Activation of Mitogen-activated Protein Kinase by the
A2A-adenosine Receptor via a rap1-dependent and via a
p21ras-dependent Pathway*

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The A2A-adenosine receptor, a prototypical Gα-coupled receptor, activates mitogen-activated protein (MAP) kinase in a manner independent of cAMP in primary human endothelial cells. In order to delineate signaling pathways that link the receptor to the regulation of MAP kinase, the human A2A receptor was heterologously expressed in Chinese hamster ovary (CHO) and HEK293 cells. In both cell lines, A2A agonist-mediated cAMP accumulation was accompanied by activation of the small G protein rap1. However, rap1 mediates A2A receptor-dependent activation of MAP kinase only in CHO cells, the signaling cascade being composed of Gαs, adenyl cyclase, rap1, and the p68 isoform of B-raf. This isoform was absent in HEK293 cells. Contrary to CHO cells, in HEK293 cells activation of MAP kinase by A2A agonists was not mimicked by 8-bromo-cAMP, was independent of Gαs, and was associated with activation of p21ras. Accordingly, overexpression of the inactive S17N mutant of p21ras and of a dominant negative version of mSos (the exchange factor of p21ras) blocked MAP kinase stimulation by the A2A receptor in HEK293 but not in CHO cells. In spite of the close homology between p21ras and rap1, the S17N mutant of rap1 was not dominant negative because (i) overexpression of rap1(S17N) failed to inhibit A2A receptor-dependent MAP kinase activation, (ii) rap1(S17N) was recovered in the active form with a GST fusion protein comprising the rap1-binding domain of raftGDS after A2A receptor activation, and (iii) A2A agonists promoted the association of rap1(S17N) with the 68-kDa isoform of B-raf in CHO cells. We conclude that the A2A receptor has the capacity to activate MAP kinase via at least two signaling pathways, which depend on two distinct small G proteins, namely p21ras and rap1. Our observations also show that the S17N version of rap1 cannot be assumed a priori to act as a dominant negative interfering mutant.

Adenosine is ubiquitously released from metabolically active cells and is rapidly generated in the extracellular space by dephosphorylation of ATP that has been released from nerve terminals as a cotransmitter. Hypoxia leads to accumulation of excessive amounts of the nucleoside. In the extracellular space, adenosine serves as an autacoid that regulates the function of virtually every organ and tissue via four different classes of G protein-coupled receptor subtypes, designated A1, A2A, A2B, and A3-adenosine receptor (1). These differ by their affinity for the endogenous agonist as well as by their pharmacological specificity. In addition, they operate through distinct signaling mechanisms. The A1- and A3-adenosine receptors control most, if not all, their cellular effectors via pertussis toxin-sensitive G proteins of the Gs and Go family; in contrast, both A2A- and A2B-adenosine receptors couple to Gi and thereby stimulate cAMP formation (2).

In primary cultures of endothelial cells from various species and vascular beds, adenosine stimulates proliferation (3, 4), an observation that is consistent with a possible role of adenosine in angiogenesis (5). In human endothelial cells derived from the umbilical vein, the mitogenic effect is mediated by the A2A-adenosine receptor (6); however, the available evidence indicates that growth stimulation is not mediated by cAMP, because forskolin, an activator of adenyl cyclase, and membrane-permeable cAMP analogs such as 8-Br-cAMP inhibit endothelial cell growth (6, 7). In addition, the endothelial A2A-adenosine receptor stimulates phosphorylation of the MAP kinase isoforms erk1 and erk2 (8) and of p70S6 kinase (9), effects that cannot be mimicked by 8-Br-cAMP.

It has long been appreciated that elevated levels of cAMP inhibit the proliferation of many cells (10). This effect is thought to arise, at least in part, from inhibition of the interaction of p21ras with the downstream kinase ras-f1, an effect that leads to cAMP-mediated suppression of the MAP kinase pathway (11–14). However, in some cells activation of PKA does lead to activation of MAP kinase, the best studied example being PC12 cells; in this pheochromocytoma cell line, the stimulation of MAP kinase by cell-permeable analogs of cAMP has been linked to activation of B-raf via rap1, another member of the ras-like family of small monomeric GTP-binding proteins (15). These findings predict that receptor-induced adenyl cyclase activation ought to activate MAP kinase in a manner dependent on rap1 but independent of p21ras. This prediction has been verified in S49 mouse lymphoma cells where the β2-adrenergic receptor requires Go and rap1, but not p21ras, to signal to MAP kinase (16). In contrast, in HEK293 cells, the

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1 The abbreviations and trivial names used are: 8-Br-cAMP, 8-bromo-cAMP; MAP kinase, mitogen-activated protein kinase (two isoforms of which exist: erk1 and erk2); HA, influenza hemagglutinin epitope; Mek1, MAP kinase kinase; PKA, protein kinase A (cAMP-dependent protein kinase); PKC, protein kinase C; GST, glutathione S-transferase; NECA, 5′-N-ethylcarboxamidoadenosine; GS21680, N-ethylcarboxamido-2-[4-(2-carboxethyl)phenylethyl]adenosine; CPA, N9-cyclopenyladenosine; XAC, xanthine amine congener; PD098059, 2-amino-3-(methoxyflavone; GF 109203X, bisindoylmaleimide I; bFGF, basic fibroblast growth factor; PDEu, phorbol 12,13-dibutyrate; H89, N[2-(1p-bromocinnamyl)amino]ethyl]-5-isouquinolinesulfonamide; CHO, Chinese hamster ovary.
β3-adrenergic receptor has recently been shown to activate MAP kinase via a complex pathway that involves PKA-dependent phosphorylation of the receptor; this modification causes the G protein specificity of the receptor to switch from Gs to Gi; the resulting activation of Gi, in turn, generates enough free G protein βγ-dimers to support the stimulation of MAP kinase via tyrosine kinase-dependent activation of p21ras (17). It is not clear if this model of signal transduction is generally applicable to all Gs-coupled receptors. In the present work, we have investigated the mechanism by which the A2A-adenosine receptor, a typical Gs-coupled receptor, regulates MAP kinase after heterologous expression in two cell lines, i.e. HEK293 and CHO. In both cell lines, cAMP was generated and MAP kinase was activated in response to agonist stimulation. However, MAP kinase activation was achieved via distinct signaling pathways in the two cell lines. In CHO-A2A cells, MAP kinase activation involved Gαs, cAMP, PKA, rap1, and B-rap; in contrast, in HEK-A2A cells, MAP kinase activation was independent of Gs and cAMP and required p21ras.

EXPERIMENTAL PROCEDURES

Materials—Guinea nucletides, adenosine deaminate, basic fibroblast growth factor (bFGF), the C125A anti-hemagglutinin mouse monoclonal antibody, and Complete® protease inhibitor tablets were from Roche Molecular Biochemicals. CGS21680 was from Tocris Cookson Ltd. (Bristol, United Kingdom). Hesperidin was from Boehringer (Mannheim, Germany); rolaprim and XAC were obtained from Research Biochemicals (Natick, MA). The materials required for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. Fetal calf serum was from PAA Laboratories (Linz, Austria), Dulbecco’s modified Eagle’s medium, non-essential amino acids, β-mercaptoethanol, and G418 (Genetin) were obtained from Life Technologies, Inc. Ham’s F-12 medium was from BioConcept (Allschwil, Switzerland). cAMP, 8-Bromo-cAMP, protein A-Sepharose, CPA, DNase I, cholaer toxin, forskolin, pertussis toxin, L-glutamine, DFBu, penicillin G, streptomycin, soybean trypsin inhibitor, lysosome, Triton X-100, and thrombin were purchased from Sigma. The Mek1 inhibitor PD98059 and affinity-purified rabbit antisera recognized the GTP-bound forms of erk1 and erk2 or all forms of erk1/erk2 from New England Biolabs Inc. (Beverly, MA). The inhibitor of protein kinase A H89 was from Alexis Corp. (Laufelfingen, Switzerland). The Micro BCA® protein assay reagent kit was from Pierce. Buffers and salts were from Merck (Darmstadt, Germany). [3H]Adenine was from NEN Life Science Products. Gibbons-Sepharose (GSH-Sepharose) preequilibrated in pull-down buffer (50 mM Tris, 200 mM NaCl, 2 mM β-glycerophosphate, 10 mM p-nitrophenol phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₃, 10 mM NaF, pH adjusted to 7.4 with HCl, 1% Nonidet P-40, 0.1% SDS, 250 units/ml aprotinin, 40 μg/ml leupeptin). The cellular debris was removed by centrifugation at 10,000 g for 10 min, and the total protein content was measured photometrically using biocinchonic acid (Micro BCA kit, Pierce). Aboce corresponded to 2.5–5 × 10⁶ cells per well. The B-Luc reporter plasmid contained 300 bp of the luciferase gene upstream of the promoter and coding region, driven by a cytomegalovirus-based promoter. The reporter plasmid was cotransfected with an equal amount of Renilla luciferase plasmid (pRL-SV) and an appropriate control plasmid to monitor transfection efficiency. The vectors were transfected into COS-7 cells using Fugene™ reagent (Boehringer). The immunoreactive bands on nitrocellulose blots were detected by chemiluminescence using SuperSignal chemiluminescence substrate from Pierce.

Cell Culture and Cellular Transfections—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium, CHO cells were grown in Ham’s F-12 nutrient mixture at 5% CO2 and 37 °C. Culture media were supplemented with 10% fetal calf serum, 2 mM l-glutamine, β-mercaptoethanol, non-essential amino acids, 100 units/ml penicillin G, 100 units/ml streptomycin. Medium for culture of CHO cells were supplemented with 0.2 mg/ml Geneticin (G418) in order to maintain the selection pressure. For transient transfections, HEK-A2A cells were plated at a density of 8 × 10⁵ cells/60-mm dish and transiently transfected with 5–10 μg of the desired cDNAs using the calcium phosphate precipitation method. CHO-A2A cells were plated at a density of 10⁶ cells/10-cm dish and transiently transfected using the polycationic liposome SuperFect™ reagent. Co-transfection of pEGFP-C1, a vector carrying a red-shifted variant of wild-type green fluorescent protein cDNA from the jellyfish Aequorea victoria, served as a control to monitor transfection efficiency by fluorescence microscopy. When required in cotransfections with several plasmids, the appropriate empty vectors were added to keep the amount of DNA/dish constant. The medium was changed to remove excess DNA precipitates 3–18 h after transfection. Serum starvation and incubation with adenosine deaminase was initiated on day 1 or 2 after transfection, prior to the MAP kinase assay; at this time point, transfected (and control) CHO-A2A cells were also replated; cells from one transfection on a 10-cm dish were divided onto four 6-cm dishes. The subsequent starvation under serum-free conditions lasted for 24 h. For HEK- and CHO-A2A, respectively, transfected MAP kinase stimulation was done as described below. Primary cultures of human endothelial cells were isolated from umbilical veins and propagated as described previously (7).

Stimulation of MAP Kinase Phosphorylation and Immunoblots—Cells were grown to confluence on 60-mm dishes, and rendered quiescent by serum starvation for 24–48 h prior to MAP kinase assays in order to minimize basal activity. The starving medium was supplemented with 1 IU/ml adenosine deaminase to remove any endogenously produced adenosine. Pretreatment of cells with cholera toxin was also done for 48 h in starving medium containing adenosine deaminase. If not otherwise indicated, cells were subsequently stimulated by addition of medium containing or lacking agonists at 22 °C for 5 min. Control incubations were carried out in order to verify that the carry-over of dimethyl sulfoxide, which resulted in final concentrations of <0.1%, neither affected the basal levels of MAP kinase phosphorylation nor the response to agonists. The exposure to agonists or vehicle was terminated by rapidly rinsing with ice-cold phosphate-buffered saline; thereafter, the dish was immediately immersed in liquid nitrogen. After rapid thawing, cells were lysed by addition of 80 μl of lysis buffer (50 mM Tris, pH 7.4, 1 mM Na3VO4, 10 mM NaF, 1% Nonidet P-40, 2 g/ml leupeptin). The cellular lysates for 1 ha t4° C with glutathione-Sepharose (GSH-Sepharose) preequilibrated in pull-down buffer (50 mM Tris, 200 mM NaCl, 200 mM Na2SO4, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor). Sepharose beads were washed three times in order to remove excess GST fusion protein. Cells were prepared for the assay in a manner similar to that outlined above for MAP kinase assays (serum starvation and incubation with adenosine deaminase for 24–48 h); if...
Cells were grown to confluence in six-well plates. [3H]Adenine (0.1 mCi/ml) was added 18 h prior to stimulation of the cells and was maintained throughout the subsequent incubation at the same concentration. Cholera toxin (0.03–1 μg/ml) was added for the last 30 min. Cholera toxin (0.05–1 μg/ml) was added for the time periods indicated in the figure legends. Assays were performed in triplicate. The formation of [3H]cAMP was quantified according to Salomon (21).

Each experiment reported was carried out at least three times.

**RESULTS**

**Activation of Adenyl Cyclase and of MAP Kinase by the Heterologously Expressed Human A2A Adenosine Receptor**—Incubation of stably transfected HEK-A2A and CHO-A2A cells with the A2A-selective agonist CGS21680 resulted in a substantial accumulation of cAMP (Fig. 1A); this effect was not observed in untransfected control cells (shown for CHO in Fig. 1A). The potency of CGS21680 in inducing formation of [3H]cAMP was comparable in both cell lines (EC50 = 32 ± 6 nM; Fig. 1A). Maximum levels of [3H]cAMP accumulation were about 2-fold higher in HEK-A2A than in CHO-A2A. In both, CHO-A2A and HEK-A2A cells (Fig. 1, B and C), CGS 21680 stimulated MAP kinase activity over a concentration range that was reasonably similar to that required for adenyl cyclase activation and that was comparable to that required for MAP kinase activation in primary human endothelial cells (Fig. 1B, A).

Activation of MAP kinase was mediated by the A2A adenosine receptor, because (i) the A1-selective agonist CPA was substantially (100–1000-fold) less potent (shown for CHO-A2A in Fig. 1B, A); assay conditions were as in Fig. 1(B, A)), CGS 21680 stimulated MAP kinase activity over a wide concentration range of CGS21680 (shown for CHO-A2A in Fig. 1D); (ii) the adenine receptor antagonist XAC prevented the activation of MAP kinase over a concentration range such that at 12 h after addition of cholera toxin cAMP had returned to basal levels; these remained unchanged over the next 36 h. Immunoblots with a Gαs-specific antiserum confirmed that the levels of Gαs were greatly reduced (data not shown). In the presence of PD098059, an inhibitor of MAP kinase phosphorylation (Fig. 1B, A), the adenine nucleotide pool in HEK-A2A (■), CHO-A2A (○), and CHO-wild type (●) cells was metabolically labeled with 0.1 mCi/ml [3H]adenine for 18 h; the cells were subsequently stimulated with the indicated concentrations of CGS21680 for 30 min in the presence of 100 μM rolipram and of 1 unit/ml adenosine deaminase. The incubation was terminated by the addition of 2.5% perchloric acid; [3H]cAMP was isolated by sequential chromatography on Dowex AG 50W-X4 and neutral alumina. Data represent the means of triplicate determinations. Data shown in Fig. 1 are means ± S.D from three determinations.

**Role of G Proteins**—Cholera toxin-induced ADP-ribosylation of Gαi impairs the ability of the protein to cleave GTP. This leads to irreversible activation of Gαi, resulting in a pronounced stimulation of downstream effectors; in most cells, the effect is reversed due to down-regulation of Gαi after long term toxin treatment (22). We exposed CHO-A2A and HEK-A2A cells to cholera toxin for time spans varying from 10 min to 48 h and measured the generation of [3H]cAMP (Fig. 1B). Interestingly, a different time course was observed in the two cell lines. In HEK-A2A cells (Fig. 2A, right panel), accumulation of cAMP rose rapidly; the peak was reached after 3 h of exposure to cholera toxin and was followed by a decrease of cAMP accumulation such that at 12 h after addition of cholera toxin cAMP had returned to basal levels; these remained unchanged over the next 36 h. Immunoblots with a Gαi-specific antiserum confirmed that the levels of Gαi were greatly reduced (data not otherwise stated, incubation with agonists was carried out for 5 min, followed by rapidly rinsing with ice-cold phosphate-buffered saline and addition of pull-down buffer to achieve cell lysis. Cell lysates were cleared by centrifugation (10,000 × g for 10 min). The resulting supernatants were incubated together with the GSH-Sepharose beads (50 μl of a 1:1 slurry containing about 10 μg of immobilized GST fusion protein) for 1 h to allow for the association of activated p21ras with the effector-GST fusion protein. Samples were washed three times in modified radioimmune precipitation buffer, resuspended in Laemmli sample buffer, and applied to SDS-polyacrylamide gels; p21ras or rap1 was visualized using specific antibodies in a dilution of 1:500.

**A2A and Cholera Toxin-mediated Cellular cAMP Accumulation**— CGS21680 was comparable in both cell lines (EC50 = 6 μM PD098059 prior to stimulation with the indicated concentrations of CGS21680 for 30 min in the presence of 100 μM rolipram and of 1 unit/ml adenosine deaminase. The incubation was terminated by the addition of 2.5% perchloric acid; [3H]cAMP was isolated by sequential chromatography on Dowex AG 50W-X4 and neutral alumina. Data represent the means of triplicate determinations. Data shown in Fig. 1 are means ± S.D from three determinations. Data shown in Fig. 1 are means ± S.D from three determinations. Data shown in Fig. 1 are means ± S.D from three determinations. Data shown in Fig. 1 are means ± S.D from three determinations. Data shown in Fig. 1 are means ± S.D from three determinations.
shown). In contrast, in CHO-\textit{A}2\textit{A} cells, the initial peak of cAMP production at 3 h was rather small; unlike HEK-\textit{A}2\textit{A} cells, cAMP levels increased subsequently and a pronounced elevation that lasted from 24 to 48 h was observed (Fig. 2A, left panel). A down-regulation of \textit{G}\textsubscript{\textit{a}} was also not detected in CHO-\textit{A}2\textit{A} cells by immunoblots, even if cells were exposed for 48 h to cholera toxin concentrations up to 1 \textit{m}g/ml (data not shown). Accordingly, agonist-induced generation of cAMP after 48 h of pretreatment with cholera toxin remained unaffected in CHO-\textit{A}2\textit{A} (cf. filled bars in the left panel of Fig. 2B), but was virtually abolished in HEK-\textit{A}2\textit{A} cells (Fig. 2B, right panel). We are currently unable to explain the resistance of \textit{G}\textsubscript{\textit{a}} in CHO-\textit{A}2\textit{A} cells to down-regulation by cholera toxin. More importantly, the loss of \textit{G}\textsubscript{\textit{a}} and of the receptor-mediated cAMP increase in HEK-\textit{A}2\textit{A} was exploited to determine, if MAP kinase stimulation by the \textit{A}2\textit{A} receptor required \textit{G}\textsubscript{\textit{a}}. As can be seen in the right panel of Fig. 2C, the response of MAP kinase to the \textit{A}2\textit{A} agonist was readily detectable in HEK-\textit{A}2\textit{A} cells that had been preincubated for 48 h with cholera toxin. This suggests that in HEK cells the \textit{A}2\textit{A}-adenosine may recruit other signaling mechanisms in addition to or in the place of \textit{G}\textsubscript{\textit{a}} i that trigger the MAP kinase cascade.

In contrast, after pretreatment with cholera toxin, the basal level of MAP kinase phosphorylation was substantially increased in CHO-\textit{A}2\textit{A} cells and CGS21680 failed to induce any further stimulation of MAP kinase (Fig. 2C, left panel). Activation of PKC by the phorbol ester \textit{P}2\textit{B} still augmented MAP kinase phosphorylation in cholera toxin-treated CHO-\textit{A}2\textit{A} cells (Fig. 2C, left panel); hence, we rule out that the absence of an \textit{A}2\textit{A} agonist effect reflects a general unresponsiveness of the cells. It is more likely that the high basal level of MAP kinase phosphorylation is caused by the persistent elevation of cAMP levels, which occlude any further stimulation of MAP kinase by CGS21680 on MAP kinase (see below).

In HEK293 cells, the \textit{\beta}-adrenergic receptor switches its \textit{G} protein coupling specificity from \textit{G}\textsubscript{\textit{b}}, to \textit{G} upon PKA-dependent phosphorylation; this allows for pertussis toxin-sensitive activation of MAP kinase in response to \textit{\beta}-adrenergic agonists (17). We have therefore tested whether the \textit{A}2\textit{A}-adenosine receptor-mediated MAP kinase activation was dependent on pertussis toxin-sensitive \textit{G} proteins. However, ADP-riboisylase of \textit{G}\textsubscript{\textit{b}} by pertussis toxin pretreatment of CHO-\textit{A}2\textit{A} (Fig. 3A, middle panel) and of HEK-\textit{A}2\textit{A} cells (Fig. 3A, bottom panel) had no effect on MAP kinase phosphorylation following agonist exposure. In experiments carried out in parallel under identical conditions, the incubation of stably transfected cells with pertussis toxin completely suppressed signaling by prototypical \textit{G} coupled receptors such as the \textit{A}2\textit{A}-adenosine and \textit{D}2-dopamine receptors and more than 99% of the \textit{G}\textsubscript{\textit{b}} subunits were found to be ADP-riboisylated (data not shown; see also Ref. 24).
HEK-A2A cells were also incubated with the combination of cholera toxin (300 ng/ml, 48 h) and pertussis toxin (100 ng/ml, 24 h). This harsh treatment caused many cells to detach from the plastic support; nevertheless, in the remaining adherent cells MAP kinase stimulation was still seen upon CGS21680 stimulation (data not shown). Finally, PKC isoforms can be activated in HEK-A2A cells. We have therefore transiently cotransfected CHO-A2A and HEK-A2A cells with plasmids encoding the dominant negative ras(S17N) mutant and the HA-tagged p44 MAP kinase (HA-erk1). In CHO-A2A cells, expression of dominant negative ras(S17N) mutant and the HA-tagged p44 MAP kinase resulted in MAP kinase activation (Fig. 4A). This observation is consistent with a role of rap1 in linking the A2A-adenosine receptor to MAP kinase in CHO-A2A cells. In contrast, 8-Br-cAMP (and forskolin) failed to activate MAP kinase in HEK-A2A cells (see Fig. 3B); hence, it appears unlikely that rap1 participates in A2A agonist-induced MAP kinase activation, although the protein is expressed and is activated in HEK-A2A cells.

Because p21ras plays a pivotal role in the activation of MAP kinase by both tyrosine kinase receptors and G protein-coupled receptors, we have searched for increase in GTP-bound p21ras following activation of the receptor in both CHO-A2A and HEK-A2A cells. The minimal ras-binding domain of raf-1 (amino acids 51–131) was employed as a bait to pull down activated rap1 from control cells (i.e. cells maintained in the presence of adenosine deaminase) and from cells stimulated with agonists. Activation of rap1 was seen after stimulation with CGS21680, 8-Br-cAMP and thrombin; the extent of stimulation was comparable in both cell lines (Fig. 4A). This observation is consistent with a role of rap1 in linking the A2A-adenosine receptor to MAP kinase in CHO-A2A cells. In contrast, 8-Br-cAMP (and forskolin) failed to activate MAP kinase in HEK-A2A cells (see Fig. 3B); hence, it appears unlikely that rap1 participates in A2A agonist-induced MAP kinase activation, although the protein is expressed and is activated in HEK-A2A cells.
MAP Kinase Activation by the A2A Receptor

Fig. 4. Pull-down of activated rap1 with a ralGDS-RBD/GST fusion protein (A) and of p21<sup>ras</sup> with a rap1(S17N) in CHO-A2A and HEK-A2A cell lysates. Growth-arrested cells were stimulated for 5 min with 1 μM CGS 21680 (C), 0.1 unit/ml thrombin (T), 100 μM 8-Br-cAMP (S), 5 ng/ml bFGF + 5 units/ml heparin (F), or vehicle (A). Cell lysates were incubated with GST fusion proteins (ralGDS-RBD in panel A; rap1(S17N) in panel B) immobilized on GSH-Sepharose beads as outlined under “Experimental Procedures.” Proteins bound to the matrix were released by denaturation; 25% aliquots were applied onto SDS-polyacrylamide gels; rap1 and p21<sup>ras</sup> were detected by immunoblotting with the appropriate antiserum and antibody, respectively. Diagrams shown in A and B were obtained by densitometric quantification of the immunoreactivity in 3–4 experiments; error bars indicate S.E.M.

not prevent the activation of MAP kinase in CHO-A2A cells irrespective of the activator tested (Fig. 5, left panel). The dominant negative action of rap1(S17N) has been questioned recently (30). Our observations are also inconsistent with a dominant negative effect of rap1(S17N) for the following reasons: if cells transiently expressing a HA-tagged version of rap1(S17N) were stimulated with agonists, HA-rap1(S17N) was readily pulled down with ral-RBD-GST from lysates of stimulated cells. The electrophoretic mobility of HA-rap1(S17N) is lower than that of endogenous rap1. Furthermore, HA-rap1(S17N) can easily be discriminated from endogenous rap1 by employing the antibody directed against the HA epitope for immunodetection (Fig. 6A). The extent of activation of HA-rap1(S17N) and of endogenous rap1 was comparable (cf. Fig. 4A). We have also corroborated that rap1(S17N) is not inactive by assessing its ability to associate with an endogenous effector, namely with B-raf (see below).

Activation of B-raf in CHO-A2A Cells—Activation of B-raf is thought to be essential in linking cAMP-dependent activation of PKA to stimulation of MAP kinase (15, 31). Provided that the expression of B-raf is distinct in CHO-A2A and HEK-A2A cells, this may account for the difference in signaling pathways controlled by the A2A-adenosine receptor in the two cell lines. Immunoblotting of whole cell lysates with a polyclonal antibody directed against the carboxyl terminus of B-raf showed that the 68-kDa isoform of B-raf was expressed in CHO-A2A but not in HEK-A2A cells (lanes dys. in Fig. 6B). The functional role of this B-raf isoform was assessed as follows. HA-tagged rap1(S17N) was transiently expressed in CHO-A2A and HEK-A2A cells; cells were stimulated by the A2A agonist CGS21680 (or with thrombin as a positive control), and the level of rap1(S17N)-associated B-raf was detected by immunoprecipitation with an antibody directed against the HA epitope followed by immunoblotting of B-raf for (Fig. 6B, lanes A, C, and T). In samples from CHO-A2A, association of B-raf to HA-rap1(S17N) was stimulated by CGS21680. In HEK-A2A cells, neither CGS21680 nor thrombin induced an association of the p68 isoform of B-raf with HA-rap1(S17N) (Fig. 6B), although both compounds strongly activate rap1 in HEK-A2A (see Fig. 4A). Multiple isoforms (up to 10) can be generated from the B-raf gene by alternative splicing (32). However, we have not detected any additional isoform (other than p68 B-raf) in the immunocomplex with HA-tagged rap1. We note that, after stimulation of CHO-cells with thrombin, the levels of p68 B-raf complexed to HA-rap1(S17N) were not significantly increased (left panel in Fig. 6B). If a HA-tagged version of wild type rap1 was employed, comparable levels of p68 B-raf were recovered in the immunoprecipitates after stimulation with thrombin and CGS21680 (data not shown). It is also evident that the levels of active HA-rap1(S17N) formed after thrombin stimulation were substantially lower than those observed with endogenous rap1 (cf. Figs. 4A and 6A). Taken together, these observations indicate that the rap1(S17N) exerted a dominant negative effect on the signal generated by the thrombin receptor but not on that of the A2A receptor.

DISCUSSION

G protein-coupled receptors control the activity of the MAP kinase cascade via several mechanisms; these include a βγ-dimer-mediated activation of non-receptor tyrosine kinases (33), Ca<sup>2+</sup> mobilization (34) and activation of PKC isoforms (35), signaling via phosphatidylinositol 3-kinase (36), and cAMP-dependent activation of PKA (15, 16, 26). In the present study, we show that the A2A-adenosine receptor controls at least two distinct signaling pathways that lead to MAP kinase activation: it is evident from our observations that the cellular complement of signaling components determines which pathway is
utilized. In CHO cells, the A_2A-adenosine receptor regulates MAP kinase phosphorylation via a cascade composed of G_{as}, adenylyl cyclase, PKA, rap1, p68 B-raf, and Mek1. In contrast, in HEK293 cells, G_{as}, cAMP, and rap1 do not participate in the MAP kinase response because the p68 isoform of B-raf is not available; the A2A-adenosine receptor rather relies on activation of p21_{ras}. The β2-adrenergic receptor, which is endogenously expressed in HEK 293 cells, activates p21_{ras} via G_{i}; this results from a PKA-dependent phosphorylation of the receptor, which switches its G protein-specificity from G_{as} to G_{i}. ng/ml bFGF + 5 units/ml heparin (F), 0.1 mM 8-Br-cAMP or vehicle (A).

Cell lysates (600 μg) were subjected to immunoprecipitation with a monoclonal anti-hemagglutinin antibody. Immunodetection of activated HA-erk1 was accomplished with the antiserum directed against phosphorylated MAP kinase as in Fig. 1; n.d., not determined. The diagrams shown under the immunoblots were obtained by densitometric quantification of the immunoreactivity in three experiments (error bars indicate S.E.).
terminus of the receptors (54, 55). The \( \Delta \alpha_{2} \)-adenosine receptor has an extended carboxyl terminus, which plays an ill-defined role in signaling (56, 57). Its potential role in MAP kinase activation is currently being explored.

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