of several proteins to this complex in response to IGF-I (9). These proteins are required for IGF-IR signaling and include IRS-1 and 2, Src, PP2A, Shp-2, and the p85 subunit of PI3-kinase (9).

Although RACK1 associates with multiple signaling proteins (1), it is not known which proteins or how many proteins interact with RACK1 at any given time to promote integrin or IGF-I signaling. The different kinetics of recruitment and release of these proteins from RACK1 in response to IGF-I stimulation suggests that IGF-I-mediated competitive interaction of signaling proteins with RACK1 may be an important mechanism of its scaffolding function in IGF-I Receptor and integrin signalling. We recently demonstrated that IGF-I-mediated cell migration requires a mutually exclusive association of RACK1 with the serine threonine phosphatase PP2A and β1 Integrin (14). PP2A is associated with RACK1 when cells are cultured without serum, but PP2A becomes rapidly released upon IGF-I stimulation at the same time as β1 integrin becomes recruited to the complex. Ligation of integrins is necessary for release of PP2A. The binding site for both of these proteins resides in the C-terminus of RACK1 between WD repeats 4-7. These observations suggest that β1 integrin competes with and displaces PP2A from binding to RACK1 in response to IGF-I stimulation, and that this competition is a necessary component for the integration of IGF-IR and adhesion signalling during the proliferation and migration of tumour cells.

PP2A has a well established role in regulating the MAP kinase pathway (15-18) and can negatively regulate growth factor signaling by preventing Shc phosphorylation in response to EGF and IGF (19,20). In addition, IGF-I stimulation transiently suppresses total cellular PP2A activity and overexpression of RACK1 increases PP2A activity. However, whether RACK1 can directly regulate PP2A activity or act to target PP2A to specific locations in the cell remains unknown.

To determine if competition between PP2A and β1 integrin for binding to RACK1 is essential for IGF-I signalling, here we aimed to identify the nature of the binding site for PP2A and β1 integrin in RACK1 and to assess if this binding site is essential for the function of RACK1 in the IGF-IR context. We found that both PP2A and β1 integrin bind to a cluster of amino acids within WD 7 of RACK1 that encompasses a tyrosine at position 302. Mutation of Y302 was sufficient to disrupt the binding of both PP2A and β1 Integrin to RACK1 and was sufficient to abrogate the effects of RACK1 overexpression on increasing IGF-I-mediated cell proliferation, and migration and suppressing PP2A activity.

These findings provide a mechanism for IGF-I-mediated regulation of RACK1 function in the integration of IGF-IR and adhesion signaling, and suggest that the competitive interaction of proteins with RACK1 may be a general feature of its scaffolding role in signal propagation.

**MATERIALS AND METHODS:**

**Reagents and antibodies.**
Recombinant IGF-I was purchased from Pepro Tech. Inc. (Rocky Hill, NJ). Anti-RACK1 and anti-PP2A monoclonal antibodies were from BD Transduction Laboratories (Heidelberg, Germany). Anti-IGF-IR polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin monoclonal antibody was from Sigma Ireland Ltd, (Dublin, Ireland). Recombinant PP2A was purchased from Upstate Biotechnology, anti-integrin β1 integrin was purchased from Chemicon (CA, USA) cat no. AB1952, and purified human integrin was purchased from Chemicon, cat no. CC1012.

**Cell culture, transfection, and IGF-I-mediated stimulation of MCF-7 cells.**
The MCF-7 breast carcinoma cell line, R+, and R- cell lines (Mouse embryonic fibroblast cell lines derived from IGF-IR knockout mouse (21) were maintained in Dulbecco’s Modified Eagle Medium.
(DMEM) (Biowhittaker, Verviers, Belgium), supplemented with 10% (v/v) FBS, 10mM L-Glu, and 5 mg/ml penicillin/streptomycin. Where indicated, MCF-7 cells, R+ and R- cells were transiently transfected with pcDNA3HA/ RACK1 wt, RACK1 FAGY-AAA, RACK1 Y302F, or empty pcDNA3 vectors (8μg of DNA) using LipofectAMINE transfection reagent (Life Technologies). After 24 hours in culture the transfected cells were seeded into 24 well plates for monitoring cell proliferation. For analysis of PP2A activity, the cells were then washed with PBS and starved from serum for 4 hours before being stimulated with IGF-I for the indicated times.

RNA interference.
The siRNAs targeted to the untranslated region of RACK1 were purchased from Dharmacon. The sequences are: siRNA utr1 sense 5’ AGGAGAGGUUGUGUAGUUUU U, siRNA utr2 sense 5’ AGAAACUAGCACCACAACCUCUCUU, siRNA utr3 sense 5’ GAGAGGUUGUGUAGUUUUU. MCF-7 cells were seeded at 50% confluency and were transfected with the indicated siRNA concentration using the oligofectAMINE transfection reagent (Invitrogen) following the manufacturer’s instructions as described previously (14). Cells were cultured for a further 24 hours, after which time the cells were assessed for PP2A activity. Alternatively, the cells were re-transfected with the pcDNA3 HARACK1 vectors described above. siRNA 1 and siRNA 2 have been described previously (14).

RACK1 truncation mutations
C-terminal RACK1 truncation mutations were generated by PCR and designed so that they could be cloned “in frame” into a pcDNA3 vector containing the HA coding sequence. HA-RACK1 wt was used as a template using the following primers: RACK1 forward 5’ ATCGGTGCACCATGAGCAGAT G, ΔWD7 5’ CATTCTAGATTCATCTACAAAATTTCTT, RACK1 ΔWD6 5’ CATTCTAGAGATCCCATAACATGG C. After confirmation of the DNA sequence and “in frame” cloning with the HA tag, the RACK1 truncation plasmids pcDNA3-HARACK1 ΔWD7 and pcDNA3-HARACK1 ΔWD6 were prepared for transfection using Qiagen Midi-prep plasmid preparation kits.

FAGY-AAA and Y302F mutation
The cloning of pcDNA3 HARACK1 has already been described (8). For mutation of the FAGY sequence of amino acids in RACK1, the section of RACK1 from BamH1 (cut position 610) to the end of RACK1 together with an Apa1 site at the 3’ end was synthesized (with FAGY changed to AAAF) and sequenced by GenScript, (Piscataway, NJ, USA) in the pUC57 plasmid. The BamH1 to Apa1 fragment (352bp) was cut from pUC57 and ligated into pcDNA3 HARACK1 which had already been digested with BamH1 and Apa1 to generate pcDNA3 HARACK1 AAAF. To generate the Y302F single mutation, a 2 step PCR reaction procedure was employed using pcDNA3 HARACK1 as a template together with the following primers: Primer 1, 5’ TCTCCAGATGGATTCCTTG, Primer 2, 5’ CGAAGGCAAACACCTTTACAC, Primer 3, 5’ GTCCGTGAAGCCAGCAAAACAG, Primer 4, 5’ AAGGGGCCCCACTAGCTGTGCACAT. The PCR product was cloned into Gem T-easy for sequencing. When the mutation was confirmed, the BamH1/Apa1 piece with the Y302F mutation was digested out and ligated into pcDNA3 HARACK1 which had already been digested with BamH1 and Apa1 to generate pcDNA3 HARACK1 Y302F.

Preparation of cellular protein extracts and immunoprecipitation.
Cellular protein extracts were prepared by washing cells with PBS and then scraping into lysis buffer consisting of Tris HCl, pH 7.4, 150mM NaCl, 1% NP40 plus the tyrosine phosphatase inhibitor Na3VO4 (1mM), and the protease inhibitors PMSF (1mM), pepstatin (1μM) and aprotinin (1.5μg/ml). After incubation at 4°C for 20 minutes nuclear and cellular debris were removed by micro-centrifugation at 14,000
rpm for 15 minutes at 4°C. For immunoprecipitation of HA tagged proteins, extracts from stimulated or unstimulated cells were initially pre-cleared using BSA coated protein G agarose beads (15 μl beads per 400 μg of total protein in 700 μl lysis buffer) by incubation at 4°C for 1 hour with gentle rocking. The lysates were recovered from the beads by centrifugation at 3,000 rpm for 3 minutes and transferred to fresh tubes for incubation with primary antibody (3 μg of each antibody) overnight at 4°C with gentle rocking. Immune complexes were obtained by adding 20 μl of Protein G agarose beads for 3 hours at 4°C. The beads were washed (x 3) with ice-cold lysis buffer and the immune complexes were then removed from the beads by boiling for 5 minutes in 20 μl of 2X SDS PAGE sample buffer for electrophoresis and western blot analysis.

Western blot analysis.
All protein samples for western blot analysis were resolved by SDS-PAGE on 4-20% gradient gels and then transferred to cellulose membranes, which were blocked for 1 hour at room temperature in TBS containing 0.05% Tween 20 (TBS-T) and 5% milk (w/v). All primary antibody incubations were performed overnight at 4°C. Secondary antibody incubations were carried out at room temperature for 1 hour. Where indicated, membranes were stripped by incubation in 62.5 mM Tris-Cl, 1% SDS and 0.7% 2-mercaptoethanol for 30 minutes at 50°C followed by extensive washing in 0.2% and 0.05% TBS-T. Secondary antibodies conjugated with horseradish peroxidase were used for detection with enhanced chemiluminescence (Super Signal from Pierce, Rockford, IL) for detection of HA tagged RACK1 truncations. In all other cases we used Alexa Fluor 680 and 800-coupled anti-rabbit and anti-mouse antibodies (LI-COR Biosciences Cambridge, UK) for Western blot analyses and detection was performed using an Odyssey® infrared imaging system (LI-COR Biosciences Cambridge, UK).

Cell Proliferation and Plating efficiency Assays

Proliferation rates of MCF-7 cells transfected with pcDNA3HA/RACK1 wt, RACK1 FAGY-AAAF, RACK1 Y302F, or empty pcDNA3 vector were measured in monolayer culture and compared to the untransfected MCF-7 cells. Following 24 hours culture, the cells were harvested with trypsin/EDTA, washed with serum free media (SF) and re-suspended in DMEM/10% FBS at a final density of 3.0 x 10^5 cells per well in multiple wells of a 24 well plate. At regular intervals (48, 72 and 96 hours post transfection) the cells were removed from triplicate wells and the viability and numbers were assessed using trypan blue exclusion and a hemocytometer.

Spot synthesis of peptides and overlay analysis.
Peptide libraries of RACK1 were produced by automatic SPOT synthesis (22) and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments) (22-24). The interaction of the spotted RACK1 peptides with purified β1 Integrin and recombinant active PP2A was determined by overlaying the cellulose membranes with 10 μg/ml of recombinant protein as previously described (25-29). Bound recombinant PP2A and β1 integrin was detected with specific mouse (PP2A) and rabbit antisera (β1 integrin), and a secondary anti-mouse and anti-rabbit antibody coupled with horseradish peroxidase (HRP). Once the binding site of both PP2A and β1 integrin on the full length RACK1 array was determined, specific alanine scanning substitution arrays were generated for the last 19 amino acids of spot C18 as described above. To generate the peptide phosphorylated on Y302, a new array was made and the tyrosine at position 302 was replaced with a phosphorylated tyrosine residue and the array was overlayed with test proteins as above.

Phosphatase assays for PP2A activity
Cellular PP2A phosphatase activity was measured using para-nitrophenyl phosphate (p-NPP) as the substrate with the serine/threonine phosphatase assay kit (Upstate Biotechnology). Cells (R+ or
MCF-7) were serum starved for 4 hours (or when required pre-incubated with Okadaic acid (10nM) for 1 hour) prior to stimulation with IGF-I (100ng/ml) stimulation for the indicated times. Cells were lysed in ice-cold lysis buffer (50mM Tris pH 7.5, 10% glycerol, 1% NP-40, 137mM NaCl, 1μM pepstatin, 1mM PMSF, 1μM aprotinin) for 20 minutes on ice. Nuclear and cellular debris was removed by centrifugation at 14,000g at 4°C for 15 minutes. Clarified supernatants were incubated with anti-PP2A (3μg) antibodies overnight at 4°C followed by addition of 30μl pre-coated protein G agarose beads for 3 hours. Pre-coated beads were prepared by incubating beads with 1% w/v BSA in TBS-Triton X-100 for 2 hours at room temperature. Immunoprecipitates were washed twice with ice-cold lysis buffer and once with assay buffer containing 2.5mM nickel chloride and 900μg of p-NPP/ml and incubated for 45 minutes at 37°C. The amount of para-nitrophenol produced was determined by measuring the absorbance at 405nM. Assays were performed with triplicate samples.

Transwell migration assays
MCF-7 cells transfected with pcDNA3HA/RACK1 wt, RACK1 FAGY-AAAF, RACK1 Y302F, or empty pcDNA3 vector were cultured in 10cm plates. Cells were serum starved for 4 hours before removing with trypsin, washed twice and then resuspended in serum free medium. The final cell density was determined using a haemocytometer. The lower wells of the Transwell chamber apparatus were loaded with serum free DMEM supplemented with 10ng/ml IGF-I (final concentration). A 50μl volume of cell suspension containing 100,000 cells was added to each upper well. The loaded chamber was incubated for 16 or 24 or hours, at which time the chamber was removed from the incubator and disassembled. Cells on the upper surface of the membrane were removed by scraping so that only cells that had migrated through the membrane remained. The membrane was then fixed with methanol, stained with 0.1% crystal violet and air-dried. Cell counts were obtained by counting all cells and data are presented as an average of counts from 5 fields of triplicate wells for each test condition.

RESULTS

The WD 6 and 7 domains of RACK1 contain binding sites for PP2A and β1 Integrin.
We have previously demonstrated (14) that both PP2A and β1 integrin associate with the C-terminus of RACK1 (WD repeats 4-7) and that they do so in a mutually exclusive manner. In contrast, the IGF-IR was shown to interact within WD repeats 1-4 of RACK1 (14). To define the binding site of both PP2A and β1 integrin on RACK1 we initially generated C-terminal truncation mutations of HA-RACK1, in which either the entire WD7 (dWD7) or both the WD6 and WD7 domains (dWD6/7) were deleted (Fig. 1). MCF-7 cells were transfected with dWD7 and dWD6/7 or wild type (wt) HA-RACK1 as a control. Each protein was immunoprecipitated using anti-HA antibody and tested for associated PP2A, β1 integrin, and IGF-IR by western blotting. Results show that both PP2A and β1 integrin associated with HA-RACK1-wt. PP2A did not associate with dWD7, whereas a small amount of β1 integrin was associated with this mutant. However, β1 integrin did not interact with the dWD6/dWD7 mutant. The IGF-IR was shown to co-immunoprecipitate with all of the RACK1 proteins, which confirmed that the truncation mutations maintained correct folding inside the cell. Overall, from these results we conclude that the PP2A binding site resides in WD7 of RACK1 but that β1 integrin has binding sites in both WD6 and WD7 of RACK1.

Identification of the shared binding site in RACK1 for PP2A and β1 integrin as the FAGY cluster.
We next focused on identifying the precise amino acids within WD7 of RACK1 that may constitute a common binding site for PP2A and β1 integrin. To do this we employed a peptide array analysis procedure that we recently used to identify the binding site for RACK1 on PDE4D5 (26) and to identify the interaction sites on βarrestin2 for binding of PDE4D5 (25). A library of
overlapping peptides (25 mers), each shifted by 5 amino acids, encompassing the entire sequence of RACK1 was spot synthesised on membranes. Duplicate membranes were then probed with purified β1 integrin or with purified active catalytic subunit of PP2A (Fig. 2). Probing the RACK1 peptide library with purified PP2A yielded a positive reaction (indicated by dark spots) with peptide spot C18 (SADGQTLFAGYTDNLVRVW), which corresponded to a sequence in the WD7 domain of RACK1. Probing the RACK1 peptide array with β1 integrin yielded 2 positive reactions. One was in WD6 (C2) and one was in WD7 (C18), which corresponds to the same site as that for PP2A. These data are in agreement with observations from the RACK1 deletion mutants (Fig. 1), which indicated a requirement of WD7 for both PP2A and β1 integrin interaction, and also indicated an additional site in WD6 required for the interaction of β1 integrin with RACK1.

To gain further insight into the identity of specific amino acids of RACK1 required for PP2A and β1 integrin binding to RACK1 we next used alanine scanning arrays. Arrays of peptides derived from the 25-mer parent peptides corresponding to spot C18 (S292 to W310) in which each new peptide had a single alanine substitution in successive amino acids, were again probed with purified PP2A or β1 integrin. The binding of PP2A and β1 integrin to the peptide spots was measured by densitometry and presented as a percentage of the binding of each protein to the control ‘parent’ peptide. Results showed that the binding of both PP2A and β1 integrin to RACK1 was either severely disrupted or lost by alanine substitution of any one of four consecutive amino acids F299, A300, G301 and Y302 (Fig. 3). The binding of PP2A to RACK1 was also decreased when the single isolated amino acids G295, L298, N305, R308 and W310 were substituted for alanine, whereas β1 integrin binding was decreased by substitution of A293, R308 and W310. Since alanine substitution of the consecutive FAGY residues had the most deleterious effect on binding of both PP2A and β1 integrin to RACK1, we concluded that the FAGY cluster is likely to be a critical part of the binding site for both PP2A and β1 integrin.

**Tyrosine 302 in WD7 is essential for binding of both PP2A and β1 integrin to RACK1.**

We next investigated whether the FAGY amino acid cluster identified as site for both PP2A and β1 integrin binding to RACK1 in the peptide array, was important for these interactions in the context of RACK1 expressed in cells. We were also interested to determine if Y302 may be of particular importance in this sequence, since tyrosine phosphorylation of RACK1 at this site could, potentially, be a mechanism to regulate its activity as a scaffolding protein in response to IGF-I signalling. Indeed, Src has been shown to interact with RACK1, and Y246 has previously been shown to be essential for Src interaction with RACK1 (30).

Mutants of HA-RACK1, FAGY to AAAF cluster or the Y302 to F302 point mutant, were generated and transiently expressed in MCF-7 cells and compared with HA-RACK1-wt as a control. RACK1 proteins were immunoprecipitated with anti-HA antibody and assessed for associated PP2A and β1 integrin (Fig. 4A). No interaction of either PP2A or β1 integrin was observed with the FAGY cluster mutant or with the Y302F mutant. These data suggest that Y302 is essential for binding of both PP2A and β1 integrin to RACK1.

These observations together with our previous data showing that PP2A and β1 integrin bind to RACK1 in a mutually exclusive manner in response to IGF-I stimulation of cells (14) suggest that that these proteins compete for binding to Y302 in RACK1.

**PP2A binds preferentially to phosphorylated Y302 of RACK1 peptides.**

The role of tyrosine phosphorylation in regulating interactions of WD domains with other proteins is not well understood. Y246 in RACK1 is required for its functions in promoting cell adhesion and migration (30,31). Both Y246 and Y228 can be phosphorylated in vitro by Src or in vivo by over-expressed Sre, and have been proposed to act as a Src phosphorylation sites as well as a binding sites for the Src SH2 domain.
However, specific phosphorylation of RACK1 on any tyrosine in vivo in response to growth factors or physiological stimuli has not yet been demonstrated. Although we were able to detect low basal levels of tyrosine phosphorylated RACK1 in MCF-7 cells by immunoprecipitation with anti-phosphotyrosine antibodies, we were unable to identify any changes in RACK1 phosphorylation in response to IGF-I stimulation or demonstrate phosphorylation of Y302 by mass spectrometry analysis of RACK1 purified from either serum-starved or IGF-I-stimulated cells (data not shown).

Therefore, in order to determine whether phosphorylation of Y302 could influence the binding of PP2A and β1 integrin to RACK1 we carried out binding studies in vitro with phosphorylated and unphosphorylated peptides that were spot synthesized on cellulose membranes. These peptides encompassed the binding site of PP2A and β1 integrin (SADGQTFLFGYTDNLVRW) in RACK1 and contained either phosphorylated Y302 or non-phosphorylated Y302. This array was then probed with recombinant β1 integrin or purified PP2A (Fig. 4B). The result obtained demonstrated a dramatic increase in the association of PP2A with the RACK1 peptide when Y302 was phosphorylated compared to the RACK1 peptide when Y302 was not phosphorylated (Fig. 4B left panel). In contrast, β1 integrin binding to the phosphorylated and unphosphorylated RACK1 peptide appeared similar (Fig. 4B right panel).

Together, these data indicate that PP2A and β1 integrin can bind to Y302 in a RACK1 peptide and that PP2A may exhibit a profoundly increased association with RACK1 when Y302 is phosphorylated. These observations combined with the loss of association of the RACK1 Y302F mutant with both PP2A and β1 integrin in cells (Fig. 4A) suggest that reversible phosphorylation of Y302 may control competitive interaction of these proteins with RACK1. In this scenario PP2A may bind to phosphorylated RACK1, and dephosphorylation of Y302 following IGF-I stimulation may facilitate β1 integrin association.

**PP2A association with RACK1 is required for PP2A phosphatase activity in cells.**

We have previously demonstrated that disruption of the interaction between RACK1 and β1 integrin is associated with loss of IGF-I-mediated cell migration (14). However, whether RACK1 is required to regulate PP2A activity is not known. To address this we asked whether the RACK1 Y302F mutant that cannot bind to PP2A had an effect on PP2A activity. To better observe the effects of the RACK1 Y302F mutant and remove the confounding effects of endogenous RACK1 we expressed this mutant form of RACK1 in cells where endogenous RACK1 was suppressed by using siRNA directed toward the untranslated region (UTR) of RACK1. As shown in Fig. 5A, all three siRNAs tested suppressed RACK1 expression in MCF-7 cells. PP2A activity was then assessed in these cells under serum-starved conditions using p-NPP as a substrate. We used serum starved conditions because we have previously observed that PP2A activity is maximal under these conditions (14). When RACK1 expression was suppressed by siRNA-mediated knockdown, PP2A activity was decreased to levels similar to those caused by pretreatment with 1nM okadaic acid, which we and others have shown specifically inhibits PP2A (14,19,20) (Fig. 5B).

Having determined that the siRNA targeting the RACK1 UTR region suppressed endogenous RACK1 expression and PP2A activity in MCF-7 cells we next asked if this phenotype could be rescued by transiently expressing HA-RACK1 wt or mutants in which the PP2A binding site was mutated (FAGY-AAAF and Y302F). Expression of HA-RACK1 wt restored PP2A activity to levels above those observed in untransfected cells (Fig. 5C). In these cells PP2A activity was transiently suppressed in response to IGF-I-mediated stimulation of the cells as described previously (14). However, transfection of either the FAGY-AAAF or the Y302F mutant did not restore PP2A activity to levels observed in transfected cells. In these cells PP2A activity was similar to that observed in the presence of 1nM okadaic
acid. IGF-I stimulation had no effect on PP2A activity in these cells.

Taken together these results indicate that RACK1 is required for maintaining PP2A activity in cells, and interaction of PP2A with RACK1 via Y302 is required both for PP2A activity and its regulation by IGF-I.

**Y302 in RACK1 is required for IGF-I mediated Cell proliferation and Migration.**

We next assessed the effects of the RACK1 mutants that do not bind PP2A or β1 integrin on cell proliferation and migration. Previous studies have shown that the effects of RACK1 on cell proliferation are dependent on cell type. Overexpression of RACK1 suppresses proliferation of NIH-3T3 cells (32), but overexpression of RACK1 enhances proliferation of MCF-7 cells (8). These differential effects may be due to RACK1-mediated suppression of Src activity in 3T3 cells and to increased integrin signalling and Erk phosphorylation in MCF-7 cells.

Here we assessed proliferation in MCF-7 cells, R- (IGF-IR null mouse embryonic fibroblast) cells, and R+ (R-cells over-expressing IGF-IR) cells. As expected, over-expression of HA-RACK1-wt increased proliferation in MCF-7 cells. However, both the FAGY and the Y302F mutant caused decreased proliferation of these cells compared with untransfected cells (Fig. 6A). Similar results were observed with HA-RACK1-wt and FAGY mutant in R+ cells (Fig. 6B), however, neither overexpression of HA-RACK1-wt nor the FAGY mutant had any effect on the proliferation of R- cells.

We next compared the effect of HA-RACK1-wt, FAGY, and Y302 F on the ability of MCF-7 cells to migrate towards IGF-I in Transwell assays. MCF-7 cells generally do not migrate in these assays but as expected, and previously described in (14), over-expression of HA-RACK1-wt increased migration of MCF-7 cells (Fig. 6C). However, over expression of either the FAGY or the Y302F mutant resulted in no enhancement of cell migration compared to controls.

These data indicate that Y302 is essential for the effects of RACK1 on IGF-I-mediated cell proliferation and migration.

**DISCUSSION:**

This study establishes RACK1 as an essential scaffolding protein for integration of IGF-I Receptor and adhesion signalling in transformed cells. It also extends our previous observations (14) by proposing a mechanism for the mutually exclusive binding of PP2A and β1 integrin to RACK1. We have now identified a tyrosine residue in RACK1 (Y302) that is essential for the association of RACK1 with both PP2A and β1 integrin, Y302 is essential for PP2A activity and the regulation of PP2A phosphatase activity by IGF-I, and is also essential for IGF-1-mediated cell proliferation and migration. As outlined in the model shown in Fig. 7 our data propose that in adherent serum-starved cells, PP2A is associated with RACK1 via a motif that encompasses Y302 in WD7. In response to IGF-I stimulation PP2A dissociates from RACK1 and PP2A phosphatase activity decreases. Subsequently, β1 integrin becomes associated with RACK1. We speculate that the dissociation of PP2A and association of β1 integrin may be due to dephosphorylation of Y302 in response to IGF-I stimulation because PP2A displays a preference for binding to a peptide in which this tyrosine was phosphorylated, whereas β1 integrin binding displays no such preference.

This study strongly supports the concept that in response to extra-cellular stimuli, such as IGF-I, signalling proteins compete for binding to RACK1. In this way RACK1 may act as an essential “switch” for the integration of growth factor and adhesion signalling. RACK1 has been shown to interact with a diverse array of proteins and to regulate a variety of important cellular processes such as proliferation, transcription, protein synthesis and cell migration (1). However, how the binding of these proteins to RACK1 is regulated is poorly understood. Competitive interactions would be one means by which RACK1 could achieve such a diversity of function as a scaffolding protein. Amongst
the proteins that have been shown to interact with RACK1 (reviewed in (1,2), it appears that these proteins can be divided into two broad categories: Those that are constitutively associated with RACK1 such as the cAMP specific phosphodiesterase PDE4D5 (33) and the IGF-IR (8), or those that associate in a transient or competitive manner in response to cell stimulation such as PKC’s (34,35), and members of the Erk signaling pathway (7).

Phosphorylation of RACK1 on serine, and RACK1 dimerization, has recently been implicated in regulating competitive interactions of proteins. The serine phosphatase PP2 was shown to regulate formation of a RACK1 dimer formed by WD4-WD4 interaction. This dimer is required to bring the E3 ligase Elongin C to HIF1a and thereby promote HIF1a degradation (36).

Our data suggest that phosphorylation and dephosphorylation of a tyrosine in RACK1 may regulate competitive interactions of proteins to a common site. RACK1 contains six tyrosines and until now, only one of these tyrosines, (Y246) has previously been associated with function. Y246 is required for RACK1 in promoting cell spreading and migration. It can be phosphorylated by Src in vitro or upon co-expression with active Src in vivo (32) (31) (4), and it has been proposed to constitute the Src binding site. In MCF-7 cells, tyrosine-phosphorylated RACK1 could be detected at low levels, but we were unable to detect any differences in tyrosine phosphorylation of the RACK1-wt and Y302F proteins in cell lysates. We were also unable to detect phosphorylation of immunoprecipitated and gel-purified RACK1 by mass spectroscopy. Use of the NetPhos phosphorylation prediction software (37), which evaluates the probability of phosphorylation of specific tyrosines in cells, resulted in a score of 0.249, which lies below the threshold of 0.5 for phosphorylation. Interestingly, in this analysis Y246 of RACK1 has a score of 0.207, which indicates an even lower probability for phosphorylation. Thus, it appears that detection of Y302 phosphorylation in endogenous RACK1 in vivo and detection of phosphorylation of RACK1 on any tyrosine may be limited by the low signal that would be associated with phosphorylation of a single residue.

Our data from in vitro analysis suggest that Y302 phosphorylation may act to modulate the ability of a RACK1 peptide to bind to PP2A. A RACK1 peptide which was phosphorylated on Y302 shows a profoundly increased level of interaction with PP2A compared to the unphosphorylated peptide. This, combined with our observation that endogenous RACK1 in cell lysates binds to PP2A in serum-starved cells but dissociates in response to IGF-I stimulation (14), suggests that Y302 may be phosphorylated when cells are starved from serum and may become dephosphorylated in response to IGF-I stimulation.

We show that Y302 and the interaction of RACK1 with PP2A is required for the cellular activity of PP2A as well as the IGF-I-mediated decrease in cellular PP2A activity. A number of previous studies have demonstrated that IGF-I, Insulin or EGF stimulation can inhibit total cellular PP2A activity, and also promote dissociation of Shc from PP2A leading to Shc phosphorylation and activation of the Ras/MAP kinase pathway (14,19,20). In this study, we found that when RACK1 expression was suppressed with siRNA, PP2A phosphatase activity was reduced. PP2A activity could be rescued by expression of wild type RACK1, but not by expression of the FAGY or Y302 mutants that do not bind PP2A. This strongly suggests that binding of PP2A to RACK1 stabilizes PP2A activity and RACK1 is required to maintain maximal cellular PP2A activity. These results are also in agreement with our previous study (14) where we found that recombinant RACK1 could stabilize PP2A activity in cell lysates, and that over-expression of RACK1 enhanced cellular PP2A activity (14). In that previous study we observed that suppression of RACK1 with siRNA caused increased basal PP2A activity, which was not suppressed by IGF-I stimulation. This observation was unexpected and could not be readily explained at the time. To resolve this we carried out an experiment using the two oligonucleotides in the previous study and the three used in the current study. All five oligonucleotides suppressed RACK1
expression and decreased cellular PP2A enzymatic activity (not shown). Thus, all the data indicate that RACK1 is required for PP2A activity.

The PP2A holoenzyme consists of a catalytic subunit (C), a scaffolding subunit (A), and a third variable regulatory subunit encoded by the B multigene family that binds when the A and C subunits form the AC core enzyme (38). Regulatory subunits are thought to control PP2A specificity by directing the AC dimer to specific locations and by bridging the AC core dimer to phosphorylated substrates. Interestingly, the B-family subunit contains seven WD repeats and forms a β-propeller structure that is highly homologous to RACK1 (39). It is thus possible that RACK1 may act as a variable regulatory subunit of PP2A.

Transient expression of the Y302 or FAGY mutants in MCF-7 cells and R+ cells reduced the proliferation rate to below that of levels observed in cells transfected with empty vector. Thus these mutants have an apparent dominant negative effect. Since the mutants can still bind to the IGF-IR it is likely that the dominant negative effect is due to ectopically expressed mutant proteins becoming associated with the IGF-IR. This would block endogenous RACK1 from forming the complex at the IGF-IR with β1 integrin and other signalling molecules that are necessary for IGF-I-mediated proliferation. This raises the question as to whether the most important function of Y302 in RACK1 relates to the recruitment of integrin signalling or the release of PP2A and consequent suppression of PP2A activity. We predict that it is a combination of both since the release of PP2A is essential for cell proliferation and migration by facilitating the recruitment of β1 integrin to RACK1.

In summary, our results provided demonstrate that tyrosine 302 in RACK1 is required for binding of both PP2A and β1 integrin binding to RACK1 and for IGF-I-mediated cell proliferation and migration.

**Acknowledgements.**

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**REFERENCES**

Figure legends:

Figure 1
Interaction of deletion mutants of RACK1 with PP2A and β1 integrin
RACK1 truncation mutations in which the entire WD7 repeat (L270-R317, -WD7, or the entire WD6 and WD7 repeats (N222-R317 –WD6/7) were generated by PCR and were cloned “in frame” with the HA tag in pcDNA3 as described in Materials and Methods. The WD deletion mutations were transiently expressed as HA-fusion proteins in MCF-7 cells along with HA-RACK1 wt as a control. Ha-RACK1 was immunoprecipitated using the anti-HA antibody and immunoprecipitates and were assessed for associated PP2A and β1 integrin content by western blotting.

Figure 2
PP2A and β1 integrin bind to a common site in WD7 of RACK1.
An array of immobilized peptide ‘spots’ of overlapping 25-mer peptides each shifted along by five amino acids in the entire RACK1 sequence was probed with purified PP2A and β1 Integrin. Spot numbers relate to peptides in the scanned array and whose sequence is given in Table1. Interactions were detected by immunoblotting. Positively interacting peptides generate dark spots and non interacting peptides remain blank.

Figure 3
The common binding site for both PP2A and β1 integrin is the amino acid cluster 299-302 (FAGY) in WD7 of RACK1.
A peptide array based on 19 amino acids of the parent peptide corresponding to spot C18 (S292 to W310) in which each amino acid was sequentially and individually substituted for alanine was probed with purified β1 integrin and purified PP2A and the interactions were detected by immunoblotting. The binding of PP2A and β1 integrin to the peptide spots was measured by densitometry and presented as a percentage of the binding of each protein to the control ‘parent’ peptide.

Figure 4
PP2A and β1 integrin bind to tyrosine 302 in WD7 of RACK1. (A), MCF-7 cells were transfected with plasmids encoding HA-RACK1, HA-RACK1 FAGY (FAGY to AAAF) and HA-RACK1-Y302F. Cell lysates were prepared and were immunoprecipitated with anti-HA antibody followed by western blotting to detect associated PP2A and β1 integrin. (B) Peptide arrays were generated in which a 19 mer ‘parent’ sequence of RACK1, (S292ADGQTLFAGY302TDNLVRVW310), was phosphorylated on Y302. The peptide array was probed with purified PP2A and β1 integrin.

Figure 5
The interaction between PP2A and RACK1 is required for PP2A activity.
(A) MCF-7 cells were transfected with three different siRNA oligonucleotides directed against the untranslated region of RACK1 (UTR1, UTR2, UTR3) or scrambled oligonucleotide (control) and were assessed for RACK1 expression by Western blotting. (B) PP2A was immunoprecipitated from MCF-7 cells transfected with UTR1, UTR2 and UTR3 were serum and assayed for phosphatase activity using p-NPP as a substrate. Data from triplicate samples are presented, with 100% activity representing that measured in MCF-7 cells transfected with the negative control. (C) PP2A was immunoprecipitated from MCF-7 cells expressing HA-RACK1, HA-RACK1 FAGY (FAGY to AAAF) and HA-RACK1-Y302F and analysed for phosphatase activity using p-NPP as a substrate.
Figure 6
Y302 in RACK1 is required for IGF-I-mediated cell proliferation
MCF-7 cells (A) R- and R+ cells (B) were transiently transfected with HA-RACK1, HA-
RACK1 FAGY (FAGY to AAAF) and HA-RACK1-Y302F using the lipofectamine
procedure. Cells were seeded at a density of 0.3 X 10^5 per well in multiple wells of a 24 well
plate. At 24 and 48 hours, cells from the triplicate wells were collected using trypsin/EDTA,
and cell number and viability were determined by trypan blue exclusion, and data are
presented as the mean and S.D. of live cell numbers in triplicate wells. (C) MCF-7 cells
transiently transfected with HA-RACK1, HA-RACK1 FAGY (FAGY to AAAF) and HA-
RACK1-Y302F were compared for migratory potential in Transwell assays. Cells were
starved and seeded at 50,000 cells/chamber and allowed to migrate toward IGF-I for 16h.
Cells were fixed using methanol before they were stained with crystal violet and examined
under a magnification of X 10. The data are presented as the number of cells/well for
triplicate wells of each transfected cell. MCF-7 cells are included as a control.

Figure 7
Model illustrating our findings on how RACK1 controls IGF-IR-mediated cell migration.
Under serum starved conditions (left), RACK1 is associated with the IGF-IR and PP2A.
PP2A activity is high. When the cells are stimulated with IGF-I (right), PP2A is released from
RACK1 allowing for the recruitment of β1 integrin into a complex that includes the IGF-IR,
RACK1 and β1 integrin. PP2A activity is reduced. Cells proliferation and migrate.
Figure 1
### 25-mer peptide sequence

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<th>Peptide spot</th>
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Figure 3

![Graphs showing % Control Binding for PP2A and β1 Integrin](image)

- **PP2A**
  - Bars represent different peptides (CSADGQTLFAGYTDNLV RW) with varying % Control Binding.

- **β1 Integrin**
  - Similar to PP2A, showing % Control Binding for the same peptides.

**RACK1**

- Highlighted region on the graph:
  - SADGQTLFAGYTDNLVRVW

Caption:

- The Journal of Biological Chemistry
Figure 4A

Legend:

- HA-RACK1
- HA-RACK1 FAGY
- HA-RACK1 Y302F
- Control

Blots for:
- β1 Integrin
- PP2A
- IGF-IR
- HA
- IP HA
Cont = SADGQTLFAGY TDNLVRVW
$^{P}Y302 = SADGQTLFAGY^{P} TDNLVRVW$

**Probe: PP2A**

Cont | $^{P}Y302$ | no peptide

**Probe: β1 Integrin**

Cont | $^{P}Y302$ | no peptide
Figure 5C

![Graph showing PP2A Activity (%)](image-url)
Figure 6A
Figure 6B
Figure 7

- IGF-I

PP2A Active
No Cell Proliferation
No Cell Migration

+ IGF-I

PP2A Activity ↓
Cell Proliferation
Cell Migration