

Rescue of p53 Blockage by the A_{2A} Adenosine Receptor via a Novel Interacting Protein, Translin-Associated Protein X^[S]

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ABSTRACT

Blockage of the p53 tumor suppressor has been found to impair nerve growth factor (NGF)-induced neurite outgrowth in PC-12 cells. We report herein that such impairment could be rescued by stimulation of the A_{2A} adenosine receptor (A_{2A}-R), a G protein-coupled receptor implicated in neuronal plasticity. The A_{2A}-R-mediated rescue occurred in the presence of protein kinase C (PKC) inhibitors or protein kinase A (PKA) inhibitors and in a PKA-deficient PC-12 variant. Thus, neither PKA nor PKC was involved. In contrast, expression of a truncated A_{2A}-R mutant harboring the seventh transmembrane domain and its C terminus reduced the rescue effect of A_{2A}-R. Using the cytoplasmic tail of the A_{2A}-R as bait, a novel-A_{2A}-R-interacting protein [translin-associated protein X (TRAX)] was identified in a yeast two-hybrid screen. The authenticity of this interaction was verified by pull-down experiments, coimmunoprecipita-

tion, and colocalization of these two molecules in the brain. It is noteworthy that reduction of TRAX using an antisense construct suppressed the rescue effect of A_{2A}-R, whereas overexpression of TRAX alone caused the same rescue effect as did A_{2A}-R activation. Results of [³H]thymidine and bromodeoxyuridine incorporation suggested that A_{2A}-R stimulation inhibited cell proliferation in a TRAX-dependent manner. Because the antimitotic activity is crucial for NGF function, the A_{2A}-R might exert its rescue effect through a TRAX-mediated antiproliferative signal. This antimitotic activity of the A_{2A}-R also enables a mitogenic factor (epidermal growth factor) to induce neurite outgrowth. We demonstrate that the A_{2A}-R modulates the differentiation ability of trophic factors through a novel interacting protein, TRAX.

Tumor suppressor protein p53 is a nuclear, DNA-binding phosphoprotein that regulates the cell cycle (White, 1996; Levine, 1997). It is known to activate the transcription of a number of genes, including p21, Bax, Mdm2, cyclin G, and Gadd45 (Ko and Prives, 1996; Levine, 1997). Cell cycle arrest

mediated by p53 is believed to occur primarily through induction of p21, an inhibitor of cyclin-dependent kinases (Xiong et al., 1993; el-Deiry et al., 1994). Moreover, p53 regulates the transcription of various mitochondrial proteins and might greatly contribute to mitochondrial abnormalities (Sawa, 2001; Polster and Fiskum, 2004). The roles of p53 in tumor formation and apoptosis therefore have been extensively investigated. In the central nervous system, p53 has been implicated in neuronal apoptosis caused by various stresses and disorders (Herzog et al., 1998; Gilman et al., 2003; Jordan et al., 2003; Bae et al., 2005). Moreover, in a widely used neuronal model (PC-12 cells), p53 and its downstream effector (p21) were shown to play critical roles in

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ABBREVIATIONS: NGF, nerve growth factor; A_{2A}-R, A_{2A} adenosine receptor; GPCR, G protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TRAX, translin-associated protein X; FK, forskolin; CGS, CGS21680 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine; CSC, 8-(3-chlorostyryl) caffeine; CHE, chelerythrine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HS, horse serum; H89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; HEK, human embryonic kidney; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PB, phosphate buffer; PAGE, polyacrylamide gel electrophoresis; BrdU, bromodeoxyuridine; BIM, bisindolylmaleimide I; AMPK, AMP-activated protein kinase; HA, hemagglutinin; ARNO, ADP-ribosylation factor nucleotide site opener; EGF, epidermal growth factor; ANOVA, analysis of variance; EGFP, enhanced green fluorescent protein; hrGFP, Vitality humanized GFP; PKI, protein kinase inhibitor.

NGF-induced neuronal differentiation by causing cell cycle arrest (Lin et al., 1992; Eisenberg et al., 1996; Levine, 1997; Erhardt and Pittman, 1998). Accumulating evidence also suggests that p53 is critical for neuronal development, because p53 knockout mice exhibit developmental abnormalities, including neural tube defects (Armstrong et al., 1995). Suppression of the p53-mediated pathway has been reported under many conditions, including germline/somatic mutations and various types of trauma (Malkin, 2001; Jiang et al., 2003). Regulation of p53 during neuronal development, plasticity, and trauma is therefore of great interest.

Adenosine has been shown to play an essential role in modulating neuronal function via four adenosine receptors (Daval et al., 1991). The A_{2A} adenosine receptor (A_{2A}-R) belongs to the G protein-coupled receptor (GPCR) family and is involved in regulating neuronal plasticity and development (Weaver, 1993; Ribeiro, 1999; Cheng et al., 2002). We and others have previously demonstrated that in PC-12 cells, stimulation of the A_{2A}-R activates at least two major cellular signaling cascades: adenylyl cyclase/cAMP/protein kinase A (PKA) and the protein kinase C (PKC)-mediated pathways (Sobreviela et al., 1994; Chang et al., 1997; Huang et al., 2001; Charles et al., 2003). In addition, A_{2A}-R stimulation rescues the ability of PC-12 cells to proceed with NGF-evoked neurite outgrowth when the MAPK cascade is impaired (Cheng et al., 2002). In the present study, using two dominant-negative p53 mutants, we demonstrated that stimulation of the A_{2A}-R suppresses proliferation and rescues the differentiation process impaired by p53 inactivation. It is intriguing that neither the PKA- nor the PKC-mediated signaling pathway contributes to this rescue effect of the A_{2A}-R. Instead, a novel A_{2A}-R-interacting protein [the Translin-associated protein X (TRAX) (Aoki et al., 1997)] that binds to the C terminus of the A_{2A}-R might mediate the rescue effect of A_{2A}-R. TRAX was originally identified as a binding protein of Translin using a yeast two-hybrid system (Aoki et al., 1997). Translin is a protein that binds RNA and single-stranded DNA with potential functions in DNA rearrangement and repair, mitotic cell division, mRNA transport, and translational regulation (Aoki et al., 1995, 1997; Han et al., 1995; Wu et al., 1997; Hosaka et al., 2000; Ishida et al., 2002; Yang et al., 2003). TRAX is a 33-kDa protein whose amino acids have 28% identity to those of Translin (Aoki et al., 1997). Although the biological function of TRAX remains largely obscure, it forms complexes with Translin in neuronal dendrites and thus might be involved in dendritic RNA processing (Finkenstadt et al., 2000). TRAX has also been implicated in DNA repair via binding to the nuclear matrix protein, C1D, an activator of the DNA-dependent protein kinase important for DNA double-strand repair and V(D)J recombination (Erdemir et al., 2002). In the present study, we found that TRAX is a novel interacting protein of the A_{2A}-R and that overexpression of TRAX recovered the NGF-induced neurite outgrowth impaired by p53 inhibition in PC-12 cells. Moreover, down-regulation of endogenous TRAX using an antisense construct obliterated the rescue effect of A_{2A}-R. Taken together, our data suggest the involvement of TRAX in mediating the rescue effect of A_{2A}-R on neuronal differentiation in PC-12 cells.

Materials and Methods

Reagents. All reagents were purchased from Sigma Chemical (St. Louis, MO) except where otherwise specified. Forskolin (FK), CGS21680 (CGS), and 8-(3-chlorostyryl) caffeine (CSC) were obtained from Sigma/RBI (Natick, MA). Chelerythrine (CHE) and bisindolylmaleimide I (BIM) were from Calbiochem (San Diego, CA). DMEM, fetal bovine serum (FBS), and horse serum (HS) were purchased from Invitrogen (Carlsbad, CA). H89 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). NGF was obtained from Alomone (Jerusalem, Israel).

Cell Culture. PC-12 cells were originally obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (HyClone, Logan, UT) supplemented with 5% FBS (HyClone) plus 10% HS (HyClone) in an incubation chamber gassed with 10% CO₂ and 90% air at 37°C. A123, a cAMP-dependent PKA-deficient variant of PC-12 cells (Ginty et al., 1991), was kindly provided by Dr. J. A. Wagner (Cornell University Medical College, Ithaca, NY). A123 cells were maintained in DMEM supplemented with 5% (v/v) HS and 10% (v/v) FBS. HEK 293T cells were maintained in DMEM supplemented with 10% (v/v) FBS and 2 mM glutamine in an incubation chamber supplied with 5% CO₂ and 95% air at 37°C.

Transfection and Neuronal Differentiation. The expression constructs, which encode the dominant-negative mutants of p53 (R175H-p53 and R273H-p53) are described elsewhere (Bargonetti et al., 1992; Srivastava et al., 1993). The A_{2A}-R-truncated mutants were generated from pcDNA3-A_{2A}-R by PCR and were subcloned into a pcDNA3.1/V5-His vector. Primers for creating A_{2A}-R₂₅₃₋₄₁₀ were as follows: 5'-ACCATGTTCTGCTCCACGTGCCGG-3' and 5'-GGAAGGGGCAAACCTCTGAAGA-3'. The antisense construct of TRAX (TRAX_{AS}, encoding the RNA containing nucleotides 451 to 870 in the antisense direction), was also produced by PCR amplification using the following primers: 5'-GCGGCCATGCGATTGACATTTACA-3' and 5'-GCGGCGAGAAATGCCTCTTCCTG-3'. Cells were transfected using LipofectAMINE 2000 (Invitrogen) following the manufacturer's protocol. For analyzing neuronal differentiation, cells were transiently transfected with the indicated construct(s) along with 1/10 of the molar amount of an EGFP-expressing construct (Clontech, Mountain View, CA), and treated with the indicated reagent(s) for 3 days. Transfected cells were marked as EGFP-expressing cells under a fluorescent microscope with a blue filter. Cells containing neurites of at least two cell-body diameters in length were scored as neurite-bearing cells. Transfected cells that grew neurites were normalized to the number of total transfected cells and are presented as the percentage of neurite-bearing cells. In each experiment, at least 100 transfected cells were counted. HEK293T cells were transfected with the desired construct(s) at 3 × 10⁶ cells in 100-mm culture plates using LipofectAMINE 2000 as described above.

Flow Cytometry and Cell Sorting. PC-12 cells were transfected with the construct(s) of desired proteins plus hrGFP at a molar ratio of 10:1. One day after transfection, cells were harvested by centrifugation, resuspended in DMEM to a final density of 5 × 10⁶ cells/ml, and filtered through a cell strainer cap (Falcon Plastics, Oxnard, CA) to remove cell aggregates. Flow cytometry and sorting of hrGFP-positive cells were carried out using a FACSVantage instrument (BD Biosciences, San Jose, CA) with a 530 ± 15-nm bandpass filter as cells traversed the beam of an argon ion laser (488 nm, 100 mW). Data acquisition and analysis were performed with CellQuest software (BD Biosciences). Sorted cells were harvested and plated for further treatments.

cAMP Assay. Transfected and sorted PC-12 cells were plated at the density of 5 × 10⁵ cells/well (on 12-well plates) and incubated with the indicated reagent(s) for the desired period of time. Cells were washed twice with ice-cold Ca²⁺-free Locke's solution (150 mM NaCl, 5.6 mM KCl, 5 mM glucose, 1 mM MgCl₂, and 10 mM HEPES, adjusted to pH 7.4). Cellular cAMP was extracted by adding 0.3 ml of 0.1 N HCl to each well and incubating this for 10 min on ice. The

cAMP content was assayed using the ^{125}I -cAMP assay system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Production of the Polyclonal Anti-TRAX Antibody. Oligopeptides (TRAX₂₇₃₋₂₉₀, corresponding to amino acids 273–290 of mouse TRAX) were purchased from Genosys (The Woodlands, TX) and conjugated to bovine serum albumin (BSA; Sigma) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. To remove the potentially existing anti-BSA IgG, the anti-TRAX antiserum was preabsorbed with 3% BSA in phosphate-buffered saline at 4°C overnight. For double immunohistochemical staining, the anti-TRAX antibody was biotinylated as described elsewhere (Lee et al., 2003).

Immunohistochemistry and Brain Tissue Preparation. Seventy-two hours after transfection, PC-12 cells were fixed and stained with the desired primary antibody reconstituted in phosphate-buffered saline/2% goat serum at 4°C for 14–16 h. Dilution of the anti-HA antibody (Invitrogen) with anti-TRAX was at 1:1000. After extensive washing, slides were incubated with the corresponding secondary antibody conjugated with Alexa red (for the A_{2A}-R-V₅) and FITC (for TRAX) at RT for 1 h, and analyzed with the aid of a laser confocal microscope (MRC-1000; Bio-Rad, Hercules, CA).

For brain tissues, 8-week-old male C57BL/6 mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Animal Care and Use Committee of Academia Sinica. In brief, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg), and intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were carefully removed, fixed with 4% paraformaldehyde/0.1 M PB for 3–5 h, and then immersed in 30% sucrose in 0.1 M PB for 2 days. Double immunostaining of A_{2A}-R and TRAX was conducted as detailed elsewhere (Shindler and Roth, 1996) with modifications. In brief, brain sections (20 μm) were first stained with an anti-A_{2A}-R antibody (Lee et al., 2003) and visualized using a highly sensitive biotin-tyramide amplification system with Avidin-Alexa Fluor 568. The sections were then blocked sequentially using the avidin D blocking solution (Vector Laboratories, Burlingame, CA) and the biotin blocking solution (Vector Laboratories), incubated with the anti-TRAX antibody, and followed by a goat anti-rabbit IgG conjugated with Alexa Fluor 488. Patterns of double immunostaining were analyzed with the aid of a laser confocal microscope.

Western Blot Analysis. For the antisense experiments, cells were transfected with the control vector or the TRAX antisense construct (TRAX_{AS}) for 72 h. For analyzing the expression level of p21, cells were transiently transfected with the indicated construct along with 1/4 of the molar amount of an EGFP expression construct. Twenty-four hours after transfection, EGFP-positive cells were sorted and harvested using a FACS Vantage (BD Biosciences) with a 530 \pm 15-nm bandpass filter, plated in 35-mm dishes, and treated with the indicated reagent(s) for 3 days. Cells were then lysed using a lysis buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, 1 mM Na₃VO₄, 1 mM dithiothreitol, and 100 nM okadaic acid. Equal amounts of sample were separated by SDS-PAGE (Laemmli, 1970) and electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) for the Western blot analyses as described previously (Cheng et al., 2002). In general, we used a 1:1000 dilution for the anti-TRAX and anti-actin antibodies, and 1:500 for the anti-p21 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

Coimmunoprecipitation. HEK 293T cells were transfected with the A_{2A}-R-V₅ cDNA along with other desired plasmids for 72 h and lysed with ice-cold radioimmunoprecipitation assay buffer (10 mM sodium phosphate, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl). The lysates were then incubated with anti-V5 monoclonal antibody (1 μl /ml lysate; Invitrogen)

for 1 h. Protein A beads (200 μl /ml lysate) were added into the mixture and rotated gently at 4°C for 2 h. The immunoprecipitates were pelleted and washed three times with ice-cold radioimmunoprecipitation assay buffer. Lysates (input) and washed immunoprecipitates were mixed with SDS-PAGE sample buffer, boiled for 5 min, and separated on 10% SDS-PAGE, followed by Western blot analyses using the corresponding antibodies.

DNA Synthesis Assay. For the [^3H]thymidine incorporation assay, transfected and sorted cells were plated onto 12-well plates and incubated with the indicated reagents for 3 days. [^3H]Thymidine (1 mCi/ml; PerkinElmer Life and Analytical Sciences, Boston, MA) was added to the growth medium and used to label cells for 4 h at 37°C. After removing the labeling medium, cells were collected onto glass microfiber filters (Whatman, Ann Arbor, MI), washed twice with 5 ml of 0.15 M NaCl, and soaked with 5% trichloroacetic acid for 5 min. The membranes were then washed with 5% trichloroacetic acid, neutralized with 0.5 M NaOH, air-dried, and counted.

DNA synthesis was also determined using a bromodeoxyuridine (BrdU) labeling and detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. In brief, PC-12 cells were transfected with the indicated plasmids plus the expression construct of EGFP, incubated for 16–18 h, and treated with the indicated reagent(s) for 4 days. BrdU (10 mM) was then added to the culture medium for 1 h, followed by immunostaining using an anti-BrdU antibody and an anti-EGFP antibody (BD Biosciences) to identify the transfected cells. Cell immunostaining was observed and photographed using a Zeiss immunofluorescent microscope (Carl Zeiss Inc., Thornwood, NY). Totals of at least 100 cells were scored for each condition.

Results

p53 has been shown to mediate NGF-triggered cell growth arrest in PC-12 cells (Eizenberg et al., 1996; Hughes et al., 2000). In line with previous findings (Eizenberg et al., 1996), we found that overexpression of a dominant-negative p53 mutant (R175H-p53) led to suppression of NGF-evoked neurite outgrowth in PC-12 cells (Fig. 1A). It is noteworthy that activation of the A_{2A}-R using an A_{2A}-R-selective agonist (CGS) counteracted the impaired neurite outgrowth caused by R175H-p53 (Fig. 1, A and B). Expression of another dominant-negative p53 mutant (R273H-p53) that lacks DNA binding ability also inhibited NGF-evoked neurite outgrowth. Again, this blockage of neurite outgrowth could be rescued by A_{2A}-R stimulation (Fig. 1C). Pretreating PC-12 cells with an A_{2A}-R antagonist, CSC, abolished the rescue effect of CGS (Fig. 1D), indicating that CGS operates through the A_{2A}-R.

To elucidate the signaling pathway underlying the rescue effect of the A_{2A}-R, we first measured whether activation of the A_{2A}-R elevated the cellular cAMP content under the conditions tested. Transfected cells were sorted by the expression of hrGFP as described and stimulated with NGF and/or CGS. As shown in Fig. 2, regardless of the absence or presence of NGF, activation of the A_{2A}-R using CGS enhanced the cellular cAMP content at both the early stage (3 h) and the late stage (3 days) of NGF-induced differentiation. Consistent with the previously described desensitization process upon chronic stimulation (Chern et al., 1993), in all conditions tested, the cAMP level evoked by a 3-h incubation of CGS was much higher than that evoked by a 3-day incubation. We were surprised to find that p53 blockage using an R273H-p53 mutant reduced the A_{2A}-R-evoked cAMP response by approximately 50%. The mechanism by which p53

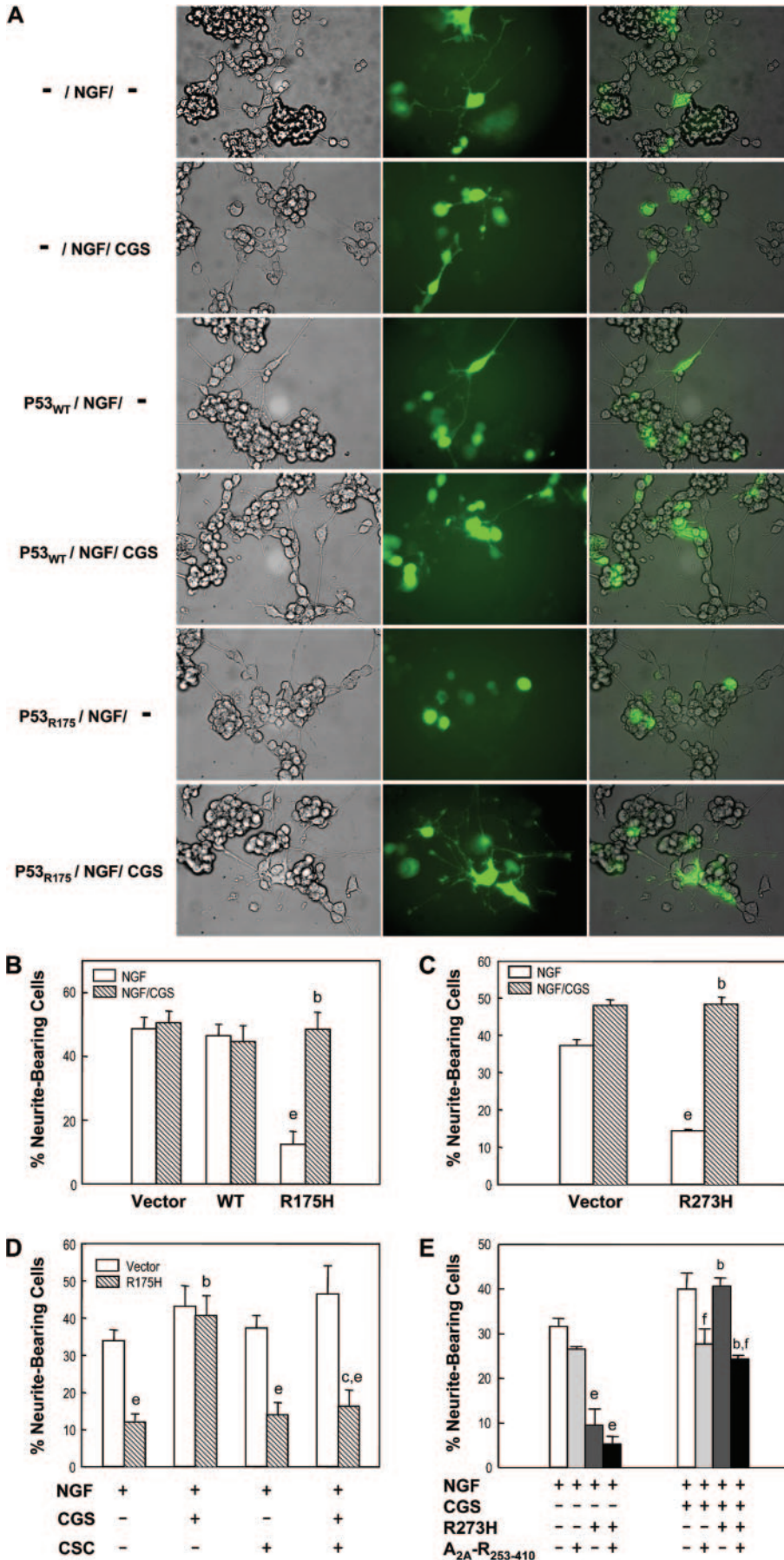


Fig. 1. Stimulation of the A_{2A} -R specifically rescued the suppression of NGF-evoked neurite outgrowth caused by p53 blockage. **A**, PC-12 cells were transiently transfected with a GFP vector, a construct encoding p53-R175H, wild-type p53, or an empty vector as indicated. One day after transfection, cells were incubated with normal growth medium containing NGF (100 ng/ml), or NGF plus CGS (1 μ M), as indicated for 72 h. Representative merged pictures (right) are phase-contrast pictures (left) overlaid with images from fluorescence microscopy (middle). **B**, NGF-evoked neurite-bearing cells transfected with the indicated plasmids treated with NGF or NGF plus CGS were quantified. **C**, PC-12 cells were transiently transfected with a control vector or with a vector encoding a p53-R273H mutant along with a GFP vector as indicated. One day after transfection, cells were incubated with normal growth medium containing NGF or NGF plus CGS for 72 h. **D**, PC-12 cells were transiently transfected with a control vector or with a vector encoding p53-R175H along with a GFP vector as indicated. One day after transfection, cells were pretreated with an A_{2A} -R-specific antagonist (CSC, 10 μ M) for 30 min before further treatment with NGF or NGF plus CGS for 3 days. **E**, expression of the C terminus of the A_{2A} -R suppressed its rescue effect. PC-12 cells were transfected with the indicated cDNAs along with a GFP vector. One day after transfection, cells were treated with NGF (100 ng/ml) or NGF plus CGS (1 μ M) for 72 h. NGF-evoked neurite-bearing cells were quantified. Data are presented as the mean \pm S.E.M. values from at least three independent experiments. **b**, specific comparison between cells treated with or without CGS in each group ($p < 0.001$; two-way ANOVA). **c**, specific comparison between cells transfected with the indicated plasmid or a control vector ($p < 0.001$; two-way ANOVA). **e**, specific comparison between cells transfected with the indicated plasmid or a control vector ($p < 0.001$; two-way ANOVA). **f**, specific comparison between cells transfected with A_{2A} -R₂₅₃₋₄₁₀ or a control vector ($p < 0.001$; two-way ANOVA).

blockage suppresses the cAMP response evoked by A_{2A}-R stimulation remains unclear.

To determine whether the cAMP-dependent kinase (PKA) mediates the rescue effect of the A_{2A}-R, we set out to determine the role of PKA. As shown in Table 1, neither of two PKA inhibitors (H89 and PKI) attenuated the rescue effect of A_{2A}-R. We also used a PKA-deficient PC-12 variant (A123) to verify the functional role of PKA in the action of A_{2A}-R. Expression of the p53-R175H mutant in A123 cells resulted in blockage of NGF-induced neurite outgrowth as occurs in parental PC-12 cells (Table 1). Most importantly, A_{2A}-R stimulation retained the ability to rescue the impaired neurite outgrowth caused by p53 blockage in A123 cells (Table 1). We next examined the involvement of PKC using two PKC inhibitors (CHE and BIM), because A_{2A}-R stimulation has been shown to activate PKCs in PC-12 cells (Lai et al., 1997; Huang et al., 2001). Similar to the effect observed with PKA inhibitors, neither CHE nor BIM alone could suppress the rescue effect of the A_{2A}-R on neurite outgrowth caused by p53 blockage (Table 2). Taken together, the rescue effect of the A_{2A}-R was both PKA- and PKC-independent.

Note that stimulation of the A_{2A}-R causes only a low and transient cAMP response as a result of desensitization of the cAMP system (Chern et al., 1993). In contrast, direct stimulation of adenylyl cyclase by FK evoked sustained and high levels of cAMP. The elevation in cAMP induced by FK (10 μ M) and CGS (1 μ M) was 10.9 ± 0.4 and 2.1 ± 0.3 nmol/10⁶ cells (mean \pm S.E.; with 20 min of incubation at RT), respectively, in PC-12 cells. It is noteworthy that FK treatment (10 μ M) also overcame the inhibitory effect of p53 blockage on

neurite outgrowth and that it was inhibited by PKI (Table 1). Thus, in contrast to the PKA-independent pathway used by A_{2A}-R stimulation, FK rescued p53 blockage via a PKA-dependent pathway.

Results described above suggest that A_{2A}-R stimulation might rescue p53 blockage through a previously uncharacterized pathway. Studies of other GPCRs have demonstrated that in addition to G proteins, GPCRs might transmit their signals via specific interacting proteins that bind to the C terminus and/or other cytosolic domains of a GPCR (Xia et al., 2003; Nickols et al., 2004). Because the C-terminal domain of the A_{2A}-R is relatively long and might exert novel function(s) (Gsandtner et al., 2005; Mijlojevic et al., 2006), we set out to determine whether the C terminus of the A_{2A}-R is involved in its rescue effect. A truncated A_{2A}-R mutant (A_{2A}-R₂₅₃₋₄₁₀), comprising the seventh transmembrane domain and the entire C terminus of the A_{2A}-R, was generated. As shown in Fig. 1E, expression of A_{2A}-R₂₅₃₋₄₁₀ reduced the rescue effect of A_{2A}-R, supporting our hypothesis that the

TABLE 1

PKA was not required for the rescue effect of A_{2A}-R

Cells were transfected with an empty vector or a construct encoding p53-R175H or/and a construct encoding PKI along with a GFP vector as indicated. One day after transfection, cells were pretreated with or without H89 (10 μ M) for 30 min before the addition of NGF (100 ng/ml), NGF plus CGS (1 μ M), or NGF plus FK (10 μ M) for 3 days. NGF-evoked neurite-bearing cells were quantified. Data represent the mean \pm S.E.M. values from at least three independent experiments.

Cell	CGS	FK	Inhibitor	Neurite-Bearing Cells	
				Vector	R175H
				%	
PC-12	—	—	—	35.4 \pm 5.2	14.3 \pm 3.2 ^a
PC-12	+	—	—	42.2 \pm 4.8	42.7 \pm 6.5 ^b
PC-12	—	+	—	39.7 \pm 1.3	42.8 \pm 9.8 ^c
PC-12	—	—	PKI	34.6 \pm 3.4	14.2 \pm 3.6 ^a
PC-12	+	—	PKI	38.4 \pm 1.8	39.0 \pm 2.7 ^b
PC-12	—	+	PKI	38.8 \pm 8.1	15.6 \pm 3.8 ^a
PC-12	—	—	H89	37.8 \pm 4.9	11.4 \pm 1.5 ^a
PC-12	+	—	H89	46.2 \pm 0.8	44.4 \pm 4.7 ^b
A123	—	—	—	43.7 \pm 1.5	22.7 \pm 1.6 ^a
A123	+	—	—	45.4 \pm 4.0	45.1 \pm 1.9 ^b

+, with the indicated drug; —, without the indicated drug.

^a Specific comparison between cells transfected with the indicated plasmid or a control vector ($p < 0.001$; two-way ANOVA).

^b Specific comparison between cells treated with or without CGS ($p < 0.001$; two-way ANOVA).

^c Specific comparison between cells treated with or without FK ($P < 0.001$; two-way ANOVA).

TABLE 2

PKC was not required for the rescue effect of A_{2A}-R

PC-12 cells were transiently transfected with an empty vector or p53-R175H along with a GFP vector. One day after transfection, cells were pretreated with the indicated inhibitor (CHE, 1 μ M; BIM, 1 μ M) for 30 min before the addition of NGF (100 ng/ml) in the absence or presence of CGS (1 μ M), as indicated, for 72 h. Values represent the mean \pm S.E.M.

CGS	Inhibitor	Neurite-Bearing Cells	
		Vector	R175H
		%	
—	—	35.2 \pm 1.2	11.1 \pm 4.1 ^b
+	—	46.4 \pm 3.5	44.7 \pm 8.2 ^a
—	CHE	37.8 \pm 2.4	14.1 \pm 4.1 ^b
+	CHE	46.2 \pm 3.5	43.7 \pm 7.1 ^a
—	BIM	37.4 \pm 5.8	13.3 \pm 2.6 ^b
+	BIM	48.1 \pm 4.1	38.4 \pm 7.5 ^a

+, with the indicated drug; —, without the indicated drug.

^a Specific comparison between cells treated with or without CGS ($p < 0.001$; two-way ANOVA).

^b Specific comparison between cells transfected with R175H or a control vector ($p < 0.001$; two-way ANOVA).

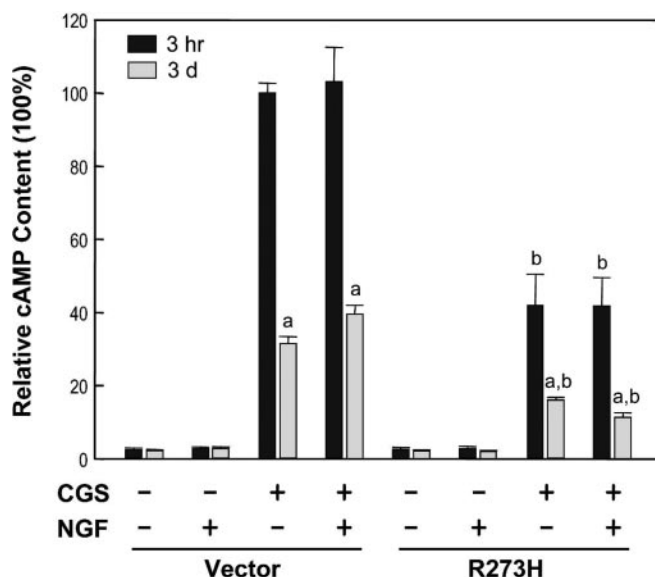


Fig. 2. Stimulation of the A_{2A}-R elevated the intracellular cAMP content in NGF-treated, R273H-p53-expressing PC-12 cells. Cells were transfected with an expression construct of R273H-p53 cells or an empty vector plus an expression vector of hrGFP. Twenty-four hours after transfection, cells were sorted by the expression of hrGFP and stimulated with the indicated reagent(s) for 3 h (closed bars) or 3 days (shaded bars). Intracellular cAMP levels were measured as described under *Materials and Methods*. Values represent the mean \pm S.E.M. of nine determinations and are expressed as percentages of the CGS-induced cAMP response in the cells transfected with an empty vector (37.0 ± 3.3 nmol/10⁶ cells). Statistical significance was evaluated by one-way analysis of variance (ANOVA). a, $p < 0.05$ compared with cells in the control, vector-transfected group. b, $p < 0.05$ compared with the cAMP level after 3 h of incubation under the same treatment conditions.

A_{2A}-R might exert its rescue effect through direct interaction with a novel protein at its C terminus. Note that expression of A_{2A}-R_{253–410} itself moderately reduced the extent of neurite outgrowth in the presence of CGS plus NGF. The C terminus of the A_{2A}-R might therefore contribute to its modulatory effect on NGF-induced neurite outgrowth as reported previously (Cheng et al., 2002).

To search for novel interacting protein(s) of the A_{2A}-R, the C terminus of the A_{2A}-R (A_{2A}-R_{290–407}) was used as bait to screen a mouse brain cDNA library using the yeast two-hybrid system. This library was chosen because of its availability and because the C terminus of rat and mouse A_{2A}-Rs are highly homologous (90% identity in amino acids). Of 5×10^6 clones, 15 independent clones expressing the reporter gene (β -galactosidase) were selected. The cDNA of each clone was analyzed by nucleotide sequencing and functional annotation using standard bioinformatic programs (Wisconsin Package, the Genetics Computer Group, Madison, WI). Among those, only one authentic cDNA clone (TRAX) was obtained. Note that the mouse and rat TRAX proteins are also highly homologous (95% identity in amino acids). TRAX was originally identified as a Translin-associated protein X (Aoki et al., 1997). Its interaction with the C terminus of the A_{2A}-R was verified by cotransforming the identified pACT2-TRAX construct with the original A_{2A}-R_{290–407} into yeast Y187 cells. Cotransformation of TRAX and A_{2A}-R_{291–407} led to expression of the reporter gene (β -galactosidase), confirming the interaction between TRAX and A_{2A}-R_{291–410} in yeast (Supplemental Materials, Fig. S1).

To further demonstrate the interaction between the A_{2A}-R and TRAX, recombinant GST and GST-TRAX proteins were purified using glutathione-Sepharose beads and were incubated with the membrane extraction of the rat striatum, which contains high levels of the endogenous A_{2A}-R (Lee et al., 2003). As shown in Fig. 3A, GST-TRAX, but not GST, successfully pulled down the striatal A_{2A}-R. The addition of excess peptide antigen (amino acids 394–410 of the A_{2A}-R) conjugated to an irrelevant protein (ovalbumin) resulted in the complete disappearance of the immunoreactive bands (Fig. 3B), confirming the specificity of the observed A_{2A}-R protein. The veracity of interaction was also confirmed by coimmunoprecipitation assays in HEK293 cells transfected with the A_{2A}-R (A_{2A}-R_{V5}) and TRAX (HA-TRAX) cDNAs. Immunoprecipitation of the A_{2A}-R protein using the V5 antibody pulled down TRAX (Fig. 3C) but not an irrelevant protein (AMPK; Fig. 3E). The transfected HA-TRAX appeared to be slightly larger (Fig. 3C) than the endogenous TRAX (~33 kDa; Fig. 4A) as a result of the presence of an HA tag at its N terminus and the 20-amino acid linker region between the HA tag and the TRAX cDNA. Colocalization of the A_{2A}-R_{V5} and HA-TRAX could also be detected (Supplemental Materials, Fig. S2). Activation of the A_{2A}-R using CGS for 2 h did not alter the amount of TRAX coimmunoprecipitated with the A_{2A}-R (Fig. 3, C and D), suggesting that interactions between the A_{2A}-R and TRAX were independent of the receptor's activity.

We demonstrated previously that, in the brain, the A_{2A}-R is enriched in the striatum and is also expressed at low levels in various regions of the brain. Because the regional expression of TRAX in the central nervous system has not yet been reported anatomically, we first set out to examine the expression of TRAX in the brain. An oligopeptide (TRAX_{273–290})

corresponding to the most C-terminal region (amino acids 273–290) of TRAX was used to produce an anti-TRAX antiserum. The anti-TRAX antiserum recognized an immunoreactive band of 33 kDa, the predicted molecular mass of TRAX, in the cytosolic fractions of the mouse cerebellum where TRAX has been reported to be expressed. Addition of

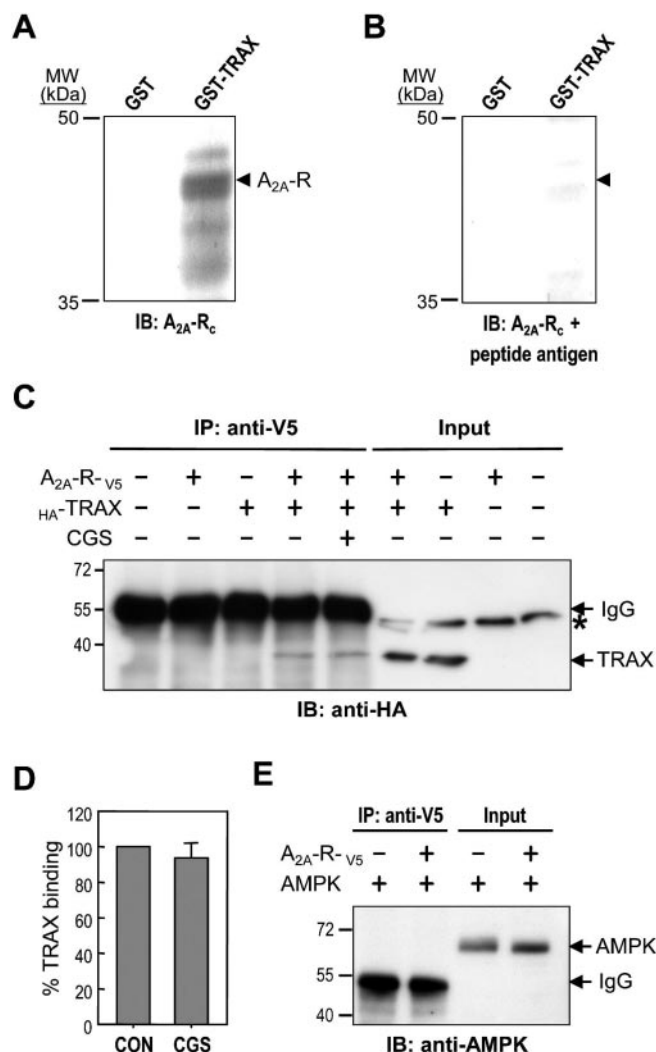


Fig. 3. The A_{2A}-R interacted with TRAX at its C-terminal domain. **A** and **B**, TRAX bound the striatal A_{2A}-R in GST pull-down assays. Striatum membrane fractions (0.4 mg) were solubilized using Nonidet P-40 (1%) and incubated with GST (1 mg) or GST-TRAX (1 mg) for 60 min at 37°C. The interacting proteins were then pulled-down using 50 μ l of glutathione-Sepharose beads. The blots were probed with an anti-A_{2A}-R antibody which recognized the C terminus of the A_{2A}-R (A_{2A}-R_C; Lee et al., 2003; **A**) and the A_{2A}-R preadsorbed with the peptide antigen (A_{2A}-R_{abs}; **B**) as indicated. **C–E**, the A_{2A}-R bound TRAX in HEK293 cells. HEK 293T cells were transfected with A_{2A}-R_{V5} cDNA along with HA-TRAX cDNA (**C**) or AMPK cDNA (**E**) for 72 h. The A_{2A}-R_{V5} and associated proteins were immunoprecipitated using the V5 antibody and analyzed for the presence of HA-TRAX or an irrelevant protein (AMPK) by Western blotting with an anti-HA or anti-AMPK antibody, respectively. Whole cell lysates (50 μ g) were run as input controls as indicated. **D**, the amount of TRAX bound to the A_{2A}-R in the absence or presence of CGS21680 (CGS) was quantified and expressed as percentages of TRAX binding in the absence of CGS. Data were generated by quantitative computed densitometry of autoradiograms from three to five independent experiments using the image analysis software package ImageQuant v.3.15 (GE Healthcare). Values represent the mean \pm S.E.M. Statistical significance was evaluated by Student's *t* test. IP, immunoprecipitation; IB, immunoblot. The asterisk (*) indicates a nonspecific band of HEK293 cells recognized by the anti-HA antibody.

excess antigen caused the complete disappearance of the TRAX-immunoreactive band (Fig. 4A). We next performed an immunohistochemical analysis of TRAX in adult mouse brains. Except for the enriched striatal expression, the expression of TRAX was similar to that of the A_{2A} -R in the brain and could be detected with different intensities in many brain areas (Supplemental Materials, Figs. S3 and S4, left). TRAX immunoreactivity was completely abolished by treatment with an excess amount of the peptide antigen, thereby verifying the specificity of TRAX (Supplemental Materials, Figs. S3 and S4, right). The overall expression profiles of the A_{2A} -R and TRAX support our hypothesis that these two molecules might be coexpressed in the brain. Indeed, double-immunohistochemical staining further demonstrated that the A_{2A} -R was colocalized with TRAX in many areas of adult mouse brains (Fig. 4).

In PC-12 cells, colocalization of the A_{2A} -R and TRAX was also evident in the presence of a p53 mutant (R273H-p53; Fig. 5A). Most importantly, transient expression of TRAX rescued the NGF-evoked neurite outgrowth impaired by p53

inhibition (Fig. 5B). Stimulation of the A_{2A} -R by CGS in TRAX-overexpressing cells did not further enhance NGF-induced neurite outgrowth, suggesting that A_{2A} -R stimulation and TRAX might use the same pathway to rescue defective neurite outgrowth. To verify whether the rescue effect of the A_{2A} -R on neurite outgrowth was mediated by TRAX, an antisense construct of TRAX (designated TRAX_{AS}) was created. Transient transfection of the antisense construct into PC-12 cells significantly diminished the expression of endogenous TRAX and markedly reduced the rescue effect of A_{2A} -R (Fig. 5C). Note that down-regulation of TRAX did not alter NGF-induced neurite outgrowth. TRAX is thus involved in the action of the A_{2A} -R but not that of NGF.

p53 has been implicated in NGF-induced cell cycle arrest (Hughes et al., 2000). We set out to examine whether A_{2A} -R stimulation also caused inhibition of proliferation assessed using [³H]thymidine incorporation assays. Transfected cells were first sorted by the expression of hrGFP and stimulated with NGF in the presence or absence of CGS for 3 days. As shown in Fig. 6, long-term treatment with NGF reduced

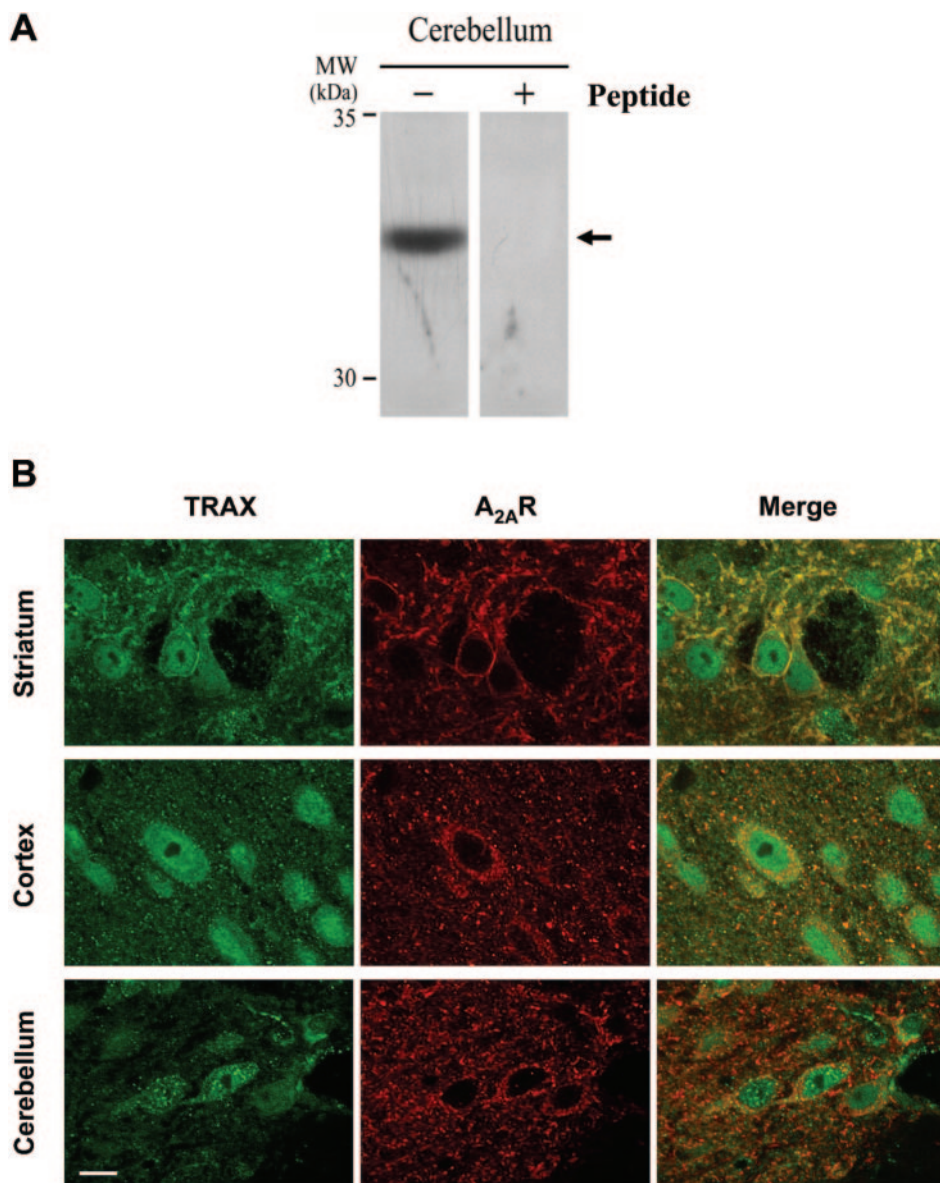


Fig. 4. Colocalization of the A_{2A} -R and TRAX in vivo. **A**, characterization of the anti-TRAX antibody. An equal amount (50 μ g) of cytosolic protein from the mouse cerebellum was loaded into each lane for Western blot analysis using the anti-TRAX antibody. The arrow indicates the TRAX band. The addition of the antigen peptide completely removed the immunoreactive band. **B**, double immunostaining of the A_{2A} -R and TRAX in various regions of the brain. TRAX was visualized by the Alexa Fluor 488 conjugated secondary antibody (green; left). The A_{2A} -R was visualized by streptavidin Alexa Fluor 568 (middle). Merged images are shown in the right. Scale bars, 6 μ m.

[³H]thymidine incorporation. As expected, p53 blockage using R273H-p53 elevated the [³H]thymidine incorporation in the presence of NGF. Most importantly, upon p53 blockage, stimulation of the A_{2A}-R using CGS lowered [³H]thymidine incorporation in the presence of NGF to a level similar to that treated with NGF alone in vector-transfected cells.

We next performed the BrdU incorporation assay to verify whether modulation of proliferation was involved in the rescue effect of A_{2A}-R. As shown in Fig. 7 and Table 3, blocking the p53-mediated pathway using a p53 mutant (R273H-p53)

reversed NGF-reduced DNA synthesis. Overexpression of TRAX caused a reduction in BrdU incorporation in a p53-independent manner. Furthermore, down-regulation of endogenous TRAX by overexpression of an antisense TRAX construct abolished the decrease in DNA synthesis mediated by A_{2A}-R activation but not by NGF treatment in either the absence or presence of p53 blockage. TRAX therefore seems to mediate the A_{2A}-R rescue effect by suppressing proliferation in a p53-independent manner.

It has been suggested that the antimitotic activity of NGF

A

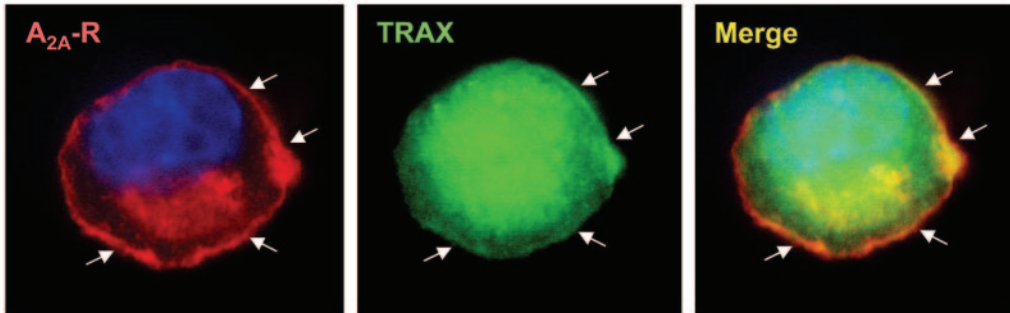
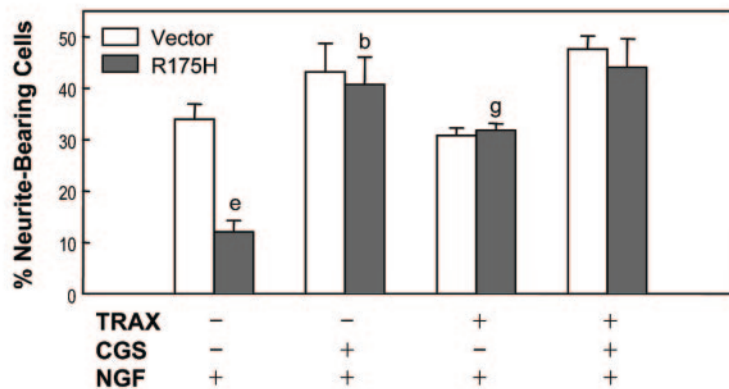
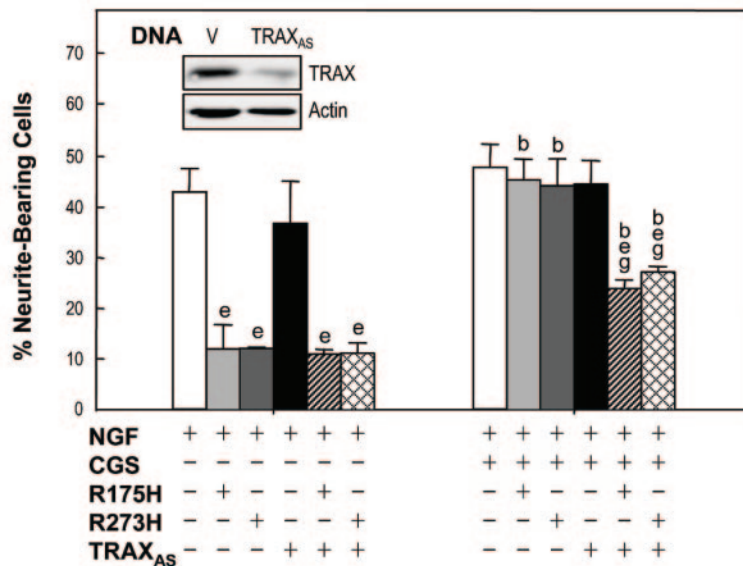


Fig. 5. TRAX mediated the rescue effect of the A_{2A}-R on damaged NGF-induced neurite outgrowth caused by p53 blockage. **A**, PC-12 cells were transfected with constructs encoding A_{2A}-R_{V5}, TRAX, and p53-R273H for 3 days. The A_{2A}-R_{V5} was visualized using streptavidin Alexa Fluor 568 (red). TRAX was visualized using the FITC secondary antibody (green). Arrows indicate the positions of colocalization. **B**, cells were transfected with the indicated plasmid(s) along with a GFP vector. **C**, cells were transfected with an antisense construct of TRAX (TRAX_{AS}) along with a GFP vector. One day after transfection, cells were treated with NGF (100 ng/ml) or NGF plus CGS (1 μM) for 3 days. Neurite-bearing cells were then quantified. Data represent the mean ± S.E.M. values from at least three independent experiments. **b**, $p < 0.001$ compared with the corresponding non-CGS-treated sample (two-way ANOVA). **e**, specific comparison between cells transfected with the indicated p53 mutant or the control vector ($p < 0.001$; two-way ANOVA). **g**, specific comparison between cells transfected with the corresponding TRAX construct or the control vector ($p < 0.001$; two-way ANOVA). The insert is a representative picture which demonstrates the expression levels of TRAX (upper) and the internal control (actin, lower) in PC-12 cells transfected with an antisense construct of TRAX (TRAX_{AS}) or an empty vector (V) by Western blot analysis.

B



C



is required for differentiation (Mark and Storm, 1997). The above findings demonstrate that A_{2A} -R stimulation and TRAX overexpression might cause cessation of proliferation

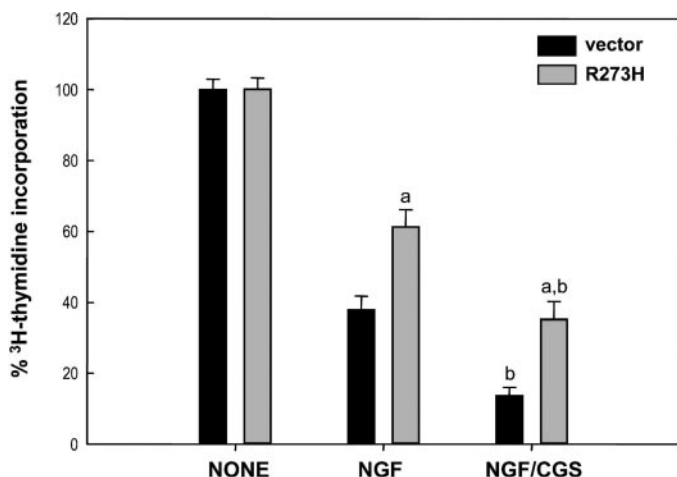


Fig. 6. A_{2A} -R stimulation restored NGF's ability to suppress proliferation in the presence of R273H-p53 ($[^3H]$ thymidine incorporation assay). PC-12 cells were transfected with an expression construct of R273H-p53 or an empty vector plus an expression vector of hrGFP. Twenty-four hours after transfection, cells were sorted by the expression of hrGFP and stimulated with the indicated reagent(s) for 3 days. $[^3H]$ Thymidine incorporation was performed as described under *Materials and Methods*. Values represent the mean \pm S.E.M. from five to seven independent experiments and are expressed as percentages of $[^3H]$ thymidine incorporation of the control, nontreated cells in each group ($17,909 \pm 2933$ cpm/ 1×10^4 cells for cells transfected with an empty vector and $12,738 \pm 3260$ cpm/ 1×10^4 cells for cells transfected with an expression construct of R273H-p53). Statistical significance was evaluated by one-way analysis of variance (ANOVA). a, specific comparison between cells transfected with an empty vector or R273H-p53 ($p < 0.05$). b, specific comparison between cells treated with NGF alone or NGF plus CGS ($p < 0.05$).

in PC-12 cells. We next examined whether A_{2A} -R activation might turn a mitogenic factor (e.g., EGF) into a differentiating factor through inhibiting proliferation. In PC-12 cells, both NGF and EGF stimulation led to a similar signal transduction pathway (Morooka and Nishida, 1998). Nevertheless, unlike NGF, EGF promoted PC-12 proliferation without evident morphological alterations (Huff et al., 1981; Maher, 1988). As shown in Fig. 8A, in combination with EGF, both an A_{2A} -R-selective agonist (CGS) and FK induced neurite outgrowth. It is noteworthy that down-regulation of TRAX using an antisense approach suppressed the neurite-inducing effect of CGS but not that of FK. In addition, overexpression of TRAX induced neurite outgrowth (Fig. 8B) and suppressed DNA synthesis (Fig. 8C) in the presence of EGF. These results support our hypothesis that stimulation of the A_{2A} -R causes a reduction in proliferation and subsequently enables EGF to trigger neuronal differentiation through a TRAX-dependent pathway.

Discussion

Many studies have shown that GPCR-interacting molecules participate in the desensitization, trafficking, targeting, signaling, fine-tuning, and allosteric regulation of GPCRs (Heuss and Gerber, 2000; Brady and Limbird, 2002; Presland, 2004). Ample evidence suggests that these interacting molecules have diverse functions and affect the action of GPCRs through direct interaction. Compared with other GPCRs, the C terminus of the A_{2A} -R is relatively long. An actin-binding protein was previously shown to interact with the C terminus of the A_{2A} -R and to contribute to the rapid desensitization process (Burgueno et al., 2003). A de-ubiquitination enzyme (Usp4) was shown to interact with the

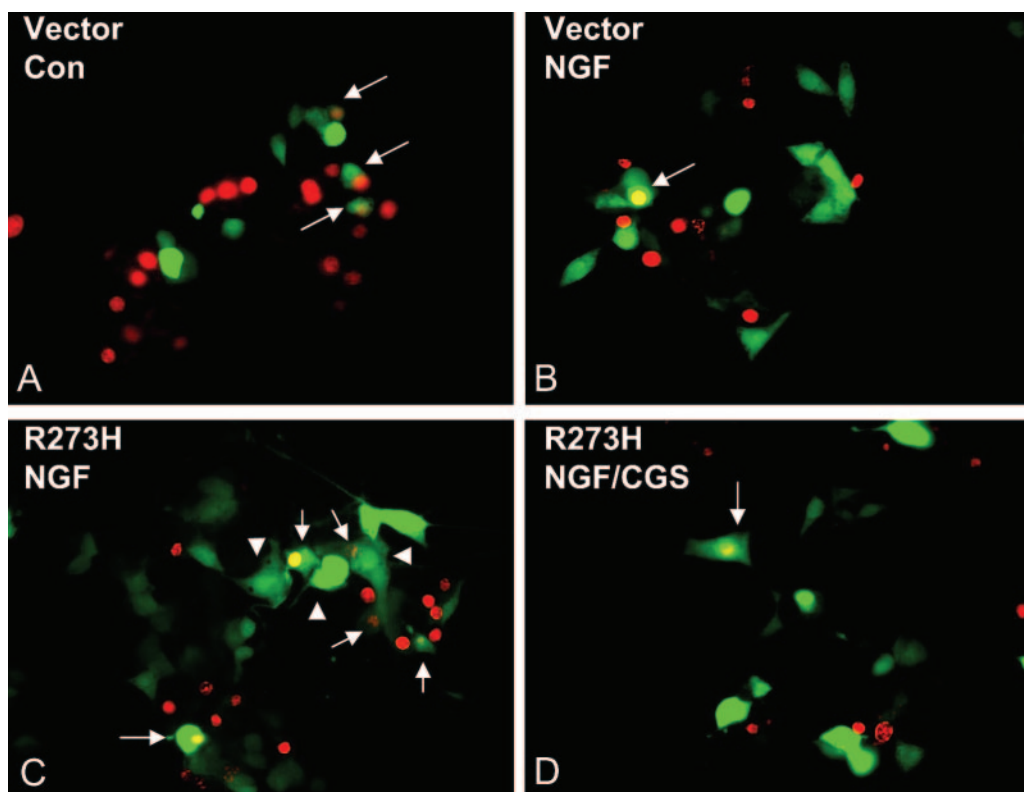


Fig. 7. A_{2A} -R stimulation restored the ability of NGF to suppress proliferation in the presence of R273H-p53 (BrdU incorporation analysis). PC-12 cells were transiently transfected with a control or a p53-R273H vector along with a GFP reporter. One day after transfection, cells were treated with NGF (100 ng/ml) or NGF plus CGS (1 μ M) for 4 days. Cells were then labeled with BrdU. Transfected cells were identified by GFP expression visualized using the FITC secondary antibody (green). BrdU incorporation was visualized using streptavidin Alexa Fluor 568 (red). Arrows indicate transfected cells positive for BrdU incorporation. To precisely visualize the BrdU incorporation in the nucleus, pictures were taken with the focus set at the mid-nuclear levels. Neurite outgrowth appeared at the bottom layer of the cells and, therefore, largely cannot be seen in the pictures. Note that approximately 40% of the NGF-treated and R273H-p53-expressing cells exhibited enlarged cell bodies (marked by arrowheads) and short neurites (with diameters of less than two cell bodies) which were not considered neurite-bearing cells in our study. Representative images from three independent experiments are shown.

A_{2A}-R. This interaction allows the A_{2A}-R to remain in the de-ubiquitinated state and to enhance the receptor's expression on the cell surface (Milojevic et al., 2006). In addition, the C terminus of the A_{2A}-R binds to a nucleotide exchange factor for ARF6 (ARNO) which mediates the sustained, G protein-independent activation of mitogen-activated protein kinase by the A_{2A}-R (Gsandtner et al., 2005). This is of particular interest because ARNO and ARF6 have been implicated in axonal elongation and branching (Hernandez-Deviez et al., 2004). These findings, together with our data presented herein, suggest that the C terminus of the A_{2A}-R seems to form complexes with multiple proteins (designated A_{2A}-R signalosome) and to mediate G protein-independent actions of the A_{2A}-R. We therefore cannot exclude the possibility that, in addition to hijacking TRAX from its association with the A_{2A}-R, expression of the A_{2A}-R C terminus might also interfere with other A_{2A}-R-interacting proteins (such as ARNO) that contribute to the rescue effect of A_{2A}-R (Fig. 1E). Nevertheless, multiple lines of evidence clearly demonstrate that TRAX plays a crucial role in the rescue effect of A_{2A}-R. First, overexpression of TRAX rescued the reduced neurite outgrowth caused by p53 blockage as did A_{2A}-R stimulation (Fig. 5B). Second, down-regulation of endogenous TRAX suppressed the ability of A_{2A}-R to rescue the p53 blockage (Fig. 5C). TRAX therefore seemed to mediate the rescue effect of A_{2A}-R by inhibiting the proliferation of PC-12 cells (Table 3; Figs. 6–8).

Accumulating evidence suggests that NGF evokes neuronal differentiation in PC-12 cells through multiple processes, including the MAPK cascade and the p53/p21 pathway (Pang et al., 1995; Hughes et al., 2000). Blocking either pathway hinders NGF-induced differentiation. Damage occurring at the MAPK cascade or the p53/p21 pathway with NGF treatment can be rescued by A_{2A}-R stimulation through distinct mechanisms. When the NGF-evoked MAPK pathway is blocked, A_{2A}-R stimulation rescues the NGF-induced neurite

outgrowth via a PKA/cAMP response element-binding protein-dependent pathway (Cheng et al., 2002). In contrast, when the NGF-induced p53 pathway is suppressed, damaged neurite outgrowth is compensated for by A_{2A}-R stimulation through a PKA-independent pathway (Fig. 9). Expression of

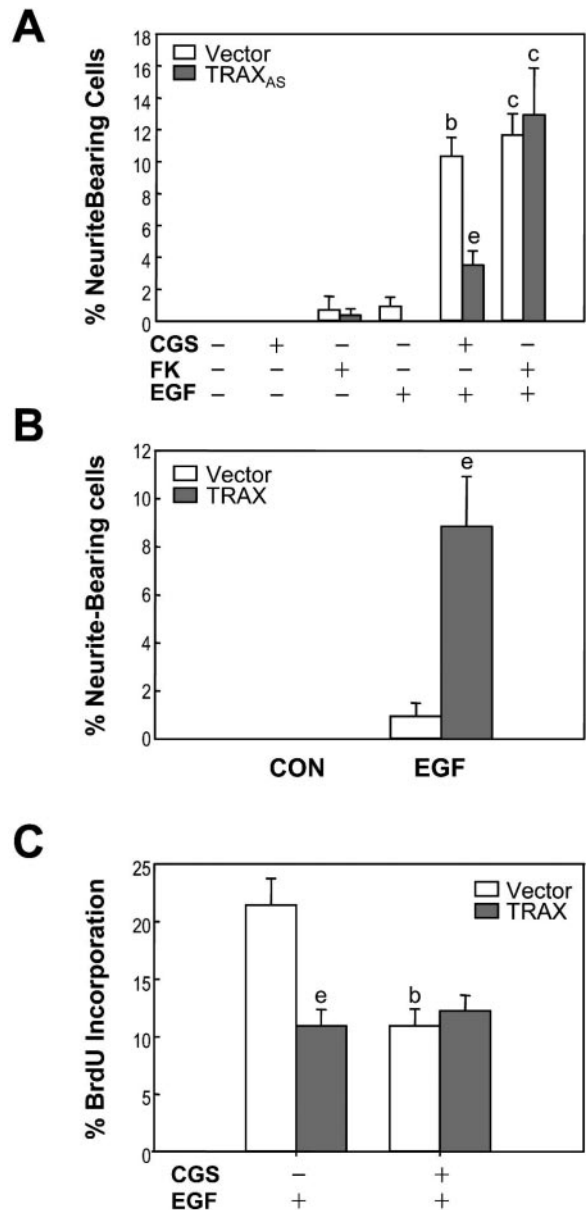


Fig. 8. A_{2A}-R stimulation enabled a mitogenic factor, EGF, to induce neurite outgrowth and to suppress DNA synthesis through a TRAX-dependent pathway. PC-12 cells were transfected with either an empty vector, an antisense construct of TRAX (TRAX_{AS}), or a TRAX expression construct, along with a GFP vector. One day after transfection, cells were treated with normal growth medium containing no additive, CGS (1 μ M), or FK (10 μ M) in the presence or absence of EGF (10 ng/ml) for 4 days as indicated. (A and B) Data are expressed as the percentage of neurite-bearing cells, and represent the mean values from three different fields. C, cells were labeled with BrdU. Data (mean \pm S.E.M.) are expressed as the percentage of transfected cells incorporating BrdU from three different fields. Transfected cells were identified by GFP expression. Three independent experiments showed similar results. b, specific comparison between cells treated with or without CGS ($p < 0.05$; two-way ANOVA). c, specific comparison between cells treated with or without FK ($p < 0.05$; two-way ANOVA). e, specific comparison between cells transfected with a control construct or a TRAX expression construct ($p < 0.05$; two-way ANOVA).

TABLE 3

Stimulation of A_{2A}-R blocked BrdU incorporation through its interacting protein, TRAX

PC-12 cells were transfected with an empty vector, a p53-R273H vector, a TRAX construct, or an antisense TRAX construct (TRAX_{AS}) as indicated along with a GFP vector. One day after transfection, cells were treated with NGF (100 ng/ml) or NGF plus CGS (1 μ M) for 4 days. Cells were then labeled with BrdU. Transfected cells were identified by GFP expression. Data (mean \pm S.E.M.) represent the percentage of transfected cells incorporated BrdU from three different fields. Three independent experiments showed similar results.

R273H	NGF	CGS	BrdU Incorporation		
			Vector	TraX	TraX _{AS}
			%		
-	-	-	26.5 \pm 2.9	11.3 \pm 1.6 ^a	19.3 \pm 1.9 ^a
-	-	+	12.9 \pm 1.0 ^b	8.8 \pm 1.6 ^a	21.2 \pm 3.0 ^a
-	+	-	2.3 \pm 0.9 ^c	5.1 \pm 1.9 ^{a,c}	5.2 \pm 2.0 ^{a,c}
-	+	+	2.9 \pm 2.0 ^c	3.7 \pm 1.8 ^c	13.4 \pm 1.6 ^{a,b,c}
+	-	-	20.5 \pm 2.8	13.0 \pm 1.2 ^a	23.1 \pm 2.4
+	-	+	15.0 \pm 0.6 ^b	13.1 \pm 0.5	19.1 \pm 0.8 ^{a,b}
+	+	-	13.9 \pm 0.8 ^{c,d}	9.4 \pm 0.5 ^{a,c,d}	15.3 \pm 1.3 ^{c,d}
+	+	+	7.3 \pm 0.6 ^{b,c,d}	6.1 \pm 0.8 ^{b,c}	12.2 \pm 0.3 ^{a,b,c}

+, with the indicated drug; -, without the indicated drug.

^a Specific comparison between cells transfected with the corresponding TRAX construct or the control vector ($p < 0.001$; two-way ANOVA).

^b Specific comparison between cells treated with or without CGS ($p < 0.001$; two-way ANOVA).

^c Specific comparison between cells treated with or without NGF ($p < 0.001$; two-way ANOVA).

^d Specific comparison between cells transfected with R273H or a control vector ($p < 0.001$; two-way ANOVA).

a dominant-negative MAPK mutant (Seth et al., 1992; Cheng et al., 2002) did not jeopardize the ability of the A_{2A} -R to rescue the blockage of neurite outgrowth caused by p53 damage (data not shown), suggesting that MAPK is unlikely to be involved. Because long-term CGS treatment led to a sustained but low level of cAMP elevation (Fig. 2), the role of cAMP is therefore interesting. Using various kinase inhibitors, we demonstrated that PKA is not involved (Table 1). Another important cAMP effector, Epac (Bos, 2003), has recently been shown to convert a PKA-mediated proliferative signal into a differentiation signal in PC-12 cells (Kiermayer et al., 2005). In addition, activation of the A_{2A} -R during hypoxia or by a prokaryotic nucleoside has been shown to induce neurite outgrowth via a cAMP-dependent pathway (Charles et al., 2003; O'Driscoll and Gorman, 2005). Although TRAX seems to play the major role in mediating the rescue effect of A_{2A} -R on p53 blockage (Fig. 5; Table 3), we cannot completely rule out the potential involvement of Epac. In the future, it will be of great interest to assess whether Epac is involved in the TRAX-mediated suppression of proliferation by A_{2A} -R stimulation.

TRAX was originally identified as a Translin-interacting protein (Aoki et al., 1997). Binding with TRAX greatly reduces the ability of Translin to bind to RNA but not to DNA (Chennathukuzhi et al., 2001). Both TRAX and Translin have been found in centrosomes and are believed to play critical roles in cell cycle controls (Castro et al., 2000; Ishida et al., 2002). Indeed, down-regulation of TRAX using the siRNA approach was shown to reduce the growth rate in HeLa cells (Yang et al., 2004), suggesting that TRAX plays a central role in proliferation. We were surprised to find that overexpression of TRAX in PC-12 caused a reduction in proliferation (Table 3). Cell-type specificity might have contributed to such a discrepancy. Recent studies have indicated that TRAX might work in concert with other interacting molecules. For example, TRAX interacts specifically with the nuclear matrix protein, C1D, an activator of DNA-dependent protein kinase (Erdemir et al., 2002) and an upstream activator of p53 (Rothbarth et al., 1999). In addition, four other interacting proteins of TRAX (i.e., snaxip1, MEA-2, Akap9, and Sun-1) that are located in the cytoplasmic fractions have been reported (Bray and Hecht, 2002). In the present study,

we identified the A_{2A} -R, a membrane protein, as a novel TRAX-interacting protein. The mechanism employed by TRAX to suppress proliferation upon A_{2A} -R activation is currently unclear. Because p21 is a major downstream target of p53 for cell cycle regulation, we performed experiments to determine whether the rescue effect of A_{2A} -R/TRAX is mediated by p21. In agreement with a previous report (Yan and Ziff, 1995), NGF treatment elevated p21 (Supplemental Materials, Fig. S6). Blocking p53 signaling using R273H-p53 markedly reduced the p21 expression level under all conditions tested. The ability of the A_{2A} -R to regulate proliferation thus seems to be independent of the p53/p21 pathway. Results of a recent study employing Translin-null mice suggest that post-transcriptional stabilization is important for the function of TRAX in proliferation (Yang et al., 2004). Because a de-ubiquitination enzyme exists in the A_{2A} -R signalosome and TRAX is a ubiquitinated protein (Yang et al., 2004; Milojevic et al., 2006), it will be of great interest to examine whether A_{2A} -R stimulation regulates the ubiquitination/expression of TRAX and subsequently affects proliferation. Another interesting aspect is that Translin and TRAX were found to bind to a nonprotein-coding RNA (BC1) and to form ribonucleoprotein particles (Muramatsu et al., 1998). These ribonucleoprotein particles are translocated to neuronal dendrites and might play a modulating role in local translation within dendrites in response to activity-dependent regulation (Kobayashi et al., 1998). The interaction between TRAX and the A_{2A} -R might thus provide a novel link that allows a membrane protein to transmit signals to the nucleus or to the translational machinery upon extracellular stimuli.

Accumulating evidence suggests that p53 is critical for embryonic development. Mice expressing no p53 exhibit a high frequency of developmental abnormalities, including early embryonic death, neural tube defects, and craniofacial malformations (Armstrong et al., 1995). Moreover, p53 has been implicated in neuronal apoptosis caused by various stresses (Herzog et al., 1998; Jordan et al., 2003) and is believed to contribute to the synaptic dysfunction of neurodegenerative diseases (Gilman et al., 2003; Jordan et al., 2003). Suppression of the p53-mediated pathway has been reported under various conditions. For example, in Huntington's disease, mutant Huntingtin aggregates recruit p53 and subsequently alter the activities of the p53-regulated promoters p21 and MDR-1 (Steffan et al., 2000; Bae et al., 2005). Furthermore, addictive drugs were found to elevate the expression of murine double minute clone 2 (MDM2), a negative regulator of p53, suggesting the involvement of p53/Mdm2 in the development of drug addiction (Jiang et al., 2003). Most importantly, germline and somatic mutations that inactivate the transactivating function of p53 have been well documented in a wide spectrum of tumor types (Malkin, 2001). Results of the present study suggest that stimulation of the A_{2A} -R might rescue the function of neurotrophins through TRAX upon p53 inactivation. Another intriguing finding reported herein is that A_{2A} -R stimulation converted a mitogenic factor (EGF) into a differentiating factor via suppression of proliferation in a TRAX-dependent manner (Fig. 7; Table 3). Note that expression of the A_{2A} -R is found in many areas of the brain (Lee et al., 2003) and has previously been implicated in neuronal development as a result of its dynamic expression during embryogenesis (Weaver, 1993). The role of the A_{2A} -R in modulating and compensating neu-

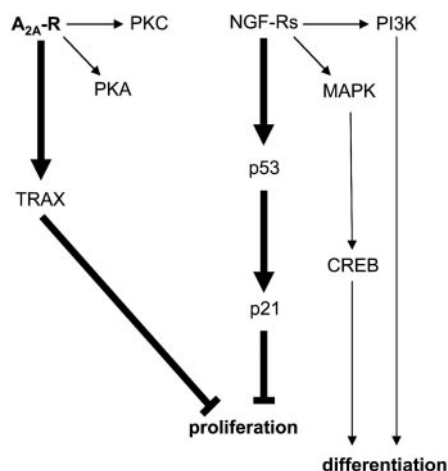


Fig. 9. Schematic representation of signaling pathways that mediate the action of A_{2A} -R action in rescuing NGF-induced neurite outgrowth abolished by blockage of the p53-mediated proliferation suppression.

ronal functions in vivo therefore might be more important than previously recognized.

In summary, we provide evidence to demonstrate that the C terminus of the A_{2A}-R interacts with TRAX to suppress proliferation in PC-12 cells. These findings add additional dimensions to the multiple signaling pathways used by GPCRs to transmit signals and modulate functions. Results of the present study also provide important insights into the cross-talk between trophic factors (differentiating or mitogenic factors) and the A_{2A}-R in neuronal trauma and neurological diseases.

Acknowledgments

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