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GROWTH SIGNALLING THROUGH THE ALPHA5BETA1 FIBRONECTIN RECEPTOR

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SUMMARY This study demonstrates that perturbation of the fibronectin receptor (FNR), a member of the integrin family of adhesion receptors, can stimulate growth of non-transformed epithelial cells but not of transformed epithelial cells. Using the non-adherent cell line FA-K562 we demonstrate that growth stimulation via FNR ligands occurs rapidly and independently of any effects on cell adhesion. Low valence FNR ligands such as glycine-arginine-glycine-aspartate-serine (GRGDS) are the most potent stimulators of the cell cycle regulatory kinase cdc2. Partial synchronization and Western blotting studies suggest that GRGDS affects cdc2/cyclin A complexes in cells in S/G2 phase of the cell cycle. These studies suggest that FNR-mediated growth control appears to be a common feature of transformation. These data suggest that the FNR may be physiologically important in growth control, especially in the presence of low valence, proteolytic degradation fragments of FN. Furthermore, escape fron FNR-mediated growth control may be a common feature of transformation. © 1995 Academic Press, Inc.

INTRODUCTION: Integrins are evolutionarily conserved heterodimeric cell surface receptors that mediate cell-cell and cell-substrate adhesion (1). It is in this functional capacity that integrins participate in embryogenesis (2), wound healing (3, 4), hemostasis, and inflammation (5,6). Integrin/ligand interactions have also recently been shown to trigger a variety of signalling events, including tyrosine phosphorylation of intracellular proteins (7-10), induction of gene expression (11-13), and ion flux (14, reviewed in 15). Abnormalities in the structure or expression of the alpha5beta1 fibronectin receptor (also known as the FNR or VLA-5) and its ligand fibronectin (FN) have been reproducibly observed in studies of transformed and neoplastic cells (16-21, rev. in 22, 23). In addition, several laboratories have demonstrated a reciprocal relationship between the amount of alpha5beta1 expressed on the cell surface and the degree of cellular transformation (24-26). These findings suggested that the FNR may contribute to growth abnormalities observed in transformed cells. A role of the FNR in growth control of normal cells is supported by the following findings. Memory T cells poised to proliferate upon re-stimulation have increased surface FNR expression relative to naive T cells (27). Transient increases in FNR expression occur during healing of skin and hepatic wounds (3, 4, 28). Finally, several studies have shown that the FNR could act as an accessory molecule for stimulating growth of lymphocytes or fibroblasts (29-31). These findings led to our earlier work demonstrating that FNR perturbation could stimulate the growth of FA-K562 cells (32). The present study extends this observation to a variety of

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non-transformed cell types and characterizes CDK, cyclin, and FNR ligand requirements for alpha5beta1-mediated growth signalling.

METHODS

CELLS AND CELL CULTURE: The previously described FA-K562 and K562 cell lines were grown in suspension in RPMI-1640 containing 5% fetal bovine serum (FBS)(GIBCO/BRL) in a humidified, 95% air, 5% CO2 incubator (25, 32). FA-K562 cells express no GRGDS-binding integrins other than the FNR (ibid. and unpublished observations). Primary keratinocytes (KC) were isolated from human neonatal foreskins essentially according to the techniques of Boyce and Ham as previously described (33). KC were only used through third passage. The papillomavirus-immortalized human epidermal cell lines FEPE1L8 and 1811T1 have been previously described (33-35). KC, FEPE1L8, and 1811T1 cells were grown in serum-free keratinocyte growth medium with additives (KGM)(Clonetics Corp). Human breast cell lines HBL100 and BT20 were obtained from the American Type Culture Collection and grown in DMEM containing 10% fetal calf serum.
ANTIBODIES AND REAGENTS: Antisera reactive with the unique C-terminal domain of cdc2 were purchased from GIBCO/BRL and used for all cdc2 immunoprecipitations. Antisera reactive with cyclins A and E were a gift from Dr. Robert Abraham (Mayo Clinic, Rochester, MN). Antibodies reactive with the alpha5 (P1D6) and beta1 (P4C10) subunits of integrins were a gift from Dr. William Carter (FHCRC, Seattle, WA)(36). Histone H1, GRGDS, and GRGES were purchased from Sigma Chemical Company (St Louis, MO). Protein G- and protein A-agarose were purchased from GIBCO/BRL. Purified plasma fibronectin (FN) was obtained from the New York Blood Center. ^{32}P-dATP (~3000 Ci/mmole) was purchased from NEN/DuPont.
CDC2 KINASE ASSAYS: Cdc2 kinase assays were performed as previously described (32). In all experiments, 10^6 cells were used for each treatment with control or test ligands. Briefly, cells incubated with FNR ligands or control ligands for various intervals were washed with ice-cold PBS, and lysed in 50 mM Tris, 250 mM NaCl, 0.5% NP-40, 1 mM PMSF, and 5 µg/ml aprotinin (defined as lysis buffer). After clarification of lysates by centrifugation (15 min x 13,000g), immunoprecipitation was performed by serial incubations with anti-cdc2 or normal rabbit sera followed by protein A-agarose beads. Agarose-associated material was washed twice in lysis buffer and twice in freshly prepared kinase buffer (20 mM Tris pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol). Agarose beads were then resuspended in 50 µl of kinase reaction mixture (kinase buffer containing 20 µg/ml histone H1, 30 µM ATP, and 100 µCi/ml ^{32}P-dATP) and incubated for 30 minutes at 37°C with rotation. Reducing sample buffer was then added and samples were boiled for 5 minutes prior to loading in lanes of a 12% polyacrylamide gel. After electrophoresis, gels were fixed, dried, and exposed to film for 30-240 minutes at -70°C. Bands corresponding to phosphorylated histone H1 migrated between 25 and 35 kDA and were quantitated using a Molecular Dynamics PhosphorImager 400E. In early experiments, sodium orthovanadate (2 mM) and sodium difluoride (5 mM) were included in the lysis buffer as phosphatase inhibitors. The ratio of kinase activity in control-treated versus GRGDS-treated cells did not change in the presence or absence of these inhibitors .
CELL CYCLE ENRICHMENT: Cdc2 kinase activities in control or GRGDS-treated cells were compared for FA-K562 cells i) grown asynchronously in 10% fetal bovine serum (FBS), ii) grown for 48-72 hours in RPMI containing 0.1% FBS (referred to as serum-starved cells), and iii) grown for 6-20 hours in the presence of serum after 72 hours of serum-starvation (referred to as released cells). Aliquots of cells to be used in kinase assays were removed, permeabilized, and incubated with propidium iodide to stain cellular DNA as previously described (32). As shown in Figure 1, serum-starved cells were enriched in G0/G1 cells and addition of serum to these serum-starved cells resulted in an initial enrichment within 6 hours of S/G2/M cells.
IMMUNOPRECIPITATION/WESTERNS: For these experiments, anti-cdc2 specific or pre-immune sera were used to quantitatively immunoprecipitate material from lysates of control or GRGDS-treated cells. Mock immunoprecipitates (containing primary antibodies and/ or protein G-agarose but no cellular proteins) were run as a negative control. Total cell lysates were loaded in one lane of each experiment as a positive blotting control. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose using an Ellard semi-dry transfer apparatus (0.8 mA/cm^2 for two hours). Blots were blocked with PBS/0.5% BSA/0.1% Triton X-100, then sequentially incubated with primary rabbit antibodies (anti-cdc2, anti-cdk2, anti-cyclin A, or anti-cyclin E, all diluted 1:1000 in blocking solution), horseradish-peroxidase-conjugated goat anti-rabbit Ig (diluted 1:10,000 in blocking solution), and Enhanced Chemiluminescence reagent (ECL, Amersham) before autoradiographic exposures using Kodak X-Omat film. In experiments designed to identify the cyclin associated with cdc2, anti-cdc2 or pre-immune sera were directly conjugated to CNBr-activated Sepharose for the initial immunoprecipitation. This protocol alteration was necessary because cyclins co-migrate with free Ig heavy chains. The blotting procedure was otherwise as described above.

PROLIFERATION ASSAYS: Cells were plated at subconfluent concentrations in wells of 96 well tissue culture plates on day -1 and allowed to adhere for 24 hours. Equimolar amounts of integrin ligands were added to wells on day 0. At intervals thereafter, quadruplicate wells were fixed with 95% EtOH, dried, stained with crystal violet (0.5% in MeOH), and observed by microscopy. Subsequently, the dye was solubilized and quantitated by measuring absorbance at 540 nm using an automated plate reader. Control experiments (Figure 4, top left panel) demonstrated that the staining intensity measured by absorbance at 540 nm increased linearly with cell number.

RESULTS: We previously reported that FNR interactions with GRGDS peptide induced cell proliferation via activation of cdc2, a cell cycle regulatory, cyclin-dependent kinase in the non-adherent FA-K562 cell line (32). Cdc2 activation was observed within hours after addition of GRGDS to FA-K562 cells. The control peptide GRGES that does not bind to the FNR had no effect on cdc2 activation (32). The FA-K562 cells used in the earlier studies express only one GRGDS-binding integrin, the alpha5beta1 FNR (25). The present study seeks information on whether primary cells and non-transformed cell lines are also susceptible to FNR-mediated growth control. In addition, this study further defines requirements for FNR-mediated growth control using FA-K562 cells as a model.

CELLS IN S/G2 PHASE OF THE CELL CYCLE SHOW MAXIMAL RESPONSES TO GRGDS.

Previous results showing GRGDS-activation of cdc2 were obtained using asynchronously grown cells (as shown in Figure 1A, left panel). As cdc2 is believed to mainly operate in G2/M, we analyzed responses of cells enriched for different phases of the cell cycle. FA-K562 cells serum-starved for 48-72 hours were enriched for G0/G1 (Figure 1A, middle panel). Addition of serum to these serum-starved cells resulted in enrichment in S/G2 cells after 6 hours (Figure 1A, right panel). The DNA staining pattern of cells released from serum-starvation was indistinguishable from that of asynchronously growing cells 48 hours after serum repletion (not shown), demonstrating that growth arrest induced by serum starvation is reversible. We measured cdc2 kinase responses to added GRGDS in these three cell populations. As before, results obtained with addition of the control peptide GRGES were indistinguishable from results obtained in control, untreated cells and no kinase activity was found in precipitates using pre-immune serum (not shown, 32). Cdc2 activity was stimulated two- to three-fold by addition of GRGDS to asynchronously grown cells (Figure 1B, left panels). In contrast, cdc2 kinase activity was reduced two-fold by addition of GRGDS to serum-starved cells (Figure 1B, middle panels). Finally, cdc2 activity was stimulated 10-fold in cells exposed to GRGDS 6 hours after release from serum starvation (Figure 1B, right panels). These data suggest that cells in late S/G2, but not cells in G0/G1, respond to exogenous GRGDS by activating cdc2.

IDENTIFICATION OF COMPONENTS OF THE CDC2/CYCLIN COMPLEX IN GRGDS-TREATED CELLS. Although antisera specific for the unique carboxyl-terminus of cdc2 were used for all immunoprecipitations, we sought additional proof that cdk2 was not responsible for our results. Figure 2A shows results of Western blots performed on cdc2 immunoprecipitates obtained from control- and GRGDS-treated cells. These experiments demonstrate that cdc2 immunoprecipitates contained no immunologically detectable contaminating cdk2. In addition, there was no difference in the levels of cdc2 (or cdk2) present in control-treated and GRGDS-treated cells (see left two lanes Figure 2A). We also probed blots of immunoprecipitated cdc2 with anti-cyclin A, anti-cyclin E, or pre-immune serum to learn which cyclin associates with cdc2 in our system (Figure 2B). The results indicate that cyclin A but not cyclin E is associated with cdc2. Data shown in Figures 1 and 2 suggest that GRGDS most likely activates cyclin A/cdc2 complexes in actively cycling cells in S/G2.

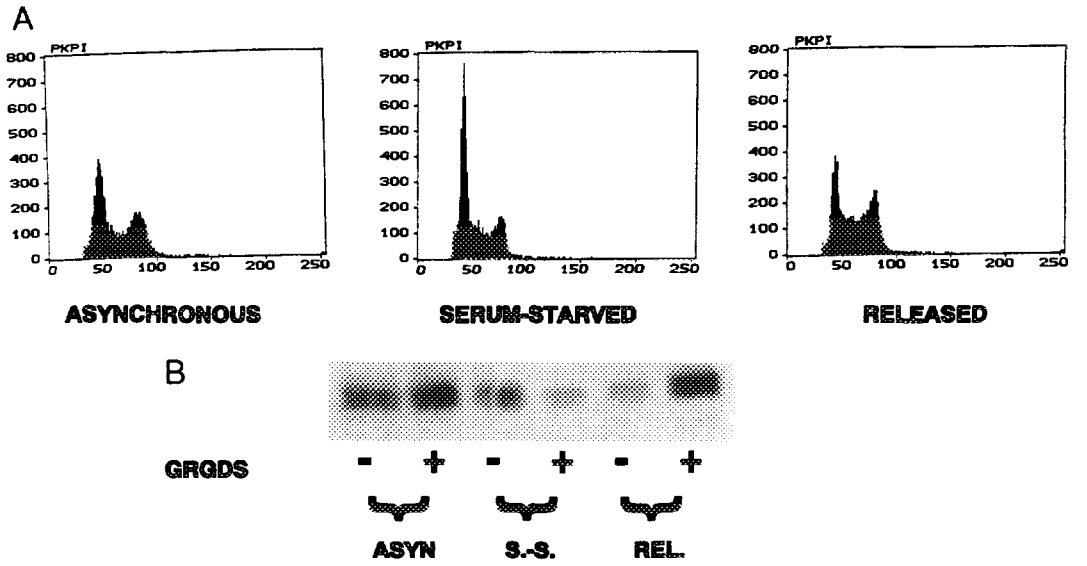

FIGURE 1. Cells in S/G2 phase of the cell cycle show maximal responses to GRGDS

Panel A shows the DNA staining profiles of asynchronous populations, G0/G1 enriched populations, and S/G2 enriched populations of FA-K562 cells, as indicated. The X axis shows intensity of propidium iodide staining in arbitrary units and the Y axis shows increasing cell numbers. Cells serum-starved under the conditions used here (48-72 hours in medium containing 0.1% FBS) were reversibly growth arrested. Six hours after release from serum starvation, enrichment of S/G2/M populations were seen (right panel).

Panel B shows histone H1 phosphorylation by immunoprecipitated cdc2 in control (-) and GRGDS-treated (+) populations of FA-K562 cells growing asynchronously (ASYN), under serum-starvation conditions (S.-S.), and 6 hours after release (REL) from serum starvation. Note that the modest increase in histone H1 phosphorylation by cdc2 in asynchronously growing cells is lost in serum-starved cells and is markedly enhanced in cells 6 hours after release from serum starvation, when increased numbers of S and G2 cells are observed. The kinase activity of immunoprecipitated cdc2 was assayed using exogenous histone H1 as a substrate (1 μg/ reaction) and 32P-gamma-dATP as a phosphate donor (10 μCi/reaction). In this and all subsequent experiments, equal cell numbers (10^6) were used for cdc2 immunoprecipitates and no histone phosphorylation was observed in immunoprecipitates using pre-immune serum.

GROWTH RESPONSES OF NON-TRANSFORMED CELLS TO GRGDS. We measured two different cell growth responses to GRGDS-- activation of the cdc2 kinase and increases in cell number-- in primary keratinocytes (KC), immortalized but non-transformed epidermal cells (FEPE1L8) (34), immortalized breast cells (HBL100) (37), and transformed epidermal (1811T1)(35) or breast cells (BT20)(37). Transformation was defined by the ability of cells to grow in an anchorage independent manner and/or to form tumors in nude mice. The addition of GRGDS stimulated cdc2 kinase activity of primary KC, and immortalized epidermal (FEPE1L8) or breast cells (HBL100)(Figure 3, left panels). No histone phosphorylation was observed in immunoprecipitates performed using preimmune sera (not shown, 32). Quantitation using a Molecular Dynamics PhosphorImager 400E showed a two-fold (HBL100, KC) to five-fold (FA-K562, FEPE1L8) induction of cdc2 by GRGDS in each experiment. In contrast, the addition of GRGDS did not stimulate cdc2 activity of transformed epidermal (1811T1) or breast cells (BT20) (37) (Figure 3, right panels). Thus, the FNR ligand GRGDS activates cdc2 in primary human KC and a variety of non-transformed human cell lines, but has no effect on cdc2 activity in transformed cell lines.

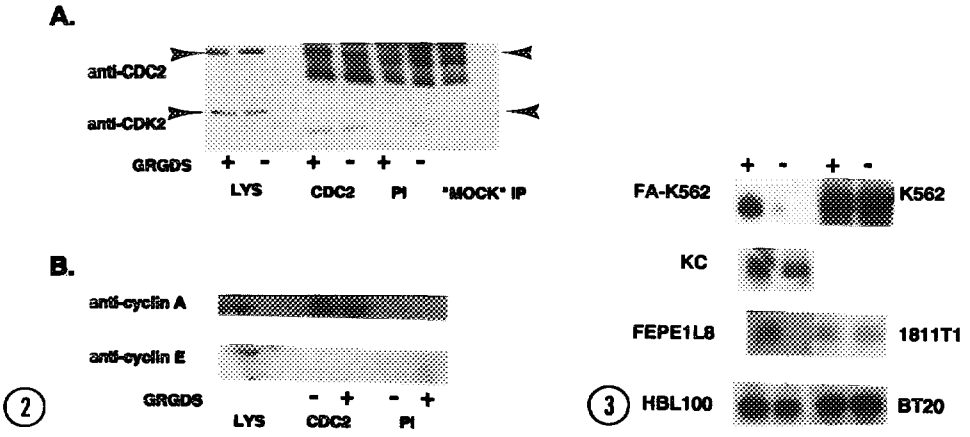

FIGURE 2. Identification of components of the cdc2/cyclin complex in GRGDS-treated cells

A. Total cellular protein (LYS), cdc2 (CDC2) or control (PI) immunoprecipitates, and "MOCK" immunoprecipitates incubated with antibody and protein G-agarose or protein G-agarose without cellular proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose and blotted with anti-cdc2 or anti-cdk2 sera to evaluate the components of immunoprecipitated complexes. As shown at the top of panel A, only cdc2 immunoprecipitates contained material co-migrating with bone-fide cdc2 (marked with arrows in top panel). Protein G-agarose "mock" immunoprecipitates did not react with blotting antibodies (see lane at far right) whereas "mock" immunoprecipitates containing anti-cdc2 and protein G-agarose contained many non-specific bands reactive with the blotting antiserum, but no cdc2. In contrast, none of the immunoprecipitates contained material that co-migrated with bone-fide cdk2 although cell lysates contained cdk2 as shown in the lower part of panel A. This demonstrates that there is no cdk2 contamination of our cdc2 immunoprecipitates and also that control and GRGDS-treated cells contain equal amounts of immunoreactive cdc2 and cdk2.

B. Cyclin A but not cyclin E was present in cdc2 immunoprecipitates. These experiments were performed as described above with two variations. First anti-cyclin A or E antisera were used to probe blots. In addition anti-cdc2 or pre-immune sera were directly coupled to CNBr-activated Sepharose and used for the initial immunoprecipitation. This was necessary as Ig heavy chain present in immunoprecipitates using unconjugated antibodies otherwise interfered with cyclin detection.

FIGURE 3. GRGDS stimulates cdc2 kinase activity in a variety of non-transformed cells

Immortalized but non-transformed FA-K562, FEPE1L8, and HBL100 cells, primary human keratinocytes (KC), and transformed K562, 1811T1, and BT20 cells were incubated for two hours with GRGDS (+) or GRGES (-) (50 nM) prior to cell lysis and cdc2 immunoprecipitation. Cdc2 activity was stimulated by GRGDS in all non-transformed cells (in left column). Phosphorimager scanning demonstrated 5-fold enhancement of cdc2 kinase activity in GRGDS-treated FA-K562 and FEPE1L8 cells, and 2-fold enhancement in KC and HBL100 cells. In contrast, cdc2 kinase activity was unaffected by added GRGDS in all transformed cells (shown in right column). Results for FA-K562 and K562, shown at the top, are included as positive and negative controls, respectively.

Unlike FA-K562 cells that express a single FNR, epithelial cells express at least three integrins that can bind GRGDS/or FN: the high affinity FNR, alpha3beta1, and alphaV- integrins (21, 38). The latter two bind collagen or vitronectin, respectively, but can bind FN and/or GRGDS with low affinity (36, 1, 39). We reasoned that proliferation induced by GRGDS but not collagen or vitronectin represented a FNR-mediated response in these cells. Therefore, cell proliferation assays were performed in the presence of various integrin ligands. GRGDS and FN stimulated the proliferation of primary KC and of non-transformed HBL100 breast cells, whereas vitronectin (VN), collagen (COL), and laminin (LAM) did not (Figure 4, right, top and middle respectively and data not shown). Thus, GRGDS is likely to stimulate growth of epithelial cells via the high affinity alpha5beta1 FNR and not via alpha3beta1 or alphaV- integrins. The correlation between FNR ligand-induced cdc2 activation and subsequent proliferation has been observed for all cells tested (Figures 3, 4, and ref. 32). In addition, the observation that

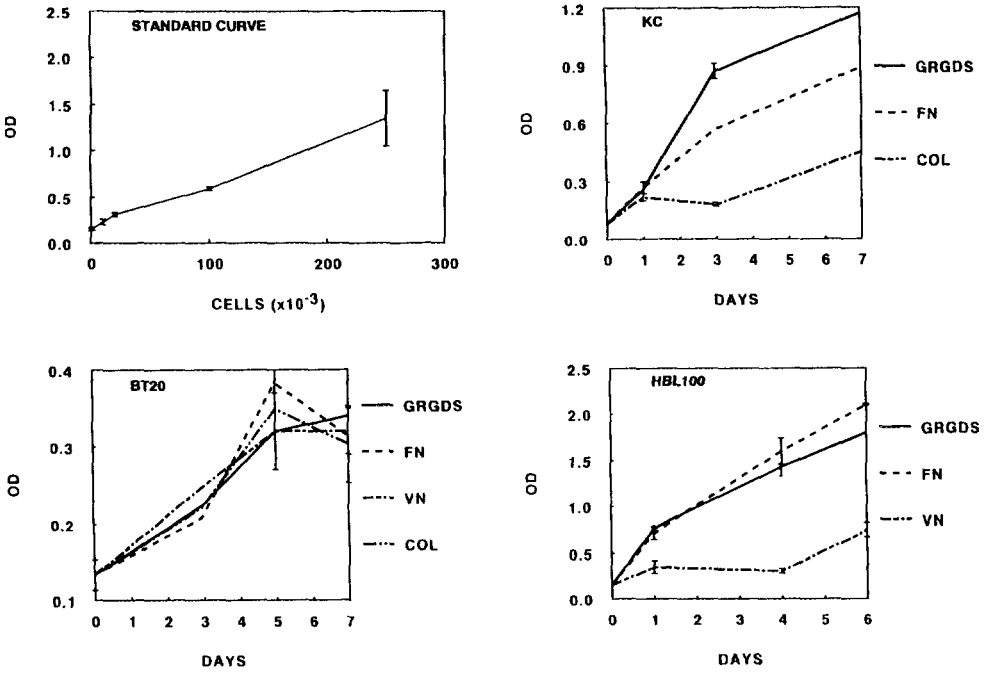

FIGURE 4. Only FNR ligands induce increases in cell numbers. The standard curve at top left shows that absorbance at 540 nm (labeled OD along Y axis) varies linearly with increasing cell number. Standard errors >5% are indicated with bars. Primary keratinocytes (KC) and immortalized HBL100 breast cells (two panels shown at right) were specifically growth-stimulated by FNR ligands, whereas transformed BT20 breast cells (left panel at left) were not. Using similar assays, we previously showed that FA-K562 cells were specifically induced to proliferate by FNR ligands, whereas K562 cells were not (32).

transformed hematopoietic, epidermal, and breast epithelial cells do not respond to FNR ligands (see Figures 3, 4; 32) suggests that the FNR may be a key growth regulator that is affected by transformation.

KINETICS OF GRGDS-INDUCED CDC2 ACTIVATION. We further characterized FNR-mediated growth signalling by using GRGDS as a model low valency FNR ligand. FA-K562 cells were used to simplify interpretation because these cells express only one integrin that can bind GRGDS. Furthermore, because FA-K562 cells are non-adherent, the effects of FNR ligand binding can be assessed independent of any effects on cell attachment. effects of FNR ligands can be attributed solely to FNR perturbation rather than to detachment from substrate. GRGDS could theoretically activate cdc2 by inducing expression of cyclins, by altering cdc2 phosphorylation, or by inducing associations between cdc2 and specific cyclins (40, 41). We analyzed the kinetics of GRGDS-induced cdc2 activation to shed light on the most likely mechanism, since only changes in cyclin expression levels should occur slowly. For these experiments, GRGDS or control peptide was added to cells in suspension, mixed by inverting the tube several times, and an aliquot containing a constant number of cells was removed immediately and at intervals thereafter. Sample processing took about one minute at 4°C, so the first time point shown in Figure 5 is labeled <1.5 minutes. Cdc2 kinase activity was higher in GRGDS-treated compared with GRGES-treated cells at all time points (Figure 5). In each of three separate experiments, GRGDS-induced cdc2 activation was apparent at the first time point and maximal at 150 to 180 minutes. As

before, controls showed no difference between non-treated and GRGES-treated cell samples (not shown). The rapid induction of cdc2 activation by GRGDS suggests that de novo synthesis of proteins or mRNA is not required and that altered cdc2 phosphorylation or cyclin associations are more likely candidate mechanisms for this phenomenon.

EFFECTS OF DIFFERENT FNR LIGANDS ON CDC2 ACTIVITY. We tested the ability of different FNR ligands to stimulate cdc2 activity of FA-K562 cells. The mAbs P1D6 and P4C10, against alpha5 and beta1, respectively (36), and FN were used. As shown in Figure 6, purified plasma FN, P1D6, and P4C10 all stimulated cdc2 activity when used at 50 nM (labeled .05). GRGDS was effective over a wide range of tested concentrations (5 nM-20 μM). However, five-fold increases in the FN concentration (to 250 nM) resulted in reduced ability to stimulate cdc2 (Figure 6 and data not shown). The relatively narrow effective concentration range for FN was observed in each of three separate experiments. In addition, although soluble P1D6 and P4C10 stimulated cdc2 activity, this activity was reduced if the cell-associated anti-FNR mAbs were cross-linked with rabbit anti-mouse IgG (labeled +2 in Figure 6). Taken together, these results suggest that FNR binding to low valence ligands stimulates growth whereas FNR binding to high valence ligands inhibits or has no effect on growth. The greater potency of low- versus high-valence ligands distinguishes FNR-mediated cdc2 activation from other integrin-mediated signalling events that require integrin clustering, such as pp125FAK phosphorylation (8-10), pH changes (14), and gene expression (11-13).

DISCUSSION: The present study demonstrates that growth stimulation induced by FNR perturbation is common to a variety of primary cells and non-transformed cell lines, including cell lines that express multiple integrins. Further, loss of growth responses to FNR ligands is shown to be a frequent occurrence in transformed cells. Together, these findings suggest that the FNR is a key growth regulator. This idea may help to explain why the FNR is frequently altered in transformed or neoplastic cells (16-26). Our findings may also be relevant to earlier observations regarding transiently increased FNR expression at wound sites (3, 4, 28). Increased FNR expression at wound margins may facilitate

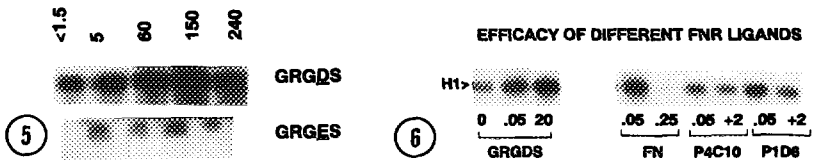

FIGURE 5. GRGDS-induced cdc2 activation occurs rapidly in FA-K562 cells. GRGDS or GRGES peptide was added to cells at time 0. Aliquots were removed and processed at intervals thereafter (indicated in minutes along the top). Processing time for the aliquot removed at time zero was approximately 60-90 seconds, and this time point is therefore labeled <1.5 minutes. Increased kinase activity was observed in GRGDS-treated samples relative to GRGES-treated samples at all time points. Maximal GRGDS-induced kinase activation was observed at between 150 and 180 minutes in all experiments. This experiment is representative of three performed.

FIGURE 6. Cdc2 stimulation by different FNR ligands. Cdc2 activation occurred in response to FNR perturbation by GRGDS, FN, P1D6 (anti-alpha5 mAb), and to a lesser extent P4C10 (anti-beta1 mAb) used at 50 nM (labeled .05 in figure). Cells were lysed 60 minutes after the addition of different FNR ligands as indicated and cdc2 kinase assays performed as described in the legend for Figure 1. GRGDS was effective when used over a wide range of concentrations (5 nM-20 μM). In contrast, FN was only effective when used at 50 nM. Cross-linking of cell-associated anti-FNR mAbs with soluble rabbit anti-mouse Ig (final concentration of 5 μg/ml) (labeled +2) reduced their ability to stimulate cdc2. This experiment is representative of three performed.

and regulate local cell proliferation. It may seem paradoxical that transformed cells have diminished FNR-mediated growth responses relative to non-transformed cells, especially given the belief that transformed cells are hyperproliferative compared with normal cells. A recently proposed model reconciles these findings by suggesting that unoccupied FNR delivers a growth inhibitory signal whereas FNR perturbation upon ligand binding delivers a growth stimulatory signal that overcomes basal growth inhibition (23). Abnormal FNR function in transformed cells could thus interfere with both putative growth suppressive signals delivered via unoccupied FNR and with ligand-dependent delivery of growth stimulatory signals. This would account for both the constitutive growth and lack of FNR-mediated growth control observed for transformed cells.

The observed negative effect of ligand multivalence on FNR-mediated growth is intriguing in view of the physiological conditions under which low valency FNR ligands would exist. Thus, only cells in contact with plasma FN (hematopoietic or endothelial cells) or cells in contact with proteolyzed FN fragments (i.e. cells at wound sites) (42) would encounter growth stimulatory, low valence FNR ligands. Such conditions would favor cellular proliferation at wound sites. Once tissue proteolysis has stopped, FNR-stimulated growth would cease. The data also suggest that FNR clustering, such as occurs at sites of mature focal contacts, would prevent or inhibit FNR-mediated growth. Conversely, the diffuse distribution of plasma membrane FNR of certain transformed cells (43, 44), of migrating KC at wound margins (3,4), and of migrating embryonic cells (2) might favor cell proliferation.

In summary, the present study advances understanding of cell cycle control, integrin-mediated signal transduction, and their interactions in several ways. First, it identifies cdc2/cyclin A as the cell cycle regulatory complex subject to modulation by exogenous GRGDS. Second, it better defines the cell cycle fraction that is responding to exogenous GRGDS. Third, it defines the FNR ligand requirements for FNR-mediated growth stimulation. Finally, and perhaps most importantly, it demonstrates that signalling via the FNR may be a general mechanism for controlling growth of non-transformed cells. This last finding, coupled with the demonstration that growth responsiveness to the FNR can be restored by minor changes in surface FNR expression levels suggests that the FNR may act as a tumor suppressor. Our future work will characterize defects in FNR in transformed cells and assess the effects of exogenous expression of FNR on growth signalling in transformed cells.

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