RSKB, a p90 ribosomal S6 protein kinase with two catalytic domains, is activated by p38- and extracellular signal-regulated kinase mitogen-activated protein kinase pathways. The sequences between the two catalytic domains and of the C-terminal extension contain elements that control RSKB activity. The C-terminal extension of RSKB presents a putative bipartite 713KKRX_721KKRKQKLRS_737 nuclear location signal. The distinct cytoplasmic and nuclear locations of various C-terminal truncation mutants supported the hypothesis that the nuclear location signal was essential to direct RSKB to the nuclear compartment. The 725APLA-732 sequence was essential for the intermolecular association of RSKB with p38. The activation of RSKB through p38 could be dissociated from p38 docking, because RSKB truncated at Ser^751 strongly responded to p38 pathway activity. Interestingly, Δ725–772_RSKB was nearly nonresponsive to p38. Sequence alignment with the autoinhibitory C-terminal extension of Ca^2+/calmodulin-dependent protein kinase I predicted a conserved regulatory 706AFN710 motif. Alkaline mutation of the key Phe^709 residue resulted in strongly elevated basal level RSKB activity. A regulatory role was assigned to Thr^709, which is located in a mitogen-activated protein kinase phosphorylation consensus site. These findings support that the RSKB C-terminal extension contains elements that control activation threshold, subcellular location, and p38 docking.

The p90 ribosomal S6 protein kinases (RSKs)^1 are a family of Ser/Thr protein kinases composed of two catalytic domains, each with canonical ATP-binding site and activation loop sequences. In addition, RSKs contain a regulatory linker sequence connecting the two kinase domains and an extended C-terminal tail. RSKs comprise RSK1–RSK3, which are stimulated through the ERK pathway (1–6); the more recently identified mitogen- and stress-activated protein kinase type 1 and RSKB, which are activated by both the p38 and ERK pathways (7–9); and RSK4 (10, 11). RSKs are involved in many diverse functions, such as regulation of glycogen metabolism by phosphorylating glycogen synthase kinase-3 and the G-subunit of protein phosphatase 1, and cell survival of cerebellar neurons through phosphorylation of BAD (reviewed in Refs. 11–13). RSKs function in the control of M phase entry of oocytes during meiosis and chromatin remodeling through histone H3 phosphorylation (14, 15). Furthermore, RSKs participate in the regulation of transcription factors and coregulators, such as CREB (3–5, 7, 8), CREB-binding protein and p300 (16), c-Fos (17), and estrogen receptor (18). Deficient mutants of RSK2 in man are linked to Coffin-Lowry syndrome, characterized by mental retardation and malformations (19). Deficient RSK4 commonly occurs in patients with X-linked mental retardation (10). Interestingly, RSKB maps to the BBS1 locus (20), which is associated with Bardet-Biedl syndrome with manifestations reminiscent of Coffin-Lowry syndrome (21), and may be a candidate BBS gene.

Many Ser/Thr kinases present a resting state characterized by an autoinhibitory conformation of the C-terminal extension. Phosphorylations, or interactions with other proteins, that relax autoinhibition, are required for the activation of these enzymes. For example, the activation of MAPKAPK2, in addition to phosphorylation of Thr^209 in the catalytic site, correlated with phosphorylation of a threonine in a PXTP motif in the C-terminal domain, which contains an autoinhibitory domain with homology to the amphiphilic A-helix of other kinases (22–24). In CaMKII, a C-terminal segment blocks the catalytic site in the absence of calmodulin; upon calmodulin binding, a threonine within that segment is phosphorylated, disrupting autoinhibition and leading to a calcium-independent active state of the enzyme (25, 26). When compared with these single-domain enzymes, RSKs with two kinase domains and regulatory sites in linker and C-terminal tail present more complex regulation. Commonly, the N-terminal kinase of RSKs phosphorylates substrates, whereas the C-terminal kinase has a role in regulating RSKs activity. For example, the stepwise activation of RSK1 involving phosphorylations of a threonine in the C-terminal activation loop and of a serine in the linker through ERK, and further phosphorylations of linker and N-terminal activation loop sites through autophosphorylation (27). RSK2 is activated through integrating signals from two independent upstream kinase pathways, ERK and 3-phosphoinositide-dependent protein kinase 1, targeting the C-terminal and N-terminal domains, respectively (28). Specific docking sites in the C-termi-
C-terminal Control Elements of RSKB

...al tail of RSKs, facilitating interaction with upstream MAPKs, in some instances were found to be essential for activation (8, 29, 30). The profound control exerted by C-terminal tail elements was further demonstrated by the constitutive activity generated by truncation or mutation in the conserved putative autoinhibitory C-terminal helix of RSK2 (31). RSK1–RSK3 interact with ERK independent of activation state and locate both to the cytoplasm and nucleus under resting conditions; upon activation, the complex translocates to the nucleus (32). Sequence comparison suggested that elements directing RSKs in general to the nuclear compartment reside in the C-terminal tail (29). Thus, the control of subcellular location, autoinhibition, and protein-protein recognition in the association with upstream MAPKs all appear to be functions of the C-terminal sequences of RSKs.

Here, we present a study of regulatory sites in the C-terminal tail of RSKB. Sequential truncations of the C-terminal tail revealed elements mediating nuclear location and p38 association. The C-terminal kinase of RSKB has sequence similarities with CaMKs. The structure of rat CaMKI (33) was of particular interest, because it presented an autoinhibited conformation with a key AFN motif adjoining a helical stretch of the C-terminal extension (26, 34); the phenylalanine of this motif played a crucial role in the binding of the C-terminal extension to the body of CaMKI and maintaining the resting state (33). This AFN motif is conserved in RSKB. Sequence alignment and structure prediction suggested a study of the role of Phe 

### EXPERIMENTAL PROCEDURES

**Reagents—**Standard reagents were from various sources as reported (8, 35, 36). Transfection procedure was from Promega (Madison, WI). CREBtide ((SRKEETREIKSR)_{10}) was purchased from Genosys Biotechnologies (Lake Front Circle, TX). Antibodies to pERK (C-20) and epotope tag FLAG (antibody M2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma, respectively. SB202190 (4-(4-fluorophenyl)-2-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole) was obtained from Dr. Wyss (Hoffmann-La Roche, Switzerland). Phorbol 12-myristate acetate was from Sigma. PD98059 was purchased from Calbiochem.

Expression Constructs, RSKB Truncations, and RSKB Mutations—Expression constructs for MAPKs and wt-RSKB were generated as described (8). Mutated MAPK/ERK kinase was obtained from Stratagene (La Jolla, CA). RSKB point mutants were generated by site-directed mutagenesis using the Altered Sites in vitro mutagenesis system (Promega) according to the recommendations of the manufacturer. Expression constructs truncated RSKB were constructed by amplification of the NotI-SalI insert of Flag-wt-RSKB/pALTER (8) by the NotI-SalI fragment of the polymerase chain reaction product using the forward primer 5'-GAAAAATACATCGACTTCCGGG and one of the following reverse primers, (i) 5'-ACTAGGAACCTGCCTGGCGCCG (Δ503-772, RSKB), (ii) 5'-ATAGAGCTACATCCACCTGCTCTC (Δ503-772, RSKB), and (iii) 5'-ATTAGACCCCGCAGGTCTGCT (Δ291-772, RSKB).

Immunostaining and Immunoblotting—One day after transfection with the indicated plasmids, HEK293 cells were harvested, and 20,000 cells/cm² were seeded on poly-l-lysine-coated coverslips and cultivated for 1 additional day in minimal essential medium containing 0.3% fetal calf serum. The slides were washed in phosphate-buffered saline, fixed for 5 min in 4% formaldehyde in phosphate-buffered saline, and processed for immunohistochemistry as previously reported (8). For p38 detection, C-20 antibody (10 μg/ml) was used as primary reagent, with Texas red-labeled goat anti-rabbit immunoglobulin from Jackson ImmunoResearch (West Grove, PA) as secondary antibody. For FLAG-tagged truncated and wt-RSKB detection, M2 antibody (10 μg/ml) was used as primary reagent, with fluorescein isothiocyanate-labeled goat anti-mouse antibody from Dako (Glostrup, Denmark) as secondary antibody. Cells were harvestd in a Leica DMRB fluorescence microscope and assigning a nuclear, mixed (i.e. strong nuclear combined with weak cytoplasmic), or homogeneous cytoplasmic staining pattern. Immunoblots were incubated with specific primary and secondary horseradish-conjugated antibodies and revealed by chemiluminescence (ECL, Amersham Farmacia Biotech) and Storm PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

**Cell Culture, Transfection, and Extract Preparation—**HEK293 (ATCC CRL 1573) cells were cultured in humidified air with 5% CO₂ at 37 °C. Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 10 μg/ml streptomycin, pH 7.4. Transfections were done by the Transfection procedure as recommended by manufacturer. In parallel transfection experiments, total amount of DNA was normalized with empty vector. In studies of cells stimulated by the cotransfected mMKK6/p38 upstream kinase pathway, a constitutively active mutant of MKK6 (mMKK6) was used (8). Transfected cells were cultured for 2 days, followed by sodium arsenite (20 μM) treatment for the last 16 h in serum-containing medium. The p38 kinase inhibitor SB202190 (10 μM) was added together with the starvation medium; a second dose of SB202190 (10 μM) was added 1 h before harvesting the cells. After stimulation, cells were washed with ice-cold phosphate-buffered saline, and extracts were prepared with lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 500 μM diithiothreitol, 1% Triton X-100, 5 mM sodium orthovanadate, 0.1% protease inhibitor mixture (Roche Molecular Biochemicals)). Cell lysates were cleared at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined using the BCA reagents (Pierce).

**In Vitro Translation and Gel Shift Assay for p38 Binding—**wt-RSKB and truncated RSKB genes were in vitro transcribed with T7 RNA polymerase and translated at 30 °C for 90 min in the presence of the rabbit reticulocyte lysate using the coupled ribosomal in vitro translation system as specified by Promega. Aliquots of reticulocyte lysate containing wt-RSKB or the various truncated RSKBs were analyzed by SDS-PAGE and autoradiography to verify expression of the in vitro translated proteins. 5–10 μl of the reticulocyte lysate were used in the p38 binding assay performed in 2 μl Tris, 30 μM HEPES, 10 μM MgCl₂, 1 mM diithiothreitol (pH 7.4) in a final volume of 25 μl. Active recombinant Flag-tagged human p38α was purified by affinity chromatography from Escherichia coli expression. 1 μg of unlabeled p38α was used in the binding assay. After incubation for 30 min at 30 °C, 5 μl of SDS-free loading buffer was added to the reaction that was then electrophoresed on a 7.5% native polyacrylamide gel at 7 mA overnight using standard SDS-PAGE running buffer (acylamide/N,N'-methylene-bisacrylamide weight ratio of 29:1). The gels were fixed for 15 min (10% acetic acid, 40% ethanol) and stained with 0.5 min N-[1,3-dihydroxy-2-naphthalenyl]methane (Sigma) containing medium. The p38 kinase inhibitor SB202190 (10 μM) was added together with the starvation medium; a second dose of SB202190 (10 μM) was added 1 h before harvesting the cells. After stimulation, cells were washed with ice-cold phosphate-buffered saline, and extracts were prepared with lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 500 μM diithiothreitol, 1% Triton X-100, 5 mM sodium orthovanadate, 0.1% protease inhibitor mixture (Roche Molecular Biochemicals)). Cell lysates were cleared at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined using the BCA reagents (Pierce).

**Immunoprecipitation and Kinase Assays—**Cell extracts were subjected to M2 immunoprecipitation followed by kinase assay using CREBtide as a substrate. Cell extracts normalized to total protein content (200 μg unless specified otherwise) were precleared twice with 30 μl of protein G-Sepharose beads and incubated at 22 °C for 30 min under constant agitation. Similarly to wt-RSKB (36), SB202190 (10 μM) added directly to the kinase reactions had no effect on the elevated basal activities of F709A- and T687E-RSKB. Furthermore, 10 μM SB202190 had no effect on stimulated F709A-RSKB activity when added to the in vitro kinase reaction with precipitates from cells activated by mMKK6/p38 cotransfection and culturing without SB202190. A minor reduction of stimulated activity (~15%) of T687E-RSKB was seen when 10 μM SB202190 was added to kinase assays with precipitates from cells activated by mMKK6/p38 cotransfection. A possible effect of SB202190 on T687E-RSKB depends on relative affinities and binding kinetics of ATP versus SB202190 for the C-terminal ATP binding site; ATP concentrations in the in vitro kinase assay and in vitro are 30 μM and in the mM range, respectively. The assay may also be influenced by coregulated p38, and the variousricky kinase of activity of distinct RSKB variants. Thus, the assay format may explain the weak reduction of stimulated T687E-RSKB activity by SB202190 independently of a hypothetical direct effect of the inhibitor. Reactions were stopped by addition of 100 μl of 0.75% phosphoric acid, and 100 μl of the mix reaction were filtered in a 96-well phosphocellulose filter plate (Millipore, Bedford, MA), washed (five times) with 100 μl of 0.75% phosphoric acid, washed once with ethanol, and air-dried. Bound radioactivity was measured in a

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To investigate whether association with p38 and Ser737 were essential to direct RSKB to the nuclear compartment. Elements of the RSKB C-terminal Tail Mediate Association with p38—RSKB was first identified as a partial cDNA encoding the sequence downstream of Leu703 (RSKBΔN1 (8)) in intracellular interaction screens using p38 as bait, indicating that essential elements for p38 docking are located in the C-terminal tail (8). To more closely define these elements, p38 coprecipitation studies were performed with the C-terminal truncation mutants of RSKB, which were introduced in HEK293 cells with and without mMKK6/His-p38 coexpression. Normalized amounts of cell lysates were precipitated with M2 antibody, and the precipitates were probed by immunoblotting with an anti-p38 antibody. As shown in Fig. 3A, wt-RSKB and Δ725–772-RSKB coprecipitated p38 independent of activation. In contrast, Δ725–727-RSBK and Δ682–772-RSBK were unable to coprecipitate p38, even though all wt and truncated RSKB were expressed at similar levels (Fig. 3B). The essential role of the Δ725APLKRRKQLRS sequence for the physical association between RSKB and p38 was confirmed in gel band shift assays (Fig. 3C). wt-RSKB and truncated RSKB were translated in vitro, incubated with and without recombinant active p38 purified from E. coli expression, and electrophoresed through nondenaturing PAGE. The retarded electrophoretic migration of RSKB in the presence of p38 was readily apparent with wt-RSKB and Δ725–772-RSKB and with the isolated C-terminal domain but absent with Δ725–727, and Δ682–772-RSKB, as well as with the isolated N-terminal domain (Fig. 3C). These findings suggested that the sequence between Ala725 and Ser737 contained elements essential for association with p38, as well as for nuclear targeting.

Truncated RSKBs Distinctly Respond to Upstream MAPK Activation—To investigate whether association with p38 and activation by p38 can be dissociated, M2 antibody precipitation kinase assays were performed with lysates from HEK293 cells that had been transfected with the various truncated RSKB...
mutants together with mMKK6/p38 cotransfection and cultured in the presence of the p38 inhibitors SB202190, as indicated in Fig. 4. $\Delta^{738-772}$-RSKB was activated by the p38 pathway to about half the extent of wt-RSKB. In contrast, the activation of $\Delta^{725-772}$-RSKB was substantially reduced to nearly the background level. Interestingly, the further truncation in $\Delta^{682-772}$-RSKB resulted in a renewed response to mMKK6/p38 activation (Fig. 4), suggesting that the lack of response of $\Delta^{725-772}$-RSKB to p38 activation did not result from the absence of those sequence elements found essential for p38 association. These data suggested that RSKB activation was under the control of C-terminal sequence elements downstream of Ser$^{682}$ that were independent of the elements mediating the strong association with p38 reflected by coprecipitation, since activation and coprecipitation could be dissected.

**Role of Phe$^{709}$ in Control of RSKB Activation**—A sequence alignment revealed that the C-terminal domains of RSKB and CaMKs have similarities. The structure of CaMKs is characterized by autoinhibited conformations in the resting state (26, 33, 34). A search through sequences of proteins deposited in the Protein Data Bank (40) revealed a particularly interesting similarity between the C-terminal extensions of rat CaMKI (33) and RSKB (Fig. 5A). The CaMKI structure presented an inhibited state with a helical stretch of the C-terminal extension pointing to the ATP and substrate binding sites. Sequence alignment revealed an AFN motif that was conserved between the C-terminal extensions of rat/human CaMKI and RSKB but not found in RSK1–RSK3 (Fig. 5A). The central Phe of this $\text{AFN}^{308}$ motif pointed into a hydrophobic pocket, thus contributing to the binding of the C-terminal extension to the body of CaMKI (Fig. 5B) (33). Further scrutiny showed that Leu$^{26}$, Phe$^{31}$, Gly$^{100}$, Gly$^{101}$, Glu$^{102}$, and Leu$^{448}$ in rat CaMKI in the resting state are in direct contact with Phe$^{307}$ (Fig. 5B); all of these contact residues, homologous to Leu$^{417}$, Phe$^{422}$, Gly$^{488}$, Gly$^{489}$, Glu$^{490}$, and Leu$^{537}$ in RSKB, respectively, are conserved in RSKB and very likely form a similar structure. In RSKB, Phe$^{709}$ is homologous to Phe$^{307}$ in rat CaMKI and was predicted to play a similarly crucial role in attaching the C-terminal extension to the body of the C-terminal kinase domain and thereby blocking access of ATP to its binding pocket.

To test this hypothesis, Phe$^{709}$ was mutated to Ala to generate F709A-RSKB. HEK293 cells were transfected with this mutant, cultured in the absence of stimuli with and without SB202190, and normalized precipitation kinase assays were performed. As shown in Fig. 6A, F709A-RSKB displayed substantially elevated basal activity in nonstimulated cells. Interestingly, the high basal F709A-RSKB activity was sensitive to SB202190 treatment of the cells (Fig. 6A). Previous studies had shown that wt-RSKB is not sensitive to SB202190 (36); the structural configuration of the ATP binding site fold required for sensitivity to pyridinyl imidazole inhibitors (44), as well as control studies showing no direct effect of SB202190 on F709A-RSKB in precipitation kinase assays practically excluded that the F709A mutation had converted RSKB into a SB202190-sensitive conformation. Thus, the inhibition documented in Fig. 6A most likely did not result from a direct effect on F709A-RSKB, the presumed target of SB202190, rather, was p38, which thus appeared to control basal F709A-RSKB activity. F709A-RSKB in HEK293 cells was then stimulated by cotransfection with mMKK6/p38 (Fig. 6B). F709A-RSKB responded to p38 pathway activation to slightly higher levels than wt-RSKB (Fig. 6B). SB202190 reduced activation through the p38 pathway to background levels. These data were corroborated by studies of HEK293 cells transfected with F709A-RSKB and stimulated by treatment with arsenite (data not shown).

The F709A mutation was then introduced into truncated $\Delta^{725-772}$-RSKB to generate $\Delta^{725-772}$[F709A]-RSKB. As shown in Fig. 7, the combination of this truncation with the F709A mutation sufficed to partially overcome the lack of response of $\Delta^{725-772}$-RSKB in nonstimulated and stimulated cells. These combined data suggested that sequence elements between Ser$^{682}$ and Asn$^{724}$ had a function in inhibitory RSKB control, which was unmasked both through the truncation at Asn$^{724}$ and through the F709A mutation. To further probe the role of Phe$^{709}$ in RSKB control, a molecular model of the C-terminal domain of RSKB was built by fitting the aligned sequence to the template of the rat CaMKI structure (33). This model was improved and refined according to standard procedures (see under “Experimental Procedures”); it presented the C-terminal extension in contact with the body of the C-terminal catalytic domain in a structural arrangement that was consistent both with the proposed homology between Phe$^{307}$ and Phe$^{709}$ in CaMKI and RSKB and with a functional role of Phe$^{709}$ as suggested by the effect of the F709A mutation on RSKB basal level activity.

$\text{Thr}^{687}$ Is a Potential Control Site of RSKB Activation—The

| TABLE I | Percentages of transfected cells with nuclear, cytoplasmic, and mixed nuclear/cytoplasmic localization of $\Delta^{662-772}$-RSKB, $\Delta^{725-772}$-RSKB, $\Delta^{682-772}$-RSKB, and wt-RSKB |
|---------|--------------------------------------------------|
|         | Nuclear | Cytoplasmic | Mixed |
| $\Delta^{662-772}$-RSKB | 17 | 71 | 12 |
| $\Delta^{725-772}$-RSKB | 19 | 76 | 5 |
| $\Delta^{682-772}$-RSKB | 65 | 27 | 8 |
| wt-RSKB | 76 | 16 | 9 |

**Fig. 2.** RSKB nuclear location is determined by the NLS element. HEK293 cells were transfected with deletion mutants $\Delta^{662-772}$-RSKB, $\Delta^{725-772}$-RSKB, and $\Delta^{682-772}$-RSKB, as indicated. All RSKB constructs were tagged with Flag epitope. After transfection cells were cultured for 1 day, seeded on poly-1-lysine-coated coverslips, and cultured for an additional day. For immunodetection, the cells were simultaneously stained with M2 and fluorescein isothiocyanate-conjugated secondary antibodies and with C-20 anti-p38 and Texas red-labeled secondary antibodies. Confocal microscopy showing two panels at higher and lower magnification are shown for each mutant, as indicated.
C-terminal RSKB sequences around Thr^{687} fits the minimal MAPK consensus phosphorylation site, ψX(S/T)P, (where ψ represents Pro or aliphatic) (45) and is adjacent to the 690-VRSDL-NATMAFN^{715} sequence containing elements of the predicted inhibitory control function. This is an intriguing parallel to MAPKAP2, in which phosphorylation of Thr^{617} contributed to activation (22); Thr^{617} is located N-terminal to the autoinhibitory A-helix of MAPKAP2, and its phosphorylation may destabilize the helix or its interaction with the hydrophobic catalytic cleft (22). Thr^{687} therefore was mutated to Glu (T687E-RSKB), to mimic the effect of phosphorylation, and to Ala (T687A-RSKB). T687E-RSKB and T687A-RSKB expression constructs were introduced in HEK293 cells, lysates were prepared, and normalized precipitation kinase assays were performed as described above. As shown in Fig. 4A, T687E-RSKB had substantially higher basal activity when compared with wt-RSKB and T687A-RSKB. Similarly to F709A-RSKB, the high basal activity of T687E-RSKB was fully sensitive to SB202190 treatment of cells (Fig. 8B). From control studies, the inhibitor had no effect on basal T687E-RSKB activity but weakly (<15%) inhibited the in vitro stimulated activity. This weak inhibition of stimulated activity may result from the distinct in vivo and in vitro assay conditions (see under “Experimental Procedures”) rather than from a direct effect of the inhibitor. Furthermore, it is very unlikely that the T687E mutation converted the C-terminal ATP binding site fold of RSKB into a SB202190-sensitive conformation (44). Thus, the presumed target of SB202190 in Fig. 8B is p38, suggesting that the high basal activity of T687E-RSKB depended on p38 activity. Furthermore, F709A-, T687A-, and T687E-RSKB were introduced in HEK293 cells that were activated by cotransfection with mMKK6/p38, and precipitation kinase assays were performed, as indicated in Fig. 5C. It was found that all mutants responded to both p38 and ERK pathway activation: F709A-RSKB reached highest activation levels, T687E-RSKB reacted similar to wt-RSKB, and interestingly, the T687A mutation was permissive. These data are supported by findings in precipitation kinase assays performed with lysates of HEK293 cells transfected with either F709A-, T687A-, or T687E-RSKB alone and activated by treatment with arsenite and phorbol myristate acetate, as indicated in Fig. 8D.

**Fig. 3.** Intermolecular association of p38 with RSKB depends on the 725APLAKRRKQKLRS^{772} sequence independent of activation. A, immunoblots revealing coprecipitated endogenous and transfected His_{6}-tagged p38 in various RSKB precipitates. HEK293 cells were transfected with Δ^{682–772}-RSKB, Δ^{725–772}-RSKB, Δ^{738–772}-RSKB, and wt-RSKB Flag fusion expression constructs (Δ^{682–772}, Δ^{725–772}, Δ^{738–772}, and wt, respectively). Cells were either stimulated by cotransfection of mMKK6/p38 or left nonstimulated, as indicated. Cell lysates were incubated with or without 1 μg of bovine serum albumin (BSA), and immunoblots were performed using CREBtide as substrate. A representative (of n = 3) experiment is shown. Incorporated radioactivity was determined in a Packard top counter (mean cpm ± S.D.). B, samples were subjected to 7.5% PAGE under native conditions to reveal retardation due to RSKB/p38 association. C, 14C-Methylated protein molecular mass markers are shown in lane 1, in kDa; shown is an autoradiogram.
DISCUSSION
RSK family kinases are regulated through complex mechanisms associated with phosphorylations in the two catalytic domain sequences, the linker sequence, and the C-terminal tail sequence. Studies of RSK1 (27, 46), RSK2 (29, 31), and RSKB and mitogen- and stress-activated protein kinase type 1 (7, 8) demonstrated the important control exerted by elements in the C-terminal sequences of RSKs. For example, a mutation of a C-terminal tyrosine converted RSK2 to a constitutively active enzyme, suggesting that a putative autoinhibitory structure element controlled the basal activity of the enzyme (31). Furthermore, a conserved element of the C terminus of RSK1–RSK3 corresponding to \(^{722}\)LAQRVRKLPSTTL in RSK1 mediated the interaction with ERK, and complex formation between RSK2 and ERK was essential for the activation of RSK2 in vivo (29). Studies of truncated and point mutations of RSKB indicated that the C-terminal extension contained several elements controlling activation. The intermolecular association of RSKB with p38 depended on an element within the \(^{725}\)APLAKRRKQKLRS\(^{737}\)-RSKB sequence that was also essential for nuclear targeting, indicating an overlap of docking site and NLS. In addition, RSKB, when truncated at Asn724, had also lost responsiveness to activation through the p38 pathway, but interestingly, the further truncation at Ser681 in D\(^{682}-772\)-RSKB largely restored responsiveness. This activation of D\(^{682}-772\)-RSKB depended on stimulation through the cotransfected MEKK6/p38 pathway and was fully sensitive to the p38 inhibitor SB202190, as indicated.

FIG. 5. Sequence alignment and structure comparison point to a crucial C-terminal AFN motif. A, alignment of C-terminal sequences of human RSK1, RSK2, RSK3, RSKB and rat/human CaMKI. The respective accession numbers in the National Center for Biotechnology Information and Swiss-Prot data bases are L07597, P51812, Q15349, AJ010119, Q63450, and Q14012. B, model of RSKB in the region of contact between the AFN motif with the body of the C-terminal catalytic domain, as predicted by fitting the aligned sequence to the CaMKI structure template (33). C, 

FIG. 6. Elevated basal activity and enhanced responses to p38 pathway stimulation of F709A-RSKB. A, HEK293 cells were transfected with F709A-RSKB and wt-RSKB Flag fusion expression constructs and cultured with the p38 inhibitor SB202190, as indicated. Kinase assays were performed with M2 antibody precipitates from normalized lysates of unstimulated cells. A representative (of \(n = 4\)) series of parallel experiments is shown (mean and S.D. of F709A-RSKB basal activity, 7.6 ± 3.2-fold). B, HEK293 cells transfected with F709A-RSKB (F709A) and wt-RSKB (wt) Flag fusion expression constructs were activated by mMKK6/p38 cotransfection (MKK6) or left unstimulated (US), and kinase assays were performed with M2 antibody precipitates from normalized cell lysates using CREBtide as substrate, as indicated. Incorporated radioactivity was determined in a Packard top counter. A representative (of \(n = 3\)) experiments is shown (mean cpm ± S.D.).

FIG. 7. The F709A mutation mitigates the lack of response of \(\Delta^{725-772}\)-RSKB to p38 pathway stimulation. HEK293 cells were transfected with \(\Delta^{725-772}\)-F709A-RSKB (\(\Delta^{725-772}\)), \(\Delta^{725-772}\)-RSKB (\(\Delta^{725-772}\)), and wt-RSKB (wt) Flag fusion expression constructs, stimulated by mMKK6/p38 cotransfection (MKK6) or left unstimulated (US), and cultured with SB202190, and kinase assays were performed with M2 antibody precipitates from normalized cell lysates, using CREBtide as substrate, as indicated. Incorporated radioactivity was determined in a Packard top counter. A representative (of \(n = 4\)) series of experiments is shown (mean cpm ± S.D.).
SB202190, suggesting that p38 phosphorylated and activated Δ682–772-RSKB, even though activation through a distinct unknown kinase pathway that would have to be under the control of p38 or sensitive to SB202190 cannot be excluded entirely.

When extrapolated to wt-RSKB, these findings suggested that in contrast to RSK2, where the formation of a complex between the upstream MAPK and the C-terminal tail was essential for activation (29), the strong physical association of RSKB with p38 and its activation through the p38 pathway are separate functions. The nonresponsiveness of Δ682–772–RSKB to p38 activation thus was likely due to the presence of inhibitory elements between Ser682 and Asn724 rather than to the absence of a p38 docking site; given the nearly complete block of Δ682–772–RSKB, these inhibitory sequences in wt-RSKB presumably are counterbalanced by elements farther downstream of Asn724.

An element within the 725–820 APLAKRRKQLR577 sequence, consistent with the nuclear location signal sequence consensus, also was essential to target RSKB to the nucleus, suggesting an overlap between p38 association and nuclear targeting sites. The preferential nuclear location of RSKB was independent of stimulation by mMKK6/p38 cotransfection, suggesting that RSKB locates constitutively to the nucleus. A similar sequence element was responsible for the nuclear location of MAPKAPK2 under resting conditions; however, in contrast to RSKB, MAPKAPK2 was exported to the cytoplasm upon activation through p38 (47, 48). The subcellular location of MAPKAPK2 appeared tightly regulated by the removal of the C-terminal putative autoinhibitory helix as a result of phosphorylation of Thr317, which may unmask an adjacent nuclear export signal (47, 48). In contrast, the present findings suggested that the cytoplasmic location of the RSKB deletion mutants truncated at Ser681 and Asn724 resulted from the absence of the nuclear location signal rather than from activation-dependent nuclear export.

The inhibited state of Δ725–772–RSKB in view of the renewed response to activation after the further truncation in Δ682–772–RSKB, pointed to the existence of an autoinhibitory sequence element(s) between residues Ser682 and Asn724. A sequence alignment focused interest on an AFN motif that was conserved between CaMKI and RSKB but absent in RSK1–RSK3. The structure of CaMKI (33) revealed a crucial role of the 306–AFN308 motif of the CaMKI C-terminal extension; the central Phe307 pointed into a hydrophobic pocket and contributed to the binding of the C-terminal extension to the body of the enzyme. This conformation of the C-terminal extension with helical stretches pointing to the ATP and substrate binding sites and blocking access of ATP to its site, related to the autoinhibited state of CaMKI. The elevated basal activity of F709A-RSKB when compared with wt-RSKB supported the view that Phe307 played a similar regulatory role in raising the activation threshold as the homologous Phe307 in rat CaMKI; this view, furthermore, is consistent with the predictions of a molecular model obtained by fitting the aligned RSKB sequence to the rat CaMKI structure template (33).

Interestingly, the introduction of the F709A mutation into Δ725–772–RSKB produced less pronounced effects on basal and stimulated activation levels than might have been expected, suggesting that the perturbation of the putative autoinhibitory element by the F709A mutation in Δ725–772–RSKB did not compensate for the absence of the activation-promoting influence of sequence elements downstream of Asn724 in wt-RSKB. Furthermore, the elevated basal activity of T687E-RSKB suggested that Thr687 is also a regulatory site, consistent with its location in a MAPK phosphorylation consensus site. Similarly to F709A-RSKB, the difference between T687E-RSKB and wt-RSKB was more readily apparent with regards to basal activity than in altered responsiveness to upstream pathway activation. Intriguingly, this elevated basal activity of both F709A- and T687E-RSKB was sensitive to SB202190 treatment of the cells. wt-RSKB is not sensitive to SB202190, and in view of the structural requirements in the ATP binding site for sensitivity to pyridinyl imidazole class inhibitors (44) and of control studies, it was practically excluded that either of the RSKB mutants had become SB202190-sensitive. Although an unknown kinase pathway between mMKK6/p38 and RSKB, which would have to be under p38 control or sensitive to SB202190, cannot be excluded entirely, the most likely target of the inhibitor in the present studies was p38. This then suggested that similar to the persistent activation of wt-RSKB in tumor necrosis factor α-stimulated cells (36), the elevated activities of both F709A- and T687E-RSKB in nonstimulated cells depended on basal p38 activity, suggesting that the two mutations lowered an activation threshold. Interestingly, Δ682–772–RSKB, lacking the putative inhibitory element between Ser682 and Asn724, but also lacking the p38 docking site, had no elevated basal activity in nonstimulated cells. Additional studies are required to elucidate whether this results from a distinct role of p38 docking in basal and burst activation of RSKB or from a location to nuclear compartment(s) with specific activation conditions.

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Fig. 8. Responses of T687E-RSKB and T687E-RSKB point to a regulatory role of Thr687. HEK293 cells were transfected with T687A-, T687E-, F709A-, and wt-RSKB Flag expression constructs, stimulated through mMKK6/p38 cotransfection or phorbol myristate acetate and arsenite treatment, and cultured for 48 h with SB202190, as indicated. M2 antibody precipitation kinase assays from ristate acetate and arsenite treatment, and cultured for 48 h with
C-terminal Elements Control Location, Activation Threshold, and p38 Docking of Ribosomal S6 Kinase B (RSKB)

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