14-3-3β Is a p90 Ribosomal S6 Kinase (RSK) Isoform 1-binding Protein That Negatively Regulates RSK Kinase Activity*

Megan E. Cavet, Stephanie Lehoux‡, and Bradford C. Berk§

From the Center for Cardiovascular Research and Department of Medicine, University of Rochester, Rochester, New York 14642

p90 ribosomal S6 kinase 1 (RSK1) is a serine/threonine kinase that is activated by extracellular signal-related kinases 1/2 and phosphoinositide-dependent protein kinase 1 upon mitogen stimulation. Under basal conditions, RSK1 is located in the cytosol and upon stimulation, RSK1 translocates to the plasma membrane where it is fully activated. The ability of RSK1 to bind the adapter protein 14-3-3β was investigated because RSK1 contains several putative 14-3-3-binding motifs. We demonstrate that RSK1 specifically and directly binds 14-3-3β. This interaction was dependent on phosphorylation of serine 154 within the motif RLSEKV of RSK1. Binding of RSK1 to 14-3-3β was maximal under basal conditions and decreased significantly upon mitogen stimulation. After 5 min of serum stimulation, a portion of 14-3-3β and RSK1 translocated to the plasma membrane, and immunofluorescence studies demonstrated colocalization of RSK1 and 14-3-3β at the plasma membrane in vivo. Incubation of recombinant RSK1 with 14-3-3β decreased RSK1 kinase activity by ~50%. Mutation of RSK1 serine 154 increased both basal and serum-stimulated RSK activity. In addition, the epidermal growth factor response of RSK1S154A was enhanced compared with wild type RSK. The amount of RSK1S154A was significantly increased in the membrane fraction under basal conditions. Increased phosphorylation of two sites essential for RSK1 kinase activity (Ser703 and Ser380) in RSK1S154A compared with RSK1 wild type, demonstrated that 14-3-3β interferes with RSK1 phosphorylation. These data suggest that 14-3-3β-14-3-3S154A interaction can involve other varied motifs and may not require phosphorylation. The predominant 14-3-3-binding motif involves a phosphoserine, but interaction can involve other varied motifs and may not require phosphorylation. Because 14-3-3 proteins exist as dimers, they can simultaneously bind multiple phosphorylation sites on the same protein (12, 13) or function as scaffolds to form protein-protein interactions (5–8, 14). Other roles of 14-3-3 proteins include regulation of interacting proteins activity, subcellular localization, and/or stability. For example, 14-3-3 proteins are known to act as a scaffold for several proteins in the mitogen-activated protein kinase cascades, including MEK1, 2, and 3 (15), and to regulate the kinase activity of Raf (16).

Work in our laboratory has shown that NHE1 is activated by mitogens through phosphorylation of serine 703 by RSK (4). Subsequently, 14-3-3β binds to this site and prevents dephosphorylation of serine 703 (17). In the present study we investigated whether RSK1 was also a binding partner for 14-3-3β because it contains a number of putative 14-3-3 interaction motifs. We found that 14-3-3β specifically interacts with RSK1 in a phosphorylation-dependent manner, and the site of interaction was identified as serine 154. RSK1-14-3-3β binding is maximal under quiescent conditions and upon mitogen stimulation; both RSK and 14-3-3β translocate to the plasma membrane where they presumably dissociate because binding to 14-3-3β is decreased. Furthermore, 14-3-3β binding is inhibitory to RSK activity, and RSK1S154A, which does not interact with 14-3-3, has increased activity both basally and upon mitogen stimulation. This study suggests...
an important role for 14-3-3 in the negative regulation of RSK kinase activity.

EXPERIMENTAL PROCEDURES

DNA Constructs and Mutagenesis—RSK1 and 14-3-3β point mutations were created using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol. GST-14-3-3β was a gift from A. J. Muslin (Washington University School of Medicine). 14-3-3β constructs were subcloned into pcDNA3.1Xpress/His vector (Invitrogen), and rat RSK1 was subcloned into pCMV-FLAG (Stratagene) or pcDNA3.1/Myc-His (Invitrogen). All of the constructs and mRNAs were sequenced (in the gene and 3′ end) to ensure that no frame shifts or other mutations had been generated.

In Vitro Translation of Full-Length RSK1—Full-length rat p90RSK1 under control of the T7 promoter in pcDNA3.1/Myc-His was transcribed and translated in vitro using the Tnt T7-coupled reticulocyte lysate system (Promega). The nascent protein was labeled using Transcend biotin-lysyl-tRNA (Promega). Briefly, 40 µl of TNT Quick Master Mix, 1 µl of methionine (1 mM), 1 µg of template DNA, and 1 µl of biotin-lysyl-tRNA were mixed in a final volume of 50 µl, incubated for 90 min at 30 °C, and immediately used for the binding assays.

Cell Culture—PS127A cells (Chinese hamster lung fibroblasts that overexpress NHE1) were a gift from Dr. J. Pouyssegur (University of Nice, Nice, France). PS127A, NIH3T3, Cos7, and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 25 mM NaHCO₃, 10 mM HEPES, pH 7.4, 50 IU/ml penicillin, 50 µg/ml streptomycin, 10% fetal bovine serum (FBS) in a 5% CO₂, 95% O₂ incubator at 37 °C. The cells were serum-starved (0% FBS) overnight prior to the experiments.

Preparation of Cell Lysates—The cell monolayers were rinsed with ice-cold phosphate-buffered saline (150 mM NaCl, 20 mM Na₂PO₄, pH 7.4) and then scraped in 1 ml of phosphate-buffered saline. After a brief centrifugation, the cells were solubilized in 1 ml of cell lysis buffer (10 mM HEPES, pH 7.4, 50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM NaVO₃, 0.5% Triton X-100 plus 1:1000 protease inhibitor mixture (Sigma)). The cells were sonicated for 20 s (in the case of pull-down studies) or needle-homogenized (in the case of coimmunoprecipitations), agitated on a rotating rocker at 4 °C for 30 min, and centrifuged at 12,000 × g for 30 min to remove insoluble cellular debris. For some pull-down experiments, phosphatase treatment of cell lysates was performed by lysing cells in CIAP buffer (20 mM Tris, pH 8, 150 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.5% Triton X-100, plus 1:1000 protease inhibitor mixture). The lysates were treated with or without 200 units of calf intestinal alkaline phosphatase (Promega) at 37 °C for 1 h. Where indicated the inhibitors NaVO₄ (1 mM) and NaF (50 mM) were added.

Immunoprecipitations and Pull-downs—For coimmunoprecipitation studies, HEK293 cells were cotransfected with FLAG-tagged RSK1 and Xpress-tagged 14-3-3β. Immunoprecipitations were carried out by pre-clearing cell lysates with protein A/G-agarose for further 12 h and treated with 20% FBS. The cells were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked in phosphate-buffered saline with 5% FBS, 0.2% bovine serum albumin. The cells were stained with anti-FLAG antibody to visualize RSK1 and anti-14-3-3β antibody and then incubated in secondary antibodies (Vecta

FIG. 1. 14-3-3β binds to RSK1 in vitro and in vivo. A, PS127 cells were lysed, and a pull-down assay was performed with GST-14-3-3, GST-14-3-3 K49Q, or GST beads. B, biotin-labeled RSK1 was synthesized in vitro translation and incubated with GST-14-3-3β or GST-14-3-3β K49Q. Proteins bound to beads were subjected to SDS-PAGE and blotted with horseradish peroxidase-streptavidin. C, HEK293 cells were transfected with FLAG-RSK1 and Xpress-14-3-3β. Cells transfected with empty vector were used as a control. The cell lysates (lanes 1 and 2) were immunoprecipitated (IP) with anti-Xpress antibody (lanes 3 and 4) or M2 anti-FLAG-agarose beads (lanes 5 and 6) and immunoblotted for FLAG-RSK1 and Xpress-14-3-3β.

X-100, sonicated, and rocked for 1 h at 4 °C and analyzed by SDS-PAGE as described above.

RSK1 Kinase Assays—Twenty milliunits of recombinant RSK1 (Upstate Biotechnology Inc.) was incubated in 30 µl of kinase buffer (30 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, and 0.2 mM/ml bovine serum albumin) overnight with 10 µg of GST-14-3-3β or GST-14-3-3β K49A. To initiate the kinase reaction, 10 µg of S6 peptide, 7.5 µM ATP, and 5 µCi of [γ-³²P]ATP (Amersham Biosciences) were added, and the reaction was incubated for 10 min at 30 °C. The reaction was terminated by spotting 25 µl of the reaction onto PS1 phosphocellulose filter paper. The filters were washed five times in 0.75% phosphoric acid and one time in acetone, and radioactive incorporation was determined by Cerenkov counting.

CREB Reporter Gene Assays—HEK293 cells were plated at 2 × 10⁵ cells/well in 24-well plates 24 h prior to transfection. The cells were transfected with different expression plasmids together with the activator plasmid for CREB (pFA2-CREB) and the reporter gene for luciferase (pFR-Luc) (Stratagene). A plasmid expressing the enzyme Renilla luciferase was used as an internal control (pRL-TK, Promega). After 24 h, the cells were serum-starved for 24 h and then stimulated as indicated. Firefly and Renilla luciferase activities were measured using a Dual Luciferase Reporter System (Promega) with a Wallac 1420 luminometer. The data are expressed as firefly luciferase normalized by Renilla luciferase activity.

Immunocytochemistry and Confocal Microscopy—Cos7 cells seeded on glass coverslips were transfected with pCMV-FLAG-RSK1 using LipofectAMINE 2000. After 24 h the cells were serum-starved for a further 12 h and treated with 20% FBS. The cells were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked in phosphate-buffered saline with 5% FBS, 0.2% bovine serum albumin. The cells were stained with anti-FLAG antibody to visualize RSK1 and anti-14-3-3β antibody and then incubated in secondary antibodies (Vector-
RESULTS

RSK1 Binds 14-3-3 in Vitro and in Vivo—Previously our laboratory has demonstrated a direct interaction between the Na+/H+ exchanger isoform NHE1 and 14-3-3 at serine 703, the site of NHE1 phosphorylation by RSK (17). Because phosphorylation by RSK on serine 703 is required for binding of the scaffolding protein 14-3-3 and RSK1 contains several putative 14-3-3-binding sites similar to the consensus 14-3-3-binding motif RSXpXpX, we investigated whether 14-3-3 could interact with RSK1. Indeed, we found that RSK1 from extracts of PS127 fibroblasts was able to bind 14-3-3 in vitro (Fig. 1A). Previously, lysine 49 has been shown to be essential for interaction with 14-3-3-binding partners Bcr, Raf, and Cbl via a phosphoserine type interaction (18, 19). Mutation of lysine 49 (K49A) in 14-3-3 abolished NHE1 interaction with 14-3-3 (17). This mutation also abolished binding of RSK1 to 14-3-3. In addition, GST alone was unable to bind to RSK1 (Fig. 1A).

To determine whether the interaction between 14-3-3 and RSK1 was direct, full-length RSK was synthesized by in vitro translation, and its ability to bind to GST-14-3-3 was determined. In vitro translated RSK bound avidly to GST-14-3-3 but bound weakly to GST-14-3-3 K49A (Fig. 1B). To demonstrate an in vivo interaction between RSK1 and 14-3-3 in mammalian cells, HEK293 cells were transiently transfected with FLAG-RSK1 and Xpress-14-3-3. Xpress-14-3-3 immunoprecipitates were prepared, and their ability to interact with RSK1 was examined by Western blot. As shown in Fig. 1C (middle panels), 14-3-3 interacted with RSK1 in vivo. The reverse immunoprecipitation (Fig. 1C, right panels) demonstrates that FLAG-RSK1 can also coprecipitate 14-3-3.

The Interaction between RSK and 14-3-3 Requires Phosphorylation—Many interactions with 14-3-3 are dependent on binding partner phosphorylation. To determine whether this is also the case for the RSK1–14-3-3 interaction, PS127 cell lysates were incubated with alkaline phosphatase for 1 h at 37°C before GST-14-3-3 pull-down. As shown in Fig. 2A, phosphatase treatment (CIAP) inhibited the binding of RSK to 14-3-3. HEK293 cells transfected with FLAG-RSK1 were treated with 1 μM staurosporine (Stauro) for 1 h before lysis and pull-down with GST-14-3-3 (Fig. 2B).
FIG. 4. Serum stimulation inhibits the interaction between RSK1 and 14-3-3β. PS127 cells were stimulated with 20% FBS for 0, 5, 10, and 20 min with and without pretreatment for 1 h with 20 μM PD98059, and the cell lysates were pulled down with GST-14-3-3β beads. A, proteins bound to beads were subjected to SDS-PAGE and immunoblotted with anti-RSK1 antibody. Relative binding of RSK1 to 14-3-3β is shown as mean ± S.E. of five experiments (bottom panel). B, densitometric analysis of relative binding of RSK1 to 14-3-3 interaction after serum stimulation. The values are normalized to the 0 time point, which was set at 100%. The results are expressed as the means ± S.E. *p < 0.05 compared with control (n = 5). C, proteins bound to GST-14-3-3β beads were subjected to SDS-PAGE and immunoblotted with anti-NHE1 antibody.

14-3-3β in the absence but not the presence of phosphatase inhibitors. Hence dephosphorylation of RSK1 abolished its ability to bind 14-3-3β. In support of this conclusion, treatment of intact cells with the broad spectrum serine/threonine protein kinase inhibitor staurosporine also decreased the binding of RSK1 to GST-14-3-3β in vitro (Fig. 2B).

Binding to 14-3-3 proteins is usually mediated via a RXXpSXP or RXXpSXp consensus motif (12, 13). RSK1 contains a number of putative 14-3-3 binding sites as shown in Fig. 3A (RLS154KEV, RLGS307GP, RDS363PGI, RGFS380FY, RDPS48EE, REAS515FV, RIS603S611GK, and RLPS732TT). RSK1 constructs with indicated serine to alanine point mutations were transiently transfected into HEK293 cells, and the possible involvement of these motifs in the RSK1-14-3-3β interaction was investigated using GST-14-3-3β pull-down and communoprecipitation assays. Pull-down experiments demonstrated that mutation of S154A within the motif RLS(154)KEV strongly reduced RSK1-14-3-3β interaction, whereas the other mutations had little or no effect (Fig. 3B). Communoprecipitation confirmed that RSK1S154A no longer bound to 14-3-3β in vivo (Fig. 3C). These data suggest that RSK1 interacts with 14-3-3β through phosphorylated serine 154.

Serum Stimulation Reduces the RSK1-14-3-3β Interaction—Because serum stimulates RSK1 activity, which then activates NHE1 through phosphorylation of serine 703 (increasing the NHE1-14-3-3 interaction) (4, 17), we investigated the effect of serum on the RSK1-14-3-3β interaction. PS127 fibroblasts were stimulated with 20% serum, and RSK association with GST-14-3-3β was determined in a pull-down assay. Binding of RSK1 to 14-3-3β was decreased by 34.6 ± 7.5% in cells stimulated for 5 min, 36.2 ± 4.1% at 10 min, and 28.2 ± 6.5% at 20 min (p < 0.05; Fig. 4, A and B). This effect was also observed in HEK293 cells and Cos7 cells overexpressing exogenous RSK1 (data not shown). Preincubating PS127 cells for 30 min with the MEK1 inhibitor PD98059 (30 μM), which prevents agonist-stimulated ERK1/2 and RSK activation, reversed this inhibition (Fig. 4, A and B). As a control, NHE1 interaction with GST-14-3-3β was verified. Binding of GST-14-3-3β to NHE1 was stimulated by serum, and this stimulation was significantly inhibited by PD98059 treatment (Fig. 4C, especially at 10 and 20 min).

Subcellular Colocalization of RSK1 and 14-3-3—14-3-3 proteins function as scaffolding proteins and have been shown to affect the subcellular localization of interacting proteins (reviewed in Refs. 7 and 14). For example, 14-3-3 can sequester proteins in the cytosol and/or target them to the plasma membrane. RSK1 has previously been shown to be located in the cytosol under basal conditions and to translocate to the plasma membrane after stimulation where it becomes fully activated (3). Therefore, we determined the subcellular distribution of 14-3-3β and RSK1 before and after serum stimulation using subcellular fractionation in PS127 fibroblasts. In the absence of serum, RSK1 and 14-3-3β were predominantly localized in the cytosol. After stimulation with 20% fetal calf serum for 5 min, there was a significant translocation of both 14-3-3β and RSK1 to the membrane fraction (Fig. 5A). In contrast, the distribution of actin was unchanged after fetal calf serum treatment (Fig. 5A). This translocation also occurred with endogenous RSK and 14-3-3β in NIH3T3 cells and in HEK293 cells expressing exogenous RSK (data not shown).

To demonstrate colocalization of RSK1 and 14-3-3β in intact cells, Cos7 cells were transfected with FLAG-RSK1, and RSK1 and 14-3-3β were visualized by immunofluorescence staining with anti-FLAG antibody and anti-14-3-3β antibody respec-
14-3-3β Regulates RSK Function

Fig. 6. Subcellular colocalization of 14-3-3β and RSK1. Cos7 cells plated on glass coverslips were transfected with FLAG-RSK1. After 36 h, the cells were either left untreated (Control) or stimulated with 20% FBS for 5 min. The cells were then fixed, permeabilized, and stained with anti-14-3-3β antibody (red) or anti-FLAG antibody for RSK1 (green). A, control Cos7 cells stained for 14-3-3β (left panel) or FLAG-RSK1 (middle panel) and overlaying of the two images (right panel). B, serum-stimulated Cos7 cells stained for 14-3-3β (left panel) or FLAG-RSK1 (middle panel) and overlaying of the two images (right panel). The cells were visualized with a Olympus IX70 Fluoview confocal microscope. Bar, 10 μm.

14-3-3β Inhibits the Kinase Activity of RSK1—It is possible that RSK1 binding to 14-3-3 could either increase or decrease kinase activity based on previous reports (reviewed in Ref. 14). Because overexpressing a dominant negative 14-3-3 construct in cells would inhibit the Raf-MEK1-ERK pathway (20) and therefore decrease RSK kinase activity, it was not satisfactory to perform this experiment. Therefore, we determined the effect of incubating active recombinant RSK1 with 14-3-3β in vitro. Recombinant RSK1 was preincubated with GST alone, GST-14-3-3β K49Q, or GST-14-3-3β for 4 h, and then a kinase assay was performed using S6 peptide as the substrate. The activity of RSK1 in the presence of GST-14-3-3β was significantly reduced by ~50% as compared with GST alone or GST-14-3-3β K49Q (Fig. 7A). This finding suggests that binding of RSK1 to 14-3-3 may suppress RSK1 activity in vivo.

To assess the functional significance of the RSK-14-3-3 binding in vivo, we compared the kinase activity of wild type RSK1 to the non-14-3-3β-binding RSK1S154A mutant using a luciferase reporter assay system to measure the activity of CREB. CREB is activated by cAMP-dependent protein kinase and by all three members of the RSK family (RSK1–3) in cells stimulated by activators of the Ras/MEK/ERK1/2 cascade (21–23). As shown in Fig. 7B, CREB activity in cells expressing RSK1 wild type (RSK1WT) was increased after serum stimulation. However, in cells overexpressing RSK1S154A, there was an 80% increase in CREB basal activity and a 114% increase in serum-stimulated activity relative to RSK1WT. This suggests that 14-3-3 binding to RSK1S154 inhibits RSK activity both basally and after activation with serum. We next investigated the effect of the RSK1S154A mutation on EGF-stimulated CREB activity. The cells were transfected with RSK1WT or RSK1S154A and stimulated for 6 h with different concentrations of EGF, after which CREB activity was measured. In cells transfected with RSK1S154A, the EGF dose-response curve was significantly shifted to the left compared with cells expressing RSK1WT (EC50 of 1 ng/ml versus 6 ng/ml; Fig. 7C), demonstrating that 14-3-3β binding inhibits EGF-stimulated RSK1 activity and thus CREB activation.

The increased CREB activity in cells transfected with RSK1S154A versus those transfected with RSK1WT combined with the finding that 14-3-3β and RSK1 interact maximally under basal conditions suggest that 14-3-3β inhibits RSK1 by preventing its activation rather than by interfering with RSK1-substrate interaction. To verify this hypothesis we used phospho-specific antibodies targeting two phosphorylation sites on RSK1, Ser380 and Ser363. Ser380 is autophosphorylated basally, and it is further phosphorylated by the carboxyl-terminal RSK1 kinase domain upon docking and activation by ERK (1, 2, 24, 25). Ser363 phosphorylation is thought to occur subsequently, after translocation of the RSK-ERK complex to the plasma membrane (3). We found that in RSK1S154A constructs, phosphorylation of both Ser380 and Ser363 was increased basally (by 27.3 ± 2.6% and 137.9 ± 10.6% respectively, n = 3) and after 5 min of serum stimulation (by 35.9 ± 3.9 and 28.1 ± 2.8%, respectively, n = 3; Fig. 8, A and B), compared with RSK1WT. These data demonstrate that 14-3-3 inhibits the activation of RSK1.

Because full activation of RSK requires plasma membrane translocation (3), the increased activity of RSK1S154A suggested an increase in the amount of this mutant at the plasma membrane compared with RSK1WT. Therefore, we performed subcellular fractionation in HEK293 cells overexpressing RSK1WT or RSK1S154A. The amount of RSK1S154A in the membrane fraction under basal conditions was increased by 28.3 ± 2.9% (p < 0.05) as compared with RSK1WT, whereas upon serum stimulation the relative amounts of RSK1WT and mutant in the membrane fraction were similar (Fig. 9, A and B).
was dependent on a phosphoserine type interaction based on three findings. First, 14-3-3β K49Q exhibited greatly reduced binding to RSK. Second, binding was inhibited by alkaline phosphatase treatment in vitro. Third, the serine/threonine kinase inhibitor staurosporine decreased the RSK1–14-3-3 interaction in vivo. 14-3-3 proteins have previously been shown to bind mainly through the motifs RXXpSXP and RXXpSXP, where p denotes phosphoserine and X is any amino acid; however, there are strong preferences for particular amino acids over others in the X positions (12, 13). To locate binding sites in RSK1 we made eight serine to alanine mutations at close matches to these consensus motifs (RLS454KEV, RLS707GSP, RGPS537PGI, RGFS569FY, RDPS577EE, RES253FV, R1630G631GK, and RKLPS727TT). Only mutation of serine 154 greatly diminished binding of RSK1 to 14-3-3β both in vitro and in vivo, suggesting that phosphorylated serine 154 is the site of 14-3-3β interaction with RSK1. Interestingly, this motif lies within a predicted coiled-coil domain of RSK (amino acids 151–173; analyzed using Lupas’s method at the EMBnet-CH website). Coiled-coil domains are known to mediate protein–protein interactions (27) and have previously been shown to be involved in the interaction of γ-aminobutyric acid, type B receptors with 14-3-3 proteins (28).

The activation of RSK is dependent on a combination of both phosphorylation and plasma membrane localization events. ERK phosphorylates RSK1 within the carboxyl-terminal domain, and the phosphoinositide-dependent kinase PDK1 phosphorylates RSK1 within the NH₂-terminal domain (1, 2). Richards et al. (3) demonstrated recently that stimulated RSK1 transiently associates with the plasma membrane before accumulating in the nucleus. They also showed that the activity of a kinase inactive RSK1 mutant lacking the ERK docking site could be restored by addition of a membrane-targeting myristoylation site to the level of myristoylated wild type RSK1. This suggests that ERK has a role in escorting RSK to a plasma membrane-associated complex, although the exact mechanism of localization of RSK remains unclear (3).

14-3-3 proteins have been shown to play an important role in the subcellular distribution of a variety of signaling proteins. They appear to have the ability to both sequester proteins in the cytosol under basal conditions. RSK1

Fig. 7. 14-3-3β regulates RSK function.

DISCUSSION

The major finding of the present study is that the serine/threonine kinase RSK1 is a 14-3-3-interacting protein whose activity is negatively regulated by 14-3-3. The association between RSK and 14-3-3β was demonstrated by (i) the ability of a GST-14-3-3β fusion protein to bind RSK1 from cell lysates, (ii) direct binding of in vitro translated RSK1 to GST-14-3-3β, and (iii) coprecipitation of transfected 14-3-3β and RSK1. An important role for phosphorylated serine was previously demonstrated using 14-3-3β mutants at lysine 49, which reduces the phosphoserine-mediated interaction of binding partners Bcr, Raf, Cbl, and Bad but not the phosphorylation-independent interaction with Bax (18, 19, 26). Mutation of lysine 49 (K49Q) in 14-3-3β also abolished phosphoserine-dependent interaction with NHE1 (17). Association of 14-3-3β and RSK1

was dependent on a phosphoserine type interaction based on three findings. First, 14-3-3β K49Q exhibited greatly reduced binding to RSK. Second, binding was inhibited by alkaline phosphatase treatment in vitro. Third, the serine/threonine kinase inhibitor staurosporine decreased the RSK1–14-3-3 interaction in vivo. 14-3-3 proteins have previously been shown to bind mainly through the motifs RXXpSXP and RXXpSXP, where p denotes phosphoserine and X is any amino acid; however, there are strong preferences for particular amino acids over others in the X positions (12, 13). To locate binding sites in RSK1 we made eight serine to alanine mutations at close matches to these consensus motifs (RLS454KEV, RLS707GSP, RGPS537PGI, RGFS569FY, RDPS577EE, RES253FV, R1630G631GK, and RKLPS727TT). Only mutation of serine 154 greatly diminished binding of RSK1 to 14-3-3β both in vitro and in vivo, suggesting that phosphorylated serine 154 is the site of 14-3-3β interaction with RSK1. Interestingly, this motif lies within a predicted coiled-coil domain of RSK (amino acids 151–173; analyzed using Lupas’s method at the EMBnet-CH website). Coiled-coil domains are known to mediate protein–protein interactions (27) and have previously been shown to be involved in the interaction of γ-aminobutyric acid, type B receptors with 14-3-3 proteins (28).

The activation of RSK is dependent on a combination of both phosphorylation and plasma membrane localization events. ERK phosphorylates RSK1 within the carboxyl-terminal domain, and the phosphoinositide-dependent kinase PDK1 phosphorylates RSK1 within the NH₂-terminal domain (1, 2). Richards et al. (3) demonstrated recently that stimulated RSK1 transiently associates with the plasma membrane before accumulating in the nucleus. They also showed that the activity of a kinase inactive RSK1 mutant lacking the ERK docking site could be restored by addition of a membrane-targeting myristoylation site to the level of myristoylated wild type RSK1. This suggests that ERK has a role in escorting RSK to a plasma membrane-associated complex, although the exact mechanism of localization of RSK remains unclear (3).

14-3-3 proteins have been shown to play an important role in the subcellular distribution of a variety of signaling proteins. They appear to have the ability to both sequester proteins in the cytosol (for example protein kinase Cβ; (29), dictyostelium myosin II heavy chain protein kinase C (30), and Raf1 (16, 31)) and to cause translocation to the plasma membrane (for example testicular protein kinase 1) (32). Data in the present study suggest that 14-3-3β may play a role in sequestering RSK1 in the cytosol under basal conditions. RSK1–14-3-3β binding is maximal when cells are quiescent, and significant colocalization can be seen using immunofluorescence.

Upon activation by serum, a portion of both RSK1 and 14-3-3β translocate to the membrane fraction, and they partially colocalize in vivo at the plasma membrane. Serum also reduces 14-3-3β binding to RSK1 by 30–40%. These results suggest that 14-3-3β translocates with RSK1 to the plasma membrane, and once there the two proteins dissociate. This dissociation may be regulated in two ways. First, phosphatases are likely to play a role in dephosphorylating the RSK1-binding site (serine 154), and second, other 14-3-3β binding partners located at the plasma membrane may compete for 14-3-3β interaction. It is interesting to note that the Na⁺/H⁺ exchanger NHE1 has previously been shown to be a RSK substrate and that upon mitogen stimulation, 14-3-3β binds at the site of RSK phosphorylation (4, 17). Therefore, once phosphorylated by RSK, NHE1 may be a competing binding protein for 14-3-3β at the membrane.

In this study, incubation of RSK1 with GST-14-3-3β inhibi-
edited RSK activity by 50%. It has been demonstrated that multiple other signaling proteins bind to 14-3-3 and that their activities are inhibited by 14-3-3. For example, 14-3-3 inhibits the activity of phosphatidylinositol 3-kinase (33), protein kinase C isoforms \( \gamma / \delta \) (29) and \( \theta / \mu \) (34), Dictyostelium myosin II heavy chain protein kinase C (30), and testicular protein kinase 1 (32). We have identified Ser154 as a major 14-3-3-binding site and found that RSK1–14-3-3 interaction is reduced in S154A mutants. Using the activity of CREB, a transcription factor downstream of Raf (1, 22) as a functional readout for RSK activity, we further demonstrated that the S154A mutation increased basal and serum-stimulated RSK activity. In addition, when a dose-response curve for EGF-dependent CREB stimulation was performed, the EC\(_{50}\) for RSK1S154A was significantly lower than that of wild type RSK1. Increased phosphorylation of two sites essential for RSK1 kinase activity (Ser\(^{380}\) and Ser\(^{363}\)) in the RSK1S154A mutant as compared with RSK1WT both basally and after mitogen stimulation suggests a role for 14-3-3\(\beta\) in the inhibition of RSK1 kinase activity by interfering with its phosphorylation.

These results suggest that 14-3-3 plays a role in maintaining RSK1 signaling fidelity. Under basal conditions it may prevent activation by weak ERK signals. It is possible that RSK1 is held in the cytoplasm in an inactive conformation by 14-3-3\(\beta\), thus blocking phosphorylation of RSK1 activation sites. Because activation of RSK requires plasma membrane localization, increased basal activity of the S154A mutant correlates well with increased amounts in the plasma membrane compared with RSK1WT. Therefore, 14-3-3 is not a major determinant of membrane translocation of RSK1, although immunofluorescence and membrane fractionation data suggest that the two proteins move in a complex to the plasma membrane before
to a greater extent than wild type Raf (16, 35). The activity of the Raf S259A mutant is increased basally and after EGF stimulation as Ser703 of NHE1 and translocates to the nucleus to phosphorylate a greater extent than wild type Raf (16, 35). The activity of the Raf S259A mutant is increased basally and after EGF stimulation.

There are two serine sites on Raf that bind to 14-3-3, suggesting 14-3-3 binding to Ser259 antagonizes Raf activity by sequestering it in the cytosol (38, 39).

Fig. 10, we propose that RSK1 and 14-3-3 dissociate. Rather, 14-3-3 is a modulator of RSK1 kinase activity. At the plasma membrane, 14-3-3 may continue to alter RSK1 activity. At the plasma membrane, 14-3-3 perhaps maintaining RSK1 in an inactive conformation. On stimulation with mitogens, both 14-3-3β and RSK1 translocate to the plasma membrane. RSK1 then phosphorylates plasma membrane substrates such as Ser703 of NHE1 and translocates to the nucleus to phosphorylate nuclear substrates.

In summary, we demonstrate for the first time that RSK1 is a 14-3-3-binding protein and suggest a functional role of the 14-3-3β/RSK1 interaction in vivo. Based on the model shown in Fig. 10, we propose that RSK1 and 14-3-3β are bound in the cytosol, maintaining RSK1 in an inactive conformation. Upon stimulation with mitogens, both 14-3-3β and RSK1 translocate to the plasma membrane. RSK1–14-3-3β complexes dissociate at the plasma membrane, allowing full activation of RSK1. Finally, RSK1 phosphorylates substrates such as Ser203 of NHE1. This study demonstrates added complexity of the cellular regulation of RSK. Further understanding of the mechanisms of 14-3-3 regulation of RSK signaling will give insights into the role of RSK in cell growth and proliferation. Because previous studies by our laboratory have demonstrated increased RSK activity in cells and tissues of hypertensive animals, it will be of interest to study the potential role of 14-3-3 in this altered RSK regulation.

REFERENCES
1. Frodin, M., and Gammeltoft, S. (1999) Mol. Cell. Endocrinol. 151, 65–77
2. Richards, S. A., Fu, J., Romanelli, A., Shimamura, A., and Blenis, J. (1999) J. Biol. Chem. 274, 20226–20234
3. Frodin, M., and Gammeltoft, S. (1999) Mol. Cell. Neurosci. 16, 358–367
4. Richards, S. A., Dreisbach, V. C., Murphy, L. O., and Blenis, J. (2001) Mol. Cell. Biol. 21, 7470–7480
5. Takahashi, E., Abe, J., Gallis, B., Aebersold, R., Spring, D. J., Krebs, E. G., and Berk, B. C. (1999) J. Biol. Chem. 274, 20226–20234
6. Fu, H., Subramanian, R. R., and Masters, S. C. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 617–647
7. Trivison, G., Shen, Y. H., and Zhu, J. (2001) Oncogene 20, 6331–6338
8. Muslin, A. J., and Xing, H. (2005) Cell Signal. 17, 703–709
9. Yaffe, M. B. (2002) FEBS Lett. 513, 53–57
10. Masters, S. C., Pederson, K. J., Zhang, L., Barbieri, J. T., and Fu, H. (1999) Biochemistry 38, 5216–5221
11. Wang, B., Yang, H., Liu, Y. C., Jelinek, T., Zhang, L., Ruoslahti, E., and Fu, H. (1999) J. Biol. Chem. 274, 13717–13724
12. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Cell 84, 889–897
13. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Atkin, A., Leffers, H., Gambin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) Cell 91, 961–971
14. van Hemert, M. J., Steensma, H. Y., and van Heusden, G. P. (2001) Biochimica et Biophysica Acta 1513, 936–946
15. Panzer, G. R., Widmann, C., Porter, A. C., Sather, S., Johnson, G. L., and Vaillancourt, R. R. (1998) J. Biol. Chem. 273, 3476–3483
16. Trivison, G., Luo, Z., and Avrich, J. (1998) Nature 394, 88–92
17. Lehoux, S., Abe, J., Florian, J. A., and Berk, B. C. (2001) J. Biol. Chem. 276, 15784–15800
18. Wang, H., Zhang, L., Liddington, R., and Fu, H. (1998) J. Biol. Chem. 273, 16297–16304
19. Zhang, L., Wang, H., Liu, D., Liddington, R., and Fu, H. (1997) J. Biol. Chem. 272, 13717–13724
20. Xing, H., Zhang, S., Weinheimer, C., Kovač, A., and Muslin, A. J. (2000) EMBO J. 19, 349–358
21. Shaywitz, A. J., and Greenberg, M. E. (1999) Annu. Rev. Biochem. 68, 821–861
22. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Science 273, 959–963
23. Xing, J., Kannoun, J. M., Xia, Z., Thiele, E. A., and Greenberg, M. E. (1998) Mol. Cell. Biol. 18, 1846–1856
24. Frodin, M., Jensen, C. J., Merienne, K., and Gammeltoft, S. (2000) EMBO J. 19, 2924–2934
25. Vink, T. A., and Ryder, J. W. (1997) Biochem. Biophys. Res. Commun. 235, 389–402
26. Nomura, S., Shimizu, S., Sugiyama, T., Narita, M., Ito, T., Matsuda, H., and Tsujimoto, Y. (2000) J. Biol. Chem. 275, 3390–3397
27. Lupas, A. (1996) Trends Biochem. Sci. 21, 375–382
28. Couse, A., Kitter, J. T., Uren, J. M., Calver, A. R., Pangallo, M. N., Walsh, F. S., and Moss, S. J. (2001) Mol. Cell. Neurosci. 17, 317–328
29. Keller, N., Liu, Y. C., Collins, T. L., Bonnefoy-Berard, N., Baier, G., Isakov, N., and Altman, A. (1996) Mol. Cell. Biol. 16, 5782–5791
30. Ratto, E., Atkin, A., and Ratto, E. (1997) Mol. Cell. Biol. 8, 1889–1899
31. Roy, S., McPherson, R. A., Apolloni, A., Yan, J., Lane, A., Clyde-Smith, J., and Hancock, J. F. (1997) Mol. Cell. Biol. 18, 3947–3955
32. Toshima, J., Shima, J., Watanabe, T., and Mizuno, K. (2001) J. Biol. Chem. 276, 43471–43481
33. Bonnefoy-Berard, N., Liu, Y. C., von Willebrand, M., Sung, A., Elcy, C., Mustelin, T., Yoshida, H., Ishizaka, K., and Altman, A. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 10142–10146
34. Hausser, A., Storz, P., Link, G., Stoll, H., Liu, Y. C., Altman, A., Pfizenmaier, K., and Johanns, F. J. (1999) J. Biol. Chem. 274, 9258–9264
35. Michael, N. R., Fabian, J. R., Mathies, K. D., and Morrison, D. K. (1995) Mol. Cell. Biol. 15, 3590–3597
36. Morrison, D. K., Heidecker, G., Rapp, U. R., and Copeland, T. D. (1993) J. Biol. Chem. 268, 17309–17316
37. Bommel, C., Radzwill, G., Moelling, K., and Hafen, E. (1997) Dev. Cell. 64, 95–104
38. Light, Y., Paterson, H., and Maras, H. (2002) Mol. Cell. Biol. 22, 4984–4996
39. Dhillon, A. S., Meikle, S., Yanez, Z., Eulitz, M., and Kohle, W. (2002) EMBO J. 21, 64–71