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Ethanol Induces Gene Expression via Nuclear Compartmentalization of Receptor for Activated C Kinase 1

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ABSTRACT

Scaffolding proteins such as receptor for activated C kinase (RACK) 1 are involved in the targeting of signaling proteins and play an important role in the regulation of signal transduction cascades. Recently, we found that in cultured cells and in vivo, acute ethanol exposure induces the nuclear compartmentalization of RACK1. To elucidate a physiological role for nuclear RACK1, the Tat protein transduction system was used to transduce RACK1 and RACK1-derived fragments into C6 glioma cells. We found that nuclear RACK1 is mediating the induction of the immediate early gene *c-fos* expression induced by ethanol. First, transduction of full-length RACK1 (Tat-RACK1) resulted in the induction of *c-fos* expression and enhancement of ethanol activities. Second, we determined that the C terminus of RACK1 (Tat-RACK1ΔN) is mediating transcription. Third, we identified a dominant negative fragment of RACK1 that inhibited the nuclear compartmentalization of endogenous RACK1 and inhibited ethanol-induction of c-fos mRNA and protein expression. Last, acute exposure to ethanol or transduction of full-length Tat-RACK1 resulted in an increase in mRNA levels of an activator protein 1 site-containing gene, *PAC1* (pituitary adenylate cyclase-activating polypeptide receptor type I), suggesting that nuclear RACK1 is involved in the regulation of the expression of genes that are altered upon acute ethanol treatment. These results may therefore have important implications for the study of alcohol addiction.

Ethanol addiction is a prevalent and costly societal problem. Understanding the biochemical and physiological mechanism of ethanol is of prime importance to develop new drugs to treat alcoholism. In recent years it has become increasingly apparent that ethanol alters signal transduction cascades that result in changes in gene expression patterns that ultimately underlie ethanol-related behaviors (Pandey, 1998; Stubbs and Slater, 1999). The changes in gene expression lead to, and manifest in, the disease state of alcoholism. Signal transduction cascades are regulated through precise compartmentalization of signaling proteins. Compartmentalization of signaling proteins such as kinases and phosphatases is achieved by their interaction with scaffolding proteins (Mochly-Rosen, 1995; Pawson and Scott, 1997). We hypothesized that ethanol is altering signaling cascades by changing the localization and function of scaffolding proteins. Indeed, we found previously that the localization and function of one such scaffolding protein, RACK1, is altered in cultured cells and in vivo upon exposure to ethanol (Ron et al., 2000). RACK1 is a scaffolding protein for kinases such as βIIPKC and Fyn (Mochly-Rosen, 1995; Pawson and Scott, 1997; Ron et al., 1999). RACK1 also interacts with substrates (Liliental and Chang, 1998; Brandon et al., 1999; Geijsen et al., 1999), as well as other signaling proteins (Liliental and Chang, 1998; Baumann et al., 2000; Mourton et al., 2001). We found that ethanol uncouples RACK1 from βIIPKC by inducing the movement of RACK1 to the nucleus. As a consequence of RACK1 nuclear localization, activation-induced βIIPKC translocation is inhibited (Ron et al., 2000).

Because acute exposure to ethanol induces a rapid movement of RACK1 to the nucleus, we assessed whether nuclear RACK1 contributes to changes in gene expression induced by ethanol. One of the genes known to be altered upon injection or consumption of ethanol in the brain is the immediate early gene (IEG) *c-fos* (Zoeller and Fletcher, 1994; Chang et al., 1995; Hitzemann and Hitzemann, 1997; Bachtell et al., 1999; Thiele et al., 2000a). The cascades mediating ethanol-induced *c-fos*

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D.-Y.H. and A.J.V. contributed equally to this work.

ABBREVIATIONS: RACK, receptor for activated C kinase; PKA, protein kinase A; PKC, protein kinase C; IEG, immediate early gene; PACAP, pituitary adenylate cyclase-activating polypeptide; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; HA, hemagglutinin; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GPDH, glycerol-3-phosphate dehydrogenase; AP-1, activator protein 1.

expression and the possible consequences of *c-fos* induction are not fully understood. We recently found that in C6 glioma cells, ethanol induces a rapid dose-dependent increase in *c-fos* expression that is dependent on cAMP/PKA signaling (Fig. 1). Because ethanol-induced movement of RACK1 to the nucleus is also mediated via cAMP/PKA pathway (Ron et al., 2000), we were

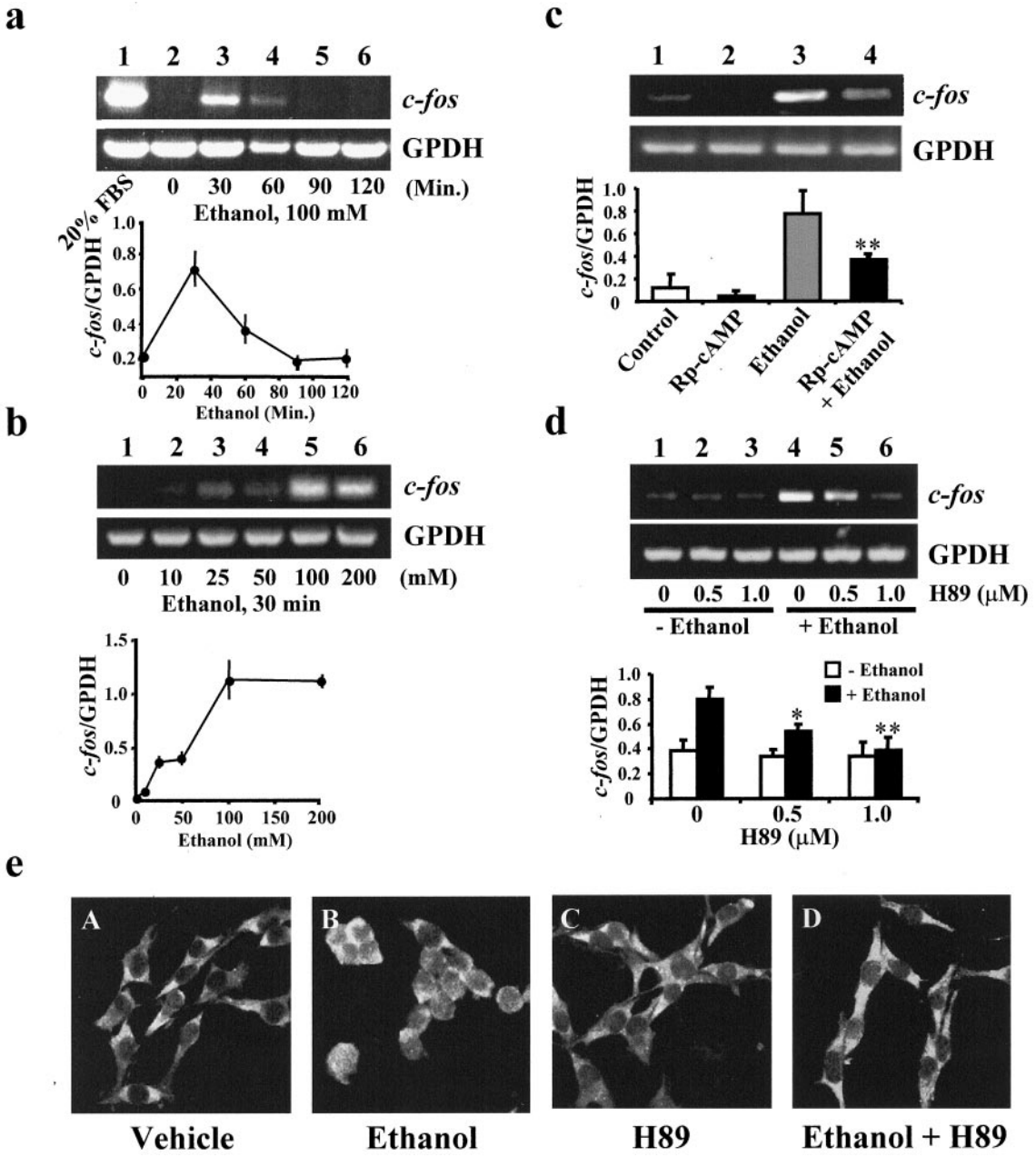

Fig. 1. Ethanol induces c-fos mRNA expression via cAMP/PKA signaling cascade. a, Ethanol induction of c-fos expression is rapid and transient. Cells were incubated in serum-free medium for 24 h and treated with 100 mM ethanol for different time points as indicated (lanes 2–6). Total RNAs were used for analysis of the expression of c-fos and GDPH genes by RT-PCR as described under *Experimental Procedures*. After completion of the PCR reaction, products were separated on an agarose gel and photographed by Eagle Eye II (Stratagene). Serum (20%) induced c-fos expression was used as a positive control (lane 1). Graph depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. b, ethanol induces c-fos expression in a dose-dependent manner. Cells were treated with different concentrations of ethanol (0–200 mM, lanes 1–6) for 30 min and gene expression analyzed by RT-PCR as described in a. Graph depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. c, ethanol induction of c-fos expression is mediated by cAMP signaling. Cells were incubated with vehicle (lanes 1 and 3) or *R*p-cAMP (20 μM, lanes 2 and 4) for 30 min and then treated with vehicle (lanes 1 and 2) or 150 mM ethanol (lanes 3 and 4) for 30 min. The expression of c-fos was analyzed by RT-PCR as described in a. Histogram depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. Statistically significant difference between ethanol and ethanol + *R*p-cAMP groups is indicated by $*226$, $P < 0.01$ (t test). d, ethanol induction of c-fos expression is mediated by PKA. Cells were pretreated with H89 at different concentrations as indicated (lanes 2 and 3 and 5 and 6) or vehicle (lanes 1 and 4) for 30 min before addition of 100 mM ethanol (lanes 4–6) for 30 min. The expression of c-fos was analyzed by RT-PCR as described in a. Histogram depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. Statistically significant differences between ethanol-treated cells in the absence of H89 and in the presence of 0.5 or 1.0 μM H89 are indicated by $*$, $P < 0.05$ and $**$, $P < 0.01$ (t test). e, ethanol-induced RACK1 nuclear translocation is mediated by PKA. C6 glioma cells were preincubated with H89 (1 μM; C and D) for 30 min at 37°C. Cells were then treated with ethanol (200 mM; B and D) for 30 min. RACK1 nuclear localization was visualized by immunofluorescence with anti-RACK1 antibody. Cells were scanned using confocal microscope and viewed at 20× magnification. Images shown are individual middle sections of projected Z series. The images are representative of two experiments.

interested to determine whether RACK1 plays a role in ethanol induction of *c-fos* expression.

We used the Tat protein transduction system to transduce RACK1 and RACK1 fragments into C6 glioma cells. The Tat-fusion protein transduction method, developed by Dowdy and colleagues, allows the delivery of proteins into cells with high efficiency (Schwarze and Dowdy, 2000; Schwarze et al., 2000). The method uses a short sequence from the human immunodeficiency virus Tat protein that facilitates the rapid transduction of proteins through membranes via an unknown mechanism of action. The method has several advantages over transfection. First, the efficiency of transduction is high. Second, transduction of proteins occurs very rapidly, allowing immediate monitoring of changes. In addition, using the protein transduction system allows the transduction of proteins regardless of protein size or cell type. Last, the method is also very attractive because it allows the transduction of proteins in vivo (Schwarze et al., 2000).

Herein, we identify nuclear RACK1 as a key player in the induction of *c-fos* mRNA and protein expression upon acute exposure of cells to ethanol. We also identify *PAC1*, the type I receptor for pituitary adenylate cyclase-activating polypeptide (PACAP), as a putative downstream gene that is altered in response to the induction of *c-fos* by ethanol via RACK1.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (high glucose, DMEM) was purchased from Invitrogen (Carlsbad, CA). *R*p-cAMP was purchased from Biolog Life Science Institute (Bremen, Germany), and H89 was purchased from Calbiochem (San Diego, CA). The protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, leupeptin, soybean trypsin inhibitor, and transferrin were purchased from Roche Applied Science (Indianapolis, IN). Ni^{2+}-nitrilotriacetic acid resin was purchased from QIAGEN (Valencia, CA). PSK(−) vectors were purchased from Stratagene (La Jolla, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). TRIzol reagent was purchased from Invitrogen. Reverse transcription kit and polymerase chain reaction (PCR) components were purchased from Promega (Madison, WI). Oligonucleotide primers were synthesized by BioSource International-Keystone Laboratories (Foster City, CA). pTAT-HA plasmid was a generous gift from Steve Dowdy (Howard Hughes Medical Center, Washington University, St. Louis, MO). Anti-RACK1 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY), anti-HA rat monoclonal antibodies were purchased from Roche Applied Science, and anti-*c-fos* polyclonal antibodies were purchased from Oncogene Science (Cambridge, MA). Texas Red-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA), and anti-mouse and anti-rabbit secondary antibodies were purchased from Roche Applied Science. TOTO-3 nuclear marker was purchased from Molecular Probes (Eugene, OR).

Cell Culture. C6 rat glioma cell line was obtained from American Type Culture Collection (Manassas, VA). The cells were grown as monolayer cultures in DMEM containing 5% FBS plus 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. The cells were subcultured every 2 to 3 days. For immunofluorescence experiments the cells were seeded at a density of 0.5×10^4 cells/ml in Nalgene Nunc four-well chamber slides (Fisher Scientific, Fair Lawn, NJ). For Western blot and RT-PCR experiments, C6 cells were split onto six-well plates at a density of 2×10^5 cells/well in DMEM containing 0.5% FBS for overnight incubation at 37°C after by incubation in serum-free DMEM for at

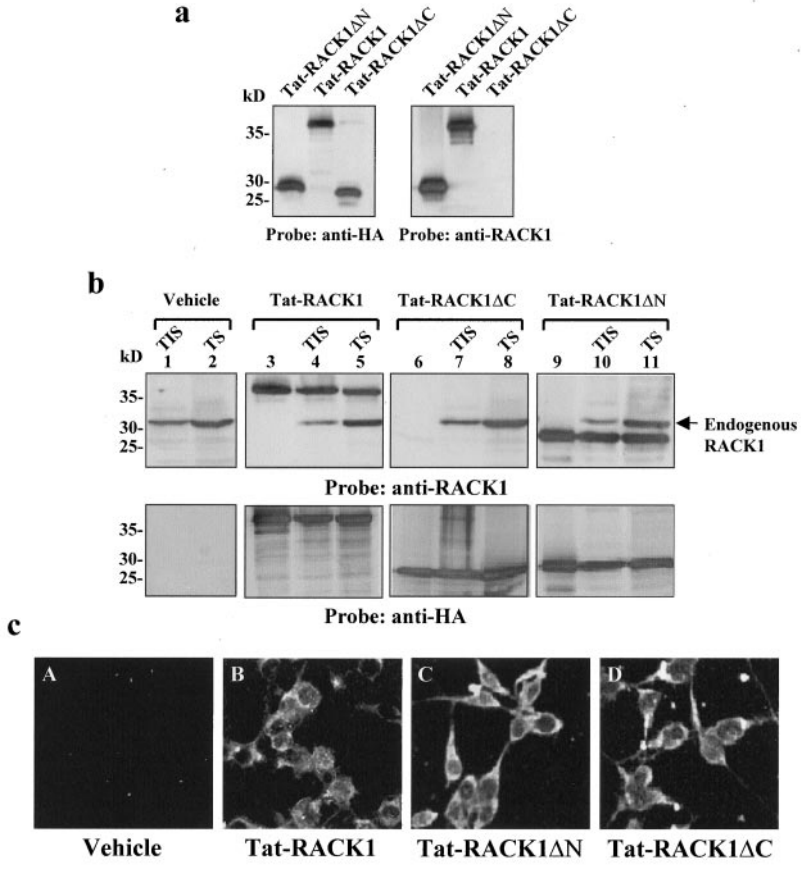

Fig. 2. Tat fusion proteins are transduced into C6 glioma cells. a, Tat-RACK1, Tat-RACK1ΔN, and Tat-RACK1ΔC were expressed in *E. coli*. The purified proteins were resolved on an SDS-PAGE gel. Tat-fusion proteins were detected with anti-HA antibodies (left) and anti-RACK1 antibodies (right). Coomassie staining (data not shown) and Western blot analysis were conduced for each batch of purified proteins. b, cells were incubated with vehicle (PBS) (lanes 1 and 2) or Tat-RACK1 (1 μM) (lanes 4 and 5), Tat-RACK1ΔC (1 μM) (lanes 7 and 8), and Tat-RACK1Δ 78 (1 μM) (lanes 10 and 11) for 30 min at 37°C. Cells were then homogenized as described under *Experimental Procedures* and the Triton-insoluble material (TIS; 20 μg, lanes 1, 4, 7, and 10) or Triton-soluble (TS; 20 μg, lanes 2, 5, 8, and 11) material was resolved on a 12% SDS-PAGE gel. The transduction of Tat-fusion proteins was detected with anti-HA antibodies (bottom). The transduction of Tat-RACK1 and Tat-RACK1ΔN were detected with anti-RACK1 antibodies (top). Purified proteins (0.5 μg; lanes 3, 6, and 9) were used as control. The images are representatives of three experiments. c, cells were incubated with vehicle (PBS; A), Tat-RACK1 (1 μM; B), Tat-RACK1Δ 78 (1 μM; C) or Tat-RACK1ΔC (1 μM; D) for 30 min at 37°C. Tat-fusion proteins were detected by immunohistochemistry using anti-HA antibodies as described under *Experimental Procedures*. Cells were scanned using confocal microscope and viewed at 20× magnification. Images shown are individual middle sections of projected Z series. The images are representatives of three experiments.

least 24 h. The medium was replaced with fresh serum-free DMEM about 6 h before treatment.

Cloning and Expression of Tat-Tagged Fusion Proteins. Tat-Kip[27] was a generous gift from Steve Dowdy (Howard Hughes Medical Center, Washington University, St. Louis, MO). The N-terminal domain of RACK1 (amino acids 1–180, RACK1ΔC) and the C-terminal domain of RACK1 (amino acids 138–317, RACK1ΔN) were amplified by PCR from a full-length RACK1 construct (Ron et al., 1995). The primers were designed with extra restriction sites for *Not*I and *Nco*I or *EcoR*1 as follows: for RACK1ΔC, upstream 5′-CGGAATGCGGCCGCCCATGGTTATGACCGAGCAAATGACCCTT-3′ and downstream 5′-GGGGCCGGAATTCCATTAAGCCAGATTCCACACCTTGA-3′; for RACK1ΔN, upstream 5′-GGAATGCGGCCGCCCATGGTTTGCAAGTACACTGTCCAGGAT-3′ and downstream 5′-GGGGCCGGAATTCCATTAGCGGGTACCAATAGTCACCTGC-3′. PCR products were digested with *Not*I and *EcoR*1 and ligated into pSK(−) for sequencing or pTatHAHis6 for expression. pTat-RACK1, pTat-RACK1ΔC, pTat-RACK1ΔN, and pTat-Kip[27] were expressed in *Escherichia coli* as described previously (Nagahara et al., 1998). Bacteria were homogenized in 20 ml of lysis buffer (8 M urea, 200 mM NaCl, and 20 mM HEPES pH 8.0) containing protease inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml soybean trypsin inhibitor, and 17 μg/ml phenylmethylsulfonyl fluoride) followed by sonication for 2 min. The homogenate was clarified by centrifugation at 4°C for 30 min and purified by using Ni^{2+}-nitrilotriacetic acid agarose beads. After incubation at 4°C for 1 to 2 h while shaking, the beads were washed three times with >6 volumes of lysis buffer containing 20 mM imidazole and then eluted with 500 mM imidazole in lysis buffer. The eluate was dialyzed overnight at 4°C against 10% glycerol in PBS.

Immunohistochemistry. C6 glioma cells were treated as indicated in figure legends. Immunostaining was performed as described previously (Ron et al., 2000). Briefly, after treatment, cells were washed in cold PBS containing 0.3% Triton X-100, fixed in cold methanol, and blocked in PBS containing 0.3% Triton X-100 and 3% normal goat serum. Immunostaining was performed with monoclonal IgM anti-RACK1 and IgG anti-HA antibodies (1:100), or polyclonal IgG *c-fos* antibodies (1:150). Staining was detected with secondary antibodies conjugated to Texas Red (1:250). Nuclei were detected with the nuclear marker TOTO-3 (1:5000). Slides were viewed with a laser scanning confocal microscope (MRC-1024; Bio-Rad, Hercules, CA). Z-field images were processed by obtaining the middle Z-field sections using NIH Image 1.61 and Adobe Photoshop (Adobe Systems, Mountain View, CA). Quantitation of the results was made as described previously (Ron et al., 2000).

Western Blot Analysis. C6 glioma cells were incubated with Tat-fusion protein in DMEM as indicated. Cells were centrifuged at 3000 rpm for 5 min and washed three times in PBS. The cell pellet was resuspended in homogenization buffer (320 mM sucrose, 1.0% Triton, 10 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl pH 7.4, and protease inhibitors), homogenized, and incubated on ice for 30 min. The extract was then centrifuged for 3 min at 4000 rpm at 4°C. Triton-insoluble and -soluble samples were resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Tat-fusion proteins were detected with rat monoclonal IgG anti-HA antibodies (1:1000). ECL Plus kit and STORM-860 (Molecular Dynamics, Sunnyvale, CA) were used to develop Western blots.

RT-PCR. C6 glioma cells were treated as indicated in figure legends. Cells were extracted with TRIzol reagent for total RNA isolation. Total RNA (1 μg) was used for reverse transcription reaction with oligo(dT)15 primer for 25 min at 42°C following the manufacturer's protocol. The expression of c-fos, c-jun, and GPDH was analyzed by PCR in 50 μl with 35 cycles (94°C, 45 s; 52°C, 30 s; and

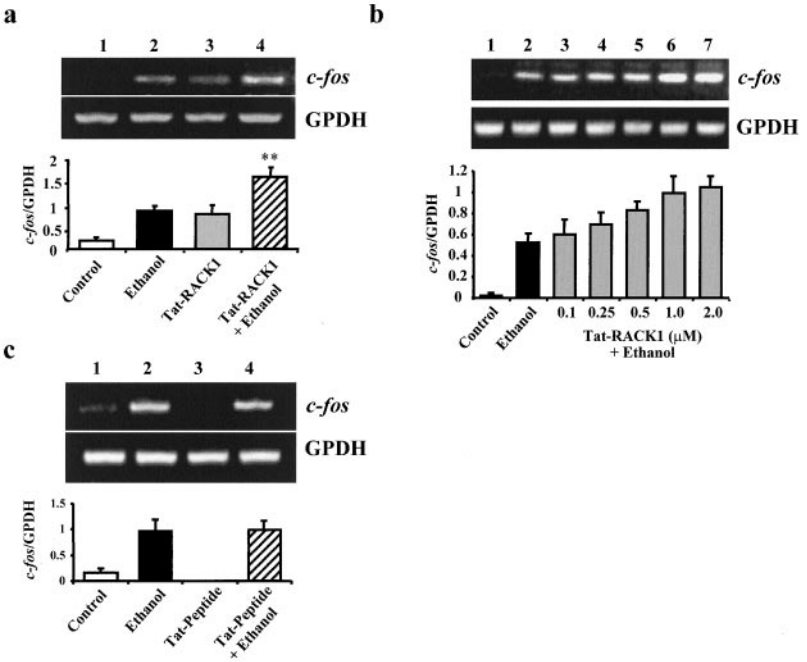

Fig. 3. Tat-RACK1 induces c-fos mRNA expression and enhances ethanol induction of c-fos. a, Tat-RACK1 enhances ethanol-induced c-fos mRNA expression. Cells were incubated in serum-free medium for 24 h and then transduced with vehicle (lanes 1 and 2) or Tat-RACK1 (1 μM; lanes 3 and 4) for 30 min. Cells were then treated with 100 mM ethanol (lanes 2 and 4) for 30 min. The expression of c-fos and GPDH was analyzed by RT-PCR as described in Fig. 1a. The c-fos/GPDH ratios were quantified by NIH Image 1.61. Histogram depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. Statistically significant difference between ethanol and ethanol + Tat-RACK1 groups is indicated by **, $P < 0.01$ (t test). b, Tat-RACK1 enhances ethanol-induced c-fos mRNA expression in a dose-dependent manner. Cells were incubated in serum-free medium for 24 h and then transduced with vehicle (lanes 1 and 2) or with increasing concentrations of Tat-RACK1 as indicated (lanes 3–7) for 30 min. The cells were treated with 100 mM ethanol (lanes 2–7) for 30 min, and the expression of c-fos and GPDH was analyzed by RT-PCR as described in Fig. 1a. The c-fos/GPDH ratios were quantified by NIH Image 1.61. c, Tat-peptide does not alter c-fos mRNA expression. Cells were incubated in serum-free medium for 24 h and then transduced with vehicle (lanes 1 and 2) or Tat-peptide (1 μM; lanes 3 and 4) for 30 min. Cells were then treated with 100 mM ethanol (lanes 2 and 4) for 30 min. The expression of c-fos and GPDH were analyzed by RT-PCR as described in Fig. 1a. The c-fos/GPDH ratios were quantified by NIH Image 1.61.

72°C, 2 min). The primers were designed as follows: c-fos, upstream 5′-ACGGAGAATCCGAAGGGAAAGGAATAAGAT-3′ and downstream 5′-AGACAAAGGAAGACGTATAAGTAGTGCAGC-3′; c-jun, upstream 5′-TAGCTGAACTGCATAGCCAGAATACGCTGC-3′ and downstream 5′-AAGCTGTGCCACCTGTTCCCTGAGCATGTT-3′; and GPDH, upstream 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and downstream 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

PAC1 was detected by RT-PCR in the same manner. The primers of *PAC1* for PCR were used as follows: upstream 5′-CTTGTACAGAAGCTGCAGTCCCCAGACATG-3′ and downstream 5′-GTGCTTGAAGTCCATAGTGAAGTAACGGTTCAC-3′. After completion of the PCR reaction, 10 μl of each product was separated on 1.8% agarose gel in Tris/acetic acid/EDTA buffer containing 0.25 μg/ml ethidium bromide, photographed by Eagle Eye II (Stratagene), and quantified by NIH Image 1.61.

Data Analysis. Digitized images of RT-PCR (photographed by Eagle Eye II; Stratagene) were quantitatively analyzed by densitometry with NIH Image 1.61 program providing peak areas. Results expressed as mean ratio of the tested genes to GPDH ± S.D. Statistical analysis was performed using Student's *t* test for significant differences.

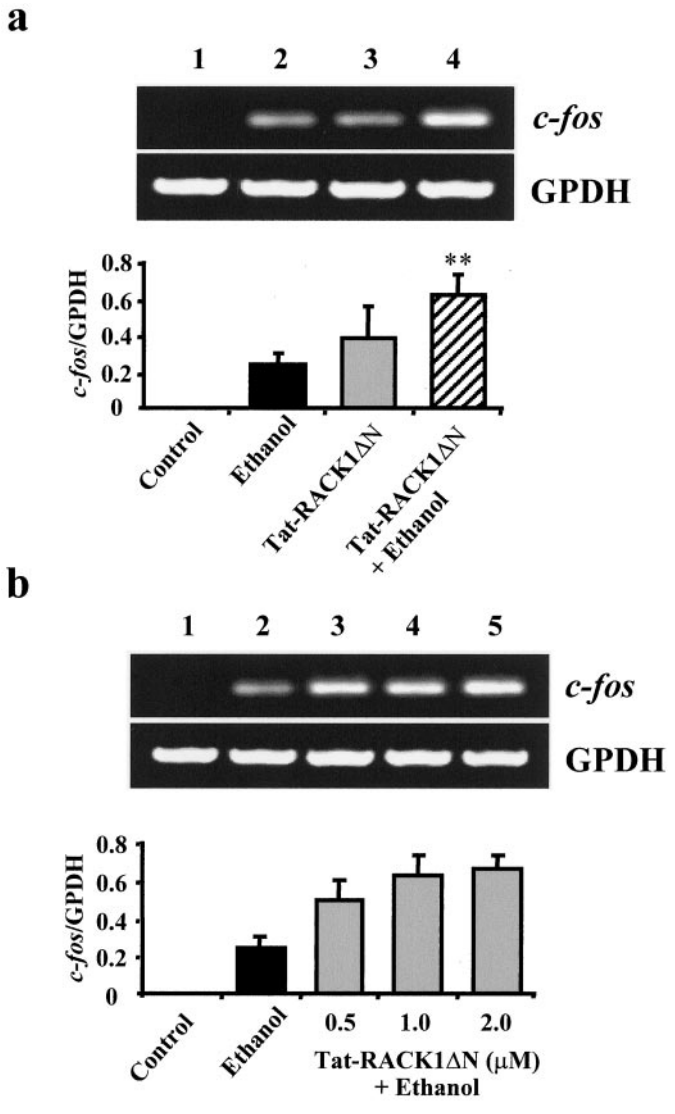

Fig. 4. Tat-RACK1ΔN induces c-fos mRNA expression and enhances ethanol induction of c-fos. a, Tat-RACK1ΔN enhances ethanol-induced c-fos mRNA expression. Cells were incubated in serum-free medium for 24 h and then transduced vehicle (lanes 1 and 2) or Tat-RACK1ΔN (1 μM; lanes 3 and 4) for 30 min. Cells were then treated with 100 mM ethanol (lanes 2 and 4) for 30 min. The expression of c-fos and GPDH was analyzed by RT-PCR as described in Fig. 1a. The c-fos/GPDH ratios were quantified by NIH Image 1.61. Histogram depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. Statistically significant difference between ethanol and ethanol + Tat-RACK1ΔN groups is indicated by **, $P < 0.01$ (*t* test). b, Tat-RACK1ΔN enhances ethanol-induced c-fos mRNA expression in a dose-dependent manner. Cells were incubated in serum-free medium for 24 h and were then transduced with vehicle (lanes 1 and 2) or with increasing concentrations of Tat-RACK1ΔN as indicated (lanes 3–5) for 30 min. The cells were treated with 100 mM ethanol (lanes 2–5) for 30 min and the expression of c-fos and GPDH was analyzed by RT-PCR as described in Fig. 1a. The c-fos/GPDH ratios were quantified by NIH Image 1.61.

Results

Recently, we observed that acute exposure of C6 glioma cells to ethanol results in a very rapid increase in the mRNA expression of the IEG *c-fos* (Fig. 1a). The induction of *c-fos* transcription by ethanol was dose-dependent and concentrations as low as 25 mM ethanol induced *c-fos* expression (Fig. 1b). Because ethanol has been linked to cAMP/PKA signaling pathway (Thiele et al., 2000b), we examined whether ethanol's induction of *c-fos* expression is mediated via cAMP/PKA signal transduction cascade. We found that the inhibitory analog of cAMP, *R*p-cAMP and the PKA inhibitor H89 reduced ethanol induction of *c-fos* expression (Fig. 1, c and d, respectively). Therefore, acute exposure of C6 glioma cells to ethanol induces a transient expression of the IEG *c-fos* via a cAMP/PKA signaling pathway.

Previously, we found that acute exposure to ethanol alters the localization and function of RACK1 in cultured cells, including C6 glioma cells and in vivo (Ron et al., 2000). Ethanol induced a rapid movement of RACK1 to the nucleus (Ron et al., 2000). Low concentrations of ethanol (5–50 mM depending on the cell type) induced RACK1 nuclear compartmentalization. We identified the cAMP/PKA signaling as the cascade responsible for RACK1 nuclear compartmentalization, because *R*p-cAMP (Ron et al., 2000) and H89 (Fig. 1e) inhibited ethanol-induced RACK1 movement to the nucleus, whereas the adenylate cyclase activator forskolin induced it (Ron et al., 2000). Because ethanol induced both *c-fos* expression and RACK1 nuclear compartmentalization via cAMP/PKA pathway, we set out to determine the possible involvement of RACK1 in the induction of *c-fos* expression by ethanol. We used the Tat protein transduction method (Nagahara et al., 1998) to transduce RACK1 and RACK1 fragments into cells. Full-length RACK1 and the RACK1 fragments: N-terminal half of RACK1 amino acids 1 to 180 (RACK1ΔC), and the C-terminal half of RACK1 amino acids 138 to 317 (RACK1ΔN) were cloned into pTatHAHis6 vector, and the recombinant proteins were expressed in *E. coli* and purified as described previously (Nagahara et al., 1998). The recombinant Tat fusion proteins were detected with anti-HA antibodies (Fig. 2a, left). Anti-RACK1 antibodies detected the full-length Tat-RACK1 and the C-terminal fragment RACK1ΔN (Fig. 2a, right).

We first determined whether Tat-fusion proteins could be transduced into C6 glioma cells. Tat-RACK1 (1 μM) and the fragments RACK1ΔC and RACK1ΔN were incubated with C6 glioma cells, and Triton-soluble and -insoluble homogenates were prepared. Samples were resolved on an SDS-PAGE gel, and the presence of the Tat-fusion proteins in the cells was detected with anti-HA antibodies (Fig. 2b, bottom)

and anti-RACK1 antibodies (Fig. 2b, top). All three fusion proteins were detected in both Triton-soluble (Fig. 2b, lanes 5, 8, and 11) and -insoluble (Fig. 2b, lanes 4, 7, and 10) homogenates. Quantification of the results shows that at least 50% of the Tat-fusion proteins were found in the Triton-soluble material, indicating the successful transduction of the fusion protein. The transduction of the Tat-fusion proteins was also determined using immunohistochemistry. Cells incubated with Tat-RACK1 (Fig. 2c, B), Tat-RACK1ΔN (Fig. 2c, C), and Tat-RACK1ΔC (Fig. 2c, D) show detectable levels of the transduced proteins throughout the cell, including the nucleus, whereas no signal was detected in control cells (Fig. 2c, A), indicating that the Tat-fusion proteins were successfully transduced into C6 glioma cells. In summary, RACK1 and RACK1 fragments expressed as Tat-fusion proteins can be transduced into cells with high efficiency.

If RACK1 is mediating ethanol induction of *c-fos* by its nuclear compartmentalization then increase in RACK1 protein levels should induce *c-fos* mRNA expression and may enhance ethanol activities. To test the hypothesis, full-length Tat-RACK1 was transduced into cells, by preincubating the protein with cells for 30 min before treatment with ethanol and *c-fos* mRNA level was measured. Tat-RACK1 induced *c-fos* expression (Fig. 3a, lane 1 versus 3), and the activities of Tat-RACK1 and ethanol were additive (Figs. 3a, lane 2 versus 3b and 4). The induction of *c-fos* expression in the absence and presence of ethanol activities are specific for the RACK1 sequence, because the transduction of the Tat-peptide did not induce or alter *c-fos* mRNA levels (Fig. 3c).

To determine which portion of the RACK1 sequence is mediating transcription, we tested the activities of the C terminus (RACK1ΔN) and the N terminus (RACK1ΔC) fragments of RACK1. We determined whether the RACK1 fragments induce *c-fos* transcription or alter ethanol-induction of *c-fos* expression. As shown in Fig. 4, the C-terminal fragment Tat-RACK1ΔN induced *c-fos* gene expression (Fig. 4a, lane 1 versus 3) and enhanced ethanol induction of *c-fos* expression in a dose-dependent manner (Fig. 4a, lane 2 versus 4 and 4b, respectively). Therefore, RACK1 is mediating the induction of *c-fos* transcription via its C terminus. Because at least a portion of Tat-RACK1ΔN is distributed in the nuclear compartment (Fig. 2b), it is highly likely that Tat-RACK1ΔN induced transcription via its nuclear compartmentalization.

Next, we tested the activities of the N-terminal portion of RACK1. Interestingly, Tat-RACK1ΔC reduced *c-fos* mRNA levels induced by ethanol (Fig. 5a, lane 2 versus 4) in a dose-dependent manner (Fig. 5b). In addition, Tat-RACK1ΔC significantly reduced c-fos protein levels (Fig. 5c, C versus D). We hypothesized that the fragment RACK1ΔC was inhibiting ethanol induction of *c-fos* expression by acting as a dom-

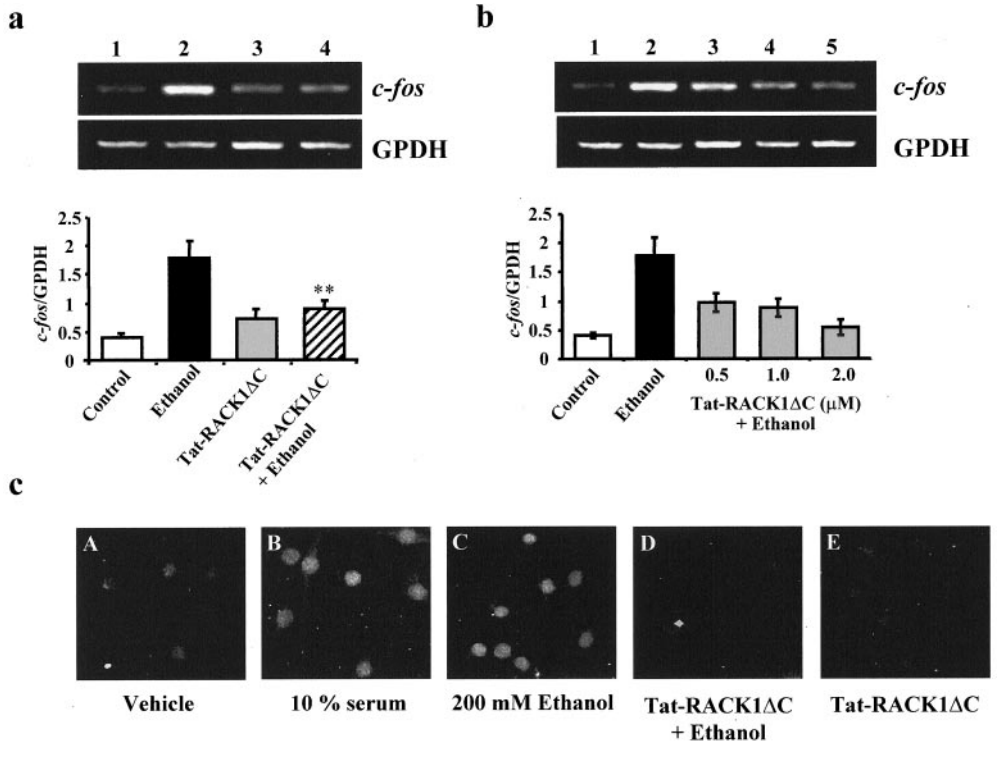

Fig. 5. Tat-RACK1ΔC decreases ethanol-induction of c-fos mRNA and protein expression. a, Tat-RACK1ΔC inhibits ethanol-induced c-fos mRNA expression. Cells were incubated in serum-free medium for 24 h and then transduced with Tat-RACK1ΔC (1 μM, lanes 3 and 4) or vehicle (lanes 1 and 2) for 30 min. Cells were then treated with 100 mM ethanol (lane 2 and 4) for 30 min. The expression of c-fos was analyzed by RT-PCR as described in Fig. 1a. Representative of images is shown at top. The c-fos/GPDH ratios were quantified by NIH Image 1.61. Histogram depicts the mean ratio (c-fos/GPDH) ± S.D. of three experiments. Statistically significant difference between ethanol and ethanol + Tat-RACK1ΔC groups is indicated by **, $P < 0.01$ (t test). b, Tat-RACK1ΔC inhibits ethanol-induced c-fos mRNA expression in a dose-dependent manner. Cells were incubated in serum-free medium for 24 h and then transduced with vehicle (lanes 1 and 2) or with increasing concentrations of Tat-RACK1ΔC as indicated (lanes 3–5) for 30 min. Cells were then treated with 100 mM ethanol (lane 2–5) for 30 min, and c-fos gene expression was analyzed as described in Fig. 1a. The c-fos/GPDH ratios were quantified by NIH Image 1.61. c, Tat-RACK1ΔC inhibits ethanol-induced c-fos protein expression. Cells were seeded in medium containing 1% FBS and then serum starved in serum-free medium for 48 h. Cells were incubated with vehicle (A–C) or Tat-RACK1ΔC (1.0 μM; D and E) for 30 min and then treated for 30 min with medium (A and E), 10% FBS (B), or 200 mM ethanol (C and D). c-fos immunoreactivity was visualized with anti-*c-fos* antibody. Cells were scanned using confocal microscope and viewed at 20× magnification. Images shown are individual middle sections of projected Z series. The images are representative of three experiments.

inant negative and preventing the nuclear targeting of endogenous RACK1. If this is true then RACK1ΔC should alter RACK1 nuclear compartmentalization induced by ethanol. We used confocal microscopy to monitor the localization of endogenous RACK1 in C6 glioma cells after transduction of Tat-RACK1ΔC. The results were quantified by measuring colocalization of RACK1 with the nuclear marker TOTO-3. As shown in Fig. 6, in unstimulated cells (Fig. 6a, top left), or cells treated with Tat-RACK1ΔC (Fig. 6a, bottom left), RACK1 is localized mainly in the cytoplasm. Incubation with 100 mM ethanol for 30 min results in the nuclear compartmentalization of RACK1 (Fig. 6a, top right, and b). However, when Tat-RACK1ΔC was transduced into cells, ethanol-induced RACK1 nuclear compartmentalization was significantly reduced (Fig. 6a, bottom right, and b). The inhibition of ethanol-induced nuclear compartmentalization of RACK1 was specific for Tat-RACK1ΔC, because a Tat-fusion protein of approximately the same size range, Tat-KIP^{27}, did not alter ethanol-induced RACK1 nuclear compartmentalization (Fig. 6b) and neither did the transduction of the Tat peptide (Fig. 6b).

To test whether RACK1 mediated *c-fos* expression is specific, we determined the activities of transduced RACK1 and fragments on serum induction of *c-fos* gene expression and on

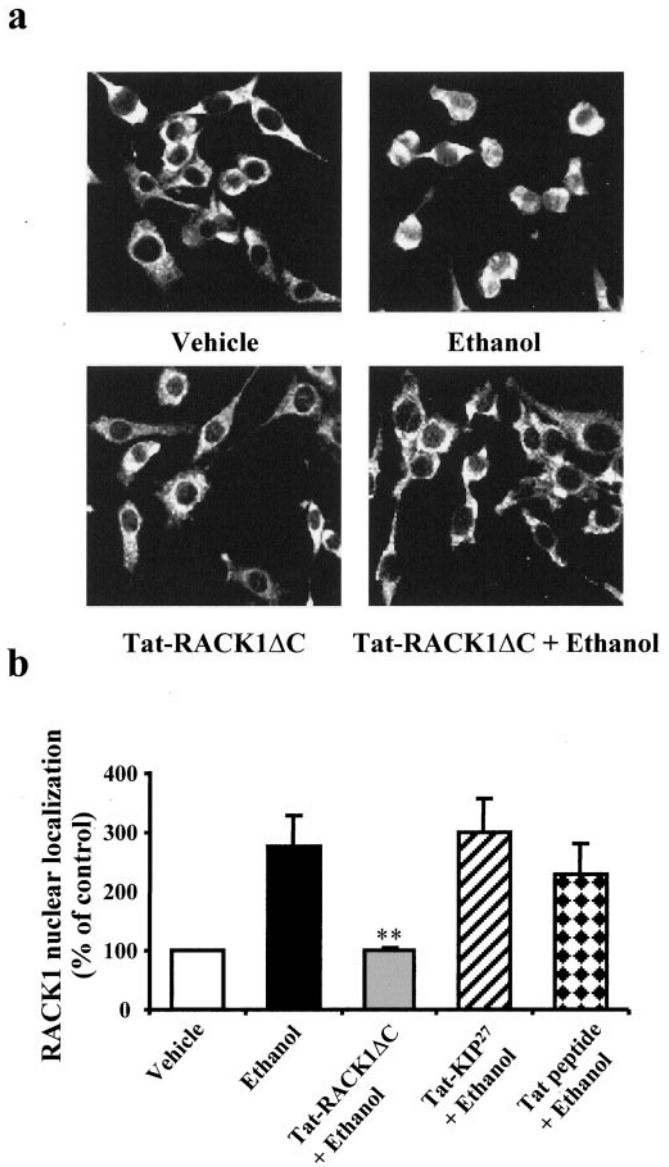

Fig. 6. RACK1ΔC inhibits ethanol-induced RACK1 nuclear compartmentalization. a, cells were incubated with vehicle (top) and Tat-RACK1ΔC (1 μM) (bottom) for 30 min at 37°C. Cells were then treated without (left) or with 200 mM ethanol (right) for 30 min. RACK1 nuclear localization was visualized by immunofluorescence with anti-RACK1 antibody. Cells were scanned using confocal microscope and viewed at 20× magnification. Images shown are individual middle sections of projected Z series. The images are representative of four experiments. b, data from immunofluorescence experiments were processed using NIH Image 1.61. Colocalization of RACK1 with the nuclear marker TOTO-3 was quantified as described previously (Ron et al., 2000). Histogram depicts the mean percentage of control ± S.D. of three experiments. Statistically significant difference between ethanol and ethanol + Tat-RACK1ΔC groups is indicated by **, $P < 0.01$ (t test).

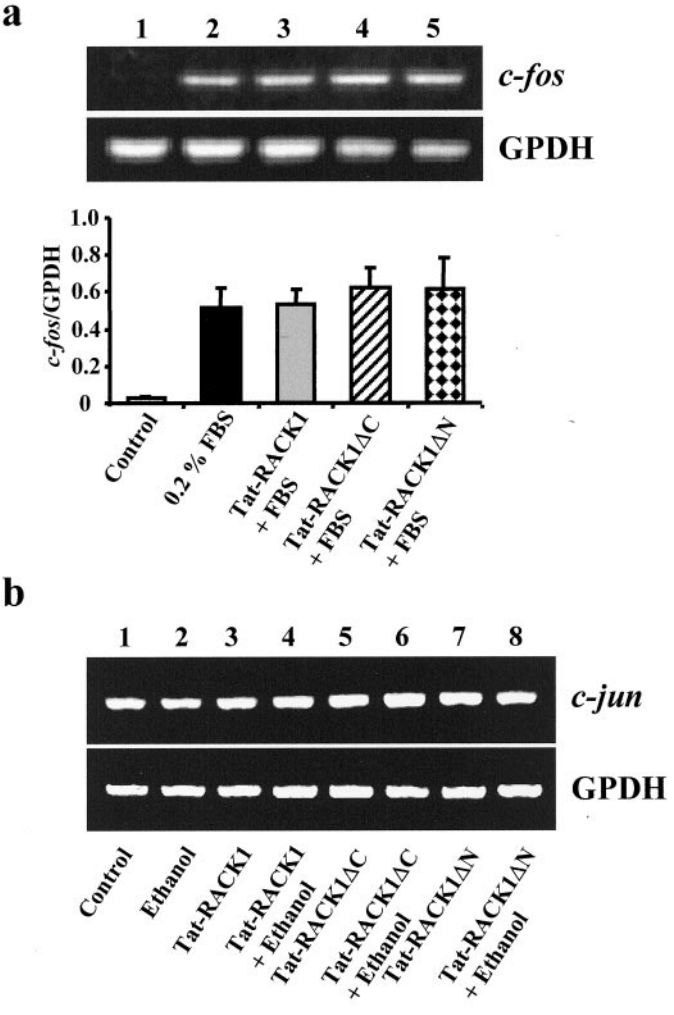

Fig. 7. Serum induction of c-fos expression does not involve RACK1, and c-jun expression levels are not regulated by RACK1. a, Tat-RACK1, Tat-RACK1ΔC, and Tat-RACK1ΔN do not alter c-fos mRNA expression induced by serum. Cells were serum-starved for 24 h and treated with vehicle (lanes 1 and 2), Tat-RACK1 (1 μM; lane 3), Tat-RACK1ΔC (1 μM; lane 4), and Tat-RACK1ΔN (1 μM; lane 5) for 30 min. The cells were then treated with 0.2% FBS for 30 min (lanes 2–5). c-fos/GPDH ratios were quantified by NIH Image 1.61. Results are mean ± S.D. of three experiments. b, Tat-RACK1, Tat-RACK1ΔC, and Tat-RACK1ΔN do not alter c-jun mRNA expression. Cells were serum-starved for 24 h and treated with vehicle (lanes 1 and 2), Tat-RACK1 (1 μM; lanes 3 and 4), Tat-RACK1ΔC (1 μM; lanes 5 and 6), and Tat-RACK1ΔN (1 μM; lanes 7 and 8) for 30 min. Cells were then treated with 100 mM ethanol (lanes 2, 4, 6, and 8) for 30 min, followed by analysis of gene expression by RT-PCR. The images are representative of three experiments.

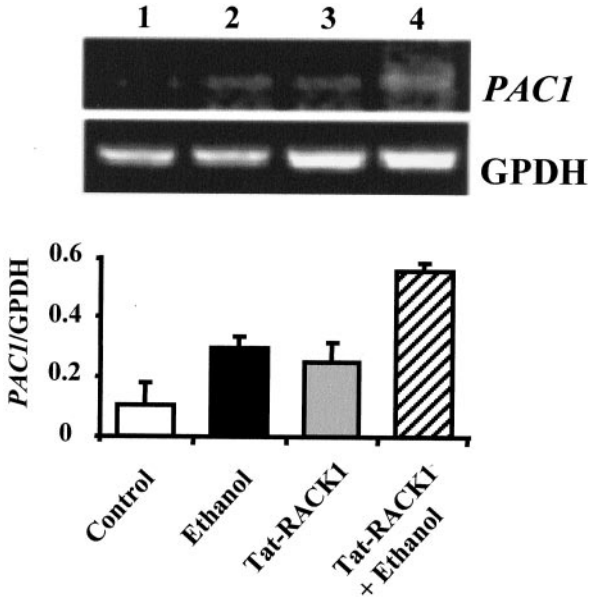

Fig. 8. Tat-RACK1 and ethanol induce mRNA expression of the *PAC1* gene. C6 cells were incubated in serum-free medium for 24 h and transduced with vehicle (lanes 1 and 2) or Tat-RACK1 (1 μM; lanes 3 and 4) for 30 min. Cells were then treated with 100 mM ethanol (lanes 2 and 4) for 30 min. *PAC1* transcription levels were determined using the reverse transcription system kit (Promega), followed by electrophoresis and photography. *PAC1*/GPDH ratios were quantified by NIH Image 1.61.

the expression of another IEG, *c-jun*. RACK1 and RACK1 fragments did not alter the induction of *c-fos* expression by serum (Fig. 7a), nor did they alter the expression levels of *c-jun* (Fig. 7b). Therefore, the function of nuclear RACK1 is likely to be specific for signaling that mediate ethanol induction of *c-fos* expression via cAMP/PKA cascade.

Last, we determined whether RACK1 affected the expression of genes that are downstream of *c-fos*. Fos is a member of the leucine zipper superfamily of transcription factors that regulate the expression of target genes by forming heterodimers with members of the Jun family of transcription factors. These complexes bind to the activator protein 1 (AP-1) consensus site and regulate the expression of a number of late-response genes. We were interested in exploring transcription of genes containing AP-1 sites. Specifically, we were interested in examining the transcription level of receptors for the neurotrophic factor PACAP, because PACAP induces RACK1 translocation to the nucleus in a cAMP/PKA-dependent manner (D. Y. He, A. J. Vagts, D. Ron, unpublished data). The PACAP pathway has been linked to ethanol sensitivity (Wand et al., 2001), and PACAP has been shown to induce *c-fos* expression via cAMP/PKA signaling pathway (Dohrman et al., 1996). Interestingly, we found that exposure to ethanol, or the transduction of Tat-RACK1 results in the induction of mRNA expression of *PAC1*, one of the receptors for PACAP (Fig. 8, lane 1 versus 3). In addition, additive activities were identified for the induction of *PAC1* when Tat-RACK1 was incubated together with ethanol (Fig. 8, lane 2 versus 4). Because the *PAC1* gene contains an AP-1 site upstream of its promoter region, it is likely that the induction of *c-fos* via RACK1 results in the induction of genes such as *PAC1* that are downstream of *c-fos*.

Discussion

In this report, we used the Tat protein transduction method to transduce RACK1 and RACK1 fragments into cells and identified a functional role for nuclear RACK1. We found that ethanol induction of *c-fos* gene expression is mediated via cAMP/PKA and the corresponding nuclear compartmentalization of RACK1.

How is RACK1 targeted to the nucleus? Although RACK1 does not have a nuclear localization sequence, its size is small enough to ensure nuclear translocation. Other scaffolding proteins have been shown to mediate activities via nuclear compartmentalization. For example, the scaffolding STE5 has been found to translocate to the nucleus to activate the mitogen-activated protein kinase kinase cascade (Mahanty et al., 1999). In addition, several endocytic proteins were recently found to translocate to the nucleus and to induce transcription (Vecchi et al., 2001). These endocytic proteins, like RACK1, contain protein-protein interaction domains that mediate the assembly of a protein complex. We identified the N-terminal fragment of RACK1 (RACK1ΔC) to act as a dominant negative and to inhibit the nuclear compartmentalization of the endogenous protein. Thus, it is possible that the RACK1ΔC is acting as a dominant negative by competing with the endogenous RACK1 for nuclear targeting. However, it is also possible that RACK1 translocation to the nucleus is dependent on binding to another protein and RACK1ΔC inhibits that interaction.

Our results strongly suggest that sequences within the C terminus of RACK1 are mediating transcription. It is unlikely though that RACK1 acts as a transcription factor itself, because it does not contain any of the consensus DNA binding motifs. However, RACK1 has been shown to directly interact with the Epstein-Barr virus *trans*-activator protein BZLF1 (Baumann et al., 2000). BZLF1 is a DNA binding protein that binds to consensus AP-1 sites and is related to *c-fos* (Farrell et al., 1989). Therefore, BZLF1 or related proteins may be the link between RACK1 and the transcription machinery. Indeed, using BLAST search, we identified two nuclear proteins with a 70-amino acid region of high homology to BZLF1; CCAAT/enhancer binding protein α (C/EBP α) (accession number AAD19575), and cAMP response element binding protein CELF (accession number B39429). Both proteins can potentially bind RACK1 in the nucleus, a process that will initiate transcription.

Exposure to ethanol and transduction of RACK1 also result in the induction of mRNA of the PACAP receptor *PAC1*. The *PAC1* gene contains an AP-1 site upstream of its promoter region. Therefore, it is likely that the induction of PAC1 is downstream to the induction of *c-fos*. Interestingly, the PACAP signaling cascade has been previously linked to ethanol. Studies using *Drosophila melanogaster* as a genetic model to identify genes that confer sensitivity to ethanol isolated the amnesiac gene that acts via cAMP signaling pathway and has homology to the mammalian PACAP sequence (Moore et al., 1998). It is therefore possible that the increase in *PAC1* expression levels by ethanol via RACK1 will enhance PACAP signaling. PACAP has been shown to act as a neurotrophic factor (Dohrman et al., 1996). Other neurotrophic factors such as glial- and brain-derived neurotrophic factors have recently been shown to have protective activities against addictive agents such as morphine and

cocaine (Berhow et al., 1996; Messer et al., 2000). Although the deletion of the PAC1 in vivo did not alter the acute sensitivity to a hypnotic dose of ethanol (Farrell et al., 1989), we predict that the induction of the *PAC1* genes mediated via the nuclear compartmentalization of RACK1 may contribute to behaviors associated with homeostatic protection against alcohol addiction, and we are currently testing the activities of RACK1 in ethanol-related behavior paradigms.

In summary, our results suggest that the scaffolding protein RACK1 is an important molecular mediator of ethanol activities. It is well established that ethanol changes signaling cascades by altering the function of kinases and phosphatases (Chandler et al., 1998; Hoek and Kholodenko, 1998). Our results imply that ethanol activities are mediated by changes in site of activity and thus function of a scaffolding protein such as RACK1. Our results also suggest that the new site of localization of RACK1 results in even more profound changes, because it induces changes in gene expression. Furthermore, changes in gene expression mediated by RACK1 may be beneficial. These findings are therefore likely to have important implications for our understanding of cellular events induced by ethanol.

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