The Mouse RACK1 Gene Is Regulated by Nuclear Factor-κB and Contributes to Cell Survival

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ABSTRACT

Receptor for activated C kinase 1 (RACK1) is a multifunctional, WD motif-containing protein important in regulating several cell surface receptors and intracellular protein kinases. To better understand its function, we cloned the mouse RACK1 gene and found it contains eight exons and seven introns, and maps to mouse chromosome 11B1.2-1.3. Promoter analysis identified NF-κB as an important transcription factor for promoter activity. In PC-12 cells, nerve growth factor (NGF), which activates nuclear factor-κB (NF-κB), maintained RACK1 levels and promoted cell survival in serum-free medium. Inhibitors of NF-κB activation blocked NGF-stimulated survival and RACK1 expression, whereas transgenic expression of RACK1 promoted survival in cells deprived of serum and NGF. Thus, RACK1 gene expression is induced by NF-κB and RACK1 contributes to NF-κB-mediated cell survival.

RACK1 is a 36-kDa protein that contains seven WD-domain motifs and is related to G protein β subunits (McCahill et al., 2002). Although originally found to act as a shuttling protein for activated PKC βII, RACK1 also binds and functionally interacts with PKCα, PKCε, the β1 subunit of GABA$_A$ receptors, the NR2B subunit of *N*-methyl-D-aspartate receptors, Src family members, integrin β subunits, the cAMP-specific phosphodiesterase E4D5, the common β chain of IL-5/IL-3/granulocyte macrophage-colony stimulating factor receptors, the β long subunit of type I interferon receptors, signal transducer and activator of transcription 1, and adenoviral E1A protein (for review, see McCahill et al., 2002). This capacity for interaction with diverse proteins suggests that RACK1 integrates signaling pathways with different physiological functions.

Several studies have found a correlation between RACK1 expression and cell growth. RACK1 levels are increased during angiogenesis in bovine corpora lutea, in cultured cord-forming aortic endothelial cells, and in blood vessels associated with human non–small-cell lung carcinomas (Berns et al., 2000). RACK1 expression is also increased in ovarian follicular cells during estrus when these cells proliferate

(Berns et al., 2000). Moreover, after ischemic injury, RACK1 levels are increased in regenerating renal tubular cells (Padanilam and Hammerman, 1997). In addition to playing a possible role in cell growth, RACK1 may also be involved in cell survival. For example, RACK1 interacts with adenoviral E1A proteins and protects mammalian cells from E1A-induced apoptosis (Sang et al., 2001). In addition, RACK1 protein levels are decreased in macrophages from aged rats in association with impaired TNF-α production, and TNF-α production is reduced in young macrophages treated with RACK1 antisense oligonucleotides (Corsini et al., 1999). RACK1 levels are also decreased in the cerebral cortex of aged rats in association with defective PKC translocation (Battaini et al., 1997) and are reduced in the frontal cortex of patients with Alzheimer's disease (Battaini et al., 1999). Therefore, decreased expression of RACK1 may contribute to cell senescence.

Because these correlations suggest that RACK1 levels modulate cell growth and survival, we cloned and characterized the mouse RACK1 gene and analyzed its promoter to identify signaling pathways that regulate RACK1 expression. We also manipulated RACK1 expression levels to directly examine its role in cell survival. Here, we report that the NF-κB transcription factor promotes expression of RACK1 and that RACK1 acts downstream of NF-κB in facilitating cell survival.

ABBREVIATIONS: RACK1, receptor for activated C kinase 1; PKC, protein kinase C; IL, interleukin; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; bp, base pair(s); BAC, bacterial artificial chromosome; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; kb, kilobase(s); PBS, phosphate-buffered saline; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; NGF, nerve growth factor; PDTC, pyrrolidine dithiocarbamate; EGFP, enhanced green fluorescent protein; Sp-1, stimulating protein-1; Z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

Materials and Methods

Cloning of Mouse RACK1 Genomic DNA. A BAC library of genomic DNA from SvJ1 embryonic stem cells (Genome Systems, St. Louis, MO) was screened with a 152-bp probe corresponding to the N-terminal part of mouse RACK1 cDNA. The probe was generated from mouse RACK1 cDNA by PCR with the following oligonucleotides: 5′-ATGACCGAGCAGATGACCCTTC-3′ and 5′-TTGGTCT-CATCTCTGGTCAG-3′. BLAST analysis showed that this probe has no significant homology with known sequences. Approximately 100 kb of plasmid DNA from one positive BAC clone was analyzed by restriction digestion by using BamHI, EcoRI, HindIII, NotI, PstI, XbaI, and XhoI, followed by Southern blot analysis with a 1.1-kb probe corresponding to most of the RACK1 cDNA. Five different BAC fragments were then subcloned into pBluescript II SK$^+$ (Stratagene, La Jolla, CA) to map 14 kb of genomic DNA and identify about 9 kb of genomic DNA sequence, including the promoter region. Subcloned DNA was sequenced by the dideoxy chain termination method by using a CEQ 2000 DNA analysis system (Beckman Coulter Inc., Fullerton, CA). DNA sequences were analyzed using MacVector 6.5.3 (Accelrys, San Diego, CA). Exon-intron boundaries were determined by identifying the differences between the genomic sequence and the cDNA sequence of mouse RACK1 (GenBank accession no. X75313). The last intron and exon were determined by PCR analysis by using genomic DNA from SvJ1 embryonic stem cells.

Primer Extension Analysis. Total RNA from NG108-15 cells was isolated using RNeasy (QIAGEN, Valencia, CA). A 35-mer primer was made corresponding to a sequence that is approximately 80 bp downstream from the transcription initiation site as predicted by software analysis (http://www.fruitfly.org/seq_tools/promoter.html). The primer sequence was 5′-GATTCCCATGATTCACTGCTGCT-CAAAGATAGCTG-3′ (DS184). The primer was end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase for 30 min at 37°C. Labeled primers were precipitated in ethanol and resuspended in 0.3 M sodium acetate. Primer (5×10^4 cpm) was annealed overnight to 45 μg of total RNA from NG108-15 cells and 45 μg of yeast tRNA as a control. cDNA was generated by incubating RNA with 50 units of monkey murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) at 42°C for 90 min. The RNA template was digested with RNase A, and the resulting products were precipitated in ethanol. The protected fragments were analyzed by electrophoresis in a 6% acrylamide gel containing 7 M urea (Invitrogen, Carlsbad, CA) at 1,000 V. The DNA sequencing ladder was prepared using the same primer to size the protected fragment.

Chromosome Localization of Mouse RACK1. Fluorescence in situ hybridization was performed by Genome Systems. DNA from a positive BAC clone containing the RACK1 sequence was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts in a solution containing 50% formamide, 10% dextran sulfate, and 2 × standard saline citrate. Specific hybridization signals were detected by incubating the hybridized samples on slides with fluoresceinated anti-digoxigenin antibodies, followed by counterstaining with 4,6-diamidino-2-phenylindole.

Northern Blot Analysis. The tissue distribution of mouse RACK1 mRNA was examined using a preblotted membrane (BD Biosciences Clontech, Palo Alto, CA). Each lane contained approximately 2 μg of purified poly A$^+$ RNA that was adjusted to a consistent β-actin signal. The RNA was run on a denaturing formaldehyde/1% agarose gel and blotted onto a positively charged nylon membrane. A 400-bp probe was generated by PCR corresponding to RACK1 cDNA (381–780 bp). The probe did not contain any significant homology with known sequences by BLAST analysis. The hybridization and washing conditions, as well as the deprobing conditions, were performed as recommended by the manufacturer.

Cell Culture and Transient Transfection Assays. NG108-15 cells were cultured in defined medium comprised of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (ratio 3:1), 2 mM glutamine, 0.1 mM hypoxanthine, 1.0 μM aminopterin, 12 mM thymidine, 25 mM HEPES, pH 7.4, trace elements [0.5 nM MnCl$_2$, 0.5 nM (NH$_4$)$_6$Mo$_7$O$_{24}$, 0.25 nM SnCl$_4$, 25 nMNa$_3$VO$_4$, 5 nM CdSO$_4$, 0.25 nM NiSO$_4$, 15 nM H$_2$SeO$_3$, 25 nM Na$_2$SiO$_3$], bovine insulin (5 μg/ml); human transferrin (50 μg/ml), and oleic acid (10 μg/ml) complexed with fatty acid-free bovine serum albumin (2 mg/ml). Cells were maintained at 37°C in 10% CO$_2$ and plated for transient transfection on 60-mm dishes at a density of 4 to 8×10^5 cells/dish. They were transfected once they became 70 to 90% confluent (~72 h after plating) by using Superfect (QIAGEN) and 5 μg of plasmid DNA. After transfection, cells were changed to growth medium containing Dulbecco's modified Eagle's medium with 10% serum plus medium supplement (JRH Biosciences, Lenexa, KS) for 24 h, after which time they were prepared for luciferase assay.

Reporter Gene Construction and Luciferase Assays. Reporter plasmids were constructed using the promoter-less luciferase reporter vector, pGL3 basic (Promega, Madison, WI). Six different serial deletion fragments were generated using *Pfu* DNA polymerase chain reaction (Stratagene, La Jolla, CA). Each fragment was created with Kpn 1 (5′ end) and Xba 1 (3′ end) restriction enzyme sites and was subcloned into the Kpn 1 and Nhe 1 site in the pGL3 basic vector. The primer sequences used are as follows: DS170, 5′-GGGGTACCCCTAGAGGCTCTGGTTGTGTTTAGGGC-3′; DS171, 5′-GGGGTACCCCTAGCATGTGGGAGTTCACCCATAG-3′; DS172, 5′-GGGGTACCCCGCTTCAGAATGCATTTGTACAGCGC-3′; DS173, 5′-GGGGTACCCCGCCTTGCTCTCCGAGTATTTCCGG-3′; DS174, 5′-GCTCTAGAGCGGAGTGGTGGCGATCTGTGTAACC-3′; and DS175, 5′-GCTCTAGAGCGCTGTACAAATGCATTCTGAAGCC-3′. The PCR generated constructs shown in Fig. 1 are as follows: pGL3 R1, DS170/DS174 (−1208 to +341, the transcription initiation site numbered as +1); pGL3 R2, DS171/DS174 (−899 to +341); pGL3 R3, DS172/DS174 (−22 to +341); pGL3 R4, DS173/DS174 (+113 to +341); pGL3 R5, DS170/DS175 (−1208 to +4); and pGL3 R6, DS171/DS175 (−899 to +4).

The construct lacking the putative NF-κB binding site in pGL3 R3 was prepared by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene). The oligonucleotides used for site-directed mutation were as follows: DS-276, 5′-GAGCAGCAGT-GAATCATGATCCCCGCCTTGCTCTCCG-3′ and DS-277, 5′-CG-GAGAGCAAGGCGGGGATCATGATTCACTGCTGCTC-3′. Cells were transfected with one of the constructed vectors or the pGL3 basic vector. Cell lysis and measurement of luciferase activity was performed using the luciferase assay system (Promega). Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Preparation of Nuclear Protein Extracts. Nuclear extracts were prepared from NG108-15 cells as described previously (Dignam et al., 1983) with minor modification. Cells were collected when cultures were confluent and then were washed in ice-cold PBS. Cells were lysed in 5 volumes of 10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mix (40 μg/ml aprotinin, 40 μg/ml leupeptin, and 25 μg/ml soybean trypsin inhibitor) on ice for 10 min. Cells were homogenized and resuspended in 5 volumes of 20 mM HEPES-KOH, pH 7.9, 25% glycerol (v/v), 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.42 M KCl, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mix. After being shaken at 4°C for 1 h and centrifuged at 20,000*g* for 10 min. The soluble extracts were separated from pellet debris and stored at −80°C until analysis by electrophoretic mobility shift assays.

Electrophoretic Mobility Shift Assays (EMSA). To detect NF-κB binding activity, we used an oligonucleotide probe that contains a potential NF-κB binding site and has the sequence 5′-TCAT-GGGAATCCCCGCCTTGCTCTCC-3′ (+103 to +128). Oligonucleotides were annealed, end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, and purified using Chroma Spin columns (BD Biosciences Clontech) before EMSA. For EMSA, 10 μg of nuclear

extract was incubated in 20 μl of mobility shift buffer (12.5 mM HEPES-KOH, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 200 ng of poly dIdC as a nonspecific competitor, 1 μg of bovine serum albumin, and 2×10^5 dpm of probe. To demonstrate specificity, nuclear extract was also incubated with a competitor oligonucleotide specific for binding NF-κB subunits (5′-AGTTGAGGGGACTTTCCCAGGC-3′) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). To identify the transcription factors present in the protein-DNA binding complexes, anti-p65 or anti-p50 (provided by Dr. Warner Greene, Gladstone

Institute, University of California, San Francisco), or anti-Sp1 (Santa Cruz Biotechnology Inc.) antibodies were added to the binding reactions. Protein-DNA complex formation and antibody supershifts were analyzed by 5% nondenaturing polyacrylamide gel electrophoresis and autoradiography.

Cell Viability Studies. PC-12 cells were plated at 5×10^5 cells/well in 35-mm six-well Falcon tissue culture flasks in DMEM containing 5% fetal bovine serum, 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin and were cultured in a humidified atmosphere containing 10% CO_2, 90%
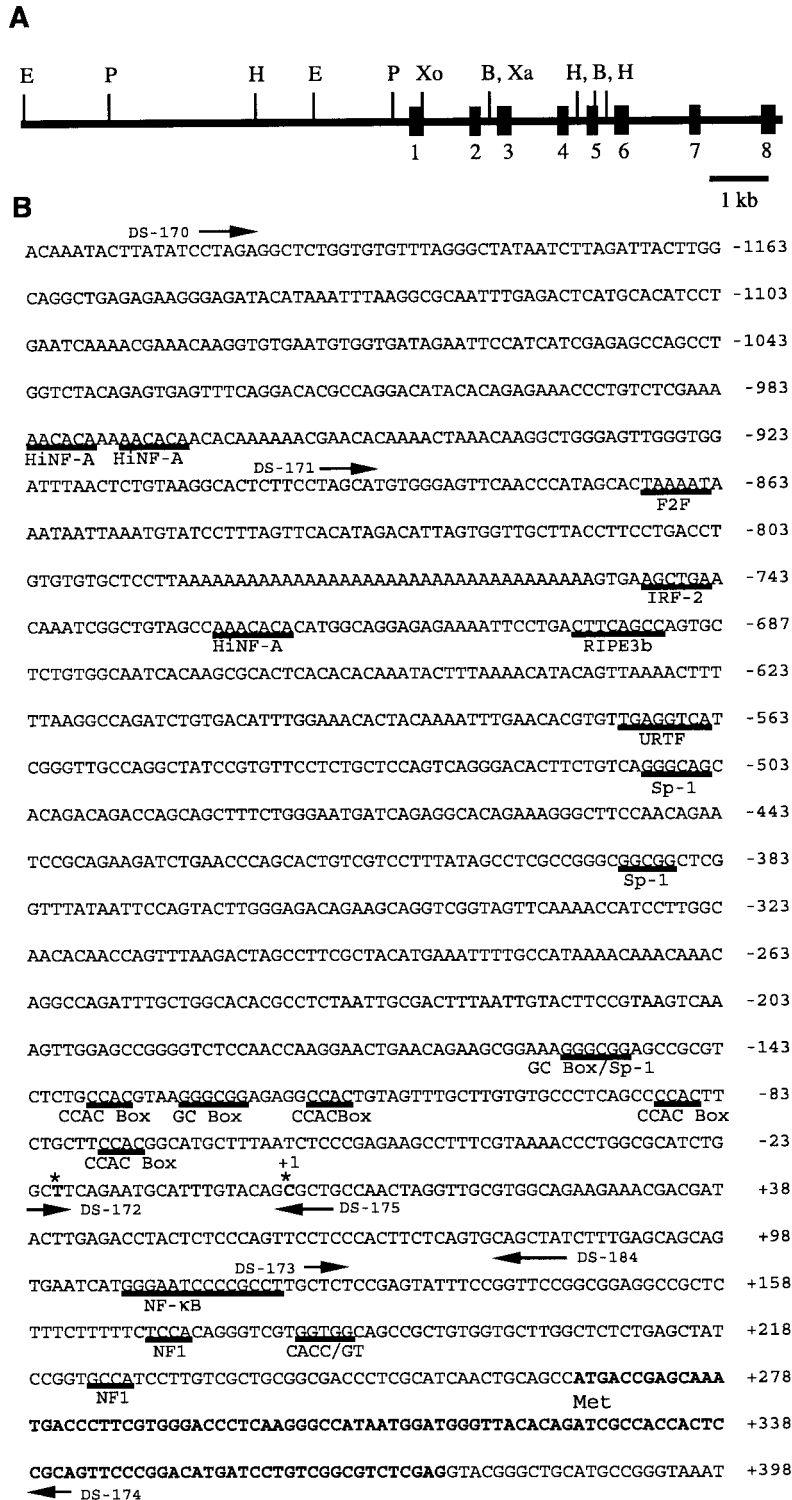

Fig. 1. Genomic structure of the mouse RACK1 gene. A, diagram of the mouse RACK1 gene showing eight exons (black boxes) and seven introns. Restriction enzyme sites are labeled as E, EcoRI; P, PstI; H, HindIII; Xo, XhoI; B, BamHI; and Xa, XbaI. B, putative promoter elements in the 5′-untranslated region of the mouse RACK1 gene. The nucleotide sequence is numbered with the major transcription start site as +1. The translation start sites (T and C) are designated with asterisks and boldface, and the translated nucleotide sequence in the first exon is indicated in boldface. Arrows indicate primers used to generate deletion constructs for analyzing 5′-flanking regions (DS170-DS175) and for primer extension experiments to identify transcription start sites (DS184). Putative regulatory elements are underlined. HiNF-A, histone nuclear factor-A; F2F, footprint II factor; IRF-2, interferon regulatory factor-2; RIPE3b, rat insulin promoter element 3b; URTF, urokinase transcription factor; NF1, nuclear factor 1.

air. After 2 days, cells were rinsed gently five times with serum free medium containing DMEM, 2 mM glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin, and then were cultured in serum-free medium without or with NGF (50 ng/ml), pyrrolidine dithiocarbamate (PDTC) (400 μM), SN50 (40 μM), scrambled SN50 (40 μM), or pan-caspase inhibitor Z-VAD-FMK (50 μM) for 24 h. PDTC and peptides were added 1 h before NGF treatment. Plates were gently rinsed with medium to remove dead cells, and remaining cells were incubated with Ca^{2+}-Mg^{2+}-free PBS for 1 min, removed from the wells, and counted using a hemocytometer. Viability was determined by trypan blue exclusion.

RACK1 Protein Expression. PC-12 cells were cultured as described for cell viability studies. Cultures were then washed with PBS and scraped from the plate in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM EGTA, 25 μg/ml each of leupeptin and aprotinin, 10 μg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride). Concentrated SDS gel sample buffer was added to a final concentration of 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromphenol blue, and 10% glycerol. Samples were boiled at 95°C for 5 min. Genomic DNA was disrupted using a 23-gauge needle and then samples were centrifuged at 10,000g for 5 min. Samples (20 μg each) were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels. Proteins were transferred to nitrocellulose membranes, which were incubated with anti-RACK1 antibody (BD Transduction Laboratories, Lexington, KY) for 12 h at 1:500 dilution. Immunoreactive bands were detected by enhanced chemiluminescence after incubation with anti-mouse IgM-peroxidase secondary antibody (Roche Diagnostics, Mannheim, Germany). RACK1 immunoreactivity was normalized to β-actin immunoreactivity detected on the same gel by using mouse anti-β-actin antibody (1:500 dilution; Sigma-Aldrich, St. Louis, MO).

NF-κB p50 DNA Binding Assay. Nuclei were extracted from PC-12 cells by using a nuclear extract kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions. DNA binding of active p50 was detected using an EZ-Detect transcription factor kit from Pierce Chemical (Rockford, IL) also according the manufacturer's instructions. This method detects active NF-κB p50 by using a synthetic oligonucleotide containing a consensus NF-κB binding site. The kit uses streptavidin-coated 96-well plates with the bound biotinylated oligonucleotide. Active NF-κB p50 binds to the consensus sequence and is then incubated with anti-p50 antibody and detected with a secondary horseradish peroxidase-conjugated antibody by using a chemiluminescent substrate and a luminometer.

Transfection of RACK1-EGFP. RACK1 cDNA was subcloned into pEGFP-N3 (BD Biosciences Clontech) to generate a vector for expression of a RACK1-EGFP fusion protein. PC-12 cells were cultured in polyornithine-treated six-well plastic cell culture plates at 1×10^5 cells/well in DMEM containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 150 μg/ml streptomycin at 37°C in a humidified atmosphere of 90% air, 10% CO_2. Cells were transfected 1 day later when using Trans IT-LT1 (Mirus, Madison, WI) according to the manufacturer's directions. After 2 days, the cells were cultured in serum-free medium for 1 to 6 days. Cultures were then rinsed in PBS and the number of surviving, EGFP-expressing cells was determined by fluorescence microscopy. The total number of cells in the same microscope field was determined by phase contrast microscopy. On average, 300 cells were examined for each experimental condition.

Results

Structure of the Mouse RACK1 Gene. The mouse RACK1 gene was identified within a 14-kb region of mouse genomic DNA (Fig. 1A; GenBank accession no. AF295529) and found to contain eight exons and seven introns. Using the "signal scan" program (http://bimas.dcrt.nih.gov/molbio/signal/), we identified several putative *cis*-acting elements in

1.5 kb of the proximal 5′-flanking sequence, including a CCAC box and sites for histone nuclear factor-A, footprint II factor, interferon regulatory factor-2, rat insulin promoter element 3b, urokinase transcription factor, Sp-1, and NF-κB transcription factors (Fig. 1B). Primer extension analysis identified two distinct transcription start sites at 265 and 285 nucleotides upstream of the translation initiation site (Fig. 2). Because the shorter mRNA species was more abundant (Fig. 2) and was also predicted by software analysis (http://www.fruitfly.org/seq_tools/promoter.html), we designated the cytosine residue at this site as nucleotide +1. Although no TATA box was predicted in proximity to either transcription start site, two GC box/Sp-1 sites and four CCAC-binding protein boxes (Smale, 1997) were found within the 150-bp upstream region. This indicates that RACK1 transcripts are driven by a TATA-less, GC-rich promoter region.

Chromosomal Localization and Expression of Mouse RACK1. In situ hybridization with the ~100-kb BAC clone containing the RACK1 gene resulted in labeling of the middle
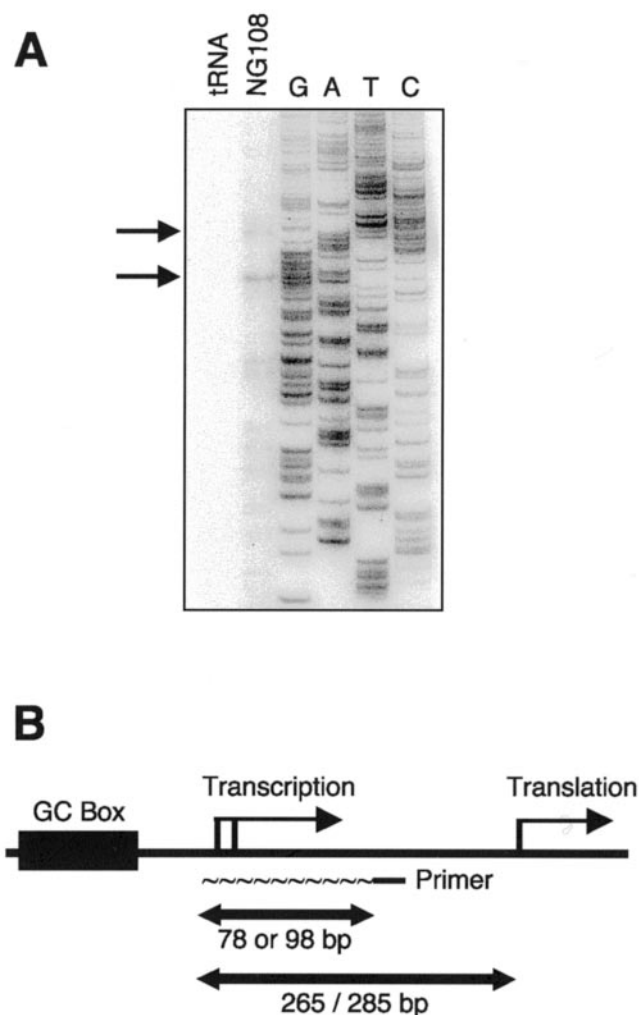

Fig. 2. Primer extension analysis. A, primer extension (NG108) and sequencing reaction products from total RNA isolated from NG108-15 cells. In the first lane tRNA was used as a negative control. Arrows indicate the sites of initiation of transcription. B, schematic diagram of the transcription start sites located 265 and 285 bp upstream of translation initiation site. Also shown is the location of primer DS184 used in the primer extension reaction relative to the transcription initiation sites.

region of chromosome 11 (Fig. 3A). Analysis of 10 specifically hybridized chromosomes demonstrated that the RACK1 gene is located at a position 34% of the distance from the heterochromatic-euchromatic boundary to the terminus of chromosome 11, an area that corresponds to band 11B1.2-1.3 (Fig. 3A). We confirmed this chromosomal location in the most recent draft sequence of the mouse genome (Ensembl gene ID = ENSMUSG00000020372). In the human genone, RACK1 is located on chromosome 5q35.3 (180.7 Mb) (Ensembl gene ID = ENSG00000146092). The location of RACK1 has not been determined in the rat genome. However, syntenic analysis and nearby gene markers from the mouse (Zfp 345a) and human (ZNF345A) genomes suggest that RACK1 resides on rat chromosome 10q 21 (http://www.informatics.jax.org/reports/homologymap).

Northern analysis showed that 1.35-kb RACK1 mRNA was present in all tissues examined (Fig. 3B), with the highest levels in liver, heart, and kidney; moderate levels in testis, spleen, lung, and brain; and a very low level in skeletal
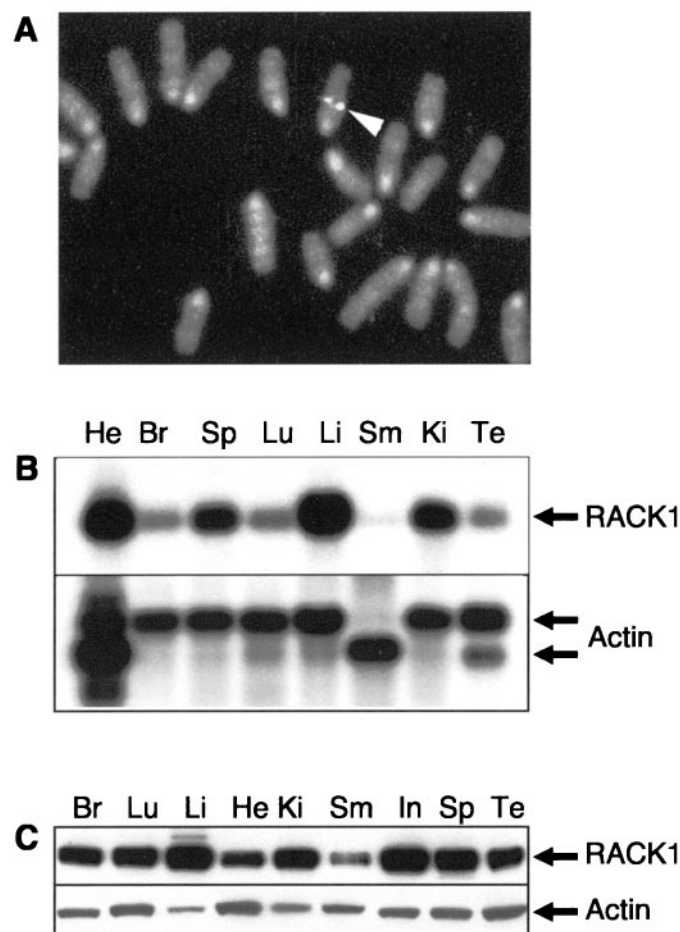
muscle. RACK1-like immunoreactivity was detected in the same tissues (Fig. 3C), although the relative abundance of mRNA and protein varied, suggesting independent rates of RACK1 protein and mRNA turnover in different tissues.

Functional Characterization of the RACK1 Promoter. To analyze proximal promoter elements, we generated a series of deleted RACK1 promoter/luciferase gene reporter constructs from the 5′-flanking region of RACK1 and used these to transfect NG108-15 cells (Figs. 1B and 4). Construct pGL3 R3 (R3), which contains an NF-κB element, produced the highest level of luciferase activity, whereas deletion of sites between −22 and +113 in pGL3 R4 markedly reduced this activity (Fig. 4). Further deletion of the proximal promoter region between +4 and +342 (constructs pGL3 R5 and pGL3 R6) abolished luciferase activity. To examine whether the proximal NF-κB site is important for RACK1 expression, we used site-directed mutagenesis to delete the three core nucleotides (GGA) from the sequence GGGAATCCCC to generate the construct pGL3 R3$^{\Delta \text{ NF-}\kappa \text{B}}$ (R3-κB). This construct produced a level of activity similar to that observed with the pGL3 R4 deletion construct (Fig. 4). These results suggest that the NF-κB binding site is an important positive regulatory element for the basal promoter activity of the mouse RACK1 gene.

NF-κB Binds to the RACK1 Promoter. Gel mobility shift assays revealed that an oligonucleotide spanning nucleotides +103 to +128 of the RACK1 promoter region containing the NF-κB binding site formed a protein-DNA complex when incubated with nuclear extracts from NG108-15 cells (Fig. 5). This protein-DNA complex was specific because the shifted bands were decreased by the presence of 50- to 200-fold excess of unlabeled NF-κB binding oligonucleotide (Fig. 5A). Protein-DNA complex bands were supershifted when antibodies against the NF-κB subunits p65 or p50 were added to the reaction, whereas addition of IgG or an anti-Sp-1 antibody did not produce a supershifted band (Fig. 5B). Anti-p50 supershifted more of the complex than did anti-p65. A control NF-κB probe (Santa Cruz Biotechnology Inc.) produced similar results (data not shown). These results suggest that NF-κB interacts with the RACK1 promoter in a region that is critical for expression of RACK1.
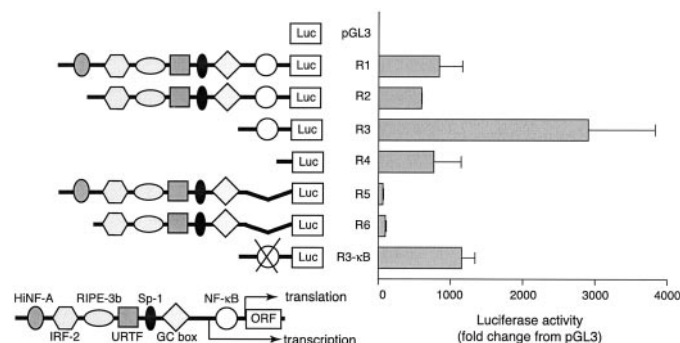

Fig. 3. Chromosomal localization and tissue expression of RACK1. A, fluorescence in situ hybridization mapping with a digoxigenin dUTP labeled BAC-RACK1 clone. A total of 80 metaphase cells were analyzed with 74 exhibiting specific labeling. Arrows indicate the position of the RACK1 gene in chromosome 11B 1.2-1.3. This location was confirmed using a probe specific for the telomeric region of chromosome 11, which cohybridized with the RACK1 BAC clone (data not shown). B, mouse multitissue Northern blot incubated with a RACK1-specific probe, stripped, and then incubated with a probe for β-actin. C, Western blot of RACK1 and β-actin immunoreactivity from various mouse tissues. Br, brain; Lu, lung; Li, liver; He, heart; Ki, kidney; Sm, skeletal muscle; In, intestine, Sp, spleen; Te, testis.

Fig. 4. Functional analysis of the 5′-flanking region of the mouse RACK1 gene. Deletion and site-directed mutagenesis analysis of the promoter region were performed with transfected NG108-15 cells. The structures of the RACK1 promoter-luciferase (Luc) reporter deletion series are shown schematically, and their relative luciferase activities in NG108-15 cells are shown at right. Each value was normalized for total protein and divided by pGL3 basic vector luciferase activity. Results are mean ± S.E. values from two experiments, each performed in triplicate.

RACK1 Promotes PC-12 Cell Survival in Serum-Free Medium. PC-12 cells rapidly undergo apoptosis in the absence of serum (Sarmiere and Freeman, 2001). NGF activates NF-κB in PC-12 cells and this is necessary for NGF-induced survival of PC-12 cells in the absence of serum (Carter et al., 1996). Because NF-κB regulates RACK1 gene expression, we examined PC-12 cells to determine whether RACK1 functions downstream of NF-κB to promote cell survival. Culture of PC-12 cells in the absence of serum for 24 h markedly decreased cell survival (Fig. 6A), but this was prevented by treatment with NGF or with the general caspase inhibitor Z-VAD-FMK (Calbiochem, San Diego, CA) which prevents apoptosis in serum-deprived PC-12 cells (Sarmiere and Freeman, 2001). The inhibitory protein IκB sequesters the majority of inactivated NF-κB in the cytosol. The metal chelator and antioxidant PDTC inhibits IκB proteolysis, thereby preventing activation and translocation of NF-κB to the nucleus (Beauparlant and Hiscott, 1996). The cell membrane-permeable peptide SN50 inhibits nuclear translocation of NF-κB, preventing NF-κB-mediated gene expression (Lin et al., 1995). PDTC and SN50 reduced survival in NGF-treated cultures to levels observed in cells cultured in serum-free medium alone (Fig. 6, A and B). However, neither PDTC nor SN50 reduced survival in cultures treated with Z-VAD-FMK. Culture in serum-free medium also decreased RACK1-like immunoreactivity and this was prevented by treatment with NGF (Fig. 6C). PDTC (Fig. 6C) and SN50 (Fig. 6D) reduced RACK1-like immunoreactivity in NGF-treated cultures to levels observed in cells cultured in serum-free medium alone. A scrambled SN50 peptide had no effect on cell survival or RACK1 immunoreactivity in NGF-treated cultures (Fig. 6, B and D). The preservation of RACK1 immu-
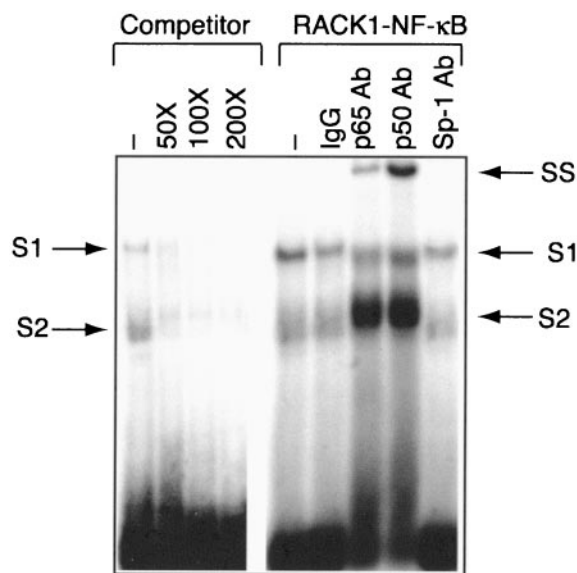

Fig. 5. Electrophoretic mobility shift analysis of the RACK1 promoter sequence. Nuclear extracts from NG108-15 cells were incubated with a ^{32}P end-labeled oligonucleotide (5′-TCATGGGAATCCCCGCCTT-GCTCTCC-3′) containing the putative NF-κB binding site from the RACK1 promoter (~+103 to +128 in Fig. 1B) in the presence (left) or absence (right) of increasing amounts of an unlabeled competitor oligonucleotide sequence specific for NF-κB. The extracts were then analyzed by electrophoresis on 5% polyacrylamide gels as described under *Materials and Methods*. S1 and S2 designate shifted bands; SS designates a supershifted band detected in the presence of antibodies to p65 or p50 NF-κB subunits.
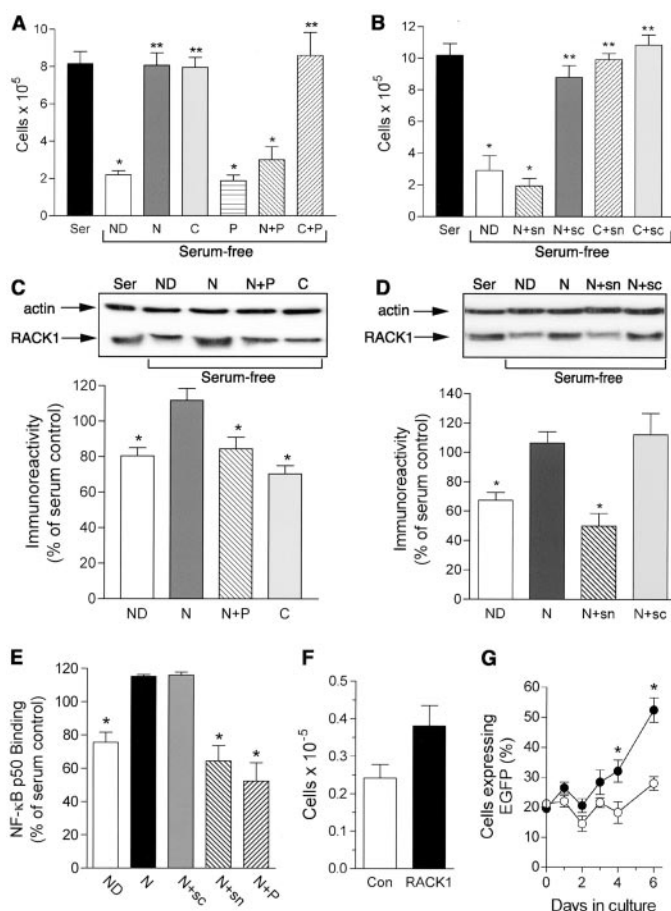

Fig. 6. RACK1 in NGF-mediated cell survival. A, number of PC-12 cells in cultures exposed to serum-containing medium for 2 days, followed by culture for 24 h in fresh serum-containing medium (Ser), serum-free medium without drugs (ND), or serum-free medium containing 50 ng/ml NGF (N), 50 μM Z-VAD-FMK (C), 400 μM PDTC (P), or a combination of these agents. There was an effect of treatment ($F_{6,91} = 28.4$, $p < 0.0001$). ∗, $p < 0.05$ compared with Ser and ∗∗, $p < 0.05$ compared with ND (Tukey test). B, number of cells in cultures exposed to serum-containing medium for 2 days, followed by culture for 24 h in Ser, ND, or serum-free medium containing NGF plus 40 μM SN50 (N+sn), NGF plus scrambled SN50 (N+sc), Z-VAD-FMK plus SN50 (C+sn), or Z-VAD-FMK plus scrambled SN50 (C+sc). There was an effect of treatment ($F_{5,30} = 31.13$, $p < 0.0001$). ∗, $p < 0.05$ compared with Ser and ∗∗, $p < 0.05$ compared with ND (Tukey's test). C, mean ± S.E.M. values from western blots of RACK1-like immunoreactivity in cells cultured for 24 h in ND or with 50 ng/ml NGF (N), NGF plus 400 μM PDTC (N+P), or 50 μM Z-VAD-FMK (C). Values were normalized to immunoreactivity measured in cells cultured for 24 h in serum. There was an effect of treatment ($F_{3,73} = 10.86$, $p < 0.0001$). ∗, $p < 0.05$ compared with NGF-treated cells (Newman-Keuls test). Top, representative Western blot with bands labeled as in the figure; the far left lane (Ser) was from cells cultured for 24 h in the presence of serum. D, mean ± S.E.M. values from western blots of RACK1-like immunoreactivity in cells cultured for 24 h in ND, or with N, N+sn, or N+sc. There was an effect of treatment ($F_{3,27} = 7.83$, $p = 0.0006$). ∗, $p < 0.01$ compared with NGF-treated cells (Newman-Keuls test). Data are mean ± S.E. values from five to nine experiments. Top, representative Western blot. E, binding of active NF-κB p50 to a synthetic oligonucleotide containing a consensus NF-κB binding site, detected in nuclear extracts from PC-12 cells cultured for 24 h in ND, or with N alone, N+sc, N+sn, or N+P. There was an effect of treatment ($F_{4,10} = 17.50$, $p = 0.0002$). ∗, $p < 0.01$ compared with NGF treated cells by Newman-Keuls test; $n = 3$ for all conditions. F, PC-12 cells were transfected with EGFP vector (Vector) or with RACK1-EGFP (transfection efficiency $17 ± 1\%$; $n = 12$), and then cultured in serum-free medium for 6 days. There were more cells in the cultures transfected with RACK1-EGFP ($p < 0.05$, two-tailed, unpaired t test). G, number of EGFP-expressing cells was calculated as a percentage of all surviving cells. There was an effect of treatment ($F_{1,88} = 22.6$, $p < 0.0001$) and day ($F_{5,88} = 14.1$, $p < 0.0001$), and an interaction between treatment and day ($F_{5,88} = 4.4$, $p = 0.0012$). ∗, $p < 0.05$ compared with EGFP-transfected cells on same test day (Bonferroni's test).

noreactivity by NGF treatment was not merely because of increased cell survival because RACK1-like immunoreactivity was not increased by Z-VAD-FMK, which prevented cell death in serum-free medium (Fig. 6C). To investigate whether these concentrations are sufficient to inhibit NF-κB in PC-12 cells, we measured activation of NF-κB p50 in nuclear extracts by using a commercially available DNA binding assay (Renard et al., 2001). In NGF-treated cells, PDTC and SN50, but not scrambled SN50, reduced NF-κB p50 DNA binding by about 50% compared with levels observed in cells treated with NGF alone (Fig. 6E). These findings indicate that NGF stimulation of NF-κB maintains expression of RACK1 in the absence of serum and suggest a role for RACK1 in NGF-mediated cell survival.

To examine whether RACK1 is sufficient to promote cell survival, we transfected PC-12 cells with a vector encoding a RACK1-EGFP fusion protein. RACK1-EGFP increased the number of cells surviving after serum withdrawal by 1.5-fold compared with cultures transfected using an EGFP vector (Fig. 6F). Moreover, 6 days after transfection, the percentage of surviving cells expressing EGFP was 2-fold greater in cultures transfected with RACK1-EGFP compared with cultures transfected with EGFP alone (Fig. 6G). These findings indicate that increased expression of RACK1 promotes cell survival in the absence of serum or NGF.

Discussion

There is nearly complete protein sequence identity between mouse, human (Guillemot et al., 1989), rat (Ron et al., 1994), bovine (Berns et al., 2000), and porcine (Chou et al., 1999) RACK1. Our functional promoter analysis and gel mobility shift assays revealed that NF-κB, acting through a binding site (GGGAATCCCC) at nucleotides +106 to +115, is an important positive regulator of RACK1 promoter activity. Recently, porcine RACK1 was cloned and seems to have an exon/intron structure similar to mouse RACK1 with eight exons and seven introns (Chou et al., 1999). The porcine promoter contains a putative NF-κB binding site GGGTATTTCC at nucleotides −137 to −128 relative to the translation start codon, suggesting that NF-κB regulates RACK1 expression in multiple species.

The NF-κB/rel family of transcription factors is activated by many stimuli, including growth factors, cytokines, UV irradiation, and oxidative stress (Roman et al., 2000). Our results predict that RACK1 expression would also be activated by these stimuli. Indeed, we found that NGF stimulates RACK1 expression in serum-deprived PC-12 cells. In many cells, activation of NF-κB is also an important consequence of PKC activation though the particular PKC isozyme involved varies among different cell types. Dissociation of IκB from the inactive NF-κB complex occurs when IκB is phosphorylated, allowing the free NF-κB complex to be translocated into the nucleus to activate target genes. IκB kinases that phosphorylate IκB are positively regulated by atypical PKCs (PKCλ/ι and PKCζ) (Lallena et al., 1999) and embryonic fibroblasts from mice lacking PKCζ show impaired NF-κB activation in response to TNF-α, IL-1, or lymphotoxin β receptor activation (Leitges et al., 2001). In contrast, in mature T lymphocytes, PKCθ couples the T cell antigen receptor signaling complex to the activation of NF-κB (Sun et al., 2000). In adipocytes, suppression of PKCα expression is

associated with decreased ability to activate NF-κB in response to phorbol ester (McGowan et al., 1996). Macrophages from mice lacking PKCε show decreased activation of NF-κB after treatment with lipopolysaccharide (Castrillo et al., 2001). Finally, PKCη seems to be required for LPS-induced activation of NF-κB in cultured astrocytes (Chen et al., 1998). These findings suggest an interesting cross talk mechanism whereby PKCs acting through NF-κB regulate RACK1 expression, which could in turn regulate PKC activity. Such cross talk may occur in the mouse heart where overexpression of PKCε increases RACK1 levels and PKCβII-RACK1 interactions in association with cardiomyopathy (Pass et al., 2001). It is not yet known whether this occurs via PKCε-induced activation of NF-κB, although this is possible because overexpression of PKCε increases NF-κB DNA binding activity in cultured rabbit cardiomyocytes (Li et al., 2000).

Our studies with NGF-treated PC-12 cells provide evidence that RACK1, acting downstream of NF-κB, functions as a survival factor in a neural cell line. NF-κB regulates the expression of several genes involved in cellular responses to injury, many of which serve neuroprotective roles such as TNF-α, TRAF1 and 2, IL-6, the β-amyloid precursor protein calbindin 28k, inhibitor of apoptosis proteins, manganese superoxide dismutase, and Bcl-2 family members (Mattson et al., 2000; Martindale and Holbrook, 2002). Our findings suggest that RACK1 constitutes an additional member of this group of genes. That RACK1 may also play a neuroprotective role in the brain is suggested by the finding that both RACK1 (Battaini et al., 1999) and NF-κB (Kaltschmidt et al., 1999) are reduced around senile plaques in brains of patients with Alzheimer's disease. Therefore, it is possible that reduced RACK1 expression and impaired PKC signaling in Alzheimer's disease brains (Battaini et al., 1999) is related to a deficit in NF-κB activity. In certain cellular contexts, NF-κB can instead promote cell death possibly by enhancing the expression of death-related gene products such as Fas ligand and p53 (Martindale and Holbrook, 2002). We do not yet know whether RACK1 can also mediate cytotoxic responses to NF-κB.

Some RACK1 binding proteins are known to be regulated by NF-κB or to interact with NF-κB signaling pathways. For example, Stat1 antagonizes NF-κB signaling in mouse primary fibroblasts (Wang et al., 2000) and human 2fTGH fibroblasts (Wang et al., 2000; Ganster et al., 2001), suggesting a mechanism by which RACK1 could function in feedback inhibition of NF-κB. A negative feedback pathway involving RACK1 may also be involved in TNF-α-mediated inhibition of neuronal N-methyl-D-aspartate receptors through NF-κB (Furukawa and Mattson, 1998) because N-methyl-D-aspartate receptor function is inhibited by RACK1 (Yaka et al., 2002). RACK1 may also participate in a feedback pathway involving Src because Src can stimulate NF-κB activation (Wooten et al., 2001), whereas Src binding to RACK1 inhibits Src kinase activity (Chang et al., 1998). Further work is required to prove whether RACK1 plays a role in such feedback pathways and whether other RACK1 binding partners are also regulated by NF-κB.

Although overexpression of RACK increased survival in serum-free medium, this effect was apparent only after 4 days in culture, suggesting that long-term effects on gene expression rather than rapid signaling events are involved in RACK1-induced survival. Recent evidence indicates that

RACK1 can regulate gene expression. For example, activation of PKA can induce movement of RACK1 to the nucleus in cultured cells (He et al., 2002) and dissociated hippocampal neurons (Yaka et al., 2003). This is associated with RACK1-dependent expression of mRNA for brain-derived neurotrophic factor (Yaka et al., 2003) and for the type 1 receptor for pituitary adenylate-cyclase-activating polypeptide (He et al., 2002), which can act as a neurotrophic factor in the central nervous system (Vaudry et al., 2002). Thus, RACK1 could promote cell survival by inducing gene expression of specific neurotrophic factor ligands and receptors.

Acknowledgments

References

Battaini F, Pascale A, Lucchi L, Pasinetti GM, and Govoni S (1999) Protein kinase C anchoring deficit in postmortem brains of Alzheimer's disease patients. *Exp Neurol* **159:**559–564.

Battaini F, Pascale A, Paoletti R, and Govoni S (1997) The role of anchoring protein RACK1 in PKC activation in the ageing rat brain. *Trends Neurosci* **20:**410–415.

Beauparlant P and Hiscott J (1996) Biological and biochemical inhibitors of the NF-kappa B/Rel proteins and cytokine synthesis. *Cytokine Growth Factor Rev* **7:**175–190.

Berns H, Humar R, Hengerer B, Kiefer FN, and Battegay EJ (2000) RACK1 is up-regulated in angiogenesis and human carcinomas. *FASEB J* **14:**2549–2558.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhäuser N, Böhm-Matthaei R, Baeuerle PA, and Barde YA (1996) Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. *Science (Wash DC)* **272:**542–545.

Castrillo A, Pennington DJ, Otto F, Parker PJ, Owen MJ, and Bosca L (2001) Protein kinase Cepsilon is required for macrophage activation and defense against bacterial infection. *J Exp Med* **194:**1231–1242.

Chang BY, Conroy KB, Machleder EM, and Cartwright CA (1998) RACK1, a receptor for activated C kinase and a homolog of the beta subunit of G proteins, inhibits activity of src tyrosine kinases and growth of NIH 3T3 cells. *Mol Cell Biol* **18:**3245–3256.

Chen CC, Wang JK, Chen WC, and Lin SB (1998) Protein kinase C eta mediates lipopolysaccharide-induced nitric-oxide synthase expression in primary astrocytes. *J Biol Chem* **273:**19424–19430.

Chou YC, Chou CC, Chen YK, Tsai S, Hsieh FM, Liu HJ, and Hseu TH (1999) Structure and genomic organization of porcine RACK1 gene. *Biochim Biophys Acta* **1489:**315–322.

Corsini E, Battaini F, Lucchi L, Marinovich M, Racchi M, Govoni S, and Galli CL (1999) A defective protein kinase C anchoring system underlying age-associated impairment in TNF-alpha production in rat macrophages. *J Immunol* **163:**3468–3473.

Dignam JD, Lebovitz RM, and Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:**1475–1489.

Furukawa K and Mattson MP (1998) The transcription factor NF-kappaB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-alpha in hippocampal neurons. *J Neurochem* **70:**1876–1886.

Ganster RW, Taylor BS, Shao L, and Geller DA (2001) Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. *Proc Natl Acad Sci USA* **98:**8638–8643.

Guillemot F, Billault A, and Auffray C (1989) Physical linkage of a guanine nucleotide-binding protein-related gene to the chicken major histocompatibility complex. *Proc Natl Acad Sci USA* **86:**4594–4598.

He DY, Vagts AJ, Yaka R, and Ron D (2002) Ethanol induces gene expression via nuclear compartmentalization of receptor for activated C kinase 1. *Mol Pharmacol* **62:**272–280.

Kaltschmidt B, Uherek M, Wellmann H, Volk B, and Kaltschmidt C (1999) Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis. *Proc Natl Acad Sci USA* **96:**9409–9414.

Lallena MJ, Diaz-Meco MT, Bren G, Payá CV, and Moscat J (1999) Activation of IkappaB kinase beta by protein kinase C isoforms. *Mol Cell Biol* **19:**2180–2188.

Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF, Camacho F, Diaz-Meco MT, Rennert PD, and Moscat J (2001) Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB Pathway. *Mol Cell* **8:**771–780.

Li RC, Ping P, Zhang J, Wead WB, Cao X, Gao J, Zheng Y, Huang S, Han J, and Bolli R (2000) PKCepsilon modulates NF-kappaB and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes. *Am J Physiol* **279:**H1679–H1689.

Lin YZ, Yao SY, Veach RA, Torgerson TR, and Hawiger J (1995) Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J Biol Chem* **270:**14255–14258.

Martindale JL and Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* **192:**1–15.

Mattson MP, Culmsee C, Yu Z, and Camandola S (2000) Roles of nuclear factor kappaB in neuronal survival and plasticity. *J Neurochem* **74:**443–456.

McCahill A, Warwicker J, Bolger GB, Houslay MD, and Yarwood SJ (2002) The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. *Mol Pharmacol* **62:**1261–1273.

McGowan K, DeVente J, Carey JO, Ways DK, and Pekala PH (1996) Protein kinase C isoform expression during the differentiation of 3T3–L1 preadipocytes: loss of protein kinase C-alpha isoform correlates with loss of phorbol 12-myristate 13-acetate activation of nuclear factor kappaB and acquisition of the adipocyte phenotype. *J Cell Physiol* **167:**113–120.

Padanilam BJ and Hammerman MR (1997) Ischemia-induced receptor for activated C kinase (RACK1) expression in rat kidneys. *Am J Physiol* **272:**F160–F166.

Pass JM, Gao J, Jones WK, Wead WB, Wu X, Zhang J, Baines CP, Bolli R, Zheng YT, Joshua IG, and Ping P (2001) Enhanced PKC beta II translocation and PKC beta II-RACK1 interactions in PKC epsilon-induced heart failure: a role for RACK1. *Am J Physiol* **281:**H2500–H2510.

Renard P, Ernest I, Houbion A, Art M, Le Calvez H, Raes M, and Remacle J (2001) Development of a sensitive multi-well colorimetric assay for active NFkappaB. *Nucleic Acids Res* **29:**E21.

Roman J, Gimenez A, Lluis JM, Gasso M, Rubio M, Caballeria J, Pares A, Rodes J, and Fernandez-Checa JC (2000) Enhanced DNA binding and activation of transcription factors NF-kappa B and AP-1 by acetaldehyde in HEPG2 cells. *J Biol Chem* **275:**14684–14690.

Ron D, Chen C-H, Caldwell J, Jamieson L, Orr E and Mochly-Rosen (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. *Proc Natl Acad Sci USA* **91:**839–843.

Sang N, Severino A, Russo P, Baldi A, Giordano A, Mileo AM, Paggi MG, and De Luca A (2001) RACK1 interacts with E1A and rescues E1A-induced yeast growth inhibition and mammalian cell apoptosis. *J Biol Chem* **276:**27026–27033.

Sarmiere PD and Freeman RS (2001) Analysis of the NF-kappa B and PI 3-kinase/Akt survival pathways in nerve growth factor-dependent neurons. *Mol Cell Neurosci* **18:**320–331.

Smale ST (1997) Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim Biophys Acta* **1351:**73–88.

Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, et al. (2000) PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature (Lond)* **404:**402–407.

Vaudry D, Rousselle C, Basille M, Falluel-Morel A, Pamantung TF, Fontaine M, Fournier A, Vaudry H, and Gonzalez BJ (2002) Pituitary adenylate cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death. *Proc Natl Acad Sci USA* **99:**6398–6403.

Wang Y, Wu TR, Cai S, Welte T, and Chin YE (2000) Stat1 as a component of tumor necrosis factor alpha receptor 1-TRADD signaling complex to inhibit NF-kappaB activation. *Mol Cell Biol* **20:**4505–4512.

Wooten MW, Vandenplas ML, Seibenhener ML, Geetha T, and Diaz-Meco MT (2001) Nerve growth factor stimulates multisite tyrosine phosphorylation and activation of the atypical protein kinase C's via a src kinase pathway. *Mol Cell Biol* **21:**8414–8427.

Yaka R, He DY, Phamluong K, and Ron D (2003) Pituitary adenylate cyclase activating polypeptide (PACAP 1–38) enhances NMDA receptor function and BDNF expression via rack. *J Biol Chem* **278:**9630–9638.

Yaka R, Thornton C, Vagts AJ, Phamluong K, Bonci A, and Ron D (2002) NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1. *Proc Natl Acad Sci USA* **99:**5710–5715.

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