Ribosomal S6 Kinase as a Mediator of Keratinocyte Growth Factor-induced Activation of Akt in Epithelial Cells

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The keratinocyte growth factor receptor (KGFR) is a member of the fibroblast growth factor receptor (FGFR) superfamily. The proximal signaling molecules of FGFRs are much less characterized compared with other growth factor receptors. Using the yeast two-hybrid assay, we have identified ribosomal S6 kinase (RSK) to be a protein that associates with the cytoplasmic domain of the KGFR. The RSK family of kinases controls multiple cellular processes, and our studies for the first time show association between the KGFR and RSK. Using a lung-specific inducible transgenic system we have recently demonstrated protective effects of KGF on the lung epithelium and have demonstrated KGF-induced activation of the prosurvival Akt pathway both in vivo and in vitro. Here we show that a kinase inactive RSK mutant blocks KGF-induced Akt activation and KGF-mediated inhibition of caspase 3 activation in epithelial cells subjected to oxidative stress. It was recently shown that RSK2 recruits PDK1, the kinase responsible for both Akt and RSK activation. When viewed collectively, it appears that the association between the KGFR and RSK plays an important role in KGF-induced Akt activation and consequently in the protective effects of KGF on epithelial cells.

INTRODUCTION

The keratinocyte growth factor receptor (KGFR) is a member of the FGFR (fibroblast growth factor receptor) family. The KGFR is expressed only in epithelial cells and it plays critical roles in the proliferation, migration, and morphogenesis of epithelial cells (Ulich *et al.*, 1994; Wilson *et al.*, 1994; Rubin *et al.*, 1995; Post *et al.*, 1996; Buckley *et al.*, 1997). The KGFR also plays important roles in skin wound healing and lung epithelial cell survival during injury (Werner *et al.*, 1994; Panos *et al.*, 1995; Yi *et al.*, 1996; Barazzzone *et al.*, 1999; Das and Olsen, 2001; Ray *et al.*, 2003). The KGFR is activated by FGF-1, FGF-3, KGF/FGF-7, and FGF-10, whereas FGFR2 is mainly activated by FGF-2/bFGF (Bottaro *et al.*, 1990; Miki *et al.*, 1991, 1992; Orr-Urtreger *et al.*, 1993). In contrast to information on signaling by other growth factor receptors, the proximal signaling molecules of FGFRs are much less characterized.

To characterize the KGFR-induced signaling pathways, we screened for proteins interacting with the KGFR cytoplasmic domain using the yeast two-hybrid assay. RSK1 is one of the proteins we identified and this interaction was confirmed in mammalian epithelial cells. The RSK (or p90RSK) family includes three members, RSK1–3, which show 75–80% similarity at the amino acid level (Frodin and Gammeltoft, 1999). RSK family members contain two heterologous kinase domains (Fisher and Blenis, 1996). The N-terminal kinase (NTK) domain belongs to the AGC group of kinases, which includes PKA, PKB/Akt, PKC, and PKG, and

is the kinase domain that phosphorylates the substrates of RSKs. The C-terminal kinase (CTK) domain and the linker are involved in the activation of the RSK NTK domain (Frodin and Gammeltoft, 1999). At the carboxyl-terminus, there is an ERK-binding site conserved in all three RSK family members (Zhao *et al.*, 1996; Smith *et al.*, 1999). The other proteins homologous to RSKs include p70^{S6K}, MSK (mitogen- and stress-activated protein kinase) and RSK-B (Frodin and Gammeltoft, 1999). Although originally identified as ribosome S6 protein kinase, S6 protein phosphorylation by RSK family proteins is very restricted and p70^{S6K} is the major physiological S6 kinase (Frodin and Gammeltoft, 1999). RSKs are activated in response to several growth factors and mitogens including EGF (Zhao *et al.*, 1996; Sassone-Corsi *et al.*, 1999), insulin, and IGF-I (Alessi *et al.*, 1995; Lazar *et al.*, 1995). The activated RSKs phosphorylate a number of proteins containing the consensus sequences (R/L)xRxxS and are involved in a wide range of cellular activities (Frodin and Gammeltoft, 1999).

Using a lung-specific inducible transgenic system, we recently showed that KGF overexpression in the lung inhibits lung epithelial cell death (Ray *et al.*, 2003). Our studies showed that KGF-mediated epithelial cell survival from oxidative stress involves the prosurvival Akt pathway (Ray *et al.*, 2003). The Akt pathway is activated by multiple growth factors. Interestingly, the kinase PDK1, which plays an essential role in Akt activation, has been shown to be recruited by RSK2, resulting in coordinated phosphorylation and activation of PDK1 and RSK2 (Frodin *et al.*, 2000). Our present studies have identified RSK1 as a KGFR-interacting protein. Expression of a RSK1 mutant lacking the ERK-binding site prevented KGF-induced Akt activation and KGF-mediated inhibition of caspase 3 activation in epithelial cells subjected

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to oxidative stress. Our results provide a framework for growth factor-induced Akt activation involving RSK.

MATERIALS AND METHODS

Reagents and Antibodies

NGF and human KGF were purchased from Roche Molecular Biochemicals (Indianapolis, IN). The MEK inhibitor U0126 was purchased from Promega (Madison, WI). Anti-Rsk1, agarose-conjugated anti-RSK1, anti-HA, antiphospho-Tyr (pY99), anti-KGFR-C (Bek, c-17), normal rabbit IgG, and protein A/G plus-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TrkA antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Plasmid Constructs and the Yeast Two-hybrid Assay

Duplex-A yeast two-hybrid system (OriGene Technologies, Inc., Rockville, MD) was used to identify KGFR-interacting proteins. The KGFR cytoplasmic domain containing the tyrosine kinase domain was cloned into the yeast expression plasmid pEG202-NLS and the resulting bait construct was named pNEGKGFRc. We first ensured that the bait construct pNEGKGFRc containing the LexA-KGFRc fusion did not activate reporter genes due to autoactivation. Autoactivation of the reporter gene *lacZ* was checked by cotransforming EGY194 with pNEGKGFRc and the reporter plasmid pSH18-34. No activation of *lacZ* was observed with the LexA-KGFRc fusion protein. To identify KGFR-interacting proteins, EGY194 was first transformed with pNEGKGFRc and pSH18-34 and the pretransformed EGY194 was next transformed with a cDNA expression library constructed from cDNA derived from a 19-day postcoital mouse embryo fused to the B42 activation domain-HA-tagged expression vector pJG4-5. After selecting clones potentially expressing interacting proteins, the specificity of the interaction was confirmed by a yeast mating assay. The expression plasmid isolated from the putative positive clone was introduced into EGY194 (a strain) and a combination of the bait plasmid (pNEGKGFRc) and reporter plasmid (pSH18-34) was introduced into EGY40 (α -strain) and used in the mating test. Appropriate controls were used in the mating assay. To obtain the RSK1 expression construct pcDNA3RSK1, the full-length RSK1 cDNA was released from clone 1 identified from the yeast two-hybrid screen by digestion with *EcoRI* and *XhoI*, followed by insertion into pcDNA3 (Invitrogen, San Diego, CA). The insert in pcDNA3RSK1 was fully sequenced. To generate HA-tagged full-length Rsk1 and a C-terminal truncated mutant (d1), a site-directed mutation was first made in pcDNA3Rsk1 to introduce a *NotI* site at the ATG start codon site (named pcDNA3Rsk1N). pcDNA3Rsk1N was digested with *XhoI*, blunt-ended with Klenow fragment, digested with *NotI*, and cloned into pCruzHAc (Santa Cruz Biotechnology) to generate pHARSK1. A DNA fragment encoding amino residues 1–675 (Rsk1d1) was amplified by PCR using pcDNA3Rsk1N as template, using the forward primer, 5'-AGGGGCT-CAGGGGCGTGGCG-3' and reverse primer 5'-GGCGATATCACTAGATC-CATGGGTGCTGCAGGACCTGC-3', digested with *NotI* and *EcoRV* and cloned into pCruzHA. The chimeric Trk-KGFR expression construct was kindly provided by Martin Sachs (MDC Max-Delbruck Center for Molecular Medicine, Berlin, Germany). The KGFR mutants were generated by site-directed mutagenesis using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The RSK1 mutant lacking the carboxyl-terminal domain was generated using standard recombinant DNA techniques.

Cell Culture, Transfection, Immunoprecipitation, and Immunoblotting

HEK 293 cells (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5). Mouse lung epithelial cells (MLE-12) cells (ATCC) were cultured in DMEM/F12 medium supplemented with 10% FBS as recommended by the supplier. MLE-12 cells were used at 90% confluency. HEK 293 cells at 70–80% confluency were transfected using GenPORTER Transfection Reagent (GTS, Inc., San Diego, CA). DNA, 10–20 μ g, was used for transfection of cells in 10-cm plates. Twenty-four hours after transfection, cells were washed and incubated in serum-free medium for 24 h before further treatment or harvesting. Cell lysates were prepared using 1 $\times$ cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 10% glycerol. Cell extracts were used for protein analysis by immunoprecipitation, Western blotting, or kinase assay essentially as described previously (Ray *et al.*, 1997). Briefly, the immunoprecipitates were washed three times, 5 min each, in ice-cold wash buffer (1 $\times$ cell lysis buffer diluted 10-fold in 20 mM Tris-HCl, pH, 7.2 containing 150 mM NaCl) followed by a final quick wash in ice-cold 20 mM Tris-HCl, pH 7.2. Immunoprecipitates were collected by centrifugation at 12,000 $\times$ g for 20 s. The immunoprecipitates were boiled in 2 $\times$ Laemmli sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting techniques.

Figure 1. Interaction of RSK1 with the cytoplasmic domain of the KGFR in yeast two-hybrid assay. (A) Schematic of the bait construct used for yeast two-hybrid screening. The important tyrosine residues conserved between the KGFR and FGFR1 are indicated. (B) Expression and tyrosine phosphorylation of the bait LexA-KGFRc fusion protein. Cell lysate from yeast cells transfected with the bait construct was immunoblotted with α -Bek (α -KGFR) antibody that recognizes the C-terminus of the KGFR or immunoprecipitated with α -Bek and immunoblotted with the α -p-tyrosine (Tyr) antibody pY99. (C) Confirmation of interaction between RSK1 and bait protein using the mating assay. Three independently transformed colonies were streaked vertically (EGY40, α strain) or horizontally (EGY194, a strain) as indicated. Clone 1 encoded a cDNA homologous to Rsk1, and clone 8 was selected as a control from the primary screening, the cDNA in which did not match with any sequence in the databases.

RSK Kinase Assay

After immunoprecipitation with anti-RSK1 antibody and washing with PBS three times, the beads were quickly washed once with 20 mM Tris-HCl, pH 7.2. The immunoprecipitated protein(s) was analyzed using the S6 kinase assay kit (Upstate Biotechnology). Briefly, the reaction was carried out at 30°C for 10 min under the following conditions: 10 μ M S6 peptide, 10 μ M [γ -³²P]ATP, 25 mM MgCl₂, 100 μ M ATP, 4 μ M PKC inhibitor peptide, 0.4 μ M PKA inhibitor peptide, 4 μ M compound R24571, 20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT. The beads were separated by centrifugation, the supernatants were transferred to a new tube, and 25- μ l samples were blotted onto p81 phosphocellulose paper square (Upstate Biotechnology). The p81 squares were washed three times with 0.75% phosphoric acid for 5 min and once with acetone for 3 min before counting in scintillation fluid using a scintillation counter.

Akt Kinase Assay

The Akt kinase assay was performed as previously described using GSK-3 protein as substrate and the Akt kinase assay kit (Cell Signaling Technology) following the manufacturer's instructions (Lu *et al.*, 2001).

RESULTS

Identification of RSK1 as a KGF Receptor-interacting Protein Using the Yeast Two-hybrid Assay

The yeast two-hybrid system was used to screen for proteins that interact with the kinase (cytoplasmic) domain of FGFR2III-b/KGFR. A murine embryonic cDNA library was screened for interactions with a chimeric bait comprising LexA and the cytoplasmic domain of the KGFR (Figure 1A).

The bait fusion protein (LexA-KGFR) was expressed in yeast cells. As shown in Figure 1B, the fusion protein displayed autophosphorylation indicating that the KGFR was in the active form in the yeast cells. Among the multiple clones identified in this assay containing candidate KGFR-interacting proteins, two clones were identical to RSK1, which contained the sequence encoding the 11 amino acids missing in the original report (Alcorta *et al.*, 1989; Spring and Krebs, 1999; Figure 1C). A third clone showed a high degree of homology to RSK2, another member of the RSK family. These data suggested that RSK family proteins have the potential to associate with the KGFR. Because RSK proteins do not contain SH2 or phosphotyrosine-binding domain (PTB) domains, the typical structural motifs that mediate association with receptor tyrosine kinases (RTKs; Sudol, 1998; Margolis *et al.*, 1999), it is possible that the association between the KGFR and RSK involves an adaptor protein or represents an unconventional interaction with a RTK. Indeed, some RTK-interacting proteins have been identified that do not interact via the conventional phosphotyrosine-SH2/PTB-type interactions. The EGFR was shown to interact with the zinc finger protein ZPR1, which involved interactions between the zinc fingers of EGFR and a region in the receptor distinct from the tyrosine-phosphorylated C-terminal region that binds SH2 and PTB proteins (Galcheva-Gargova *et al.*, 1996).

Association of RSK1 with the KGFR in Epithelial Cells

All of our studies have been carried out with RSK1. We first tested whether endogenous RSK1 and the KGFR interact in lung epithelial cells. Mouse lung epithelial cells (MLE-12) were left in the absence of KGF or were treated with KGF for different lengths of time. Anti-KGFR antibody was used to immunoprecipitate the receptor and coimmunoprecipitation of RSK1 was assessed using immunoblotting techniques. As shown in Figure 2A, treatment of lung epithelial cells with KGF induced association of RSK1 with the receptor within 5 min of receptor activation. The level of association decreased after 15 min post-KGF stimulation and was not detectable after 30 min. To be able to explore the association between the KGFR and RSK1 in more detail, particularly the importance of tyrosine kinase activity of the receptor in this association, we used expression vector for the KGFR and transfected HEK 293 cells with the expression vector. The expression vector used contained the extracellular domain of TrkA and the transmembrane domain and cytoplasmic domains of the KGFR (pTrk-KGFR). In most epithelial cell types including HEK293 cells, the KGFR is expressed at low levels and because TrkA is not expressed in epithelial cells, anti-TrkA antibody can be used where necessary to investigate interactions between the KGFR and associated proteins. As shown in Figure 2B, Trk-KGFR was found to be associated with endogenous RSK1 as shown by coimmunoprecipitation studies using anti-RSK1. The control IgG did not result in immunoprecipitation of KGFR or RSK1. Although the interaction between the endogenous proteins was only evident upon stimulation of the cells with KGF, the association between RSK1 and overexpressed Trk-KGFR was detected in both starved and stimulated cells. This was not surprising since receptor tyrosine kinases are known to undergo ligand-independent activation (autophosphorylation) due to dimerization when the protein is overexpressed, which would cause interaction with RSK1 without the need for stimulation by ligand. Taken together, these data indicated that RSK1 is associated with the KGFR in mammalian cells.

Figure 2. Association between RSK1 and the KGFR in mouse lung epithelial cells and in HEK 293 cells. (A) MLE-12 cells (90% confluent) were washed three times in serum-free DMEM/F12 medium and starved in the same medium for 5 h. Cells were then left in the absence of KGF or were stimulated with KGF (100 ng/ml) for 5, 15, and 30 min. Three milliliters of medium was used for stimulation of cells in 10-cm plates. Total cellular lysate (1.4 mg protein) was immunoprecipitated with α -KGFR (25 μ g of agarose-coupled antibody in 500 μ l total volume) and the immunoprecipitates were analyzed by Western blotting techniques using α -RSK1 to demonstrate coimmunoprecipitation of RSK1. The blot was stripped and reprobed with α -KGFR. (B) HEK293 cells were transfected with pTrk-KGFR and stimulated with NGF (100 ng/ml, 15 min) after starvation for 24 h. Total cellular lysate (~500 μ g protein) was immunoprecipitated with control IgG or α -RSK1 antibody. The immunoprecipitates were analyzed by western blotting techniques using α -KGFR to demonstrate coimmunoprecipitation of KGFR. The membrane was stripped and reprobed with α -RSK1 antibody to demonstrate equivalent immunoprecipitation of RSK1 in all samples. The bottom panel shows direct Western blot analysis with α -KGFR antibody.

ERK-mediated Activation of RSK1 in KGFR Signaling

Structurally, RSK family members are composed of an N-terminal kinase (NTK) domain, a C-terminal kinase (CTK) domain, and an ERK binding site at its carboxyl-terminus (Figure 3A; Frodin and Gammeltoft, 1999). On stimulation by some growth factors, ERK phosphorylates several serine/threonine residues in RSK family proteins, which are critical for RSK activation. To test whether RSK1 can be activated by KGF and if so whether the RSK1 kinase activity activated by KGF is mediated through ERK, starved HEK 293 cells were stimulated by KGF in the presence or absence of the MEK inhibitor U0126. RSK1 activity was increased about sevenfold upon KGF stimulation and both basal as well as KGF-induced kinase activity was inhibited by the MEK inhibitor (Figure 3B). These results indicated that ERK is required for both the basal level activity of RSK1 and the increase in RSK activity in response to KGF stimulation.

Figure 4. Detection of coimmunoprecipitation of KGFR and RSK1 using α -phosphotyrosine antibody (α -p-Tyr). HEK 293 cells were transfected with empty vector, pTrk-KGFR, pHARSK1, or a combination of pTrk-KGFR and pHARSK1d1. Cell lysates were immunoprecipitated with α -phosphotyrosine antibody (pY99). The immunoprecipitates were resolved by SDS-PAGE and immunoblotting was performed with α -RSK1 and α -KGFR.

Figure 3. ERK-mediated RSK1 activation by KGF-KGFR. (A) Schematic of wt and mutant RSK1 structure. The two kinase domains, NTK and CTK, are connected by a linker region. At the very carboxyl-terminus of RSK1 is the ERK-binding site, which was deleted in the mutant RSK1d1. (B) Activation of RSK1 by KGF stimulation in HEK 293 cells is mediated by ERK. HEK 293 cells were incubated with or without the MEK inhibitor U0126 (10 μ M for 3 h) after 24-h starvation. Cells were incubated in the presence or absence of KGF (100 ng/ml) for 15 min and immunoprecipitation was carried out with α -RSK1. RSK1 kinase activity was assayed in the immunoprecipitates. In cells transfected with HA-tagged RSK1d1, the immunoprecipitated mutant RSK1d1 showed no kinase activity whether KGF was present or not. The presence of wt and mutant RSK1 in the immunoprecipitates was confirmed by immunoblotting of SDS-PAGE resolved proteins by a combination of two antibodies, α -RSK1 and α -HA (to be able to detect both wtRSK1 and RSK1d1). The two bands in the last 2 lanes indicate immunoprecipitation of the wt and mutant RSK1 proteins. (C) RSK1 activation by wt Trk-KGFR but not by the mutant Trk-KGFR^{Y542/543F}. HEK 293 cells were transfected with different expression constructs as shown. Cellular lysates were subjected to immunoprecipitation with α -RSK1 antibody followed by kinase assay using S6 peptide as the substrate. The RSK1 protein in the immunoprecipitates was identified by immunoblotting with α -RSK1 antibody. The blot was stripped and reprobed with anti-TrkA antibody. The data shown in B and C are means $\pm$ SEM of at least three independent experiments.

A similar result was also obtained by the use of pHARSK1d1, a construct containing a deletion in the carboxyl-terminus of RSK1, which includes the ERK binding site of RSK1 (Figure 3A). When transfected into HEK 293 cells, the mutant HARSK1d1 showed no apparent kinase activity when stimulated with KGF (Figure 3B). These data indicated that interaction with ERK is required for RSK activation. We also examined the effect of overexpression of a mutant of the KGFR devoid of its tyrosine kinase activity.

In cells transfected with an empty plasmid vector, RSK1 showed very low basal level activity (Figure 3C). When RSK1 was overexpressed alone, the increase in kinase activity was 1.5-fold over baseline and in cells overexpressing pTrk-KGFR^{wt}, the endogenous RSK1 kinase activity increased by almost 3.5-fold (Figure 3C). In cells overexpressing both pTrk-KGFR^{wt} and RSK1, the kinase activity of RSK1 was increased sixfold (Figure 3C). The kinase activity of RSK1 was increased by only 1.5-fold in cells transfected with a combination of pRSK1 and the kinase inactive chimeric receptor pTrk-KGFR^{Y542/543F} (Figure 3C). All of these estimations were normalized for RSK1 protein levels in the immunoprecipitates. Taken together, these data indicated that RSK1 kinase activity can be augmented by an activated KGFR.

Coimmunoprecipitation of the KGFR and RSK1 Using Anti-Phosphotyrosine Antibody

Because the KGFR is a receptor tyrosine kinase, we used another approach to detect coimmunoprecipitation of the KGFR and RSK1, which involved immunoprecipitation with α -phosphotyrosine antibody. As shown in Figure 4, both RSK1 and the KGFR were detected in the immune complex obtained using α -phosphotyrosine antibody. Overexpression of Trk-KGFR and RSK1 significantly augmented immunoprecipitation of RSK1 with the α -phosphotyrosine antibody. These results showed that the tyrosine kinase property of the KGFR could also be exploited to demonstrate association between the KGFR and RSK1.

Association between RSK1 and the KGFR and RSK1 Tyrosine Phosphorylation Are Dependent on the Tyrosine Kinase Activity of the KGFR

The KGFR contains several tyrosine residues that are highly conserved among FGFR family members. Y352, Y655, and Y542/Y543 of the KGFR correspond to the FGFR1 conserved tyrosine residues Y463, Y766, and Y653/Y654, which are involved in binding of Shc, PLC- γ , and activation of FGFR1, respectively (Mohammadi *et al.*, 1996; Klint and Claesson-Welsh, 1999). To test the roles of these conserved tyrosine residues in the association between RSK1 and the KGFR, HEK 293 cells were transfected with Trk-KGFR mutants in

Figure 5. Tyrosine phosphorylation of RSK1 and dependence on KGFR tyrosine kinase activity. HEK 293 cells were transfected with pRSK1 and wt pTrk-KGFR, pTrk-KGFR^{Y542/543F}, pTrk-KGFR^{Y352F}, or pTrk-KGFR^{Y655F}. After starvation for 24 h, the cells were incubated with or without NGF (100 ng/ml) for 15 min and the cell lysates (300–500 μ g protein) were immunoprecipitated with α -RSK1 antibody. After SDS-PAGE, the resolved proteins were analyzed by successive immunoblotting with α -phosphotyrosine antibody (pY99), α -KGFR, and α -RSK1 antibody.

which the respective tyrosine residues were mutated to phenylalanine. Cell lysates were immunoprecipitated with α -RSK1 antibody. As shown in the middle panel, overexpression of the wt receptor and the mutants Trk-KGFR^{Y352F} and Trk-KGFR^{Y655F} resulted in phosphorylation of a 130-kDa molecule but as expected, the kinase-inactive Trk-KGFR^{Y542/543F} mutant did not show receptor phosphorylation.

As stated previously, receptor phosphorylation in the absence of the cognate ligand (NGF) is most likely due to ligand-independent dimerization and autophosphorylation of overexpressed receptor molecules. None of the mutations in the tyrosine residues affected the association with RSK1 and association was detected with or without ligand (NGF) stimulation (Figure 5). These data indicated that the association of RSK1 with the KGFR does not involve amino acid residues that in FGFR1 are known to interact with specific adaptor proteins. Also, when the proteins were overexpressed, tyrosine kinase activity of the KGFR was not required for its association with RSK1. It is possible that dimerization of the receptor alone can trigger association with RSK1. Interestingly, this analysis also revealed tyrosine phosphorylation of a 90-kDa molecule. Successive reprobing of the blot with α -TrkA and α -RSK1 antibodies showed the bands to overlap with TrkA-KGFR (130 kDa) and RSK1 (90 kDa). Although association between RSK1 and the Trk-KGFR^{Y542/543F} mutant was detectable, the kinase-inactive mutant prevented tyrosine phosphorylation of the 90-kDa RSK1 protein (Figure 5). The other mutations had no effect on receptor or RSK1 tyrosine phosphorylation.

RSK1 Is Required for Akt Activation by the KGFR

We have recently shown that KGF activates the prosurvival molecule Akt in epithelial cells *in vitro* as well as *in vivo* in the lung using transgenic mice in which KGF was expressed in an inducible, lung-specific manner (Ray *et al.*, 2003). KGF overexpression protected the lung epithelium from hypoxia-induced cell death. Moreover, a dominant-negative mutant of Akt prevented KGF-mediated protection of cells injured by oxidative stress. Thus, Akt is a downstream ef-

Figure 6. Activation of Akt in cells overexpressing RSK1. HEK 293 cells were left untransfected or transfected with pHARSK1 or pHARSK1d1. The cells were starved for 24 h before KGF stimulation (100 ng/ml for 15 min). Cell lysates (500 μ g protein) were immunoprecipitated with Akt antibody and the Akt kinase reaction was carried out using GSK-3 α/β as the substrate. After immunoprecipitation, SDS-PAGE, and transfer onto membrane, the extent of phosphorylated GSK-3 α/β was determined by immunoblotting with a phospho-GSK-3 α/β -specific antibody. The amount of Akt in the immunoprecipitates was determined by immunoblotting with α -Akt antibody. Expression of wt and mutant RSK1 was assessed by direct Western blot analysis of cell lysates with α -HA antibody.

fector molecule in protection afforded by KGF. The proteins, that mediate KGF-induced Akt activation, however, are not known. It was recently shown that RSK2, a member of the RSK family of proteins recruits PDK1 and promotes coordinated phosphorylation and activation of PDK1 and RSK2 (Frodin *et al.*, 2000). PDK1 is a kinase that is critical for the activation of Akt and related enzymes, including RSK, that regulate many physiological processes such as cell survival, proliferation, and gene expression. If RSK associates with the KGFR, it seemed logical that RSK may actually be involved in Akt activation. Indeed, recent studies indicate that ERK may be involved in Akt activation under certain circumstances and the RSK homologue MSK was shown to phosphorylate and activate Akt (Hsu *et al.*, 2001; Nomura *et al.*, 2001; Yu *et al.*, 2001). To test whether RSK1 is involved in Akt activation, we checked the Akt kinase activity in cells overexpressing wt or mutant RSK1 and stimulated by KGF. KGF stimulation caused a fivefold increase in Akt kinase activity (Figure 6). Overexpression of wtRSK1 caused a 3–5 fold increase in Akt kinase activity with or without KGF stimulation (Figure 6). In contrast, when the mutant RSK1d1 was overexpressed, KGF-induced Akt kinase activity was inhibited (Figure 6). Even though the expression of the mutant was lower than that of wt RSK1, it was still able to block KGF-induced increase in Akt kinase activity. These data indicate that activation of RSK proteins may be an integral component of KGF-induced Akt activation.

RSK1 Mutant Prevents KGF-mediated Inhibition of Oxidant-induced PARP Cleavage

Because our data suggested an important role for RSK1 in KGF-induced Akt activation, we investigated whether blocking RSK1 function would influence antiapoptotic functions induced by KGF in 293 cells. For this purpose, we treated 293 cells with H₂O₂, which has been shown to induce proapoptotic pathways in these cells (Gotoh and Cooper, 1998). Because apoptosis is associated with caspase-3 activation, a focal point of different proapoptotic pathways, we investigated the activation of both procaspase 3 and cleav-

Figure 7. The RSK1 mutant RSK1d1 acts in a dominant-negative manner to prevent inhibition of procaspase-3 and PARP cleavage by KGF. 293 cells were incubated in serum-containing medium with or without H₂O₂ (control). The MEK inhibitor PD98059 was added at a concentration of 10 μ M 30 min before addition of H₂O₂ (1 mM). 24 h before treatment with H₂O₂, the cells were transfected with pHA-RSK1d1 or empty vector. KGF (100 ng/ml) was added to the cells 30 min before the addition of H₂O₂. Cell extracts were made 4 h after H₂O₂ stimulation. Procaspase-3 or PARP cleavage was assessed by western blotting as an indicator of cellular apoptosis. β -actin expression was used as a loading control. Densitometric analysis of the PARP bands was performed, and the ratio of intensity of cleaved band to the total intensity (cleaved + uncleaved bands) $\times$ 100 was estimated as percentage of PARP cleavage. The experiment was performed twice with similar results.

age of the substrate of caspase-3, poly (ADP-ribose) polymerase (PARP), in the cells under different conditions of treatment. Also, to inhibit the functions of endogenous RSK1, we overexpressed RSK1d1, which caused impairment of KGF-induced Akt activation (Figure 6). As shown in Figure 7, treatment of cells with H₂O₂ promoted cleavage of both procaspase-3 and the substrate of caspase-3, the PARP protein. Cleavage of both proteins was inhibited in the presence of KGF. However, when the RSK1 mutant was expressed in the cells, KGF was unable to inhibit protein cleavage. The mutant alone had no effect on either protein. These results were similar to our previous observations of the ability of DN Akt to block inhibition of PARP cleavage by KGF (Ray *et al.*, 2003). Addition of the ERK inhibitor

PD98059 inhibited the effects of KGF, suggesting ERK-stimulated RSK activity to be important for KGF-induced effects.

DISCUSSION

The present study demonstrates that RSK1 associates with the KGFR in epithelial cells. In addition to RSK1, we also identified one cDNA clone encoding a protein homologous to RSK2, which could associate with the KGFR. This suggests that the association between the KGFR and RSK might be a general feature for the RSK family proteins. This is the first report demonstrating a role for RSK in growth-factor-induced Akt activation.

Recent studies indicate dual functions of RSK and Akt in the regulation of cellular processes. For example, both RSK and Akt were shown to induce phosphorylation of the negative regulator of cell cycle progression p27^{Kip1} (Fujita *et al.*, 2003). An additional level of relationship between the two molecules was revealed in studies of PDK1 activation. Akt and RSK1 share some common substrates and both can be regulated by PDK1 (Alessi *et al.*, 1997). While RSK and Akt are both substrates of PDK1, recruitment of PDK1 by RSK2 was shown to cause phosphorylation and activation of PDK1 that in turn would activate both Akt and RSK2 again illustrating a close relationship between RSK and Akt activation (Frodin *et al.*, 2000).

We have recently shown that KGF-induced Akt activation plays an important role in the protection of epithelial cells from hyperoxia-induced cell death (Ray *et al.*, 2003). An important finding in the present study is the ability of the d1 mutant of RSK1 to behave in a dominant-negative manner inhibiting KGF-induced Akt activation. Moreover, overexpression of wt RSK1 increased the basal level of activated Akt in the cells. Interestingly, association between the endogenous KGFR and RSK proteins was detected within 5 min after KGF stimulation of cells (Figure 2A) and our previous studies show Akt activation to occur between 10 and 15 min after KGF stimulation of cells (Ray *et al.*, 2003). Thus, the kinetics of KGF-RSK1 association and KGF-induced Akt activation and the ability of the RSK1 mutant to block KGF-induced Akt activation collectively suggest that an important mediator of KGF-induced Akt activation is RSK1. We also show that ERK might be involved in Akt activation. The ability of the RSK1 homologue MSK1 to phosphorylate and activate Akt was recently demonstrated (Hsu *et al.*, 2001; Nomura *et al.*, 2001; Yu *et al.*, 2001). The mechanism by which RSK regulates Akt activation remains to be determined.

RSK1 is an abundant protein and found broadly distributed in the cytoplasm, nucleus, and the membrane (Chen *et al.*, 1992 and data not shown). At the C-terminus of RSK is an ERK-docking site conserved among the RSK family members (Frodin and Gammeltoft, 1999). In quiescent cells, a fraction of inactive RSK in the cytoplasm remains associated with ERK1/2 (Blenis, 1993). A fraction of RSK was previously shown to be membrane-associated with the receptor p185 (neu) via ERK under certain circumstances (Carraway *et al.*, 1999; Fincham *et al.*, 2000). However, association between RSK and any of the FGFR family members has not been reported before this study. Among the FGFR family members, signal transduction of FGFR1 is the best studied and known proteins associated with FGFR1 are PLC- γ , FRS2 and Crk (Klint and Claesson-Welsh, 1999; Reilly *et al.*, 2000). Our data suggest that the association of the KGFR with RSK1 may not involve tyrosine residues that have been shown to be involved in interactions with PLC- γ , FRS2 and Crk. These data suggest that the RSK1-KGFR association

may represent a novel kind of protein-protein interaction between RTKs and serine/threonine kinases for regulations of RTK signal transduction. However, it remains to be determined whether this kind of interaction exists among other FGFR family members and other growth factor receptors. Interestingly, FGFR-4 immunoprecipitates were shown to contain a doublet of 85-kDa protein phosphorylated at serine residues (Vainikka *et al.*, 1996).

We have shown that RSK1 is tyrosine phosphorylated by KGFR activation. The functional significance of RSK1 tyrosine phosphorylation is unclear at the present time. Because the kinase activity of RSK1 or RSK3 was not affected by treatment with protein tyrosine phosphatase, it appears that RSK1 tyrosine phosphorylation is not required for its serine/threonine kinase activity (Zhao *et al.*, 1995 and data not shown). One possibility is that RSK1 is a dual kinase with independent serine/threonine and tyrosine kinase activities. It is also possible that the phosphorylated tyrosine residues in RSK1 might provide docking sites for phosphotyrosine-binding proteins, such as SH2- or PTB-containing proteins (Sudol, 1998; Margolis *et al.*, 1999) that control distinct KGF-mediated functions.

RSK1 is activated by the KGFR and this activation appears to be mediated via its interaction with ERK. Because of its abundance and wide distribution, RSK1 may be involved in many aspects of the biological and pathological functions of the KGFR. Many cytoplasmic and nuclear proteins contain the consensus sequences for RSK phosphorylation [(R/L)xRxxS] (Leighton *et al.*, 1995; Frodin and Gammeltoft, 1999). The cytoplasmic substrates of RSKs include proteins that regulate cell survival such as BAD, I κ B α , and GSK3 (Glycogen synthase kinase-3) and also those that are involved in cell cycle progression such as p27^{Kip1} (Fujita *et al.*, 2003). Stimulation by growth factors results in activation of both cytosolic and nuclear RSK, and part of the cytosolic pool of RSK translocates to the nucleus upon activation (Chen *et al.*, 1992). The nuclear substrates of RSKs include CREB/Ser¹³³, CREB-binding protein, c-fos, and histone 3/Ser¹⁰. RSK may have two important roles in the pro-survival effects of KGF: i) activation of Akt that results in BAD phosphorylation (Ray *et al.*, 2003) and ii) direct phosphorylation of BAD/Ser¹¹²Ser¹⁵⁵ and CREB/Ser¹³³ (Bonni *et al.*, 1999; Tan *et al.*, 2000).

In summary, we have identified RSK1 as a KGFR-interacting protein. KGFR activation leads to ERK-dependent activation of RSK1. RSK1 activation is important for KGF-induced Akt activation in epithelial cells.

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