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13. ABSTRACT *(Maximum 200 Words)*

When deprived of adhesion normal cells are unable to proliferate; instead they arrest in G1 phase of the cell cycle. Unlike normal cells, transformed cells are able to by-pass this adhesion-mediated control of cell proliferation and grow in an anchorage-independent manner. Understanding the mechanism by which the extracellular matrix controls progression through the G1 phase of the cell cycle would give us insight into the cellular mechanism that accompany carcinogenesis. We have shown, here using a human mammary epithelial model, that the expression of the early growth control gene, c-Myc, is directly regulated by cell adhesion through a 1 integrin-dependent pathway, involving the PKC, c-Src family of kinase, and MAPK. When deprived of adhesion mammary epithelial cells are unable to progress into S phase upon EGF stimulation, and they arrest in early G1. This Anchorage-dependent block correlates with the down regulation of c-Myc mRNA and protein. Restoration of the expression of c-Myc in non-adherent cells is sufficient to override this G1 arrest, allowing for the anchorage-independent decrease in levels of p27, activation of cyclin E-CDK2, and expression of E2F-1.
Our study shows that c-Myc is a crucial mediator of the adhesion-dependent control of cell cycle progression in human mammary epithelial cells.

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Introduction:

To proliferate, normal cells require two types of extracellular signals: hormonal/growth factor stimuli and adhesion to the extracellular matrix (ECM). When deprived of attachment to a solid substrate, even in the presence of growth factors, normal cells are unable to replicate their DNA, and they arrest in the G1 phase of the cell cycle (1,2). A hallmark of transformed cells, which correlates with tumorigenicity, is the loss of anchorage-dependent growth. As cells become malignant, they gain the ability to bypass the extracellular matrix control of cell proliferation.

Recent studies have linked cell adhesion to key regulatory molecules of cell cycle progression: the cyclins and their associated cyclin-dependent kinases (cdk)(1,3,4,5). A potential, and still controversial link between integrin-mediated cell adhesion and upregulation of cyclin expression and cdk activity is the c-Myc proto-oncogene, which is frequently overexpressed in breast cancer. Experiments altering Myc expression indicate that Myc is required for cell proliferation; it plays a crucial role in the progression through the G1 phase of the cell cycle in response to both serum and growth factors (6,7,8). Myc has been shown to induce cyclin E and cyclin A expression (9), as well as the activity of the cyclin E-cdk2 complex, presumably by triggering the release of the p27 and p21 kinase inhibitors (10,11), an effect strongly reminiscent of the one caused by cell adhesion.

Studies of the signaling pathways from integrin to the cell cycle machinery have been concentrating on the ones leading to the upregulation of the c-Jun and c-Fos immediate early genes, explaining the anchorage-dependent expression of cyclin D (12). Possible signaling pathways regulating c-Myc expression and function have been for the large part unexplored. The regulation of c-Myc by cell adhesion seems to be cell type-specific since c-Myc mRNA has been reported to be down regulated by lack of adhesion in endothelial cells, but does not appear

to occur, or to be only a secondary event in fibroblasts (1,13,14). To date, the role of integrins in the regulation of c-Myc expression and function has not been explored.

We have thus proposed to study whether, in human mammary epithelial cells, Myc is a link between the ECM adhesion-dependent growth and the cell cycle regulatory machinery.

Body:

During the September 97- August 98 period we had addressed Aim 2 of the original proposal:

Aim 2: To characterize the role of Myc in mediating anchorage-dependent regulation of cyclin-dependent kinase activity.

a. Investigate whether cell anchorage activates two parallel pathways, one Myc-independent leading to the expression of cyclin D1, the second involving Myc activation of cyclin E-cdk2 activity.

b. Define how Myc induces cdk2 activation, focusing on cyclin E expression, p21 and p27 expression, inhibitor association with the cyclin-cdk2 complex, and regulation of cdc25 phosphatase.

Since we had not yet shown that c-Myc expression was modulated by cell adhesion, we had shifted our hypothesis to the following: Deregulated expression of c-Myc enables mammary epithelial cells to progress through the anchorage-dependent G1 checkpoint. This Myc induced G1-S transition is achieved through the activation of G1 cyclin-CDK complex due to the down regulation of CDK inhibitors.

We had shown, using flow cytometry, that the immortalized mammary epithelial cell line 184AIN4 (15), can be synchronized and growth arrested in an G0/G1 stage of the cell cycle by EGF withdrawal. Upon readdition of EGF, cells in monolayer progress into S and G2 phase of the cell cycle. However, cells stimulated with EGF in suspension culture were unable to enter S phase. In contrast, A1N4 cells stably transfected with c-Myc under a constitutive promoter

(A1N4-Myc cells) (16) are able to replicate their DNA in suspension culture. The overriding of the adhesion-dependent G1 check point by overexpression of c-Myc was further supported at the molecular level by studies of the phosphorylation state of the retinoblastoma protein, pRb. Whereas pRb could only be detected in its hypophosphorylated form in non-adherent A1N4 cells, hyperphosphorylated forms of pRb are present in the Myc transfectants. These results confirm that A1N4-Myc cells progress through the G1-S transition in the absence of adhesion.

We then asked whether Myc is able to induce the expression and activity of the various G1 cyclin-CDK complexes in an adhesion-independent manner. Our results indicated that low levels of cyclin D-CDK4 activity was still present in non-adherent A1N4 cells and that overexpression of c-Myc does not upregulate CDK4 activity. We then examined the cyclin E-CDK2 complex. Whereas lack of adhesion completely inhibited CDK2 kinase activity, overexpression of c-Myc restored CDK2 activity to similar levels to those detected in adherent cells. Unlike the parental cells that only express very low level of cyclin E in suspension, A1N4-Myc cells upregulate cyclin E in an adhesion-independent manner. In addition, the phosphorylated form of CDK2 which correspond to the active form of the kinase was present in non-adherent A1N4-Myc cells, but was not detectable in the parental cells, confirming the difference in CDK2 kinase activity detected by *in vitro* kinase assay. This difference in kinase activity could be explained by the observation that the levels of the CDK2 inhibitor, p27 was drastically changed between the parental cells and the transfectants. Whereas in non-adherent parental A1N4 cells, p27 was present at high levels and found complexed with CDK2, in c-Myc overexpressing cells p27 was not detectable. We have further shown that p27 is not downregulated by a decrease in transcription but by a decrease in the stability of the protein, which is rapidly degraded by the ubiquitin-proteosome pathway in non-adherent A1N4-Myc cells.

We have this year (September 99-August 2000) completed this section of the study:

- We have shown that lack of adhesion prevents the induction of the expression of the late G1 transcription factor E2F-1, supporting our previous flow cytometry and pRb results. However, expression of c-Myc rescues the expression of E2F-1 in non-adherent cells confirming the ability of A1N4-Myc cells to progress through the G1-S transition in suspension culture.
- To further support our data in the A1N4/ A1N4-Myc system, and to address the direct cause and effect of c-Myc and the adhesion-dependent regulation of p27, we have generated a retrovirus expressing the full length human c-Myc, and we transiently infected the anchorage-dependent immortalized human mammary epithelial cell line, MCF-10A. Transient expression of c-Myc in MCF-10A is sufficient to prevent the upregulation of p27 when MCF-10A are deprived of adhesion.

In the September 98-August 99 period, we had addressed part of Aim 1 of the original proposal:

Aim 1: To determine whether cell adhesion to ECM components is required for Myc induction and activation.

a. Analyze whether cell adhesion alters Myc expression, more specifically Myc mRNA levels, mRNA stability, and protein levels.

b. Study the effect of cell anchorage on Myc activity. The focus will be on adhesion-dependent alteration of Myc phosphorylation, p107 association with Myc, as well as phosphorylation and expression levels of Max.

c. If cell adhesion activates Myc, we will define whether particular ligands and specific members of the β1 family of integrins mediate the regulation of Myc in epithelial cells.

Loss of adhesion in the mammary epithelial cell lines A1N4 and MCF-10A results in a decrease in c-Myc protein, which is reversible upon cell reattachment. Similarly to

c-Myc protein, an initial increase in c-Myc mRNA, following EGF stimulation, occurs both in adherent and non-adherent cells. However, after four hours, a three-fold decrease in mRNA levels can be observed in non-adherent cells, as compared to adherent cells. These results suggest that cell adhesion is required for maximal sustained expression of c-Myc in epithelial cells. Treatment with cycloheximide can reverse the downregulation of Myc mRNA in suspended cells, indicating that protein synthesis or a labile protein is required for Myc mRNA down regulation. Furthermore, treatment with Actinomcin D showed only a slight decrease in the stability of c-Myc mRNA in non-adherent cells. This observation suggests that the cell adhesion-dependent regulation of the expression of c-Myc occurs, at least in part, at the transcriptional level. These results, together with the results presented above under *Aim 2,* are described in detail in Appendix A.

Since c-Myc protein is downregulated to undetectable levels when mammary epithelial cells are deprived of adhesion, we did not address part b of *Aim 1* that proposed to investigate a modulation in Myc function, and instead addressed directly *Aim1c*. We have first examined whether specific ECM components can modulate Myc expression. In the presence of EGF, the upregulation of Myc protein is enhanced when the cells are allowed to attach onto fibronectin, as compared to tissue culture dish. We thus asked whether adhesion to ECM components only allows for optimal EGF signaling, or whether an integrin-initiated signaling pathway is cooperating with the EGF pathway. To address the later possibility, we tested whether adhesion to ECM components in the absence of growth factors can induce c-Myc expression. Adhesion of quiescent A1N4 cells to fibronectin, collagen type IV and I, but not laminin, induces the expression of c-Myc in an ECM concentration-dependent manner. Both cytoskeletal rearrangement and integrin engagement and clustering are required for the induction of c-Myc by fibronectin. Furthermore, β1 integrin function-blocking antibodies prevent the adhesion-

dependent induction of c-Myc. These results indicate that cell-adhesion to the extracellular matrix through the β1 integrin receptor can modulate the expression of c-Myc.

We have now investigated the further aims we have proposed in the 1999 annual report:

1- Identify which specific β1integrin dimer is responsible for the regulation of c-Myc
2- Identify the signaling molecules responsible for transmitting the regulatory message from the integrin to c-Myc.

Cellular adhesion to fibronectin can be mediated by α4β1, α5β1, αvβ1, αvβ5 integrins. Flow cytometry analysis indicates that the A1N4 cells express α4, α5, α2, and β1 integrin subunits, but only very low levels of αvβ5 on their surfaces. Treatment of A1N4 cells with either anti-human α5 integrin-blocking antibody (clone P1D6), or anti-human α4 integrin-blocking antibody (clone P1H4), did not impede the fibronectin-dependent induction of c-Myc, leaving the αvβ1 integrin as a strong candidate. However, since no function-blocking antibody against the αv subunit alone, or the αvβ1 dimer is available commercially, we were unable to test this hypothesis.

We have shown that adhesion of A1N4 cells to fibronectin results in the activation of c-Src and of the mitogen activated protein kinase (MAPK), each of which precede the induction of c-Myc. Pharmalogical inhibitors, specific for either the c-Src family of kinase and for MEK1, both block the adhesion-dependent induction of c-Myc. In addition, pharmacological inhibitor of PKC, as well as prolonged treated with TPA, also blocked the adhesion-dependent induction of c-Myc. These observations indicate that PKC, the c-Src family of kinases, and the MAP kinase pathway are involved in the integrin-mediated regulation of c-Myc. These results, together with the previous results mentioned under *Aim 1,* are described in detail in Appendix B.

To further support of pharmacological data on the role of c-Src in the adhesion-dependent control of the expression of c-Myc, we attempted to transfect A1N4 cells with wild type, constitutively active, and dominant negative forms of c-Src. However, these experiments were inconclusive, for technical reasons. Due to the low efficiency of transfection we could not assess the effect of these Src mutants in transient transfections. Furthermore, because the expression of these transgenes was toxic to the cells, we could not obtain stable transfectants expressing elevated levels of these Src mutants.

Key research accomplishments:

We have shown that:

- In human mammary epithelial cells, cell adhesion is required for the sustained expression of c-Myc mRNA and protein
- Adhesion to ECM components enhances the growth factor-dependent induction of c-Myc
- β1 integrin can, independent of growth factors, induce the expression of c-Myc
- Fibronectin-dependent induction of c-Myc is mediated by the PKC, c-Src family of kinases, and the Erk1/2 MAPK pathway
- In the absence of adhesion, human mammary epithelial cells arrest in early G1
- Deregulated expression of c-Myc enables A1N4 cells to bypass the anchorage-dependent G1 checkpoint and replicate their DNA in suspension culture.
- Expression of c-Myc in non-adherent human epithelial cells is sufficient to down regulate the CDK inhibitor p27, rescuing the activation of the cyclin E-CDK2 and the expression of E2F-1.

Reportable outcomes:

Abstracts

C.M. Benaud and R.B.Dickson. The role of c-myc in bypassing an anchorage-dependent G1 checkpoint in human mammary epithelial cells. 38th American Society for Cell Biology Annual Meeting, San Francisco, CA. December 1998.

C.M. Benaud and R.B.Dickson. Regulation of c-Myc by β1 integrins in mammary epithelial cells. 39th American Society for Cell Biology Annual Meeting, Washington, DC. December 1999.

C.M. Benaud and R.B.Dickson. Role of c-Myc in anchorage-independent growth. Era of Hope Meeting, Atlanta, GA. June 2000

Manuscripts

C.M.Benaud and R.B.Dickson. 2000. Regulation of the expression of c-Myc by β1 integrin in epithelial cells. Submitted

C.M.Benaud and R.B.Dickson. 2000. Adhesion-regulated G1 cell cycle arrest requires the downregulation of c-Myc. Submitted

Degree obtained

Ph.D. Thesis title: "Role of c-Myc in the adhesion dependent control of epithelial cell proliferation" July 2000. Department of Cell Biology, Georgetown University

Conclusions:

To date, most of the studies on anchorage-dependent growth regulation have been performed only on fibroblasts. However, fibroblast and epithelial cells have a very different relationship to their ECM. Primary mammary epithelial cells have been shown to receive specific survival signals from basement membrane matrix which are not

provided by attachment to plastic or type I collagen (17). Hence, epithelial cells may differ from fibroblasts in their interaction with the extracellular matrix, and the control of cell growth by the ECM may also vary.

Our results indicate that, contrary to what has been described in fibroblasts, in mammary epithelial cells c-Myc expression is directly regulated by cell adhesion. In addition, we have shown that a β1 integrin-dependent signaling pathway can directly induce the expression of c-Myc, and that both integrin and growth factors signaling pathways cooperate for maximal sustained expression of c-Myc.

We have also shown that, similar to mesenchimal systems, mammary epithelial cell growth is controlled by an anchorage-dependent G1 checkpoint. Furthermore, deregulated expression of c-Myc can override this checkpoint by downregulating the CDK inhibitor p27, activating the late G1 cyclin E-CDK2 complex, and inducing the expression of the G1/S transcription factor E2F-1. Taken together, our results show that sustained expression of c-Myc, which is required for progression through the cell cycle, requires cell adhesion. Overexpression of c-Myc, which often occurs in breast cancer, can enable human mammary epithelial cells to ignore the integrin control of Myc, and progress through G1 phase and G1/S transition, in an anchorage-independent fashion.

This study sheds light on the mechanism by which deregulated expression of c-Myc may unable mammary epithelial-derived cancer cells to proliferate in an uncontrol manner, ignoring the environmental clues provided by the extracellular matrix.

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Adhesion-Regulated G1 Cell Cycle Arrest Requires the Downregulation of c-Myc

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Running Title: Anchorage-Dependent checkpoint and c-Myc

ABSTRACT

Adhesion to the extracellular matrix is required for the expression and activation of the cyclin-cyclin-dependent kinase (CDK) complexes, and for G1 phase progression of non-transformed cells. However, in non-adherent cells no molecular mechanism has yet been proposed for the cell adhesion-dependent up-regulation of the p27 cyclin-dependent kinase inhibitor (CKI), and the associated inhibition of cyclin E-CDK2. We now show that in epithelial cells the expression of c-Myc is tightly regulated by cell-substrate adhesion. When deprived of adhesion, two independently derived mammary epithelial cell lines, 184A1N4 and MCF-10A, rapidly decrease their level of c-Myc mRNA and protein. This decrease in levels of c-Myc correlates with G1 phase arrest, as indicated by hypophosphorylation of pRb and inhibition of the activity of the cyclin E-CDK2 complex. In 184A1N4 cells, cell-substrate adhesion is required for the suppression of p27, and induction of cyclin E, E2F-1, but not cyclins D1 and D3. Enforced expression of c-Myc in non-adherent 184A1N4 and MCF-10A cells reverses the adhesion-dependent inhibition of cell cycle progression. Restoration of c-Myc in non-adherent cells induces the expression of E2F-1, and hyperphosphorylation of pRb in response to EGF treatment. In addition, expression of c-Myc results in the anchorage-independent activation of the CDK2 complex, the associated upregulation of cyclin E, and the destabilization and degradation of p27 by the ubiquitin-proteasome pathway. Our study thus suggest that c-Myc is the link between cell adhesion and the regulation of p27 and cyclin E-CDK2. Furthermore, we describe a role for c-Myc in adhesion-mediated regulation of E2F-1.

INTRODUCTION

To proliferate, normal cells require two types of extracellular signals: hormonal/growth factor stimuli and adhesion to the extracellular matrix (ECM). When deprived of attachment to a solid substrate, even in the presence of growth factors, normal cells are unable to replicate their DNA, and they arrest in the G1 phase of the cell cycle (17,33,39). Recent studies have linked cell adhesion to key key types of regulatory molecules required for the progression through the cell cycle: the cyclins and their associated cyclin-dependent kinases (CDK) (13,64).

Mitogenic stimulation of quiescent, adherent cells first induces the expression the cyclin D family of proteins in mid-G1, which is followed by an increase in levels of cyclin E in late G1. Cyclins D1/2/3 and cyclin E associate with and activate CDK4/6 and CDK2, respectively (50). The cyclin D-CDK4/6 complexes and the cyclin E-CDK2 complex collaborate in the phosphorylation of the pRb family of proteins, triggering their release from the repressive pRb family-E2F transcriptional complex (3,22,32). Once Rb-free, the E2F complex transactivates the expression of cyclin E, cyclin A, and a number of targets required for S phase progression and DNA replication (8,19,38). A common mechanism by which anti-mitogenic signals inhibit the activity of the cyclin-CDK complex, and thus block cell cycle progression, is through the induction of the cyclin dependent kinase inhibitors (CKI). Whereas the INK4 family of proteins specifically inhibits the cyclin D-CDK4 complex, the CIP/KIP inhibitors, which include p21, p27 and p57, are more ubiquitous. They associate both with the cyclin D-CDK4 complex and the cyclin E-CDK2 complex (51).

In fibroblasts, loss of adhesion results in the down regulation of cyclin D (4,64) and cyclin A (2,18,53,64), as well as inhibition of the activity of the cyclin E-CDK2 complex, due to the accumulation of the CDK inhibitors, p21 and p27, and their association with the cyclin E-CDK2 complex (13,64). This inhibition of CDK activity prevents pRb phosphorylation, and arrests the cells in the G1 phase of the cell cycle.

Studies of the signaling pathways bridging cell adhesion to extracellular substrates to the cell cycle machinery, have concentrated, to date, on the regulation of expression of cyclin D.

Specifically, they have highlighted the role of adhesion-dependent activation of the MAP kinase pathway in the induction of cyclin D1 (44). Furthermore, the cyclin D1 promoter is regulated by both the jun-fos (AP-1) and β–catenin-TCF transcription factors, and the expression and activation of these transcriptional complexes are regulated by cell-substrate adhesion-dependent pathways (6,9,10,15,37,52), explaining the adhesion requirement for the expression of cyclin D. In contrast to cyclin D, the signaling pathway leading to CDK2 inhibition in non-adherent cells is largely unresolved, and the exact mechanism by which cell adhesion regulates the accumulation of cyclin-dependent kinase inhibitor, p27, remains to be elucidated.

A potential, but unexplored link between integrin-mediated cell adhesion, enhanced expression of cyclins, and elevated activity of CDKs is the c-Myc early response gene. Experiments altering the expression of c-Myc indicate that expression of this protooncogene is required for cell proliferation, and plays a crucial role in progression through the G1 phase of the cell cycle (11,12,20,23). c-Myc induces cyclin E and cyclin A expression (2,42,45,58), as well as the activity of the cyclin E-CDK2 complex, presumably by triggering the release of the kinase inhibitors p27 (34,42,58,62), an effect strongly reminiscent of the one caused by cell adhesion in fibroblasts (13,64).

Possible signaling pathways regulating expression and function of c-Myc have been largely unexplored. The role of cell adhesion in the regulation of c-Myc itself is not well established. Studies examining c-Myc messenger RNA in fibroblasts have shown no immediate alteration in c-Myc mRNA levels when cells are deprived of adhesion (4,9). A decrease in c-Myc mRNA could only be detected after 24 hrs of suspension culture of BALB/3T3 fibroblasts. However, adhesion may still play a role in the regulation of the transcription of c-Myc in these cells; replating the cells, once they have downregulated c-Myc, induces an increase in c-Myc mRNA (9). In contrast, when placed in suspension culture, endothelial cells rapidly downregulate (within 1 hr) c-Myc mRNA. These differences in the regulation of c-Myc mRNA in response to adhesion may reflect various degrees of suppresion of senescence of the model studied, or a cell-type specificity of the effect. Therefore, the precise role(s) of cell adhesion on the regulation of c-Myc mRNA and protein need

to be further explored. Furthermore, the function of c-Myc in the anchorage-dependent regulation of the G1 checkpoint has not been studied. We have now examined the role of cell adhesion in EGF-dependent regulation of human mammary epithelial cell cycle progression, and investigated the role of c-Myc in mediating the adhesion-dependent control of proliferation.

Our results indicates that human mammary epithelial cells respond to a disruption of cell-substrate adhesion by a profound decrease in the levels of c-Myc mRNA and protein. The G1 arrest under non-adherent conditions correlates with a decrease in the cellular levels of E2F-1, a decrease in the expression of cyclin E, and an inhibition of the cyclin E-CDK2 complex. Using both stable and transient transfection of c-Myc, we show that increased expression of c-Myc in non-adherent cells can revert the adhesion-dependent block at multiple points, allowing cells to progress through the G1/S transition. Enforced expression of c-Myc in non-adherent cells is sufficient to allow anchorage-independent induction of E2F-1, and to promote the activation of the cyclin E-CDK2 complex, closely coupled to the increase in cyclin E and destabilization of p27. These results suggest that the decrease in the amount of c-Myc might be an important step in the adhesion-dependent G1 checkpoint of epithelial cells.

MATERIALS AND METHODS

Cell culture and flow cytometry:

184A1N4 is a non-tumorigenic cell line derived from a primary culture of human mammary epithelial cells that were immortalized with benzo(a)pyrene (provided by Dr. M.R.Stampfer, university of California, Berkeley) (56). These cells were then transformed with a retroviral expression vector containing the mouse c-Myc oncogene, giving rise to the stably transfected 184A1N4-Myc cell line, which overexpresses the Myc protein (provided by Dr D.Salomon, NCI, NIH) (60). Cells were plated at low density (6.6×10^3 cells $/cm^2$) and allowed to adhere in complete medium, IMEM (Gibco BRL, Rockville, MD), supplemented with 0.5% fetal bovine serum (Gibco BRL), 0.5 μg/ml hydrocortisone (Sigma, St Louis, MO), 5 μg/ml insulin (Biofluids, Rockville, MD) and 10ng/ml epidermal growth factor (EGF) (Collaborative Biomedical Research, Waltham, MA) for 5-7hrs. Cells were then washed twice with phosphate buffered saline (PBS), and maintained in a low serum, EGF-free medium (IMEM (Gibco BRL), supplemented with 0.5% fetal bovine serum (Gibco BRL), 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Biofluids)) for 48-72 hrs to synchronize the cells in G0/G1 (57). Quiescent cells were then subjected to stimulation by EGF (10ng/ml), either in suspension or in monolayer culture. A second non-tumorgenic, mammary epithelial line, MCF-10A (Michigan Cancer Foundation) (54) was maintained in 50:50 % IMEM:HAM F12 (GIBCO BRL) supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Biofluids) and 10ng/ml EGF. For suspension culture, cells were trypsinized and transferred to agarose-coated. To prevent cell adhesion, tissue culture dishes were coated with boiling 0.8% agarose (5ml/100mm dishes; 10ml/150mm dishes). The agarose was then equilibrated overnight with an equal volume of medium. For cell cycle studies, cells were collected, fixed in citrate buffer/ DMSO, and their nuclei stained with propidium iodine (61). The DNA content was analyzed by flow cytometry (Lombardi Cancer Center Flow Cytometry Core). Cycloheximide and Actinomycin D were purchased from Sigma; ALLM and ALLN from Calbiochem (La Jolla, CA).

Retroviral transfection:

The PLXSN retroviral vector and the PLXSN vector containing the cDNA encoding the human c-Myc (PLXSN-MYC) (provided by Dr. M.R. Stampfer) were transfected into GP+E86 cells by calcium phosphate, followed by glycerol shock. The next day the packaging cells expressing the PLXN or the PLXSN-MYC ecotropic viruses were changed to selection medium containing 600μg/ml G418 (Mediatech, Inc., Herndon VA). When the cells became confluent, the virus-containing supernatant was collected and used to infect PA317 amphotropic packaging cells (ATCC, Rockville, MD). Following infection, PA317 cells producing PLXSN and PLXSN-Myc retroviruses were selected in the presence of 500μg/ml G418. PA317 cells producing high titers of retroviruses were subcloned.

For transient transfection of MCF-10A, $2x10^5$ cells in 100 mm dishes were infected with 5 ml of supernatant containing a high titer of either PLXSN or PLXSN-MYC retrovirus, supplemented by 8μg/ml polybrene (Sigma). After two hrs at 37°C, 5ml of MCF-10A growth meduim containing 8 μg/ml polybrenene was added to the cells. The next day, the supernatant was removed from the cells, and the cells were fed with MCF-10A growth medium. Cells were trypsinized 48hrs following infection, and transferred to suspension plates.

Western blotting:

For preparations of whole cell extracts, adherent cells were scrapped in PBS, and non-adherent cells collected and pelleted by centrifugation (1500xg). The cell pellets were lysed on ice in lysis buffer (1% TritonX-100, 50mM Tris, pH 8.0, 150mM NaCl, 1mM EDTA, 50mM sodium fluoride, 0.5 mM sodium orthovanadate, 60μg/ml aprotinin, 10μg/ml leupeptin, 1 μg/ml pepstatin, 10μg/ml PMSF). Cellular debris were removed by centrifugation for 10 min at 14,000Xg. Equal amounts of proteins, as determined by the BCA protein micro assay (Pierce, Rockford, IL), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto PVDF membranes (Millipore, Bedford, MA). For immunoblotting, membranes were blocked in PBS-0.1% Tween 20 with 5% milk overnight at 4°C. The blots were then

incubated with the primary antibody for 1 hr at room temperature, followed by a 1 hr incubation with either a 1:10,000 dilution goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Biorad, Hercules, CA), or a 1:5000 dilution of anti-rabbit HRP-linked secondary antibody (Amersham, Arlington Heights, IL). Immunoreactive proteins were detected with chemiluminescent substrate (Pierce). The following antibodies were used: monoclonal 9E10 anti-human c-Myc, anti-pRb (G3-245) and anti-p21 (6B6) from Pharmingen (San Diego, CA), polyclonal anti-c-Myc (C-19), anti-CDK2 (M2), anti-CDK4 (C-22), anti-cyclin D3 (C-16), and anti-E2F-1 (C-20) from Santa Cruz (Santa Cruz, CA), anti-p27Kip1 from Transduction Laboratories (Lexington, KY), and anti-cyclin E from UBI (Lake Placid, NY).

Northern blotting:

To prepare total cellular RNA, 10^6 cells were collected and resuspended in 2 ml of Rnazol (Tel-test, Inc., Friendswood, TX). The RNA was prepared according to the manufacturer instructions. 10μg of total RNA was separated on 1% agarose-formaldehyde gels and transferred onto N-Hybond nylon membranes (Amersham). Blots were cross linked by UV light exposure (UV stratalinker, Stratagene, La Jolla, CA) and prehybridized in 50%formaldehyde, 5X SSPE, 5X Denhardt, 0.5% SDS, and 0.2 mg/ml heat denatured salmon sperm DNA at 42°C for 6hrs. Cyclin D1 and p27 mRNA expression were detected using randomly primed radiolabeled probes (Pharmacia, Piscataway, NJ) generated from cyclin D1 and p27 cDNA (gifts from Dr S.Reed, Scripp, San Diego, CA). Probes were heat denatured, added to the pre-hybridization solution, and hybridized overnight at 42°C. Blots were reprobed for GAPDH as loading control.

***in vitro* kinase assay:**

CDK2 and CDK4 were immunoprecipitated overnight at 4°C from 100μg of total cell lysates proteins with anti-CDK2 (M2) and anti-CDK4 (C-22) antibodies, respectively. The antibodies were collected with 40μl of 50% protein A sepharose (Pharmacia) for 1hr at 4°C. The immunoprecipitates were washed 3 times in lysis buffer, followed by two washes in kinase buffer

(50mM TrisHCl pH7.5, 10mM $MgCl_2$, 1mM DTT), and then resuspended in 30µl of kinase buffer. The samples were incubated at 30°C for 15-30min with 20µM ATP, 5-10µCi [γ^{32}] ATP (3000Ci/mmol, Amersham), and 1µg Histone H1 (Sigma) as a substrate for CDK2, or 1µg recombinant pRb (pRb769 from Santa Cruz, Inc) as a substrate for CDK4. The kinase reaction was stopped by addition of 2X loading buffer. After boiling, the kinase reactions were separated on a 10% SDS-PAGE, the gel dried, and the phosphorylated substrates detected by autoradiography.

Pulse-chase analysis:

$1.5x10^6$ quiescent cells were stimulated with 10ng/ml of EGF in suspension for 4 hrs. Cells were then deprived of methionine for 20 min, pulse labelled in suspension for 1 hr with 125 µCi [^{35}S] methionine (NEN, Boston, MA), washed once with PBS, and then chased for up to 3 hrs in non-radioactive, complete medium. Cells were lysed and protein concentration quantified with BCA reagent (Pierce). p27 was immunoprecipitated from 150µg total cell extracts using p27 polyclonal antibody (C19) from Santa Cruz Inc, overnight at 4°C overnight. The immunoprecipitate was collected with protein A sepharose and washed 5 times with lysis buffer. Immunoprecipitates were separated on SDS-PAGE and labeled proteins visualized by fluorography. For densitometry analysis exposed x-ray films were scanned using DNA35 scanner (PDI/Biorad) and the image analyzed with the ImageQuant software from Molecular Dynamics.

RESULTS

Cell cycle distribution of non-adherent A1N4 and MCF-10A

To examine the role of cell adhesion in the regulation of cell cycle progression in human mammary epithelial cells, we first analyzed the cell cycle distribution of two immortalized mammary epithelial cell lines when deprived of adhesion, 184A1N4 (referred to hereafter as A1N4) and MCF-10A,. Exponentially growing A1N4 and MCF-10A cells were trypsinized and maintained in suspension culture for 48 hours. The DNA content of adherent and non-adherent cells was examined by flow cytometry after propidium iodine staining of their nuclei (figure 1A). In contrast to adherent cells, which were present in all the phases of the cell cycle, more than 80% of non-adherent A1N4 and MCF-10A contained 2N DNA, and 10-15% of the cells 4N DNA. Less than 3% of the non-adherent cells had a DNA amount representative of S phase. These results show that, when deprived of adhesion, most of the cells arrest in G1, and that a minor population of cells arrests in G2/M. These observations suggest the presence of two adhesion-dependent regulation checkpoints, one in G1, and one in the G2 phase of the cell cycle. To further investigate the G1 phase adhesion-dependent checkpoint, we synchronized A1N4 cells in G0/G1 phase by EGF deprivation, and then restimulated the quiescent cells to enter the cell cycle by addition of 10ng/ml of EGF (figure1B) (35). Unlike their adherent conterparts, A1N4 cells stimulated with EGF in suspension culture were unable to progress into S phase and were blocked in G1phase (figure 1B).

Regulation of the expression of c-Myc by cell adhesion

To investigate the hypothesis that c-Myc is a critical mediator in the anchorage-dependent G1 checkpoint, we first examined the effect of cell adhesion on mitogen-dependent regulation of expression of c-Myc. Similar to the effect of serum in fibroblasts (59), exposure of adherent quiescent A1N4 cells to EGF induced a strong increase in the levels of c-Myc protein within 1hr of stimulation (figure 2A). Elevated amounts of c-Myc proteins were maintained for at least 8 hrs. When A1N4 cells were stimulated with EGF in suspension, a weaker increase in c-Myc still occurred. However, by 4 hrs of suspension culture, c-Myc became undetectable by Western blot, indicating a strong decrease in the amount of c-Myc when cells are deprived of adhesion (figure

2A). The direct effect of adhesion on c-Myc levels was confirmed allowing cells, maintained in suspension for 4 hrs, to reatttach (figure 2.B). The level of the c-Myc protein started to increase within 2 hrs of cell adhesion, and by 4 hrs the expression of c-Myc reached a level comparable to that present in cells grown in monolayer. To ensure that the regulation of c-Myc by adhesion is not a particularity of the A1N4 cell line, we also tested another human mammary epithelial cell line, the MCF-10A (figure 2C). When exponentially growing MCF-10A are deprived of adhesion, their level of c-Myc protein is decreased within 2 hrs, and c-Myc became undetectable by immunoblotting after 6 hrs of suspension culture.

We asked whether this decrease of c-Myc occurred at the mRNA level (figure 2D). The initial induction of Myc mRNA, following EGF stimulation, was similar in both adherent and non-adherent cells. However, after 4 hrs, a 3-fold decrease in the levels of mRNA was observed in suspension culture, as compared to adherent cells. These results suggest that cell adhesion is required for maximal, sustained expression of c-Myc in epithelial cells. Treatment of cells with cycloheximide reversed the decrease of c-Myc mRNA in suspended cells, indicating that protein synthesis or a labile protein is required for down regulation of c-Myc mRNA. Furthermore, treatment of cells with Actinomycin D indicated that the decrease in c-Myc mRNA was not due to a decrease in its stability (data not shown). Therefore, these data suggest a transcriptional regulation of the c-myc gene by cell adhesion. However, we cannot exclude the presence of a post-transcriptional mode of regulation. These results indicate that cell adhesion is required both for the expression of c-Myc in cycling epithelial cells, and for the EGF-induced, sustained expression of c-Myc, when quiescent cells are stimulated to re-enter the cell cycle.

c-Myc prevent early G1 arrest in non-adherent cells

To test whether this decrease in amount of c-Myc is responsible for the G1 phase block observed in non-adherent epithelial cells, we used A1N4 cells stably transfected with c-Myc under a constitutive promoter (A1N4-Myc cells) (60). In suspension culture, A1N4-Myc cells express levels of c-Myc within 1.5 -fold order of magnitude, compared to adherent A1N4 cells (figure 3), indicating that the level of c-Myc restored in non-adherent cells is likely to be physiologically

relevant. The ability of A1N4-Myc cells to progress through the G1 phase in suspension culture was assessed by the phosphorylation of the retinoblastoma protein (pRb) and the expression of the E2F-1 transcription factor (figure 4). As adherent cells progress through G1, pRb is progressively phosphorylated at multiple sites by the G1 cyclin-dependent kinases (22,32). In early G1, pRb is present in its hypophosphorylated form, which can be separated by SDS-PAGE from its hyperphosphorylated form, characteristic of late G1 (figure 4A). In quiescent A1N4 cells, pRb is only detected in its hypophosphorylated, faster migrating form, which shifts to the hyperphosphorylated form, following EGF stimulation of cells grown in monolayer. In contrast, EGF-stimulated, non-adherent cells failed to hypophosphorylate pRb, confirming at the molecular level that non-adherent A1N4 cells are blocked in early G1. However, A1N4-Myc cells hyperphosphorylated pRb under non-adherent conditions, indicating that deregulated expression of c-Myc enables cells to override the adhesion-dependent, early G1 phase checkpoint.

Levels of E2F-1 can also be used as a marker of G1 phase progression, since the expression of this transcription factor is induced in mid to late G1 (3,27). The E2F-1 protein starts to be detectable within 6 hrs following EGF stimulation of quiescent, adherent A1N4 cells, and its level is markedly increased by 12hrs, a time corresponding to the G1/S transition. Reflecting the G1 arrest, the expression of E2F-1 is not induced in non-adherent A1N4 cells. Adherent A1N4-Myc cells contain 2.5-fold higher levels of E2F-1 after 12 hrs of EGF stimulation. Although delayed, the expression of E2F-1 is rescued in non-adherent A1N4-Myc cells, where by 12 hrs it is expressed at levels comparable to adherent A1N4 cells (figure 4B). These observations support our pRb phosphorylation results, indicating that non-adherent cells expressing c-Myc progress through the adhesion-dependent G1 check point to the G1/S transition.

c-Myc is not required for the expression and activation of the cyclin D-CDK4 complexes

Since pRb is phosphorylated in non-adherent A1N4-Myc cells, we asked whether c-Myc is able to induce the expression and activity of the various G1 cyclin-CDK complexes in an adhesion-independent manner. We first examined the cyclin D-CDK4 complexes (figure 5). The transcription of Cyclin D1 is decreased in some fibroblasts deprived of adhesion, resulting in a

decrease in cyclin-CDK4 activity (64). Northern blot analysis indicated that, although in suspension culture a lag period is required for EGF-induced upregulation of cyclin D1 mRNA, by 12 hrs equivalent levels of cyclin D1 transcripts could be detected in both adherent and non-adherent A1N4 cells (figure 5A). Similar kinetics in the induction of cyclin D1 mRNA were observed in A1N4-Myc cells, which expressed slightly higher levels of cyclin D1 mRNA (1.5-fold increase) than A1N4 cells (figure 5A). Western blot analysis for cyclin D3 showed an identical delay in the induction of cyclin D3 protein, caused by a lack of adhesion. Nevertheless, both A1N4 and A1N4-Myc cells expressed similar amount of cyclin D3, in an anchorage-independent manner (figure 5B). This observation, that non-adherent cells induce cyclin D1 and D3 in response to EGF, indicates that the adhesion-dependent checkpoint in A1N4 cells is in the early-mid G1 phase. The level of CDK4 protein was constant in both A1N4 and A1N4-Myc cells, regardless of whether they were quiescent, stimulated with EGF in monolayer, or in suspension (figure 5.B). In addition, an *in vitro* kinase assay, where the cyclin D-CDK4 complex was immunoprecipitated with a CDK4 antibody and incubated with recombinant Rb protein as a substrate, indicated that the levels of CDK4 kinase activity were similar in non-adherent A1N4 and A1N4-Myc cells (figure 5C). Therefore, we concluded that cyclin D-CDK4 activity is still present in non-adherent A1N4 cells, and that overexpression of c-Myc does not alter the activity of the CDK4 complexes.

c-Myc prevents the inhibition of the cyclin E-CDk2 complex in non-adherent cells

We next examined the cyclin E-CDK2 complex (figure 6). To assess the activity of cyclin-dependent kinase 2 (CDK2), we immunoprecipitated CDK2 from total cell lysates. The kinase activity of these immunoprecipitates was tested by an *in vitro* kinase assay, using histone H1 as an exogenous substrate. Whereas lack of adhesion completely inhibited CDK2 kinase activity, overexpression of c-Myc in non-adherent cells restored CDK2 activity to levels similar to those detected in adherent cells (Figure 6A). To explain these differences in kinase activity between non-adherent A1N4 and A1N4-Myc cells, we examined by Western blotting the levels of positive and negative regulators of CDK2 present in total cell lysates. Unlike the parental cells, that only expressed very low level of cyclin E, A1N4-Myc cells induced cyclin E in suspension (figure 6B).

In adherent, EGF-stimulated A1N4 cells, CDK2 migrates as a doublet on SDS-PAGE. Others have reported that the faster migrating form correspond to a molecules containing a stimulatory phosphorylation on threonine 160 (13,16). Similarly to quiescent A1N4 cells, CDK2 was predominantly present in its slower migrating form in non-adherent A1N4 cells. However, the faster migrating form of CDK2 was strongly detectable in non-adherent A1N4-Myc cells (figure 6B), confirming the difference in CDK2 kinase activity observed in the *in vitro* kinase assay. This difference in kinase activity could be explained, at least in part, by the difference in the level of the CDK2 inhibitor, p27, which was significantly decreased in the A1N4-Myc cells (figure 6B). In contrast to the non-adherent A1N4 cells, where the total levels of p27 were strongly increased, no increase in p27 was detectable in the A1N4-Myc cells. Moreover, co-immunoprecipitation studies indicated that p27 is found complexed with CDK2, but not with CDK4 in non-adherent A1N4 cells (figure 6C). The amount of p27 associated with the CDK2 complex was decreased by 3-4-fold in non-adherent A1N4-Myc cells, where the CDK2 complex was mostly free of p27. The effect of both cellular adhesion and deregulated expression of c-Myc is specific to p27, since the levels of the CDK inhibitor p21 did not differ, comparing the two culture conditions (figure 6B). Together, the increased expression of cyclin E, the phosphorylation of CDK2, and the decreased association of p27 with the CDK2 complex may contribute to the induction of CDK2 activity in non-adherent A1N4-Myc cells.

c-Myc destabilizes the p27 CKI in non-adherent cells

CDK2 kinase activity, observed in A1N4-Myc, cells is correlated with a decrease in the steady state level of the p27 protein. p27 is predominantly regulated at the post-transcriptional level, and the p27 protein has been shown to be rapidly degraded by the proteasome-ubiquitin pathway (24,40). To further investigate the mechanism by which the level of p27 are decreased in non-adherent A1N4-Myc cells, we first compared the steady state levels of p27 mRNA between A1N4 and A1N4-Myc cells. Non-adherent A1N4-Myc cells showed no significant decrease in total p27 mRNA (figure 7A). To determine whether the levels of p27 were regulated by a post-transcriptional mechanism, the stability of the p27 protein was examined by pulse-chase analysis

(figure 7B). Non-adherent A1N4 and A1N4-Myc cells were labeled for 1 hour with [S^{35}] methionine, and chased in non-radioactive medium for up to 180 mins. Our results indicated that the half-life of p27 in suspended A1N4 cells was of about 100 mins. In contrast, the half-life of p27 protein decreased to less than 35 mins in A1N4-Myc cells. To examine whether the rapid degradation of the p27 protein observed in A1N4-Myc cells was caused by the ubiquitin-proteasome pathway, we treated A1N4-Myc cells, in suspension culture, with the proteasome inhibitor ALLN (figure 7C). Since the ALLN peptide aldehyde also inhibits calpains I and II, and cathepsin B and L, we also treated cells with a control peptide, ALLM, which has an equivalent inhibitory affinity for calpains I and II, and cathepsin B and L, but has no effect on the proteasome. Incubation with the proteasome inhibitor ALLN specifically led to a 3-fold increase in the p27 protein, indicating that the proteasome-dependent degradation contributes to the decreased levels of p27 present in non-adherent A1N4-Myc cells.

Since the A1N4-Myc cells are stably transfected with the c-myc gene, we wished to ensure that the alteration observed in the levels of p27 was a direct consequence of elevated levels of expression of c-Myc, and not due to selection in tissue culture. In addition, we wished to show that this effect of c-Myc on adhesion-dependent regulation of p27 is not a pecularity of the A1N4 cell line. Therefore, we extended our study to the anchorage-dependent MCF-10A cells. We transiently transfected MCF-10A with the PLXSN retroviral construct carrying human c-myc under a constitutive promoter (figure 7, D). When deprived of adhesion, MCF-10A cells increased their levels of p27 protein. This induction in the levels of p27 correlated with a decrease in c-Myc protein (figure 2 and 7, D), and with G1 arrest (figure 1). Expression by retroviral infection of c-Myc in MCF-10A cells was sufficient to prevent the increase in the levels of p27 under non-adherent culture conditions (figure 7, D). Similar to the non-infected MCF-10A cells, the level of p27 was increased in non-adherent control cells infected with retrovirus carrying the PLXSN vector alone.

DISCUSSION

Although c-Myc is known to be critical for the progression of the G1/S phase of the cell cycle, particularly in response to growth factor stimuli, its role is unclear in the regulation of cell cycle progression in response to cell-substrate adhesion. In this report, we analyze the role of c-Myc in the anchorage-dependent G1 checkpoint in human mammary epithelial cells (figure 8). We show that the expression of c-Myc is tightly regulated by cell adhesion in human mammary epithelial cells. In non-adherent cells the decrease in the levels of c-Myc correlates with G1 cell cycle arrest. However, restoration of the expression of c-Myc in non-adherent epithelial cells allows these cells to bypass the adhesion requirement for the expression and activation of key targets at the G1/S transition. Expression of c-Myc in non-adherent cells prevents the stabilization of p27, restores the expression of cyclin E, and thereby results in the adhesion-independent activation of the CDK2 complex and the hyperphosphorylation of pRb. In addition, the expression of c-Myc in non-adherent cells rescues the expression of E2F-1 in response to the EGF stimuli. Taken together, these results suggest that c-Myc mediates the adhesion-dependent regulation of the cyclin E-CDK2, complex and of the E2F-1 transcription factor.

Previous reports have shown considerable variation, based on the model used, on the effect of cell adhesion on the expression of c-Myc mRNA. We found that cell adhesion is not necessary for the initial, immediate induction of c-Myc by EGF. Rather, cell adhesion is required for the sustained expression of c-Myc in cycling cells. In the absence of adhesion, A1N4 and MCF-10A epithelial cells rapidly decrease their levels of c-Myc, contrasting with reports in BALB/3T3 cells,and in primary cultures of human skin fibroblasts (4,9). Cell adhesion in mammary epithelial cells regulates the expression of c-Myc, at least in part, at the level of transcription, since a 3-fold decrease in Myc mRNA is observed in non-adherent cells. This decrease in c-Myc cannot be accounted for by a decrease in its mRNA stability. However, an additional post-trancriptional mechanism of regulation might play a role, since the decrease in the c-Myc protein is greater than that the one observed at the level of c-Myc mRNA. c-Myc can be regulated both at the levels of translation initiation and of protein stability (14,46,55). It is tempting to speculate that adhesion

may alter the stability of c-Myc, since c-Ras can stabilize c-Myc (47), and since signaling by c-Ras is adhesion-dependent (25,31).

To address whether regulation of c-Myc is required for the adhesion-dependent checkpoint, we compared A1N4 cells with stably transfected A1N4-Myc cells, which constitutively express murine c-Myc. Enforced expression of c-Myc in non-adherent cells is sufficient to restore pRb phosphorylation. Both the cyclin D-CDK4 complex and the cyclin E-CDK2 complex participate in the phosphorylation of pRb (22,32), and their expression and activity is regulated by cell adhesion (13,64). The transcription of cyclin D1 is regulated by cell adhesion in some fibroblast systems (64), but not others (13,53). In our human mammary epithelial A1N4 cells, the induction of cyclin D1 mRNA is growth factor-dependent, but adhesion-independent. Although cell adhesion is not nescessary for the induction of cyclin D1, it alters the kinetics of accumulation of cyclin D1. The peak expression of cyclin D1, following EGF stimulation, is delayed by 6 hrs in cells maintained in suspension, as compared to those in monolayer. This induction of cyclin D1 indicates that non-adherent A1N4 cells are still responsive to EGF, and are able to progress through mid-G1 phase in an anchorage-independent manner. In addition to the expression of cyclins D1 and D3, two other lines of evidence indicate that the cyclin D-CDK4 complex is not mediating the anchorage-dependent control of G1 phase progression, nor is this complex a target of c-Myc. First, the levels of the CDK4 inhibitor p16 are low in the A1N4, and remain unaffected by either adhesion or c-Myc (data not shown). Furthermore, the CDK4 complex is active in non-adherent A1N4 cells, and this activity is not altered by c-Myc.

In contrast, we found that cell adhesion is strictly required for the activation of CDK2 in A1N4, and expression of c-Myc can reverse that effect. Our results suggest that the ability of c-Myc to rescue the activity of CDK2 in non-adherent cells is mediated through at least two steps. First, the amount of the positive regulator of CDK2, cyclin E, is increased. Second, the level of the CDK2 inhibitor, p27, is decreased. In contrast to fibroblasts which induce cyclin E under non-adherent conditions (7,13,53,64), the expression of cyclin E is adhesion-dependent in the A1N4 cells. Expression of c-Myc in suspended cells is sufficient to restore the amount of cyclin E to a

level similar to the one present in adherent cells. Consequently, increased availability of cyclin E may allow its association and activation CDK2. Deregulated expression of c-Myc results in an increase in the level of cyclin E under non-proliferative conditions, such as serum starvation and confluence arrest (26,58), where the levels of cyclin E are normally very low. In contrast, c-Myc does not affect the level of cyclin E in exponentially growing cells (26). Similarly, upon EGF stimulation, adherent A1N4 and A1N4-Myc cells do not display any significant difference in the induction of cyclin E. However, expression of c-Myc in non-adherent cells promotes the expression of cyclin E. This difference in the effect of c-Myc, based on the proliferative status of the cells, suggests that c-Myc may play a role in the de-repression of cyclin E, rather than its direct induction. The ability of c-Myc to relieve transcriptional repression has recently been described for the cyclin D2 gene (5). Alternatively, the trancription of cyclin E in non-adherent cells may be driven by E2F-1, whose expression is rescued in A1N4-Myc cells. The cyclin E promoter contains an E2F binding site, and E2F-1 overexpression increases the level of cyclin E (8,38). Our current data do not indicate whether the expression of E2F-1 precedes the expression of cyclin E. However, the 2.5-fold increase in levels of E2F-1 detected in adherent A1N4-Myc cells does not result in an increase in the levels of cyclin E, suggesting that E2F-1 alone may not be implicated in the regulation of cyclin E by c-Myc.

In addition to the decrease in cyclin E, the increase in the levels of the CDK inhibitor, p27, participates in the inhibition of the CDK2 complex in non-adherent cells. Similar to previous reports, the absence of adhesion results in a 3-4-fold increase in association of p27 with CDK2. This magnitude of increased binding of p27 to CDK2 has been reported to be sufficient to inhibit CDK2 (21). Expression of c-Myc in non-adherent cells decreases the levels of p27 associated with CDK2 to levels similar to those observed in adherent, proliferating A1N4 cells. c-Myc is believed to indirectly induce the activation of the cyclin E-CDK2 complex by inducing the transient interaction of p27 with a "heat labile" protein, and thus, displacing p27 from the CDK2 complex (34,42,58,62). Two mechanisms have been proposed to drive the release of p27 from the CDK2 complex. First, a c-Myc-dependent increase in cyclin D1 and D2 may titer p27, displacing this

CKI from the cyclinE-Cdk2 complex to the cyclinD-CDK4 complex (5,41). However, in other models, cyclin D expression is not altered by c-Myc, and is not required for the activation of cyclinE-CDK2 complex. Nevertheless, c-Myc induces the dissociation of CDK2-phosphorylated p27 from the cyclin E-CDK2 complex. Although not required for c-Myc-dependent activation of cyclin E-CDK2 complex, the phosphorylation and release of p27 from the CDK2 complex is believed to be the first step for targeting of p27 for degradation through the ubiquitin-proteosome pathway (34,40,42). Even though the A1N4-Myc cells express a slightly higher level of cyclin D1 mRNA than the parental A1N4 cells, this does not influence the distribution of p27. We could not detect any p27 associated with CDK4 by co-immunoprecipitation, even though we could detect the association of p27 with a minor population of the CDK2 complexes in non-adherent A1N4-Myc cells. Therefore, c-Myc appears to induce the release of p27 from the CDK2 complex, which leads to its degradation through the ubiquitin-proteasome pathway, rather than its displacement from the CDK2 complex to the CDK4 complex. In agreement with this hypothesis, the stability of p27 is sharply decreased in non-adherent A1N4-Myc cells. In addition, treatment of non-adherent A1N4-Myc cells with a proteasome inhibitor results in a 3-fold increase in p27. In A1N4, c-Myc specifically regulates the levels of p27. The level of another member of the same family of CDK inhibitors, p21, was not altered by adhesion or c-Myc. We were unable to assess the effect of adhesion and c-Myc on the third member of this CDK family, p57, since A1N4 cells do not express this CKI (36).

In both A1N4 and MCF-10A cells, the expression of c-Myc and of p27 are inversely correlated. Extracellular, anti-mitogenic signals also elicit a similar pattern. TGF-β-induced growth arrest of epithelial and endothelial cells, and growth inhibition due to cell-cell contact, each of which are believed to be mediated by the induction of p27, also result in the decrease of c-Myc levels (1,28,43,49). Mutual exclusion of c-Myc and p27 expression, in addition to the ability of c-Myc to decrease the level of p27, suggests that a decrease in levels of c-Myc is a general mechanism whereby anti-mitogenic signals induce p27 stabilization and cell cycle arrest.

Ectopic expression of c-Myc has been reported to rescue the expression of cyclin A in non-adherent Rat1 fibroblasts (2). We have made a similar observation in the A1N4 cells. Expression of cyclin A is adhesion-dependent, and deregulated expression of c-Myc can induce the expression of cyclin A in non-adherent A1N4 epithelial cells (data not shown). The results presented in this report suggest a mechanism whereby c-Myc influences the expression of cyclin A in non-adherent cells. Repression of the transcription of cyclin A in non-adherent cells is mediated by the E2F binding site in the cyclin A promoter (53). Serum stimulation of quiescent cells results in a switch from the transactivation-repressive, E2F-p107 complex to free E2F and the E2F-p107-cyclin E complex under adherent conditions. In contrast, in non-adherent cells, this switch does not occur, thereby maintaining the inhibition of cyclin A transcription. In addition, p27 prevents the activation of the cyclin A promoter by blocking the association of cyclin E-CDK2 with the E2F-p107 complex (63). Therefore, the decrease in the levels of p27, the increase in the levels of cyclin E, the activation of CDK2, and the phosphorylation of members of the Rb family of proteins by c-Myc may all be implicated in the regulation of transcripton of the cyclin A gene. Furthermore, enforced expression of c-Myc in non-adherent cells rescues the expression of E2F-1. This c-Myc-dependent induction of E2F could provide free E2F to drive the transcription of cyclin A. These data are consistent with our recent observation of c-Myc-associated expression of E2F-1 and cyclin A in transgenic mouse mammary tumorigenesis *in vivo* studies (30).

How c-Myc participates in the induction of E2F-1 is not clear. E2F-2, an E2F family member which has a cell cycle-dependent pattern of expression similar to E2F-1, has been proposed to be a direct target of c-Myc, based on the finding that the E2F-2 gene contains a c-Myc-responsive E box in its promoter (48). However, no c-Myc-binding element has yet been identified in the E2F-1 promoter. Alternatively, c-Myc could induce the expression of E2F-1 through the inactivation of the Rb family of pocket proteins. In quiescent cells, the transcription of E2F-1 is repressed by the binding of the Rb family-E2F complexes to the E2F binding site in the E2F-1 promoter (3). This repression may not be released when cells are stimulated with EGF in suspension, thereby preventing the expression of E2F-1 that is normally observed in adherent cells late in G1. In

response to EGF, phosphorylation of pRb in non-adherent, c-Myc-transfected cells may release the trancriptional repression of the E2F-1 gene, and allow for anchorage-independent expression of E2F-1. Our data indicate that non-adherent cells are still responsive to EGF, and that EGF is required for c-Myc-induced G1 phase progression in these cells.

Not only do cyclin E-CDK2 and E2F reciprocally influence the expression and activity of each other, but also they collaborate in the induction of S phase and of DNA replication (29). Our results show that both cyclin E-CDK2 and E2F-1 are regulated by cell adhesion in epithelial cells. Furthermore, c-Myc can bypass the adhesion control of G1/S phase transition at multiple points by restoring the activity of cyclin E-CDK2, as well as the expression of E2F-1. Thus, a decrease in the level of c-Myc in non-adherent cells is likely to be a critical event to prevent anchorage-independent progression into S phase.

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Figure 1: Flow cytometry of cell cycle distribution in adherent and non-adherent HMEC. A. Exponentially growing A1N4 and MCF-10A cells were refed fresh medium, either as a monolayer, or in suspension for 48 hrs. Cells were collected, their nuclei stained with propidium iodine, and their DNA content analyzed by flow cytometry. B. Quiescent A1N4 cells (A) were stimulated with EGF as a monolayer for 20 hrs (B) and 24 hrs (C), or in suspension for 24 hrs (D). Cell DNA content was analyzed by flow cytometry, as above.

Figure 2: Regulation of c-Myc expression by cell adhesion in human mammary epithelial cells. A. Quiescent A1N4 cells were restimulated to enter the cell cycle with EGF, either as a monolayer or in suspension. Cells were collected at the indicated times, following EGF stimulation. B. A1N4 cells maintained in suspension for 4 hrs in the presence of EGF, were transferred to tissue culture dishes, and was allowed to reattach for the indicated times. C. Non-synchronized MCF-10A cells were refed fresh medium either as a monolayer (att) or in suspension (susp) for the indicated times. Whole cell lysates were prepared and cells analyzed for levels of c-Myc by western blotting (A, B, and C). The lower band detected by the c-Myc antibody in figure C migrates at the same size than MycS (46Kd). D. Quiescent A1N4 cells were stimulated with EGF as a monolayer or in suspension for the indicated times. Total RNA was analyzed for c-Myc mRNA levels by Northern blot hybridization. Equal RNA loading was verified using a probe for glyceraldehyde phosphate dehydrogenase (GAPDH).

Figure 3: Expression of c-Myc in non-adherent A1N4-Myc cells. Quiescent A1N4 and A1N4-Myc cells were stimulated with EGF for 6hrs and 15hrs, either as a monolayer (A6, A15) or in suspension (S6, S15). Total cell lysates were analyzed for levels of c-Myc by immunoblotting with a polyclonal c-Myc antibody.

Figure 4: Myc overexpression induces pRb phosphorylation and E2F-1 expression in non-adherent A1N4 cells. Quiescent (0) A1N4 and A1N4-Myc cells were stimulated for the

indicated times with EGF, either as a monolayer (A) or in suspension (S). Whole cell lysates were analyzed by western blotting for pRb (A) and E2F-1 (B). The slower migrating band represents the hyperphosphorylated form of pRb (Rb-P).

Figure 5: Cyclin D-CDK4 complex expression and activity are not affected by c-Myc Quiescent (0) A1N4 and A1N4-Myc cells were stimulated for the indicated times with EGF either as a monolayer (A) or in suspension (S). Cells were collected, and either total RNA isolated, or total cell lysates prepared. A. Northern blot analysis of cyclin D1 mRNA, showing both cyclin D1 major (4.5Kb) and minor (1.5 Kb) transcripts. Blots were reprobed with GAPDH probe as a loading control. B. Western blot analysis for cyclin D3 and CDK4. C. CDK4 was immunoprecipitated from total cell lysates, and the immunoprecipitate incubated with [γP^{32}] ATP and recombinant Rb as an exogenous substrate. The *in vitro* kinase reaction was separated by SDS-PAGE and the gel exposed to x-ray film.

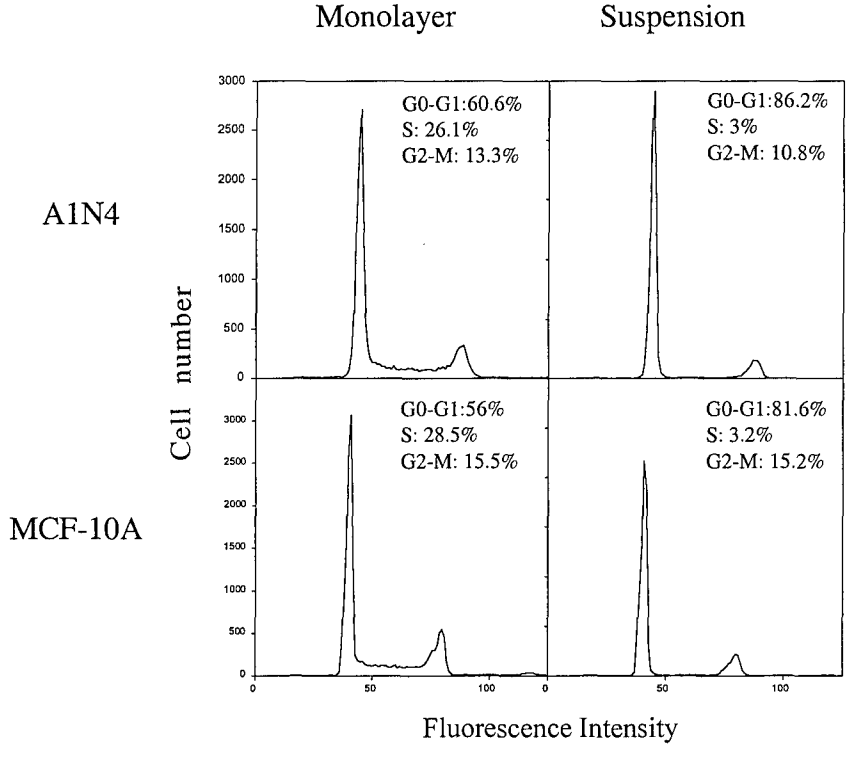
Figure 6: Overexpression of c-Myc upregulates cyclin E-CDK2 activity in non-adherent A1N4 cells. Quiescent (0) A1N4 and A1N4-Myc cells were stimulated with EGF for 12 hours, either as a monolayer (A) or in suspension (S). A. CDK2 *in vitro* kinase assay. CDK2 imunoprecipitates were incubated with [γP^{32}] ATP and histone H1 as an exogenous substrate. The kinase reaction was separated by SDS-PAGE and phosphorylated histone detected by radiography. B. Total cell lysates were analyzed by western blotting for the levels cyclin E, CDK2, p27 and p21. CDK2 migrates on SDS-PAGE as a doublet. C. CDK2 and CDK4 complexes were imunoprecipitated from total cell lysates with anti-CDK2 and anti-CDK4 antibodies, respectively. The immunoprecipitates were frationated on SDS-PAGE and the blot analyzed by Western for the presence of p27. The blot was then reprobed with an anti-CDK2 antibody.

Figure 7: c-Myc alters the post-translational regulation of p27 in non-adherent cells. A. Quiescent A1N4 and A1N4-Myc cells were stimulated with EGF for 12 hrs, either as a monolayer

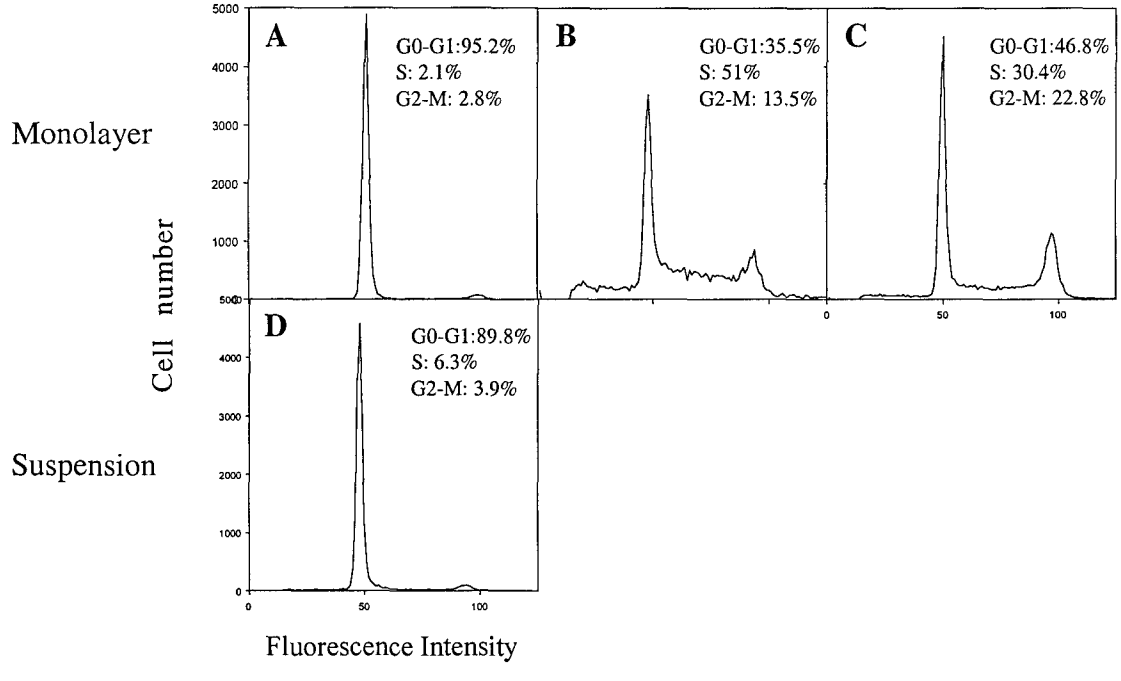
(A) or in suspension (S). Total RNA was used for Northern blot analysis of p27 mRNA levels. Ethidium bromide staining of the gel showed the 18S and 28S ribosomal RNA as a loading control. B. A1N4 and A1N4-Myc cells, stimulated with EGF in suspension for 4 hrs, were pulse labelled with [S^{35}] methionine for 1 hr, and chased with non-radioactive medium for the indicated times. p27 was immunoprecipitated from total cell lysate, the immunoprecipitate was frationated on SDS-PAGE, and radioactive p27 visualized by fluorography (left panel). The signal intensity in the left panel was quantified by densitometry and plotted against chase time. The signal intensity at time zero represents 100% (right panel). C. A1N4-Myc cells were stimulated for 12 hrs in suspension with EGF alone or in the presence of the calpains and proteosome inhibitors ALLN, ALLM, or the DMSO vehicle. A1N4 cells stimulated with EGF for 12hrs in suspension were used for comparaison. Levels of p27 present in total cell lysate were determined by western blot. D. Exponantially growing MCF-10 cells were transiently transfected using a retrovirus carrying either PLXSN vector control or PLXSN carrying human c-Myc (PLXSN-MYC). 48 hrs after the infection, transfected, as well as non-transfected MCF10A were trypsinized and maintained in suspension in complete medium for the indicated times. Total cell lysates were analysed by Western blotting for the levels of c-Myc and p27.

Figure 8: Model for the role of c-Myc in mediating the adhesion dependent G1 checkpoint

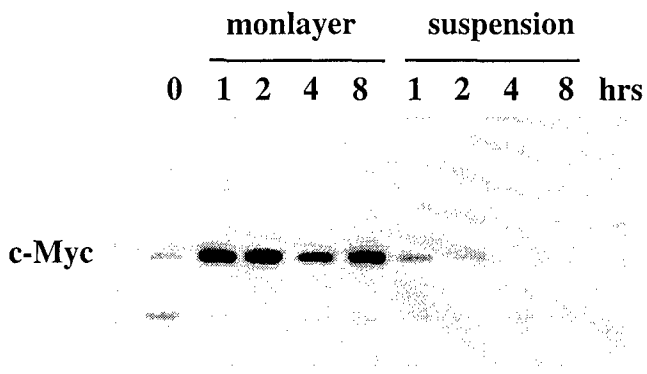
A


Monolayer
Suspension
A1N4
MCF-10A
Cell number
G0-G1:60.6%
S: 26.1%
G2-M: 13.3%
G0-G1:86.2%
S: 3%
G2-M: 10.8%
G0-G1:56%
S: 28.5%
G2-M: 15.5%
G0-G1:81.6%
S: 3.2%
G2-M: 15.2%
Fluorescence Intensity

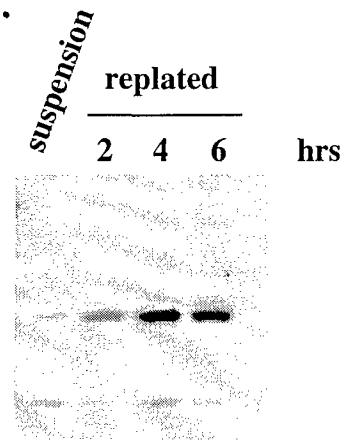
B


Monolayer
Suspension
Cell number
A
G0-G1:95.2%
S: 2.1%
G2-M: 2.8%
B
G0-G1:35.5%
S: 51%
G2-M: 13.5%
C
G0-G1:46.8%
S: 30.4%
G2-M: 22.8%
D
G0-G1:89.8%
S: 6.3%
G2-M: 3.9%
Fluorescence Intensity

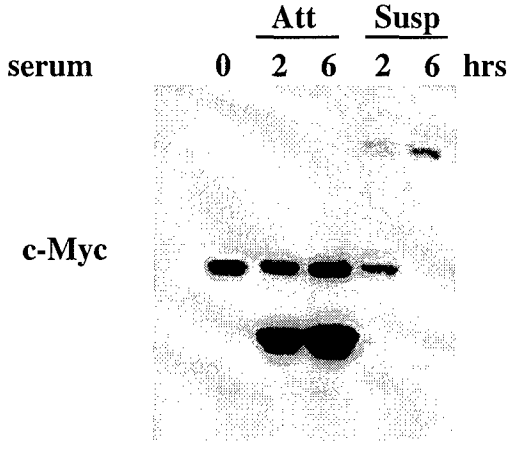
A.


monlayer
suspension
0 1 2 4 8 1 2 4 8 hrs
c-Myc

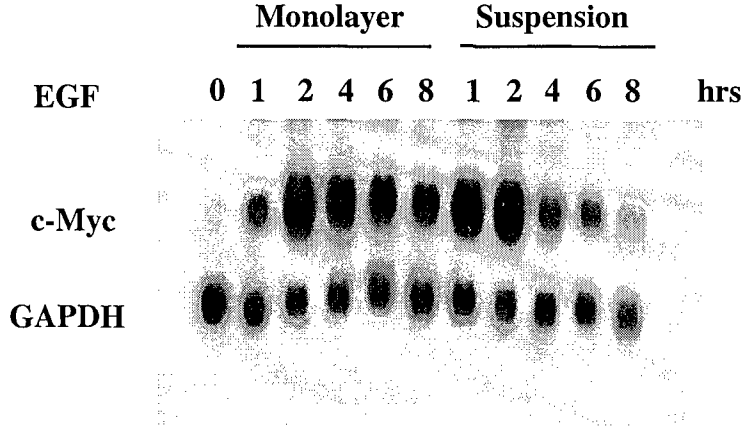
B.

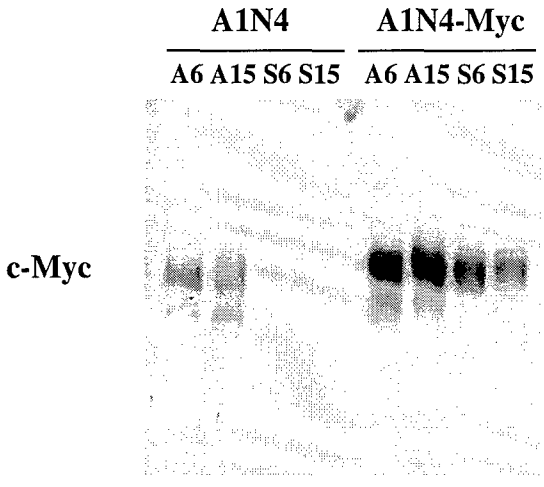

suspension
replated
2 4 6 hrs

C.

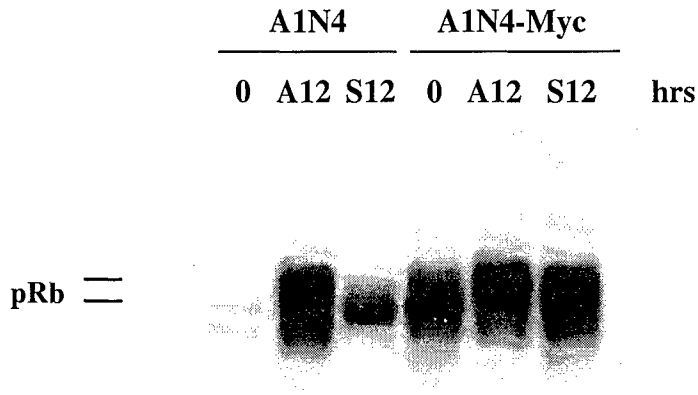

Att
Susp
serum
0 2 6 2 6 hrs
c-Myc

D.

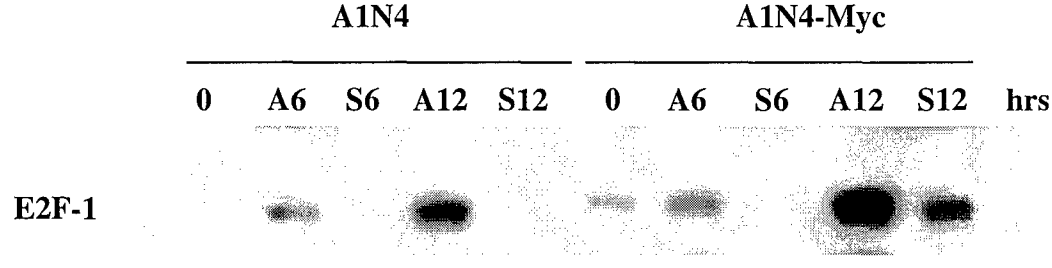

Monolayer
Suspension
EGF
0 1 2 4 6 8 1 2 4 6 8 hrs
c-Myc
GAPDH


A1N4
A1N4-Myc
A6 A15 S6 S15
A6 A15 S6 S15
c-Myc

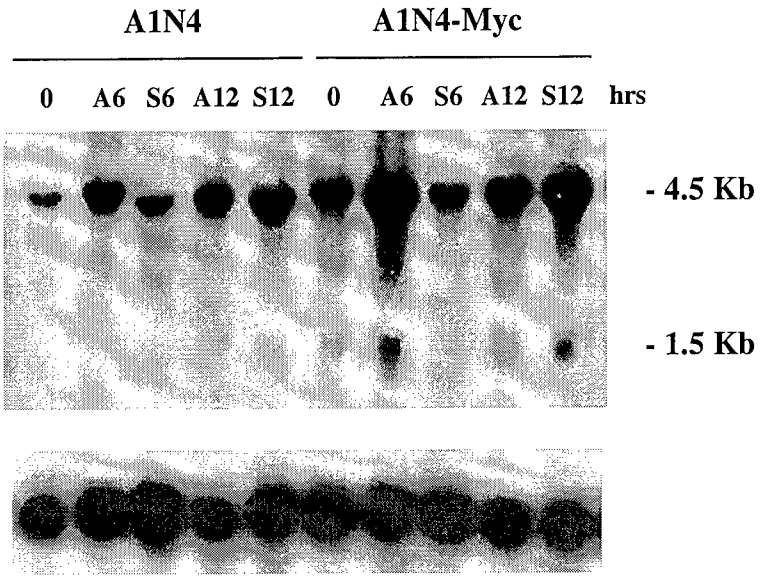
A.


A1N4
A1N4-Myc
0 A12 S12 0 A12 S12 hrs
pRb

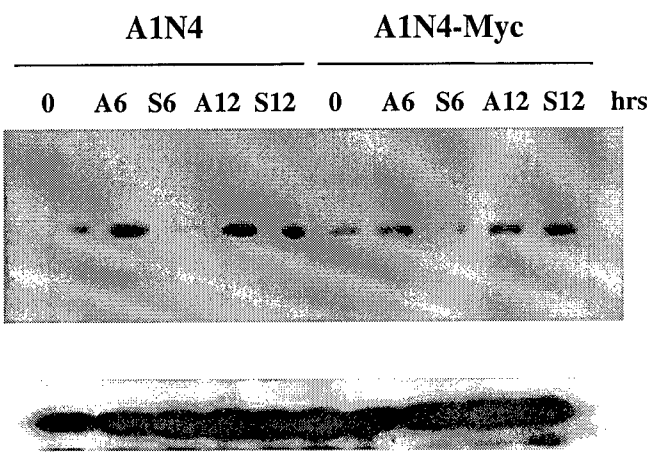
B.


A1N4
A1N4-Myc
0 A6 S6 A12 S12 0 A6 S6 A12 S12 hrs
E2F-1

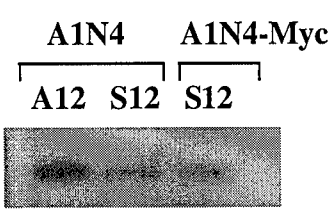
A


A1N4
A1N4-Myc
0 A6 S6 A12 S12 0 A6 S6 A12 S12 hrs
- 4.5 Kb
- 1.5 Kb
Cyclin D1 mRNA
GAPDH

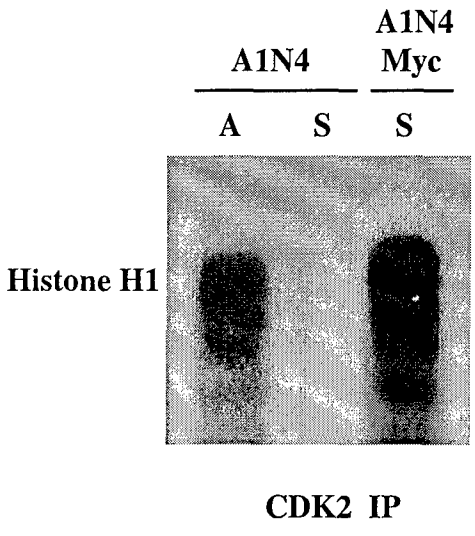
B


A1N4
A1N4-Myc
0 A6 S6 A12 S12 0 A6 S6 A12 S12 hrs
Cyclin D3
CDK4

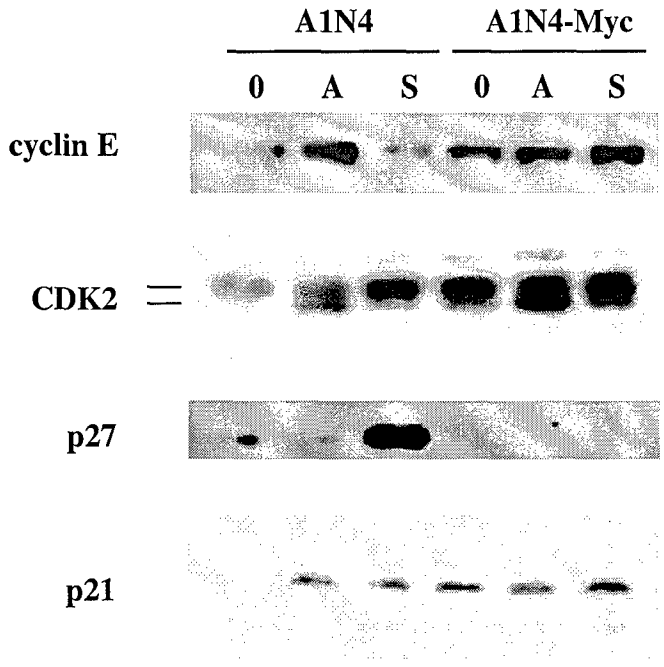
C


A1N4
A1N4-Myc
A12 S12 S12
Rb

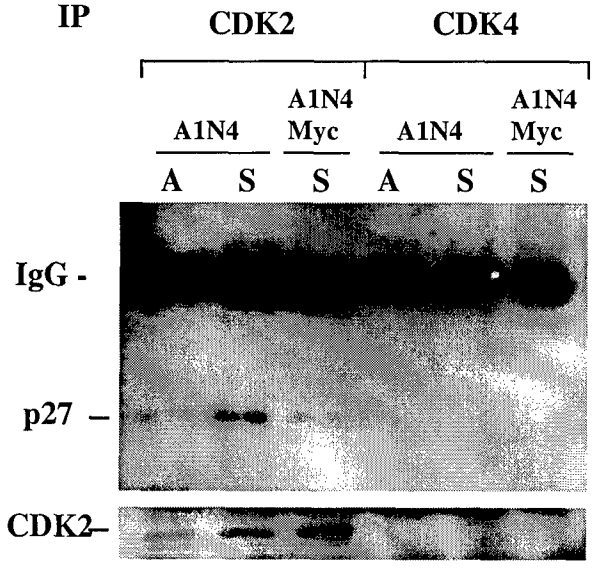
A.


A1N4
A1N4 Myc
A
S
S
Histone H1
CDK2 IP

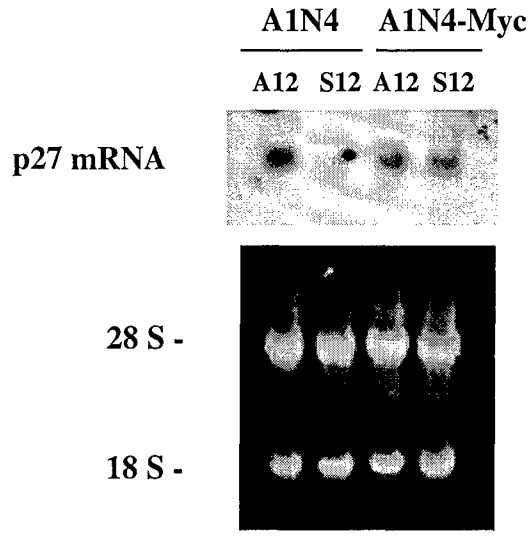
B.


A1N4
A1N4-Myc
0
A
S
0
A
S
cyclin E
CDK2
p27
p21

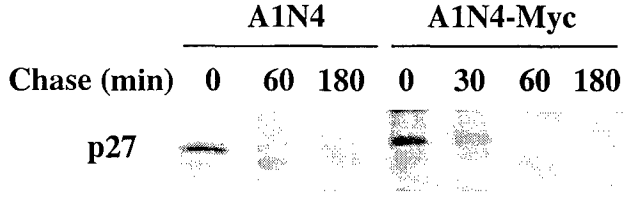
C.

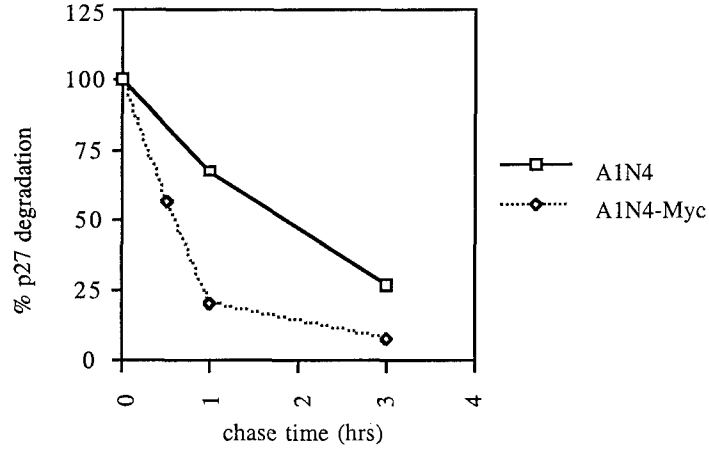

IP
CDK2
CDK4
A1N4
A1N4 Myc
A1N4
A1N4 Myc
A
S
S
A
S
S
IgG -
p27 –
CDK2–

A.

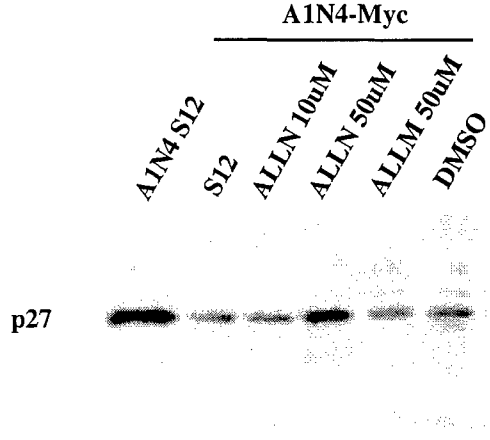

A1N4
A1N4-Myc
A12
S12
A12
S12
p27 mRNA
28 S -
18 S -

B.

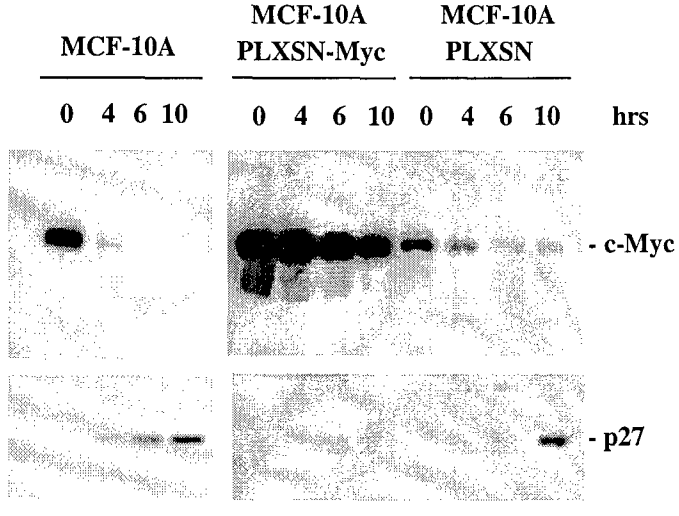

A1N4
A1N4-Myc
Chase (min)
0
60
180
0
30
60
180
p27

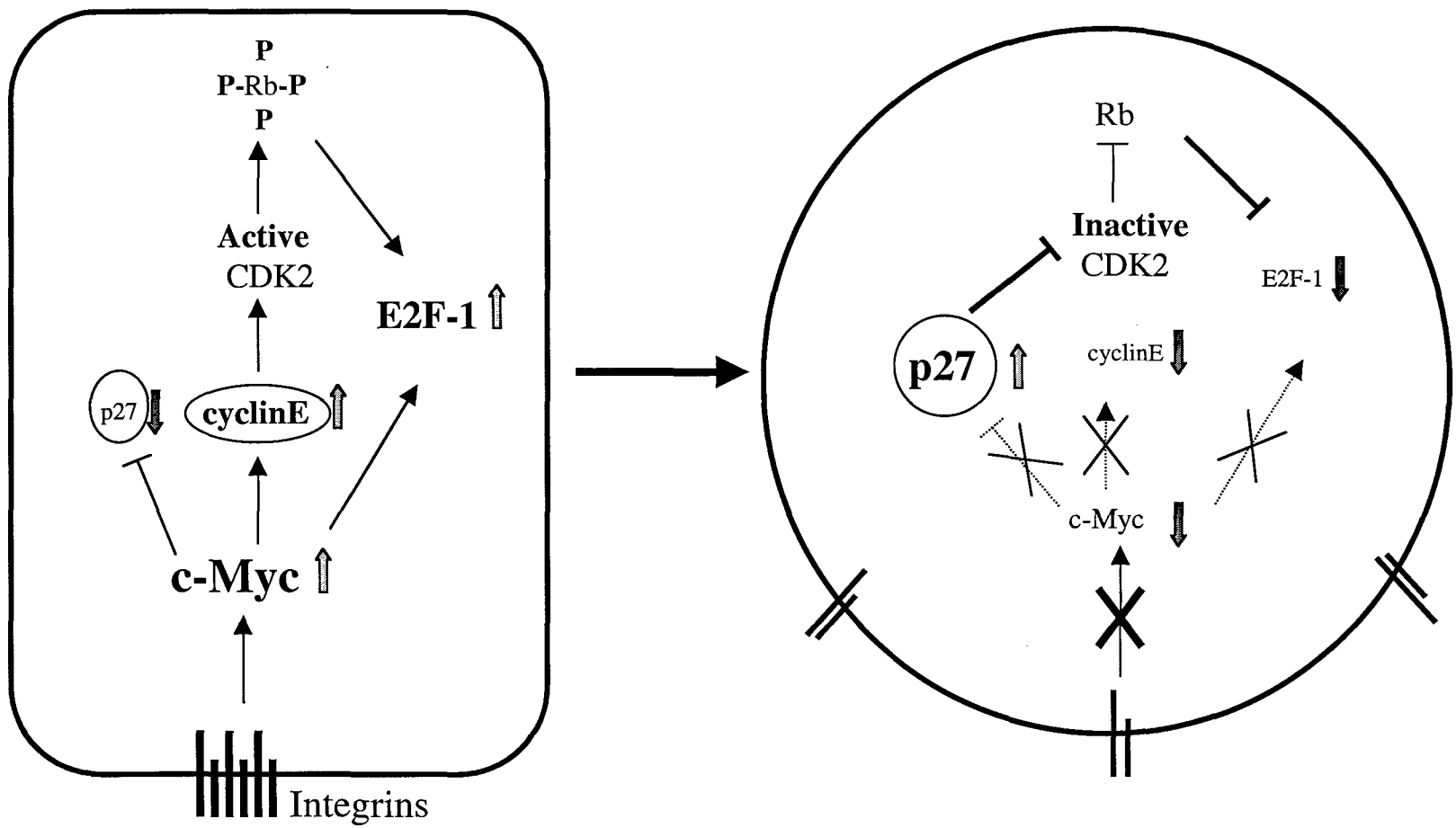

125
100
75
50
25
0
% p27 degradation
0
1
2
3
4
chase time (hrs)
A1N4
A1N4-Myc

C.


A1N4-Myc
A1N4 S12
S12
ALLN 10uM
ALLN 50uM
ALLM 50uM
DMSO
p27

D.


MCF-10A
MCF-10A PLXSN-Myc
MCF-10A PLXSN
0
4
6
10
0
4
6
10
0
4
6
10
hrs
- c-Myc
- p27



Adherent

Non-adherent

Regulation of the expression of c-Myc by β1 integrins in epithelial cells

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Running Title: regulation of c-Myc by β1 integrin

Key words: c-Myc, adhesion, integrin, Src, PKC, MAPK

ABSTRACT

Cell adhesion promotes cellular proliferation through the regulation of gene expression, including the immediate early genes. However, the precise role of cell adhesion in the regulation of the c-Myc proto-oncogene is not clear, and the adhesion-dependent signaling pathway(s) regulating the expression of c-Myc has yet been defined. We now show that integrin signaling directly regulates the expression of c-Myc in the mammary epithelial cell line 184A1N4 (A1N4). Adhesion of quiescent A1N4 cells to fibronectin, and to collagen types IV or I, induces the expression of c-Myc in an ECM concentration-dependent fashion. Cytoskeletal rearrangement, and integrin engagement and integrin clustering are required for the induction of c-Myc by fibronectin. Furthermore, β1 integrin function-blocking antibodies prevent the adhesion-dependent induction of c-Myc. Adhesion of A1N4 cells results in the activation both of c-Src and of the Erk 1/2 mitogen-activated protein kinase (MAPK), each of which precedes the induction of c-Myc. Pharmacological inhibitors specific for either the c-Src family of kinases or for MEK1 block the adhesion-dependent induction of c-Myc. These observations indicate that β1 integrins regulate the expression of c-Myc through the activation of the Src family of tyrosine kinases and the MAK kinase pathway.

INTRODUCTION

Cell adhesion to the extracellular matrix cooperates with hormones and growth factors to regulate cell proliferation. Members of the β1 and β4 families of integrins have been implicated for the control of cell proliferation, both *in vitro* and *in vivo* (Mortarini et al., 1995; Udagawa et al., 1995; Murgia et al., 1998). Integrin engagement and subsequent integrin aggregation lead to the formation of focal adhesions. These events not only enhance the activation of growth factor receptors, but also induce multiple interconnecting signaling pathways (Miyamoto et al., 1996; Moro et al., 1998; Giancotti and Ruoslahti, 1999). A number of signaling molecules are recruited and activated at the sites of focal adhesions, including FAK, PKC, the c-Src family of kinases, and the MAP kinases. PKC has been shown to modulate the activity of c-Src and of the MAP kinase pathway (Vuori and Ruoslahti, 1993; Schlaepfer et al., 1998; Miranti et al., 1999; Howe and Juliano, 1998). In addition, the activation of MEK is greatly decreased in c-Src knockout cells, indicating that the MAP kinase pathway may mediate some of the effects of c-Src (Schlaepfer et al., 1997). These signaling pathways have been implicated in the regulation of cell cycle progression. Overexpression of Ras or Src results in anchorage-independent cell growth. However, the molecular mechanisms by which adhesion-dependent signaling pathways contribute to the regulation of the cell cycle still need to be further explored.

One of the mechanisms by which integrin-mediated adhesion participates in the control of cell cycle progression is through the regulation of the immediate early genes. Cell adhesion is strictly required for the expression of c-Jun and c-Fos (Dike and Farmer, 1988; Dike and Ingber, 1996). In contrast, some discrepancies are present in the literature on the role of cell adhesion in the regulation of c-Myc. While cell-substrate adhesion modulates the levels of c-Myc mRNA in bovine capillary endothelial cells, no immediate decrease in c-Myc message is observed in mouse BALB/c3T3 and primary fibroblasts when deprived of adhesion.

While the adhesion-induced signaling pathways leading to the expression and activation of the Jun/Fos transcription factor have been extensively studied, the integrin-induced signaling pathway(s) regulating the expression of c-Myc/Max have not yet been delineated. Growth factors induce the expression of c-Myc, notably via the "c-Src pathway" (Barone and Courtneidge, 1995). In addition, the MAP kinase pathway has been shown to mediate regulation of the expression of c-Myc in response to v-Abl and CSF-1 (Weber et al., 1997; Cheng et al., 1999). Cell adhesion signaling may have some similarities to growth factor signaling. Therefore, some components(s) in an adhesion signaling pathway(s) may function to potentiate or synergize with growth factor signaling to allow cells to overcome a threshold level required to induce the expression of c-Myc. Alternatively, cell adhesion may provide additional, independent signals to supplement the growth factor effect on expression of c-Myc.

In this study we have examined the effect of interaction of cell-ECM components on the expression of c-Myc in human mammary epithelial cells. We have shown that cellular adhesion to specific extracellular matrix components modulates the expression of c-Myc. In addition, we have demonstrated the presence of a β1 integrin-signaling pathway that promotes expression of the c-Myc protein, independent of growth factors. This integrin-induced regulation of c-Myc is mediated by the PKC, c-Src-family of kinases and Erk 1/2 MAP kinase.

MATERIAL AND METHOD

Reagents and antibodies

184A1N4 (referred to hereafter as A1N4) is a non-tumorgenic cell line derived from a primary culture of human mammary epithelial cells that were immortalized with benzo(a)pyrene (provided by Dr. M.R.Stampfer, University of California, Berkeley) (Stampfer and Bartley, 1985). For c-Myc western blotting, antibody from the c-Myc 1-9E10.2 hybridoma (ATCC, Rockville, MD) (Evan et al., 1985) was used. Blocking

antibody against human β1 integrin (M13) was purchased from Bohringer Mannhein (Indianapolis, IN). Total Erk1/2 MAP kinase was detected with the anti-MAPK1-2 polyclonal antibody from Upstate Biotechnology (Lake Placid, NY), and the phosphorylated form was detected with the monoclonal antibody E10 against phospho Erk1/2 (New England Biolab, Beverly, MA). The pharmacologic inhibitors Herbimycin A and Phorbol 12-Myristate 13-Acetate, were purchased from Sigma (St Louis, MO), PP1 and PD98059, H89 and Staurosporine from Biomol (Plymouth Meeting, PA), and Calphostin C was purchased from Alexis Corporation (Pittsburgh, PA).

Cell culture and cell adhesion to ECM

A1N4 cells were plated at low density (6.6×10^3 cells /cm^2) and allowed to adhere in complete medium, IMEM (Gibco BRL, Rockville, MD), supplemented with 0.5% fetal bovine serum (Gibco BRL), 0.5 μg/ml hydrocortisone (Sigma, St Louis, MO), 5 μg/ml insulin (Biofluids, Rockville, MD) and 10ng/ml epidermal growth factor (EGF) (Collaborative Biomedical Research, Waltham, MA) for 5-7hrs. Cells were then washed twice with phosphate buffered saline (PBS), and maintained in a low serum, EGF-free medium (IMEM from Gibco BRL, supplemented with 0.5% fetal bovine serum (Gibco BRL), 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Biofluids) for 48-72 hrs to synchronize the cells in G0/G1 (Stampfer et al., 1993).

Quiescent cells were then detached with 10mM EDTA in PBS, washed in synchronization medium, and unless specified otherwise in the figure legend, they were resuspended in synchronization medium without EGF and transferred to ECM-coated petri dishes or to fibronectin-suspension culture.

ECM-coated plates were prepared by coating 60mm bacterial petri dishes with either 1μg/cm^2 of human fibronectin diluted in PBS, 1μg/cm^2 collagen type IV diluted in 0.05N HCl, or 5μg/cm^2 collagen type I diluted in 0.02N acetic acid (Collaborative Biomedical

Research, Becton Dickinson, Franklin Lakes, NJ) for 1hr at room temperature. The plates were then blocked for 30min-1hrs with 1% BSA and washed three times with PBS.

For the stimulation of cells in suspension with fibronectin, quiescent cells were resuspended in 2 ml of synchronization medium, containing either 5-10 μg/ml of fibronectin or microbeads coated with either fibronectin or BSA (5 beads/cells), and maintained in suspension rotating at 37°C for 1 hr. Fibronectin-coated magnetic beads were prepared as described by Plopper and Ingber (Plopper and Ingber, 1993). $3x10^6$ tosyl-activated, 4.5 μm diameter microbeads (Dynal, Lake Success, NY) were coated with 8 μg of fibronectin or 1% BSA (Fraction V, Sigma) in carbonate buffer pH 9.4 at 37°C overnight. Beads were washed and free tosyl groups were blocked for 4 hours by rotating at 37°C with 0.2M Tris pH 8.0. Beads were then blocked in synchronization medium containing 1% BSA for 30 min and washed twice with medium before being added to the suspension of cells.

Cell lysis and immunoblotting

For preparations of whole cell extracts, adherent cells were scraped in PBS, and non-adherent cells collected and pelleted by centrifugation (1500xg). The cell pellets were lysed on ice in lysis buffer (1% TritonX-100, 50mM Tris, pH 8.0, 150mM NaCl, 1mM EDTA, 50mM sodium fluoride, 0.5 mM sodium orthovanadate, 60μg/ml aprotinin, 10μg/ml leupeptin, 1 μg/ml pepstatin, 10μg/ml PMSF). Cellular debris was centrifuged for 10 min at 14,000Xg. Equal amounts of proteins, as determined by the BCA protein micro assay (Pierce, Rockford, IL), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto PVDF membranes (Millipore, Bedford, MA). For immunoblotting, membranes were blocked in PBS-0.1% Tween 20 with 5% milk overnight at 4°C. The blots were then incubated with the primary antibody for 1 hour at room temperature, followed by a 1 hr incubation with either a 1:10,000 dilution goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Biorad,

Hercules, CA), or a 1:5000 dilution of anti-rabbit HRP-linked secondary antibody (Amersham, Arlington Heights, IL). Immunoreactive proteins were detected with chemiluminescent substrate (Pierce, Rockford, IL). Equal protein loading was confirmed by amino black staining of the membrane.

c-Src in vitro kinase assay

Cells attached to fibronectin were lysed in lysis buffer containing 0.5% sodium deoxycholate and 0.1% SDS. c-Src was immunoprecipitated with anti-c-Src antibody (GD11)(Upstate Biotechnology). The immunoprecipitates were resuspended in kinase buffer (20mM HEPES, pH 7.0, 10mM $MnCl_2$, 10μM sodium orthovanadate, 1mM DTT) containing 2μg of acid denatured rabbit muscle enolase as an *in vitro* substrate and 5μCi [γ^{32}P] ATP (3000 Ci/mmole) (NEN, Boston, MA). The samples were incubated at 30°C for 10 min. The reaction was stopped by adding 2X SDS sample buffer, and the samples were boiled and resolved by SDS-PAGE. The gel was stained with Coomassie Blue (BioRad) to verify equal loading of the substrate; the extent of phosphorylation was determined by autoradiography.

RESULTS

Adhesion of epithelial cells to ECM components modulates their level of c-Myc

To investigate the role of cell-substrate adhesion in the regulation of the c-Myc early gene in human mammary epithelial cells, we used a non-transformed human mammary epithelial cell line, A1N4. When maintained in low serum in the absence of epidermal growth factor (EGF), the A1N4 cells arrest in a quiescent G0/G1 state, which correlates with a low level of c-Myc expression. Stimulation of these cells in monolayer with EGF results in a rapid induction of the c-Myc protein (Stampfer et al., 1993) (Figure 1). However, in contrast to adherent cells, non-adherent A1N4 cells cannot maintain elevated levels of c-Myc

in response to the EGF stimuli (Figure 1). In order to evaluate further the role of cell adhesion in the regulation of c-Myc, we tested the ability of ECM components to alter the levels of c-Myc in cells exposed to EGF. Non-adherent cells that expressed low levels of c-Myc, were allowed to re-attach to untreated plastic tissue culture dishes or to fibronectin-coated petri dish. Attachment of the cells to fibronectin specifically and rapidly enhanced the induction of c-Myc, as compared to cells adhering to plastic (Figure 1).

Cellular adhesion to ECM components induces the expression of c-Myc in the absence of growth factors

Since adhesion of A1N4 cells to ECM components enhances the EGF-induced expression of c-Myc, we wished to test the hypothesis that cell adhesion, independent of growth factor receptor pathways, participates in the regulation of the expression of c-Myc. Therefore, we asked whether adhesion of quiescent A1N4 cells to a ECM-coated plates can induce the expression of c-Myc in the absence of exogenous EGF (Figure 2). Bacterial petri dishes were coated with 1μg/cm^2 of ECM, and then blocked with BSA to prevent the non-specific adhesion of cells. Quiescent A1N4 cells were detached with EDTA and replated onto either fibronectin, collagen type I, or collagen type IV -coated plates in synchronization medium containing no EGF. Western blots indicated that the levels of c-Myc were transiently increased following adhesion and spreading of cells on both fibronectin, collagen type I, and collagen type IV (Figure 2a).

More precise analysis of the dynamics of the induction of c-Myc by fibronectin indicated that the levels of c-Myc start to increase at 30 min following adhesion to fibronectin, peaking by 1 hr, and remaining elevated for up to 3 hrs (Figure 2b). No induction of c-Myc was detected when adherent quiescent cells were refed with synchronization medium lacking EGF, or when the cells detached and maintained in suspension for 1 hr (data not shown). Furthermore, cell adhesion on fibronectin induced the expression of c-Myc protein in a fibronectin concentration-dependent manner (Figure 2c). The expression of c-Myc reached a maximum when the cells were plated on a

fibronectin concentration of 1μg/cm^2. Further increases in fibronectin concentration did not result in additional increases in levels of c-Myc (data not shown).

Cytoskeletal rearrangement and integrin aggregation are required for the induction of c-Myc

To examine whether the integrity of the cytoskeleton is required for adhesion-dependent induction of c-Myc, cells were treated with the inhibitor of actin filament polymerization, cytochalasin D, before plating them onto fibronectin. Cytochalasin D treatment strongly decreased, but did not totally block, the fibronectin-dependent induction of c-Myc (Figure 3a). Treatment of cells with the DMSO solvent, as a control, did not alter the increase of c-Myc induced by cell adhesion to fibronectin.

We next asked whether interaction of cells with fibronectin could induce c-Myc expression in suspension cultures. Incubation of non-adherent cells with soluble fibronectin, at concentrations of up to 10μg/ml, failed to induce the expression of Myc (Figure 3B, left panel). In the absence of a solid substrate, binding of integrins to soluble fibronectin may fail to induce integrin aggregation and the formation of focal adhesion complexes, which are necessary to initiate integrin-mediated signal transduction pathways. A previous report from Plopper and Ingber has shown that 4.5μm microbeads coated with fibronectin can induce the formation of focal adhesion complexes. Focal adhesion proteins are recruited in the areas surrounding the sites of interaction of the cell with fibronectin-coated beads (Plopper and Ingber, 1993). We used this model to test whether fibronectin can induce the expression of c-Myc in non-adherent cells. Quiescent A1N4 cells were incubated in suspension for 1 hr with 5 fibronectin-coated magnetic beads per cell. Cells associated with the fibronectin-coated beads were then pelleted and lysed. Total cell lysates were analyzed by Western blotting for the level of c-Myc protein (Figure 3b, right panel). Interaction of cells with fibronectin-coated beads induced the expression of c-Myc in suspension. The amount of induction of c-Myc protein in cells interacting with the fibronectin-coated beads, even though significant, was lower than the level of induction

observed in cells adhering to fibronectin-coated plates. One possible explanation is that the number of beads present in suspension may be limiting for maximal induction of the expression of c-Myc. In addition, the cell spreading and the cytoskeletal rearrangement resulting from cell-surface interactions may be needed for optimal signaling. To control for non-specific effects of the beads, we incubated A1N4 cells in suspension with magnetic beads coated with BSA. BSA-coated beads did not significantly induce expression of c-Myc (Figure 3b).

β1 integrins engagement induces the expression of c-Myc

To specifically address the role of integrins in the stimulation of the expression of c-Myc following cellular adhesion to fibronectin, we used integrin-specific blocking antibodies. Cellular adhesion to fibronectin can be mediated by α4β1, α5β1, αvβ1, and αvβ3 integrin heterodimers. Flow cytometric analysis indicates that A1N4 cells express α4, α5, α2, β1 integrin subunits, but only very low levels of αvβ3 on their cell surface, suggesting that αvβ3 integrin is not implicated in the adhesion dependent-regulation of c-Myc in A1N4 cells (data not shown). To directly test the involvement of β1 integrins in the regulation of c-Myc, we used a function-blocking antibody against the β1 integrin subunit, mAb13 (Akiyama et al., 1989; Yamada et al., 1990). A1N4 cells were pre-incubated for 20 min with either β1-integrin blocking antibody or with rat IgG as a control. The cell-antibody mix was then transferred to fibronectin-coated dishes for 1 hr. Treatment of cells with β1 integrin-blocking antibody, but not with rat IgG, diminished by 10-fold the fibronectin-dependent upregulation of c-Myc (Figure 4a). In addition, fibronectin-dependent induction of c-Myc was blocked in an antibody concentration-dependent manner (Figure 4b). Addition of either anti-human α5 integrin blocking-antibody (clone P1D6), or anti-human α4 integrin blocking-antibody (clone P1H4), which bind to α5 and α4 integrins respectively, on the surface of A1N4 cells did not impede fibronectin-dependent induction of c-Myc (data not shown). Similarly, the blocking antibody against α2β1, a laminin receptor highly expressed on the surface of A1N4 cells, did not block the induction of c-

Myc in response to cellular adhesion to fibronectin (data not shown). This indicates that the inhibition induced by mAb13 was not the result of steric hindrance blocking cell adhesion. Taken together, our results demonstrate that adhesion to fibronectin can induce the expression of c-Myc independent of the growth factor pathways. Furthermore, fibronectin-dependent stimulation of c-Myc expression is driven by a β1 integrin pathway.

Role of c-Src in fibronectin-dependent induction of c-Myc

We next wished to investigate the effector molecules involved in the regulation of c-Myc by β1 integrins. A possible candidate is the c-Src phosphotyrosine kinase. The c-Src kinase has been previously implicated in the regulation of c-Myc by growth factor receptors (Barone and Courtneidge, 1995). In addition, c-Src is recruited at the sites of the focal adhesion, where it phosphorylates on tyrosine residues a number of signal transduction proteins (Clark and Brugge, 1995; Schlaepfer et al., 1997). However, no previous studies have addressed the role of c-Src in integrin-mediated control of the expression of c-Myc. In agreement with the role of c-Src in integrin-mediated signal transduction, adhesion and spreading of quiescent A1N4 cells on fibronectin induce the activity of c-Src kinase, peaking at 1hr following attachment (Figure 5a).

To test the role c-Src in the adhesion-dependent induction of c-Myc, we treated quiescent A1N4 cells with the phosphotyrosine inhibitor, Herbimycin A, and with the c-Src family-specific pharmacological inhibitor, PP1 (Pyruzolopyrimidine). Both inhibitors, but not the DMSO vehicle control, blocked in a dose-dependent fashion the induction of c-Myc, following adhesion to fibronectin (Figure 5b). These results suggest that the c-Src family of tyrosine kinases is required in fibronectin-dependent induction of c-Myc.

Involvement of MEK in adhesion-dependent regulation of c-Myc

The Raf/Mek/MAP Kinase pathway has been implicated in the regulation of proliferation by adhesion (Roovers et al., 1999). Furthermore, this pathway has been shown to mediate some of the effects of c-Src (Hakak and Martin, 1999; Schlaepfer et al., 1998; Aziz et al., 1999). To determine if the activity of MEK1 is required for fibronectin-mediated

induction of c-Myc, we examined the effect of the specific MAP kinase kinase inhibitor PD98059 (Alessi et al., 1995). Treatment of quiescent A1N4 cells with this inhibitor prevented the fibronectin-dependent induction of c-Myc in an inhibitor dose-dependent manner (Figure 6a). Western blot analysis, using an antibody specific for the phosphorylated form of the MEK1 substrates, Erk1 and Erk2, indicated that Erk1/2 is transiently phosphorylated in A1N4 cells, within 30 min of adhesion to fibronectin. This activation of MEK, following adhesion to fibronectin, precedes the induction of c-Myc (Figure 6b). Treatment of cells with 50 μM PD98059 prevents the phosphorylation of Erk1/2, demonstrating that the inhibitor effectively blocks fibronectin-induced activation of MEK1 (Figure 6b). The membranes were stripped and reprobed with an antibody against total Erk1/2 to verify that the variations observed in Erk 1/2 phosphorylation levels are not due to a modulation in the expression of Erk (Figure 6b). These results indicate that the increase in MEK1 activity, following cell adhesion onto fibronectin, correlates with expression of c-Myc. Furthermore, inhibition of MEK1 can block adhesion-dependent induction of c-Myc.

Involvement of PKC in the adhesion-dependent induction of c-Myc

Several lines of evidences have implicated PKC in integrin-mediated events. PKC has been proposed to be an upstream modulator of the c-Src and MAPK pathways (Vuori and Ruoslahti, 1993; Miranti et al., 1999; Schlaepfer et al., 1998). We used two PKC inhibitors, Staurosporine and Calphostin C, to address the role of PKC in the adhesion-dependent regulation of c-Myc. Inhibition of PKC with Calphostin C entirely blocked fibronectin-induced expression of c-Myc, and treatment of A1N4 cells with Staurosporine inhibited by 80% the induction of c-Myc, following adhesion (Figure 7). In contrast, the PKA inhibitor, H89 did not alter the induction of c-Myc. In addition, 24 hrs of pretreatment of A1N4 cells with TPA, which decreases the levels of the TPA responsive PKC, blocked the induction of c-Myc in response to adhesion, although it did not affect the ability of A1N4 cells to adhere and spread onto fibronectin (Figure 7).

DISCUSSION

Although cell adhesion-initiated signaling pathways have been extensively studied, many gaps remain to link these signaling cascades to the regulation of cell proliferation. In this study, we have demonstrated the existence in human mammary epithelial cells of an ECM receptor (integrin)-initiated signaling pathway leading to the expression of c-Myc, independent of growth factor receptor signaling. Adhesion of quiescent A1N4 cells to the basement membrane component, collagen type IV, and to the stromal components, fibronectin and collagen type I, induced the expression of c-Myc. In addition, we deliniated an adhesion-induced signaling pathway regulating the induction of c-Myc.

The β1 integrins have been implicated in the regulation of proliferation of mammary epithelial cells, both *in vitro* and *in vivo*. Disruption of β1 integrin function results in a decrease in mammary epithelial cell proliferation both in a transgenic mouse model and in tissue culture (Faraldo et al., 1998; Weaver et al., 1997). In addition, the cytoplasmic tail splice variant of β1 integrin, integrin β1C, which is normally expressed in non-proliferating, differentiated epithelial cells, has been described as a growth inhibitor (Fornaro et al., 1998; Manzotti et al., 2000). We now provide evidence for one of the molecular mechanism by which β1 integrins regulate epithelial cell proliferation. Using a function-blocking antibody, we have demonstrated that β1 integrins mediate the induction of c-Myc in response to cell adhesion to fibronectin. Furthermore, the enforced expression of β1C integrin results in an increased in the levels of p27, suggesting that it is an upstream regulator of p27 (Fornaro et al., 1999). Interestingly, c-Myc induces the degradation of p27. Therefore, β1C integrin may function as a naturally occurring, dominant negative form of β1 integrins. β1C may inhibit integrin-mediated induction of c-Myc, resulting in the stabilization of p27.

Our results indicate that adhesion-dependent regulation of c-Myc is mediated through PKC, the c-Src family of tyrosine kinases, and the MAP kinase pathway. It could be possible that the effect of these kinases on the expression of c-Myc is linked to their role in the organization of the cytoskeleton. However, specific inhibition of phosphatidylinositol 3-

kinase, whose activity is also regulated by cell adhesion and which has been implicated in cell migration, does not alter the induction of c-Myc (data not shown). Furthermore, treatment of A1N4 with TPA, which does not reduce cell adhesion and spreading on fibronectin, still blocks the induction of c-Myc. Together, these observations suggest that PKC, Src and MAPK are involved in a direct adhesion-dependent signaling pathway inducing the expression of c-Myc.

Previous studies have indicated that c-Src is involved in the transcriptional regulation of c-Myc in response to growth factors (Barone and Courtneidge, 1995). In addition, it has been suggested that the Ras/Raf/MEK/MAPK pathway may play a role in the regulation of AP-1 expression and function, whereas c-Src regulates the expression of c-Myc (Barone and Courtneidge, 1995; Roussel et al., 1991). However, other studies have suggested a connection between this MAP kinase pathway and the expression of c-Myc. Transfections of murine fibroblasts with conditionally active c-Raf and H-Ras result in an increase in c-Myc mRNA (Lloyd et al., 1989; Kerkhoff et al., 1998). Ki-Ras has also been implicated in the regulation of c-Myc in colon cancer cells (Shirasawa et al., 1993). In addition, the Ras/Raf/MEK pathway mediates the induction of c-Myc by CSF-1 and v-Alb (Weber et al., 1997; Cheng et al., 1999). In fact, both the Raf/MEK/MAPK pathway and v-Src have been shown to complement a defective mutant colony-stimulating factor-1 receptor in NIH3T3. Furthermore, in that model v-Src requires the activation of MEK to induce the expression of c-Myc (Aziz et al., 1999). It has also been suggested that c-Src is an upstream activator of the MAP kinases in the integrin signal transduction pathways. Multiple, non-linear pathways are believed to link integrin-induced activation of c-Src and the activation of the MAPK pathway. Through its association and phosphorylation of FAK, c-Src creates a binding site for the Grb2 adaptor protein (Schlaepfer et al., 1998; Schlaepfer and Hunter, 1997). Furthermore, c-Src induces Shc phosphorylation, and the c-Src substrate p130Cas enhances Grb2 association with Shc. This event has been shown to mediate c-Src-induced

activation of serum response elements through the Ras/MEK/ERK pathway (Hakak and Martin, 1999; Schlaepfer et al., 1998).

Our results reported here, together with the experiments described above, suggest the following model (Figure 8). Cell adhesion to fibronectin through β1 integrin results in the activation of c-Src. c-Src, through FAK/p130CAS/Shc/Grb2 adaptor proteins, participates in the activation of the Ras/Raf/MEK/ERK signaling cascade, which in turn induces the expression of c-Myc. Our results do not exclude the possibility that c-Src can also induce c-Myc, independent of the MAP kinase pathway. Indeed, several signals are believed to cooperate with the MEK/MAP kinase pathway, in response to CSF-1, to induce the expression of c-Myc (Cheng et al., 1999). c-Src can possibly regulate the expression of c-Myc, independent of the MAPK pathway, through the activation of the STAT family of transcription factors, since c-Src can activates STAT-3 independent of ERK activation, and since STAT 3 has been suggested to drive the transcription of c-Myc (Goi et al., 2000; Kiuchi et al., 1999). We also provide evidence, using PKC inhibitors, that PKC is implicated in the adhesion-dependent regulation of c-Myc. PKC is activated following cell adhesion to fibronectin, and can modulate the integrin-induced activation of c-Src and of the Ras/Raf/MEK/ERK pathway (Vuori and Ruoslahti, 1993; Schlaepfer et al., 1998; Miranti et al., 1999; Howe and Juliano, 1998).

The molecular mechanisms by which c-Src and the MAP kinase pathway regulate the expression of c-Myc are not yet fully characterized, and their understanding requires further studies. Both c-Src and MAPK have been shown to regulate the levels of c-Myc mRNA suggesting transcriptional regulation. Elements in the c-Myc promoter that confer adhesion-responsiveness to the c-Myc gene still need to be defined. Two transcription factors might participate in the cell adhesion-dependent regulation of the transcription of c-Myc. First, the ERK1/2 phosphorylates and activates the Ets family of transcription factors. Ets-1 has been shown to induce the expression of reporter genes, under the control of the c-Myc promoter, through the binding of the E2F site in the promoter of c-Myc (Roussel et al.,

1994). In addition, c-Src, independent of the MAP kinase pathway, might regulate the transcription of the c-myc gene through the activation of the STAT3 transcription factor (Kiuchi et al., 1999). Alternatively, the MAPK pathway has been shown to increase the phosphorylation of histone H3. This histone phosphorylation has been associated with increased transcription of the early genes, including c-Myc (Chadee et al., 1999).

An additional, post-transcriptional regulation of c-Myc by cell adhesion should not be excluded. It is tempting to speculate that cell adhesion may alter the stability of c-Myc, since c-Ras has been shown to stabilize c-Myc (Sears et al., 1999), and since we have shown that the MAP kinase pathway regulates the levels of c-Myc protein in response to fibronectin.

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Figure 1: Adhesion to fibronectin enhances EGF-dependent induction of c-Myc. Quiescent A1N4 cells were trypsinized, resuspended in medium containing 10ng/ml of EGF, and either replated onto tissue culture dishes (A), or held in suspension for 4 hrs (S). The cells maintained in suspension were transferred to either plastic tissue culture dishes (TC), or dishes coated with fibronectin (FN), in the presence of EGF. Cells were allowed to adhere for the indicated times. Non-adherent cells were washed away with PBS, and the attached cells were lysed, and analyzed by western blotting for their levels of c-Myc.

Figure 2: Adhesion of epithelial cells to ECM components induces the expression of c-Myc, independently of growth factors. (**a**) Quiescent A1N4 cells were detached and transferred, in the absence of EGF, to petri dishes coated with fibronectin (FN), collagen type IV (Col IV), collagen type I (Col I), or BSA (petri) for the indicated times. Cell lysates were analyzed by western blotting for levels of c-Myc. (**b**) Quiescent A1N4 cells (Q) were detached (t=0), and replated in synchronization medium without EGF on 1μg/cm^2 fibronectin-coated plates for the indicated time. (**c**) Fibronectin concentration-dependent increases in c-Myc. Quiescent cells were plated for 1 hr onto dishes coated with the indicated concentrations of fibronectin. Whole cell extracts were prepared and analyzed by western blotting for expression of c-Myc.

Figure 3: Role of the cytoskeleton and integrin clustering in fibronectin-induced expression of c-Myc. (**a**) Quiescent A1N4 cells (Q) were pretreated for 10 min with 1μg/ml cytochalasin D, an equivalent amount (0.1%) of dimethyl sulfoxide (DMSO), or left untreated. Cells were then replated on fibronectin-coated dishes for 1 hr in the presence of cytochalasin D, DMSO, or left untreated. After 1hr, the cells were lysed and cell extracts were subjected to SDS-PAGE and immunoblotting with anti-c-Myc antibody. (**b**) Quiescent cells (Q) were either plated on fibronectin-coated plates (adherent) or maintained in suspension in the presence of soluble fibronectin (left panel), or in the presence of

microbeads coated with either fibronectin (FN) or BSA (right panel). Cells were collected, and whole cell lysates were prepared and analyzed by western blot for levels of c-Myc.

Figure 4: β1 integrin mediates the fibronectin-induced expression of c-Myc. (**a**) Effect of β1 blocking antibody on induction of c-Myc. Quiescent cells were detached and incubated for 20 min on ice in the presence of 10 μg/ml β1 blocking antibody (mAb13), 10μg/ml IgG, or no antibody, and transferred to fibronectin-coated dishes for 1hr. Cells were lysed, and total cell extracts were analyzed by western blot for level of c-Myc. (**b**) Concentration-dependent effects of the β1 blocking antibody on the induction of c-Myc. Cells were pre-incubated with the indicated concentrations of β1 blocking antibody before allowing them to attach onto fibronectin-coated dishes.

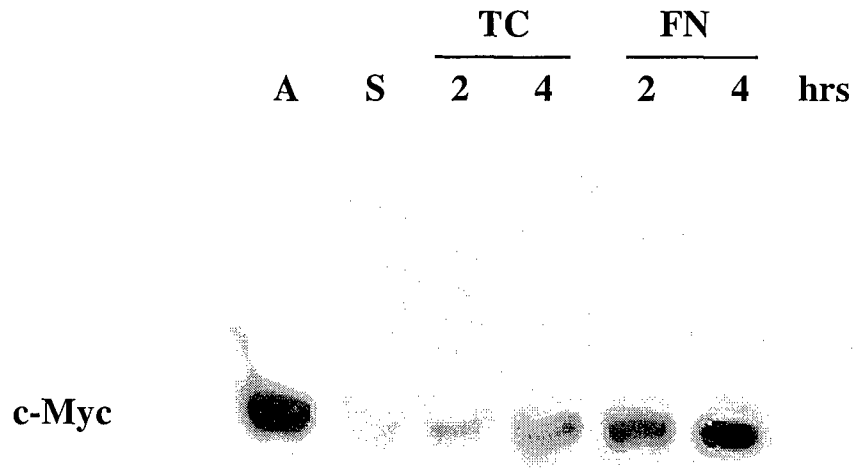
Figure 5: Effect of pharmacological inhibitors of c-Src on fibronectin-induced expression of c-Myc. (a) Quiescent A1N4 cells were plated on fibronectin for the indicated times. c-Src was immunoprecipitated and incubated *invitro* with [γP^{32}] ATP and enolase, as an exogenous substrate. The kinase assays were separated on SDS-PAGE, and phosphorylated enolase detected by autoradiography. **(b)** Quiescent A1N4 cells (Q) were pre-treated with the indicated concentrations of Herbimycin A or 0.1% DMSO for 16 hrs or with PP1 for 30 min. Cells were then detached and replated in the presence of Herbimycin A, PP1, DMSO, or no addition onto fibronectin-coated petri dishes for 1 hr. Adherent cells were lysed and analyzed by western blotting for induction of c-Myc.

Figure 6: Effect of MEK1 inhibition on fibronectin-induced expression of c-Myc. (**a**) Quiescent A1N4 cells (Q) were preincubated for 1 hr with the indicated concentration of the MEK1 inhibitor PD98059, an equivalent concentration of the DMSO vehicle, or left untreated. Cells were then detached, and replated in the presence of PD98059, DMSO, or without treatment on fibronectin-coated dishes for 1 hr. Whole cell lysates were made and

analyzed by western blotting for c-Myc levels. (**b**) Quiescent A1N4 cells (Q) were preincubated for 1 hr with 50 μM PD98059, 0.05% DMSO, or left untreated. Cells were then detached, and allowed to adhere to fibronectin-coated dishes for either 30 min or 1 hr, in the presence of PD98059, DMSO, or in the absence of treatment. Cells were lysed, and lysates analyzed by western blotting for c-Myc and phosphorylated form of Erk1/2. Blots were stripped and reprobed for total Erk1/2.

Figure 7: Effect of PKC inhibition on fibronectin-dependent c-Myc expression. Quiescent A1N4 cells (Q) were pretreated with the indicated concentration the PKC-specific pharmacological inhibitors Staurosporine or Calphostin C, the PKA inhibitor H89, or 0.1% DMSO for 1 hr. Alternatively, PKC levels were downregulated by incubating quiescent cells with 100ng/ml of phorbol 12-Myristate 13-acetate (PMA), or a equivalent amount of the vehicle alone, ethanol (EtOH) for 24 hrs. Cells were then replated onto fibronectin-coated dishes for 1 hr in the presence of Staurosporine, Calphostin C, H89, DMSO, TPA or ethanol. Adherent cells were collected, lysed, and analyzed by western analysis for levels of c-Myc.

Figure 8: Model for the fibronectin-dependent signaling pathway regulating the expression of c-Myc. Adhesion of epithelial cells to ECM components through β1 integrins ligation and aggregation induces the activation of c-Src. PKC may mediate the integrin-induced activation of c-Src. c-Src has been proposed to activate the Raf/MEK/ERK kinase pathway through tyrosine phosphorylation of FAK or Shc. Both c-Src and MEK activation following β1 integrin engagement induce the expression of c-Myc, in either a linear or a parallel pathway(s).


TC
FN
A
S
2
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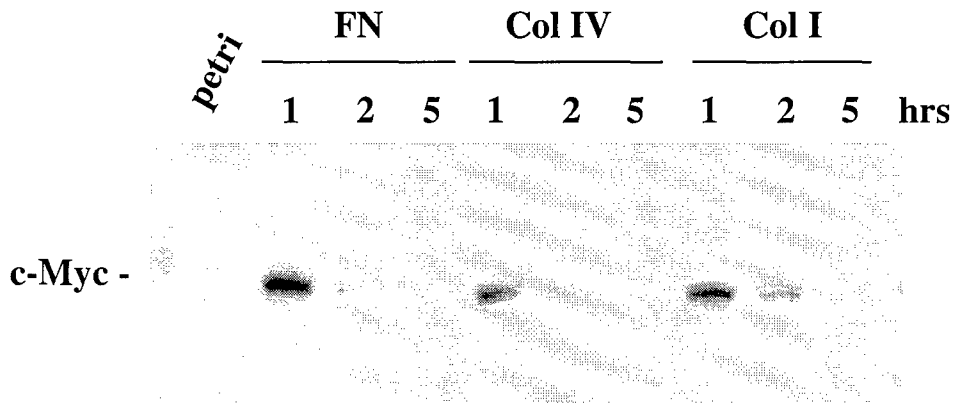
a.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Col IV
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1 2 5 1 2 5 1 2 5 hrs
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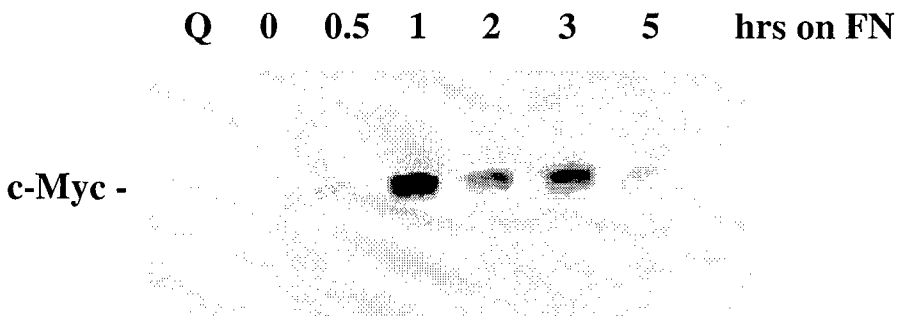
b.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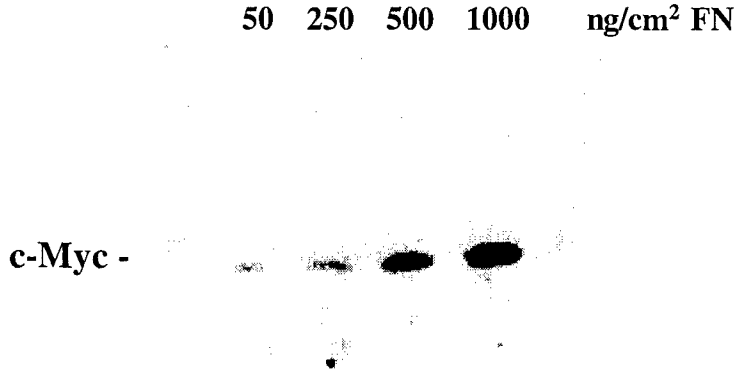
c.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c-Myc -

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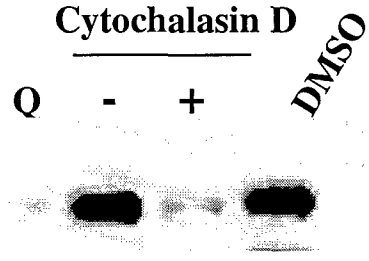
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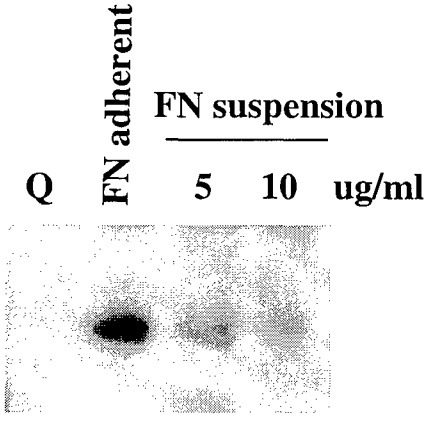
Q
-
+
DMSO

b.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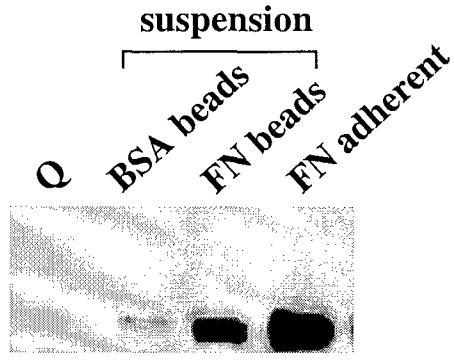
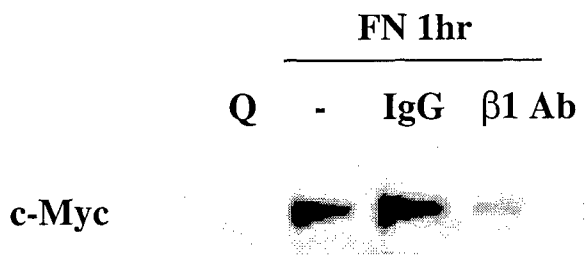
Q
5
10
ug/ml


suspension
Q
BSA beads
FN beads
FN adherent

a.



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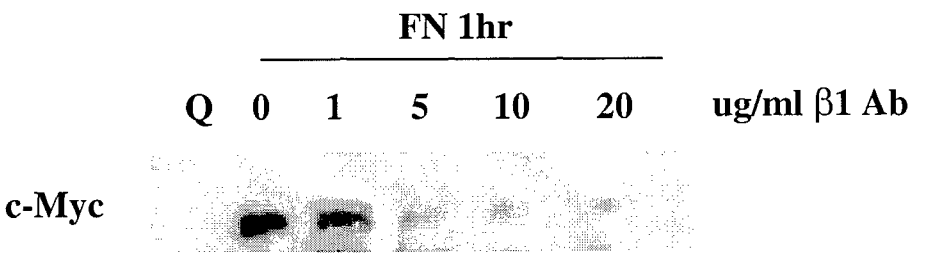

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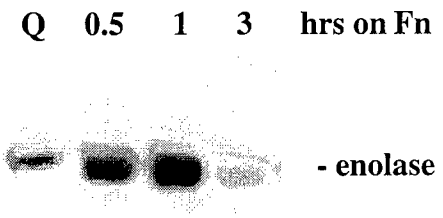
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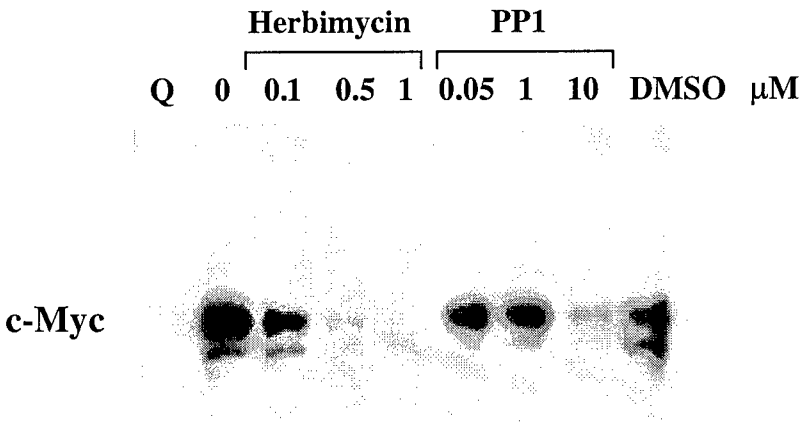
- enolase

b.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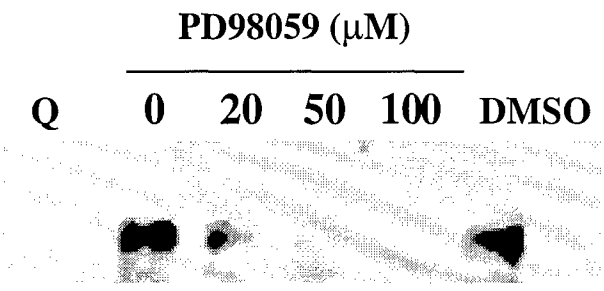
PP1
Q 0 0.1 0.5 1 0.05 1 10 DMSO μM
c-Myc

a.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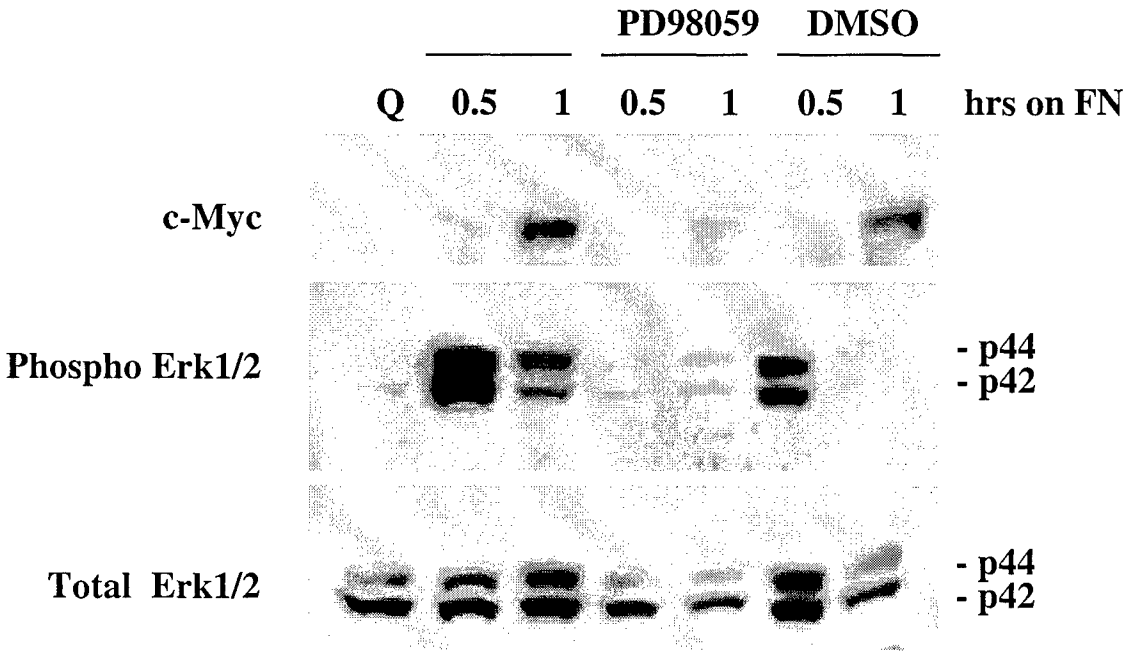
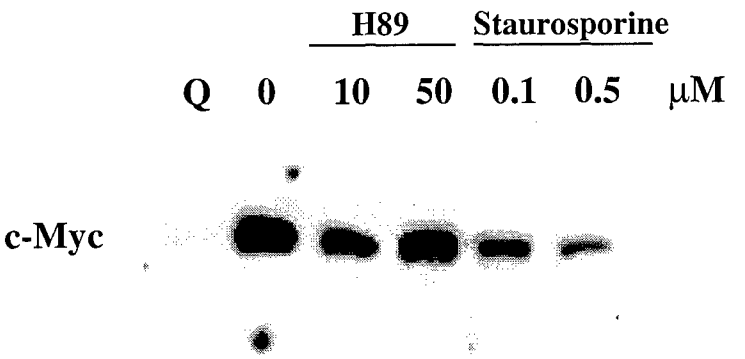
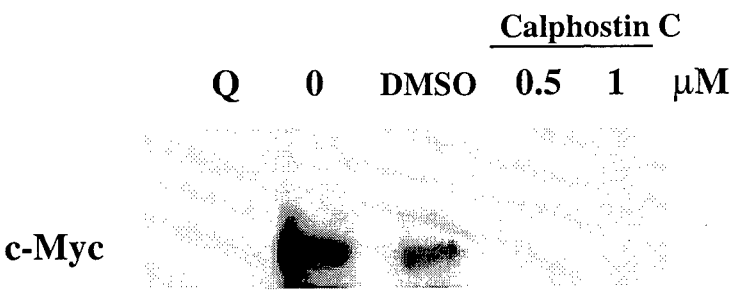
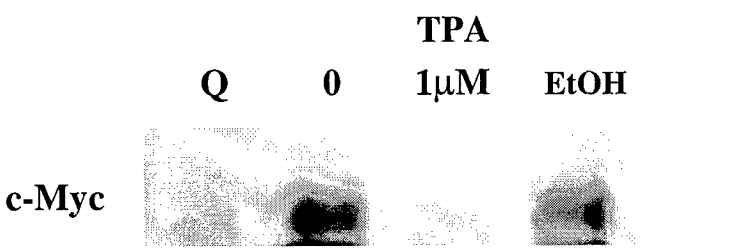
Q
0
20
50
100
DMSO
c-Myc

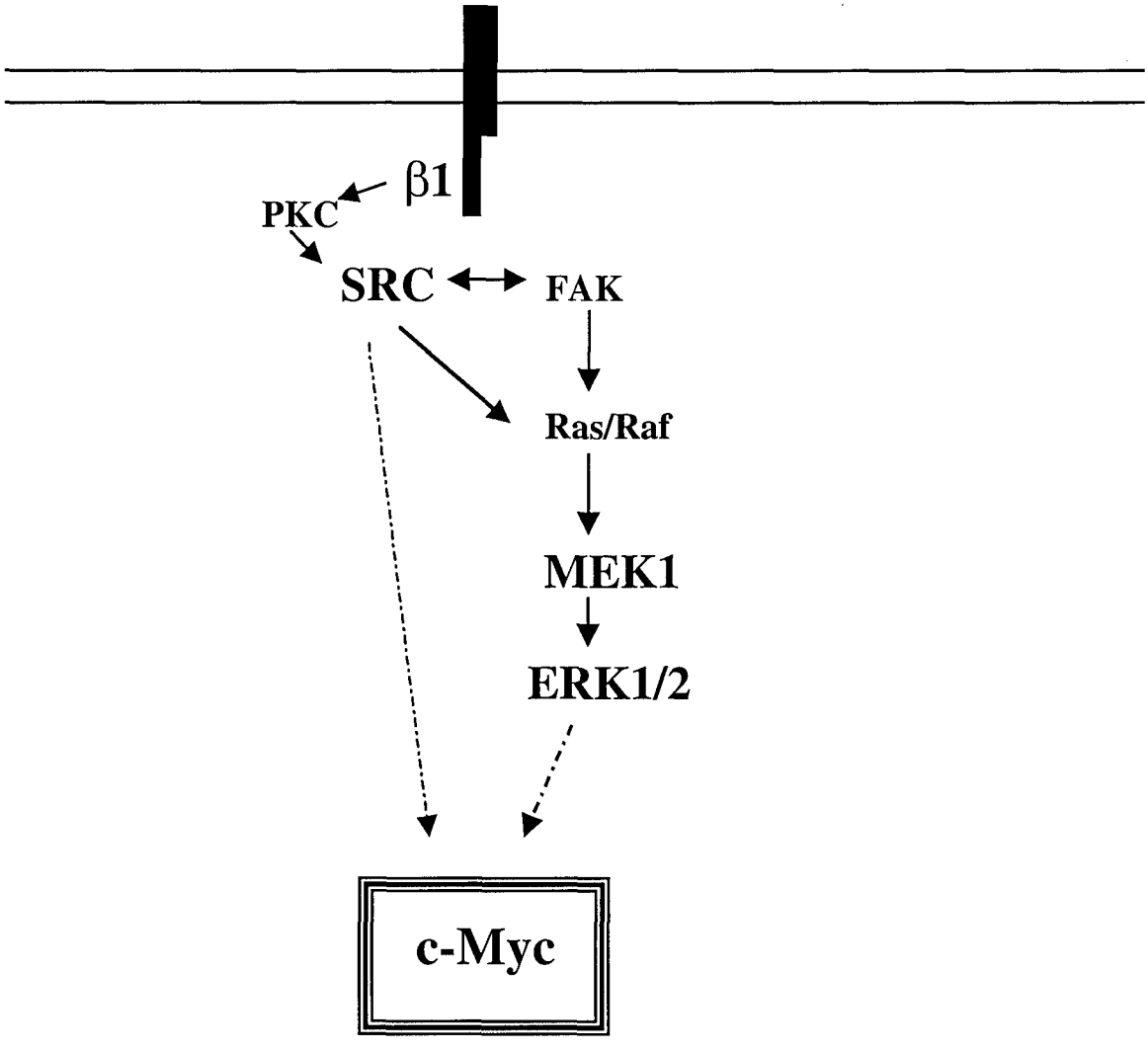
b.


PD98059
DMSO
Q
0.5
1
0.5
1
0.5
1
hrs on FN
c-Myc
Phospho Erk1/2
- p44
- p42
Total Erk1/2
- p44
- p42


H89
Staurosporine
Q 0 10 50 0.1 0.5 μM
c-Myc


Calphostin C
Q 0 DMSO 0.5 1 μM
c-Myc


TPA
Q 0 1μM EtOH
c-Myc


β1
PKC
SRC
FAK
Ras/Raf
MEK1
ERK1/2
c-Myc