Extracellular ATP Induces Anchorage-independent Expression of Cyclin A and Rescues the Transformed Phenotype of a Ras-resistant Mutant Cell Line*

(Received for publication, August 26, 1996, and in revised form, October 17, 1996)

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Anchorage-independent growth is characteristic of neoplastic cells, but the signal transduction pathways that mediate this phenotype are poorly understood. Several important cell cycle events are dependent on cell-substratum adhesion in non-transformed cells, including activation of G1 cyclin-dependent kinases and expression of cyclin A; the adhesion requirement of these events is abrogated in Ras-transformed cells. The ER-1-2 mutant rat fibroblast cell line is: 1) resistant to Ras-mediated, anchorage-independent growth; 2) defective in Ras-mediated, adhesion-independent expression of cyclin A, but not adhesion-independent activation of cyclin-dependent kinases; and 3) rescued for Ras-induced, anchorage-independent growth by ectopic expression of cyclin A. We report here that extracellular ATP induces adhesion-independent expression of cyclin A and rescues growth in soft agar by ER-1-2 cells that express Ras. ADP, AMP and the non-hydrolyzable analog adenosine 5′-[(β,γ-iminodiphosphate) are also effective, but adenosine is not. Adenine nucleotide-induced growth in soft agar is inhibited by reactive blue 2, an antagonist of some P2 purinoceptors. ATP does not induce adhesion-independent expression of cyclin A in ER-1-2 or control rat fibroblasts that do not express Ras, indicating a requirement for additional Ras-regulated signals for expression of this gene; one such signal may lead to phosphorylation of the retinoblastoma protein, pRB, and related proteins. These results suggest that extracellular ATP could play a role in the multistage carcinogenic process in vivo.

The ability of neoplastically transformed cells to proliferate in the absence of cell-substratum adhesion (i.e. anchorage-independent growth) is the best in vitro correlate of tumorigenicity (1). It is reasonable to hypothesize, therefore, that the molecular mechanisms that underlie anchorage-independent growth are related to the mechanisms that mediate some of the aggressive growth properties of malignant tumors. Despite the widespread use of colony formation in soft agar as an assay for the transformed phenotype, the signal transduction pathways that oncogenes use to drive this aberrant form of cell proliferation are poorly understood. We have developed a system that allows analysis of oncogene-induced, anchorage-independent growth independently from several other transformation-associated phenotypes (2). The Rat 6 fibroblast-derived, somatic cell mutant line ER-1-2 responds to expression of the Ras oncoprotein with alterations in cell morphology and gene expression that are nearly indistinguishable from those of a control cell line, PKC3-F4 (2, 3). Unlike PKC3-F4 cells, however, ER-1-2 cells fail to form colonies in soft agar in response to Ras (2). ER-1-2 cells are also resistant to anchorage-independent growth induced by v-Src and v-Raf, and this phenotype is dominant in somatic cell hybridizations.

We and others have previously demonstrated that several important cell cycle events are dependent on cell-substratum adhesion of fibroblast cell lines, including activation of G1 cyclin-dependent kinases (Cdk) (as measured by pRB phosphorylation, and cyclin E- and A-dependent kinase activities) and expression of the cyclin A gene (4–7). PKC3-F4 and NIH 3T3 cells that express oncogenic Ras proliferate in non-adherent cultures, and all of these cell cycle events occur in the absence of adhesion in the Ras-transformed derivatives (5). In contrast, ER-1-2 cells that express Ras (ER-1-2/ras cells) possess G1 cdk activities when cultured without adhesion, but remain almost fully adhesion-dependent for expression of cyclin A (5). Furthermore, ectopic expression of cyclin A is sufficient to rescue anchorage-independent growth of ER-1-2/ras cells, but does not induce anchorage-independent growth of PKC3-F4 or ER-1-2 cells, presumably because these cells still lack G1 cdk activity in the absence of adhesion (5). These data therefore indicate that: 1) oncogenic Ras can supplant the adhesion requirement of cellular functions that are necessary for cell cycle progression, 2) the adhesion dependence of G1 cdk activity can be dissociated from the adhesion dependence of cyclin A expression, and 3) the ability of Ras to direct expression of cyclin A in the absence of cell-substratum adhesion may be a critical, but insufficient, aspect of its transforming properties.

Taken together, the results of several studies suggest that cell-substratum adhesion is likely to regulate multiple signaling pathways that lead independently to activation of G1 Cdks and expression of cyclin A (4, 5, 7). The observation that, in the ER-1-2 cell line, Ras is able to overcome the adhesion requirement of G1 cdk activation, but not the adhesion requirement of cyclin A expression, suggests that Ras also signals via multiple pathways to achieve anchorage-independent growth. This is consistent with numerous recent reports, which indicate that

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Ras has multiple direct effectors and relies on multiple pathways to achieve cell transformation (8–10). The signal transduction pathways utilized by Ras to overcome the adhesion requirement of Cdk activation and expression of cyclin A are not known. ER-1-2/ras cells, which are apparently defective only in anchorage-independent expression of cyclin A, display constitutively active mitogen-activated protein kinase (MAPK), and the transformed phenotype of these cells is not rescued by ectopic expression of activated alleles of MAPK kinase, Rac1 or RhoA. Thus, the pathways controlled by these established mediators of Ras’s transforming potential (11–14) may not directly regulate, or be sufficient for, anchorage-independent expression of cyclin A.

Extracellular adenine nucleotides have received considerable attention as signal transducing ligands for a growing family of plasma membrane receptors, termed P2 purinoceptors (for review, see Refs. 15 and 16). Two different types of P2 receptors have been described: P2X receptors, which are structurally distinctive, ligand-gated ion channels; and P2Y receptors, which are G protein-coupled receptors with seven transmembrane domains. At least seven different P2X receptors and seven different P2Y receptors have been molecularly cloned, and additional receptors have been characterized pharmacologically (see Refs. 16–18 and references therein). Extracellular ATP, operating via P2 purinoceptors, can elicit an extremely wide range of biological effects in different systems (15). Of particular interest to the studies reported here are the observations of Heppel and colleagues that extracellular ATP can act as a mitogen or co-mitogen for a number of different fibroblast cell lines (19–21). We have recently described a low molecular weight, hydrophilic, heat-and protease-resistant, secreted factor (22) that is inactivated by UV light (UV) of 260 nm wavelength, indicating the presence of one or more critical UV-labile chemical bonds. Based on its chemical properties, the hypothesis that TRF might be an adenine nucleotide was tested. We report here that extracellular ATP rescued anchorage-independent growth of ER-1-2/ras cells (22). Interestingly, TRF can be destroyed by ultraviolet light (UV) of 260 nm wavelength, indicating the presence of one or more critical UV-labile chemical bonds. Based on its chemical properties, the hypothesis that TRF might be an adenine nucleotide was tested.

EXPERIMENTAL PROCEDURES

Materials—ATP, ADP, AMP, GTP, UTP, AMP-PNP, adenosine, and the calcium ionophore A23187 were all from Boehringer Mannheim. α,β-Methylene-ATP (α,β-Me-ATP), β,γ-methylene-ATP (β,γ-Me-ATP), β,γ-imidodiphosphate (β,γ-iP), α,β-methylene ATP, (β,γ-Me-ATP), P1,P2(dadenosine-5') triphosphate (AppppA), 12-O-tetradecanoylphorbol-13-acetate, thapsigargin, reactive blue 2, and 8-bromo-cAMP were all from Sigma. 2-Methylthio-ATP (2-Me-S-ATP) and pyridoxal-phosphate-6-azophenyl-2'-4'-disulfonyl acid (PPADS) were from Research Biochemicals International.

Cell Culture—All cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) plus 10% calf serum and enhanced chemiluminescence (ECL) system (Amersham Corp.). Northern blot analysis of total cellular RNA extracted with Trizol (Life Technologies, Inc.) was performed as described (2).

RESULTS

Rescue of Anchorage-independent Growth of ER-1-2/ras Cells By ATP and Structural Analogs—Because we had previously characterized TRF as a low molecular weight, non-protein, non-lipid secreted factor (22) that is inactivated by UV light,2 we have tested various cell-derived factors with similar chemical properties for their ability to rescue anchorage-independent growth of ER-1-2/ras cells. Table I and Fig. 1 show that inclusion of 50 μM ATP in the agar medium induced colony formation of ER-1-2/ras cells and two independent subclones of ER-1-2/ras (designated ERRC1 and ERRC6), but did not induce colony formation in the non-Ras-expressing PKC3-F4 or ER-1-2 cell lines. Furthermore, ATP did not enhance colony formation by the fully transformed PKC3-F4/ras cells (Table I and Fig. 1), nor did it enhance the growth of ER-1-2/ras cells cultured on plastic dishes (data not shown). Thus, similar to TRF and ectopic expression of cyclin A (5, 22), extracellular ATP specifically rescued anchorage-independent growth of ER-1-2/ras cells.

| Cell line | Growth in soft agar (colonies/10⁴ cells) |
|-----------|----------------------------------------|
| PKC3-F4   | 0                                       |
| PKC3-F4/ras | 2442                                    |
| ER-1-2    | 0                                       |
| ER-1-2/ras | 0                                       |
| ERRC1     | 0                                       |
| ERRC6     | 0                                       |

- A total of 10,000 cells of each cell line were seeded into 0.3% agar, in the presence or absence of ATP (50 μM). Macroscopic colonies were scored after 2 weeks of growth. Values represent averages and indicate determinations that differed by less than 5%. The experiment was repeated at least three times for each cell line; representative data are shown.

2 J.-J. Yang and R. S. Krauss, unpublished observations.
To begin to examine whether adenine nucleotides might be acting via a P₂ purinoceptor, we tested the effects of inhibitors of these receptors on soft agar colony formation by ER-1-2/ras cells mediated by adenine nucleotides (50 μM) or partially purified TRF. There is currently a lack of highly specific antagonists for the various different P₂ purinoceptors. However, re-active blue 2 and PPADS are considered to be relatively specific antagonists for P₂Y and P₂X receptors, respectively (16). Fig. 2B demonstrates that reactive blue 2 inhibited adenine nucleotide-induced colony formation with an IC₅₀ of ~20 μM. In contrast, TRF-mediated colony formation was not affected by reactive blue 2 at concentrations up to 100 μM, demonstrating that the effect of reactive blue 2 on adenine nucleotide-mediated colony formation was specific and not due to toxicity to the cells (Fig. 2B). These data also indicate that TRF and adenine nucleotides exerted their effects on ER-1-2/ras cells via distinct mechanisms. PPADS did not inhibit colony formation induced either by ATP or by TRF (data not shown).

We next tested various ATP analogs and other nucleotides for their ability to stimulate anchorage-independent growth of ER-1-2/ras cells over the dose range 0.01–100 μM (Fig. 2C). β,γ-Me-ATP was as effective as ATP at higher doses, but was reproducibly less efficacious at concentrations <20 μM. In contrast, α,β-Me-ATP, 2-Me-S-ATP, UTP, GTP, and AppppA were all inactive in this assay (Fig. 2C and data not shown). Because α,β-Me-ATP, 2-Me-S-ATP, and UTP are more potent agonists than ATP for certain purinoceptors (16), and the assay used here involved chronic exposures, it was possible that these compounds were desensitizing a signaling system responsible for inducing anchorage-independent growth and that their inactivity was therefore artifactual. To address this point, the ability of α,β-Me-ATP, 2-Me-S-ATP, or UTP to inhibit colony formation of ER-1-2/ras cells by 50 μM ATP was tested over the dose range 1–100 μM. None of these three compounds were able to inhibit ATP-mediated colony formation at any dose tested, ruling out a desensitization role for these analogs (data not shown). Finally, we tested several second messenger mimetics in this system: the phorbol ester 12-O-tetradecanoylphorbol-
Extracellular ATP Induces Anchorage-independent Expression of Cyclin A—The ER-1-2 cell line is defective in Ras-mediated, anchorage-independent expression of cyclin A, and ectopic expression of cyclin A rescues colony formation of ER-1-2/ras cells in soft agar (5). It was of interest, therefore, to test whether ATP induced anchorage-independent expression of the endogenous cyclin A gene in ER-1-2/ras cells. Because it is not possible to recover viable, non-adherent cells from soft agar cultures, we utilized a methylcellulose culture system that allows for nearly quantitative recovery of intact cells cultured under non-adherent conditions (5). It has been demonstrated previously that the growth properties in soft agar and methylcellulose of the cell lines used in these studies are nearly identical (5). Furthermore, ATP induced colony formation of ER-1-2/ras cells in methylcellulose cultures at a similar frequency as in soft agar cultures, and this colony formation was inhibited by reactive blue 2 (data not shown). As shown in Fig. 3A, ER-1-2/ras cells expressed only trace levels of cyclin A protein in methylcellulose cultures that were not supplemented with ATP, but addition of 50 μM ATP induced expression of cyclin A in such cultures. This response was not an indirect effect due simply to ATP driving the ER-1-2/ras/cyclin A in such cultures. This response was not an indirect one, ATP occurred at the RNA level (Fig. 3).

To examine the time course of cyclin A induction by ATP, ER-1-2/ras cells were inoculated into methylcellulose-containing medium and incubated for 24 h to allow some decay of adhesion-mediated cyclin A production. ATP was then added to the cultures and cyclin A protein levels analyzed at various time points thereafter. As shown in Fig. 3C, cyclin A levels continued to decrease over the next 6 h in both ATP-treated and untreated cultures. There was only a small effect on cyclin A expression between 3 and 12 h after exposure to ATP, but by 24 h post-treatment cyclin A was fully induced.

We next asked whether ATP could induce cyclin A expression in the PKC3-F4 and ER-1-2 cell lines, which were not induced to grow in soft agar by this compound. As seen in Fig. 3D, expression of cyclin A was completely adhesion-dependent in these two cell lines, and ATP did not lead to expression of cyclin A in non-adherent, methylcellulose cultures of these cells. Thus, additional Ras-mediated signals were required for anchorage-independent expression of cyclin A. A role for the E2F transcription factor has been implicated in the induction of the cyclin A gene near the G1/S phase border (23). Because E2F activity is strongly influenced by the phosphorylation status of pRB family members (24), and pRB phosphorylation is an adhesion-dependent event (5, 7), we investigated the effects of ATP on pRB phosphorylation in non-adherent cultures of PKC3-F4, ER-1-2, and ER-1-2/ras cells. Similar to our previous observations, pRB was hypophosphorylated in extracts prepared from non-adherent cultures of PKC3-F4 and ER-1-2 cells (Fig. 3E). Consistent with its inability to induce cyclin A or soft agar colony formation in PKC3-F4 and ER-1-2 cells, addition of ATP to these cells in the absence of adhesion had no effect on the phosphorylation status of pRB. It is possible, therefore, that one of the additional Ras-mediated events that is necessary for anchorage-independent expression of cyclin A is phosphorylation of pRB family members and, by implication, resultant alterations in E2F activity. As expected, pRB was hyperphosphorylated in ER-1-2/ras cells with or without treatment with ATP (Fig. 3E).

TRF did not induce anchorage-independent expression of cyclin A (Fig. 3A), yet it rescued colony formation in soft agar of ER-1-2/ras cells (22). These data suggest that TRF exerted its
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FIG. 4. Additive effects of ATP and TRF on soft agar colony formation by ER-1-2/ras cells. ER-1-2/ras cells were inoculated into soft agar medium containing either ATP (50 μM), a maximally effective dose of partially purified TRF, or both. Values represent averages of triplicate determinations ± one standard deviation. The experiment was performed twice; representative data are shown.

DISCUSSION

Anchorage-independent growth is the best in vitro correlate of tumorigenicity (1); identification of the oncprotein-driven signaling pathways that mediate this aberrant form of cell proliferation is therefore necessary for molecular elucidation of the transformed phenotype. Recent studies indicate that several biochemical events that are necessary for cell cycle progression are dependent on cell-substratum adhesion in non-transformed cells. These events include activation of G1 cyclin-Cdk complexes (as measured by pRB phosphorylation, and cyclin E- and A-dependent kinase activities) and expression of the cyclin A gene (5–7). NIH 3T3 and Rat 6-derived fibroblast cell lines that are transformed by Ras display both G1 Cdk activities and expression of cyclin A even when cultured under non-adherent conditions (5). The functional relevance of these observations is demonstrated in the ER-1-2 somatic cell mutant line, which is resistant to Ras-induced, anchorage-independent growth (2). ER-1-2/ras cells exhibit G1 Cdk activities in non-adherent cultures, but are defective for expression of cyclin A under such conditions (5). Ectopic expression of cyclin A is sufficient to rescue growth in soft agar of ER-1-2/ras cells, but is not sufficient to induce anchorage-independent growth of Rat 6-derived lines that do not express Ras. Thus, Ras-mediated events other than anchorage-independent expression of cyclin A are also required for growth in soft agar; one such event is likely to be loss of the adhesion requirement for G1 Cdk activity.

With such functionally relevant end points as anchorage-independent expression of cyclin A now identified, it should be possible to dissect the signaling pathways utilized by Ras to drive anchorage-independent growth. In particular, it should be possible to exploit the ER-1-2/ras cell line toward this end. Constitutively activated forms of components of these pathways would be predicted to rescue growth in soft agar of these cells in the same fashion that ectopic expression of cyclin A did, provided that they function downstream of the defective signaling point in these cells. We have recently observed that ER-1-2/ras cells display constitutively active MAPK and, further, that the transformed phenotype of these cells is not rescued by ectopic expression of activated alleles of MAPK kinase, Rac1 or RhoA, each of which is required for transformation by Ras (11–14). Thus, it is possible that the pathways controlled by these proteins may not directly regulate, or be sufficient for, anchorage-independent expression of cyclin A. Alternatively, the defective signaling point in ER-1-2/ras cells might reside between one of these proteins and the cyclin A gene. We report here that an extracellular signaling agent, ATP, induced anchorage-independent expression of cyclin A and rescued growth in soft agar in ER-1-2/ras cells. These data argue that a pathway that leads to induction of the cyclin A gene in the absence of the normal adhesion requirement is functional in these cells and can be activated by extracellular adenine nucleotides. The observations that: 1) an antagonist of P2Y purinoceptors (reactive blue 2) blocked the effects of adenine nucleotides on agar growth, and 2) a non-hydrolyzable ATP analog (AMP-PNP) was fully efficacious, suggest that the adenine nucleotides might have exerted their effects via activation of a purinoceptor. If this is the case, we are not aware of any cloned or characterized receptor that displays a similar pharmacological profile (16).

Expression of the cyclin A gene is dramatically up-regulated in non-transformed cells at the G1/S border, and cyclin A is required for the G1/S transition and progression through both S and G2 phases (for review, see Refs. 25 and 26). In fibroblasts, expression of cyclin A requires signals from both growth factors and cell-substratum adhesion, and occurs as late as 18 h after adhesion, quiescent cells are treated with growth factors (25, 26). The cyclin A gene promoter must therefore integrate information from multiple signaling pathways that has accrued over a considerable period of time. It is striking that cyclin A was induced in an adhesion-independent manner in ER-1-2/ras cells by treatment with a soluble, extracellular signaling factor, ATP. Perhaps ATP- and integrin-mediated signaling pathways overlap in Rat 6 cells. Signals provided by ATP treatment were not sufficient to induce cyclin A, however, since PKC3-F4 and ER-1-2 cells that did not express Ras did not respond, nor were they stimulated to grow in soft agar. Thus, additional Ras-mediated events were necessary for anchorage-independent expression of cyclin A, which, as described above, was itself insufficient to drive proliferation of suspended cells. Because non-adherent PKC3-F4 and ER-1-2 cells displayed only hypophosphorylated pRB even when treated with ATP, and E2F activity has been implicated in cyclin A gene induction (23), one likely possibility for an additional required event is phosphorylation of pRB family members and concomitant alterations in E2F activity. Taken together, it appears that the putative dominant mutation in ER-1-2 cells acts very specifically to block a Ras-regulated signaling pathway that, in cooperation with other Ras-regulated events, leads to anchorage-independent expression of cyclin A and therefore to growth in soft agar. We propose that signals provided by ATP, possibly via activation of a purinoceptor, feed into this pathway at a point downstream of the point of blockade in ER-1-2 cells. The requirement for multiple Ras-regulated signaling and cell cycle events for anchorage-independent growth is consistent with the notion that anchorage-independent growth is the most stringent in vitro criterion of transformation and has a very high correlation with tumorigenicity (1).

We originally tested ATP for its effects on ER-1-2/ras cells because the chemical properties of TRF might be consistent with those of a nucleotide (22). The effects of TRF were not, however, inhibited by reactive blue 2, nor did TRF induce cyclin A expression, despite its ability to rescue growth in soft agar of...
these cells (22). It is concluded that TRF is very likely not an adenine nucleotide. The mechanism of action of TRF remains unknown; the fact that it can drive proliferation of cells that produce only trace levels of cyclin A underscores the complexity of the perverse growth properties of oncogene-transformed cells.

Further analysis of ATP-mediated signaling in ER-1-/ras cells could provide insight into the signaling pathways utilized by Ras to induce anchorage-independent growth in wild-type cells. The observations presented here may have further implications, as well. The ability of extracellular adenine nucleotides to influence a cell cycle event that is strongly linked to the transformed phenotype suggests that these compounds could play a role in multistage carcinogenesis in vivo. For example, early in the carcinogenic process, it is possible that cells could acquire the ability to carry out the activation of G1 Cdns without adhesion signals, but not yet the ability to express cyclin A under such conditions. Exposure to adenine nucleotides might serve as a tumor promotion-like stimulus to such cells.

Acknowledgments—We thank Rick Assoian, Michele Pugano and Jim Roberts for gifts of antibodies. We also thank Hank Sadowski, Jim Manfredi, Xiangwei Wu, and Mark Frankel for comments on the manuscript.

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