The small G protein RAP1 and the kinase B-Raf have been proposed to link elevations of cAMP to activation of ERK/mitogen-activated protein (MAP) kinase. In order to delineate signaling pathways that link receptor-generated cAMP to the activation of MAP kinase, the human A₂A-adenosine receptor, a prototypical Gₛ-coupled receptor, was heterologously expressed in Chinese hamster ovary cells (referred to as CHO-A₂A cells). In CHO-A₂A cells, the stimulation of the A₂A-receptor resulted in an activation of RAP1 and formation of RAP1-B-Raf complexes. However, overexpression of a RAP1 GTPase-activating protein (RAP1GAP), which efficiently clamped cellular RAP1 in the inactive GDP-bound form, did not affect A₂A-agonist-mediated MAP kinase stimulation. In contrast, the inhibitor of protein kinase A H89 efficiently suppressed A₂A-agonist-mediated MAP kinase stimulation. Neither dynamin-dependent receptor internalization nor receptor-promoted shedding of matrix-bound growth factors accounted for receptor internalization nor receptor-promoted shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors accounted for shedding of matrix-bound growth factors. Here, β-arrestin functions as adapter protein for the recruitment of SRC and for the assembly of a large signaling complex. This model has been primarily developed with the β₂-adrenergic receptor (10) and predicts that stimulation of MAP kinase by the receptor is blocked by abrogating dynamin and SRC. (iv) Finally, G protein-coupled receptors may promote transactivation of tyrosine kinase receptors by causing the shedding of matrix-bound growth factors; this effect depends on the activation of matrix metalloproteases (11).

Although G protein-coupled receptors may recruit multiple and redundant pathways to stimulate MAP kinase (12), it is evident that some of the proposed mechanisms are mutually exclusive. It is also difficult to understand why protein kinase A is required for CAMP-dependent stimulation of MAP kinase (13) if GTP-activated RAP1 is formed by the action of Epac (7, 8). In the present study, we have therefore tested the predictions of the four models; we compared the action of receptor-independent elevations of cAMP (by membrane-permeable analogues and forskolin, the direct activator of adenylyl cyclase) with the effect of the A₂A-adenosine receptor. This Gₛ-coupled ERK, extracellular signal-regulated protein kinase; HA tag, influenza hemagglutinin-epitope tag; Mek1, MAP kinase kinase; GST, glutathione S-transferase; CGS21680, N-ethylcarboxamido-2-[4-(2-carboxyethyl)phenylethyl]adenosine; PDBu, phorbol 12,13-dibutyrate; H89, N-[2-(4-bromocinnamyl)amino]ethyl-5-isoquinolinesulfonamide; PMSF, phenylmethylsulfonyl fluoride; PP1, [4-amino-5-(4-methylphenyl)-7-(4-butylnonyl)pyrazolo[3,4-d]pyrimidin)]; CHO, Chinese hamster ovary; 8-Br-cAMP, 8-bromo-cAMP; GFP, green fluorescent protein; EGF, epidermal growth factor; GAP, GTPase-activating protein.
receptor activates MAP kinase in CHO cells in a manner dependent on cAMP (14). Our experiments show that RAP1 and dynamin-dependent receptor endocytosis are dispensable for receptor and cAMP-dependent activation of MAP kinase; similarly, MAP kinase stimulation cannot be accounted for by transactivation of a receptor tyrosine kinase due to release of a matrix-bound release growth factor. In contrast, SRC (or an SRC-like kinase) plays an essential role, but it is downstream of protein kinase A.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adenosine deaminase, basic fibroblast growth factor, 12CA5 anti-hegammaglutinin mouse monoclonal antibody, and enzymes for DNA manipulation were from Roche Molecular Biochemicals. CGS21680 was from Tocris Cookson Ltd. (Bristol, UK). Hepes was from BioMol (Munich, Germany). The materials required for SDS-PAGE were from Bio-Rad. Fetal calf serum was from PAA Laboratories (Linz, Austria); Dulbecco’s modified Eagle’s medium, Opti-MEM medium, horse serum, non-essential amino acids, β-mercaptoethanol, and G418 (geneticin) were obtained from Invitrogen. Ham’s F-12 medium was from BioConcept (Allschwil, Switzerland). Collagen was from Biomedical Technologies Inc. (Stoughton, MA). Centrifuge tubes and tissue culture plates were from Greiner (Viena, Austria) and from Corning Costar (Acton, MA). Forkolin, 8-Br-cAMP, t-glutamic acid, penicillin G, streptomycin sulfate, 100 U/ml penicillin, 100 µg/ml streptomycin, leupeptin, and thrombin were from Sigma. Aprotinin, PP3, and PDBu were from Calbiochem. PP1 was from Alexis Biochemicals (San Diego, CA). The inhibitor of protein kinase A H89 was from Alexis Corp. (Laeufelfingen, Switzerland). The Micro BCA® protein assay reagent kit was from Pierce. Buffers and salts were from Merck. Glutathione-Sepharose and protein G-Sepharose was from Amersham Biosciences. Polyclonal rabbit antibodies against p42 and p44 MAP kinases were purchased in Laennec with an ELISA kit and visualized by horseradish peroxidase-linked secondary antibodies. Anti-mouse or antirabbit immunoglobulin antibodies were from Amersham Biosciences. The immunoreactive bands were visualized by enhanced chemiluminescence using horseradish peroxidase-linked secondary antibodies.

**Cell Culture and Cellular Transfections**—CHO and HEK293 cells were propagated and transfected as outlined earlier (14). SYF and SYF + c-SRC cells were maintained in Dulbecco’s modified Eagle’s medium at 5% CO2 and 37 °C supplemented with 10% fetal calf serum, 2 mM t-glutamine, β-mercaptoethanol, non-essential amino acids, 100 units/ml penicillin G, and 100 µg/ml streptomycin. PC12 cells were plated onto collagen-coated culture dishes and propagated in Opti-MEM medium containing 10% horse serum, 5% fetal calf serum, 2 mM t-glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin.

For co-culture of CHO or CHO-A2A with reporter CHO cells, expressing reporter MAP kinase, CHO cells were transiently transfected with a plasmid expressing a HA-tagged reporter MAP kinase. After 24 h, the transfected reporter CHO cells were seeded with CHO-A2A or control CHO cells (ratio 1:1) and allowed to adhere for 12 h. Thereafter, the cells were rendered quiescent by withdrawing serum for 12 h, and MAP kinase assays were subsequently performed as described below.

**Stimulation of MAP Kinase Phosphorylation, Immunoprecipitation, and Immunoblotting—Confluent cell layers (in 6-cm dishes) were rendered quiescent by serum starvation for 12–24 h. The starving medium was replaced with 1 unit/ml adenosine deaminase to remove endogenously produced adenosine; 30 min prior to MAP kinase assays the medium was again changed against prewarmed (37 °C) medium in order to minimize basal activity. If not otherwise indicated, cells were subsequently stimulated by addition of medium containing or lacking agonists and maintained at 37 °C for 5 min. Control incubations were carried out in order to verify that the carry-over of dimethyl sulphoxide, 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 (in mM: 50 Tris, 40 β-glycerophosphate, 100 NaCl, 10 EDTA, 10 µ-nitrophenol, 1 PMSF, 1 Na3VO4, 10 NaF, pH adjusted to 7.4 with HCl), 1% Nonidet P-40, 0.1% SDS, 250 units/ml aprotinin, 40 µg/µl leupeptin. The cellular debris was removed by centrifugation at 10,000 × g for 10 min, and the total protein content was measured photometrically using bicinchoninic acid (Micro-BCA kit, Pierce). Aliquots corresponding to 2.5 µg of total protein were dissolved in Laemmli lysis buffer containing 30 mli dithiothreitol and applied to SDS-polyacrylamide gels (monomer concentration 10–15% acrylamide, 0.26–0.4% bisacrylamide). MAP kinase phosphorylation was assayed by incubating nitrocellulose blots with an antiserum that recognizes only the dually phosphorylated forms of p42 and p44 MAP kinase; in order to rule out that the differences observed were due to the application of unequal amounts of lysates, control blots were also probed with an antiserum recognizing both the unphosphorylated (inactive) and phosphorylated (active form). The immunoreactive bands were visualized by enhanced chemiluminescence using horseradish peroxidase-linked secondary antibodies. Immunodetection of the other proteins was performed in an analogous manner, using the appropriate antibodies or antisera. In several instances the monoclonal antibody directed against the HA epitope and protein G-Sepharose that had been pre-equilibrated in lysis buffer; HA-tagged or GAFF-tagged ERK1 were used interchangeably with equivalent results. Co-immunoprecipitations of HA-tagged RAP1 with B-RAF were done in a similar manner.

**Full-Down Assays for the Determination of RAP1 Activation—**GST fusion protein of the minimal RAP1-binding domain of RatGDS (rat-RBD, see Ref. 15) were expressed in Escherichia coli (strain BL21DE3); following induction by isopropyl-thio-β-galactosidase, bacterial lysates were prepared as described. GST fusion proteins were immobilized by incubating bacterial lysates for 1 h at 4 °C with GSH-Sepharose pre-equilibrated in RIPA buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS) supplemented with 2 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µM PMSF. The Sepharose beads were washed 3 times in order to remove excess GST fusion protein. Cells were prepared for the assay in a similar way as outlined above for MAP kinase assays if 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 RIPA buffer to achieve cell lysis. Cell lysates were cleared by centrifugation (20,000 × g for 1 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 RAP1 with the RatGDS-GST fusion protein. Samples were washed 3 times in RIPA buffer, resuspended in Laemmli sample buffer, and applied to SDS-polyacrylamide gels; RAP1 was visualized using specific antibodies in a dilution of 1:250. If HA-tagged RAP1 was co-transfected, the assay was carried out in a similar way as described except that the transfection procedure preceded the assay and that the immunoblot was done with the 16B12 monoclonal antibody directed against HA tag sequence. Each experiment was at least carried out three times.

**RESULTS**

**Time Course of RAP1 and MAP Kinase Activation following Stimulation by an A2A-agonist—**Receptor-dependent stimulation of MAP kinase can be transient (i.e. a monophasic activation that fades after a few minutes) or result in sustained activation. If RAP1 were important for regulation of MAP ki-
nase by the A_{2A}-adenosine receptor, the kinetics of activation should be reasonably similar. To test this prediction, the time course of MAP kinase activation (Fig. 1A) and of RAP1 activation (Fig. 1B) was determined in CHO cells that heterologously expressed the human A_{2A}-adenosine receptor (CHO-A_{2A} cells). Addition of the A_{2A}-selective agonist CGS21680 resulted in biphasic stimulation of MAP kinase phosphorylation. An early increment in the phosphorylation of the endogenous p42 and p44 (ERK2 and ERK1) isoforms of MAP kinase was seen at 5–10 min and was followed by a sustained phase of activation after 60–90 min (Fig. 1A). It is evident from Fig. 1B (left panel) that GTP-loaded RAP1A was also detected with biphasic kinetics. We also verified by co-immunoprecipitation that receptor-dependent activation of RAP1 (by CGS21680 or by thrombin as a positive control) resulted in formation of a complex between of RAP1 with its putative effector B-Raf (Fig. 1B, right panel). RAP1 is under the control of cAMP-dependent exchange factors (7, 8). Thus, protein kinase A should be dispensable for both activation of RAP1 and stimulation of MAP kinase. This was clearly not the case. Formation of GTP-bound RAP1 was insensitive to the PKA-inhibitor H89 (Fig. 1C, lower panel); in contrast, activation of MAP kinase was suppressed by H89 (Fig. 1C, upper panel). In order to directly activate cAMP-dependent effectors, we also performed a similar set of experiments with the membrane-permeable cAMP analogue 8-Br-cAMP; the results were equivalent to those shown in Fig. 1, A–C, for the A_{2A}-agonist CGS21680 (data not shown).

Inhibitory Effect of RAP1GAP on A_{2A}-agonist-stimulated RAP1 Activation but Not on MAP Kinase Activation—The time course of RAP1 activation and the kinetics of MAP kinase phosphorylation were compatible with a cause and effect relationship. Similarly, the fact that following stimulation by an A_{2A}-agonist, RAP1 associated with B-Raf, which is a MAP kinase kinase and thus, by definition, upstream of ERK1/2. These observations clearly argued for a role of RAP1 in mediating the stimulatory effect of the A_{2A}-agonist on MAP kinase. However, the protein kinase A inhibitor H89 discriminated between stimulation of MAP kinase phosphorylation and GTP loading of RAP1. This indicated a requirement for protein kinase A rather than for an exchange factor of the Epac family. It has been argued that protein kinase A acted upstream of RAP1 activation, possibly on the RAP1 exchange factor C3G (16). In order to address this discrepancy, we employed RAP1GAP, a member of the GAP family that regulates RAP1 (17). The intrinsic GTPase activity of small G proteins is very low, and it is, in most cases, accelerated by a GTPase-activating protein (GAP) that provides one or more residues required for catalysis. Thus, GAPs switch off the active, GTP-bound state. We transiently expressed an HA-tagged version of RAP1GAP together with epitope versions of reporter RAP1 (Fig. 2A) and ERK1 (Fig. 2B). Overexpression of HA-tagged RAP1GAP efficiently prevented the accumulation of GTP-bound RAP1 after stimulation of CHO-A_{2A} cells by the A_{2A}-agonist (Fig. 2A, top row). Cells that expressed HA-RAP1GAP (middle row in Fig. 2A) synthesized less HA-RAP1 (Fig. 2A, bottom row). However, GTP-bound RAP1 was below the detection limit, even if HA-RAP1GAP-transfected cells had been stimulated by the A_{2A}-agonist. In contrast, GTP-loaded RAP1A was detected under basal conditions in vector-transfected control cells; it is evident from a comparison of the level of total (bottom row in Fig. 2A) and GTP-bound RAP1 (top row, Fig. 2A) that the lower level of reporter HA-RAP1 in HA-RAP1GAP-transfected cells clearly still would have sufficed to allow for the detection of receptor-dependent activation. Although the accumulation of GTP-bound RAP1 was completely abrogated by the overexpression of RAP1GAP, it did not have any appreciable effect on the phosphorylation of a co-transfected reporter MAP kinase in response to stimulation by CGS21680 (Fig. 2B, cf. lanes C in vector and HA-RAP1GAP-transfected cells). Similar observations were also made if cells were stimulated with the membrane-permeable cAMP analogue 8-Br-cAMP (Fig. 2B, lanes labeled 8Br). These results were difficult to reconcile with published data, namely that RAP1GAP attenuated MAP kinase activation by the β_{2}-adrenergic receptor (16). We therefore also transiently co-expressed the β_{2}-adrenergic receptor in CHO-A_{2A} cells together with the reporter MAP kinase in the absence and presence of HA-RAP1-GAP. By contrast with MAP kinase stimulation with the A_{2A}-agonist (and 8-Br-cAMP), RAP1GAP did diminish isoproterenol-stimulated phosphorylation of the reporter MAP kinase construct (Fig. 2C, cf. lanes labeled I and C for isoproterenol and CGS21680, respectively). Finally, we ruled out that any differences observed are due to a variation in the amount of reporter MAP kinase in the immunoprecipitates.
the reporter MAP kinase, and the dynamin-dependent formation of endocytotic vesicles (19), the infected with dynamin K44A, a dominant negative suppressor of isoproterenol, not shown). Fig. 3 (which did not alter the response to the agonists CGS21680 and B from a representative experiment that was reproduced twice. CHO-A2A cells were transiently transfected with plasmids encoding RAP1 (A), as in B, and C. CHO-A2A cells were transiently co-transfected with HA-tagged RAP1GAP or empty vector with an HA-tagged RAP1 (1.5 μg/6-cm dish each). After the cells had reached confluency (24 h), they were maintained in medium containing adenosine deaminase in the absence of serum for 24 h. Subsequently cell were incubated with 1 unit/ml adenosine deaminase in the absence (lanes A) and presence of 0.5 μM CGS21680 (lanes C) for 5 min. The GST pull-down was done as indicated under “Experimental Procedures”; aliquots (30%) of each sample (top row) and 20 μg of the corresponding cellular lysate (middle and bottom rows) were applied to SDS-polyacrylamide gels. Immunoblotting (IB) was performed with monoclonal 16B12 anti-HA antibody. Data are from a representative experiment that was reproduced twice. B and C, CHO-A2A cells were transiently co-transfected with plasmids encoding HA-tagged RAP1GAP or empty vector with an HA-tagged p44 MAP kinase (1.5 μg/6-cm dish each) and β2-adrenoreceptor (or the appropriate empty vector; also at 1.5 μg/dish; C) and subsequently maintained as in A. After serum starvation, the cells were incubated with adenosine 1 unit/ml deaminase (lanes A), 0.5 μM CGS21680 (lanes C), 100 μM 8-Br-cAMP (lanes Br), or 1 μM isoproterenol (lanes I) in C for 5 min. The HA-tagged reporter MAP kinase was immunoprecipitated (IP) from cellular lysates as outlined under “Material and Methods.” Aliquots (30%) of the immunoprecipitates were applied onto SDS-polyacrylamide gels. Immunoblotting was done with anti-sera directed against the carboxyl-terminus of MAP kinase (IB, anti P-ERK) and the carboxyl-terminus of MAP kinase (IB, anti ERK).

because comparable levels were detected with an antiserum that recognizes holo-ERK1/2 (bottom rows in Fig. 2, B and C).

Involvement of Endocytosis and of Transactivation—Activation of MAP kinase by β2-adrenoreceptors is dependent on receptor internalization (18). In order to test if this was also true for the A2A-receptor, CHO-A2A cells were transiently co-transfected with dynamin K44A, a dominant negative suppressor of dynamin-dependent formation of endocytotic vesicles (19), the reporter MAP kinase, and the β2-adrenoreceptor. As a control, we employed the plasmid encoding wild type dynamin (which did not alter the response to the agonists CGS21680 and isoproterenol, not shown). Fig. 3A shows that the response to the β2-agonist isoproterenol was blunted in cells that expressed dynamin K44A; in contrast, dynamin K44A did not have any appreciable effect on the stimulation of reporter MAP kinase by the A2A-adenosine receptor (Fig. 3A, lanes C). Two additional manipulations highlighted the fundamental difference between activation of ERK1/2 by the β2-adrenoreceptor and A2A-adenosine receptor; neither overexpression of the carboxyl-terminus of the β-adrenergic receptor kinase nor of phosducin, which both act as scavengers for free βγ, impaired A2A-receptor-stimulated MAP kinase activation (data not shown). In contrast, receptor-generated Gβγ is important for MAP kinase activation by the β2-adrenergic receptor (20). Taken together the observations indicated that there was a fundamental difference in the mechanism by which the A2A-adenosine receptor and the β2-adrenergic receptor impinged on MAP kinase. We have therefore explored if the A2A-adenosine receptor signaled to MAP kinase via transactivation. Originally, receptors with tyrosine kinase activity were proposed to act as scaffolds for the assembly of signaling complexes and to thereby support MAP kinase activation by G protein-coupled receptors, and this was referred to as transactivation; more recently, the emphasis has shifted to the ability of G protein-coupled receptors to promote the release of cell surface-bound growth factors, e.g. heparin-binding EGF, via activation of a matrix metalloprotease (11). By definition, this type of stimulation is paracrine stimulation; we have therefore used a coculture system that was analogous to the one originally employed by Prenzel et al. (11; see also ref. 21); CHO-A2A or control CHO cells were mixed at 1:1 ratio with reporter CHO cells that harbored an epitope-tagged MAP kinase and seeded at high density. Growth factor release and the ensuing para-

![Fig. 2](Image 61x474 to 285x728)

**Fig. 2.** Effect of RAP1GAP overexpression on activation of RAP1 (A) and of MAP kinase phosphorylation by CGS21680 and 8-Br-cAMP (B) and by isoproterenol (C) in CHO-A2A cells. A, CHO-A2A cells were transiently co-transfected with plasmids encoding HA-tagged RAP1GAP or empty vector with an HA-tagged RAP1 (1.5 μg/6-cm dish each). After the cells had reached confluency (24 h), they were maintained in medium containing adenosine deaminase in the absence of serum for 24 h. Subsequently cell were incubated with 1 unit/ml adenosine deaminase in the absence (lanes A) and presence of 0.5 μM CGS21680 (lanes C) for 5 min. The GST pull-down was done as indicated under “Experimental Procedures”; aliquots (30%) of each sample (top row) and 20 μg of the corresponding cellular lysate (middle and bottom rows) were applied to SDS-polyacrylamide gels. Immunoblotting (IB) was performed with monoclonal 16B12 anti-HA antibody. Data are from a representative experiment that was reproduced twice. B and C, CHO-A2A cells were transiently co-transfected with plasmids encoding HA-tagged RAP1GAP or empty vector with an HA-tagged p44 MAP kinase (1.5 μg/6-cm dish each) and β2-adrenoreceptor (or the appropriate empty vector; also at 1.5 μg/dish; C) and subsequently maintained as in A. After serum starvation, the cells were incubated with adenosine 1 unit/ml deaminase (lanes A), 0.5 μM CGS21680 (lanes C), 100 μM 8-Br-cAMP (lanes Br), or 1 μM isoproterenol (lanes I) in C for 5 min. The HA-tagged reporter MAP kinase was immunoprecipitated (IP) from cellular lysates as outlined under “Material and Methods.” Aliquots (30%) of the immunoprecipitates were applied onto SDS-polyacrylamide gels. Immunoblotting was done with anti-sera directed against the carboxyl-terminus of MAP kinase (IB, anti P-ERK) and the carboxyl-terminus of MAP kinase (IB, anti ERK).

![Fig. 3](Image 316x490 to 546x728)

**Fig. 3.** A, effect of dynamin and its dominant negative interfering mutant dynamin K44A on receptor-induced activation of MAP kinase in CHO-A2A cells. CHO-A2A cells were transiently transfected with plasmids encoding wild type (wt) dynamin or dynamin K44A with HA-tagged p44 MAP kinase and β2-adrenoreceptor (1.5 μg/6-cm dish each). After the cells had become confluent (24 h), they were maintained in medium containing adenosine deaminase in the absence of serum for an additional 24-h period. Subsequently, cells were incubated with 1 unit/ml adenosine deaminase (lanes A), 0.5 μM CGS21680 (lanes C), 1 μM isoproterenol (lanes I), or 1 μM PDBu (lanes P) for 5 min. The HA-tagged reporter MAP kinase was immunoprecipitated (IP) from cellular lysates as outlined under “Experimental Procedures.” Immunoblotting (IB) was performed as indicated in legend to Fig. 2. B, transactivation of reporter MAP kinase transfected in CHO cells by CHO-A2A and CHO cells upon stimulation with CGS21680. CHO cells, transiently transfected with HA-tagged reporter MAP kinase, were co-cultured with CHO-A2A or CHO cells until reaching confluency. After maintaining cells in serum-deprived medium for 12 h, cells were stimulated with 0.5 μM CGS21680 for 5 and 10 min; immunoprecipitation and immunodetection of the levels of bisphosphorylated and total ERK/MAP kinase was performed as indicated under “Experimental Procedures.”
cultures that lacked A2A-receptor-bearing cells (Fig. 3). We therefore tested incubated CHO-A2A cell in the presence or absence of PP1 for 15 min. Thereafter the cells were stimulated in the presence of 0.5 μM CGS21680 (lanes CGS) or 1 μM PDBu (lanes P) for 5 min. 

**Blockage of SRC Family Kinases or Ablation of SRC Blunts MAP Kinase Stimulation by the A2A-adenosine Receptor and cAMP**—The observations presented so far suggested that cAMP-dependent activation of PKA played an essential role in linking the A2A-adenosine receptor to the MAP kinase cascade. However, Gαs (9) and JAK2, but the concentrations required are substantially higher (i.e. in the micromolar range; Ref. 23). As mentioned earlier, over a similar concentration range, the inactive analog of PP1 did not affect MAP kinase stimulation (Fig. 5B). 

**Erk Activation by cAMP**—The observations presented so far suggested that cAMP-dependent activation of PKA played an essential role in linking the A2A-adenosine receptor to the MAP kinase cascade. However, Gαs (9) and JAK2, but the concentrations required are substantially higher (i.e. in the micromolar range; Ref. 23). As mentioned earlier, over a similar concentration range, the inactive analog of PP1 did not affect MAP kinase stimulation (Fig. 5B).
cells verified that these murine fibroblasts responded to elevations of cAMP by forskolin with an increase in MAP kinase phosphorylation (Fig. 6A, top), a response seen with the membrane-permeable cAMP analog 8-Br-cAMP and upon transient expression of the human A2A-adenosine receptor (not shown). Furthermore, in NIH3T3 fibroblasts, PP1 inhibited MAP kinase stimulation by forskolin (Fig. 6A) or 8-Br-cAMP (not shown). We therefore employed murine embryonic fibroblasts that were devoid of SRC, YES, and FYN (SYF cells) to evaluate the importance of SRC family kinases in the cAMP-dependent activation of MAP kinase. In SYF cells, we observed a pronounced reduction in the capacity of forskolin to activate MAP kinase (Fig. 6B). In cells, in which c-SRC was reintroduced via retroviral infection (SYF + c-SRC), the response to forskolin was restored. The stimulation by the phorbol ester PDBu (lanes P in Fig. 6) was used as an internal control; it is evident that the response to PDBu was reasonably similar in NIH3T3 cells, SYF cells, and SYF + c-SRC cells.

Although it is difficult to compare the levels of signaling molecules across different cell types, it is worth pointing out that the levels of SRC in SYF + c-SRC cells were not vastly different from those seen in PC12 cells (Fig. 6C), i.e. in a cell line in which MAP kinase stimulation via cAMP is a physiological response controlled by the endogenously expressed A2A-adenosine receptor. Accordingly, in SYF + c-SRC inhibition by PP1 of cAMP-dependent stimulation of MAP kinase was observed over a concentration range (Fig. 6D) that was similar to that observed in PC12 cells (Fig. 5A, middle). In fact, a comparison of all concentration-response curves showed that PP1 inhibited cAMP-dependent formation of phosphorylated MAP kinase with essentially identical IC₅₀ values (range 5–10 nM) in CHO-A₂A, PC12, and SYF + c-SRC irrespective of whether cAMP accumulation had been induced by activation of the A₂A-adenosine receptor or by direct activation of adenylyl cyclase (Fig. 6E).

Protein kinase A phosphorylates SRC on Ser¹⁷ (25). Accordingly, we employed c-SRC(S17A), a mutated version of SRC in which Ser¹⁷ was replaced by Ala, to test whether PKA-dependent phosphorylation of SRC was required for MAP kinase stimulation. SYF cells were transiently co-transfected with a plasmid encoding c-SRC or c-SRC(S17A) and a vector driving the expression of the reporter MAP kinase. As expected, addition of forskolin resulted in increased levels of phosphorylated MAP kinase in cells that expressed c-SRC (Fig. 6F, top); in contrast, in cells that contained c-SRC(S17A) forskolin failed to stimulate MAP kinase phosphorylation over the unstimulated control.
trol (cf. lanes F and U in Fig. 6F, top). The observed differences were not accounted for by different levels of reporter MAP kinase or SRC expression (Fig. 6F, middle and bottom).

DISCUSSION

It has long been appreciated that intracellular cAMP levels impinge on cell growth and cell survival (26); the effects range from inhibition of proliferation and apoptosis to trophic stimulation and differentiation. Because of the many targets of cAMP-dependent protein kinase, it has been notoriously difficult to pinpoint the underlying molecular mechanisms. More recently, a plausible mechanism was proposed that suggested that MAP kinase stimulation by cAMP was elicited via activation of RAP1 which, in its activated GTP-loaded form, combined with B-RAF (6). This model is attractive because RAS and RAP1 are closely related (with RAP standing for RAS proximate) and because in vitro B-Raf can be activated by RAP1 (5); thus, RAP1-dependent regulation of B-Raf is congruent with RAS-dependent activation of c-Raf (= RAF-1). Finally, the discovery of Epac-exchange factors that are direct targets of cAMP seemingly closed the gap between adenyl cyclase and RAP1 (7, 8) such that the cascade upstream of Mek1 can be delineated as consisting of receptor Gs adenyl cyclase/cAMP-Epac-RAP1-B-RAF. However, our observations are inconsistent with this model; the experiments relied on the use of both the A2A-adenosine receptor, a prototypical Gs-coupled receptor (27), which stimulates MAP kinase in CHO cells via cAMP and Gs (14, 28), and on stimuli that acted downstream of the receptor and Goalpha (by employing forskolin as a direct activator of adenyl cyclase isomers and the membrane-permeable cAMP analog 8-Br-cAMP). The rationale for using these two approaches is the evidence that receptor-generated cAMP may be compartmentalized and elicit effects distinct from those produced by downstream stimuli (29). Our data unequivocally show that protein kinase A and an SRC family kinase are essential components of the signaling cascade that links cAMP to MAP kinase. In contrast, RAP1 is dispensable for cAMP-dependent stimulation of MAP kinase irrespective of the source of cAMP because the deactivation of RAP1 by RAP1GAP did not affect stimulation of ERK1/2. Earlier work (30) also found that the extent of RAP1 activation did not show any correlation to the level of MAP kinase phosphorylation. We therefore conclude that cAMP generated in response to a prototypical Gs-coupled receptor (the A2A-adenosine receptor) or to forskolin (a direct activator of adenyl cyclases) does lead to complex formation between RAP1 and B-RAF but that these complexes are irrelevant to activation of ERK1/2 by cAMP, possibly because complexes formed between RAP1 and B-RAF are segregated from Mek, the kinase upstream of MAP kinase. It is not clear which biological response is elicited by this reaction, but it is evident that RAF kinases have targets other than Mek1 (31, 32).

Our work also confirmed that MAP kinase stimulation by the beta2-adrenergic receptor was abrogated if RAP1 was clamped in the inactive conformation by overexpression of RAP1GAP (16). These findings are in marked contrast to the observations obtained in parallel by directly raising cAMP or by activating the A2A-adenosine receptor. This seeming contradiction can be resolved as follows: the beta2-adrenergic receptor is rapidly (i.e. within minutes) internalized, and dynamin-dependent internalization is important for stimulation of MAP kinase (18). In contrast, the A2A-adenosine receptor is not internalized to an appreciable extent over the time frame required to induce MAP kinase phosphorylation2 (see also Ref. 34); accordingly, the interfering mutant dynamin-K444A did not affect the response to A2A-agonist stimulation. It is also worth noting that, when visualized in living cells, active GTP-bound RAP1 mainly resides on intracellular vesicles (35). We therefore propose that the role of RAP1 in MAP kinase activation is indirect, possibly by controlling the assembly of signaling complexes on intracellular vesicles, and contingent on receptor internalization.

In the three cell lines investigated (CHO-cells, PC12 cells, and NIH3T3/murine embryonic fibroblasts) in which elevations of cAMP caused MAP kinase phosphorylation, the response was inhibited by the inhibitor of SRC family kinase PP1 (but not by its inactive analog P3); obviously, these findings argue for a role of SRC or SRC-like kinases upstream of MAP kinase. This argument is further supported by three sets of observations: (i) in SYF cells that are deficient in the three ubiquitous SRC-like kinases, the MAP kinase response to elevated cAMP was strongly impaired; (ii) expression of SRC restored the ability of forskolin to stimulate MAP kinase phosphorylation; (iii) the IC50 values of PP1 were similar in the three cell lines investigated in which cAMP promoted MAP kinase phosphorylation regardless of whether cAMP was raised by the endogenous receptor (PC12 cells) or the heterologously expressed receptor (CHO-A2A) or by forskolin (SYF+c-SRC). Several distinct sites of action have been proposed for SRC (or SRC-like kinase) in the downstream cascade controlled by Gs-coupled receptors. SRC binds directly to the beta2-adrenergic but not the beta2-adrenergic receptor, and this interaction supports activation of MAP kinase (36). However, in adipocytes, i.e. the cellular background in which the beta2-adrenergic receptor occurs physiologically, the ability of the beta2-receptor to stimulate MAP kinase is fully accounted for by its ability to raise cAMP (37). It is worth pointing out that, for the A2A-adenosine receptor, this was also true not only in CHO cells (where the receptor had been introduced by stable transfection) but in PC12 cells, which endogenously express the receptor. With the beta2-adrenergic receptor, beta-arrestin serves as a docking site for SRC (10), and SRC-dependent phosphorylation of dynamin is essential for MAP kinase activation (38). It is evident that these mechanisms of recruiting are restricted to receptors that can directly bind SRC-like kinases or that require dynamin-dependent internalization. SRC (9) and the SRC-like kinase LCK (39) have been reported to be directly activated by Goalpha. This provides for a more general mechanism of activation. In endothelial cells (38) and HEK293 cells (14), stimulation of MAP kinase is independent of Goalpha. Accordingly, the SRC inhibitor PP1 did not block MAP kinase activation. However, direct activation of SRC (or SRC-like kinase) by Goalpha fails to explain (i) the ability of forskolin and 8-Br-cAMP to stimulate MAP kinase in an SRC-dependent manner and (ii) the ability of the protein kinase inhibitor H89 to block stimulation of MAP kinase. It has long been known that the major site of serine phosphorylation is on Ser17 of SRC (25); this is also the site targeted by protein kinase A (41), and PDK-1-dependent phosphorylation of SRC on Ser17 promotes its release from the plasma membrane (33, 42). This may facilitate signaling by redirecting SRC to a subset of specific substrates (40). Thus, the action of PDK-1 may be accounted for, at least in part, by a direct effect on SRC (or an SRC-like kinase). There are, however, examples of specialized cells in which MAP kinase stimulation by cAMP does not rely on PKA (and is independent of RAP1) (43). We also do not intend to claim that the link between protein kinase A and SRC (or a SRC-like kinase) is the only mechanism that accounts for MAP kinase stimulation; there was, for instance, still a slight stimulation of MAP kinase by forskolin in SYF cells. However, our data provide firm evidence for an important role of

2 O. Kudlacek, and M. Freissmuth, unpublished observations.
protein kinase A upstream of SRC (or SRC-like kinases) and that this is an essential component in the signaling cascade that links Gα-coupled receptors to stimulation of MAP kinase.

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