RSK-B, a Novel Ribosomal S6 Kinase Family Member, Is a CREB Kinase under Dominant Control of p38α Mitogen-activated Protein Kinase (p38αMAPK)∗

(Received for publication, May 29, 1998, and in revised form, August 6, 1998)

Benoit Pierrat‡‡, Jean da Silva Correia‡, Jean-Luc Mary, Mar Tomás-Zuber, and Werner Lesslauer¶

From the Department of Preclinical Research, Central Nervous System Diseases, F. Hoffmann-LaRoche Ltd., CH-4070 Basel, Switzerland

A novel ribosomal S6 kinase (RSK) family member, RSK-B, was identified in a p38αMAPK-baited intracellular interaction screen. RSK-B presents two catalytic domains typical for the RSK family. The protein kinase C-like N-terminal and the calcium/calmodulin kinase-like C-terminal domains both contain conserved ATP-binding and activation consensus sequences. RSK-B is a p38αMAPK substrate, and activated by p38αMAPK and, more weakly, by ERK1. RSK-B phosphorylates the cAMP response element-binding protein (CREB) and c-Fos peptides. In intracellular assays, RSK-B drives cAMP response element- and AP1-dependent reporter expression. RSK-B locates to the cell nucleus and co-translocates p38αMAPK. In conclusion, RSK-B is a novel CREB kinase under dominant p38αMAPK control, also phosphorylating additional substrates.

Eucaryotic cells exposed to intercellular communication or environmental signals respond with growth, adaptation, or death, all of which require restructuring of specific patterns of gene expression. The external signals are transduced to intracellular effectors by pathways that link cell surface and nuclear events and process transient input to short as well as more permanent response. Mitogen-activated protein kinases (MAPKs)† are ubiquitous and highly conserved elements of signal pathways under the control of growth factor, cytokine, and G protein-coupled receptors (reviewed in Refs. 1–4). A first group of MAPKs, the extracellular signal-regulated kinase family (ERKs), initially was discovered to mediate hormone and growth factor effects on proliferation and differentiation (5). A 54-kDa kinase was reported that shared with ERKs activation through tyrosine and threonine phosphorylation and a requirement for proline C-terminal to the Ser/Thr phosphorylation site, but differed with regard to substrate specificity, the 54-kDa species being the more potent c-Jun kinase (6); molecular cloning revealed a second kinase family, the c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (7, 8). A third pathway, architecturally similar to the ERK cascade, but primarily stimulated by cellular stress and cytokines was identified, with a homologous kinase called p38αMAPK designating the pathway (9–13). All MAPK families, ERK, JNK, and p38αMAPK are activated by concomitant phosphorylation of threonine and tyrosine residues in TEY (14, 15), TPY (7, 8), and TGY (9, 10, 13) sites, respectively, a few residues N-terminal to the conserved APE sequence in kinase subdomain VIII (16).

Early discoveries assigned functions in cell growth and stress to MAPKs, suggesting redundant roles in basic cell functions. However, ERK, JNK, and p38αMAPK families are encoded by multiple genes, with further diversification by alternative mRNA splicing into a growing number of isoforms. At least five isoforms of the p38αMAPK family are currently known. Some isoforms show a pronounced preference in tissue expression and selective interaction with upstream kinases and downstream substrates, pointing to highly specialized functions (17, 18). The functional independence of the ERK, JNK, and p38αMAPK pathways is documented by their selective activation through distinct upstream kinases (2, 19). Opposing effects of ERK as compared with JNK or p38αMAPK pathway activation have been reported, e.g. shifts in the dynamic balance between activation of JNK/p38αMAPK and concomitant inhibition of ERK may induce apoptosis in rat PC12 cells (20). Activation of p38αMAPK-dependent pathways has been linked to stress-induced apoptotic death in neutrophils and to excitoxicity in rat cerebellar granule cells (21, 22). Many studies place the p38αMAPK and JNK pathways in the context of “sounding the alarm” activity and of toxicity (for review, see Refs. 23–25). However, even mild environmental stimuli further increase the high basal JNK activity in the brain (26), consistent with physiologic JNK functions.

Various p38αMAPK substrates have been identified; MAP kinase-activated protein kinases (MAPKAPK2, -3, and -5), kinases called Mnk1 and PRAK, and transcription factors, such as ATF-2 and MEF2C, are p38αMAPK substrates (10, 27–30, 32). Although many p38αMAPK-controlled functions concern transcriptional regulation, post-transcriptional control of cytokine production by p38αMAPK also has been reported (12). Presently known substrates of JNKs are transcription factors such as c-Jun and ATF-2 (8, 34, 35). The ERK pathway activates all three currently known isoforms of RSK (RSK1, -2, and -3; Refs. 36 and 37) and transcription factors such as Elk1 (38). ERKs and RSKs are coordinately regulated (39), associate (40, 41),
FIG. 1. A, schematic alignment and amino acid sequence identity of the respective protein kinase C- and calcium/calmodulin kinase-like catalytic domains of RSK-B, human RSK1 (p90RSK1), p70S6K, and MAPKAPK2. Conserved residues of the ATP-binding and activation sites are highlighted. B, amino acid sequence alignment of RSK-B, RSK1 (p90RSK1), and MAPKAPK2. Conserved amino acid residues are highlighted. Residues are numbered according to RSK-B.
and co-translocate to the cell nucleus (42, 43), where RSKs phosphorylate substrates such as CREB (44).

The present study aimed to dissect the many different functions of the p38α MAPK MAPK pathway by identifying downstream p38α MAPK substrates. We performed an intracellular interaction trap screen in yeast, using a Gal4 DNA binding domain-p38α MAPK construct as bait, and identified a novel kinase, termed RSK-B, similar to the RSK family and containing two complete catalytic domains. RSK-B associates with and is activated by p38α MAPK. It is also activated, albeit more weakly, by ERK1, and may represent a convergence point between the p38α MAPK and ERK1 pathways. RSK-B controls CREB and AP-1 activity in luciferase reporter construct studies, and CREB and c-Fos are RSK-B substrates. RSK-B is nuclear and localizes p38α MAPK to the nucleus.

**EXPERIMENTAL PROCEDURES**

Reagents and Materials—Standard reagents were from various sources as reported (45, 46). Antibodies to RSK1, -2, and -3 and to epitope tags FLAG (antibody M2) and His6 (anti-X-express) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), International Biotechnology (New Haven, CT), and Invitrogen (Leek, The Netherlands), respectively. Transfectam procedure was from Biosepra. Luciferase assay kit was from Promega. PathDetect cis expression plasmids carrying luciferase genes under the control of CRE (x4) and AP-1 (x7) binding elements (pCRE-luc, pAP1-luc), and expression plasmids carrying constitutively active mutants of MEKK (380–672; mMEKK), and MEK1 (S218/222E, D32–51; mMEK1) under cytomegalovirus promoter;
PathDetect trans expression plasmids carrying SV-40 and Gal4 (1–147) DNA binding domain fused to the activation domain of CREB (1–283), and the luciferase reporter gene under the control of Gal4 binding element (x4) (Gal4-CREB) were from Stratagene (La Jolla, CA). FLAG M2 affinity gel was from Eastman Kodak (New Haven, CT).

Yeast Two-Hybrid Screen and Isolation of RSK-B cDNA—Intracellular interaction trap plasmids pA2, pGAD424, pT7D1, pVA3, and pCl1 were from CLONTECH. Plasmids pT7D1 and pVA3 encoding Gal4-activation domain-SV40 large T-antigen and Gal4 binding domain-p53 fusion protein, respectively, were used as a positive control in the two-hybrid assays and to eliminate false positives. A Xhol-BamHI cDNA fragment encoding p38α(MAPK) was polymerase chain reaction amplified and cloned into frame in pGBT9 vector which contains an unique XhoI site in the polylinker at position 873. An EcoRI-Smal fragment of the initial 1.3-kb RSK-B cDNA fragment (RSK-BAN2) from the two-hybrid screen, a human placenta Agt11 library (CLONTECH) screen and 5’-RACE techniques yielded full-length 3.2-kb RSK-B cDNA according to standard protocols (47). Sequences were analyzed using the GCG Sequence Analysis software package (Madison, WI).

Expression Constructs and Mutagenesis—A mammalian expression vector for FLAG-tagged RSK-B was engineered by introducing RSK-B cDNA into modified pALTER plasmid (Promega). An NdeI linker (5′-TATGCTACCCATGGACTACAAGGACGATGACGATAAGCA-3′) containing KpnI site, Kozak (48), and FLG epitope sequences was inserted in the NdeI site 5′ to RSK-B cDNA in pGAD424 vector. The KpnI-SalI fragment from this vector was ligated into the KpnI-SalI sites of pALTER to result in FLAG-tagged RSK-B in pALTER. The same strategy was used for RSK-BAN1 and RSK-BΔc expression plasmids. RSK-B(K65A), RSK-B(K440A) and RSK-B(S360E) were generated by site-directed mutagenesis using the Altered Sites in vitro mutagenesis system (Promega). Wild-type MKK6 was polymerase chain reaction-amplified and cloned into pALTER, and the active MKK6 mutant mMKK6(S207G/T211G) was constructed using the the same strategy. p38α(MAPK), ERK1 and JNK1/J2 were subcloned into pEBV-His expression vector (Invitrogen) as His6-epitope tag construct. Transient expression of all epitope-tagged proteins was tested in HEK 293 cells. Recombinant RSK-B and RSK-BAN1 were expressed in HEK 293 cells transfected with the respective FLAG-fusion vectors. FLAG M2 affinity gel was equilibrated in lysis buffer, washed once in lysis buffer with 300 mM NaCl, 400 mM K2HPO4, and incubated with transfected cell lysate (800 μg of total protein, 25 μl of affinity gel) for 1 h at 4 °C. FLAG-tagged protein was eluted by adding 20 μl of 10 mM Tris, pH 7.4, 150 mM NaCl, 500 μg/ml FLAG peptide, 400 μg/ml bovine serum albumin for 1 h at 30 °C with agitation, and recovered from 14,000 rpm supernatant. By SDS-PAGE, FLAG-tagged proteins and bovine serum albumin were the major components.

Mammalian Cell Culture, Transfection, and Luciferase Assays—HEK 293 (ATCC CRL 1573) and HeLa cells (ATCC CCL2) were cultured in humified air with 5% CO2 at 37 °C. HEK 293 cells were maintained in minimal essential medium, 10% fetal calf serum, 100 units/ml penicillin, 10 μg/ml streptomycin, pH 7.4. For recombinant protein expression in HEK 293 cells, 3 × 105 cells in 25×cm2 flasks were transfected with 3 μg of DNA with the Transfectam procedure. HeLa cells for reporter construct assays were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 100 units/ml penicillin, 10 μg/ml streptomycin, pH 7.4. For recombinant protein expression in HeLa cells, 5 × 106 cells/well in six-well plates were transfected with the specified amounts of plasmid DNA with the DEAE-dextran method. Luciferase assays were done as recommended by Promega. Luciferase activity was measured with a Packard top count luminesimeter.

Commonprecipitation and Kinase Assays—Cell lysates were prepared as reported (45, 46). Lysate aliquots (50 μg of total protein) were precleared (three times) for 20 min at 4 °C with 1 μg of antibody-agarose beads and incubated with 1 μg of either anti-FLAG or anti-p38α(MAPK) antibodies for 1 h at 4 °C under constant agitation. Immune complexes were allowed to bind to 13 μl of protein A-Sepharose beads for 1 h at 4 °C and washed, and immunoblots were performed as reported (45, 46). Precipitation kinase assays were carried out as reported without adding exogenous substrate (45, 46). SDS-PAGE “in-gel” kinase assays were performed as described (45) using MBP, CREB(tide YRERLISRPSSYRK) and Fos peptide (RHKSSHSSNESR) as substrates. Extracts with 500 μg of total protein were precipitated with 30 μg of M2 as described above. Samples were separated in 10.5% SDS-PAGE containing 200 μg/ml MBP, CREB(tide), or Fos peptide co-polymerized in the running gel.

Immunofluorescence—HEK 293 cells were plated on poly-L-lysine coated coverslips. After 24 h, cells were transfected with various expression constructs as above. After 24 h of culture, the cells were serum-starved for 24 h in minimal essential medium containing 0.3% fetal calf serum. The coverslips were fixed with 4% formaldehyde in PBS for 5 min, followed by sequential washes with PBS and 1% horse serum in PBS. The coverslips were incubated with antibody (10 μg/ml) for 1 h in the dark, and washed with PBS. Secondary labeled antibodies were applied for 1 h in the dark, followed by PBS washes. To visualize nuclei, the coverslips were placed in H3O2 for 2 min, treated with bisbenzimide (1.25 μg/ml H3O2) for 1 min, and washed once with H2O. Coverslips were dried, mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and viewed with a Leica confocal fluorescence microscope.

RESULTS

A p38α(MAPK) Bait Identifies RSK-B in Intracellular Interaction Trap Screen—A 1.3-kb cDNA insert containing an open reading frame encoding 160 amino acid residues (RSK-BAN2, amino acids 603–772) was isolated from a human placenta cDNA-Gal4 activation domain fusion library using a full-length p38α(MAPK)-Gal4 DNA binding domain fusion construct as bait. 5’-RACE and cDNA library screen techniques yielded the par-
ent 3.2-kb cDNA with an open reading frame encoding a 772-amino acid protein, termed RSK-B. The presumed translational start codon is preceded by a 5'-CCGCC sequence consistent with an optimal initiation context (46). The 733-nucleotide 3'-nontranslated sequence contains an AATAAA polyadenylation signal 30 nucleotides in front of the poly(A) tail. The predicted RSK-B amino acid sequence revealed similarity with Caenorhabditis elegans putative protein kinase C54G4 (50% overall amino acid identity) and human RSK1 (49) (Fig. 1); two complete catalytic protein-Ser/Thr kinase domains with 53% and 43% identity with RSK1 in the N- and C-terminal domains, respectively, were predicted. The C-terminal domain is similar to MAPKAPK-2, and the N-terminal domain to MAPKAPK-2, and the N-terminal domain to MAPK, confirming the C-terminal RSK-B sequence as a regulatory domain.

Western blot analyses revealed that RSK-B is widely expressed in tissues and various cell lines (data not shown).

Fig. 4. A, activation of RSK-B through p38αMAPK and ERK1, but not through JNK1. HEK 293 cells were co-transfected with indicated plasmids and cultured for 2 days. Standard M2 precipitates of cell lysates were analyzed by MBP-in-gel kinase assay in parallel (lanes 1-10), and immunoblotting with M2 (lane 11) and anti-p38αMAPK (lane 12) antibodies. MBP-incorporated radioactivity of specific bands quantified by PhosphorImager and activation factors determined: (i) p38αMAPK enhanced RSK-B activities (lanes 4 versus 3) by 6.7-fold (monomer) and 10.7-fold (dimer) (representative of n = 6 experiments), (ii) ERK1 enhanced RSK-B activity (lanes 7 versus 6) by 1.4-fold (monomer) and 1.7-fold (dimer) (representative of n = 2 experiments; activation of p38αMAPK, ERK1, and JNK1 in these protocols is shown in Fig. 3C). B, sensitivity of RSK-B activity to p38αMAPK and ERK1 pathway inhibition. HEK 293 cells were co-transfected with indicated plasmids and cultured for 2 days in parallel; 10 μM FHPI or PD098059 was added overnight prior to cell lysis. MBP-in-gel kinase assay of M2 precipitates under standard conditions. MBP-incorporated radioactivity of specific bands quantified by PhosphorImager. Setting RSK-B mono- and dimer bands of lane 1 to 100%, FHPI reduced activities of RSK-B monomer and dimer to about 15% (lanes 2 versus 1). Compared with lane 1, RSK-B activities in lane 3 were 39% and 38%, and in lane 4 were 25% and 24% for monomer and dimer bands, respectively.

Novel RSK Family CREB Kinase under p38αMAPK Control
FIG. 5. A, RSK-B drives AP1- and CRE-dependent luciferase reporter expression. HeLa cells were co-transfected with either pAP1-luc or pCRE-luc together with RSK-B (or empty pALTER vector), and control, mMKK6, mMEK1, or mMEKK as indicated (1 μg). Luciferase assays were performed with cell lysates after 2 days of culture. **Top panels**, relative luciferase activity, normalized to pALTER of control to reveal baseline luciferase activity in control, mMKK6, mMEK1, or mMEKK transfectants. **Bottom panels**, luciferase induction factor normalized to pALTER of each control, mMKK6, mMEK1, or mMEKK transfection, to reveal the RSK-B attributable luciferase activity. A representative experiment (n = 4) is shown.

B, RSK-B activity is necessary for luciferase response in either pCRE-luc or pAP1-luc co-transfectants. HeLa cells were co-transfected with mMKK6, wild type, or mutated RSK-B, and either pCRE-luc or pAP1-luc, as indicated (1 μg). The cells were cultured for 2 days, and luciferase...
To investigate a possible stimulus-enhanced association of RSK-B with either p38αMAPK, ERK1/2, or JNK1, HEK 293 cells were co-transfected with (i) RSK-B, p38αMAPKHis6, and mMKK6, and (ii) with RSK-B together with either mMEK1 or mMEKK. Cell lysates of these various transfectants were precipitated with M2, and immunoblot analyses were performed with anti-p38αMAPK, -ERK1/2, and -JNK1 antibodies (Fig. 2B). No enhancement of RSK-B association with p38αMAPK and ERK1 was seen, suggesting a stimulus-independent association of RSK-B with endogenous/transfected p38αMAPK and ERK1. Co-precipitation of mMEKK did not lead to JNK1 co-precipitation, consistent with a lack of RSK-B and JNK1 interaction in the presence and absence JNK pathway activation.

**RSK-B Is a p38αMAPK Substrate—**RSK-B could be produced in recombinant expression only in low yield and furthermore, had a strong tendency for degradation and aggregation. This is in close parallel to the excellent study of Dalby et al. (52), who reported that recombinant RSK1 can only be expressed in very low yield, was highly degraded and could not be activated by ERK. To investigate whether RSK-B is a p38αMAPK substrate, we made therefore first use of the pronounced association of RSK-B and p38αMAPK and tested RSK-B phosphorylation by co-precipitated p38αMAPK in an *ex vivo* type experiment. RSK-B, RSK-BΔN1, and RSK-BΔC were precipitated by M2 from HEK 293 transfectants and assayed as substrates of co-precipitated p38αMAPK in standardized *in vitro* kinase assays, and 32P incorporated in specific bands was PhosphorImager counted (Fig. 3A). Activation of the p38αMAPK pathway by mMKK6/p38αMAPKHis6 co-precipitation resulted in a more than 5-fold increase in 32P-incorporation in RSK-B when compared with RSK-B from unstimulated cells. The C-terminal catalytic domain, RSK-BΔN1, showed a similar p38αMAPK stimulus-enhanced 32P-incorporation, whereas the N-terminal domain, RSK-BΔC, did not show any 32P-incorporation, consistent with a lack of interactivity with p38αMAPK. The specific inhibitor of p38αβMAPK, FHIP1 (12, 55), added to the *in vitro* assay at 10 μM reduced RSK-B and RSK-BΔN1 phosphorylation practically to background level, whereas the specific MEK1 inhibitor PD098059 (56) (10 μM) did not reduce 32P incorporation into RSK-B and RSK-BΔN1 (Fig. 3A). Thus, although RSK-B background phosphorylation by undefined activities occurs, its p38αMAPK stimulus-dependent increase, which furthermore is blocked by the specific inhibitor FHIP1, strongly supports the view that RSK-B is a p38αMAPK substrate.

These data were corroborated by results of standard kinase assays using aliquots of p38αMAPK, ERK1/2, and JNK, which were precipitated in parallel from HEK 293 cells co-transfected with p38αMAPKHis6, mMKK6, ERK1His6, mMEK1, or JNKHis6α/β mMEKK, respectively, and affinity-purified RSK-BΔN1 and RSK-B from small scale recombinant HEK 293 transient expression as substrates (Fig. 3, B and C). Phosphorylation of full-length RSK-B by p38αMAPK was enhanced 2.5-fold when compared with a parallel assay performed in the presence of 10 μM FHIP1 (Fig. 3B). RSK-BΔN1 phosphorylation was enhanced by activated p38αMAPK and ERK1 3.1- and 1.5-fold, respectively, when compared with a consistent background phosphorylation in the p38αMAPK lane as a control (Fig. 3B). In independent studies, 3–7-fold p38αMAPK-dependent inductions of RSK-BΔN1 phosphorylation were found, consistent with a phosphorylation level in such assays found by other authors (31). Amounts of p38αMAPK, ERK1, and JNK equivalent to those used in the kinase assays were probed for activation in Western blots using antibodies selectively reacting with the respective activated phosphorylated kinases (46) (Fig. 3C).

**Control of RSK-B Activity by MAPK Pathways—**To differentiate the roles of p38αMAPK, ERK and JNK in RSK-B activation, upstream kinases of the three pathways were co-transfected with RSK-B in HEK 293 cells, and MBP in-gel kinase assays of M2 precipitates of cell lysates were performed under standard conditions (Fig. 4A). First, investigating the p38αMAPK pathway, it was found that p38αMAPK in mMKK6/p38αMAPK/RSK-B co-transfectants enhanced RSK-B activity about 10-fold when compared with mMKK6/RSK-B transfectants (Fig. 4A, lane 4 versus lane 3; see legend). In all gels, RSK-B bands of the expected apparent molecular mass of RSK-B monomers and higher molecular mass bands were seen (see Fig. 4A, Western, lane 11). The latter bands, which may result from aggregation occurring after cell lysis, from their gel location tentatively were designated RSK-B dimers. As expected, mMKK6 co-transfection also resulted in the activation of endogenous and transfected p38αMAPK (Fig. 4A, lanes 3 and 4, and Western, lane 12). The requirement for transfected exogenous p38αMAPK to achieve RSK-B activation is a peculiarity of the HEK 293 cells used (see below). Second, investigating the ERK pathway, ERK1His6 in mMEKK1/ERK1His6/RSK-B co-transfectants enhanced RSK-B activity about 1.5–2-fold (Fig. 4A, lane 7 versus lane 6; see legend), supporting a functional connection between ERK1 and RSK-B already suggested by co-precipitation (Fig. 2). Finally, no connectivity between RSK-B and the JNK1 pathway was detected in JNK1βHis6α, mMEKK, and JNK1β1His6α/mMEKK RSK-B co-transfectants, consistent with the negative co-precipitation data.

To confirm the role of the p38αMAPK and ERK1 pathways in RSK-B activation, HEK 293 cells co-transfected with RSK-B together with either p38αMAPKHis6/mMKK6 or ERK1His6/mMEKK1 were treated overnight with 10 μM FHIP1 or 10 μM PD098059, and RSK-B activity was investigated in MBP in-gel kinase assays of M2 precipitates of cell lysates (Fig. 4B). FHIP1 treatment practically abolished RSK-B activity in RSK-B+p38αMAPKHis6+mMKK6 co-transfectants. Interestingly, PD098059 treatment also caused a partial RSK-B inhibition in RSK-B+mMEK1+ERK1His6α co-transfectants, supporting a link between the ERK pathway and RSK-B.

In *Vivo* Substrates of RSK-B Point to a Role in Transcriptional Control—RSK-B in *vivo* substrates were investigated in reporter gene assays. First, HeLa cells were co-transfected with AP1- or CRE-dependent luciferase reporter constructs, and RSK-B with or without either mMKK6, mMEK1, or mMEKK. The cells were cultured for 2 days and lysed, and the lysates were assayed for luciferase activity. As expected, mMKK6, mMEK1, and mMEKK in the absence of RSK-B had various effects on luciferase activities (Fig. 5A, top panels). However, transfected RSK-B substantially enhanced AP1- and CRE-dependent luciferase responses, if the p38αMAPK pathway was activated by mMKK6 co-expression, whereas activation of the ERK and JNK pathways had less and no significant effects, respectively (Fig. 5A, bottom panels). None of the RSK-B mutants, RSK-B/K65A, RSK-B/K440A, or RSK-B/K65A+K440A, was active (Fig. 5B). The RSK-B activity depended on endogenous p38αMAPK, since treatment of mMKK6+RSK-B HeLa cell co-transfectants with 10 μM FHIP1 overnight or for 6 h practi-
Novel RSK Family CREB Kinase under p38 MAPK Control

(A) Graph showing relative luciferase activity with different treatments: control, FHP1, PD98059, MKK6, MKK6/FHP1, MKK6/PD98059. The bars represent different conditions with pAlter and RSK-B.

(B) Graph showing relative luciferase activity with unstimulated, TNF, and Mkk6 conditions. The bars represent different treatments: pAlter, RSK-B, RSK-B(K66A), RSK-B(K40A), RSK-B(S80C).

(C) Gel images showing CREB and Fos peptide in gel kinase with different treatments: RSK-B, RSK-1, RSK-2, RSK-3, p38-His+mMKK6. The molecular weights are indicated on the left.
cally abolished luciferase expression, whereas similar treatment with PD098059 enhanced luciferase activity (Fig. 5C).

RSK-B Is a New CREB Kinase—To more precisely define the functional link between RSK-B and luciferase responses, HeLa cells were transfected with RSK-B with or without mMKK6, together with expression vectors encoding a luciferase reporter (Fig. 6). A, RSK-B drives CREB-phosphorylation dependent reporter gene response. Left panel, HeLa cells were co-transfected with RSK-B (or empty pALTER vector) with or without mMKK6, together with the [Gal4]5-luc and Gal4-CREB reporter (1 μg) and cultured for 2 days. RSK-B and RSK-B + mMKK6-transfected cells were left untreated (control, MKK6), treated with 10 μM FHIP (FHIP, MKK6/FHIP) or 10 μM PD098059 (PD98059, MKK6/PD98059) for 6 h prior to lysis and luciferase assay. Right panel, HeLa cells transfected with RSK-B (or empty pALTER vector) together with the [Gal4]5-luc and Gal4-CREB reporter were cultured for 2 days and treated with either 10 μM FHIP, 100 ng/ml TNFα, or 100 ng/ml TNFα + 10 μM FHIP 6 h prior to lysis. Luciferase assays of cell lysates. A representative experiment (n = 2) is shown. B, luciferase response depends on stimulated RSK-B activity. HeLa cells were co-transfected with wild type or mutated RSK-B (or empty pALTER vector) together with the [Gal4]5-luc and Gal4-CREB reporter. The RSK-B pathway was activated by treating cells with 100 ng/ml TNFα or by mMKK6 co-transfection, or left unstimulated. Luciferase assays of cell lysates as indicated. A representative experiment (n = 2) is shown. C, RSK-B phosphorylates CREB and c-Fos peptides. Tagged RSK-B was precipitates from RSK-B + mMKK6-transfected HEK 293 cells and analyzed in kinase assays with CREB and c-Fos peptides as substrate. RSK1, 3 precipitates were included as controls.
gene controlled by GAL4 binding site repeats (GAL4-luc) and a GAL4-CREB activation domain fusion protein (GAL4-CREB). The cells were cultured for 2 days, lysed, and assayed for luciferase activity. The luciferase response was substantially enhanced in RSK-B- and mMKK6 co-transfectants (Fig. 6A). Treatment of the cells with 10 μM FHIP for 6 h or overnight completely abolished this response, whereas similar treatment with PD98059 had no significant effect. In parallel studies, HeLa cells were transfected with RSK-B and treated with TNFα for 6 h (Fig. 6A). TNFα markedly stimulated the luciferase response, which depended on p38MAPK activity as demonstrated by sensitivity to treatment of cells with 10 μM FHIP 30 min prior to TNFα. RSK-B activity was essential for this luciferase response, since inactive RSK-B mutants were ineffective in TNFα-stimulated and mMKK6 co-transfected cells (Fig. 6B). To demonstrate that CREB-dependent reporter activation can result from a direct interaction of RSK-B and CREB, RSK-B precipitates from HEK 293 cell transfectants were analyzed by CREBtide in-gel kinase assay (Fig. 6C), which revealed that RSK-B phosphorylated CREBtide and, in parallel studies, c-Fos peptide.

**Nuclear Location of RSK-B**—The potential nuclear localization signal toward the RSK-B C terminus prompted an investigation of the subcellular location of RSK-B by M2 immunostaining (Fig. 7). RSK-B in HEK 293 transfectants was exclusively nuclear. Interestingly, double staining for RSK-B and co-transfected p38MAPKHis6 showed that RSK-B and p38MAPKHis6 co-localized to the nucleus, whereas cells transfected with p38MAPKHis6 in the absence of RSK-B showed a broad and diffuse staining of nucleus and cytoplasm. Upon co-transfecting RSK-BAN1 and p38MAPKHis6, the C-terminal RSK-B domain and p38MAPKHis6 also co-localized to the nucleus, whereas the N-terminal domain RSK-BN1 remained in the cytoplasmic compartment. The co-transfection of p38MAPKHis6 was necessary, because the staining of endogenous p38MAPK was weak and did not allow to draw conclusions about subcellular distribution. A similar nuclear co-localization was observed between RSK-B and ERKHis6, but not with JNK1His6 in parallel immunostainings of respective transfectants (data not shown).

**DISCUSSION**

Here, we present a novel RSK family CREB kinase, RSK-B, which is under dominant control of p38MAPK. Other members of the RSK family, RSK1, -2, and -3, have been shown to phosphorylate CREB at serine 133, necessary for CREB-dependent transcriptional activation, in response to activation through the ERK pathway (37, 44). CREB phosphorylation through the p38MAPK pathway was also reported, and MAPKAPK2 was proposed as effector molecule even though the activity of MAPKAPK2 to phosphorylate CREB in vitro was questioned (37, 58). The activation of the p38MAPK pathway was found causally related to apoptosis induced by trophic factor withdrawal in PC12 cells and fibroblasts (20, 59) More recently, it was found in nerve growth factor-stimulated PC12 cells using kinase inhibitors that ERK and p38MAPK pathway signals activate CREB, and MAPKAPK2, or possibly MNK1, were discussed as potential effector molecules (37). While we cannot rule out that MAPKAPK2 or MNK1 indeed may have had such activity, the present data support the view that RSK-B, given its potent p38MAPK-dependent CREB kinase activity, may have in fact been the enzyme responsible for CREB activation in the previous studies (37). The activation of RSK-B through ERK1 is weaker than through p38MAPK, but this convergence of the p38MAPK and ERK pathways onto RSK-B is intriguing and reminiscent of similar convergence shown for the ternary complex factor (60). The targeted cloning of RSK-B used p38MAPK as probe, and all functional studies relate to p38MAPK transfections; at present it is not known if and to what extent RSK-B interacts with other p38MAPK isoforms.

RSK-B in addition to CREB is capable to activate other transcription factors as shown by its activity to drive AP1-dependent reporter genes. It cannot be ruled out that the AP-1 response resulted from indirect activation, e.g., the expression of the c-fos gene while being critically controlled by the serum response element also depends on CREB activation (61, 62). Although RSK-B did not phosphorylate c-Jun (data not shown) and, in general, c-Jun is not considered a substrate of RSK family members (23), a c-Fos peptide was a RSK-B substrate, consistent with the view that RSK-B may signal through c-Fos phosphorylation. The notion that RSK-B functions in transcriptional control is also consistent with its apparently exclusive nuclear location at the sensitivity of immunostaining. The other RSK family members show a stimulus-induced translocation to the cell nucleus (43). ERK and p38MAPK reside in the cytoplasm, and in cell body and dendrites of neuronal cells (42, 63), and they co-localize with these RSKs to the cell nucleus upon cell stimulation. This is consistent with the present finding that RSK-B, or RSK-BAN1, co-localized p38MAPK to the nuclear compartment; further studies must show whether the exclusive nuclear location of RSK-B is physiologic or results from transfection and overexpression.

The distinct pathway connectivities of RSK1, RSK2, RSK3, and RSK-B indicate that RSKs exert selective functions. This conclusion is further supported by the fact that marked differences in tissue and cell-specific RSK1, -2, and -3 expression have been reported (49). Furthermore, spontaneous mutations in the RSK2 gene, resulting in RSK2 proteins with impaired kinase activity, have been found to be associated with severe psychomotor retardation, facial and digital dysmorphisms, and skeletal deformations in Coffin-Lowry syndrome (64); neither RSK1 and RSK3 nor, presumably, RSK-B are capable to fully complement RSK2 in these patients. The role of MAPKs in brain and in cognitive functions was the focus of many recent studies (65–67). A fascinating role of MAPKs is emerging from studies of neuronal activity-dependent modification of synaptic connections in the adult nervous system (68, 69), and in long term facilitation in Aplysia (70). Although much evidence points to a role of p38MAPK pathways in cellular growth, toxicities, and inflammation, which all may involve RSK-B, the involvement of MAPKs and CREB in cognitive functions, and the link of RSK-B and CREB, is intriguing (70–73).

**Acknowledgments**—We gratefully acknowledge the excellent technical assistance of Monique Dellenbach and Christelle Bubendorff. We are indebted to Drs. R. Hochstrasser, G. Ju, M. Segato, U. Utans, and P. C. Wyss (all of Hoffmann-LaRoche) for help in confocal microscopy, gift of reagents, cloning, and photography.

**REFERENCES**

1. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556
2. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
3. Waskiewicz, A. J., and Cooper, J. A. (1995) Curr. Opin. Cell Biol. 7, 798–805
4. Gutkind, J. S. (1998) J. Biol. Chem. 273, 1839–1842
5. Boulot, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePina, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) Cell 65, 663–675
6. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Wootbett, J. R. (1991) Nature 353, 670–673
7. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) Nature 353, 670–673
8. Dejerard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Fong, Y. W. (1994) Cell 77, 1027–1037
9. Freshney, R. W., Ullrich, M., and Fong, Y. W. (1994) Cell 78, 1027–1037
10. Rouse, J., Cohen, P., Trigun, S., Morange, M., Alonso-Llamazares, A., and Nebreda, A. R. (1994) Cell 78, 1027–1037
11. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) Science 265, 808–811
12. Lee, C. J., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., and Landvatter, S. W, ...
Novel RSK Family CREB Kinase under p38 MAPK Control

Zhou, Y., Bjerbaek, C., Weremowicz, S., Morton, C. C., and Moller, D. J. (1995) Mol. Cell. Biol. 15, 4533–4536

Xiong, J., Muzyka, R. D., and Greenberg, M. E. (1996) Science 273, 959–963

Kalb, A., Bluthmann, H., Moore, M. W., and Lesslauer, W. (1996) J. Biol. Chem. 271, 28097–28104

da Silva, J., Pierrat, B., Mary, J.-L., and Lesslauer, W. (1997) J. Biol. Chem. 272, 28373–28380

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kozak, M. (1984) Nucleic Acids Res. 12, 857–872

Miller, D. E., Xia, C. H., Tang, W., Zhu, A. X., and Jakubowski, M. (1994) Am. J. Physiol. 266, 351–359

Johns, S. W., Erikson, E., Blinis, J., Maller, J., and Erikson, R. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3377–3381

Grove, J. T., Banerjee, P., Balusubramaniam, A., Coffey, P. J., Prise, D. J., Avruch, J., and Woodgett, J. R. (1991) Mol. Cell. Biol. 11, 5541–5550

Daly, K. N., Morrice, N., Caudwell, F. B., Avruch, J., and Cohen, P. (1998) J. Biol. Chem. 273, 1496–1505

Vik, T. A., and Ryder, J. W. (1997) Biochem. Biophys. Res. Commun. 235, 398–402

Stokoe, D., Caudwell, B., Cohen, P. T., and Cohen, P. (1993) Biochem. J. 296, 843–849

Eyres, P. A., Craxton, M., Morrice, N., Cohen, P., and Goedert, M. (1998) Chem. Biol. 5, 321–328

Alesi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494

Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P., and Com, M. J. (1996) EMBO J. 15, 4629–4642

Isakov, M., Winter, K., Ado, T., Schmid, W., Sachsenmaier, C., Engel, K., Gaestel, M., Rahmshofer, H. J., and Herrlich, P. (1997) EMBO J. 16, 1099–1102

Kummer, J. L., Rao, P. K., and Heidenreich, K. A. (1997) J. Biol. Chem. 272, 12655–12660

Price, M. A., Cruzalez, F. H., and Treisman, R. (1996) EMBO J. 15, 6552–6460

Vandekerckhove, J., Sassone-Corsi, P., and Verma, I. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9474–9478

Sheng, M., Dougan, S. T., McFadden, G., and Greenberg, M. E. (1988) Mol. Cell. Biol. 8, 2787–2796

Fiore, R. S., Bayer, V. E., Pelech, S. L., Posada, J., Cooper, J. A., and Baraban, J. M. (1993) Neuron 8, 463–472

Trivier, E., de Cesare, D., Jacquot, S., Pannetier, S., Zackai, E., Young, I., Monni, F. L., Sassone-Corsi, P., and Hanauer, A. (1996) Nature 384, 567–570

Murphy, T. H., Worley, P. F., and Baraban, J. M. (1991) Neuron 7, 625–635

Riscica, L., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994) Neuron 12, 1297–1221

Wilk-Blassczak, M. A., Stein, B., Xu, S., Barbosa, M. S., Cobb, M., and Belardetti, F. (1998) J. Neurosci. 18, 112–118

English, J. D., and Sweatt, J. D. (1996) J. Biol. Chem. 271, 24329–24332

English, J. D., and Sweatt, J. D. (1997) J. Biol. Chem. 272, 19103–19106

Martin, K. C., Michael, D., Rose, J. C., Barb, M., Casadio, A., Zhu, H., and Kandel, E. R. (1997) Neuron 18, 899–912

Martin, K. C., and Kandel, E. R. (1996) Neuron 17, 567–570

Frank, D. A., and Greenberg, M. E. (1994) Cell 79, 5–8

Deisseroth, K., Bitto, H., and Tien, R. W. (1996) Neuron 16, 89–101