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Dissection of Signal Transduction Pathways Mediated by Ras for the Induction of Anchorage-Independent Growth

by

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Abstract

Dissection of Signal Transduction Pathways Mediated by Ras for the Induction of Anchorage-Independent Growth

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Inappropriate control of Ras proteins will lead to cellular transformation, including anchorage-independent growth, which is a strong correlate of tumorigenicity. Ras proteins exert their biological effects by interacting with effector molecules through an effector loop (residues 32 to 40). There is a growing number of Ras-mediated-effector pathways that have been identified. To understand the relationship between Ras, cell-substratum adhesion, and the cell cycle machinery, we have used three Ras effector loop mutants (12V, 35S, 12V, 37G, and 12V, 40C) to dissect the signaling pathways generated by Ras required for cell cycle progression and stimulation of anchorage-independent growth. Anchorage-independent growth of cells depends on abrogation to the normal adhesion requirement of several cell cycle events: 1) activation of G_1 cyclin-dependent kinases (as measured by phosphorylation of the retinoblastoma protein and by cyclin E-dependent kinase activity); and 2) cyclin A expression. These effector site mutants of Ras failed to induce anchorage-independent growth and certain cell cycle events of cultured fibroblast cells, indicating that multiple pathways generated by Ras signal to the cell cycle machinery, which together promote cell cycle progression in non-adherent cultures of cells.

A mutant rat fibroblast cell line (ER-1-2) that fails to form colonies in soft agar when infected with a v-H-*ras* - expressing retrovirus, yet still undergoes transformation-related changes in morphology and gene expression in response to this oncogene was also studied. This cell line is resistant to Ras because it does not support Ras-mediated expression of cyclin A under non-adherent growth condition. We tested whether secreted factors play an important role in controlling anchorage-independent growth of ER-1-2/*ras* cells. A low molecular weight, hydrophilic, heat-and protease-resistant, UV-sensitive, secreted factor (designated TRF) that specifically rescued anchorage-independent growth of ER-1-2/*ras* cells was identified. We then tested small molecules that have similar chemical properties to TRF. We found that extracellular ATP, most likely acting via a P_2 purinoceptor, also rescues anchorage-independent growth of ER-1-2/*ras* cells. Furthermore, ATP, but not TRF, induces expression of cyclin A in non-adherent cultures of ER-1-2/*ras* cells.

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Chapter 1

Introduction

Growth Factor-Mediated Signal Transduction

Tremendous progress in the field of cell transformation has been made through studies focusing on the mitogenic signal transduction pathways that control cellular growth and differentiation. Most of the proto-oncogenes encode proteins that are components of these pathways. Somatic mutations of these genes, as well as tumor suppressor genes, play a key role in the development of neoplastic diseases (1,2). Therefore, it has become very important to understand how signals can be transmitted from exterior of a cell through the cytoplasm into the nucleus, which controls the decision for a cell to divide or differentiate. Many mitogenic growth factors bind to tyrosine kinase receptors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or fibroblast growth factor (FGF)-receptors. These protein tyrosine kinase receptors play an important role for the control of cell growth or differentiation, which depends on cell background (3,4). Abnormal control of the expression or mutations in these receptors will lead to neoplastic transformation or defects in differentiation. Binding of ligand to its receptor results in the induction of the dimerization and autophosphorylation of these receptors on tyrosine residues (5), which, in most cases, recruit and phosphorylate Src homology domain 2 (SH2)-containing signal molecules (2,6).

The SH2 domain-containing family of proteins includes the p85 component of phosphoinositide 3-Kinase (PI3-K) pathway; phospholipase C-γ in the protein kinase C pathway (7); Src family kinases; p120-GAP, Shc, and Grb2 in the Ras pathway (8); and the p91STAT pathway (9,10). The specificity of the interactions between receptors and these SH2 domain-containing signal molecules is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residue (11).

PI3-K is a lipid kinase and is able to phosphorylate phosphoinositide at the 3′ position of the inositol ring. The PI3-K comprises a catalytic (p110) and regulatory (p85) subunit (12). The tyrosine phosphorylated receptors, such as PDGF, are capable of binding to the SH2 domains of p85 (13). This results in a few fold increase of PI3-K activity (14,15). The products of PI3-K might serve as second messengers.

Activation of PLC-γ would stimulate Ptdins turnover pathway, which leads to the production of intracellular inositol-(1,4,5)P_3 (IP_3) and diacylglycerol (DG). IP_3 will then serve as second messenger that binds to its receptor located on the endoplasmic reticulum (ER). This step will trigger the release of calcium from ER to cytosol and activate calcium-calmodulin-sensitive kinases. The production of DG will lead to activation of PKC. This Ptdins pathway is able to activate multiple cellular events including changes in cytosolic pH, potassium level, and transcription of certain genes (2).

A predominant example of a ligand-receptor activation pathway is the so called " ras-mitogen-activated-protein kinase (Ras/MAPK) " pathway. In this pathway the SH2 containing adaptor molecule Grb2 binds to the tyrosine phosphorylated and activated receptor and then couples to Ras via the guanine nucleotide exchange factor, SOS. Recent studies show that in stimulated cells Grb2 is bound to SOS through its two SH3 domains. SOS then catalyzes exchange of GDP for GTP on Ras and initiates activation through sequential phosphorylation of c-raf-1, MAPK kinase, MAPK and $p90^{rsk}$ (16-20). The latter two kinases are capable to translocate to nucleus and may phosphorylate transcription factors such as $p62^{TCF}$/elk-1, c-jun, c-myc, and NF-IL6 (21-24).

Beside these transcription factors, which exist in the nucleus, transcription factors, such as p91STAT, exist in cytoplasm prior to receptor activation and associate with their cognate receptor upon activation. Followed by the association with receptor, STAT proteins are then phosphorylated on a conserved tyrosine residue. Either types of receptors, with or without intrinsic kinase activity, could lead to phosphorylation of these STAT proteins. Receptors lacking intrinsic kinase activity involve specific members of the JAK (Janus kinase) family to phosphorylate STAT proteins. This step is crucial for the dimerization and allows them to leave the receptor and migrate to nucleus as transcription factors

(10,25).

Ras and Its Effectors

It has been indicated that Ras plays an essential role in the regulation of cellular proliferation and differentiation. Ras antibody microinjection experiments and experiments utilizing dominant-negative Ras mutants indicate that Ras proteins are important for cellular proliferation and differentiation. The normal state of Ras is in GDP bound form and the activation of such protein is stimulated by guanine nucleotide exchange factors, such as SOS and Ras-GRF, for the exchange of GDP for GTP on Ras. In mammalian cells, at least two proteins, p120-GAP (GAP, GTPase-activating protein) and NF1, stimulate the intrinsic weak GTPase activity of Ras. Therefore, these proteins can act as negative regulators for Ras function. Mutation of GTPase domain on Ras causes the constitutive activation of such protein in which the turnover rate of GTP to GDP is slower than wild type (26-28). Introduction of such mutated Ras gene into established cell lines in culture causes the neoplastic transformation of such cell lines, resulting in morphological changes, serum- and anchorage-independent growth. The latter is the most stringent cell culture assay for the test of cellular transformation (29,30).

Ras proteins exert their biological effects by interacting with effector molecules through a domain called the effector loop. There is a growing number of Ras effectors identified by methods of affinity binding experiments, yeast two-hybrid system, and genetic mutation of either Ras or candidate effectors. A region of Ras that interacts with the effectors has been delineated by a set of mutations in constitutively active Ras. The mutation in this region will ablate the biological function of Ras without interfering with the GTP binding activity. This region is located in between residues 32 and 40 and defined as the effector domain (31-33). The studies of the signal transduction of Ras have explored its effectors, such as raf, PI3-K, and others.

There are many potential Ras effectors that have been identified in vertebrates including Raf, MEKK1, p120-GAP, PI3-K, PKCζ, Ral-GDS, AF-6 and Rin 1 (32-35). Cytosolic Raf can associate with GTP loaded Ras proteins through the effector domain (31,36). The mechanism for the activation of Raf through the binding to Ras is still unclear. How Raf receives signals and becomes activated has become an interesting issue. A 14-3-3 protein family member might be involved in the binding and activation of Raf kinase activation (37-39). The activated Raf will then phosphorylate the MEK protein kinase on serine, which results in the activation of such kinase. MEK is a dual specificity threonine/tyrosine kinase, which would catalyze the phosphorylation and activation of ERK/MAP kinases (40-44). These kinases will then translocate into nucleus and phosphorylate transcription factors, which control the expression of genes required for cell cycle progression (22,45-48).

Other than Raf, MEKK1 is another serine/threonine kinase that binds to Ras. GTP-bound Ras binds MEKK1 through its carboxy-terminal kinase domain (49). Additionally, dominant inhibitory Ras reduces the activity of MEKK1. This indicates that MEKK1 lies downstream of Ras (50). MEKK1 activates different signaling pathway downstream of Ras and it does not phosphorylate MEK in vivo, but rather functions as upstream regulator of stress-activated protein kinase SAPK/JNK (51,52). One of the MEK family, SEK, is activated by MEKK1. SEK directly phosphorylates and activates stress-activated protein kinase, SAPK/JNK. The transactivating domain of c-jun is phosphorylated by SAPK/JNK. Phosphorylated c-jun forms homodimers or heterodimers with c-fos, has potent AP-1 activity and regulates the expression of a number of genes (50,51,53,54). However, dominant inhibitory Rac attenuates the activation of JNK by Ras and growth factors, which questions whether MEKK1 is a direct effector of Ras (55).

p120-GAP was the first vertebrate protein identified that associates with GTP bound form of Ras (26). It has been identified as negative regulator of Ras signaling pathway by accelerating intrinsic Ras GTPase activity. Cellular transformation by overexpressing normal Ras is inhibited by overexpression of p120-GAP (56). The presence of SH2, SH3 and PH domains, however, indicates that p120-GAP could be Ras effector as well as regulator (33-35). Because SH2, SH3 and PH domains are recognized sites for protein-protein interaction, therefore, it is possible that downstream signals are generated by p120-GAP through these domains.

Not only is PI3-K able to be associated with activated tyrosine kinase receptors, but it has been implicated that PI3-K is a downstream effector of Ras. Ras has been shown to bind directly to p110 subunit of PI3-K and this binding requires GTP (14,57-59). It was also shown in an in vitro reconstitution system that GTP-bound form of Ras can increase the lipid kinase activity of PI3-K directly (33). This indicates that PI3K activity can be regulated directly by Ras in cells. PI3-K may provide a link between Ras and Rho family GTPase. ADP-ribosylation of Rho inhibits activation of PI3-K and PI3-K appears to function upstream of Rac, possibly, by generating PI[3,4,5]P_3, which activates Rac-GEF (15,60). A 60 kDa serine/threonine kinase, Akt/PKB, was identified and suggested to be a candidate downstream target of PI3-K. The carboxy-terminal catalytic domain of Akt/PKB is a kinase related to both PKA and PKC, whereas the amino-terminal domain has a PH domain, which is likely involved in either protein-protein or protein-lipid interactions. It has been shown that PI(3,4,5)P_3, but not PI or PI(4,5)P_2, is able to activate Akt/PKB in vitro. Wortmannin, a specific inhibitor of PI3-K, inhibits the activation of Akt/PKB and dominant-negative mutant of PI3-K inhibits growth factors-, such as PDGF, induced activation of Akt/PKB. These results strongly indicate Akt/PKB as a downstream target of PI3-K (7,61,62).

PKCζ is an atypical PKC isoform that is calcium independent and insensitive to diacylglycerol. PKCζ has been reported as mitogenic in murine fibroblasts and essential for Ras-dependent oocyte maturation in *Xenopus*. The immunoprecipitation experiment has shown that Ras and PKCζ could be coprecipitated in PDGF-treated NIH3T3 cells. Dominant negative Ras(N17) inhibits growth factor stimulated PKCζ activity (63). These results suggested the direct or indirect activation of PKCζ by GTP-bound Ras.

Ral-GDS is a guanine nucleotide exchange factor for the Ras-like GTPases RalA and RalB. Mammalian cells stimulated by EGF increased the association of Ras and Ral-GDS. It has been reported recently that Ras could stimulate the exchange activity of Ral-GDS in cells (64-66). These results make Ral-GDS as a candidate effector for Ras. The function of Ral is not well understood, but it has been implicated in the control of phospholipase D.

AF-6 is a 180kDa Ras-interacting protein and it was purified by H-Ras affinity column chromatography. The N-terminal domains of AF-6 interact with GTP-bound of Ras. AF-6 has a GLGF motif, which is found among several proteins located in cell-cell interaction sites (67). This structural feature of AF-6 suggests that it locates at the junction of plasma membrane and cytoskeleton. The function of AF-6 remains unclear.

The mammalian Rin1 protein was identified from human cDNA encoded protein that interfered with Ras signal transduction in yeast. Rin1 was also found to preferentially bind to GTP-bound Ras and can compete with Raf for Ras binding (68,69). The growing list of Ras-binding proteins indicate that Ras generates multiple signaling pathways to regulate cell proliferation or differentiation.

Ras Effector Site Mutants

Oncogenic Ras can promote cell cycle progression, but the intracellular signal transduction pathways generated by Ras that signal to cell cycle machinery are poorly understood. There is increasing evidence that Ras may mediate its actions through the activation of multiple downstream effector pathways. These multiple Ras-regulated pathways contribute to cell transformation. Mutations in the Ras effector loop (residues 32 to 40) can lead to selective disruption of Ras interaction with some effectors but not with others. These Ras effector site mutants may be useful for the separation of the abilities of Ras to interact with different downstream targets. Three Ras effector loop mutants (12V, 35S; 12V, 37G; and 12V, 40C) have been used to dissect the signaling pathways generated by Ras required for cell transformation and other phenomena. The signaling pathways generated by these Ras mutants are different. Of the three most well characterized pathways (e.g. Raf, RalGDS, and PI3-K), 12V, 35S Ras activates only the Raf pathway, 12V, 37G Ras interacts only with Ral-GDS, and 12V, 40C Ras activates only the PI3-kinase pathway (31,32,65,70,71). Genetic evidence from use of these effector loop Ras mutants indicates that Ras mediates transformation by activating all three of these pathways. Ras effector loop mutant (12V, 37G) retained the ability to synergistically enhance the transforming activity of another Ras effector loop mutant (12V, 35S). This indicates that both Raf-dependent and Raf-independent pathways are required for full Ras transformation. Moreover, Ras effector loop mutants, 12V, 37G, and 12V, 40C, showed synergistic induction of transformed foci, suggests that at least two distinct Raf-independent pathways that promote Ras transformation (71). Futhermore, ectopic expression of activated Ras (12V) in rat embryo fibroblast (REF-52) cells induces membrane ruffling, MAP kinase activation, and stimulation of DNA synthesis. Ras effector loop mutant (12V, 40C) was defective for MAP kinase activation and stimulation of DNA synthesis, but retained the ability to induce membrane ruffling. Another mutant (12V, 35S) was defective for stimulation of membrane ruffling and induction of DNA synthesis, but retained the ability to induce MAP kinase activity. Expression of both mutants together resulted in a stimulation of DNA synthesis, indicating that at least two distinct Ras effector pathways have synergistic effects for the mitogenic activity of Ras (70). To disect signals required for anchorage-independent growth induced by Ras, we transfected NIH 3T3 and Rat 6 cells with contructs expressing activated forms of H-Ras (12V) or Ras effector site mutants (12V, 35S; 12V, 37G; and 12V, 40C). The results will be discussed in a later chapter.

Adhesion/Integrin-Mediated Signalling

The extracellular matrix (ECM) is a potent regulator of cell growth, migration and apoptosis. The major ECM protein include fibronectin, collagen, vitronectin and laminin. Integrins, as the receptors for the ECM, play a key role in mediating signals from the ECM. When cells adhere to the ECM, cell surface integrins recognize RGD sequences within ECM proteins and form focal adhesions (72). Thus, focal adhesion sites contain aggregated integrins that interact with components of ECM. On the cytoplasmic side of focal adhesions, integrins interact with cytoskeleton proteins. The aggregated integrins lead to the induction of cascades of intracellular signals that include increases in intracellular pH (73-75), intracellular calcium, inositol lipid synthesis (76), tyrosine phosphorylation of focal adhesion kinase (FAK) (77), activation of Src-family kinases and components of the Ras signal transduction cascade, and activation of a serine kinase called integrin-linked kinase-1 (ILK-1). It has also been demonstrated that integrins can mediate the activation of PKC (78), MAPK (79), PI3-K (80), and NF-κB (81). Among integrin-mediated signalling events, FAK plays a central role. FAK appears to bind directly to integrins and becomes activated by autophosphorylation. It is also phosphorylated further by the Src kinase (82). A number of signalling molecules then bind to FAK and many are phosphorylated by it. These signalling molecules include Grb-2 and p85 subunit of PI3-K. The proteins phosphorylated by FAK also include the Src substrates Cas (82) and the cytoskeleton-associated proteins paxillin, tensin, and cortactin. Integrin-mediated signals also synergize with signals from growth factors. Therefore, both extracellular factors jointly regulate cell proliferation, differentiation, and other processes.

Cell Cycle Regulation and Anchorage-Independent Growth

The regulation of cell proliferation should be precisely controlled in order to distribute genetic information equally to daughter cells. Cell proliferation is controlled by extracellular factors that initiate cascades of events that lead to cell growth and division. As growth factor treated cells progress through G_1 phase, they pass the restriction point, R (83), to continue cell cycle progression. R is the point at which cell cycle progression becomes independent of growth factors. Cell cycle progression is regulated by cyclin-dependent protein kinases (CDKs), which regulate a number of target molecules by phosphorylation. These downstream molecules carry out the steps that lead ultimately to DNA replication and mitosis. These CDKs are sequentially regulated by their partners, cyclins D, E, A, and B. In addition, CDKs are regulated by other kinases and phosphatases (for example, Weel kinase, Cdc25 phosphatase, and CAK, and CDK inhibitory proteins (84-91). Therefore, CDK activity depends on cyclin binding, phosphorylation, and the binding of CDK inhibitory proteins. The misregulation of such cell cycle regulatory proteins might lead to transformation. The major G_1 phase CDKs are CDK4, CDK6, and CDK2, and these CDKs are inactive in the absence of their cyclin partner. CDK4/6 are activated by the binding of D type cyclins (D1, D2, or D3) and cyclin E binds to CDK2 (88,92). The activation of these CDKs is required for G_1 phase progression (88,91,92). Cyclin A is induced at or near the G_1/S boundary and it binds to CDK2 in S phase, which is involved in S phase progression (93,94). Mitogenic growth factor treated cells increase the activity of cyclin D-CDK4/6 and cyclin E-CDK2. The expression of D-type cyclins and the activity of cyclin E-CDK2 can also be regulated by cell adhesion to substratum (95). A loss of cyclin D1-dependent kinase activity before R point prevents many cultured cell lines from entering S phase. However, its absence later in the cell cycle has no effect. Therefore, cyclin D-dependent kinases must phosphorylate some substrates whose activity before R point is required for G_1 progression. The retinoblastoma tumor suppressor protein (RB) and the related pocket proteins p107 and p130 are targets of these G_1 cyclin associated kinases (88,96-102). As a result of phosphorylation of RB family proteins, E2F family proteins are released from the hyperphosphorylated pRB. The E2F family encompasses two distantly related subfamilies, E2F and DP. The E2F family includes five E2F members and three DP members. E2F/DP heterodimer formation facilitates binding to RB family proteins. This heterodimer is an active transcription factor, when released from hyperphosphorylated RB family proteins (103-106). E2F promotes the transcription of genes, including cyclin A gene, necessary for entry and progression through the S phase of cell cycle. RB is also thought to inhibit S phase entry by collaborating with E2F to be transcription repressors, which repress promoters containing E2F sites. The formation of pRB and E2F/DP complexes are indeed transcriptional repressors which contribute to pRB-dependent G_1 arrest (107). All the events described above are regulated jointly by growth factors and cell anchorage to the extracellular matrix (ECM) (Fig1).

In addition to cyclin binding, G_1 CDKs activity are regulated by CDK-inhibitors (CKIs). Two families of small proteins that interact with and inhibit the activation of cyclin-Cdks has been described. One family of proteins includes: $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$,which bind to cyclin E-Cdk 2, cyclin A-Cdk 2 and cyclin D-Cdk 4/6 (108-112). The other family proteins including: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, which bind and inhibit CDK4 and CDK6, but not other CDKs (113-115). Cell anchorage is involved in reducing the steady levels of $p21^{cip1}$ and $p27^{kip1}$ (86,87,116,117). Therefore, cell adhesion to ECM and growth factors work together to decrease protein levels of $p21^{cip1}$ and $p27^{kip1}$ that titrate these proteins from cyclin E-CDK2, allowing for the activation of cyclin E-CDK2 activity.

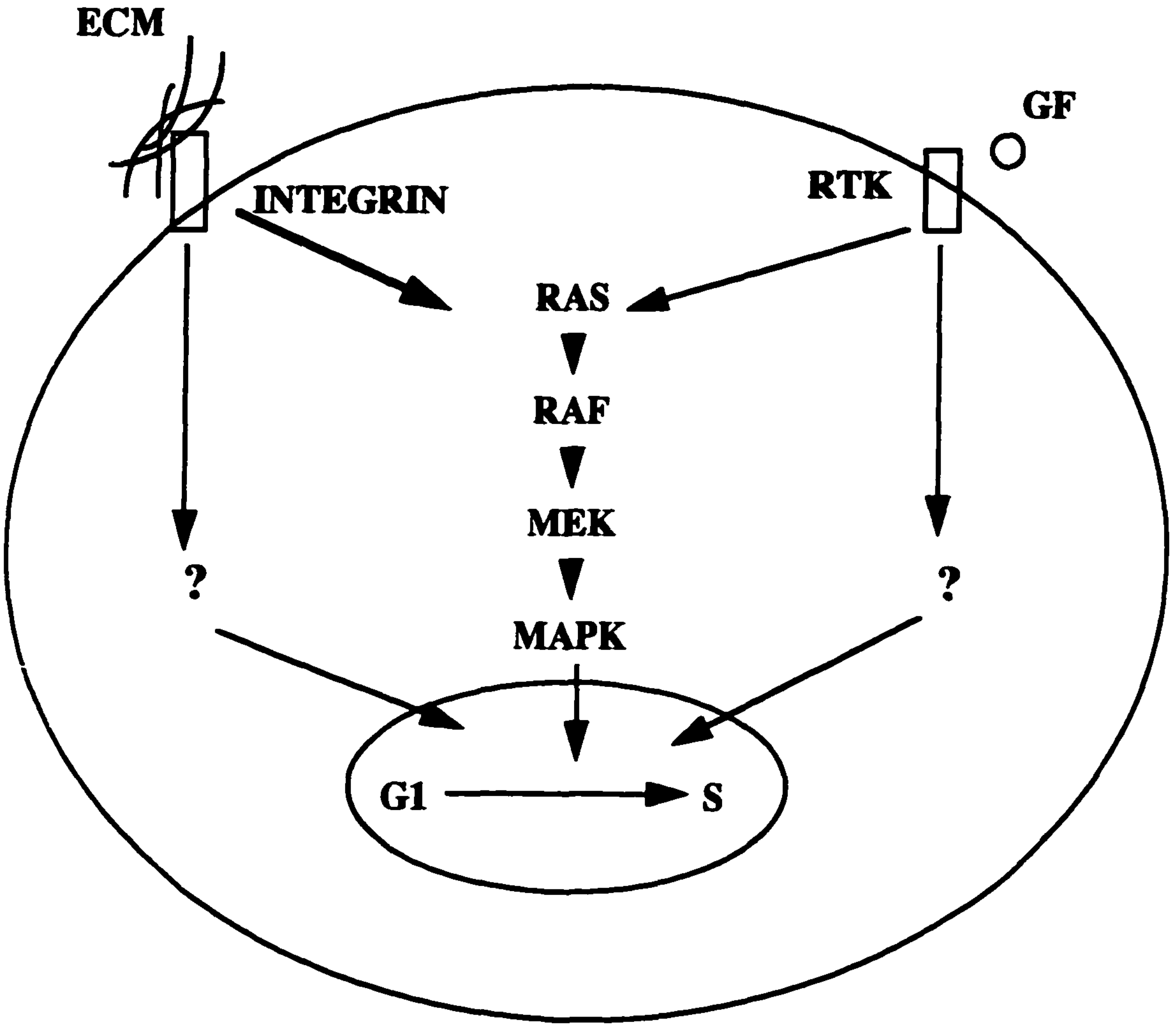

Figure 1: Integrin and growth factor collaboration. Distinct and common signals generated by growth factors and integrins synergize in the regulation of cell cycle progression.

Cell anchorage strongly controls cell proliferation, differentiation, and apoptosis. The interaction of cells with the extracellular matrix is mediated by the integrin family of surface proteins. Upon binding to extracellular matrix proteins, integrins transmits signals into cells and rearrangement of cytoskeletal architecture. Cells transformed by oncogenes, however, are able to grow in the absence of contacts with extracellular matrix. These oncogenes presumably provide signals normally transmitted from extracellular matrix via integrins. Cell cycle machinery could be the ultimate target for anchorage-independent cell cycle progression. We and others previously demonstrated that several important cell cycle events are required for the anchorage-independent growth of fibroblast cell lines, including activation of G_1 Cdks (as measured by pRB phosphorylation, and cyclin E- and A-dependent kinase activities) and expression of the cyclin A gene (95,117-120). Oncogenic Ras proteins abrogate the anchorage-dependence of these cell cycle events. In summary, cyclin D1 is induced in the presence of growth factors and the adhesion of cells on ECM. The ECM and growth factors also cooperate to decrease the steady levels of $p21^{cip1}$ and $p27^{kip1}$. Thus, cyclin D-CDK4/6 and cyclin E-CDK2 become active and then phosphorylate pRB, p107, and p130, allowing for E2F-dependent cyclin A expression. Beside E2F-dependent cyclin A expression, there is an E2F-independent, adhesion-regulated requirement for cyclin A expression (118,121). Cyclin A-CDK2 activity is required for S phase progression of cell cycle.

Oncogene-Resistant Mutant Cell Line

My advisor, Dr. Krauss, previously isolated a mutant cell line (ER-1-2) derived from rat embryo fibroblasts that overexpress protein kinase C ß1 (122). In contrast to a control cell line (PKC3-F4), ER-1-2 cells fail to form colonies in soft agar when infected with a v-H-*ras*-expressing retrovirus. Interestingly, when ER-1-2 cells were infected with this Ras retrovirus they displayed, like control cell lines, a morphologically transformed phenotype, despite their inability to grow in soft agar. This mutant cell line is genetically dominant, and resistant to growth in soft agar induced by *src, ras,* and *raf*. This phenotype leads to the hypothesis that there is a blockade downstream of a Ras-mediated signal transduction pathway that is specific for anchorage independent growth (Fig 2). It suggests that the signals generated by Ras for morphological transformation can be branched from the signals required for anchorange-independent growth. Consistent with studies by others, there are at least two signaling pathways generated by Ras in which one could lead to morphological transformation and the other could be required for cellular proliferation. Because a predominant signal transduction pathway for cellular growth is "Ras-MAPK pathway", we tested if the pathway is blocked in this mutant cell line. According to our studies, MAPK activity in the mutant cell line was the same as in the control cell line, PKC3-F4, in response to Ras (J. J. Y. and R. S. K., unpublished data). We found that most of primary and secondary response genes, such as c-jun, jun B, fos B, c-myc, collagenase,

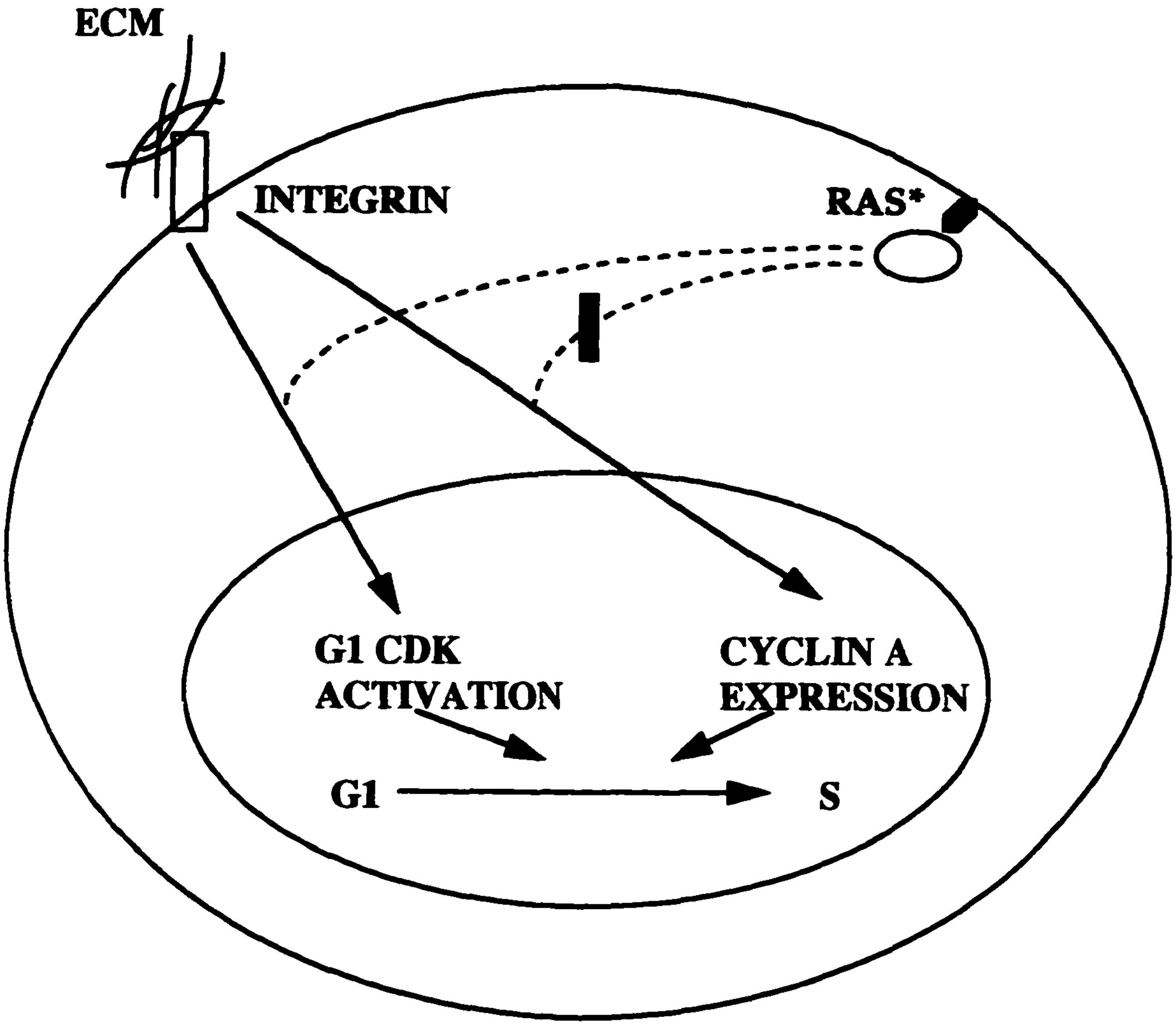

Figure 2: Ras generates multiple signalling pathways that lead to the cell cycle machinery. The activation of G1 CDKs and expression of cyclin A are normally anchorage-dependent. Oncogenic Ras abrogates the anchorage-dependent activation of these events. A blockade downstream of Ras signalling pathways prevents the expression of anchorage-independent cyclin A in ER-1-2/*ras* cells.

ornithine decarboxylase, osteopontin, stromelysin, cathepsin L, and insulin-like growth factor I genes, are constitutively expressed in ER-1-2 and control cells after infection with a Ras virus (123). Expression of many of these genes is likely to require MAPK activity. We also tested another pathway, JNK pathway, in this mutant cell line in response to Ras. We found that JNK activity, like MAPK activity, was not affected in the mutant line, when compared to the control cell line (J. J. Y. and R. S. K., unpublished data). This indicates that the signal transduction from Ras through MAPK/ or JNK is intact. Thus, the defect of the signal transduction pathway in this mutant cell line may be further downstream than MAPK/ or JNK. It is also possible that the defect is not in "ras-MAPK" pathway or "JNK pathway" but in a different signalling pathway(s) branched from Ras which is required for the anchorage-independent growth.

As described above, the anchorage-independent growth of tumor cells does not need signals generated from adhesion molecules. The proliferation of normal cells, however, requires signals of both adhesion and growth factors. Important targets for those signals in cells are the cell cycle machinery. In order to explore the defect of ER-1-2/ras cells for anchorage-independent growth, we tested the expression of cell cycle machinery components. We were able to demonstrate that several cell cycle events are required for cellular anchorage-independent growth in ER-1-2 and PKC3-F4 cells. These events are: pRB phosphorylation, cyclin E-dependent kinase activity, and expression of cyclin A. All three events occurred in *ras*-expressing PKC3-F4 cells in the absence of adhesion. These data indicate that *ras* oncogene abrogates the anchorage-dependence of these events. We found that suspended ER-1-2/*ras* cells displayed hyperphosphorylation of pRB and cyclin E-dependent kinase activity, but expression of cyclin A in these cells remained adhesion dependent. Thus, signals generated by Ras that lead to pRB phosphorylation and cyclin E-dependent kinase activity could be dissociated from the signals needed for cyclin A expression. The infection of ER-1-2 cells with a retroviral vector containing a cyclin A cDNA rescues the growth in soft agar of such cells in response to Ras (118). Thus, the expression of cyclin A requires signal(s) from adhesion or signals generated by oncogenic Ras. This mutant cell line, therefore, is valuable for studing the regulation of anchorage-independent growth, a phenotype that correlates with tumorigenesis.

Purinoceptors

It has been reported that nucleotides, such as ATP, ADP, AMP, UTP, and adenosine, can act as extracellulars factors by binding to specific receptors located on cell membrane, and these receptors have been classified and named purinoceptors. These receptors are either serpentine receptors coupled to G proteins or intrinsic ion channels. Purinoceptors are categorized into two subclasses: P_1 and P_2. The agonist preferences for P_1 receptors are adenosine>AMP>>ADP, ATP. P_2 receptors have the opposite order of preferences. P_2 purinoceptors have been classified into two subtypes, P_{2X} (P_{2X1}-P_{2X7}) and P_{2Y} (P_{2Y1}-P_{2Y7}) according to the relative potency of various nucleotide agonists, where the P_{2X} family are receptors that act as multisubunit, ligand-gated ion channels and the P_{2Y} family are seven transmembrane, G protein-coupled receptors (124-128). It has also been reported that ATP is a mitogen and acts synergistically with other growth factors on many cell lines. The mitogenic effect of ATP on Swiss 3T3 cells is via the stimulation of the production of the second messenger cAMP, and on IMR-90 human fibroblasts is via the suppression of arachidonate metabolism (129-131). Futhermore, many types of transformed cells and tumor cells express P_2-purinoceptors.

It is also important to address the sources of extracellular ATP. It has been proposed that there are three major sources of extracellular nucleotides released from cytoplasm. The most obvious source of extracellular ATP is cytosolic ATP (~3-5 mM in most cells), which can be released on the sudden breakage of intact cells. Two additional sources are 1) exocytotic release of granule ATP, and 2) the release of cytosolic ATP via intrinsic plasma membrane channels or pores (124).

The work presented in this thesis is divided into three chapters. In Chapter Three, we determined that anchorage-independent growth of cultured fibroblast cell lines induced by Ras requires multiple effector-mediated pathways integrated to the cell cycle machinery. As described above, the induction of several cell cycle events are necessary for oncogene-mediated, anchorage-independent growth. Among these events, the expression of cyclin A plays a critical role. We also found that Ras effector site mutants synergized with ectopic expression of cyclin A gene to induce anchorage-independent growth. In Chapter Four, we asked if there is a secreted activity that could restore the the ability of the mutant cell line (ER-1-2) to form colonies in soft agar in response to *ras*. We found that conditioned medium (CM) from non-transformed rat fibroblasts contains an activity that specifically corrects the defect in this mutant cell line. The major activity in CM, designated transformation-restoring factor (TRF), is ~1300 molecular weight, lipid insoluble, and heat, protease, acid and base stable. It is, however, UV (260nm) sensitive. In Chapter Five, we tested various factors that have chemical properties similar to TRF for the ability to rescue anchorage-independent growth of ER-1-2/*ras* cells. We found that extracellular ATP induces colony formation of ER-1-2/*ras* cells and activates a signaling pathway that leads to the anchorage-independent expression of cyclin A.

Chapter 2

General Method

Cell Culture - Rat 6- and NIH 3T3-derived cell lines were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) plus 10% bovine calf serum or 5% fetal bovine serum, respectively, as previously described (1,2). Cells producing high titer recombinant v-H-*ras* virus were kindly provided by K. Marcu. TGFβ1 and EGF were purchased from Gibco and prepared according to the manufacturer's instructions. For soft agar assays, 10,000 cells of the indicated type were suspended in 2 ml of 0.3% Noble agar (Difco) in DMEM containing 5% calf serum and overlayed above a layer of 2.5 ml of 0.5% agar in the same medium on a single well of a six well dish. The cultures were then overlayed with 2 ml DMEM plus 5% calf serum and 0.3% agar every three-four days. At the end of two weeks, colonies were stained with the vital stain INT (Sigma) for 72 hr at 37° C, and colonies were counted under a low power inverted light microscope. When feeder layers of cells were used they were seeded into the wells at a concentration of 1/20 confluence; the following day the medium was removed, and a standard bottom agar layer, warmed to 37° C, was overlayed onto the adherent cells. The bottom agar layer was allowed to solidify at room temperature, and the plates were kept at 37° C until top agar containing indicator cells was overlayed above the bottom agar (generally 4-5 hr later). When potential chemical inhibitors of ATP- or TRF-induced anchorage-independent growth were tested, cells were pretreated with the inhibitor for one hour while growing on tissue culture plates and then inoculated into agar medium with ATP or TRF and inhibitor. For subsequent feedings, the inhibitors were added to one half the standard feeding volume and the medium was then overlaid on the agar cultures. After a one hour incubation at 37° C, an additional half-volume containing ATP or TRF was added and the cultures were placed back in the incubator. NRK cells were grown under the same conditions as Rat 6 cell-derived cell lines. Assays of growth of NRK cells in soft agar were performed as described above, except that 5% dialyzed fetal calf serum was used in place of bovine calf serum, and the cells were fed once a week for two weeks. To recover cells cultured under non-adherent conditions, preparative methylcellulose cultures were used in place of soft agar cultures (2). Briefly, 1×10^5 cells were inoculated into 10 ml of DMEM containing 5% calf serum and 1.3% methylcellulose, in a 50 ml conical tube. The tubes were then placed in a water-jacketed CO_2 incubator at 37° C. Three days later, the medium was diluted with 40 ml of ice-cold phosphate-buffered saline (to solubilize the methylcellulose), and the cells were recovered by gentle centrifugation. Recovery of cells by this method was nearly quantitative, and cell viability was >98%, as determined by trypan blue dye exclusion assays. The growth properties in soft agar and methylcellulose of the cell lines used in these studies are nearly identical (2).

Immunoblotting - Cells from monolayer or methylcellulose suspension cultures were harvested in lysis buffer (50 mM Tris-HCl, pH 8.0/250 mM NaCl/1% NP-40, 2 mM EDTA) containing 1 mM PMSF, 10 ng/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate. Total proteins were then separated on

SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham) and the membranes were probed with specific antibodies. After extensive washing (with 40 mM Tris-Hcl, pH 8.0/50 mM NaCl/1 mM EDTA), the blots were reprobed with horseradish peroxidase-conjugated secondary antibody and specific protein bands visualized with the ECL chemiluminescent detection system (Amersham). Immunoblotting was performed with the following antibodies, from the indicated sources; anti - H-Ras (SC-35; Santa Cruz Biotechnology), anti-cyclin D (06-13T; UBI), anti-cyclin E (SC-481; Santa Cruz Biotechnology), anti-cyclin A (courtesy of R. Assoian, University of Miami; and M. Pagano, New York University), anti-human cyclin A (clone 6E6; Novocastra Laboratories), anti-human cyclin E (06-134; UBI), anti-pRB (14001A; PharMingen), anti-p27^{kip1} (A. Koff, Memorial Sloan-Kettering Cancer Center), and anti-p21^{cip1} (SC-397; Santa Cruz Biotechnology).

Chapter 3

Ras Signals to the Cell Cycle Machinery via Multiple Pathways to Induce Anchorage-Independent Growth

Abstract

Several specific cell cycle activities are dependent on cell-substratum adhesion in non-transformed cells, and the ability of the Ras oncoprotein to induce anchorage-independent growth is linked to its ability to abrogate this adhesion requirement. Ras signals via multiple downstream effector proteins, a synergistic combination of which may be required for the highly altered phenotype of fully transformed cells. We describe here studies on cell cycle regulation of anchorage-independent growth that utilize Ras effector loop mutants in NIH 3T3 and Rat 6 cells. Stable expression of activated H-Ras (12V) induced soft agar colony formation by both cell types, but each of three effector loop mutants (12V,35S; 12V,37G; and 12V,40C) were defective in producing this response. Expression of all three possible pairwise combinations of these mutants synergized to induce efficient anchorage-independent growth of NIH 3T3 cells, but only the 12V,35S + 12V,37G and 12V,37G + 12V,40C combinations were complementary in Rat 6 cells. Each individual effector loop mutant partially relieved adhesion dependence of pRB phosphorylation, cyclin E-dependent kinase activity and expression of cyclin A in NIH 3T3, but not Rat 6, cells. All pairwise combinations of effector loop mutants that were synergistic in producing anchorage-independent growth in either cell line, also led to efficient abrogation of the adhesion requirement for these cell cycle activities. Ectopic expression of cyclins D1, E or A synergized with individual effector loop mutants to induce soft

agar colony formation in NIH 3T3, but not Rat 6, cells, cyclin A being particularly effective. Taken together, these data indicate that that Ras utilizes multiple pathways to signal to the cell cycle machinery, and that these pathways synergize to supplant the adhesion requirements for specific cell cycle events, leading to anchorage-independent growth.

Introduction

Ras proteins are small guanine nucleotide binding proteins that play a central role in signal transduction pathways that regulate cell proliferation (1). Wild-type Ras proteins are activated transiently, via guanine nucleotide exchange mechanisms, in response to a wide variety of extracellular signalling agents (2). When in the GTP-bound state, Ras is capable of binding to several different established and potential effector proteins, including members of the Raf, phosphatidylinositol 3 (OH)-kinase (PI(3)K), and Ral guanine nucleotide dissociation stimulator (RalGDS) families, Rin, protein kinase Cζ, AF6, and the GTPase-activating proteins, $p120^{GAP}$ and neurofibromin (3,4). Binding to Ras leads, directly or indirectly, to activation of these effectors, which in turn activate downstream signalling cascades. Thus, Ras may be viewed as a hub from which multiple pathways radiate. Activating mutations in Ras result in constitutive signalling to these downstream elements, and such mutations are observed with high frequency in human tumors (5).

Expression of mutated, oncogenic Ras in cultured rodent fibroblast cell lines induces a highly pleiotropic response, including alterations in cell morphology, loss of contact inhibition, changes in gene expression, decreased dependence on serum growth factors, and the ability to proliferate in the absence of adhesion to a substratum (i.e., anchorage-independent growth). Many of these phenotypes can be dissociated from one another. For example, introduction of Ras oncoprotein into quiescent Swiss 3T3 cells led to both morphological transformation and DNA synthesis, but only the induction of DNA synthesis required activation of protein kinase C (6). Furthermore, a Rat 6 fibroblast-derived mutant cell line, ER-1-2, responded to stable expression of the v-H-*ras* oncogene with alterations in morphology and gene expression that were nearly indistinguishable from those observed with a matched control cell line, yet failed to form colonies in soft agar in response to *ras* (7,8).

The studies cited above raise the possiblity that different aspects of the transformed phenotype might be controlled by distinct combinations of Ras-regulated pathways. Evidence for this notion has been elegantly provided through the use of Ras effector loop mutants. Certain point mutations in this region (amino acids 32-40 in H-Ras) render Ras defective for binding specific effector proteins, while remaining competent for binding and activating others, albeit at lower than wild-type efficiency (9,10). Several individual mutants were defective in transformation assays, but, when coexpressed, complemented each other (9-11). These studies have implicated at least three effector proteins as potential synergistic mediators of transformation by Ras: Raf, PI(3)K, and RalGDS (9-15). Raf stimulates the mitogen-activated protein kinase (MAPK) cascade, leading to phosphorylation and activation of transcription factors and other proteins (16). PI(3)K stimulates cortical actin rearrangement via the small GTP binding protein Rac (10). Ras also activates the c-Jun N-terminal kinase (JNK) cascade in a Rac-dependent manner (17), but the role of PI(3)K in Ras-mediated activation of this pathway is not yet established. The mechanisms by which RalGDS contributes to the transformed phenotype are not known, but may involve both Ral-dependent and -independent pathways (12,14,18). It should be mentioned that Raf, MAPK, PI(3)K and Rac are each required for Ras to exert its full powers of transformation (10,19-21) and, under certain conditions, transform fibroblasts on their own (21-24).

We have undertaken an analysis of the mechanisms by which Ras inappropriately drives cell cycle events to induce anchorage-independent growth. Anchorage-independent growth is the best *in vitro* correlate of tumorigenicity (25), but the effector pathway or pathways utilized by Ras to produce this phenotype are still largely unknown. We and others demonstrated that several important cell cycle events were dependent on cell-substratum adhesion of non-transformed fibroblast cell lines, including: 1) activation of G_1 cyclin-dependent kinases (Cdks; as measured by cyclins D- and E-dependent kinase activities and phosphorylation of pRB family members); and 2) expression of the cyclin A gene (26-31). Certain other cell cycle events, such as expression of cyclin E, were not regulated by adhesion. Strikingly, all of these cell cycle activities occurred in the absence of adhesion in Ras-transformed cell lines (26,31). In contrast, ER-1-2 cells that expressed v-H-*ras* (ER-1-2/*ras* cells), which failed to proliferate in soft agar, possessed G_1 Cdk activities when cultured without adhesion, but remained almost completely adhesion-dependent for expression of cyclin A (26). Importantly, ectopic expression of cyclin A rescued anchorage-independent growth of ER-1-2/*ras* cells, but did not induce anchorage-independent growth of control or ER-1-2 cells, presumably because these cells still lacked G_1 Cdk activities in the absence of adhesion (26). Anchorage-independent activation of G_1 Cdks and expression of cyclin A are therefore likely to be functionally relevent endpoints in determination of the transformed phenotype.

Expression of the cyclin A gene is dependent on G_1 Cdk activity (32). Results from the ER-1-2 cell system and other studies (26,28,33,34) indicate, however, that there is an additional, adhesion- and Ras-regulated function that is also required for cyclin A expression, and that this function is at least partly dissociable from the mechanisms by which adhesion and Ras regulate G_1 Cdk activity. It is possible, therefore, that multiple Ras effector pathways are required to supplant adhesion-mediated cell cycle regulation and induce growth in soft agar. To test this hypothesis more directly, we have stably expressed Ras effector loop mutants in Rat 6 and NIH 3T3 fibroblasts and analyzed the cells for: 1) growth in soft agar; and 2) the ability to drive specific cell cycle events in the absence of cell-substratum adhesion. These studies indicate that Ras signals to the cell cycle machinery via several pathways, and that a combination of pathways is required to produce efficient anchorage-independent growth.

Material and Methods

Transfections and Retroviral Infections - pDCR-*ras*(12V), pDCR-*ras*(12V35S),pDCR-*ras*(12V37G), and pDCR-*ras*(12V40C) encode the indicated mutant Ras alleles under the control of the cytomegalovirus promoter, and also contain the selectable marker gene *neo*. These reagents were originally developed by White, Wigler and colleagues (9), and were kindly provided by D. Bar-Sagi (SUNY-Stonybrook). Rat 6 and NIH 3T3 cells were transfected with a total of 20 μg of these plasmids, either singly or in combination, by the calcium phosphate technique (35). Transfected cultures were selected in G418-containing medium (400 μg per ml for Rat 6-derived cell lines and 700 μg per ml for NIH 3T3-derived cell lines), and drug-resistant colonies were pooled and analyzed as described in Results.

Production of pBabePuro-based recombinant retroviruses that encode human cyclins D1, E or A, and infection of Rat 6 and NIH 3T3 cell lines, were performed as previously described (26). Infected cultures were selected in puromycin-containing medium (5 μg per ml for Rat 6-derived cell lines and 7.5 μg per ml for NIH 3T3-derived cell lines), and drug-resistant colonies were pooled and analyzed as described in Results.

Cyclin E-Dependent Kinase Assays - For *in vitro* cyclin E- dependent kinase assays, cyclin E was immunoprecipitated from 500 μg of total cellular protein (from lysates prepared as described above) with polyclonal anti-cyclin E antibody (SC-481; Santa Cruz Biotechnology), and the immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 μM Tris-HCl, pH 7.4, 4 μM $MgCl_2$). The washed immunoprecipitates were then incubated with kinase buffer, 2 μg histone H1, 1 μM ATP, and 5 μCi [γ-^{32}P]ATP in a final volume of 16 μl for 30 min at 37° C. The products of the reaction were separated on a 12% SDS-polyacrylamide gel. The gel was then dried and exposed to X-ray film.

Results

Phenotypic Effects of Ras Effector Loop Mutants on NIH 3T3 and Rat 6 Cells - To test the hypothesis that multiple Ras effector pathways might contribute to the induction of anchorage-independent growth, the NIH 3T3 and Rat 6 cell lines were transfected with expression vectors for fully activated H-Ras (12V), three H-Ras effector loop mutants that also contained the activating 12V mutation (12V,S35; 12V,37G; 12V,40C), or an empty vector as a control. Of the three effector proteins for which there is evidence of a role in Ras-mediated transformation, the 12V,S35 mutant binds to Raf, but not PI(3)K or RalGDS; the 12V,37G mutant binds to RalGDS, but not Raf or PI(3)K; and the 12V,40C mutant binds to PI(3)K, but not to Raf or RalGDS (9,10,12,14). The expression vector also contained the selectable marker gene *neo*; multiple G418-resistant colonies were selected, pooled and tested for expression of Ras protein by Western blotting with anti-Ras antibody. As shown in Figure 1A, endogenous levels of Ras protein were at the limit of detection in empty vector controls (designated *neo*), but each Ras mutant was expressed at a similar, easily detectable level.

The various transfectants were then tested for the ability to form colonies in soft agar. As shown in Table I and Figure 2, the *neo* controls of both NIH 3T3 and Rat 6 cells remained as single cells when cultured in suspension. As expected, expression of H-Ras 12V led to production of large, macroscopic colonies

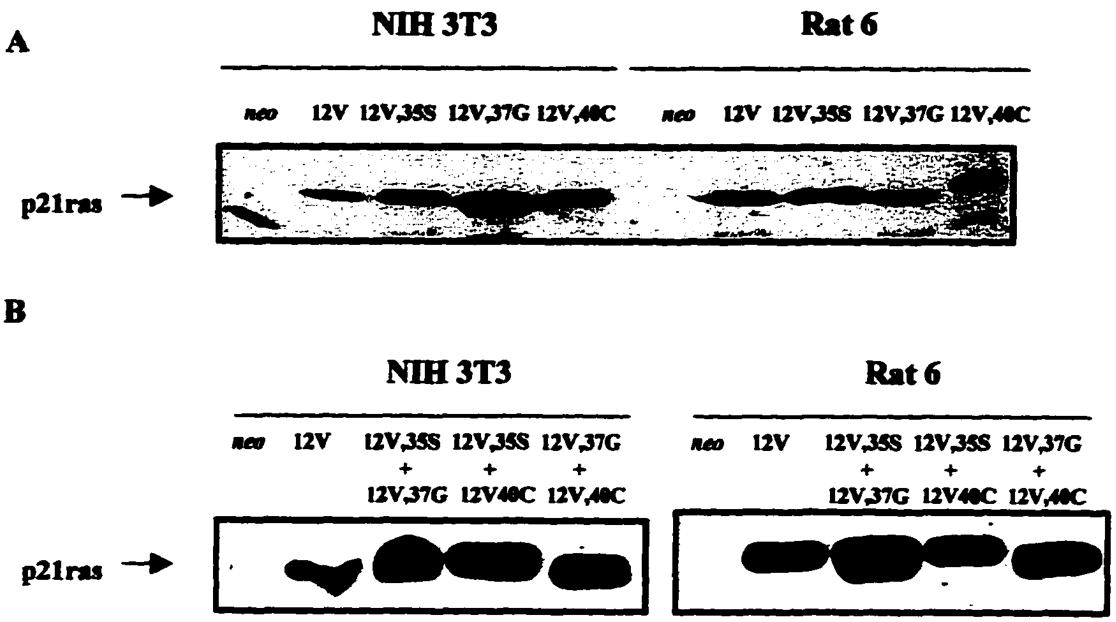

Figure 1: Expression of H-Ras 12V and effector loop mutant proteins in NIH 3T3 and Rat 6 cells. (**A**) NIH 3T3 and Rat 6 cells were stably transfected with expression vectors for the indicated proteins. (**B**) NIH 3T3 and Rat 6 cells were transfected with combinations of expression vectors for the indicated proteins. Cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-Ras antibodies.

TABLE I: Induction of Anchorage-Independent Growth by H-Ras and Various H-Ras Effector Loop Mutants

Vector	Colony-Forming Efficiency (%)[a]	
	NIH 3T3	Rat 6
neo	0	0
12V	2.2	1.9
12V,35S	0*[b]	0*
12V,37G	0*	0*
12V,40C	0*	0*
12V,35S + 12V,37G	1.9	1.7
12V,35S + 12V,40C	1.4	0*
12V,37G + 12V,40C	0.6	0.5

[a] A total of 10,000 cells were seeded into 0.3% agar. Macroscopic colonies (>0.2 mm diameter) were scored after two weeks of growth. Values represent averages of duplicate determinations that differed by less than 5%. The experiment was repeated at least twice for each transfection; data from several experiments are normalized to a single value from the 12V transfectant; the range for 12V over numerous experiments was from 1-5%.

[b] The asterisk indicates that, while no macroscopic colonies were formed, microscopic colonies of 8-12 cells were formed with an efficiency of ~1.5% (see text for details).

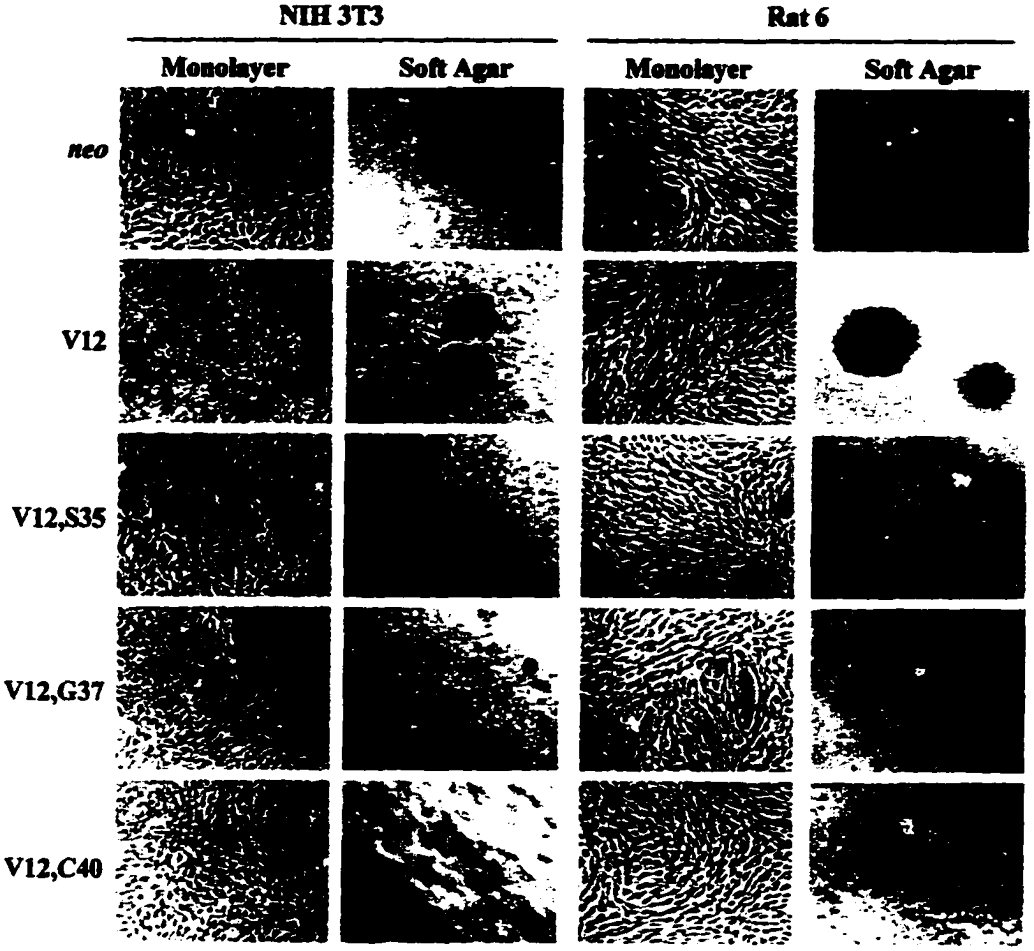

Figure 2: Morphology in adherent culture and soft agar colony formation of NIH 3T3 and Rat 6 cells that express H-Ras 12V and effector loop mutants. NIH 3T3 and Rat 6 cells that expressed *neo* or the indicated H-Ras constructs were cultured on tissue culture dishes (monolayer) or in soft agar as described under "Experimental Procedures" and photographed. Magnification, X40 for monolayer cultures; X40 for soft agar cultures.

in both cell lines. In contrast, each effector loop mutant was severely impaired at inducing anchorage-independent growth, producing no macroscopic colonies at all in either NIH 3T3 or Rat 6 cells. The effector loop mutants were not completely devoid of activity, however, as all three mutants led to the formation of very small colonies (~8-12 cells) in both cell types.

The data described above suggested that more than one Ras-regulated signalling pathway may be required for efficient induction of anchorage-independent growth. We therefore tested the ability of pairwise combinations of Ras effector loop mutants to stimulate soft agar colony formation by NIH 3T3 and Rat 6 cells. Combinations of 12V,35S + 12V,37G; 12V,35S + 12V,40C; and 12V,37G + 12V,40C were transfected into each cell line and, again, G418-resistant colonies were pooled, analyzed for expression of H-Ras, and tested for growth in soft agar. Figure 1B demonstrates that each double transfectant produced abundant immunoreactive H-Ras protein, presumably a mixture of the two transfected mutants.

Coexpression of all three pairwise combinations of effector loop mutants in NIH 3T3 cells led to production of macroscopic colonies in soft agar. The combination of 12V,35S + 12V,37G was nearly as efficient as the fully-transforming 12V, while the other two combinations were somewhat less effective. In each case, however, the colonies formed by coexpression of two effector loop mutants were only about one-half the diameter of those formed by V12 (0.5 mm vs. 1.0 mm, respectively). These data indicate that Ras can induce the formation of macroscopic colonies in soft agar with reasonable efficiency in the absence of binding to Raf, or PI(3)K, or RalGDS, but not, most likely, in the absence of binding to any two of these effectors.

Similar to NIH 3T3 cells, the combined expression of 12V,35S + 12V,37G in Rat 6 cells produced a number of colonies that was only slightly lower than that observed with 12V (Table I), and these colonies were, on average, only half the diameter of those formed with 12V (0.5 mm vs. 1.0 mm, respectively). The combination of 12V,37G + 12V,40C also exhibited complementation in colony formation but, again, was not as effective as 12V,35S + 12V,37G; furthermore, these colonies averaged only about 0.3 mm in diameter. In contrast to NIH 3T3 cells, the combination of 12V,35S + 12V,40C was ineffective at inducing colony formation in Rat 6 cells, beyond the microcolonies each mutant produced individually. These data suggest that a function supplied by the 12V,37G mutant, but not the other two mutants, may be necessary, but not sufficient, for induction of anchorage-independent growth of Rat 6 cells.

The effects of the different H-Ras proteins on cell morphology was also examined (Figure 2). NIH 3T3 cells that expressed 12V displayed a typical transformed phenotype: the cells were rounded, refractile, and disorganized, as compared to the *neo* control transfectants, which grew as a flat monolayer. Cells that expressed each of the effector loop mutants resembled the *neo* controls much more closely than the 12V-expressing cells, but each also exhibited somewhat higher cell density and refractility. The effect of the H-Ras proteins on Rat 6 cell morphology was less pronounced than on NIH 3T3 cells. The 12V-expressing Rat 6 cells grew in a swirling pattern and to higher density than that observed with the *neo* control cells, but the effector loop mutant-expressing cells were indistinguishable from these control transfectants (Figure 2).

Effects of Various Ras Mutants on Adhesion-Mediated Expression and Regulation of Cell Cycle Proteins - Phosphorylation of pRB, cyclin E-dependent kinase activity, and expression of cyclin A are all fully dependent on cell anchorage in several different non-transformed cell types, including NIH 3T3 and Rat 6 (26-31). Oncogenic Ras abrogates the adhesion-dependence of these activities in both cell types, and its ability to do so is apparently linked to its ability to drive anchorage-independent growth (26,31). We therefore asked to what extent, if any, might expression of the Ras effector loop mutants relieve adhesion-dependence of these events. 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 (26). It has been demonstrated previously that the growth properties in soft agar and methylcellulose of the fibroblast lines used in these studies are nearly identical (26,36). Furthermore, the proliferative behavior of the effector loop mutant-expressing cells in methylcellulose culture was very similar to that in soft agar (data not shown).

Similar to previous reports (26,28), pRB phosphorylation, cyclin E-dependent kinase activity, and cyclin A expression were all adhesion-dependent in *neo* control, and adhesion-independent in the 12V-expressing, NIH 3T3 cells (Figure 3A-C). Interestingly, each of the three effector loop mutants induced detectable levels of pRB phosphorylation in the absence of adhesion (Figure 3A). The level of activity was in the order: 12V,35S > 12V,40C > 12V,37G, with the 12V,35S mutant displaying activity nearly as robust as that seen with the fully-transforming 12V. All three mutants also induced cyclin E-dependent kinase activity under anchorage-independent culture conditions (Figure 3B). In this case, none of the effector loop mutants were as effective as 12V, but 12V,35S and 12V,40C were each more effective than 12V,37G. Finally, all three mutants also induced expression of cyclin A in an adhesion-independent manner, but at a level significantly lower than 12V. In particular, expression of 12V,37G or 12V,40C in NIH 3T3 cells led to production of only trace levels of cyclin A. It is concluded that, in NIH 3T3 cells, Ras proteins that are defective in binding any two of the three effectors implicated in transformation are still able to abrogate, in part, adhesion-mediated regulation of the cell cycle. The remaining signalling capabilities of such defective Ras proteins are not, however, sufficient

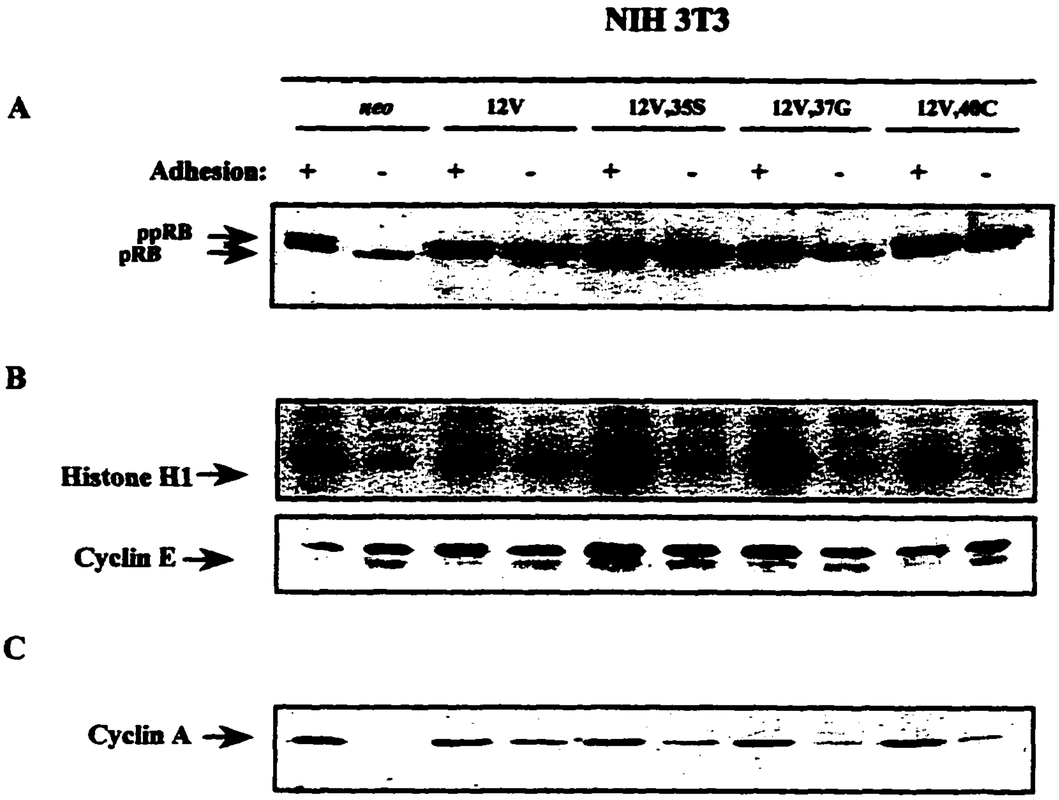

Figure 3: Analysis of adhesion-regulated cell cycle activities in NIH 3T3 cells that express H-Ras 12V and effector loop mutants. Cells expressing *neo* or the indicated H-Ras constructs were grown on tissue culture plates (+ adhesion) or in preparative methylcellulose cultures (- adhesion) and were analyzed by immunoblotting or immunoprecipitation and kinase assay as follows: (**A**) pRB phosphorylation; blots were probed with a specific anti-pRB antibody. The hyperphosphorylated (ppRB) and hypophosphorylated (pRB) forms are distinguished by their mobilities and are indicated by arrows. (**B**) Cyclin E-dependent kinase activity; cyclin E was immunoprecipitated and analyzed for associated histone H1 kinase activity. The bottom panel shows a Western blot of cyclin E, demonstrating approximately equivalent levels of cyclin E in the different cell types under the various culture conditions. (**C**) Expression of cyclin A; blots were probed with a specific anti-cyclin A antibody. Each experiment was performed at least twice on two independent extracts from each cell line. Representative data are shown. See Experimental Procedures for additional details.

to override fully the adhesion requirement for cell proliferation, as reflected by molecular markers (Figure 3) and colony formation in soft agar (Table 1 and Figure 2).

The ability of individual effector loop mutants to abrogate, even partially, the anchorage-dependence of cell cycle events was much less pronounced in Rat 6 cells than it was in NIH 3T3 cells. As with NIH 3T3 cells, pRB phosphorylation, cyclin E-dependent kinase activity, and cyclin A expression were all adhesion-dependent in *neo* controls, and adhesion-independent in the 12V-expressing cells (Figure 4A-C). Expression of each of the three effector loop mutants in Rat 6 cells, however, led to just trace levels of pRB phosphorylation and cyclin E-dependent kinase activity when the cells were cultured in suspension (Figure 4A and 4B). Furthermore, these cells remained completely dependent on adhesion for production of cyclin A (Figure 4C).

Cells transfectanted with combinations of different effector loop mutants, most of which showed complementation in inducing colony formation in soft agar (Table 1), were also assessed for pRB phosphorylation and expression of cyclin A under adherent and non-adherent culture conditions. In NIH 3T3 cells transfected with 12V,35S + 12V,37G or 12V,35S + 12V,40C, both of which gave a significant level of agar colony formation, anchorage-independent phosphorylation of pRB and production of cyclin A were only slightly lower than

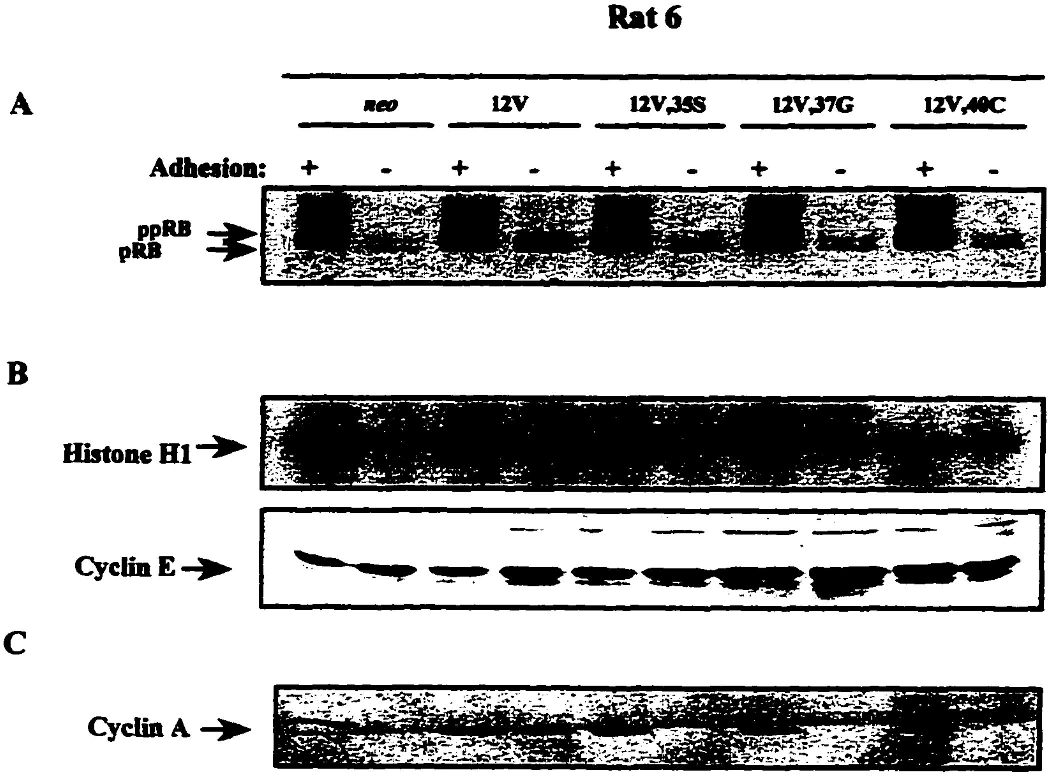

Figure 4: Analysis of adhesion-regulated cell cycle activities in Rat 6 cells that express H-Ras 12V and effector loop mutants. Cells expressing *neo* or the indicated H-Ras constructs were grown on tissue culture plates (+ adhesion) or in preparative methylcellulose cultures (- adhesion) and were analyzed by immunoblotting or immunoprecipitation and kinase assay as follows: (A) pRB phosphorylation; blots were probed with a specific anti-pRB antibody. The hyperphosphorylated (ppRB) and hypophosphorylated (pRB) forms are distinguished by their mobilities and are indicated by arrows. (B) Cyclin E-dependent kinase activity; cyclin E was immunoprecipitated and analyzed for associated histone H1 kinase activity. The bottom panel shows a Western blot of cyclin E, demonstrating approximately equivalent levels of cyclin E in the different cell types under the various culture conditions. (C) Expression of cyclin A; blots were probed with a specific anti-cyclin A antibody. Each experiment was performed at least twice on two independent extracts from each cell line. Representative data are shown. See Experimental Procedures for additional details.

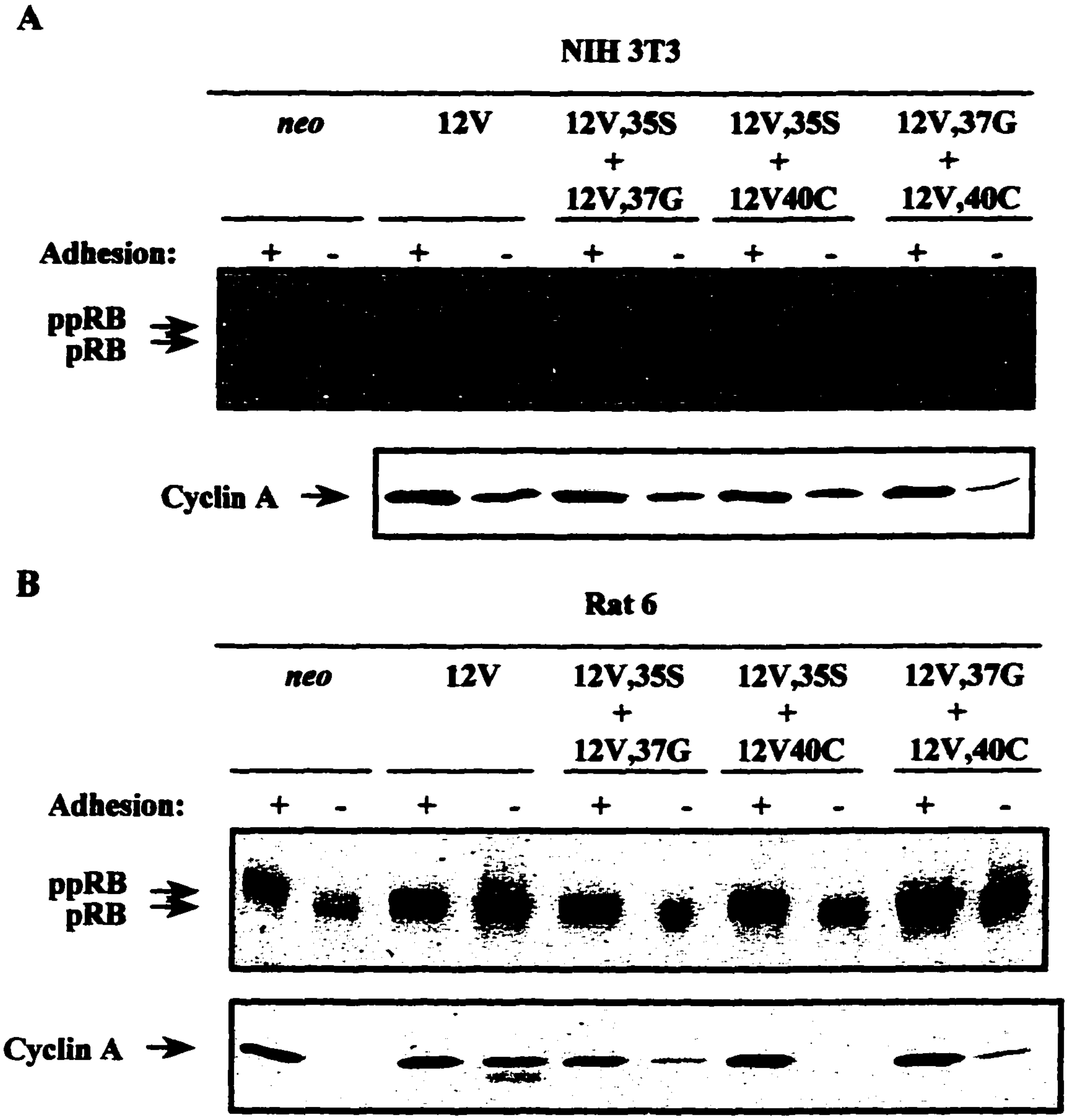

Figure 5: Analysis of adhesion-regulated cell cycle activities in NIH 3T3 and Rat 6 cells that express H-Ras 12V and various combinations of effector loop mutants. Cells expressing the indicated H-Ras constructs were grown on tissue culture plates (+ adhesion) or in preparative methylcellulose cultures (- adhesion) and were analyzed by immunoblotting with specific antibodies directed against pRB (top panels) or cyclin A (bottom panels), as indicated. The hyperphosphorylated (ppRB) and hypophosphorylated (pRB) forms are distinguished by their mobilities and are indicated by arrows. (A) NIH 3T3 cell derivatives. (B) Rat 6 cell derivatives. Each experiment was performed twice.

that observed with 12V alone (Figure 5A). Additionally, the 12V,37G + 12V,40C transfectant, which was a less effective complementation pairing, was also less able to overcome the adhesion dependence of these actvities (Figure 5A). A similar pattern was observed in Rat 6 cells. The 12V,35S + 12V,37G and 12V,37G + 12V,40C double transfectants both produced colonies in soft agar and, unlike the single transfectants, displayed significant levels of pRB phosphorylation and cyclin A expression in the absence of adhesion (Figure 5B). In contrast, the 12V,35S + 12V,40C combination, which did not synergize to form colonies in soft agar in Rat 6 cells, showed lower levels of phosphorylated pRB, and did not express cyclin A at all (Figure 5B).

Several different mechanisms play a role in regulation of G_1 Cdk activity by cell-substratum adhesion (26-31). It has been reported previously that suspension of NIH 3T3 cells led to decreased amounts of cyclin D1 and elevated amounts of the Cdk inhibitor, $p27^{kip1}$ (28,30). We therefore assessed the effects of 12V and effector loop mutants on adhesion-mediated regulation of the levels of these proteins in NIH 3T3 cells. As shown in Figure 6, expression of cyclin D1 was partially adhesion-dependent in the *neo* controls. In contrast, 12V-expressing cells possessed very high levels of cyclin D1, regardless of their state of adhesion. A significant fraction of the 12V-induced increase in cyclin D1 levels was observed with 12V,35S, suggesting a role for Raf in mediating this response. This is consistent with previous reports implicating MAPK in transcriptional regulation of the cyclin D1 promoter (37). 12V,37G-expressing cells also displayed elevated levels of cyclin D1 that were unaltered by loss of adhesion, but the 12V,40C mutant was largely without effect on cyclin D1.

In contrast to a previous report (30), we found that levels of $p27^{kip1}$ in NIH 3T3 cells were essentially unaffected by either adhesion or Ras (Figure 6). We therefore investigated the effects of adhesion and Ras on the levels of a different Cdk inhibitor, $p21^{cip1}$. $p21^{cip1}$ was induced by loss of adhesion in *neo* control cells, but was present at even higher levels in 12V-transformed cells, whether these cells were cultured in monolayer or suspension (Figure 6). $p21^{cip1}$ levels were influenced by effector loop mutants in a fashion that was similar to that observed with cyclin D1. 12V,35S and 12V,37G each led to elevated levels of $p21^{cip1}$, regardless of adhesive state, whereas 12V,40C was again without significant effect. $p21^{cip1}$, like cyclin D1, is encoded by a mitogen-inducible gene (38), and its constitutive expression by Ras-transformed cells is therefore not surprising. The results presented in Figure 6 suggest that similar pathways might regulate production of cyclin D1 and $p21^{cip1}$. This is of interest since $p21^{cip1}$ has been implicated in assembly of cyclin D-Cdk4 complexes (39).

We have previously reported that the levels of cyclin D1, $p27^{kip1}$ and $p21^{cip1}$ were not regulated by adhesion in Rat 6-derived cell lines (26), and,

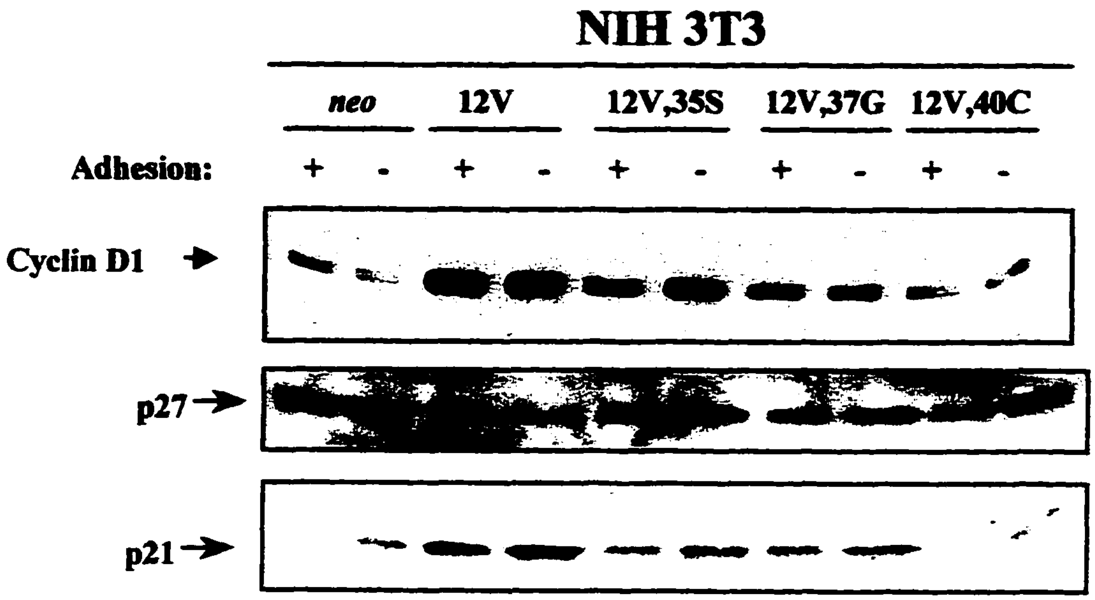

Figure 6: Effects of adhesion and various Ras proteins on expression of cyclin D1, $p27^{kip1}$ and $p21^{cip1}$ in NIH 3T3 cells. Cells expressing *neo* or the indicated H-Ras constructs were grown on tissue culture plates (+ adhesion) or in preparative methylcellulose cultures (- adhesion) and were analyzed by immunoblotting with specific antibodies to cyclin D1, $p27^{kip1}$ or $p21^{cip1}$, as indicated. Each experiment was performed at least twice on two independent extracts from each cell line. Representative data are shown.

therefore, the effects of the various Ras mutants on production of these proteins were not investigated in suspension cultures of this cell line. (The effects of Ras mutants on production of cyclin D1 in adherent cultures is, however, presented below.)

Cooperation Between Ras Effector Loop Mutants and Cyclins in Inducing Anchorage-Independent Growth - Expression of cyclins D1 and A are adhesion-dependent in several cell types (26-28). Ectopic expression of each of these cyclins influenced adhesion-mediated regulation of cell cycle progression, although in most cases, it was not sufficient to induce colony formation in soft agar (26,28,40). The Ras-mediated increase in cyclin D1 levels, observed in this and other studies (Figure 6 and (26,41)), is thought to be important for transformation by this oncogene. Furthermore, ectopic expression of cyclin A rescued anchorage-independent growth of transformation-resistant ER-1-2/*ras* cells (Ref. (26), and see Introduction). Thus, it was of interest to test whether ectopic expression of cyclins D1, E or A could complement individual Ras effector loop mutants in the induction of anchorage-independent growth. NIH 3T3 and Rat 6 cells that expressed *neo*, 12V, or each of the three effector loop mutants were infected with recombinant retroviruses that drive expression of human cyclins D1, E or A cDNAs from the viral long terminal repeat (26). The parental vector, pBabePuro (42), also confers resistance to the drug puromycin. The cells were infected with one of the cyclin-expressing viruses or, as a control, a virus that lacked a cDNA insert. The infectants were selected in puromycin-containing medium, and puromycin-resistant colonies were then pooled and analyzed for expression of the various exogenous cyclins and colony formation in soft agar.

As we have reported previously, it was more difficult to achieve ectopic expression of cyclin A than cyclins D1 or E (26); many of the colonies that intially emerged following infection of either the NIH 3T3 or Rat 6 cell derivatives with the cyclin A virus died. Nevertheless, ectopic producers of each of the three cyclins were obtained from the pooled infectants. As can be seen in Figure 7A, NIH 3T3 derivatives infected with the cyclin D1 virus displayed enhanced levels of cyclin D1, when analyzed with an antibody that recognized both the endogenous mouse protein and the exogenous human protein. The 12V derivative had only about two-fold more cyclin D1 immunoreactivity than its matched control infectant population, but the *neo* cells and each of the effector loop mutant-expressing cells all produced significantly enhanced levels of cyclin D1 (Figure 7A; the low level of cyclin D1 seen in the control virus-infected, 12V,35S-expressing cells, relative to that observed with these cells in Figure 6, was due to underloading of this lane in this particular experiment). Ectopic expression of cyclins E and A was detected with antibodies that were specific for the exogenous human proteins (Figure 7A).

The NIH 3T3 infectants were then tested for their ability to form

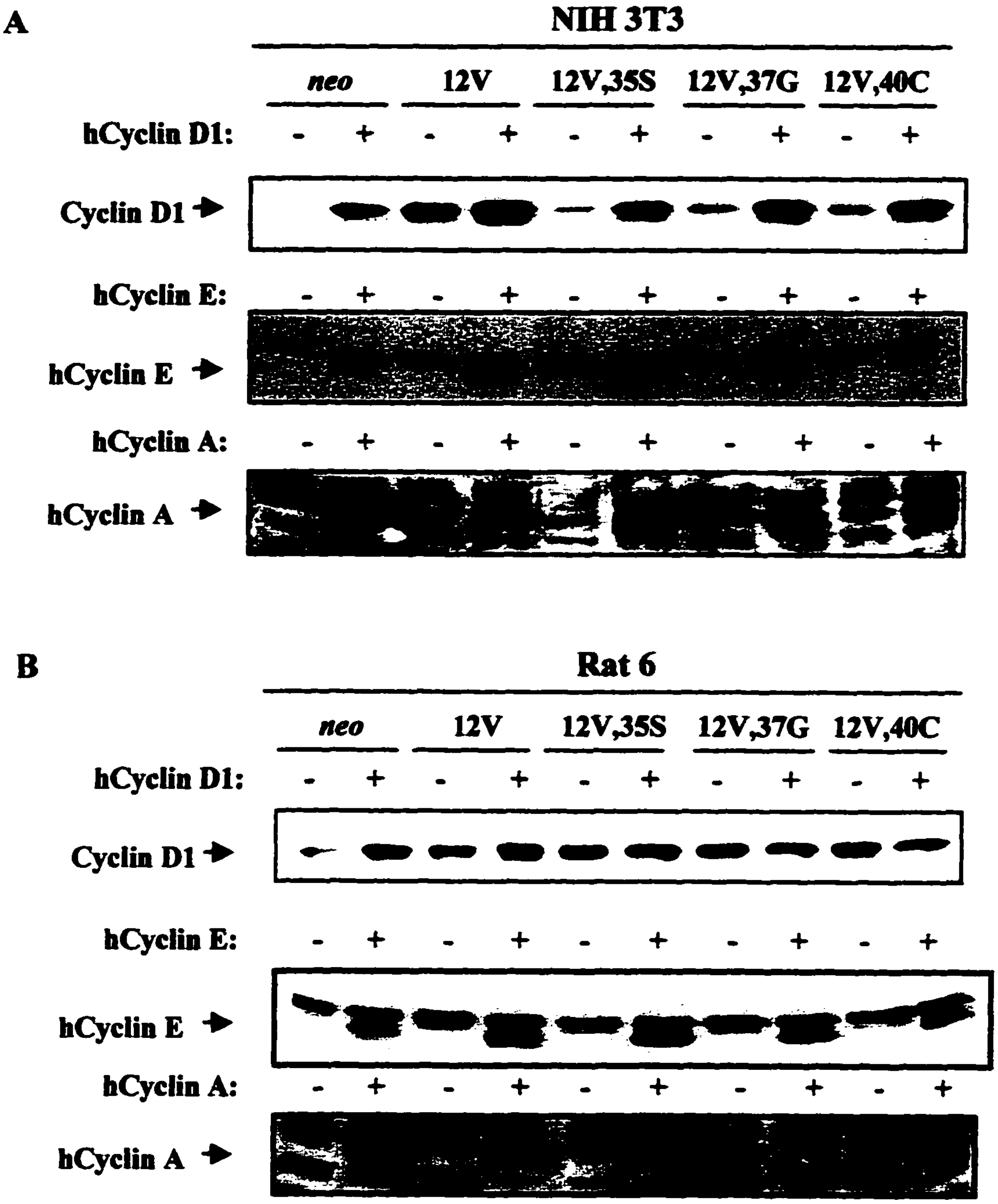

Figure 7: Analysis of ectopic expression of cyclins D1, E and A in NIH 3T3 and Rat 6 cell derivatives. NIH 3T3 or Rat 6 cells that express *neo*, H-Ras 12V or various effector loop mutants were infetced with recombinant retroviruses that either lacked a cDNA insert (-) or that harbored a human (h) cyclin D1, hcyclin E, or hcyclin A cDNA, (+). Cell extracts were analyzed by immunoblotting with antibodies specific for cyclin D1, human cyclin E or human cyclin A. Specific bands are indicated by the arrows. The hcyclin A band migrates between two nonspecific, cross-reactive bands. (A) NIH 3T3 cells. (B) Rat 6 cells.

macroscopic colonies in soft agar. As can be seen in Table II, ectopic expression of cyclins D1, E, or A was not sufficient to induce anchorage-independent growth of the *neo* control cells. Infection of the 12V cells with the control retrovirus (+pBabePuro) did not increase the efficiency of colony formation of these cells (compare Table I and Table II). In contrast, infection of the 12V cells with any of the cyclin-expressing viruses led to enhanced growth in soft agar: the 12V/cyclin D1, 12V/cyclin E, and 12V/cyclin A cells displayed 5.4-, 2.1-, and 2.3-fold enhanced colony-forming efficiencies, respectively. These data indicate that the levels of each cyclin are rate-limiting for anchorage-independent growth, even in cells that express abundant oncogenic Ras protein. Surprisingly, the 12V/cyclin D1 cells showed an increase in colony-forming efficiency (5.4-fold) that was out of proportion to the level of overproduction of cyclin D1 (~2-fold, assuming that the anti-cyclin D1 antibody recognized mouse and human proteins equally well). Possible reasons for this phenomenon are described in the Discussion.

Stable overproduction of cyclin D1 in the various Ras effector loop mutant-expressing NIH 3T3 cells led to a low, but significant, level of colony formation in soft agar relative to the number of colonies observed with the V12/cyclin D1 cells, which are the most appropriate cell type for comparison (Table II). The order of sensitivity to cyclin D1-induced colony formation was 12V,37G > 12V,40C > 12V,35S, with these infectants achieving 21%, 14% and 9% the efficiency seen with V12/cyclin D1 cells, respectively. Ectopic production of cyclin E had a

TABLE II: Cooperation Between Ras Effector Loop Mutants and Cyclins in Inducing Anchorage-Independent Growth of NIH 3T3 Cells

Cell Line	Colony-Forming Efficiency (% +/- SD)[a]			
	+pBabePuro[b]	+Cyclin D1	+Cyclin E	+Cyclin A
neo	0	0	0	0
12V	2.2 +/- 0.1	12.1 +/- 1.0	4.7 +/- 0.5	5.1 +/- 0.6
12V,35S	0*[c]	1.0 +/- 0.1	0.4 +/- 0.1	2.0 +/- 0.2
12V,37G	0*	2.5 +/- 0.1	1.2 +/- 0.1	1.7 +/- 0.4
12V,40C	0*	1.7 +/- 0.2	0*	3.7 +/- 0.3

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[a] A total of 10,000 cells were seeded into 0.3% agar. Macroscopic colonies (>0.2 mm diameter) were scored after two weeks of growth. Each infection with cyclin-expressing viruses was performed twice; each pool of infectants was then analyzed twice in duplicate. Values represent averages of all determinations +/- one standard deviation. See text for further details.

[b] +pBabePuro, cells infected with a retroviral vector lacking a cDNA insert; +cyclin D1, E, or A, cells infected with a retroviral vector that drives expression of a human cyclin of the indicated type.

[c] The asterisk indicates formation of microscopic colonies (see legend to Table I).

similar effect on the 12V,37G and 12V,35S-expressing cells, yielding 26% and 9% the number of colonies seen with V12/cyclin E cells. In contrast, the 12V,40C-expressing NIH 3T3 cells were not complemented by infection with the cyclin E virus, despite production of easily detectable levels of human cyclin E (Figure 7A). Ectopic expression of cyclin A had a much more pronounced effect on anchorage-independent growth of the effector loop mutant-expressing cells than did cyclins D1 or E. The order of sensitivity to cyclin A-induced colony formation was 12V,40C > 12V,35S > 12V,37G, with these infectants producing 71%, 39% and 34% the number of colonies produced by V12/cyclin A cells, respectively (Table II). These data are consistent with the notion that cyclin A plays a key role in the induction of anchorage-independent growth (26,27,36).

We also performed a similar set of experiments with the various Ras-expressing Rat 6 cell derivatives. In contrast to NIH 3T3 cells, expression of each effector loop mutant in Rat 6 cells led to an increase in cyclin D1 levels that was similar to that seen with expression of 12V (Figure 7B; see Figure 6 for data on NIH 3T3 cells). Infection with the cyclin D1-expressing virus failed to increase significantly the production of cyclin D1 by these cell lines, perhaps as a result of this already high level. Infection with the cyclin E- and A-expressing viruses did lead to production of the human proteins in all of the Rat 6 cell derived lines, at levels comparable to that observed with the NIH 3T3 infectants (Figure 7B). In contrast to the NIH 3T3 cells, however, coexpression of any of the three Ras effector loop mutants with any of the three cyclins failed to result in formation of macroscopic colonies (data not shown). Thus, cyclins D1, E and A were able to complement, with some degree of specificity, the Ras effector loop mutants in the induction of anchorage-independent growth of NIH 3T3, but not Rat 6, cells.

Discussion

Stable expression of oncogenic Ras in rodent fibroblast cell lines induces a wide array of responses referred to collectively as the transformed phenotype. Such responses are thought to be of relevance to the role of Ras in human cancers and include alterations in cell morphology, growth factor requirements and gene expression. A response that shows an excellent correlation with tumorigenicity is anchorage-independent growth, measured as the ability to form colonies in semi-solid medium (25). Recent studies have indicated that Ras functions by binding to multiple effector proteins that, in turn, activate distinct downstream signalling pathways (3,4). A major task is to discern which effector pathways contribute to which aspects of the transformed phenotype. Ras effector loop mutants, which are defective for binding specific effector proteins, while remaining competent for binding and activating others, are particularly well suited to such investigations (9). In this study, we have exploited Ras effector loop mutants to ask whether multiple Ras-regulated pathways are involved in the induction of anchorage-independent growth. It has previously been shown that several cell cycle events, including activation of G_1 Cdk activities and expression of cyclin A, are dependent on cell adhesion in non-transformed fibroblasts (26-31), and that anchorage-independent growth most likely depends on the ability of Ras to supplant this requirement for adhesion (26,31,36). The effector loop mutants were therefore also used to assess whether multiple pathways might signal to the cell cycle machinery in cells cultured in suspension. We report here that although oncogenic Ras (12V) induced formation of colonies in soft agar by both NIH 3T3 and Rat 6 cells, each of three effector loop mutants (12V,35S; 12V,37G; and 12V,40C) had almost completely lost this ability. Pairwise combinations of these mutants, however, synergized to produce growth in soft agar by both cell types. The most likely explanation for these observations is that multiple Ras-mediated pathways are required for efficient induction of anchorage-independent growth. Synergy between specific effector loop mutants has also been observed previously in assays of focus formation, and induction of DNA synthesis following microinjection (9-11,13).

The ability of individual Ras effector loop mutants, and combinations of such mutants, to abrogate the adhesion-dependence of specific cell cycle events is consistent with the conclusion drawn above. In Rat 6 cells, expression of individual mutants produced only trace levels of pRB phosphorylation and cyclin E-dependent kinase activity in the absence of cell-substratum adhesion. Furthermore, none led to expression of cyclin A under non-adherent conditions. In contrast, coexpression of 12V,35S + 12V,37G or 12V,37G + 12V,40C led to both colony formation in soft agar and loss of the anchorage requirement for these cell cycle activities. This correlation also held for the combination of 12V,35S and 12V,40C which did not lead to either colony formation or adhesion-independent cell cycle activities in Rat 6 cells. These data suggest that an effector that binds to 12V,37G, but not the other two mutants, may be required for induction of anchorage-independent growth of this cell line. Two potential effectors that fit this description are RalGDS and Rin1 (12,43).

NIH 3T3 cells responded to the effector loop mutants in a manner that was generally similar to that observed with Rat 6 cells. The NIH 3T3 line was, however, distinct from Rat 6 in two important ways. First, in NIH 3T3 cells, all three mutants were partially able to drive pRB phosphorylation, cyclin E-dependent kinase activity and expression of cyclin A in the absence of cell-substratum adhesion. This is striking, and indicates that, at least in these cells, Ras can signal to the cell cycle machinery without binding to any two of the three effectors thus far implicated in transformation: Raf, PI(3)K, and RalGDS. It is not yet clear whether signalling by any of these pathways alone is sufficient to drive partial, anchorage-independent activation of the cell cycle machinery, because the effector mutants used in this study may bind to proteins that could also participate in these actions. For example, when assayed in the yeast two-hybrid system, 12V,37G bound to Rin1, and all three mutants bound to AF6 (11,43).

NIH 3T3 cells that expressed 12V,37G or 12V,40C produced only trace levels of cyclin A, providing a reasonable explanation for their inability to form macroscopic colonies in soft agar (26,36). The 12V,35S-expressing cells, however, displayed higher levels of hyperphosphorylated pRB and cyclin A in suspension culture than did cells that expressed the other two mutants, yet were equally impaired at forming colonies. Thus, there may be additional requirements for efficient induction of anchorage-independent growth. Whether these represent cell cycle events other than those measured in this study, or metabolic activities, such as anaerobic glycolysis (44), is not known. The synergy in soft agar colony formation seen with NIH 3T3 cells that expressed combinations of mutants was, however, reflected in enhanced adhesion-independent activation of the cell cycle machinery, suggesting that, if not actually sufficient, these cell cycle activities are very likely to be necessary for anchorage-independent growth.

A second difference between NIH 3T3 and Rat 6 cells was that, in the former cell line but not the latter, Ras effector loop mutants synergized with ectopic expression of various cyclins in the formation of colonies in soft agar. Cyclins D, E and A are each required for the G_1-to-S phase transition, and the levels of these proteins are rate-limiting for G_1 phase progression (40,45-49). Taken together, these data strongly suggest one important reason individual effector loop mutants are impaired at inducing anchorage-independent growth is that, in the absence of adhesion-mediated signals, they are insufficient to activate fully the cell cycle machinery that controls G_1 phase progression and the G_1-to-S phase transition. Overall, cyclin A was much more effective at synergizing with the effector loop mutants than were cyclins D1 or E. This is consistent with our previous conclusion that the ability of Ras to drive expression of cyclin A in the absence of cell adhesion is likely to be critical to the induction of anchorage-independent growth by this oncogene (26,36). The failure of ectopic cyclin expression to complement individual effector loop mutants in Rat 6 cells may be due to the much weaker ability of these mutants to supplant the adhesion requirements of all the cell cycle activities investigated, relative to NIH 3T3 cells.

In addition to synergizing with Ras effector loop mutants, all three cyclins enhanced the efficiency of the 12V-expressing NIH 3T3 cells to form colonies in soft agar. This suggests that the level of each cyclin is rate-limiting for anchorage-independent growth, even in cells that express abundant oncogenic Ras protein. A peculiar result that arose from the ectopic cyclin expression studies was that a ~2-fold enhancement of cyclin D1 levels led to a greater than 5-fold increase in colony-forming efficiency. Cell-substratum adhesion is required for maximal production of cyclin D1 in non-transformed NIH 3T3 cells, via both transcriptional and translational mechanisms (28). 12V-transformed NIH 3T3 cells displayed a much higher amount of cyclin D1 than their non-transformed counterparts, even when the cells were deprived of adhesion. It should be pointed out, however, that colony forming assays require cells to proliferate for two weeks. Because it is not feasible to recover and analyze cultures grown in semi-sold medium for prolonged periods of time, we and others have typically analyzed the effects of adhesion on expression of cell cycle proteins and activities just 24-72 hours after suspension (26-31). It is possible that production of proteins such as cyclins D1 and A remain partially adhesion-dependent in Ras-transformed cells, and that the time course with which their expression decreases following suspension of these cells is such that the effect is missed at the relatively early time points that are usually analyzed. By this reasoning, it is possible that the amount of cyclin D1 produced from the endogenous gene decreases over a two week assay period to a level that would raise the contribution of the retrovirally-produced cyclin D1 to a proportion closer to the observed effects on colony formation. A similar phenomenon may underlie the very potent effects of what appear to be relatively low levels of ectopically produced cyclin A. We are currently attempting to develop protocols that will allow this problem to be addresed experimentally. Alternatively, because the 12V-transformed cells are a polyclonal population, it is possible that the disproportionate response is due to heterogeneity in sensitivity to the enhancing effects of ectopic cyclin expression, although this seems less likely.

The conclusions drawn in this report, based on analysis of effector loop mutants in two independent cell lines, are consistent with those derived from most other recent studies; i.e., multiple pathways contribute to, and may be required for, full transformation by Ras (9-15). There are, however, some exceptions worth mentioning. Khosravi-Far et al. reported that stable expression of the 12V, 35S; 12V,37G; and 12V,40C mutants each led to growth in soft agar and tumorigenicity (11). These results were obtained with a strain of NIH 3T3 cells (UNC) that is particularly sensitive to transformation by activated Rho family members, but not, apparently, with a second strain (CSHL) (9,11). In another report, Stang et al. investigated an exhaustive panel of effector loop mutants and concluded that the Raf pathway alone may be sufficient for transformation of Rat 2 cells (50). This may be due to a particular sensitivity of this cell line to this pathway or, possibly, to the fact that transformation was scored only by examination of the morphology of cells in drug-resistant colonies and not by more stringent assays such as growth in soft agar. However, in the limited number of mutants analyzed in the present study, Ras-mediated morphological alterations and anchorage-independent growth were not dissociated, as they have been able in certain somatic cell mutants (7).

The failure of the 12V,40C mutant to induce morphological transformation is also worth noting. Microinjection of this construct into several different cell lines caused rearrangement of cortical actin and membrane ruffling to a degree similar to that observed with 12V alone (10,13). This suggests that the requirements for stable morphological transformation and for the cytoskeletal alterations measured in these short term assays may not be identical. Alternatively, the main pathways involved may be similar, but additional layers of regulation may be imposed on cells that are challenged with stable, as opposed to transient, expression of Ras oncoprotein.

In summary, multiple Ras signalling pathways appear to be required to induce anchorage-independent growth, a hallmark of the transformed phenotype. These pathways collaborate to supplant the adhesion requirements of specific cell cycle events which presumably drive cell proliferation in the absence of cell-substratum adhesion. It is hoped that the cell lines developed in this study will facilitate identification of the Ras-mediated pathways involved in anchorage-independent growth and the biochemical mechanisms by which they connect to the cell cycle machinery.

Chapter 4

Transformation-Restoring Factor: A Low Molecular Weight, Secreted Factor Required For Anchorage-Independent Growth of Oncogene-Resistant Mutant Cell Lines

Abstract

We have previously described two independent mutant rat fibroblast cell lines that fail to form colonies in soft agar when infected with a v-H-*ras* -expressing retrovirus, yet still undergo transformation-related morphological alterations in response to this oncogene. We report here that conditioned medium (CM) from non-transformed rat fibroblasts contains an activity that specifically corrects this defect in the mutant cell lines, rendering them capable of anchorage-independent growth in response to *ras*. The major activity in CM, designated transformation-restoring factor (TRF), is ~1300 molecular weight, lipid insoluble, and heat, protease, acid and base stable. Latent activity, distinct from TRF, is also present in CM; several lines of evidence indicate that transforming growth factor (TGF) β is responsible for this activity. TRF, however, cannot substitute for TGFβ in the phenotypic transformation of NRK cells. TRF activity is decreased in CM of control cells transformed by *ras*, and this response to *ras* is retained by the mutant cell lines. We propose that whereas wild-type cells transformed by *ras* may constitutively activate a TRF-regulated pathway, thus becoming independent of TRF for growth in soft agar, these mutants have acquired dependence on an exogenous supply of TRF for this aspect of the transformed phenotype. Cellular activities regulated, directly or indirectly, by TRF may be effectors of the anchorage-independent growth property that is a hallmark of transformed rodent fibroblasts.

Introduction

Most proto-oncogenes encode proteins that are components of mitogenic signal transduction pathways that control cellular growth and differentiation (1), and somatic mutation of such genes, as well as of tumor suppressor genes, plays a critical and rate-limiting role in the development of neoplastic diseases (2). Tremendous progress has been made in understanding how mitogenic signals are transmitted from the exterior of the cell, through the cytoplasm and into the nucleus, where resultant changes in gene expression govern a cell's decision to divide. Many mitogenic growth factors bind to receptor tyrosine kinases; upon ligand-induced dimerization and autophosphorylation, such receptors recruit and (usually) phosphorylate SH2 domain - containing signalling molecules. Pathways activated in such fashion include those controlled by the SH2 domain proteins phospholipase Cγ, phosphatidylinositol-3-OH kinase, *src* family kinases, and STAT family of transcription factors (1,3,4). Most prominantly, the SH2 domain adaptor protein GRB2 controls the so-called "Ras - mitogen-activated protein kinase (MAPK)" pathway. GRB2 couples to Ras via the guanine nucleotide exchange factor SOS, and SOS-catalyzed exchange of GTP for GDP on Ras p21 initiates activation of a linear cascade of protein-serine/threonine kinases, including c-Raf-1, MAPK kinase, MAPK and p90^{rsk} (5-9). The latter two kinases are capable of translocation into the nucleus where they may phosphorylate transcription factors such as p62^{TCF}/elk-1, c-Jun, c-Myc and NF-IL6 (10-14).

Mutations in these pathways can cause transformation of cultured cells and contribute to the genesis of human cancers. Despite detailed knowledge of how mitogenic signals are transduced, however, little is known about the mechanisms by which such mutations lead to maintenance of the transformed phenotype. For example, activating mutations in receptor tyrosine kinases, *ras* and other proto-oncogenes not only decrease cells' requirements for exogenous growth factors but also lead to permanently altered cell morphology and anchorage-independent growth; the molecular basis of these phenotypes is largely unknown. We have chosen a molecular genetic approach, i.e., the analysis of revertants of oncogene-transformed cells, to gain insight into how aberrations in such pathways result in neoplastic transformation. The study of revertants, in this case somatic cell mutants, offers a powerful means to unravelling complex biochemical pathways. Specifically, mutants can often reveal important activities that are difficult to detect in wild-type genetic backgrounds and can be instrumental in dissecting the multiple branches of complex signal transduction networks.

My advisor, Dr. Krauss, isolated two oncogene-resistant mutant cell lines, designated as ER-1-2 and R-1a (15). These two cell lines, like a matched control cell line (PKC3-F4), overexpress PKC. In contrast to PKC3-F4 cells, these two cell lines fail to form colonies in soft agar in response to oncogenes such as *ras*, *raf*, and *src*. Moreover, these two mutant cell lines display morphological transformation in monolayer cultures in response to *ras*. Thus, signals generated by *ras* for morphological transformation could be dissociated from those for anchorage-independent growth. Additionally, both mutant cell lines had defects in induction of the metallothionein (MT) I and II genes in response to $ZnSO_4$ and other compounds, suggesting alterations in gene expression may be linked to the defect in these mutant cell lines.

We describe here a low molecular weight factor present in conditioned medium from wild-type Rat 6 cells that specifically corrects the defect in the R-1a and ER-1-2 mutant cell lines, restoring the ability of these cell lines to proliferate in soft agar in response to the v-H-*ras* oncogene.

Materials and Methods

Preparation of Conditioned Medium (CM)-Cells grown in DMEM plus 10% calf serum were grown on 150 mm plates to near confluence, washed twice with PBS and refed with one-half volume of fresh medium containing either 5% or 0% calf serum. This medium was harvested 48 hr later, passed through a 0.45μ filter and either used immediately or frozen at -70° C. When used in the soft agar assay, this CM made up 50% of the final volume (fresh medium, agar and serum were added for a final concentration of 1x DMEM, 0.3% agar and 5% or 10% calf serum, depending on whether the CM contained 0% or 5% serum, respectively). This medium was considered 1x CM. Except where noted, ER-1-2 cells infected with a v-H-*ras* virus were used as indicator cells for testing the activity of CM. When different cell lines were compared for their production of TRF activity in CM (Figure 4A), the CM volume was normalized to cell number per plate. A final concentration of 0.5x CM was used for the experiment shown in Figure 4A; this was determined to be on the linear portion of the CM dose response curve for the cell lines shown.

Dialysis, Fractionation and Treatments of CM-Dialysis of CM was performed with ~3500 MW cutoff dialysis tubing (Spectrum), and was against fresh medium for 24 hr with one change of dialysis medium. To recover the low MW fraction for testing in the soft agar assay, Centricon-3 centrifugal microconcentrators (Amicon) were employed according to the manufacturer's instructions. The filtrates and retentates were then tested in the soft agar assay. Size exclusion chromatography was performed on hand-packed Bio-Gel P2 columns (Bio-Rad; diameter, 2 cm; height, 25 cm) that were packed and equilibrated with 50 mM ammonium bicarbonate and run at a flow rate of 8 ml/hour. CM harvested from C1-D3 cells was lyophilized and redissolved in 50 mM ammonium bicarbonate at 1/30 its original volume. This concentrated CM was applied to the column and eluted with 50 mM ammonium bicarbonate. Fractions (0.4 ml) were collected, monitored for absorbance at 280 nm and pooled. The pooled fractions were lyophilized, redissolved in DMEM, passed through a 0.2μ filter and tested in the soft agar assay.

Protease sensitivity of TRF was analyzed by treatment of CM with either proteinase K (50 mg/ml; Boehringer Mannheim) or pronase (0.5 mg/ml; Sigma) for either two or 18 hours at 37° C, followed by application to a Centricon-3 microconcentrator. Filtrates were recovered and tested in the soft agar assay. To test the effectiveness of the proteolysis, an aliquot of protease-treated CM was concentrated and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels, and the gels were stained with Coomassie Blue. Protease-treated CM lost all visibly staining material under these conditions. To test acid and base stability, microconcentrator filtrate and retentate fractions were brought to pH 2.5 or 12 with 10N HCl or 10N NaOH, respectively. After incubation for 2 hours, samples were reneutralized with 10N NaOH or 10N HCl, and tested in the soft agar assay.

Samples that were treated with acid and immediately reneutralized were used as controls. This increased salt concentration resulted in a ~20 percent decrease in colony forming efficiency of ER-1-2/*ras* cells; the data presented in Table 4 are normalized to reflect this. To test solubility in organic solvents, samples of CM were extracted twice with equal volumes of chloroform/methanol/acetic acid (100/200/4) or with ethyl acetate that had been saturated with fresh serum free medium. For some experiments with ethyl acetate, the CM was acidified to pH 3.0 with HCl prior to extraction (this pH is sufficient to protonate the carboxylic acid groups of fatty acids). The organic layers were pooled, evaporated under reduced pressure in the dark, and redissolved in a small volume of DMSO. The reconstituted material was then tested in the soft agar assay. The extracted aqueous phase was also lyophilized, reconstituted in fresh medium and tested for activity.

Results

Correction of the Revertant Phenotype with Conditioned Medium-Because the revertant cell lines display a loss of expression of MT, and presumably other, genes, we hypothesized that loss of expression of a secreted growth factor in these cell lines might prevent their acquisition of the anchorage-independent phenotype. We tested this hypothesis by asking if control cells secrete an activity capable of restoring the ability of the revertant cells to form colonies in soft agar in response to the v-H-*ras* oncogene. Table 1 describes all the cell lines used in this study with regard to the exogenous genes they harbor and their ability to grow in soft agar with or without expression of the v-H-*ras* oncogene. Initial experiments employed a "feeder" layer of C1-D3 cells grown underneath 0.5% bottom agar as a source of such factors, with control and revertant cell lines tested for their ability to grow in a layer of 0.3% top agar in response to infection with high titer (~5 x 10^6 ffu/ml) recombinant v-H-*ras* virus. As previously described (15) and shown in Figure 1, the control C1-D3 and PKC3-F4 cells formed colonies in control soft agar cultures when infected with the v-H-*ras* virus, while the R-1a and ER-1-2 revertant cell lines did not. It should be noted that Weinstein and coworkers have reported that R6-PKC3 and PKC3-F4 cells form small colonies in agar medium containing 10% serum, and that these cells form about 5-fold more colonies in soft agar in response to v-H-*ras* than do C1-D3 cells (15-17). The experiments presented here were performed in agar medium containing 5%

TABLE 1: Cell Lines Used in this Study[a]

Cell Line	Exogenous Genes	Growth in Soft Agar[b]	Growth in Soft Agar in Response to *ras*[b]
C1-D3	*neo, hph*	—	++
PKC3-F4	*neo, hph, PKCβ1*	+/—	++++
R-1a	*neo, hph, PKCβ1,* MT-T24 c-H-*ras* (silent)	—	—
ER-1-2	*neo, hph, PKCβ1,* MT-T24 c-H-*ras* (silent)	—	—
C1/T24	*neo,* T24 c-H-*ras*	++++	N.A.[c]

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[a] The references for these cell lines are Krauss *et al.* (1992) and Hsiao *et al.* (1989).

[b] —, no growth; +/—, formation of microscopic colonies in medium containing 10% serum and no growth in medium containing 5% serum; ++, formation of macroscopic colonies at low frequency in 10% serum; ++++, formation of macroscopic colonies at high frequency in 10% serum. See references in footnote a and text for details.

[c] N.A., Not applicable.

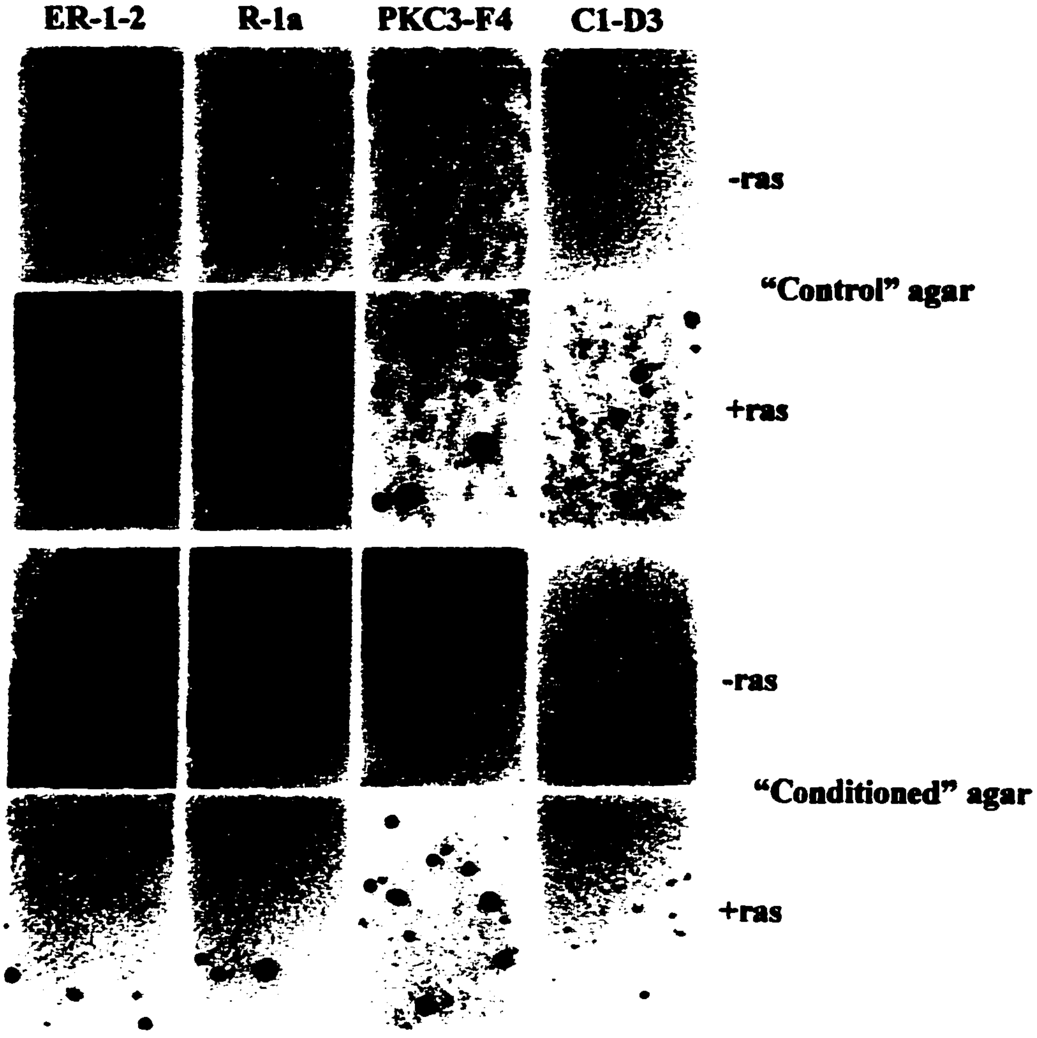

Figure 1: Soft Agar Colony Formation by Control and Revertant Cell Lines in Response to v-H-*ras*, in the Presence or Absence of a Feeder Layer of C1-D3 Cells. "Control" agar cultures lacked a feeder layer; "Conditioned" agar cultures had a feeder layer. Magnification, 40x. See text for details.

serum; under these conditions PKC3-F4 cells fail to grow and their enhanced sensitivity to v-H-*ras* is evident only as an increase in colony size (Figure 1).] When a feeder layer of non-transformed C1-D3 cells was placed underneath the bottom agar, the colony forming efficiency of control v-H-*ras* - expressing C1-D3 and PKC3-F4 cells was not substantially altered (~15-20 percent cloning efficiency). In contrast, the presence of a feeder layer dramatically restored the ability of the v-H-*ras* - expressing R-1a and ER-1-2 mutant cell lines to proliferate in soft agar. Furthermore, the feeder layer did not support growth of C1-D3, PKC3-F4 or revertant lines that did not express v-H-*ras*. Thus, C1-D3 cells secrete a factor that specifically corrected the defect in the two revertant cell lines. Under these conditions, the colony forming efficiencies of R-1a/*ras* and ER-1-2/*ras* cells were ~20 and ~50 percent that of PKC3-F4/*ras* cells, respectively. Similar results were obtained with four independent populations of v-H-*ras* infectants and when a v-*raf* - expressing virus was used in place of the v-H-*ras* virus (data not shown). Furthermore, addition of cell-free conditioned medium (CM) obtained from C1-D3 cells to the soft agar also restored the ability of the revertants to proliferate in the semi-solid medium in response to the v-H-*ras* oncogene (see below). When four independent colonies of ER-1-2/*ras* cells were isolated from CM-supplemented soft agar cultures and expanded into cell lines, each, like the parent ER-1-2/*ras* population, was morphologically transformed but could not grow in soft agar unless the semi-solid medium was again supplemented with CM (data not shown). Moreover, C1-D3 cell CM had no effect on expression of the virus-encoded v-H-*ras* p21 in the ER-1-2/*ras* clones, as demonstrated by Western blotting analyses (Figure 2). To characterize further the activity(ies) secreted by C1-D3 cells, the remainder of the studies presented here utilized cell free CM; because the ER-1-2/*ras* cells gave a more robust response in the feeder layer assay than did the R-1a/*ras* cells, the former cells were chosen for these analyses.

For initial characterization of C1-D3 cell CM, the effect of the presence or absence of serum during conditioning of the medium by these cells over a period of 48 hours was tested, as was the effect of heat treatment on the activity of the CM. Table 2 demonstrates that when the variously derived and treated CM were added to the soft agar growth assay of ER-1-2/*ras* cells, these cells formed colonies the same way they did in response to a feeder layer. CM derived from C1-D3 cells grown in 5% serum had higher activity than serum-free CM from these cells, suggesting that cells grown in the presence of serum synthesized greater levels of the secreted activity(ies) than did cells starved of serum. Interestingly, heat treatment (100° C, 5 min) had little effect on the activity of the 5% serum-containing CM, but led to an increase, usually 2.5 - 3.0 - fold, in the activity of the serum-free CM. Furthermore, the soft agar colonies of ER-1-2/*ras* cells that were formed with heat-treated, serum-free CM were larger than those formed with unheated, serum-free CM. This indicates that both free and latent (i.e., heat-activatable) activities were present in C1-D3 cell CM. It is significant that over the course of numerous experiments the frequency of soft agar colony

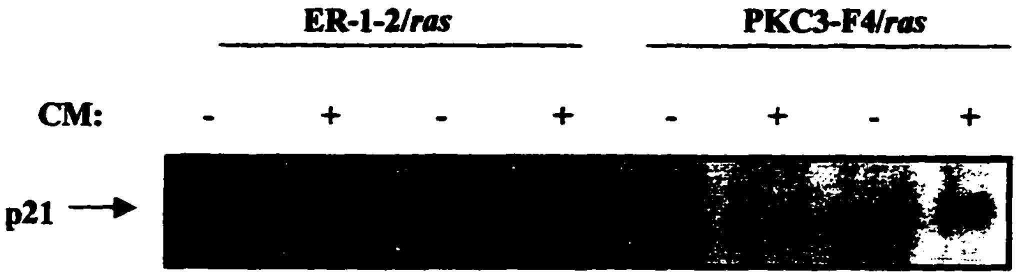

Figure 2: Western Blot Analysis of v-H-*ras* p21 Protein in Clones of ER-1-2/*ras* and PKC3-F4/*ras* Cells Treated With or Without C1-D3 CM. Forty micrograms of total cellular protein was fractionated on a 0.1% SDS-12.5% polyacrylamide gel, transferred to nitrocellulose and probed with anti-ras p21 antibody. See Methods for details. CM, C1-D3 cell conditioned medium; the numbers 1 and 2 represent independent clones of ER-1-2/*ras* and PKC3-F4/*ras* cells.

TABLE 2: Growth in Soft Agar of ER-1-2/ras Cells Cultured with Different Types of CM

Source of CM	% Serum in CM	Heat Treatment[a]	# Colonies/Well[b]
None	—	-	0
C1-D3 cells	5%	-	1551
C1-D3 cells	5%	+	1869
C1-D3 cells	0%	-	489
C1-D3 cells	0%	+	1860

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[a] CM was heated at 100° C for 5 minutes.

[b] A total of 10,000 ER-1-2/*ras* cells were seeded into 0.3% soft agar that contained no additions or addition of CM as noted in the first three columns. Macroscopic colonies (>0.2 mm diameter) were scored after two weeks of growth.

formation by ER-1-2/*ras* cells grown with either serum-containing CM or heat-activated, serum-free CM was always 50 - 100% of the frequency observed with the control PKC3-F4/*ras* cells (i.e., 15-20% cloning efficiency), indicating nearly complete correction of the mutant phenotype in these cells.

To ascertain whether the activities found in C1-D3 cell CM were of large or small molecular weight, serum-containing and serum-free CM were dialyzed against a large volume of fresh, non-conditioned medium with ~3500 molecular weight (MW) cut-off dialysis tubing. In two separate experiments, 85-90% of the activity from 5% serum-containing CM or serum-free CM was lost upon dialysis, suggesting that the major activity in CM was a low molecular weight compound. To confirm and extend these observations in a fashion that allowed recovery of the low molecular weight fraction, centrifugal microconcentrators equiped with a 3000 MW cut-off filter (Centricon-3; Amicon) were employed. Only the serum-free CM could be analysed with these filters because serum rapidly clogged them. Table 3 shows that the majority of C1-D3 CM activity was recovered in the <3000 MW filtrate of such microconcentrator preparations; over the course of ten such preparations the filtrate fraction yielded between 250 - 780 colonies per 10^4 ER-1-2/*ras* cells seeded into soft agar, similar to unfractionated, serum-free C1-D3 CM. Very little activity was present in the >3000 MW retentate. Heat treatment of the filtrate fraction demonstrated that this activity was heat stable, and that no latent activity was present in this fraction. In contrast, the retentate fraction

TABLE 3: Fractionation of C1-D3 Cell CM by Centricon-3 Microconcentrators

Condition	Fraction[a]	Heat Treatment After Fractionation[b]	Heat Treatment Before Fractionation	Relative Colony Formation[c]
1	<3000 MW	-	-	100
2	>3000 MW	-	-	6
3	<3000 MW	+	-	97
4	>3000 MW	+	-	49
5	<3000 MW	-	+	121
6	>3000 MW	-	+	57
7	<3000 MW plus	-	-	89
	>3000 MW	-	-	
8	<3000 MW plus	-	-	222
	>3000 MW	+	-	

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[a] Fraction refers to the retentate (>3000 MW) or filtrate (<3000 MW) fractions derived from centrifugation of C1-D3 cell CM through a Centricon-3 (3000 MW cut-off filter) microconcentrator.

[b] CM fractions were heated at 100° C for 5 minutes.

[c] A total of 10,000 ER-1-2/*ras* were seeded into 0.3% soft agar in the presence of the indicated C1-D3 cell CM fractions; macroscopic colonies (>0.2 mm diameter) were scored after two weeks of growth. Over the course of several experiments, the value of the <3000 MW fraction (set here at a relative value of 100) ranged from 250 - 780 colonies/well. Data from a representative experiment are shown.

contained latent activity that was generally 35-50% that of the filtrate fraction. When CM was heated prior to fractionation, a similar amount of activity was present in the retentate, while no additional activity was found in the filtrate, indicating that the activated latent factor was a higher MW factor, distinct from the free activity found in the filtrate fraction (Table 3). The addition of untreated retentate fraction to the filtrate did not stimulate the filtrate's activity, but addition of heated (i.e., activated) retentate to the filtrate had a mild synergistic effect (Table 3). Similar to what we observed with unheated and heat-activated serum-free CM, the filtrate and heated retentate mixture usually yielded 2.5 - 3.0 - fold the number of colonies obtained with filtrate alone; furthermore, the soft agar colonies of ER-1-2/*ras* cells obtained from these mixtures were larger than those obtained from either fraction alone. Taking these data together, we conclude that the major activity in C1-D3 cell CM was a heat stable, low MW factor(s), and that additional, weaker activity resided in a larger MW, non-dialyzable factor that was present largely or exclusively in latent form. Combining these two factors resulted in activity that was slightly greater than the additive effects of the factors in isolation.

Characterization of the Low MW Activity in C1-D3 CM-Because the major activity present in unheated CM was found in the low molecular weight fraction, we sought to gain information about the properties of the active factor(s) in this fraction. Treatment of serum-free C1-D3 cell CM with either of two non-specific proteases, proteinase K and pronase, followed by isolation of the <3000 MW microconcentrator filtrate fraction, revealed that the activity was completely resistant to the action of these enzymes (Table 4). Thus, the factor is either not a polypeptide or is very resistant to proteolysis. Likewise, the activity was not destroyed when the <3000 MW fraction was incubated at pH 2.5 or pH 12, followed by reneutralization prior to testing in the soft agar assay. Finally, the activity remained in the aqueous phase when serum-free C1-D3 cell CM was extracted with ethyl acetate or chloroform/methanol/acetic acid, indicating that the active factor is probably not a lipid.

To gain a more accurate estimate of the MW of the factor, size exclusion chromatography was performed on serum-free C1-D3 CM with Bio-Gel P2 columns and appropriate molecular weight markers (fractionation range 100-1,800 daltons). Column fractions were collected and tested for the ability to stimulate anchorage-independent growth of ER-1-2/*ras* cells. Figure 3 shows a representative chromatogram. The majority of the activity reproducibly eluted as a single peak just after the vitamin B12 (cyanocobalamin) marker (MW 1,350); like the crude <3000 MW microconcentrator filtrate fraction, this activity was stable to heat treatment (data not shown). Although it cannot be ruled out that this peak contains more than one compound, for the purposes of nomenclature in this report we have designated this material transformation-restoring factor (TRF). Less than 15% of the total activity eluted in the void volume with protein markers that were too large to be separated with a P2 gel. Heat treatment of this fraction

TABLE 4: Effect of Various Treatments on Activity of C1-D3 Cell CM Filtrate Fraction

Condition[a]	Colonies/Well[b]
Non-conditioned medium	0
Conditioned medium, <3000 MW fraction	780
plus proteinase K pretreatment	885
plus pronase pretreatment	1052
ethyl acetate extract	0
ethyl acetate extract of acidified medium	0
chloroform/methanol/acetic acid extract	0
incubation at pH 2.5	713
incubation at pH 12	564

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[a] C1-D3 CM or C1-D3 CM microconcentrator filtrate fraction were treated as described in Methods. Each condition was tested at least twice, and in the case of the proteases, three or four times. Representative data from single experiments are shown.

[b] A total of 10,000 ER-1-2/*ras* were seeded into 0.3% soft agar in the presence of the indicated, treated C1-D3 cell CM filtrate fractions. Macroscopic colonies (>0.2 mm diameter) were scored after two weeks of growth.

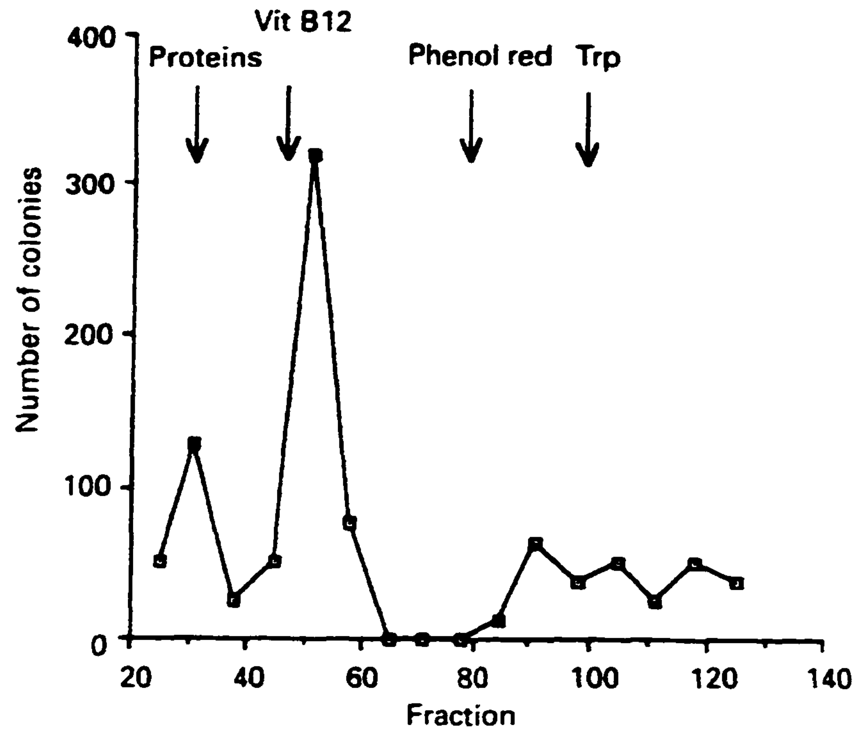

Figure 3: Size Exclusion Chromatography of C1-D3 Cell CM. CM was fractionated on a Bio-Gel P2 column as described in the text and fractions were tested for their ability to stimulate soft agar colony formation by ER-1-2/*ras* cells. The elution pattern of markers is denoted by the arrows [proteins (a mixture of thyroglobulin, 670,000 MW; bovine gamma globulin, 158,000 MW; chicken ovalbumin, 44,000 MW; and equine myoglobin, 17,000 MW); Vitamin B12, 1,350 MW; phenol red, 354 MW; tryptophan (trp), 204 MW].

did, however, reveal the latent activity, further confirming the higher molecular weight nature of this activity. Finally, a smaller amount of activity eluted as a series of small peaks in the very low MW range (~100-300 daltons). This activity was destroyed by heat; its role in the total activity present in the microconcentrator filtrate fraction is unclear since the activity present in the filtrate was almost completely heat-resistant (Table 3). This very low MW material was not studied further.

Regulated Production of TRF Activity in Various Rat 6 Cell Derivatives-Because the two revertant cell lines required an exogenous source of TRF to form colonies in soft agar in response to the v-H-*ras* and v-*raf* oncogenes, the level of TRF activity in CM obtained from R-1a and ER-1-2 cells was measured and compared to several control cell lines. Serum-free CM was harvested from the control C1-D3, PKC3-F4 and C1/T24 cells, as well as from revertant R-1a and ER-1-2 cells that either did or did not express the v-H-*ras* oncogene and was then tested for the ability to stimulate soft agar colony formation of ER-1-2/*ras* cells (Figure 4A; see Table 1 for a description of the cell lines). To our surprise, CM from non-transformed C1-D3 cells, partially transformed PKC3-F4 cells and highly transformed C1/T24 cells revealed an inverse correlation between the ability of these cells' CM to stimulate anchorage-independent growth of ER-1-2/*ras* cells and their degree of transformation; PKC3-F4 cells had about one-third the activity of C1-D3 cells, while the *ras*-transformed C1/T24 cells had negligible activity. Both revertant cell lines had a level of activity similar to that of PKC3-F4 cells, and this activity was completely or largely lost when these two cell lines expressed v-H-*ras*. Additionally, each of the cell lines had a similar amount of heat-activatable latent activity (data not shown). Thus, R-1a and ER-1-2 cells produced similar amounts of TRF activity as the most appropriate control cell line, PKC3-F4 cells (recall that the revertants overproduce PKCβ1), and responded "normally" to expression of a *ras* oncogene (i.e., by reducing the amount of TRF activity in the CM).

One possible explanation for these results is that PKCβ1- and *ras*-expressing cells take up and internalize TRF more efficiently than non-transformed cells, thus depleting their medium of this factor. To test this possiblity, we added C1-D3 cell CM to fresh plates of C1/T24 or C1-D3 cells for 48 hours and measured the activity present in these "reconditioned" media (Figure 4B). Filtrate fractions of CM "re-conditioned" by C1/T24 (which do not produce TRF activity) and C1-D3 cells had a similar amount, or roughly double the amount, of the activity in filtrates of the original C1-D3 cell CM, respectively. Thus, TRF was not significantly cleared from culture medium, and the transformed cells actually secreted less TRF activity than did the non-transformed cells. These data also suggest that TRF may exert its effects from the exterior of the cell, perhaps through a plasma membrane receptor. We conclude, therefore, that in the revertant cell lines, signals generated by an activated *ras* oncogene or by overexpression of PKCβ1 that decrease secreted TRF activity were intact. These data are also

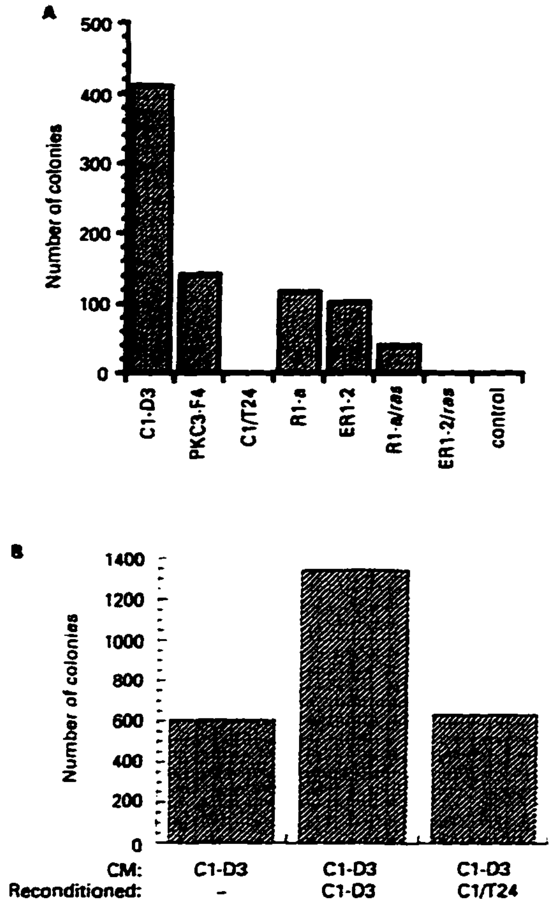

Figure 4: **(A) Formation of Colonies in Soft Agar by ER-1-2/*ras* Cells Induced by CM from Various Cell Sources. A total of 10,000 ER-1-2/*ras* cells were seeded into 0.3% soft agar containing either no CM (control) or CM from the indicated cell sources; macroscopic colonies were scored after two weeks of growth. See text for details. (B) "Reconditioning" of C1-D3 CM by fresh C1-D3 and C1/T24 cells. C1-D3 cell CM was added to fresh plates of (i.e., reconditioned by) C1-D3 cells, C1/T24 cells or plates containing no cells (-). See text for details.**

consistent with the observation that the revertants required both v-H-*ras* expression and exogenous TRF to proliferate in soft agar (Figure 1 and Table 2); R-1a and ER-1-2 cells secreted sufficient TRF activity to proliferate in soft agar *prior* to infection with the v-H-*ras* virus (Figure 4A) but, like wild-type cells, they sharply reduced the amount of TRF activity they produced after v-H-*ras* expression, thus necessitating an exogenous supply. Apparently, *ras* oncogene expression in control cells both down-regulated TRF production and relieved the requirement for this factor. The regulated production of TRF activity may offer potential insights into the nature of the mutant phenotype of the R-1a and ER-1-2 cell lines (see Discussion).

Because v-H-*ras* - expressing cell lines shut off production of TRF activity, but TRF was necessary for the revertants to respond to this oncogene, we asked whether addition of C1-D3 cell CM filtrate fraction had any effect on the ability of anchorage-independent cells to form colonies in soft agar. C1-D3/*ras* and PKC3-F4/*ras* cell populations reproducibly formed about twice the number of colonies in the presence of the filtrate fraction than in its absence (data not shown). When four individual clones of PKC3-F4/*ras* cells were isolated and tested in this fashion, the colony-forming efficiency in soft agar of each clone was mildly stimulated (stimulation range: 127 - 197 percent among different clones). Thus, even though expression of v-H-*ras* repressed production or activity of TRF, v-H-*ras* - transformed cells still responded positively, if quite modestly, to its effects.

The Latent Activity in CM is Transforming Growth Factor (TGF) β-During the course of studies on the pH stability of TRF (see above), we noticed that the latent activity present in the >3000 MW microconcentrator retentate fraction could be activated by acid and alkali, as well as by heat treatment (data not shown). Latent, secreted, mitogenic or transforming activity towards fibroblasts that can be activated by pH extremes or heat is a signature property of members of the TGFβ family of growth factors (18). We chose, therefore, to test whether recombinant TGFβ1 could stimulate anchorage-independent growth of ER-1-2/*ras* cells, in the presence and absence of the C1-D3 cell CM microconcentrator filtrate fraction. Figure 5 shows that TGFβ1 stimulated soft agar colony formation of ER-1-2/*ras* cells in a dose-dependent fashion, and that, like the heat-activated CM microconcentrator retentate fraction, this activity was stimulated by the addition of the CM filtrate fraction. The activity of a maximal dose of recombinant TGFβ1 [1.2 ng/ml; higher concentrations did not further increase colony formation (not shown)] plus CM filtrate was nearly identical to that of heat-treated CM of the same preparation from which the filtrate used in this experiment was derived (Figure 5). Recombinant TGFβ1 did not stimulate anchorage-independent growth of C1-D3, PKC3-F4, or ER-1-2 cells that had not been infected with the v-H-*ras* virus, nor did it substantially augment soft agar colony formation in C1-D3/*ras* or

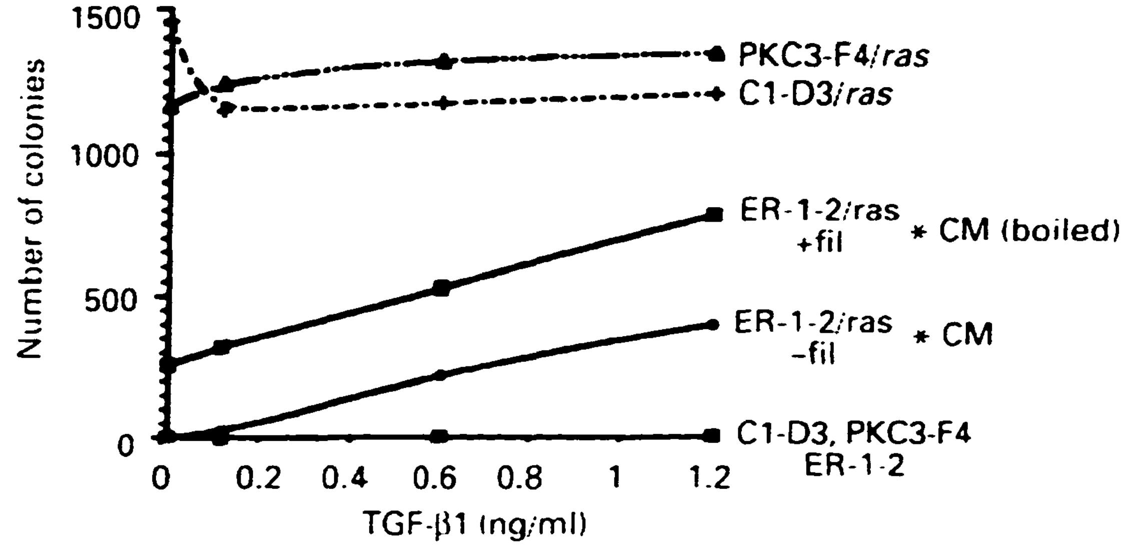

Figure 5: The Effect of TGFβ1 on Soft Agar Colony Formation by Various Cell Lines. A total of 10,000 cells of the indicated type were seeded into 0.3% soft agar containing the indicated concentration of recombinant TGFβ. ER-1-2/*ras* cells were incubated with (+fil) or without (-fil) C1-D3 cell CM filtrate fraction. Macroscopic colonies were scored after two weeks of growth. Also shown (indicated by the asterisks) are the number of colonies formed by unfractionated C1-D3 cell CM (CM) and heat-treated CM [CM (boiled)] that were of the same preparation as that from which the filtrate used in the ER-1-2/*ras* cultures was derived, and that were tested at the same time. Data shown are representative of at least two different experiments for all conditions. See text for further details.

PKC3-F4/*ras* cells (Figure 5).

Having shown that recombinant TGFβ1 had activity similar to the latent activity present in CM, it was of interest to know whether the latent activity in CM had TGFβ-like activity in a different system. A standard assay for TGFβ tests this factor's ability, in combination with epidermal growth factor (EGF), to stimulate anchorage-independent growth of NRK cells (19,20). It was also of interest to test whether TRF had TGFβ-like or EGF-like activity in this established system. The retentate, when heat-activated, substituted for TGFβ, but not EGF, in the NRK assay (21). These data, in combination with the heat, acid and alkali activation studies, very strongly suggest that the latent activity secreted by C1-D3 cells is one or more members of the TGFβ family. Interestingly, the CM filtrate fraction had neither TGFβ-like, nor EGF-like activity on NRK cells, even when treated with heat (21). TRF, therefore, has a novel activity with regard to induction of anchorage-independent growth, that is at least partially distinct from the activities of these growth factors. These data also demonstrate that the microconcentrator filtrate fraction was free of active or latent TGFβ.

Discussion

This paper describes studies on the R-1a and ER-1-2 revertant cell lines, which fail to form colonies in soft agar when infected with a v-H-*ras* - expressing retrovirus yet still undergo transformation-related morphological alterations in response to this oncogene (15). We have found that addition of CM derived from non-transformed C1-D3 fibroblasts to the semi-solid agar medium specifically corrected the defect in these mutant cell lines, restoring their ability to proliferate in an anchorage-independent manner in response to expression of v-H-*ras*. The major activity in CM, designated TRF, could be recovered as a factor of ~1300 MW that was not extractable into organic solvents and was heat, protease, acid and base stable. TRF did not induce anchorage-independent growth of C1-D3, PKC3-F4 or revertant cell lines that did not express an activated oncogene, and stimulated only a very modest increase in the ability of *ras*-transformed control cell lines to form colonies in soft agar. The mutant genetic background of these revertant cell lines was therefore necessary to reveal TRF activity. As such, this factor may be previously unidentified or, if known, its role in mediating the transformed phenotype may be unrecognized. These data suggest that, at least in response to *ras*, a pathway controlled directly or indirectly by TRF may be a necessary though not sufficient effector of the anchorage-independent phenotype that is a hallmark of transformed cells. The very small size of TRF was unexpected, but an extensive literature dating back to at least 1960 testifies to the importance of serum- and CM-derived dialyzable factors in cellular growth and transformation (22-27). Very few of these factors have been identified, however, and it is unclear whether any may be identical or related to TRF. Recent technological advances indicate that structural studies on limited quantities of biological materials are now feasible (28), and structural identification of TRF, as well as further studies on its mechanism of action, are currently under investigation.

Latent (i.e., heat-activatable) activity was also present in CM, and several lines of evidence indicate that one or more members of the TGFβ family were responsible for this activity. TRF did not, however, substitute for TGFβ (or EGF) in the phenotypic transformation of NRK cells. The putative pathway controlled by TRF must therefore be at least partially distinct from those controlled by these two factors. TGFβ was generally not as effective as TRF in stimulating ER-1-2/*ras* cells to grow in soft agar, but the activity of a combination of TRF and TGFβ was somewhat greater than would be predicted for an additive effect. It is possible that TGFβ signals via multiple pathways, one of which is held in common with TRF. By this reasoning, the sum total of TGFβ signaling would be necessary for its role in transforming NRK cells, while the putative common pathway would be sufficient to correct the defect in the revertant cell lines.

A critical aspect to this study was the finding that production of TRF activity was sharply reduced in *ras*-expressing cell lines relative to non-transformed C1-D3 cells, including both fully transformed cells and *ras*-expressing revertants. This observation suggests that synthesis (or activity) of TRF is regulated, and raises the question of why *ras*-transformed cells would halt production of a factor upon whose actions the oncogene apparently relies. The acquisition by the revertants of dependence on an exogenous supply of TRF must also be explained. The phenotype of R-1a and ER-1-2 cells, and data of other investigators, indicates that the signals generated by an activated *ras* oncogene that control morphological alteration can be dissociated from those that control mitogenesis or anchorage-independent growth (15,29-34). Furthermore, the signaling pathways that maintain the fully-transformed state are likely to be multi-branched. We hypothesize that one signaling pathway downstream of activated Ras p21 can constitutively activate a pathway regulated by TRF that is necessary for growth in soft agar, while a distinct pathway represses TRF production. The fact that the *ras*-expressing revertants responded to an exogenous supply of TRF suggests that the defect in these cell lines may reside in this putative pathway that bridges Ras-mediated signals with the TRF-regulated pathway, which itself remains intact. Thus, in the revertant cell lines Ras may no longer supply the TRF signal, but the provision of exogenous TRF restores these cells to anchorage-independence. Apparently, other *ras*-mediated events are also required for anchorage-independent growth, because TRF alone is not sufficient to induce this phenotype. The signals that control the *ras*-mediated decrease in production of TRF are unknown, but must be at least partially distinct from the predicted defective pathway, because these signals have been maintained in the revertants.

This hypothesis would explain both the requirement of the revertant cell lines for TRF and the observation that *ras*-transformed control cells proliferate in an anchorage-independent fashion without making substantial TRF activity themselves. Because the revertant cell lines have retained a wild-type morphological response to *ras*, as well as down-regulation of TRF activity, it is possible that the pathway(s) that control these morphological alterations may also regulate TRF production. While these hypotheses are attractive, the present data obviously do not rule out other mechanisms.

In summary, we have described a low molecular weight factor, TRF, that specifically corrects the defect in oncogene-resistant mutant cell lines. Structural identification of TRF, and analysis of its mechanism(s) of action, should provide insights into how oncogenes induce anchorage-independent cell proliferation.

Chapter 5

Extracellular ATP Induces Anchorage-Independent Expression of Cyclin A and Rescues the Transformed Phenotype of a Ras-Resistant Mutant Cell Line

Abstract

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 G_1 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 AMP-PNP are also effective, but adenosine is not. Adenine nucleotide-induced growth in soft agar is inhibited by reactive blue 2, an antagonist of some P_2 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*.

Introduction

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 G_1 cyclin-dependent kinases (Cdk's[1]) (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 G_1 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 G_1 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 G_1 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 G_1 Cdk's 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 G_1 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 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, Racl or RhoA[2]. Thus, the pathways controlled by these established mediators of Ras' 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 P_2 purinoceptors (15,16). Two different types of P_2 receptors have been described: P_{2X} receptors, which are structurally distinctive, ligand-gated ion channels; and P_{2Y} receptors, which are G protein-coupled receptors with seven transmembrane domains. At least seven different P_{2X} receptors and seven different P_{2Y} receptors have been molecularly cloned, and additional receptors have been characterized pharmacologically (16-18). Extracellular ATP, operating via P_2 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 (designated Transformation-Restoring Factor, TRF) that specifically rescues 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[2]. 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. Furthermore, ATP induced expression of cyclin A in non-adherent cultures of ER-1-2/*ras* cells. Contrary to our original hypothesis, however, TRF does not appear to be an adenine nucleotide and exerted its effects on anchorage-independent growth via a mechanism distinct from that used by ATP.

Material and Methods

Materials - ATP, ADP, AMP, GTP, UTP, adenylyl-imidodiphosphate (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), 12-O-tetradecanoylphorbol-13-acetate, P^1,P^4-di(adenosine-5′) tetraphosphate (AppppA), thapsigargin, reactive blue 2 and 8-Br-cAMP were all from Sigma. pyridoxal-phosphate-6- azophenyl-2′,4′-disulphonic acid (PPADS) and 2-Methylthio-ATP (2-Me-S-ATP) were from Research Biochemicals International.

Partial Purification of TRF - TRF was partially purified by fractionating serum-free conditioned medium derived from rat 6 fibroblasts by size exclusion chromatography on a Bio-Gel P2 column (Bio-Rad) as previously described (22). Active fractions were pooled, lyophilized, dissolved in water and sterile-filtered prior to use.

RNA Analyses - Northern blot analysis of total cellular RNA extracted with Trizol (Gibco/BRL) was performed as described (5).

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 1 and Figure 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 PKC-3-F4 or ER-1-2 cell lines. Furthermore, ATP did not enhance colony formation by the fully-transformed PKC3-F4/*ras* cells (Table 1 and Figure 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. We next tested the ability of ATP and other adenine nucleotides to rescue growth in soft agar of ER-1-2/*ras* cells over the dose range 0.01-100 μM (Figure 2A). ATP, ADP and AMP were all equipotent, with an EC_{50} of <10 μM, and colony formation was observed at doses as low as 1 μM. The non-hydrolyzable ATP analog, AMP-PNP, was as effective as ATP itself, indicating that hydrolysis of ATP was not necessary for its effects in this system (Figure 2A). In contrast,

TABLE I: Rescue of growth in soft agar of ER-1-2/*ras* cells by extracellular ATP

	Growth in Soft Agar (colonies/10^4 cells)[a]	
Cell Line	- ATP	+ ATP
PKC3-F4	0	0
PKC3-F4/*ras*	2442	2432
ER-1-2	0	0
ER-1-2/*ras*	0	499
ERRC1	0	474
ERRC6	0	691

[a] 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 (>0.2 mm diameter) were scored after two weeks of growth. Values represent averages of duplicate determinations that differed by less than five percent. The experiment was repeated at least three times for each cell line; representative data are shown.

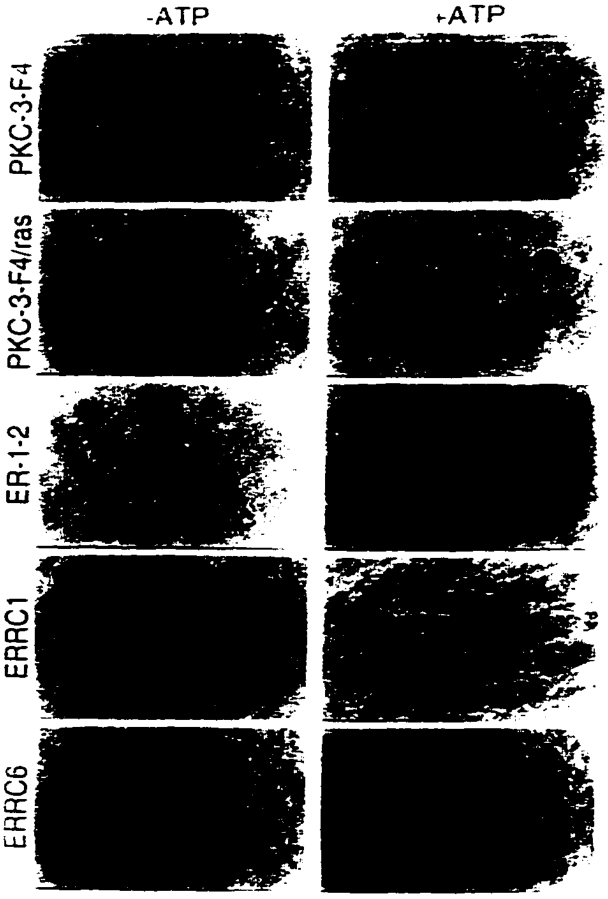

Figure 1: Soft agar colony formation by various cell lines in the presence or absence of ATP. Cells were cultured as described in Experimental Procedures and the legend to Table I. See Table I for quantitation of results. Magnification, 40x.

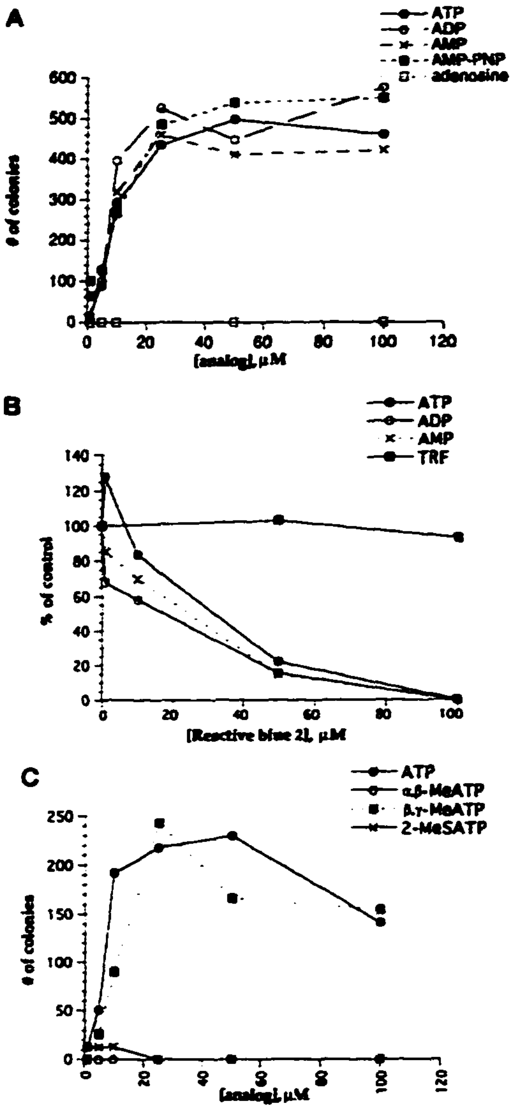

Figure 2: Pharmacological characterization of the effects of ATP on soft agar colony formation of ER-1-2/*ras* cells. (A) Dose-response curves for ATP, ADP, AMP, AMP-PNP and adenosine; (B) Dose-response curves for reactive blue 2-mediated inhibition of colony formation by ATP, ADP, AMP (each used at 50 μM) and partially purified TRF; (C) Dose-response curves for ATP, α,β-Me-ATP, β,γ-Me-ATP and 2-Me-S-ATP. Each data point represents the average of duplicate determinations that differed by less than five percent. For (A) and (C) the data points represent 1, 5, 10, 25, 50 and 100 μM concentrations for each analog; the 0.00, 0.01 and 0.1 μM concentrations for each analog yielded no colonies and the data points are not shown on the graph. The experiments shown in (A) and (B) were performed three times, and the experiment shown in (C) performed twice, with very similar results; representative data are shown.

adenosine was inactive at concentrations up to 100 μM (Figure 2A).

To begin to examine whether adenine nucleotides might be acting via a P_2 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 P_2 purinoceptors. However, reactive blue 2 and PPADS are considered to be relatively specific antagonists for P_{2Y} and P_{2X} receptors, respectively (16). Figure 2B demonstrates that reactive blue 2 inhibited adenine nucleotide-induced colony formation with an IC_{50} 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 (Figure 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 (Figure 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 (Figure 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-13-acetate, thapsigargin, arachidonic acid and the calcium ionophore A23187 were all inactive in stimulating anchorage-independent growth of ER-1-2/*ras* cells (data not shown). 8-Br-cAMP also did not stimulate growth of ER-1-2/*ras* cells and, in fact, potently inhibited anchorage-independent growth of the fully transformed PKC3-F4/*ras* cells (data not shown).

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 Figure 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* cells through the cell cycle because partially purified TRF did not induce cyclin A in these cells (Figure 3A), despite the fact that it rescued colony formation to a similar extent as ATP (ref. (22) and see Figure 4). The specificity of this response was further underscored by the observation that ATP did not alter the levels of cyclins D1 and E in methylcellulose cultures of ER-1-2/*ras* cells (Figure 3A). Northern blot analyses demonstrated that the induction of cyclin A by ATP occurred at the RNA level (Figure 3B). To examine the time course of cyclin A induction by

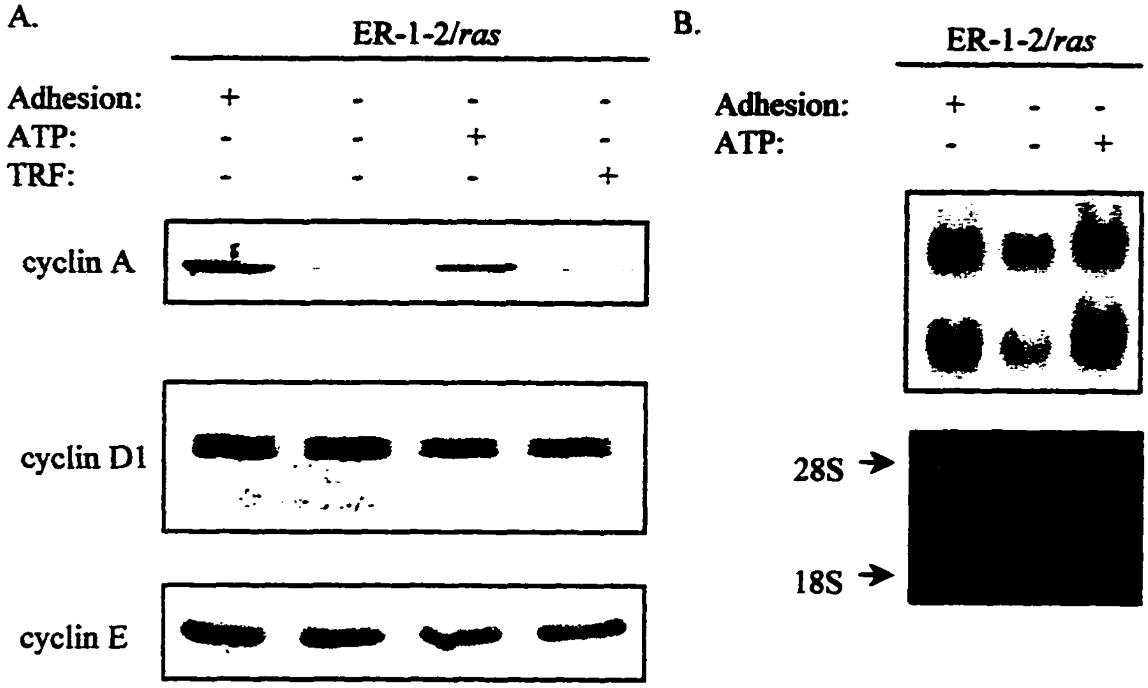

Figure 3-(A, B): Effects of ATP on anchorage-independent expression of various cell cycle proteins in ER-1-2/*ras*, PKC3-F4 and ER-1-2 cell lines. Cells were grown on tissue culture plates (+ adhesion) or in preparative methylcellulose cultures (-adhesion), in the presence (+) or absence (-) of ATP or partially purified TRF. Cells were then harvested, lysed and 50 µg of total cellular protein analyzed by Western blotting and probing with specific antibodies (**A**, **C**, **D** and **E**), or 10 µg of total cellular RNA analyzed by Northern blotting and hybridization with a cyclin A cDNA probe (**B**). **A**, Expression of cyclin A, D1 and E proteins in ER-1-2/*ras* cells. **B**, expression of cyclin A mRNA in ER-1-2/*ras* cells. The two bands represent the 3.0 and 1.8 kb cyclin A mRNA's. The ethidium bromide-stained gel, featuring the 28S and 18S rRNA bands, is shown as a loading control. **C**, Time course of ATP-induced, anchorage-independent expression of cyclin A protein in ER-1-2/*ras* cells. **D**, Expression of cyclin A protein in PKC3-F4 and ER-1-2 cells. The lower molecular mass protein band present in the adherent ER-1-2 cell sample is an apparent degradation product of cyclin A. **E**, Phosphorylation status of pRB. The hyperphosphorylated (ppRB) and hypophosphorylated (pRB) forms are distinguished by their mobilities and are indicated by arrows.

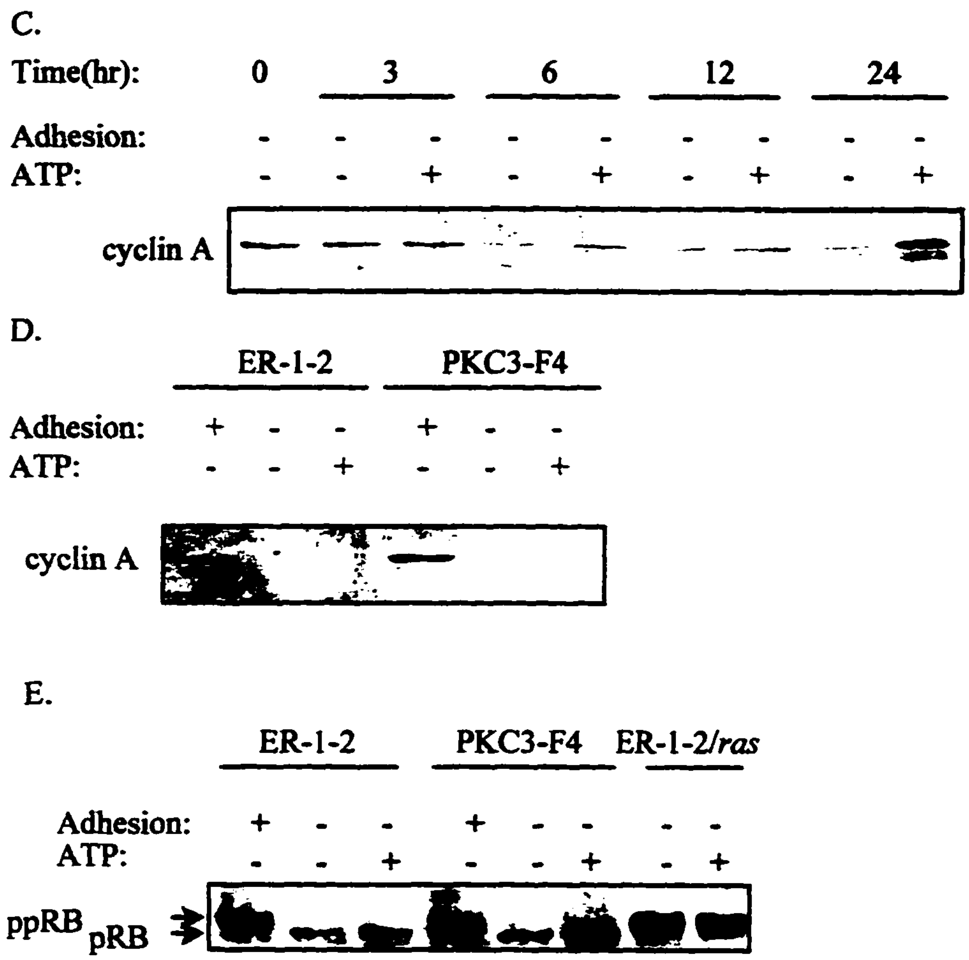
C.
Time(hr): 0 3 6 12 24
Adhesion: - - - - - - - - -
ATP: - - + - + - + - +
cyclin A
D.
ER-1-2
PKC3-F4
Adhesion: + - - + - -
ATP: - - + - - +
cyclin A
E.
ER-1-2
PKC3-F4
ER-1-2/ras
Adhesion: + - - + - - - -
ATP: - - + - - + - +
ppRB
pRB

Figure 3-(C, D, E)

ATP, ER-1-2/*ras* cells were inoculated into methylcellulose-containing medium and incubated for 24 hours 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 Figure 3C, cyclin A levels continued to decrease over the next 6 hr in both ATP-treated and untreated cultures. There was only a small effect on cyclin A expression between 3 and 12 hours after exposure to ATP, but by 24 hours 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 Figure 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 G_1/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 (Figure 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 (Figure 3E).

TRF did not induce anchorage-independent expression of cyclin A (Figure 3A), yet it rescued colony formation in soft agar of ER-1-2/*ras* cells (22). These data suggest that TRF exerted its effects via a mechanism distinct from that used by ATP, which did induce cyclin A. If this were true, it would be predicted that the effects of TRF and ATP would be at least additive. To test this hypothesis, 50 μM ATP (a saturating dose; see Figure 2A) was mixed with a maximally effective dose of partially purified TRF (22), and the number of soft agar colonies formed by ER-1-2/*ras* cells treated with the mixture was compared to the number formed with each agent alone. As can be seen in Figure 4, the mixture of ATP and TRF had a slightly more than additive effect when compared to either compound alone.

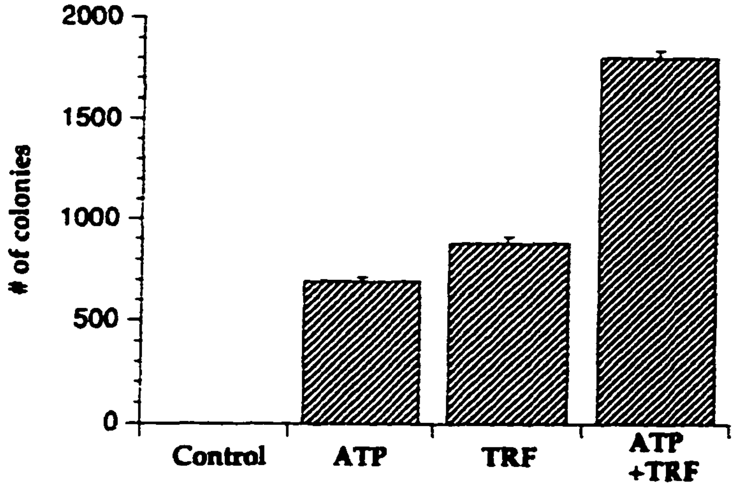

Figure 4: Additive effects of ATP and TRF on soft agar colony formation by ER-1-2/*ras* cells. ER-1-2/*ras* cells were inoculated into soft agar medium containing either ATP (50 μM), a maximally effective dose of partially purified TRF, or both. Values represent averages of triplicate determinations +/- 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 oncoprotein-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 G_1 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 G_1 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 G_1 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 G_1 Cdk activity.

With such functionally relevant endpoints 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 towards 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[2], 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 P_{2Y} 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 G_1/S border, and cyclin A is required for the G_1/S transition and progression through both S and G_2 phases (25,26). In fibroblasts, expression of cyclin A requires signals from both growth factors and cell-substratum adhesion, and occurs as late as 18 hr after adherent, 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 possiblity 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-1-2/*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 aquire the ability to carry out the activation of G_1 Cdk's 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.

Chapter 6

Concluding Remarks

Two cell cycle events, G_1 CDK activation and the expression of cyclin A, are essential for cell cycle progression. Cell adhesion to the ECM is required for the stimulation of both events. When cells adhere to the ECM, cell surface integrins are aggregated. The aggregated integrins generate at least two distinct signalling pathways for the stimulation of these events. Oncogenic Ras promotes cell cycle progression by generating multiple signalling pathways that fit into the pathways generated by integrins and stimulate both cell cycle events in the absence of adhesion. ER-1-2/*ras* cells remain anchorage-dependent for expression of cyclin A. Therefore, the defect is in a pathway downstream of Ras for the induction of cyclin A expression. ATP activates its cognate receptor and then generates signals for the induction of cyclin A expression. We predict that activated purinoceptors stimulate the dissociation of G protein subunits. The activated G proteins increase the intracellular second messenger molecules, for example calcium, diacylglycerol, and IP_3, which might fit into the signalling pathway that is generated by integrin for the induction of cyclin A expression. However, the mechanism for the stimulation of cell cycle progression by TRF is still unclear (Fig 1). Perhaps TRF works by activating events downstream of cyclin A.

This dissertation presents work aimed at furthering understanding of the relationship between signal transduction pathways mediated by Ras and cell cycle regulation. Aberrations in either one of these events are frequently associated

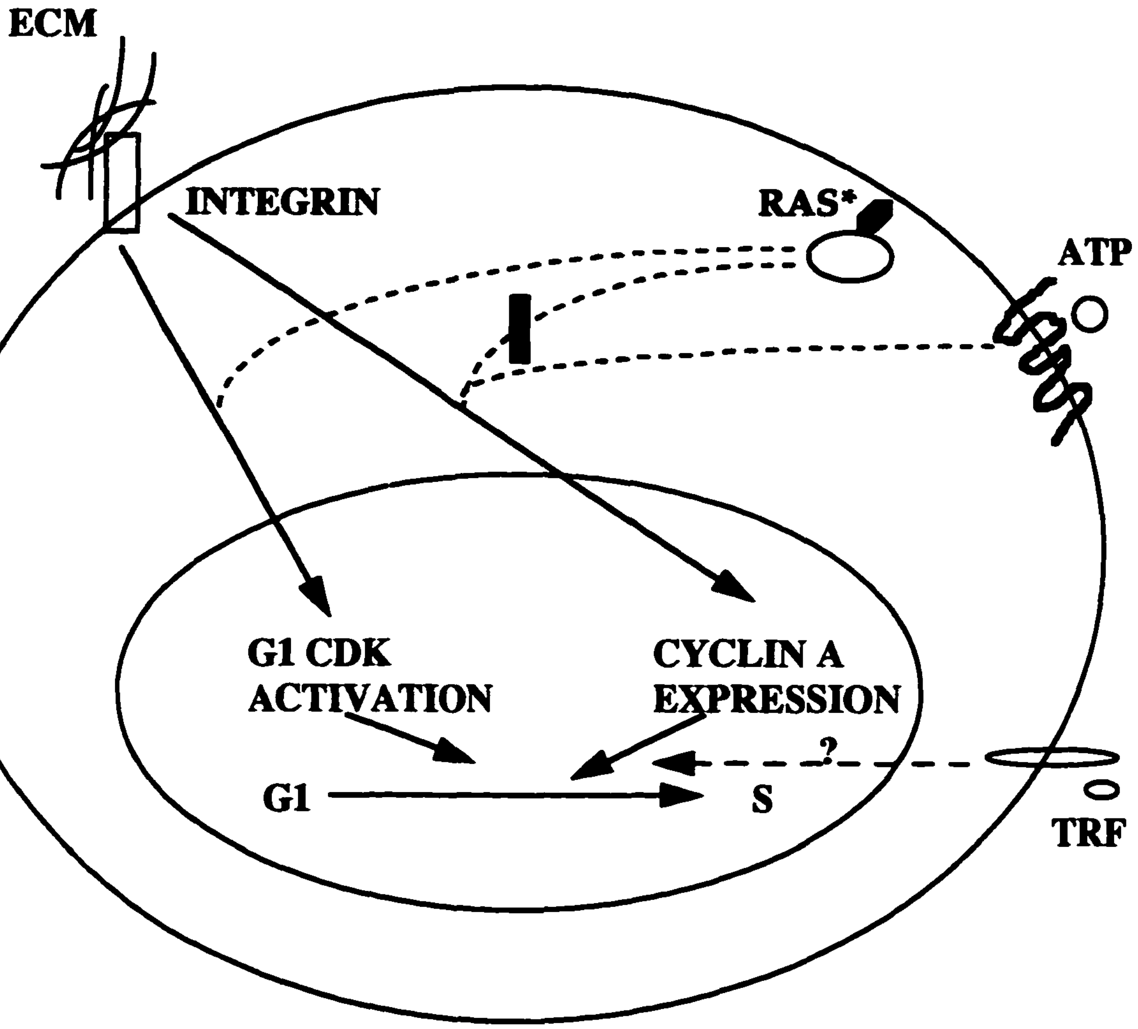

Figure 1: ATP and TRF rescue the defect of ER-1-2/*ras* cells through different mechanisms. Signals generated by activation of a putative purinoceptor bypass the blockade of Ras signalling to induce the expression of cyclin A. TRF works through an unknown mechanism, possibly by activating events downstream of cyclin A.

with neoplastic growth. Activated forms of Ras play a key role during the formation of tumors in vivo, as well as the transformation of cell lines in culture leading to changes in cell morphology, gene expression, and stimulation of anchorage-independent growth. The last property is the best correlate of tumorigenicity. Therefore, the understanding of downstream targets of Ras-mediated signal transduction pathways have become an important issue. We used fully activated Ras and three Ras effector loop mutants to address the possibility that multiple Ras-mediated signaling pathways are required for anchorage-independent growth and activation of several cell cycle events. These cell cycle events include: 1) pRB phosphorylation; 2) cyclin E-dependent kinase activity; and 3) cyclin A expression. Each of three effector loop mutants lost their ability to induce formation of colonies in soft agar, and at least partially lost the ability to activate these cell cycle events. Coexpression of these mutants promotes anchorage-independent growth and activation of these cell cycle events. Thus, multiple Ras-mediated pathways are required for anchorage-independent growth. However, the effector loop mutants used in this study may bind to several effectors. We should address the question of whether signaling by any of these pathways, Raf, RalGDS, and PI(3)K, alone is sufficient to partially activate these cell cycle events. Transfection of constitutively activated forms of these cDNAs into fibroblast cells can help answer this question. To test the hypothesis that multiple pathways are required for anchorage-independent growth, each Ras effector loop mutant can be cotransfected with each of these effector cDNAs.

It has been suggested that anchorage-dependent activation of cyclin D1-Cdk4/6 correlates with the anchorage-mediated induction of cyclin D1 mRNA. Therefore, another interesting question raised in this study is that the activity of cyclin E-Cdk2 is adhesion-dependent, but cyclin E and Cdk2 protein levels were not changed between adherent and non-adherent cultures. Therefore, it should be tested whether posttranslational modification of this kinase would participate in this regulation. Two different types of modifications should be studied in the future. First, because $p21^{cip1}$ and $p27^{kip1}$ protein levels increased in non-adherent cultures of some cells but not others (for example Rat 6), identification of putative novel cyclin E-Cdk2 regulatory proteins might be attempted. The question of binding ratio of CKIs with G_1 Cdks between adherent and non-adherent culture also should be addressed. For example, low amount of $p21^{cip1}$ promotes the assembly of cyclin D 1-Cdk4 complexes, whereas high levels of $p21^{cip1}$ inhibit kinase activity. Second, the phophorylation status of cyclin E-Cdk2 in adherent and non-adherent cultures should be investigated. Hence, phosphopeptide mapping and phosphoamino acid analysis should be performed to confirm the phosphorylated or dephosphorylation status of Cdk2.

We discussed earlier that TRF specifically corrected the defect of ER-1-2/*ras* cells. TRF is ~1300 molecular weight, lipid insoluble, heat, protease, acid and base stable, but destroyed by UV(260nm) light. However, the identity of TRF is still a mystery. To identify TRF, multiple chromatographic and spectroscopic procedures will need to be performed.

During the analysis of TRF, we also tested various compounds that have chemical properties similar to TRF. As we described above, adenine nucleotides specifically restored the anchorage-independent growth of ER-1-2/*ras* cells. However, TRF is not ATP. ATP induced anchorage-independent growth of ER-1-2/*ras* cells through the induction of the cyclin A gene. ATP and adenosine, can act as extracellular factors by binding to specific purinoceptors. Because the induction of anchorage-independent growth of ER-1-2/*ras* cells by ATP was blocked by a P_{2Y} purinoceptor antagonist, reactive blue 2, we proposed that the receptor for ATP located on ER-1-2/*ras* cells could be a serpentine receptor coupled to G proteins. Most of the P_2 purinoceptors have been classified pharmacologically. Receptors can be classified according to their activation by ATP analogs with different preferences. The agonist preference for the putative receptor located on ER-1-2/*ras* cells is different from that of other known receptors. We therefore predict that this receptor could be a new, unidentified subtype of P_2 purinoceptor. In order to isolate this new subtype of P_2 purinoceptor, degenerate oligonucleotide primers could be designed based on the best conserved segments among all known P_2 receptor sequences. These primers could then be used to amplify novel receptor cDNA fragments by low stringency RT-PCR starting with total mRNA from ER-1-2/*ras* cells. Once a novel P_2 receptor has been cloned, it would be important to study signal transduction pathways generated by this receptor, particularly those that might lead to cyclin A gene expression.

In summary, it is concluded that the work presented in this dissertation has provided evidence for anchorage-independent growth induced by Ras through multiple pathways to multiple targets of the cell cycle machinery. Extracellular signaling molecules, such as ATP , exert theirs effects on cells through receptors that generate signals that are able to cross-talk with signals generated by Ras. Cell cycle progression is regulated jointly by growth factors and integrins and activated Ras abrogates some of these requirements to promote abnormal growth. It is hope that this dissertation will stimulate futher investigation on understanding the role of multiple signal transduction pathways onregulation of the cell cycle machinery.

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