RACK1 Is an Insulin-like Growth Factor 1 (IGF-1) Receptor-interacting Protein That Can Regulate IGF-1-mediated Akt Activation and Protection from Cell Death*

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The insulin receptor and insulin-like growth factor 1 receptor (IGF-1R), activated by their ligands, control metabolism, cell survival, and proliferation. Although the signaling 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 β-chain as bait. This screen identified Receptor for Activated C Kinases (RACK1) 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. Overexpression of RACK1 in either R+ fibroblasts or MCF-7 cells inhibited IGF-1-induced phosphorylation of Akt, whereas it enhanced phosphorylation of Erks and Jnks. Src, the p85 subunit of phosphatidylinositol 3-kinase, and SHP-2 were all associated with RACK1 in these cells. Interestingly, the proliferation of MCF-7 cells was enhanced by overexpression of RACK1, whereas 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 signaling and suggest that RACK1 has a particular role in regulating Akt activation and cell survival.

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. They are tetrameric receptors made up of two α-subunits that bind the ligands insulin, IGF-1 or IGF-2, and two β-subunits that share high homology in their kinase domains (reviewed in Ref. 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 signaling pathway that includes insulin-related substrate 1 (IRS-1), phosphatidylinositol 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 inter-linked, 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 and 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-Akt or other signaling pathway by IGF-1 and insulin, there is a limited understanding of how the activity of the IR and IGF-1R is 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 signaling (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 (22–25). These indicated that domains of the IGF-1R C terminus with distinct amino acid sequences from similarly located domains in the IR are required for 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 signaling molecules or regulating receptor function have not yet been elucidated. To address this we undertook a screen for

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¶ The abbreviations used are: IGF-1, insulin-like growth factor 1; IGF-1R, insulin-like growth factor 1 receptor; IR, insulin receptor; RACK1, identified receptor for activated C kinases; PI3-K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; IRS-1, insulin-related substrate 1; HA, hemagglutinin; Erk, extracellular signal-regulated kinase; PKC, protein kinase C.
proteins that could interact with the IGF-1R by using the yeast two-hybrid system.

In this report we identify RACK1, a homologue of the β-subunit of heterotetrameric G proteins (26, 27), as an interacting protein for the IGF-1R 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. Overexpression 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 to negatively regulate activation of the PI3-K pathway. Thus, RACK1 may have a broad role in regulating glucose metabolism and cell survival.

EXPERIMENTAL PROCEDURES

Materials—Recombinant IGF-1 was purchased from PeproTech (Rocklin, NJ). The anti-IGF-1R and anti-IR, anti-HIF-2, and anti-ERs monoclonal antibodies and the anti-IRS-2 monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-IGF-1R monoclonal antibody ND122 was from ImmunoGen (24). The anti-phospho-Akt, anti-Akt polyclonal antibodies, and the anti-phospho-p42/p44 MAP kinase monoclonal antibody were from Cell Signaling Technology (Beverly, MA). The anti-phosphotyrosine monoclonal antibody, 4G10, the anti-Erk-2, anti-p85, and anti-IRS-2 monoclonal antibodies, and 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 Roche Molecular Biochemicals, and the anti-actin monoclonal antibody was purchased from Sigma.

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 IGF-1R (amino acids 930–1337 (26)) 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 yeast 880p-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 screening for growth of clones that had expressed activated LacZ (medium: Ura−, and 5-bromo-4-chloro-3-indolylmethylsulfonyl fluoride (1 mM), pepstatin (1 μg/ml), and aprotinin (1.5 μg/ml). After incubation at 4 °C for 20 min, nuclear and cellular debris were removed by micro-centrifugation at 14,000 rpm for 15 min at 4 °C.

For immunoprecipitation of endogenous or transfected proteins, protein extracts from stimulated or unstimulated cells were initially precleared by incubating the beads with 15 μl of beads per 400 μg of DNA) using agarse gel electrophoresis. Banda of the expected length were cut out and ligated into the pKs vector, which had been digested with XhoI and XhoI and analyzed by agarose gel electrophoresis. The expected length RACK1 in-frame with the HA coding sequence that was previously (22). The β-chain of the IGF-1R (wild type, K1003R, and Y1316F) was subcloned into the expression vector pIREs (CLONTECH) using BamHI restriction sites on either end as described previously (20). The double tyrosine 1162/1221 to phenylalanine mutant (Y1162F/Y1221F), the serine 1248 to alanine mutant (S1248A), and the serine 1252 to alanine (S1252A) mutant were generated by site-directed mutagenesis using the QuikChangeTM 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 sequence of the mutants, the fragments were subcloned into the pIREs expression vector already containing a wild type IGF-1R β-chain sequence by using the BamHI and HindIII restriction sites. Full-length IGF-1R harboring each of these mutations in the pcdNAS vector was obtained by digestion of pcdNAS 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 procMv 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 knockout mouse), R+ cells (R− cells that have been re-transfected with the IGF-1R (29)), and COS cells were all maintained in Dulbecco’s modified Eagle’s medium (Bio-Whittaker, Verviers, Belgium), supplemented with 10% (v/v) calf serum, 10 mM l-Glu, and 5 mg/ml penicillin/streptomycin. COS cells or R+ cells were transfected with pcDNA3/HA-RACK1 or empty pcDNA3 vectors (4 μg of DNA) using LipofectAMINE Plus, (Invitrogen). After 24 h in culture the transfected R+ cells were split into 6-well plates or 10-cm plates and cultured for an additional 18 h, at which time cells were starved for 3 h 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 h after transfection cells were split into medium containing G418 (1 mg/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 overexpressing HA-RACK1 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 1 mg/ml G418. For analysis of signaling responses cells were washed and starved from serum for 3 h.

Preparation of Cellular Protein Extracts and Immunoprecipitation—Cellular protein extracts were prepared by washing cells with phosphate-buffered saline and then scraping into lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 plus the tyrosine phosphatase inhibitor Na3VO4 (1 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 μM), and aprotinin (1.5 μg/ml).

For immunoprecipitation of endogenous or transfected proteins, protein extracts from stimulated or unstimulated cells were initially precleared by incubating the beads with 15 μl of beads per 400 μg of total protein in 700 μl of lysis buffer) by incubation at 4 °C for 1 h with gentle rocking. The lysates were recovered from the beads by centrifugation at 3,000 rpm for 3 min 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 h at room temperature and were pelleted by centrifugation at 3,000 rpm for 3 min at 4 °C. The beads were washed (3 times) with ice-cold lysis buffer and then either used for in vitro kinase assay or removed from the beads by boiling for 5 min in 20 μl of 2× 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 h 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% β-mercaptoethanol for 30 min at 50 °C followed by extensive washing in 0.2% TBS-T. Secondary antibody conjugated with horseradish peroxidase was used for detection with enhanced chemiluminescence (SuperSignal from Pierce) or ECL+ (Amer sham Biosciences) following the manufacturers’ instructions.

Assays for Proliferation and IGF-1-mediated Protection from Cell Death—MCF-7 Neo and MCF-7 cells overexpressing HA-RACK1 (clone A1, B1, and C1) were cultured in Dulbecco’s modified Eagle’s medium
supplemented with 10% fetal calf serum (complete medium) at 3 × 10^4 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 min. The cell pellets were then resuspended in 100 μl of medium and counted using a hemocytometer and trypan blue exclusion. Data are presented as the mean and S.D. 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 h the cells were washed once in phosphate-buffered saline and re-incubated in serum-free medium supplemented with either no additives, with added IGF-1 (100 ng/ml), with etoposide (0.5 μM), or with IGF-1 + etoposide. After a further 36 h 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 4 h and then stimulated with IGF-1 for 0, 15, or 30 min. 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 (50 mM Hepes, pH 7.4, 10 mM MgCl_2, 10 mM MnCl_2) and then resuspended in 25 μl of a kinase reaction mixture containing ATP (0.03 mM final concentration), 2 μl of [γ-32P]ATP (5 μCi/μl), and 2 μl of poly(Glu-Tyr) (Sigma). Following a 20-min incubation period, samples of reaction mixture (5 μl) were removed to fresh tubes containing 9 μl of H_2O and 35 μl of 20 mM EDTA, pH 7.4. Triplicate samples were then transferred to glass microfiber filters in 24-well plates and washed extensively with ice-cold trichloroacetic acid (10%) containing 10 mM Na_2HPO_4. Following a final wash with 70% ethanol, the filters were dried, and 32P was measured in a scintillation counter (Beckman Instruments). The data are presented as the mean and S.D. of counts/min 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 β-chain fused to the LexA DNA-binding protein as bait. Preliminary experiments demonstrated that this IGF-1R β-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 PI3-K, Csk, Grb-10, and Shc, most of which were identified previously (30–32) as IGF-1R-interacting proteins by other investigators.

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 β-chain in yeast, but unlike Shc, it was also observed with a kinase-inactive (K1003R) mutant of the IGF-1R β-chain (data not shown) RACK1 was originally identified in brain (26) and is a known adapter protein for the IGF-1R (29). Cells were starved from serum and then stimulated with IGF-1 for the indicated times, at which times the IGF-1R was immunoprecipitated and assayed 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 β-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 (36). We were therefore interested to determine whether 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 endoge-
Coated protein G-agarose beads as a control (Co). 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 β-chains, either wild type (wt) or the indicated point mutants. After 36 h the cells were lysed, and the lysates were immunoprecipitated with anti-IGF-1R polyclonal antiserum or with antibody-coated protein G-agarose beads as a control (Co). The precipitated proteins were then resolved by Western blotting to detect IGF-1R β-chains, either wild type or the indicated mutants) and with a plasmid encoding full-length RACK1, an IGF-1R-interacting protein, Regulates Akt Activation

To investigate further the residues in the IGF-1R necessary for interaction with RACK1, we investigated a series of IGF-1R β-chain mutants transiently expressed in R- fibroblasts. From each transiently transfected cell population, endogenous RACK1 association with the IGF-1R β-chain was analyzed by Western blotting with anti-RACK1 antibody. 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 confirm that the pattern of interaction with the mutant β-chain receptors was physiologically relevant, the experiments were also performed with full-length IGF-1R (wild type and mutants) transiently transfected into R- cells. Results are shown in Fig. 2C and demonstrate that, as was observed with the β-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.

**Overexpression of RACK1 Enhances Erk and JNK Activation by IGF-1 but Decreases Akt Activation**—Because 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. Overexpression of RACK1 has been shown previously to be inhibitory to the growth of 3T3 fibroblast cells, and this is associated with its ability to sequester Src (35). Like these investigators we were unable to obtain clones of 3T3 fibroblasts that stably overexpressed HA-RACK1. However, we were able to generate clones of MCF-7 cells that stably overexpressed HA-RACK1.

To determine whether RACK1 influences the signaling responses from the IGF-1R, we analyzed this in R- cells transiently overexpressing HA-RACK1 and MCF-7 cells stably overexpressing HA-RACK1. IGF-1-induced phosphorylation of Erks was assessed as a measure of MAP kinase activation; phosphorylation of Akt was assessed as a measure of PI3-K 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 min in vector-transfected R- cells and peaked by 10 min, was greatly reduced in HA-RACK1-transfected R- cells. By contrast, phosphorylation of Erks was enhanced at 5 min 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 min of IGF-1 stimulation in the control cell but was clearly detectable by 30 min in the HA-RACK1-expressing R- cells (Fig. 3A). Levels of HA-RACK1 overexpression were confirmed by staining with the anti-HA antibody. These results indicate that the PI3-K pathway is inhibited by overexpression 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 were increased in the HA-RACK1-overex-
pressing cells compared with vector-expressing controls (Fig. 3B). This suggests that IGF-1R activity is enhanced by overexpression of HA-RACK1.

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

Overall the data indicate that RACK1 overexpression increases phosphorylation of IRS-1 and Shc by IGF-1. At the same time it attenuates activation of Akt but enhances activation of MAP kinase 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.

**Overexpression of RACK1 Enhances the Proliferation Rate of MCF-7 Cells but Decreases IGF-1-mediated Protection from Apoptosis**—The data above indicate that overexpression of RACK1 abrogates IGF-1-mediated activation of the PI3-K 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, we and others (37) observed that RACK1 is inhibitory to the growth of fibroblasts but apparently does not inhibit MCF-7 cells. Therefore, we asked whether overexpression 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 overexpressing HA-RACK1, triplicate cultures of each of the three clones A1, B1, and C1 were assessed for accumulated cell numbers in medium supplemented with fetal bovine serum compared with vector-expressing cells, Neo. This demonstrated that in each MCF-7 cell clone overexpressing 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 overexpression of RACK1 provides a proliferative advantage to these tumor cells, which is the opposite effect to that observed in fibroblasts. The enhanced 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 apo-
assessed for viability and number using trypan blue exclusion. Data are
added. After a further 36 h in culture, the cells were harvested and
determined by trypan blue exclusion, and data are presented as mean and
trypsin/EDTA and centrifuged. Cell numbers and viability were deter-
ined time points cells from triplicate wells were removed using

Three clones of MCF-7 cells overexpressing HA-RACK1 (A1,
B1, and C1) and one overexpressing vector (Neo) were seeded at a
density of $3 \times 10^4$ per ml in multiple wells of 24-well plates. At the
indicated times cells from triplicate wells were removed using
trypsin/EDTA and centrifuged. Cell numbers and viability were deter-
dined by trypan blue exclusion, and data are presented as mean and S.D. of live cell numbers in triplicate wells.

Altogether the data demonstrate that overexpression 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
Overexpress RACK1—The status of IGF-1-mediated activation
of the PI3-K and MAP kinase pathways is differentially af-
ected in HA-RACK1-overexpressing 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 inter-
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altered in MCF-7 cells overexpressing RACK1. To do this, in
vitro kinase assays were performed with IGF-1R immunopre-
cipitated 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 toward poly(Glu-Tyr) was slightly higher
in the MCF-7/HA-RACK1 cells compared with control cells in
the unstimulated state. IGF-1 stimulation caused an
increase in kinase activity toward poly(Glu-Tyr) in
these cells compared with vector controls. This indicates that
IGF-1 only afforded a very slight increase in cell number. This
indicates that MCF-7 cells overexpressing 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.

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 overexpressing 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.

Fig. 5. In vitro kinase activity of IGF-1R is enhanced in
RACK1-overexpressing cells. MCF-7 cells either expressing empty
vector (Neo) or HA-RACK1 (clone A1) were starved from serum for 3 h
and then stimulated with IGF-1 for the indicated times. Cells were
lysed, and the lysates were immunoprecipitated using an anti-IGF-1R
polyclonal antisera and protein G-agarose beads. The beads were then
washed in kinase buffer (see “Experimental Procedures”) and resus-
pered in a kinase reaction mix that contained kinase buffer, $[^{32}P]ATP$,
and an exogenous peptide substrate poly(Glu-Tyr). After a 20-min in-
cubation the reaction mix was removed and precipitated on fiberglass
filters using trichloroacetic acid, and after extensive washing the filters
were dried and counted by liquid scintillation counting. Inset shows the
levels of IGF-1R in each sample determined by Western blotting with
an aliquot of the immunoprecipitated proteins.

ptosis in MCF-7 cells overexpressing RACK1. Cells were cul-
tured in serum-free medium and were then treated with eto-
poside in the presence or absence of IGF-1. As can be seen in
Fig. 4B, in serum-free medium IGF-1 stimulated an increase in
cell number, which was ~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-overexpressing cells is increased in serum-supple-
mented medium. When cells were treated with etoposide a
similar decrease in cell number occurred in both Neo and A1
cells, which indicates a similar level of cell killing. However,
IGF-1 rescued the Neo cells and also increased the cell number

Fig. 4. Proliferation is enhanced, but protection from eto-
poside killing is decreased in MCF-7 cells overexpressing
HA-RACK1. Three clones of MCF-7 cells overexpressing HA-RACK1 (A1,
B1, and C1) and one overexpressing vector (Neo) were seeded at a
density of $3 \times 10^4$ 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 deter-
dined by trypan blue exclusion, and data are presented as mean and S.D. of live cell numbers in triplicate wells.

B

Control
IGF-1
Etoposide
Etoposide + IGF-1

Original number of cells
0
2
4
6
8
10
Number of Cells (x 10^4)

Neo
HA-RACK1

![Graph showing the number of cells over time for different conditions.](image)

The status of IGF-1-mediated activation
of the PI3-K and MAP kinase pathways is differentially af-
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in the MCF-7/HA-RACK1 cells compared with control cells in
the unstimulated state. IGF-1 stimulation caused an
increase in kinase activity toward poly(Glu-Tyr) in
these cells compared with vector controls. This indicates that
overexpression 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 this enhanced IGF-1R activity.

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 signaling pathways is through recruitment of Src, which has been shown previously to phosphorylate the IGF-1R on key sites that stimulate its activation (38) or through sequestration of proteins necessary to activate the different signaling pathways. Sequestration of Src by RACK1 has been suggested previously (37) to account for the inhibition of fibroblast growth. 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 Fig. 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 min there was a significant decline in the amount of Src associated with RACK1, and by 30 min 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 MAP kinase activation, but it may contribute to JNK activation after 30 min of IGF-1 stimulation. However, because 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 been shown previously 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 min of IGF-1 stimulation. At this time Akt activation is diminished (Fig. 3). RACK1 does not interact with She in these cells (not shown). Overall the data indicate that the decreased Akt activation observed in the presence of RACK1 overexpression 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 overexpression has a negative effect on activation of the PI-3K 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 composed 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 (40–49). Recent evidence suggests that RACKs act as a β-propeller structure where each WD repeat forms a different blade on the β-propeller (27). Interestingly the WD repeats in RACK1 are conserved from chlamydomonas to humans and can be used by viruses to interact with cellular proteins (27, 44).

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 (37), phospholipase Cγ, PTPμ (47), cAMP-specific phosphodiesterase-4 (43),...
the B-subunit of integrins, and certain pleckstrin homology domains in vitro, including dynamin and β-spectrin (27). Only a subset of these interactions depends 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 (48) and the common β-chain of the interleukin-5/interleukin-3/GM-CSF receptor (43), and it is thought to promote signaling from these receptors by its ability to also associate with signal transducers and activators of transcription.

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-1R or IR function.

Overexpression of RACK1 led to enhanced IGF-1R kinase activity and IGF-1-induced phosphorylation of IRS-2 and Shc. This raised the following interesting question: why does overexpression of RACK1 lead to increased MAP kinase and JNK activation but decreased Akt activation? Because the interaction of RACK1 with the IGF-1R apparently does not change in response to kinase activation, the effects on signaling 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 PCK5 (47, 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 been proposed previously (37) as the growth inhibitory mechanism for RACK1 in NIH-3T3 cells. We also observed RACK1 interaction with c-Src in R+ cells and MCF-7 cells, but the complex dissociated completely after 15 min of IGF-1 stimulation. Thus, Src is associated with RACK1 during the 5–10-min post-IGF-1 stimulation that Akt and Erk become phosphorylated. Dissociation of Src could contribute to enhanced IGF-1-mediated JNK activation observed at 30 min because we have found previously (50) that JNK is activated by IGF-1 in a PI3-K and MAP kinase-independent manner. 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 been shown previously (51) 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 platelet-derived growth factor, fibroblast growth factor, or epidermal growth factor. In addition sequestration of Gβγ-subunits could block IGF-1R mitogenic activity but had no effect on insulin or IGF-1R mitogenic activity (52). Our observation that RACK1 overexpression enhances IGF-1-mediated activation of MAP kinase suggests that RACK1 may act like or assist βγ-subunits to enhance directly 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 signaling can confer a growth advantage on MCF-7 cells, inhibition of Akt activation by overexpression 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 signaling 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 (22, 29, 53, 57). It has also been demonstrated that activation of the class 2 PI3-Ks via IRS-1 may be a unique signal from the IR to regulate glucose metabolism (57). Thus, activation of PI3-K 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. Because its interaction with the IGF-1R is dependent on serine 1248, which is also conserved in the IR at position 1262, the effects of RACK1 on the PI3-K 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, 58) and cause insulin resistance. Tumor necrosis factor can stimulate phosphorylation of serine 307 in IRS-1 (59) and insulin resistance. Although a series of kinases including PKCε, IKKβ, PKCα, and JNK have been implicated (1, 21), it is not known which kinase directly phosphorylates this serine. Because RACK1 is a receptor for the C kinases, it is also possible that its associated kinases are involved in tumor necrosis factor α-mediated insulin resistance. It will be of interest to investigate whether a serine kinase is necessary to maintain RACK1 interaction with the IR or IGF-1R 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.

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Addendum—When this paper was in review another group identified RACK1 as an IGF-1R-interacting protein (60).

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