RACK1 is an IGF-1 Receptor interacting protein that can regulate IGF-1-mediated Akt activation and protection from cell death.

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Summary

The insulin receptor (IR) and insulin like growth factor 1 receptor (IGF-1R) activated by their ligands control metabolism, cell survival, and proliferation. Although the signalling pathways activated by these receptors are well characterized, regulation of their activity is poorly understood. To identify regulatory proteins we undertook a two-hybrid screen using the IGF-1R beta chain as bait. This screen identified RACK1 (Receptor for Activated C Kinases) as an IGF-1R-interacting protein. RACK1 also interacted with the IGF-1R in fibroblasts and MCF-7 cells, and with endogenous Insulin receptor in Cos cells. Interaction with the IGF-1R did not require tyrosine kinase activity or receptor autophosphorylation, but did require serine 1248 in the C-terminus. Over-expression of RACK1 in either R+ fibroblasts or MCF-7 cells inhibited IGF-1-induced phosphorylation of Akt, while it enhanced phosphorylation of Erks and Jnks. Src, the p85 subunit of PI-3 kinase and SHP-2 were all associated with RACK1 in these cells. Interestingly, the proliferation of MCF-7 cells was enhanced by over-expression of RACK1, while IGF-1-mediated protection from etoposide killing was greatly reduced. Altogether the data indicate that RACK1 is an IGF-1R interacting protein that can modulate receptor signalling, and suggest that RACK1 has a particular role in regulating Akt activation and cell survival.
Introduction

The insulin and IGF-1 Receptors (IR and IGF-1R) belong to a family of tyrosine kinase receptors that also includes the Insulin-related receptor (IRR). They are tetrameric receptors made up of two alpha subunits that bind the ligands insulin, IGF-1 or IGF-2, and two beta subunits that share high homology in their kinase domains (reviewed in (1)).

These receptors are homologous to a receptor found in the nematode Caenorhabditis elegans and in drosophila, and they activate an evolutionarily conserved metabolic and survival signalling pathway that includes insulin related substrate 1 (IRS-1), phosphatidyl inositol-3-kinase (PI3-K), the serine/threonine kinase Akt, and the Forkhead family of transcription factors (2-5).

There is considerable overlap in IR and IGF-1R function. The IR has a primary role in regulating glucose metabolism and also promotes cell survival and growth (1). The IGF-1R can regulate metabolism, it is critical for growth during development, it promotes cell survival, and it has an additional role in facilitating cellular transformation and cancer progression (6). Cell survival and glucose metabolism are tightly interlinked, because glucose metabolism is essential for Akt-mediated survival stimulated by IGF-1 and other growth factors (7, 8). In addition the potential of the IR and IGF-1R polypeptide chains to associate and form hybrid receptors (9-11), gives them the capacity to either compensate for or to inactivate one another. Lack of function of either the IR or the IGF-1R can cause diabetes in mouse models (12).

The IGF-1R has a well-documented role in cancer development and progression (6). Signals from the IGF-1R can enhance tumor cell survival, growth, and increase...
expression of genes that mediate invasion and metastasis (6, 13, 14). The dependence of
tumor cells on IGF-1R function is supported by the observations that inhibition of IGF-
1R function by antibodies (15); triple helix formation (16); or antisense strategies (17)
can reverse the transformed phenotype and lead to cell death.

Although there is a huge body of literature focusing on activation of the PI3-
kine/Act or other signalling pathway by IGF-1 and insulin, there is a limited
understanding of how the activity of the IR and IGF-1R are regulated. Tyrosine
phosphatases including LAR and PTP-1B regulate IR kinase activity and glucose
metabolism (18, 19). Recently we found that PTP-1B can also regulate IGF-1R kinase
activity and function in transformed cells (20). Another regulatory mechanism for the IR
and IGF-1R is proposed to operate through serine phosphorylation of the receptors or
IRS-1 (21). However although specific serines on IRS-1 are associated with inhibition
of insulin signalling (21) it is not known which serines in the IR or IGF-1R are
phosphorylated, or how they could negatively regulate the activity of these receptors. It
is also not known if there are regulatory mechanisms that act uniquely on the IGF-1R or
the IR. Specific regulatory mechanisms could be very important in distinguishing signals
necessary for the maintenance of normal cells from those necessary for cancer
progression.

A hint that the IGF-1R and IR have different signals and regulation came from
previous studies with mutants of the IGF-1R. These indicated that domains of the IGF-
IR C terminus with distinct amino acid sequences from similarly located domains in the
IR are required or have a regulatory effect on the anti-apoptotic and transforming activity
of the IGF-1R (22-25). The functions of the C terminus in recruiting signalling molecules or regulating receptor function have not yet been elucidated. To address this we undertook a screen for proteins that could interact with the IGF-1R by using the yeast 2-hybrid system.

In this report we identify RACK1, a homologue of the beta subunit of heterotrimeric G proteins (26, 27) as an interacting protein for the IGF-IR and IR. RACK1 associated with the IGF-1R and the IR in a tyrosine kinase independent manner, but did not interact with an IGF-1R that had serine 1248 mutated to alanine. Over-expression of RACK1 in R+ fibroblasts or in MCF-7 cells resulted in enhanced receptor kinase activity, phosphorylation of IRS-2 and Shc, as well as enhanced phosphorylation of Erks and JNK. However IGF-1 induced phosphorylation of Akt was greatly inhibited. Interestingly, although RACK1 enhanced the growth rate of MCF-7 cells it inhibited IGF-1-mediated protection from etoposide killing. Altogether the data indicate that RACK1 interacts with the IGF-1R and the IR to negatively regulate activation of the PI3-kinase pathway. Thus, RACK1 may have a broad role in regulating glucose metabolism and cell survival.

MATERIALS AND METHODS

MATERIALS: Recombinant IGF-1 was purchased from Pepro Tech. Inc. (Rocky Hill, NJ). The anti-IGF-1R and anti-IR, anti-SHP-2, and anti-c-Src polyclonal antibodies and the anti-c-Src monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-IGF-1R monoclonal antibody ND122 was from ImmunoGen, Inc (24).
The anti phospho-Akt, anti-Akt polyclonal antibodies, and the anti phospho-p42/44 MAP kinase monoclonal antibody were from Cell Signaling Technology, (Beverly, MA). The anti-phospho tyrosine monoclonal antibody, 4G10, and the anti-Erk-2, anti-p85, and anti-IRS-2 monoclonal antibodies; the anti-phospho-Jun and anti-Shc polyclonal antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-Shc and anti-RACK1 monoclonal antibodies were from BD Transduction Laboratories (Heidelberg, Germany). The anti HA antibody, 12CA5 was from Boehringer (Mannheim, Germany) and the anti-actin monoclonal antibody was purchased from Sigma Ireland Ltd, (Dublin, Ireland).

**Yeast two-hybrid screen**

The yeast two-hybrid screen was carried out using the reagents and protocols from the MATCHMAKER LexA two-hybrid system (Clontech, Palo Alto, CA). To be used as bait, the cDNA encoding the cytoplasmic domain of the wild type (WT) IGF-1R (AA 930-1337 (29) was fused to the LexA sequence in the yeast expression vector pLexA under the control of the ADH1 promoter. This plasmid was then transformed into the yeast strain EGY48 [p80P-lacZ], which harbored the lacZ reporter plasmid, and transformants were selected using Ura-, His- medium. These yeast cells were subsequently transformed with a cDNA library derived from fetal brain expressed in the pB42AD vector, and simultaneously subjected to nutritional selection and selection for growth of clones that had that expressed activated LacZ (medium: Ura-, His-, Trp-, leu- gal + , Raf + BU salts, and X-Gal). Blue colonies that expressed potential
interacting proteins were isolated and subjected to further selection for a true interaction using Shc as a positive control for an IGF-1R interacting protein, and for interaction with a kinase inactive IGF-1R beta chain (K1003A). The yeast plasmid DNA was recovered and transformed into the E. coli strain KC8 by electroporation. The cDNA inserts were subjected to PCR analysis to exclude known IGF-1R interacting proteins including Grb-10 and p85. Unidentified inserts were then sequenced and compared with the DNA databases using BLAST analysis.

**RACK1 and IGF-1R sub-cloning and mutants.**

Sequence analysis of the two clones expressing RACK1 in the pB42AD vector revealed that one of them encoded the full length RACK1 protein. To obtain full length RACK1 in frame with the HA epitope tag oligonucleotides primers complimentary to the 5’ and 3’ ends of RACK1 were designed that incorporated the restriction sites SalI and XbaI: RACK1 forward 5’-TCGGTCGACCCATGACTGAGCAGATG, and RACK1 reverse 5’-CATTCTAGACTAGCGTGCCAAT. PCR products were digested with SalI and XbaI and analyzed by agarose gel electrophoresis. Bands of the expected length were cut out and ligated into the pKS vector, which had been with digested SalI and Xba. After confirmation of the DNA sequence RACK1 was liberated from this vector using SalI/XbaI and ligated into a pcDNA3 vector containing the HA coding sequence that had been digested with XhoI/XbaI.

The pcDNA3 vectors encoding IGF-1R full-length receptor, the kinase dead mutant (K1003R), and the tyrosine 1316 to phenylalanine mutant (Y1316F) were
previously described (22). The beta chain of the IGF-1R (wild type, K1003R, and Y1316F) was sub-cloned into the expression vector pIRES (Clontech) using BamHI restriction sites on either end as previously described (20). The double tyrosine 1162/1221 to phenylalanine mutant (Y1162/1221F), the serine 1248 to alanine mutant (S1248A), and serine 1252 to alanine (S1252A) mutant were all generated by site-directed mutagenesis using “The Transformer” TM kit from Clontech. The template used was a pKS vector encoding a fragment of the IGF-1R from the unique HindIII restriction site in the kinase domain to the stop codon (20). After verification of the mutants’ sequence the fragments were sub-cloned into the pIRES expression vector already containing a wild type IGF-1R beta chain sequence by using the HindIII and BamHI restriction sites. Full length IGF-1R harboring each of these mutations in the pcDNA3 vector was obtained by digestion of pcDNA3 vector expressing wild type IGF-1R with HindIII and BamHI, and then replacing it with the fragment containing each mutation. The sequence of all IGF-1R-encoding plasmids was verified by DNA sequencing. A prcCMV plasmid encoding the full-length insulin receptor was kindly provided by Kenneth Siddle, University of Cambridge, UK.

Cell culture and transfection.

The MCF-7 breast carcinoma cell line, R- cells (fibroblasts derived from the IGF-1R knock out mouse), R+ cells (R- cells that have been re-transfected with the IGF-1R (30)), and Cos cells were all maintained in Dulbecco’s modified Eagle Medium (DMEM) (Biowhittaker, Verviers, Belgium), supplemented with 10% (v/v) fetal calf serum, 10mM
L-Glu, and 5 mg/ml penicillin/streptomycin. Cos cells or R+ cells were transiently transfected with pcDNA3/HA-RACK1 or empty pcDNA3 vectors (4µg of DNA) using Lipofectamine Plus, (Life Technologies). After 24 hours in culture the transfected R+ cells were split into 6 well plates or 10cm plates and cultured for an additional 18 hours, at which time cells were starved for 3 hours and stimulated with IGF-1, and protein extracts were prepared for immunoprecipitation or western blot analyses.

To generate stable transfectants of HA-RACK1 MCF-7 cells were transfected as described for R+ cells, but 24 hours after transfection cells were split into medium containing G418 (1mg/ml) and maintained for 14 days, with regular replenishment of medium and drug. At this time individual clones were selected, expanded, and screened for expression of HA Rack by western blotting. Clones of MCF-7 cells stably over-expressing HA-RACK1 were maintained in DMEM medium supplemented with 1 mg/ml G418. For analysis of signalling responses cells were washed and starved from serum for 3 hours.

**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 endogenous or transfected proteins protein extracts from stimulated or unstimulated cells was initially pre-cleared using BSA coated Protein G agarose beads (15µl beads per 400µg 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 mins and transferred to fresh tubes for incubation with primary antibody (3µg of each antibody) overnight at 4°C with gentle rocking. Immune complexes were obtained by adding 20µl of Protein G agarose beads for 3 hours at room temperature and were pelleted by centrifugation at 3,000 rpm for 3 mins at 4°C. The beads were washed (x 3) with ice cold lysis buffer and then either used for in vitro kinase assay or removed from the beads by boiling for 5 mins in 20µl 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 nitrocellulose 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 overnight at 4°C. Secondary antibody incubations were carried out at room temperature. Where indicated, membranes were stripped by incubation in 62.5 mM Tris-Cl, 1% SDS and 0.7% Beta mercapto ethanol for 30 mins 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) or ECL+ (Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturers instructions.

**Assays for proliferation and IGF-1-mediated protection from cell death.**

MCF-7 Neo and MCF-7 cells over expressing HA-RACK1 (Clone A1, B1 and C1) were cultured in DMEM supplemented with 10% FCS (complete medium) at 3x10^4 cells per well in multiple wells of a 24 well plate. To monitor cell growth at intervals, attached cells were removed from triplicate wells to eppendorf tubes using trypsin-EDTA, and were centrifuged at 3,000 rpm for 3 mins. The cell pellets were then resuspended in 100µl of medium and counted using a hemocytometer and trypan blue exclusion. Data are presented as the mean and standard deviation of counts from triplicate wells.

To assess cell survival in response to IGF-1 in the presence or absence of the apoptosis-inducing drug etoposide, cells were seeded in complete medium. After 18 hours the cells were washed once in PBS and re-incubated in serum free medium supplemented with either no additives, or with added IGF-1 (100ng/mL), or with etoposide (8.5µM), or with IGF-1 + etoposide. After a further 36 hours in culture the cells were assessed for viability and cell number in triplicate wells as described for proliferation assays.

**In vitro kinase Assays**

MCF-7 cells were starved from serum for four hours and then stimulated with IGF-1 for 0, 15, or 30 minutes. Cell lysates were prepared and immunoprecipitated with anti-IGF-
1R polyclonal antisera as described above. Protein-G agarose complexes obtained from immunoprecipitations were washed in kinase buffer (50mM Hepes pH 7.4, 10mM MgCl₂, 10mM MnCl₂) and then resuspended in 25μl of a kinase reaction mixture containing ATP (0.03 mM final conc.), 2μl of ³²P ATP (5μCi/μl) and 2μl of poly (Glu:Tyr) (Sigma). Following a 20 min. incubation period, samples of reaction mixture (5μl) were removed to fresh tubes containing 9μl H₂O and 35μl of 20 mM EDTA pH 7.4. Triplicate samples were then transferred to glass microfibre filters in 24 well plates and washed extensively with ice cold TCA (10%) containing 10mM Na₂HPO₄. Following a final wash with 70% ethanol, the filters were dried and ³²P was measured in a scintillation counter (Beckman). The data are presented as the mean and standard deviation of cpm for triplicate samples.

RESULTS

Identification of RACK1 as an IGF-1R interacting protein.

In order to identify novel IGF-1R interacting proteins, a yeast two-hybrid screen was performed using the cytoplasmic portion of the IGF-1R beta chain fused to the LexA DNA binding protein as bait. Preliminary experiments demonstrated that this IGF-1R beta chain was autophosphorylated when expressed in yeast. It could also interact with a series of proteins derived from B cell and HELA cell cDNA libraries including the p85 subunit or PI-3 kinase, Csk, Grb-10, and Shc, most of which were previously identified as IGF-1R interacting proteins by other investigators (31-33).
From a screen of a brain-derived cDNA library the only known protein that interacted with the IGF-1R was a cDNA encoding RACK1/GNB2L1. RACK1 interaction was observed with a kinase active IGF-1R beta chain in yeast, but unlike Shc, it was also observed with a kinase inactive (K1003R) mutant of the IGF-1R beta chain (data not shown) RACK1 was originally identified in brain (26) and is a known adapter protein for the IFNα receptor (34). It has the potential to interact with several proteins through its 7 WD repeat motifs (35).

To determine whether RACK1 interacts with the IGF-1R in mammalian cells the cDNA encoding RACK1 was sub-cloned in frame with the epitope tag HA at the N terminus into the mammalian expression vector pcDNA3. Cos cells were then transiently co-transfected with pcDNA3 plasmids encoding either HA-RACK1 or full length IGF-1R. The IGF-1R was immunoprecipitated from cells using a polyclonal antibody that detects an epitope in its C terminus and then analyzed for associated HA-RACK1 by immunoblotting with anti-HA antibody. To determine if activated PKCs influenced the interaction of RACK1 with the IGF-1R (as it does with PKC and Src (36)) the transfected cells were treated with the phorbol ester PMA to activate PKC or were left untreated. The results shown in Fig. 1A demonstrated that HA-RACK1 was present in the IGF-1R immunoprecipitates at equivalent amounts in the presence or absence of PMA. This indicates that HA-RACK1 associates with the IGF-1R in Cos cells, and this interaction is not altered by PMA.

We next investigated whether endogenous RACK1 interacts with the IGF-1R and how this is affected by ligand stimulation of the IGF-1R. To do this we used R+ cells
(R- fibroblasts derived from the IGF-1R knock out mouse that were re-transfected with the IGF-1R (30). Cells were starved from serum and then stimulated with IGF-1 for the indicated times, at which times the IGF-1R was immunoprecipitated and assessed for both phosphotyrosine content and associated endogenous cellular RACK1 by western blotting. As can be seen in Fig. 1B RACK1 was associated with the unphosphorylated form of the IGF-1R. Upon IGF-1 stimulation the IGF-1R beta chain underwent autophosphorylation, as detected by phosphotyrosine staining, and similar amounts of RACK1 protein were associated with the phosphorylated receptor. This indicates that RACK1 is associated with the IGF-1R in either the inactive or active state, and this interaction does not require autophosphorylation of the IGF-1R.

**RACK1 interacts the insulin receptor, with kinase inactive IGF-1R, but not with a serine mutant of the IGF-1R.**

Several proteins that interact with the IGF-1R also interact with the IR, but there is at least one IGF-1R interacting protein that was identified in a yeast two-hybrid system that does not interact with the IR. (37). We were therefore interested to determine if RACK1 could interact with the IR as well as with the IGF-1R. To test this, the endogenous IR or IGF-1R proteins were immunoprecipitated from Cos cells, and the immunoprecipitates were investigated for associated endogenous RACK1 by western blotting. As can be seen in Fig. 2A RACK1 interacted with both the IR and IGF-1R in Cos cells. This suggests that RACK1 interacts with amino acid residues or receptor domains that are common to both the IR and IGF-1R.
To further investigate the residues in the IGF-1R necessary for interaction with RACK1 we investigated a series of IGF-1R beta chain mutants transiently expressed in R- fibroblasts. From each transfected cell population endogenous RACK1 association with the IGF-1R beta chain was analyzed by western blotting with anti-RACK1 antibody. As can be seen in Fig. 2B RACK1 interacted with a wild type IGF-1R beta chain, but also with a kinase inactive (K1003R) mutant of the beta chain. This confirms the result obtained in the yeast two-hybrid system and the observation that RACK1 interacts with un-stimulated IGF-1R (Fig. 1B). RACK1 also interacted with IGF-1R beta chains containing mutated tyrosines, the Y1316F mutant or the double tyrosine mutant Y1162/1222F. However, RACK1 did not interact with an IGF-1R beta chain mutated at a C terminal serine, S1248A, whereas it retained interaction with another beta chain mutated at serine, S1252A. Levels of expression of the mutants and wild type IGF-1R beta chains receptors were similar as shown by western blotting with anti-IGF-1R antibody.

To confirm that the pattern of interaction with the mutant beta chain receptors was physiologically relevant the experiments were also performed with full length IGF-1R (WT and mutants) transiently transfected into R- cells. Results are shown in Figure 2C and demonstrate that, as was observed with the beta chain proteins, interaction of endogenous RACK1 was not observed with the S1248A mutant whereas RACK1 interacted with the other mutants tested.

Altogether, these data demonstrate that RACK1 interacts with both the IR and the IGF-1R. The interaction with the IGF-1R is not dependent on an active tyrosine kinase.
nor does it require a number of tyrosines in the IGF-1R. However, RACK1 interaction requires serine 1248 in the C terminus of the IGF-1R. Interestingly this serine is conserved in the IR at amino acid position 1262, which suggests that it could mediate RACK1 interaction with both receptors.

Over-expression of RACK1 enhances Erk and JNK activation by IGF-1, but decreases Akt activation.

Since RACK1 can interact with the IGF-1R in the absence of kinase activation, this suggests that RACK1 acts to negatively regulate receptor activity rather than to mediate signaling responses in response to ligand binding. Over-expression of RACK1 has previously been shown to be inhibitory to the growth of 3T3 fibroblast cells, and this is associated with its ability to sequester Src (36). Like these investigators we were unable to obtain clones of 3T3 fibroblasts that stably over-expressed HA-RACK1. However, we were able to generate clones of MCF-7 cells that stably over-expressed HA-RACK1.

To determine if RACK1 influences the signaling responses from the IGF-1R we analyzed this in R+ cells transiently over-expressing HA-RACK1 and MCF-7 cells stably over-expressing HA-RACK1. IGF-1-induced phosphorylation of Erks was assessed as a measure of MAP kinase activation; phosphorylation ofAkt was assessed as a measure of PI3-kinase activation; and c-Jun phosphorylation was assessed as a measure of JNK activation.

As can be seen in Fig. 3A, IGF-1–induced phosphorylation of Akt, which was
induced by 5 minutes in vector-transfected R+ cells and peaked by 10 minutes, was greatly reduced in HA-RACK1-transfected R+ cells. By contrast, phosphorylation of Erks was enhanced at 5 minutes and was more sustained in the HA-RACK1-expressing cells than in the vector controls. Under these conditions phosphorylation of c-Jun was not detectable within 30 minutes of IGF-1 stimulation in the control cells, but was clearly detectable by 30 minutes in the HA-RACK1-expressing R+ cells (Fig. 3A). Levels of HA-RACK1 over-expression were confirmed by staining with the anti-HA antibody. These results indicate that the PI3-kinase pathway is inhibited by over-expression of RACK1 in R+ cells, whereas the MAP kinase and JNK pathways are enhanced.

To assess the effects of RACK1 on IGF-1R activity and substrate phosphorylation Shc and IRS-2 were immunoprecipitated from R+ and assessed for phosphorylation in response to IGF-1 stimulation by western blotting with anti-phosphotyrosine antibody. This demonstrated that both IRS-2 and Shc phosphorylation was increased in the HA-RACK1 over-expressing cells compared with vector-expressing controls (Fig. 3B). This suggests that IGF-1R activity is enhanced by over-expression of HA-RACK1.

We next investigated the effects of RACK1 over-expression on signalling responses in MCF-7 cells. Three clones of MCF-7 cells over-expressing HA-RACK1 were isolated (clones A1, B1, and C1). Analysis of endogenous RACK1 and HA-RACK1 expression levels are shown in Figure 3C and indicate that approximately 2 fold higher levels of HA-RACK1 are expressed in these clones. IGF-1-mediated activation
of the PI3 kinase and MAP kinase pathways was investigated in all three clones with similar results, and this is shown in Figure 3D for clone A. As was seen with R+ cells phosphorylation of Akt was decreased in response to IGF-1 stimulation whereas phosphorylation of Erks was enhanced.

Overall the data indicate that RACK1 over-expression increases phosphorylation of IRS-1 and Shc by IGF-1. At the same time it attenuates activation of Akt, but enhances activation of MAPK and JNKs. This suggests that RACK1 has a selective role in modulation of IGF-1R signaling and that it has a negative regulatory effect on the Akt pathway.

**Over-expression of RACK1 enhances the proliferation rate of MCF7 cells but decreases IGF-1-mediated protection from apoptosis.**

The data above indicate that over-expression of RACK1 abrogates IGF-1-mediated activation of the PI3-kinase pathway, but enhances the MAP kinase pathway in both fibroblasts and MCF-7 cells. These pathways are activated in survival and proliferative responses from the IGF-1R. However, others (38) and we observed that RACK1 is inhibitory to the growth of fibroblasts, but apparently does not inhibit MCF-7 cells. Therefore, we asked whether over-expression of RACK1 had an effect on the proliferation rates or IGF-1-mediated protection from apoptosis in MCF-7 cells.

To compare the proliferation rates of MCF-7 cells over-expressing HA-RACK1 triplicate cultures of each of the three clones A1, B1, and C1 were assessed for accumulated cell numbers in medium supplemented with FBS compared with vector-
expressing cells, Neo. This demonstrated that in each MCF-7 cell clone over-expressing HA-RACK1 the rate of cellular proliferation was increased and the doubling time was approximately twice as high as in Neo cells (Fig. 4A). This suggests that over-expression of RACK1 provides a proliferative advantage to these tumor cells, which is the opposite effect to that observed in fibroblasts. The enhance growth correlates with enhanced IGF-1-mediated activation of MAP kinases (Fig. 2), and also suggests that decreased Akt activation does not affect the growth of MCF-7 cells.

We next investigated IGF-1-mediated protection from apoptosis in MCF-7 cells over-expressing RACK1. Cells were cultured in serum free medium, and then treated with etoposide in the presence or absence of IGF-1. As can be seen in Figure 4B, in serum free medium IGF-1 stimulated an increase in cell number, which was approximately 30% greater in the HA-RACK1 A1 clone than in the Neo cells. This is in agreement with the observations in Fig. 4A that the proliferation rate of the HA-RACK1 over-expressing cells is increased in serum-supplemented medium. When cells were treated with etoposide a similar decrease in cell number occurred in both Neo and A1 cells that indicates a similar level of cell killing. However, IGF-1 rescued the Neo cells and also increased the cell number in these cultures by over 100%. By contrast in the A1 cells IGF-1 only afforded a very slight increase in cell number. This indicates that MCF-7 cells over-expressing HA-RACK1 have diminished IGF-1-mediated protection from etoposide killing. Since this decrease can be correlated with the decrease in IGF-1-induced phosphorylation of Akt observed in these cells, it suggests that the blunted Akt activation is responsible for the lack of protection from etoposide killing.
Altogether the data demonstrate that over-expression of RACK1 enhances the proliferative rate of MCF-7 cells in the presence of serum or IGF-1, but inhibits IGF-1-mediated protection from induction of cell death.

**IGF-1R kinase activity is enhanced in MCF-7 cells that over-express RACK1**

The status of IGF-1-mediated activation of the PI3-kinase and MAP kinase pathways is differentially affected in HA-RACK1 over-expressing cells, and this may be responsible for the inhibition of fibroblast cell growth as well as the enhanced proliferation combined with decreased protection from apoptosis in MCF-7 cells. However, we were also interested to determine whether the kinase activity of the IGF-1R is altered in MCF-7 cells over-expressing RACK1. To do this *in vitro* kinase assays were performed with IGF-1R immunoprecipitated from MCF-7 cells by measuring incorporation of $^{32}$P-ATP into the peptide substrate poly (Glu: Tyr). Results shown in Fig. 5 demonstrate that the basal tyrosine kinase activity of the IGF-1R towards poly: (glu-tyr) was slightly higher in the MCF-7/ HA-RACK1 cells compared to control cells in the unstimulated state. IGF-1 stimulation caused an approximately 25% greater increase in kinase activity towards poly (glu-tyr) in these cells compared with Vector controls. This indicates that over-expression of RACK1 in MCF-7 cells enhances IGF-1R kinase activity. This could contribute to the enhanced IRS-2 and Shc phosphorylation observed in R+ cells, to the enhanced MAP kinase and JNK activation, and to the enhanced proliferative rates observed with these cells (Fig. 4). However, it is remarkable that IGF-1-induced Akt phosphorylation is diminished so much even in the presence of
Association of Src, p85 and SHP-2 with RACK1.

One way in which RACK1 could enhance IGF-1R kinase activity and differentially modulate IGF-1R activated signalling pathways is through recruitment of Src, which has previously been shown to phosphorylate the IGF-1R on key sites that stimulate its activation (36,38), or through sequestration of proteins necessary to activate the different signaling pathways. Sequestration of Src by RACK1 has previously been suggested to account for the inhibition of fibroblast growth (38). Therefore we investigated Src recruitment to endogenous RACK1 in response to IGF-1 stimulation in R+ cells and in MCF-7 cells. Similar results were obtained for both cell lines and are shown in Figure 6A. Src was found to be associated with RACK1 in unstimulated cells, but in response to IGF-1 stimulation the Src protein was slowly released. By 15 minutes there was a significant decline in the amount of Src associated with RACK1 and by 30 minutes there was no Src associated with RACK1. This indicates that the RACK1-Src complex is responsive to and is altered by IGF-1 stimulation. However, the kinetics of Src dissociation is much slower than those for Akt activation or Erk activation in response to IGF-1. This suggests that Src activity does not account for the increase in MAPK activation, but it may contribute to JNK activation after 30 minutes IGF-1 stimulation. However, since the kinetics of Src release is similar in both R+ and MCF-7 cells we conclude that Src is not responsible for the differential effects of RACK1 on cell growth in fibroblasts and MCF-7 cells.
We next investigated whether the decreased activation of Akt observed with overexpression of RACK1 was correlated with sequestration of proteins that promote activation of this pathway, the p85 subunit of PI-3K and SHP-2, which has previously been shown to associate with p85 and to be essential for IGF-1-mediated activation of Akt (39). To do this HA-RACK1 was immunoprecipitated from transiently transfected R+ cells and then analyzed for associated p85 and SHP-2 by western blotting. The results shown in Fig. 6B demonstrate that p85 and SHP-2 are both associated with RACK1 in the absence of IGF-1 stimulation and remain associated in response to 5 minutes IGF-1 stimulation. At this time Akt activation is diminished (Fig. 3). RACK1 does not interact with Shc in these cells (not shown). Overall the data indicate that the decreased Akt activation observed in the presence of RACK1 over expression can be correlated with the sequestration of Src, p85, and SHP-2 by RACK1.

**Discussion:**

We have identified RACK1 as an IGF-1R and IR interacting protein whose over-expression has a negative effect on activation of the PI-3 kinase pathway, but has a positive effect on activation of the MAP kinase and JNK pathways. This was correlated with increased proliferation rates but decreased IGF-1-mediated protection from etoposide killing in MCF-7 cells. RACK1 interaction with the IGF-1R occurred independently of tyrosine kinase activity but it required serine 1248, which is also present in a conserved amino acid stretch in the IR. This suggests that RACK1 is a regulator of
IGF-1R and IR function.

RACKs are a family of proteins that share homology with the B subunits of heterotrimeric G proteins and are also members of an ancient group of regulatory proteins, which are made up of a series of Trp-Asp (WD repeats). RACKs generally are comprised of 5-7 WD repeats (seven in the case of RACK1), which confers on them the potential to act as a scaffold or adapter proteins (40-42). Recent evidence suggests that RACKs act as a beta propeller structure where each WD repeat forms a different blade on the beta propeller (43). Interestingly the WD repeats in RACK1 are conserved from chlamydomonas to humans, and can be used by viruses to interact with cellular proteins (44,45).

RACKS were originally identified as molecules that bind only to activated forms of PKC, facilitating their translocation and anchoring to membranes or cytoskeletal structures in proximity to its substrates (26,46,47). However, RACK1 has also been found to interact with Src family members (38), phospholipase Cγ, PTPμ (47), cAMP specific phosphodiesterase-4 (43), the beta subunit of integrins, and certain pleckstrin homology domains in vitro, including dynamin and beta spectrin (27). Only a subset of these interactions depend on PKC stimulation suggesting that RACK1 can facilitate signaling complexes in response to distinct cellular stimuli. RACK1 also associates with the type 1 interferon receptor and the common beta chain of the IL-5/IL-3/ GM-CSF receptor (49) and it is thought to promote signalling from these receptors by its ability to also associate with STATs (34).

The observation that RACK1 interacts with kinase inactive and unphosphorylated
IGF-1R combined with the finding that serine 1248 is necessary for the interaction suggests that RACK1 associates with the inactive receptor and remains there when the receptor kinase becomes activated. We cannot rule out the possibility that other serines or domains in the receptor are also involved in RACK1 interaction, but mutation of the single amino acid (serine 1248) is sufficient to disrupt RACK1 association. This suggests that serine 1248 in the IGF-1R or its cognate serine 1262 in the IR could act as regulatory sites on these receptors. It is not known if these serines are phosphorylated in vivo, or how they contribute to IGF-IR or IR function.

Over-expression of RACK1 led to enhanced IGF-1R kinase activity and IGF-1-induced phosphorylation of IRS-2 and Shc. This raised the interesting question: Why does over-expression of RACK1 lead to increased MAP kinase and JNK activation but decreased Akt activation? Since the interaction of RACK1 with the IGF-1R apparently does not change in response to kinase activation the effects on signalling modulation are likely to be mediated through proteins that are already associated with RACK1 in unstimulated cells. Our observation that two proteins that promote IGF-1-mediated activation of AKT, the p85 subunit of PI-3K and the phosphatase SHP-2, are constitutively associated with RACK1 suggests that sequestration of these proteins may be the cause of the observed Akt inhibition. However, it is possible that other RACK1 interacting proteins are also involved in regulating Akt activity. For example the regulatory phosphatase PTPμ interacts with RACK1 and was found to be active in regulating focal adhesions via PKCδ (48,49). It will be necessary to do a complete analysis of RACK1 associated proteins in response to IGF-1 or insulin stimulation of
cells to get a comprehensive picture of how RACK1 regulates signaling from these receptors.

Sequestration of Src has previously been proposed as the growth inhibitory mechanism for RACK1 in NIH-3T3 cells (38). We also observed RACK1 interaction with c-Src in R+ cells and MCF-7 cells, but the complex dissociated completely after 15 minutes IGF-1 stimulation. Thus, Src is associated with RACK1 during the 5-10 minutes post IGF-1 stimulation that Akt and Erks become phosphorylated. Dissociation of Src could contribute to enhanced IGF-1-mediated JNK activation observed at 30 minutes because we have previously found that JNK is activated by IGF-1 in a PI3-kinase and MAP kinase independent manner (50). In all of our experiments the differential effects of RACK1 on activation of the Akt and the MAP kinase pathway were correlated with different consequences for the growth and survival of fibroblasts and MCF-7 cells. It has previously been shown that βγ subunits of G proteins can activate MAP kinases in a PKC-independent manner in response to IGF-1R or IR stimulation, but not in response to PDGF, FGF, or EGF (52). In addition sequestration of Gβγ subunits could block IGF-1R mitogenic activity, but had no effect on insulin or IGF-1R metabolic activity (53). Our observation that RACK1 over-expression enhances IGF-1-mediated activation of MAP kinase suggests that RACK1 may act like or assist βγ subunits to directly enhance IGF-1-mediated activation of MAP kinase and JNKs, and also to promote cellular growth in MCF-7 cells. It is also noteworthy that although enhanced MAP kinase signalling can confer a growth advantage on MCF-7 cells inhibition of Akt activation by over-expression of RACK1 is sufficient to block IGF-1-
mediated protection from the cytotoxic insult of etoposide. This suggests that reduced Akt activation by itself is not sufficient to halt the growth of these tumor cells because the MAP kinase pathway is hyperactive, but in combination with an apoptotic signal from etoposide reduced Akt activation can inhibit cell survival and growth.

RACK1 interacts with both the IGF-1R and IR, so it is likely that it can regulate signalling from both receptors. Although the IR and IGF-1R have many overlapping functions they can have significant differences in signaling output (53-56), some of which has been attributed to tissue distribution, differences in the structure or amino acid sequence of the receptors, or to the usage of different kinds of G proteins (57, 53, 22, 29).

It has also been demonstrated that activation of the class 2 PI3-kinases via IRS-1 may be a unique signal from the IR to regulate glucose metabolism (58). Thus, activation of PI3-kinase and Akt may be more important for metabolism and cell survival than for cellular proliferation or growth of tumor cells. If RACK1 selectively regulates the Akt pathway, then it may have a particular role in regulating glucose metabolism and cell survival. Since its interaction with the IGF-1R is dependent on serine 1248, which is also conserved in the IR at position 1262, the affects of RACK1 on the PI3-kinase pathway may be dependent on the activity of cellular serine kinases that phosphorylate this serine in the IR or IGF-1R.

Serine phosphorylation of the IR, IGF-1R and IRS-1 has been proposed to be a negative regulatory mechanism (21, 59) and cause insulin resistance. TNF can stimulate phosphorylation of serine 307 in IRS-1 (59) and insulin resistance. Although a series of kinases including PKCζ, IKKB, PKCα, and JNK have been implicated (1, 21) it is not
known which kinase directly phosphorylates this serine. Since RACK1 is a receptor for the C kinases it is also possible that its associated kinases are involved in TNFα-mediated insulin resistance. It will be of interest to investigate whether a serine kinase is necessary to maintain RACK1 interaction with the IGF-1R or IR and whether RACK1 has a role in insulin resistance.

In summary we have identified RACK1 as an IGF-1R and IR interacting protein that has the potential to enhance activation of the MAP kinase or JNK pathways, but that inhibits Akt activation. Thus RACK1 may be an important regulator of cell survival and metabolism.

Acknowledgements:

We are grateful to Gary Loughran and Anthony Lyons for assistance with cloning IGF-1R mutants, and to Rajeeva Singh and Tom Chittenden for helpful discussions. This work was supported by research grants from Enterprise Ireland and the Irish Cancer Society.

Note: When this paper was in review another group identified RACK1 as an IGF-1R-interacting protein (ref 60):

References:
29. Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T.,


Figure Legends

Figure 1

RACK1 interacts with IGF-1R beta chain in Cos cells and with full length IGF-1R in R+ cells. (A) Cos cells were transiently transfected with plasmids encoding either HA-RACK1 or the IGF-1R beta chain. After 36 hours cell lysates were prepared, precleared, and subjected to immunoprecipitation with an anti-IGF-1R polyclonal antiserum. The precipitated proteins were then resolved by SDS-PAGE and transferred to nylon membranes for western blotting with an anti-HA antibody to detect HA-RACK1. The blots were stripped and re-probed for IGF-1R content using an anti-IGF-1R monoclonal antibody. (B) R+ cells were starved for 3 hours and then stimulated with IGF-1 for the indicated times. Cell lysates were prepared and the IGF-1R was immunoprecipitated and then analysed for IGF-1R levels, phosphotyrosine content, or associated RACK1 by western blotting.

Figure 2

RACK1 association with the insulin receptor and IGF-1R mutants. (A) Cos cells were lysed and the lysates were split into two samples that were immunoprecipitated using either anti-IR or anti-IGF-1R antibodies. The precipitated proteins were then resolved on the same gel and transferred to a nylon membrane that was sequentially probed with anti-IGF-1R, anti-IR, or anti-RACK1 antibodies. (B) R- cells were transiently transfected with plasmids encoding the indicated IGF-1R beta chains, either wild type or the indicated point mutants. After 36 hours the cells were lysed and the lysates were
immunoprecipitated with anti-IGF-1R polyclonal antisera or with antibody coated protein G agarose beads as a control (Co). The precipitated proteins were then analyzed by western blotting to detect IGF-1R beta chain or associated cellular RACK1. (C) R-cells were transiently transfected with plasmids encoding full length IGF-1Rs (either wild type or the indicated mutants), and with a plasmid encoding full length IR. After 24 hours the cells were lysed and analysed for association of RACK1 with IGF-1R or IR as described for A and B.

Figure 3

Analysis of IGF-1-mediated activation of PI3-kinase, MAP-kinase, and Jun kinase pathways. (A) R+ cells were transiently transfected with either empty pcDNA3 vector or pcDNA3 encoding HA-RACK1. After 36 hours cells were starved for 3 hours, then stimulated with IGF-1 for the indicated times and lysed. The lysates were then resolved by SDS PAGE and analysed by western blotting with anti-phospho-Akt, anti-phospho-Erk, and anti-Phospho-c Jun antibodies. The blots were then stripped and reprobed with anti-Akt, anti-HA and anti-Actin antibodies to demonstrate equal loading. (B) R+ cells transiently expressing either vector or HA-RACK1 were starved and stimulated with IGF-1 for the indicated times. Cell lysates were prepared and immunoprecipitated with anti-IRS-2 and anti-Shc antibodies. The immunoprecipitates were then subjected to western blotting with anti phosphotyrosine antibody and the blots were re-probed with either anti-IRS-2 anti-Shc antibody. (C) MCF-7 cells were transfected with either pcDNA3 empty vector or HA-RACK1 and clones stably over-expressing HA-RACK1.
were isolated as described in Methods. Expression of HA-RACK1 (lower arrow) and endogenous RACK1 (upper arrow) was measured in these clones by western blotting with anti-RACK1 antibody. (D) Cells were starved from serum for 4 hours and then stimulated with IGF-1 for the indicated times. To measure phosphorylation of Akt and Erks cell lysates were prepared and subjected to western blotting with the indicated antibodies.

Figure 4

*Proliferation is enhanced but protection from etoposide killing is decreased in MCF-7 cells over-expressing HA-RACK1.* Three clones of MCF-7 cells over-expressing HA-RACK1 (A1, B1, C1) and one over-expressing Vector (Neo) were seeded at a density of 3x10^4 per ml in multiple wells of 24 well plates. At the indicated time points cells from triplicate wells were removed using trypsin/EDTA and centrifuged. Cell numbers and viability were determined by trypan blue exclusion and data are presented as mean and standard deviation of live cell numbers in triplicate wells. (B) To measure IGF-1-mediated protection from etoposide killing MCF-7/Neo or MCF-7/HA-RACK1 cells (clone A1) were seeded in multiple wells of a 24 well plate at 3x10^4 per ml. After 18 hours culture cells were washed and resuspended in serum free medium either with no additions (control), or with IGF-1 added, or with etoposide (8.5µM) added, or with IGF-1 + etoposide added. After a further 36 hours in culture the cells were harvested and assessed for viability and number using trypan blue exclusion. Data are presented as mean and standard deviation of live cell number from triplicate wells.
Figure 5

*In vitro* kinase activity of IGF-1R is enhanced in RACK1-over-expressing cells. MCF-7 cells either expressing empty vector (Neo) or HA-RACK1 (clone A1) were starved from serum for three hours and then stimulated with IGF-1 for the indicated times. Cells were lysed and the lysates immunoprecipitated using an anti-IGF-1R polyclonal antisera and protein G agarose beads. The beads were then washed in kinase buffer (see materials and methods) and resuspended in a kinase reaction mix that contained kinase buffer, $^{32}$P ATP, and an exogenous peptide substrate poly-(Glu: Tyr). After a 20 minute incubation the reaction mix was removed and precipitated on fibreglass filters using TCA, and after extensive washing the filters were dried and counted by liquid scintillation counting. Panel insert shows the levels of IGF-1R in each sample determined by western blotting with an aliquot of the immunoprecipitated proteins.

Figure 6

Dissociation of RACK1 and Src in response to IGF-1 stimulation, and association of p85 and SHP-2 with RACK-1. (A) R+ cells (top panel) or MCF-7 cells (bottom panel) were starved for 4 hours and stimulated with IGF-1 for the indicated times. Cells were lysed and immunoprecipitated with anti-Src polyclonal antisera. The precipitated proteins were then analyzed by western blotting for RACK1 or Src using anti-RACK1 and anti-Src monoclonal antibodies. In the middle panel the expression of the IGF-1R and
phosphorylation status of the IGF-1R beta chain are shown by western blot analysis of the lysates of R+ cells. (B) R+ cells were transiently transfected with HA-RACK-1, cultured for 48 hours starved for four hours and stimulated with IGF-1 for the indicated times. Cell lysates were prepared and immunoprecipitated with anti-HA or control antibody (Co). The immunoprecipitates were then subjected to western blotting with either anti-p85 or anti-SHP-2 antibody and blots were re-probed with anti-HA antibody. A sample of cell lysate is included to show total cellular levels of these proteins.
**Figure 1**

**A**

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<tr>
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<td>37 KD</td>
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**B**

IP: IGF-1R

| 100 KD | IGF-1R |
| 100 KD | Phosphotyrosine |
| 37 KD | RACK-1 |

IGF-1 (min.)
**Figure 3**

**A**

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**B**

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Figure 3

C

D

Neo  A1  B1  C1

37 KD

HA-RACK-1  RACK-1

Neo  Clone A1

P-Akt

P-Erk

Akt

HA

Actin

IGF-1 (min.)

0  5  10  15  30  0  5  10  15  30
Figure 4

A

Number of Cells ($\times 10^6$) vs. Hours

Clone B1
Clone C1
Clone A1
Neo

B

Number of Cells ($\times 10^6$)

Control
IGF-1
Etoposide
Etoposide + IGF-1

Original number of cells

Neo
HA-RACK-1
Figure 5
Figure 6

A

R+ cells

IP: Src
RACK-1

Src

Cell lysate
IGF-1R

Phosphotyrosine

IGF-1 (min.)

MCF-7 cells

IP: Src
RACK-1

Src

IGF-1 (min.)

B

Co

HA

Cell lysate

p85

SHP-2

HA

IGF-1 (min.)
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Patrick A. Kiely, Anagha Sant and Rosemary O'Connor

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