Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 15084–15089, December 1998
Pharmacology

Identity of adenylyl cyclase isoform determines the rate of cell cycle progression in NIH 3T3 cells

MARTINE J. SMIT, DENNIS VERZIJL, AND RAVI IYENGAR*

Department of Pharmacology, Mount Sinai School of Medicine, New York, NY 10029

Communicated by Lutz Birnbaumer, University of California, Los Angeles, CA, October 5, 1998 (received for review May 28, 1998)

ABSTRACT **Cell cycle progression is regulated by cAMP in several cell types. Cellular cAMP levels depend on the activity of different adenylyl cyclases (ACs), which have varied signal-receiving capabilities. The role of individual ACs in regulating proliferative responses was investigated. Native NIH 3T3 cells contain AC6, an isoform that is inhibited by a variety of signals. Proliferation of exogenous AC6-expressing cells was the same as in control cells. In contrast, expression of AC2, an isoform stimulated by protein kinase C (PKC), resulted in inhibition of cell cycle progression and increased doubling time. In AC2-expressing cells, platelet-derived growth factor (PDGF) elevated cAMP levels in a PKC-dependent manner. PDGF stimulation of mitogen-activated protein kinases 1 and 2 (MAPK 1,2), DNA synthesis, and cyclin D1 expression was reduced in AC2-expressing cells as compared with control cells. Dominant negative protein kinase A relieved the AC2 inhibition of PDGF-induced DNA synthesis. Expression of AC2 also blocked H-ras-induced transformation of NIH 3T3 cells. These observations indicate that, because AC2 is stimulated by PKC, it can be activated by PDGF concurrently with the stimulation of MAPK 1,2. The elevation in cAMP results in inhibition of signal flow from the PDGF receptor to MAPK 1,2 and a significant reduction in the proliferative response to PDGF. Thus, the molecular identity and signal receiving capability of the AC isoforms in a cell could be important for proliferative homeostasis.**

Homeostasis of proliferation is a complex process that is determined by a balance between external signals triggering proliferation and intracellular components regulating this process. Many mitogens trigger proliferation by activation of Ras (1–4). Ras activates Raf, initiating the protein kinase cascade to mitogen-activated protein kinases 1 and 2 (MAPK 1,2) (extra cellular signal-regulated kinases 1 and 2) ultimately triggering mitogenesis. The Ras/MAPK 1,2 pathway has been shown to transduce signals from growth factors to the cell cycle machinery (5–8). Key mediators of cell cycle progression through the G_1 phase to the S phase are the cyclins (D and E) that form complexes with specific cyclin-dependent kinases (9–11). Cyclin D1 appears to play a major role in cell cycle progression because inhibition of its expression resulted in growth arrest whereas overexpression shortened the G_1 phase (6). Inhibition of the MAPK 1,2 pathway blocks mitogen-induced expression of cyclin D1, and sustained activation of the MAPK 1,2 pathway is required for continued accumulation of cyclin D1, thus suggesting a role for MAPK 1,2 in cell cycle progression (5–8).

In many cell types, intracellular cAMP affects proliferation. Growth-stimulatory effects of cAMP have been reported in some cell types whereas growth inhibitory effects are observed in others (12–15). Elevation of intracellular levels of cAMP, by $G\alpha_s$-mediated activation of adenylyl cyclase (AC), by addition of 8-Br-cAMP, or forskolin, suppresses mitogenic signaling through protein kinase A (PKA) phosphorylation of Raf (16–19). Recently, cyclin D1 also has been found to be phosphorylated by PKA *in vitro* and has been described as a target of the cAMP inhibitory effects (20, 21). Hence, cAMP blocks transfer of signal from the growth factor receptor and Ras to MAPK 1,2 and thus inhibits proliferation. Regulation of intracellular levels of cAMP is controlled largely at the level of its synthesis by the ACs. Nine ACs have been cloned and characterized (22, 23). They are expressed in a tissue-selective manner and have different signal-receiving capabilities. ACs that are stimulated by Ca^{2+} or protein kinase C (PKC) are expressed mostly in neuronal and postmitotic cells. On the other hand, many peripheral cells express AC isoforms that are not stimulated by signals other than $G\alpha_s$ and are inhibited by a variety of signals. Because the different AC isoforms vary in their ability to receive and integrate signals, we were interested in determining whether the identity of AC isoforms present in a cell affected cell cycle progression and proliferation. We were particularly interested in adenylyl cyclase 2 (AC2) because it can be stimulated by PKC (24–26), and, hence, presumably by growth factors. We determined whether growth factor stimulation of AC2, and the subsequent elevation of cAMP, could modulate growth factor signaling through other pathways and thus regulate proliferation. For this, we expressed AC2 in NIH 3T3 cells and studied the consequences of its expression.

MATERIALS AND METHODS

Materials. Platelet-derived growth factor (PDGF), Ro-31–8220, and Ro-20–1724 were purchased from Calbiochem, cyclin D1 antibody was from Santa Cruz Biotechnology, MAPK antibodies were from New England Biolabs, and fluorescein isothiocyanate-conjugated anti-BrdU was from Becton Dickinson. DMEM and Lipofectamine were obtained from GIBCO/BRL, and bovine calf serum was from HyClone. Phorbol 12-myristate 13-acetate (PMA), 4α phorbol, propidium iodide, and BrdU were from Sigma, [^{3}H]thymidine was from NEN, and [2,8-^{3}H]adenine was from ICN.

Cell Culture. NIH 3T3 cells were grown at 37°C, 5% CO_2 in DMEM containing 5% calf serum supplemented with L-glutamine (2 mM) and 50 units/ml penicillin and 50 μg/l streptomycin. NIH 3T3 cells were stably transfected with the vector pcDNA3 or pMam-neo (inducible promoter) containing the AC2 or AC6 cDNA or vector without insert by using Lipofectamine. Clonal cell lines were obtained by selection with G-418 (0.8 mg/ml). Stable cell lines were maintained in DMEM with 0.4 mg/ml G-418. Expression of the insert was induced by dexamethasone (1 μM) and was monitored by measurement of accumulation of cAMP. Clonal cell lines were treated with dexamethasone (1 μM) on alternative days for 2 weeks before

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AC, adenylyl cyclase; MAPK 1,2, mitogen-activated protein kinases 1 and 2; PDGF, platelet-derived growth factor; PKC, protein kinase C; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate.

*To whom reprint requests should be addressed at: Department of Pharmacology, Box 1215, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. e-mail: iyengar@msvax.mssm.edu.

performance of experiments (27). For the coexpression experiments, AC2-expressing cells were cotransfected with the dominant negative subunit of PKA (pHL-REV$_{AB}$neo, 10 μg) (AB) (30) or wild-type PKA regulatory subunit (pHL-REV$_{wt}$neo, 10 μg) (WT) and the hygromycin resistance gene (pRSV1.1, 1 μg). Hygromycin resistant clones were assessed for PDGF-induced [^{3}H]thymidine incorporation.

Accumulation of cAMP. Cells were labeled with [^{3}H]adenine (2 μCi/ml) for 24 hr. Cells were preincubated for 30 min with the phosphodiesterase inhibitor Ro-20174 (0.3 mM), and accumulation of cAMP was measured for 30 min in the absence or presence of the stimulus. Accumulation of cAMP was measured as described (19) and was expressed as [^{3}H]cAMP/([^{3}H]ATP + [^{3}H]cAMP) ($\times 10^3$).

Reverse Transcription–PCR of AC Isoforms. Degenerate primers (sense 5′ KIKTIG, antisense 3′ WG(N/K)TVN) encompassing a conserved domain within the ACs, with unique sizes for each of the family members (AC2, 291 bp; AC5/6 273 bp), were used to identify the ACs present in NIH 3T3 cells, as described by Premont (28). Poly(A) mRNA was isolated and reverse transcribed by using an oligo(dT) primer, and the first strand was used for the PCR reaction. The amplified products were identified on a 4% NuSieve gel (FMC).

Cell Growth Curve. Control and AC2-expressing cells were seeded in 24-well plates (6,000 cells/well). The number of cells was determined every day for a period of a week by using a hemacytometer.

Cell Cycle Analysis. Cell cycle progression into S phase was determined by incorporation of BrdU and propidium iodide into DNA. Cells were plated at a density of 0.2×10^6 cells, were serum-starved for 24 hr, and were restimulated with DMEM containing 5% calf serum for the indicated time periods. One hr before harvesting, cells were pulse-labeled with 10 μM BrdU, were fixed in 70% ethanol (30 min, 4°C), were washed, were fixed with paraformaldehyde (0.25% in PBS), and were RNase (0.5 mg/ml in PBS)-treated for 20 min at 37°C. Thereafter, cells were treated with 0.1 M HCl/0.5% Triton X-100 for 10 min at room temperature, were boiled for 10 min, and were placed on ice for 10 min. Next, cells were double-stained with fluorescein isothiocyanate-conjugated anti-BrdU (30 min) and 10 μg/ml propidium iodide. The percentage of cells in different cell cycle phases of exponentially growing cells was determined by fixing them in 70% ethanol (30 min, 4°C). The cells were washed with PBS, were treated with 100 μg/ml RNase (Boehringer Mannheim) in PBS for 20 min at room temperature, and were stained with 5 μg/ml propidium iodide in PBS supplemented with 0.1% sodium citrate and 0.1% Triton X-100 for at least 10 min in the dark. Cell cycle analysis was performed on a fluorescence-activated cell sorter (FACscan, Epics Profile II, Becton Dickinson). Data were analyzed by using ELITE software (Coulter).

Phosphorylation of MAPK 1,2. MAPK activity was determined by using an antibody that recognizes the phosphorylated (and activated) forms of MAPK 1,2. Growth-arrested control and AC2-expressing cells were treated with or without PDGF (20 ng/ml) for 5 min, were lysed in RIPA buffer (1× PBS/1% NP-40/0.5% sodium deoxycholate/0.1% SDS) and were sonicated. Cell extracts were separated on a 10% SDS/PAGE gel, were blotted electrophoretically onto Immobilon P membrane, were blocked and incubated with the anti-phospho MAPK 1,2 antibody, and were detected by chemiluminescence as described by the manufacturer's protocol (New England Biolabs). As a control, antibodies to nonphosphorylated MAPK 1,2 were used.

DNA Synthesis. Control and AC2-expressing cell lines were serum-starved for 24 hr, followed by an 18-hr incubation period with increasing concentrations of PDGF. [^{3}H]thymidine (0.5 μCi/ml) was added, and incorporation into DNA for 6 hr was measured as described (19).

Colony Formation in Soft Agar. PMN-1 and AC2-A cells were transfected with 0, 0.2, or 1 μg of H-ras plasmid (pT24), hygromycin resistance gene (1 μg pRSV1.1), and salmon sperm DNA (14 μg). Hygromycin-resistant clones were plated on soft agar to assess colony formation. Cells (2×10^3) were suspended in 3 ml of medium (DMEM/5% calf serum) containing 0.3% agar. The mixture was added over a layer of 0.5% agar in DMEM in a 6-well plate. Cells were fed weekly with 1 ml of DMEM/5% calf serum and dexamethasone (1 μM). Three weeks later, plates were stained with the vital stain 2-(-*p*-isodophenyl)-3-(-*p*-nitrophenyl)-5-phenyltertazolium chloride hydrate for 2 days. Colonies larger than 0.15 mm in diameter were scored.

RESULTS

Expression of Exogenous AC2 and AC6 in NIH 3T3 Cells: Effect on Proliferation and Cell Cycle. To identify the ACs expressed in NIH 3T3 cells, we performed reverse transcription–PCR analysis by using degenerate primers encompassing a conserved domain within the ACs, with unique sizes for each of the family members (28). Identification of the amplified products showed only a strong 273-bp band, a size corresponding to AC5 or AC6, and no other bands corresponding to the other isoforms (Fig. 1, lane a). Because AC9, like AC6, is thought to be expressed ubiquitously (29), we checked with AC9 specific primers as to whether this isoform was expressed in NIH 3T3 cells. However, no detectable expression of AC9 isoform was found (data not shown). When exogenous AC2 was expressed in NIH 3T3 cells, a band at 291 bp corresponding to AC2 was observable (Fig. 1, lane b).

We transfected NIH 3T3 cells, expressing AC6 endogenously, with either the pcDNA3 vector alone or vector-containing epitope-tagged (Flag) AC2 or AC6 cDNA to obtain cell lines with comparable expression levels. Clonal cell lines expressing AC2, AC6, or empty vector were selected by neomycin resistance. Clonal cell lines expressing AC2 or AC6 showed increased forskolin-stimulated AC activity as compared with empty vector-transfected cell lines (Fig. 2*A*). Initial experiments showed that AC2-expressing cells grew significantly slower than AC6-expressing cells (Fig. 2*B*). In contrast, expression of exogenous AC6 did not affect the rate of proliferation as compared with vector transfected cells (Fig. 2*B*, inset). These findings were corroborated further by cell cycle analysis. Cells were growth-arrested (serum-starved for 24 hr) to reenter the cell cycle synchronously and were restimulated for 12 hr with medium containing 5% serum (Fig. 2*C*). The AC2-expressing cells showed an arrest in the G_1 phase as measured by BrdU labeling. Cells expressing exogenous AC6, on the other hand, entered the S phase at a rate comparable to that of vector-transfected (endogenous AC6-expressing) cells. Similar observations were obtained for other AC2 and AC6-expressing clonal cell lines, indicating that the observed effects are not caused by clonal variation (data not shown). Thus, overexpression of AC6 does not affect mitogenic properties of NIH 3T3 cells. However, AC2-expressing cells reside longer in the G_1 phase and, hence, proliferate more slowly.

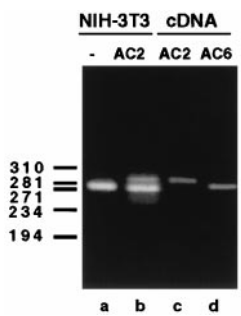
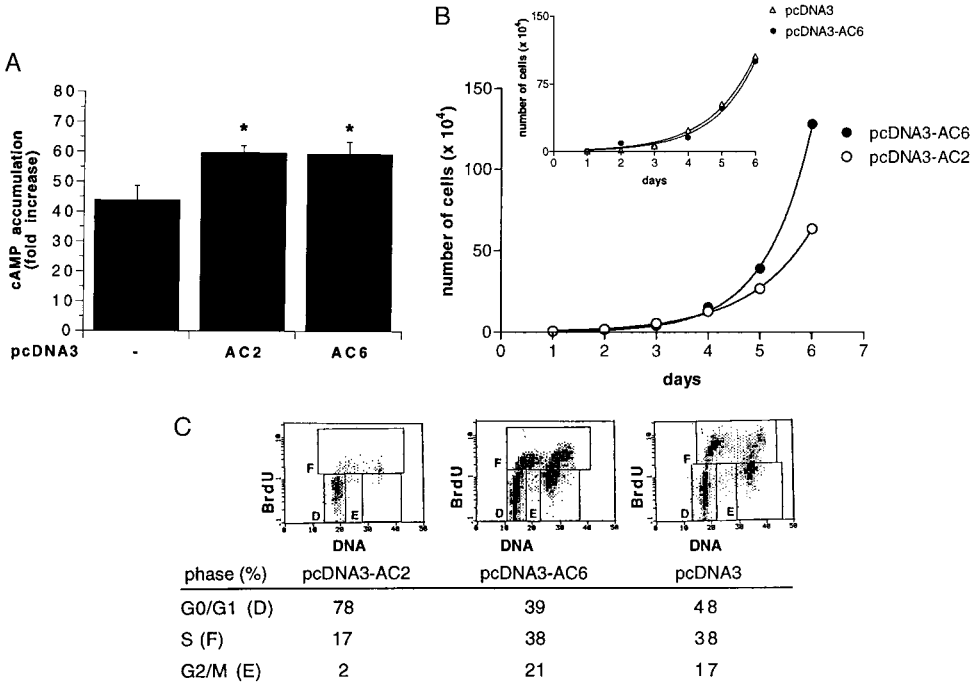


FIG. 1. Identification of AC isoforms expressed in control (native) and AC2-expressing NIH 3T3 cells. Lanes a and b represent amplified PCR products from control (native) and AC2-expressing cells (AC2 under an inducible promoter). In lanes c and d, positive cDNA controls (pcDNA3-AC2 and AC6 vector) for AC2 (lane c, 291-bp band) and AC6 (lane d, 273-bp band) are shown.



phase (%)	pcDNA3-AC2	pcDNA3-AC6	pcDNA3
G0/G1 (D)	78	39	48
S (F)	17	38	38
G2/M (E)	2	21	17

FIG. 2. Expression of AC2 and AC6 isoforms in NIH 3T3 cells: effects on forskolin-induced cAMP accumulation (*A*), cell proliferation (*B*), and cell cycle (*C*). (*A*) Cells were stimulated with or without forskolin (10 μM) for 30 min, and cAMP accumulation was measured. Values represent fold increase above basal [basal control (pcDNA3), 3.8; AC2, 3.0; and AC6-expressing cells, 3.1], means ± SEM ($n = 6$). The asterisk indicates a significant difference ($P < 0.05$) from control (pcDNA3) cells as determined by a Student's *t* test. (*B*) Growth curves of clonal cell lines stably expressing the epitope-tagged AC2 (open circles) and AC6 (closed circles) isoform or empty vector (pcDNA3, open triangles) (inset). The experiment is a representative of three experiments. Values are means of triplicate determinations with a standard error smaller than 5%. (*C*) Distribution of a population of cells in different phases [G_0/G_1 phase (D), G_2/M population (E), and S-phase population (F)] after 24 hr of growth arrest followed by 12 hr of stimulation with DMEM containing 5% serum. BrdU uptake as measured by fluorescein isothiocyanate fluorescence is depicted on the *y* axis. DNA content as measured by propidium iodide fluorescence is depicted on the *x* axis. The data are representative of three experiments.

Ectopic Expression of AC2 By Using an Inducible Promoter: Effect on Cell Proliferation. Because AC2 expression was shown to affect cell proliferation, clonal cell lines were derived from NIH 3T3 cells transfected with a vector containing an inducible promoter to regulate the expression of AC2. NIH 3T3 cells were transfected with the pMam-neo vector containing AC2 cDNA or vector without insert. Expression of the insert was induced by dexamethasone (1 μM) and was monitored by measurement of accumulation of cAMP. Research in our own and other laboratories has demonstrated that AC2 is activated by PKC (24–26). Hence, AC2-expressing clonal NIH 3T3 cell lines were isolated for their capability to increase cAMP levels in response to phorbol ester (PMA) treatment. Control cells (PMN-1) expressing the empty vector did not show an increase in cAMP on PMA treatment (1 μM) (Table 1). Two clonal cell lines (AC2-A and AC2-B) showing a 1.7- and 1.5-fold increase of cAMP on phorbol ester treatment were selected (Table 1) and were used for further experiments. Expression of AC2 inhibited cell division in these clonal cell lines (Fig. 3*A*). Doubling times increased from 21 hr for control to 25 hr for AC2-expressing cells. When AC2-expressing cells were grown in medium containing 10% calf serum, instead of 5%, suppression of proliferation was even more pronounced (data not shown). Clonal cell lines showing an even higher increase (>2-fold) of cAMP levels on PMA exposure displayed a marked delay in cell growth within weeks and could not be used for further experiments because they could not be propagated on a continuing basis.

Effect of AC2 Expression on PDGF-Induced cAMP Accumulation, MAPK 1,2 Activity, and Cell Proliferation. Inhibition of proliferation by increased serum suggests that growth factors in the serum may affect activation of AC2. Activation of growth factor receptors such as the PDGF receptor stimulate several downstream effectors, including Ras, phospholipase C-γ, and phosphatidylinositol 3 kinase (1–4). Because AC2 is activated by PKC (24–26), growth factors could stimulate AC2 through receptor tyrosine kinase-signaling pathways and raise levels of cAMP. Because increasing cellular cAMP has been shown to suppress signaling through MAPK 1,2 pathways (16–19), simultaneous elevation of cAMP with activation of the MAPK 1,2-signaling pathways could inhibit mitogenic signaling in AC2-expressing cells. To test this hypothesis, we examined the effects of PDGF on cAMP accumulation and MAPK 1,2 activity in control and AC2-expressing cells. In control cells, PDGF did not increase cAMP levels. In clonal cell lines expressing AC2, PDGF stimulated cAMP production (Fig. 3*B*). To determine whether this effect was mediated through PKC, AC2-expressing cells were preincubated with the PKC inhibitor Ro-31–8220. Preincubation of AC2-expressing cells with the PKC inhibitor blocked the PDGF-induced accumulation of cAMP (Fig. 2*C*). These data indicate that the PDGF-induced elevation of cAMP in AC2-expressing cells is PKC-dependent.

In control cells, PDGF induced a maximal MAPK 1,2 response after a 5-min stimulation, as determined by an increase in phosphorylation of MAPK 1,2 by using an antibody against the

Table 1. Accumulation of cAMP in control and AC2-expressing cells

Cell lines	Basal	PMA	Fold
PMN-1	2.5 ± 0.2	2.9 ± 0.3	
AC2-A	2.7 ± 0.3	4.5 ± 0.5*	1.7
AC2-B	2.2 ± 0.3	3.3 ± 0.4*	1.5

Clonal cell lines were treated with dexamethasone (1 μM) on alternative days for 2 weeks. Accumulation of cAMP was measured for 30 min in the absence or presence of 1 μM PMA. Values are means ± SEM of triplicate determinations of at least four independent experiments. The asterisk indicates a significant difference ($P < 0.05$) from nontreated cells as determined by a Student's *t* test.

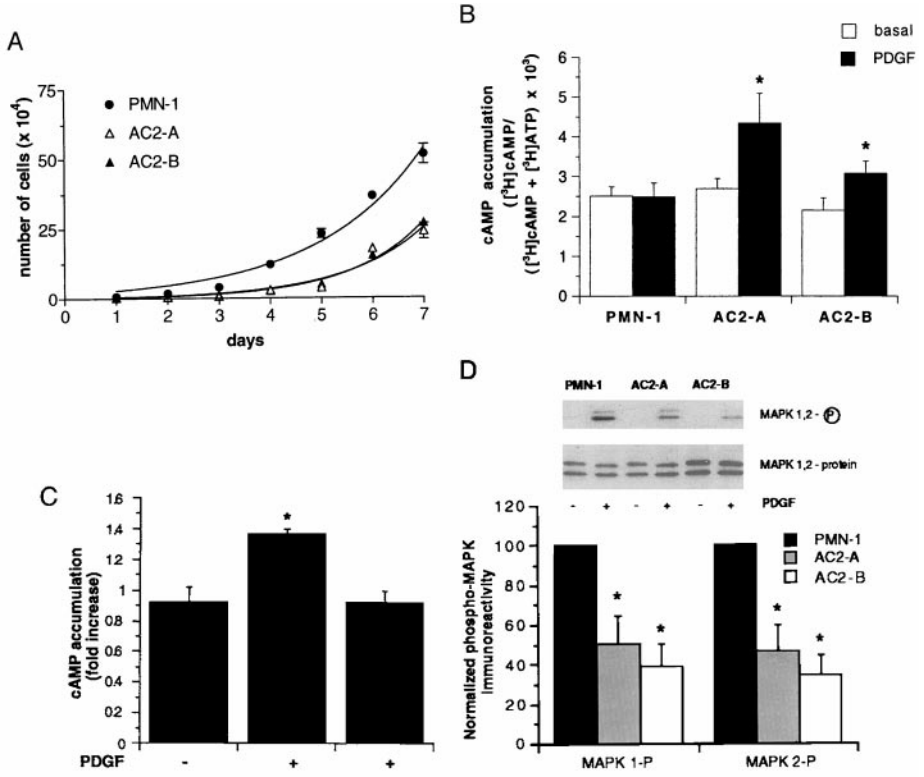


FIG. 3. Ectopic expression of AC2 by using an inducible promoter: effects on cell proliferation (*A*), on PDGF-induced cAMP accumulation (*B* and *C*), and on MAPK 1,2 activity (*D*). (*A*) Growth curves of clonal cell lines cells stably expressing AC2 (open triangles, clonal cell line A; closed triangles, clonal cell line B) or empty vector PMN-1 (closed circles). Cells were treated with dexamethasone (1 μM) every other day for 2 weeks. Values are means ± SEM of three separate experiments performed in triplicate. (*B* and *C*) Cells were stimulated with or without PDGF (20 ng/ml) for 30 min, and, in *C*, AC2-expressing cells were preincubated with or without the PKC inhibitor Ro-31–8220 (10 μM) for 30 min. Values are means ± SEM of triplicate determinations of at least four independent experiments. The asterisk indicates a significant difference ($P < 0.05$) from nontreated cells as determined by a Student's *t* test. (*D*) Immunoblots of phospho-MAPK 1,2 and total MAPK 1,2 in extracts from cells treated with or without PDGF (20 ng/ml) for 5 min after 24 hr of serum starvation (upper blot). Equal aliquots were used. The immunoblots are representative of three experiments. Quantification of phospho-MAPK 1,2 (upper band p44, MAPK 1, lower band p42, MAPK 2) (lower part). Increase in MAPK 1,2 phosphorylation was corrected for total MAPK 1,2 protein. Values represent means ± SEM of three independent experiments. The asterisk indicates a significant difference ($P < 0.05$) from control cells as determined by a Student's *t* test.

phosphorylated forms of MAPK 1,2. PDGF-stimulated MAPK 1,2 activity was reduced markedly in AC2-expressing cells (Fig. 3*D*, upper blot). Total levels of MAPK 1 and MAPK 2 were similar in both the control and AC2-expressing cell lines (Fig. 3*D*, lower blot). The biochemical effects of AC2 expression are noteworthy in that the relatively small increases in cAMP levels results in a very substantial decrease in MAPK 1,2 activation.

When cells were growth-arrested to reenter the cell cycle synchronously, AC2-expressing cells showed a delayed onset into the S phase on PDGF exposure (Fig. 4*A*). In addition, exponentially growing AC2-expressing cells showed an increased distribution in the G_1 phase and decreased distribution in the S and G_2/M phase as compared with control cells (Table 2). Because growth arrest is associated with a decreased expression of cyclin D1 (5–7), we determined the level of cyclin D1 expression after PDGF treatment. In control cells, a maximal increase in cyclin D1 expression was observed after 8 hr of PDGF stimulation (Fig. 4*B*). In AC2-expressing cells, however, cyclin D1 expression was reduced markedly at 4 and 8 hr and was not detectable after 24 hr of PDGF exposure. Even after 10 and 12 hr of PDGF exposure, cyclin D1 expression levels remained low in AC2-expressing cells (data not shown). In contrast, no differences in the level of $p27^{Kip1}$ or c-myc, two other cell cycle regulators which are modulated by cAMP, or in the pattern of cyclin E were observed in control and AC2-expressing cells (data not shown).

The inhibition of cell cycle progression should result in decreased DNA synthesis. Hence, we tested for the effect of AC2 expression on PDGF stimulation of [^{3}H]thymidine incorporation into DNA. DNA synthesis was reduced by 50% in AC2-expressing cells (Fig. 4*C*). This decrease is a result of a drop in maximal effect without altering the position of the PDGF concentration effect curve. These data imply that elevation of cAMP in AC2-expressing cells as a result of PKC activation is sufficient to inhibit mitogen-induced DNA synthesis. To definitively ascertain that the effect of AC2 expression is mediated by cAMP and activation of PKA, we coexpressed the dominant negative mutant of PKA (AB) in AC2-expressing cells. The dominant negative regulatory subunit blocks activation of PKA in NIH 3T3 cells (30). As a control, we coexpressed the wild-type regulatory subunit (WT). Expression of the dominant negative and not the wild-type PKA regulatory subunit resulted in a complete reversal of the suppressive effect of AC2 on PDGF-induced DNA synthesis (Fig. 3*D*). These data indicate that the antimitogenic effects of AC2 are mediated through cAMP and PKA.

The Effect of AC2 Expression on H-ras-Induced Transformation of NIH 3T3 Cells. When altered, many gene products that regulate cell cycle progression are important determinants of transformation (31). Because AC2 slows down cell cycle progression, it is possible that AC2 could affect transformation by dominant oncogenes. Hence, we determined the capability of H-ras to transform AC2-expressing cells. Cotransfection of control cells with different amounts of H-ras (0.2 and 1 μg) led to anchorage-independent cell growth in soft agar (Fig. 5). However, H-ras was largely unable to transform AC2-A (Fig. 5)- and AC2-B (reduction 50%)-expressing cells.

DISCUSSION

It is now well recognized that signals may be routed through multiple pathways within the cell and that interactions between

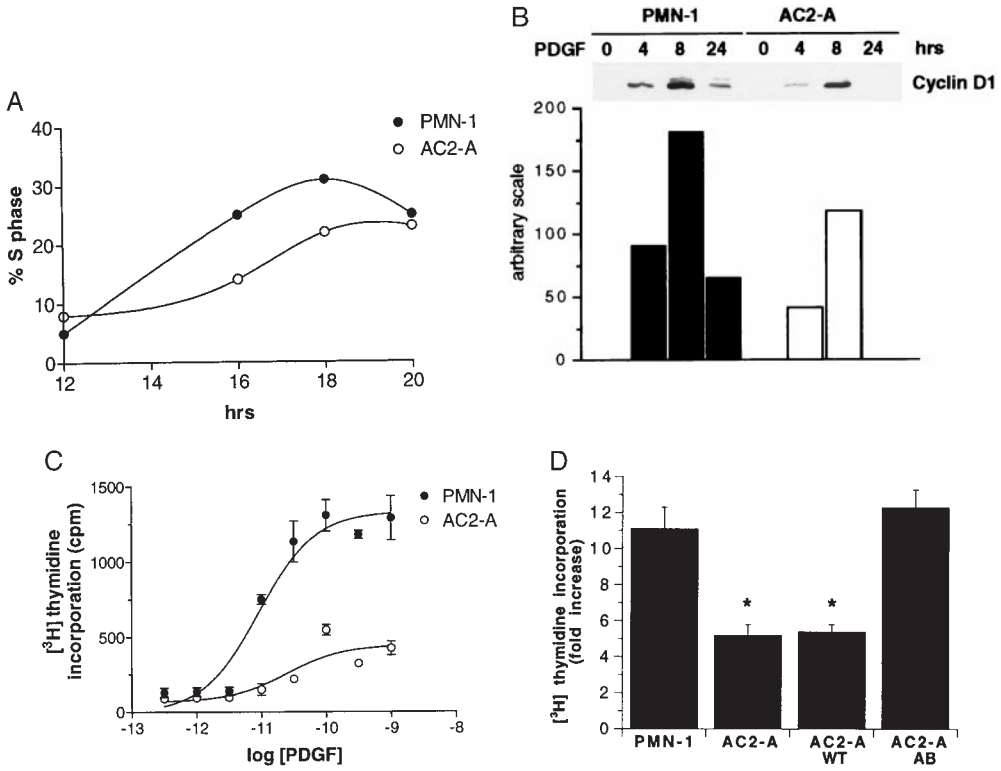


FIG. 4. Cell cycle analysis (*A*), cyclin D1 expression (*B*), DNA synthesis (*C*), and role of PKA (*D*) in control (PMN-1, closed circles) and AC2-expressing (AC2-A, open circles) cells. (*A*) Cell cycle progression into S phase was measured after cells were growth-arrested for 24 hr by serum starvation and were restimulated with DMEM containing 5% calf serum for the indicated times. (*B*) Immunoblot of cyclin D1 in extracts from serum-starved cells (solid bars, PMN-1; open bars, AC2-expressing cells) treated with PDGF (20 ng/ml) for 0, 4, 8, and 24 hr. (*C*) PDGF-stimulated incorporation of [^{3}H]thymidine in control and AC2-expressing cells. Values are means ± SEM of three separate experiments performed in triplicate. (*D*) Effect of expression of the dominant negative (AB) or wild-type (WT) PKA regulatory subunit on PDGF (20 ng/ml)-induced DNA synthesis in PMN-1 and AC2-A clonal NIH 3T3 cells. Values are means ± SEM of three separate experiments performed in triplicate. For *A*, *B*, and *C*, representative results of three experiments are presented.

signaling pathways are often critical for evoking integrated cellular responses. The routing of signals through multiple pathways depends on the capability of the components in individual pathways to receive the signal. Thus, isoform diversity becomes a defining criterion in determining signal routing. Fig. 6 *A* and *B* summarizes the pathways we have analyzed in this study in which the type of AC present determines whether PDGF elevates cAMP levels. NIH 3T3 cells express endogenous AC6, an isoform not stimulated by PKC (22, 23), and, hence, PDGF does not elevate cAMP levels in the native system. On expression of AC2, an isoform stimulated by PKC (24–26), PDGF stimulates cAMP production. This occurs in few native systems as well. In airway smooth muscle cells that were shown to express AC2, PDGF also was shown to elevate cAMP levels modestly in a PKC-dependent way (32). The elevation of cAMP in AC2-expressing NIH 3T3 cells, by PDGF, has important consequences for the biological actions of PDGF. Elevation of cAMP and activation of PKA results in an inhibition of signal flow from Ras to MAPK 1,2 at the level of Raf (16–19). Continuous activation of MAPK 1,2 has been shown to stimulate the expression of cyclin D1, and this elevation of cyclin D1 levels is at least in part responsible for cell cycle progression in fibroblasts (5–8). Our data show that PDGF induces cyclin D1 expression in NIH 3T3 cells, and it is proposed that MAPK1, 2, acting through increases in cyclin D1 levels, contributes to the proliferative response. In AC2-expressing cells, PDGF induction of cyclin D1 levels is reduced greatly, and this is accompanied by an increase in the proportion of cells in G_1 and in reduced proliferation. This is summarized in Fig. 6*B*. Here, AC2 connects, by PDGF regulation, the cAMP and Ras-MAPK 1,2 pathways. This leads to a short circuit of signal flow from the PDGF receptor to MAPK 1,2 and in the resultant proliferative homeostasis. Other growth factors present in the serum that

Table 2. Cell cycle analysis in control and AC2-expressing cells

Cell cycle	PMN-1, %	AC2-A, %	AC2-B, %
G1	66.2 ± 4.0	81.1 ± 1.8*	75.6 ± 3.1*
S	20.3 ± 3.1	8.3 ± 0.9*	13.7 ± 2.8*
G2/M	11.0 ± 2.2	4.6 ± 0.3*	7.3 ± 1.5*
Apoptosis/necrosis	2.7 ± 0.8	5.9 ± 1.6*	2.4 ± 1.0

Percentage of exponentially growing control (PMN-1) and AC2-expressing (AC2-A/B) cells in different phases was determined by a FACscan. Values are means ± SEM of triplicate determinations of at least four independent experiments. The asterisk indicates a significant difference ($P < 0.05$) from control cells as determined by a Student *t* test.

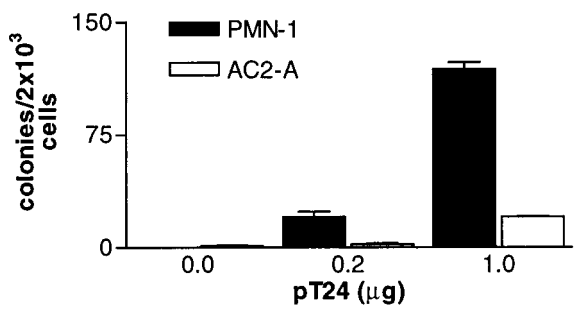


FIG. 5. The effect of AC2 expression on H-ras induced transformation of NIH 3T3 cells. The graph shows the number of colonies formed in soft agar by clonal cell lines PMN-1 (solid bars) and AC2-A (open bars) cotransfected with the indicated amounts of H-ras plasmid (pT24) and hygromycin resistance gene. The experiment is representative of two experiments. Values are means ± SEM.

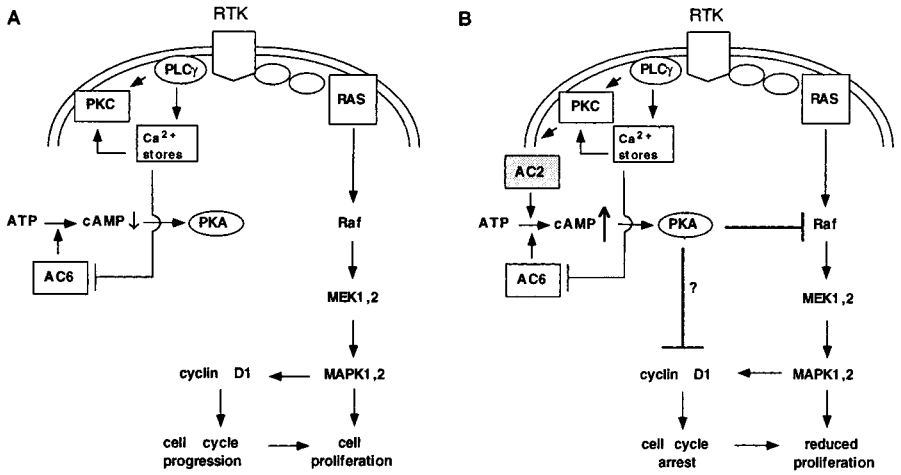


FIG. 6. Integration of tyrosine kinase signaling to both cAMP and MAPK 1,2 pathways, resulting in cell cycle arrest and the reduction of MAPK 1,2 triggered proliferation. *A* shows signaling pathways from receptor tyrosine kinase through MAPK 1,2 and cyclin D1 resulting in proliferation. In the presence of the endogenously expressed AC6, activation of the tyrosine kinase pathway leads to a transient inhibition of cAMP through an increase of intracellular Ca^{2+}. As a result, PKA would be inhibited, not affecting the growth-factor mediated signal transmission through the Raf/MAPK 1,2 pathway and the cell cycle. In *B*, the ectopic expression of AC2 could result in a PKC-mediated increase in cAMP. The resultant inhibition of signal transmission to MAPK 1,2 by inhibition of Raf and cyclin D1 can result in cell cycle arrest and in the reduction of the proliferative signal. For reasons of simplicity, PKC-regulation of Raf is omitted. RTK, receptor tyrosine kinase; PLCγ, phospholipase C-γ.

activate receptor tyrosine kinase signaling pathways are thought to induce proliferative homeostasis by the proposed mechanism. Thus, the molecular identity of upstream components and their signal integrating capabilities are likely to play a key role in determining both the amplitude and duration of the signal communicated into the cell. In this context, it is noteworthy that AC2 and AC7, which are stimulated by PKC, are expressed largely in postmitotic neuronal cells and platelets. In contrast, most cells that retain proliferative capability do not express significant levels of those AC isoforms that have the ability to be activated by growth factors. Thus, the types of ACs present in a cell are likely to be among the factors that determine proliferative homeostasis.

That specific isoforms of AC can function as homeostatic elements is surprising. Although the coupling properties of AC2 have been well characterized in cell-free systems, it was not clear whether these coupling characteristics result in an altered biological effect or if compensatory mechanisms render the expression of AC2 irrelevant. These experiments show that expression of AC2 ectopically can have profound biological consequences.

The conditional regulation of proliferation in AC2-expressing cells may be useful in therapeutic modalities. Our data show that expression of AC2 blocks H-ras-induced transformation of NIH 3T3 cells. Elevation of cellular cAMP by expression of activated $G\alpha_s$ blocks transformation *in vitro* and tumor formation *in vivo* in both fibroblasts and mammary epithelial cell lines (19, 33). Also in these studies, modest increases in cAMP induced suppression of mitogenic signaling pathways. Lowering the positive signal by negative integration of signaling pathways below the threshold that triggers the biological response may be sufficient to induce suppression. In addition, cAMP analogs also have been shown to have tumor suppressor activity (34). Transformation of many cell types is associated with increased receptor tyrosine kinase–Ras–MAPK 1,2 signaling. Selective expression of AC2 by germ-line therapy might possibly reduce the incidence of cancer in high risk populations.

We thank A. Lu, T. Santore, and I. George for technical assistance, Drs. P. Ram, R. Premont, M. Sugrue, and S. Aaronson for valuable discussions, and Dr. S. McKnight for the wild-type and dominant negative PKA regulatory subunit plasmids. M.J.S. was supported by a fellowship from the Netherlands Organization for Scientific Research and National Institutes of Health fellowships T32 DA 07135-17 and F32 CA75623–01. This research is supported in part by National Institutes of Health Grant GM-54508.

1. Blenis, J. (1993) *Proc. Natl. Acad. Sci USA* **90,** 5889–5892.
2. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556.
3. Egan, S. E. & Weinberg, R. A. (1993) *Nature (London)* **365,** 781–783.
4. Schlessinger, J. (1993) *Trends Biochem. Sci.* **18,** 273–275.
5. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. & Pestell, R. G. (1995) *J. Biol. Chem.* **270,** 23589–23597.
6. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R. & Pouyssegur, J. (1996) *J. Biol. Chem.* **271,** 20608–20616.
7. Weber, J. D., Hu, W., Jefcoat, S. C., Jr., Raben, D. M. & Baldassare, J. J. (1997) *J. Biol. Chem.* **272,** 32966–32971.
8. Weber, J. D., Raben, D. M., Phillips, P. J. & Baldassare, J. J. (1997) *Biochem. J.* **326,** 61–68.
9. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F. & Sherr, C. J. (1993) *Genes Dev.* **7,** 1559–1571.
10. Lukas, J., Bartkova, J., Welcker, M., Petersen, O. W., Peters, G., Strauss, M. & Bartek, J. (1995) *Oncogene* **10,** 2125–2134.
11. Grana, X. & Reddy, E. P. (1995) *Oncogene* **11,** 211–219.
12. Rozengurt, E. (1986) *Science* **234,** 161–166.
13. Dumont, J. E., Jauniaux, J. C. & Roger, P. P. (1989) *Trends Biochem. Sci.* **14,** 67–71.
14. Heldin, N. E., Paulsson, Y., Forsberg, K., Heldin, C. H. & Westermark, B. (1989) *J. Cell. Physiol.* **138,** 17–23.
15. Magnaldo, I., Pouyssegur, J. & Paris, S. (1989) *FEBS Lett.* **245,** 65–69.
16. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Webber, M. J. & Sturgill, T. W. (1993) *Science* **262,** 1065–1068.
17. Häfner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M. & Kolch, W. (1994) *Mol. Cell. Biol.* **14,** 6696–6703.
18. Cook, S. J. & McCormick, F. (1993) *Science* **262,** 1069–1072.
19. Chen, J. & Iyengar, R. (1994) *Science* **263,** 1278–1281.
20. L'Allemain, G., Lavoie, J. N., Rivard, N., Baldin, V. & Pouyssegur, J. (1997) *Oncogene* **14,** 1981–1990.
21. Sewing, A. & Muller, R. (1994) *Oncogene* **9,** 2733–2736.
22. Iyengar, R. (1993) *FASEB. J.* **7,** 768–775.
23. Smit, M. J. & Iyengar, R. (1998) *Adv. Second Messenger Phosphoprotein Res.* **32,** 1–21.
24. Jacobowitz, O., Chen, J., Premont, R. T. & Iyengar, R. (1993) *J. Biol. Chem.* **268,** 3829–3832.
25. Yoshimura, M. & Cooper, D. M. F. (1993) *J. Biol. Chem.* **268,** 4604–4607.
26. Jacobowitz, O. & Iyengar, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10630–10634.
27. Kroll, S. D., Chen, J., De Vivo, M., Carty, D. J., Buku, A., Premont, R. T. & Iyengar, R. (1992) *J. Biol. Chem.* **267,** 23183–23188.
28. Premont, R. T. (1994) *Methods Enzymol.* **238,** 116–127.
29. Premont, R. T., Matsuoka, I., Mattei, M.-G., Pouille, Y., Defer, N. & Hanoune, J. (1996) *J. Biol. Chem.* **271,** 13900–13907.
30. Clegg, C. H., Correll, L. A., Cadd, G. G. & Mcknight, G. S. (1987) *J. Biol. Chem.* **262,** 13111–13119.
31. Orr-Weaver, T. L. & Weinberg, R. A. (1998) *Nature (London)* **392,** 223–224.
32. Pyne, N. J., Moughal, N., Stevens, P. A., Tolan, D. & Pyne, S. (1994) *Biochem. J.* **304,** 611–616.
33. Chen, J., Bander, J. A., Santore, T. A., Chen, Y., Ram, P. T., Smit, M. J. & Iyengar, R. (1998) *Proc. Natl. Acad. Sci USA* **95,** 2648–2652.
34. Cho-Chung, Y. S. (1992) *Biochem. Soc. Trans.* **20,** 425–430.