Fibronectin Receptor Modulates Cyclin-dependent Kinase Activity*

(Received for publication, June 23, 1992)

B. Erhan Symington
From the Fred Hutchinson Cancer Research Center, Division of Basic Sciences M477, Seattle, Washington 98104

The high affinity fibronectin receptor (FNR) is expressed by hematopoietic cells, fibroblasts, and proliferating epithelial cells. Expression of this integrin is altered by chemical and viral transformation, suggesting that FNR dysfunction may play a role in growth control. This study demonstrates that exposing FA-K562 cells to glycine-arginine-glycine-aspartate-serine (GRGDS), a peptide ligand of the FNR, specifically stimulates p34/cdc2- and cyclin A-associated kinase activities. This occurs within 2 h of peptide addition. The 110-kDa form of the retinoblastoma protein appears within 3 h of GRGDS addition, consistent with activation of a G1/S kinase. DNA staining profiles demonstrate that GRGDS induces cell cycle progression within 24 h. Increased anchorage-independent growth is subsequently observed in GRGDS-treated FA-K562 cells. The control peptide, GRGES, which cannot bind the FNR, has none of these effects. This demonstrates that an extracellular integrin ligand can regulate cell proliferation. Furthermore, these results suggest that integrins link the extracellular environment and intracellular growth regulators.

The fibronectin receptor (FNR) (also termed α5β2, VLA-5, and CD49e/CD29) is an integrin adhesion receptor (1, 2). The minimal sequence in fibronectin specifically recognized by this FNR is arginine-glycine-aspartate (RGD) (1, 2). Arginine-glycine-glutamate (RGGE) does not bind to the α5β2 FNR. Although initially thought to mediate cell-substrate adhesion to fibronectin only, recent studies have demonstrated a role for the FNR in growth control. For example, FNR overexpression has been associated with loss of anchorage-independent growth of human erythroleukemia cells and hamster fibroblasts (5, 6). T cell proliferation can be induced by ligation of CD3 and either the α5β2 or α5β1 FNR (7, 8). This study examines whether the α5β2 FNR alone plays a role in cell growth regulation. The FA-K562 cell line was used for most of these studies. This stable human cell line expresses 5-fold more cell-surface FNR than the parental K562 cell line (5). The FNR is the only RGD-binding receptor expressed by both K562 and FA-K562 cell lines. Thus, FA-K562 cells provide a model for studying potential effects of FNR/peptide ligand interactions on cell growth.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—K562 cells were obtained from the American Type Culture Collection. The FA-K562 subline, selected for its ability to adhere to fibronectin, has been previously described (6). Both cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in a humidified 5% CO2, 95% air incubator.

**Soft Agar Colony Formation**—Cells were washed and plated at a concentration of 104 cells/ml in RPMI 1640 medium containing 20% fetal bovine serum, 1% Difco Bacto-agar, and 10 μg/ml GRGDS or GRGES (Peninsula Laboratories, Inc.). Colonies were counted 14-21 days later. Cloning efficiencies were calculated as the percent of plated cells that formed macroscopic colonies.

**Cell Cycle Analysis**—Cells were incubated with or without GRGDS or GRGES peptide (10 μg/ml) in RPMI 1640 medium, 10% fetal calf serum (5 × 106 cells/ml). Cell aliquots (106) were removed at 0 or 24 h, washed, and sequentially incubated in lysis buffer (10 mM Tris, 30 mM NaCl, 20 mM MgCl2, pH 7.4), followed by lysis buffer containing 1% Nonidet P-40 (5 min each on ice). RNase (Boehringer Mannheim) was then added at 50 units/ml, and the cells were incubated for 30 min at 37°C. Propidium iodide was added to a final concentration of 40 μg/ml, and the cells were incubated for 30 min on ice prior to analysis using an EPICS V flow cytometer interfaced to an MDADS computer. Data were analyzed using REPROT software (True-Tracks Inc., Seattle, WA). Cell doublets and debris were excluded from analysis by gating on integrated propidium iodide to peak propidium iodide.

**In Vitro Kinase Assays**—FA-K562 cells were incubated for 2 h in RPMI 1640 medium, 10% fetal calf serum containing 10 μg/ml GRGDS or GRGES peptide and solubilized in 50 mM Tris, pH 7.4, 250 mM NaCl, 0.5% Nonidet P-40. A cell density of ~103 cells/100 μl. Cell lysates were reacted with anti-cdc2, anti-cdk2, anti-cyclin E (9), or normal rabbit serum (1:50 final dilution), followed by protein A-agarose beads. The first two antisera were raised against the unique carboxyl terminus of cdc2 or cdk2, and can recognize both free and cyclin-bound cdk2 (9). In three experiments, cyclin A or E was immunoprecipitated using a mouse monoclonal or rabbit antiserum (9), respectively. Beads were washed, resuspended in 50 μl of kinase buffer (20 mM Tris, 10 mM MgCl2, 1 mM dithiothreitol, 30 μM ATP, 10 μCi of [γ-32P]ATP (3000 Ci/mmol; Amerham Corp.) with or without 1 μg of histone H1 (reaction Sigma)), and incubated for 30 min at 37°C prior to solubilization in SDS-PAGE sample buffer and electrophoresis on 12% polyacrylamide slab gels. Gels were fixed in 10% methanol, 10% acetic acid; dried; and exposed to Kodak X-Omat film for 30-120 min. Kinase activity was quantitated by two methods (densitometry of developed films and Cerenkov counting of excised gel bands), both of which gave equivalent results.

**Retinoblastoma Protein Labeling and Immunoprecipitation**—Cells (5 × 104/ml) were washed, resuspended in methionine-free RPMI 1640 medium containing 200 μCi/ml Tran35S-label (ICN), and incubated with 10 μg/ml GRGDS or GRGES. Cells (5 × 104/ml) were solubilized in lysis buffer (50 mM HEPS, 250 mM NaCl, 0.1% Nonidet P-40, pH 7.0) and spun at 15,000 rpm in an Eppendorf microcentrifuge. Cell lysates were sequentially incubated at 4°C with 10 μg/ml anti-retinoblastoma protein monoclonal antibody (NZ91) (Pharmingen, San Diego, CA) or irrelevant IgG2 isotype control, rabbit anti-mouse IgG, and protein A-agarose beads. Beads were then washed four times in 10 mM Tris-HCl, 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, pH 7.5; resuspended in sample buffer; and analyzed by 7% SDS-PAGE.

**RESULTS AND DISCUSSION**

A key difference between FA-K562 and the parental cell line is that K562 cells grow well in soft agar, whereas FA-K562 cells do not (cloning efficiencies of >95 and <10%, respectively) (8). Thus, FA-K562 cells appear to be growth-
inhibited compared to parental K562 cells. GRGDs and GRGES have no effect on the soft agar growth of K562 cells (96 versus 99% cloning efficiency, respectively) (Fig. 1). In contrast, GRGDs, the FNR-binding peptide, stimulates the soft agar growth of FA-K562 cells (from 3 to 98% cloning efficiency) (Fig. 1). GRGES has no effect on FA-K562 soft agar colony formation. In previous studies, anti-FNR antibodies stimulated the soft agar growth of FA-K562 cells in a specific and concentration-dependent manner (5). Taken together, these results suggest that FNR perturbation (or occupancy) stimulates the growth of FA-K562 cells.

The effects of GRGDs peptide on cell cycle progression of FA-K562 and K562 cells was examined to evaluate the mechanism for colony growth in GRGDs-treated FA-K562 cells. FA-K562 and K562 cells were incubated for 4 or 24 h with GRGDs or control GRGES or with no peptide. The DNA staining profiles of cells treated for 24 h with GRGDs or GRGES peptide are shown in Fig. 2. The DNA profiles of untreated or GRGES-treated FA-K562 and K562 cells were identical (data not shown). No differences in the DNA staining profiles of any treatment group were observed after only 4 h. Incubation with GRGDs decreased the proportion of G1 phase FA-K562 cells (from 52 to 36%) and increased the proportion of S phase FA-K562 cells compared to untreated or GRGES-treated cells (Fig. 2, left). In contrast, incubation with GRGDs had no effect on cell cycle progression of K562 cells (Fig. 2, right). Thus, peptide effects on both the cell cycle and anchorage-independent growth were limited to GRGDs peptide and the FNR-overexpressing FA-K562 line. These results indicate that increased DNA synthesis occurred within 24 h of exposure to GRGDs. This interval was found to be even shorter when [3H]thymidine uptake assays were performed. Five hours after the addition of peptides, [3H]thymidine uptake was 11-fold higher for GRGDs-treated than for control and RGD-treated FA-K562 cells. As shown in Fig. 3, little kinase activity was found associated with cyclin E isolated from control or RGD-treated cells. Longer exposures of autoradiographs showed that cyclin E-associated kinase activity was unchanged by RGD treatment. Kinase activity was detected in cyclin A immunoprecipitates from control cells. Modest but consistent increases in cyclin A-associated kinase activity were observed in RGD-treated compared with control-treated cells in each of three experiments. These findings are compatible with a number of interpretations. Immunoprecipitation followed by Western blotting was performed to determine whether RGD treatment caused an association between cyclin A and cdc2 kinase. Cyclin A or control immunoprecipitates from control or RGD-treated cells were probed with antisera to cdc2 or cdk2. The reverse of this experiment, where cdc2 and irrelevant antibody immunoprecipitates were probed with anti-cyclin A, was also performed. These experiments demonstrated that the RGD-induced increase in cdc2 kinase activity was not due to cyclin A-associated cdc2 (data not shown).

A number of different cyclins can associate with the p34/cdc2 kinase (9, 13). Cyclin B-cdc2 complexes have been implicated in the control of mitosis, whereas complexes of cyclin E or A with cdc2 act at the G1/S transition (9, 13). Kinase activities associated with cyclins A and E were measured in control and RGD-treated FA-K562 cells. As shown in Fig. 3, little kinase activity was found associated with cyclin E isolated from control or RGD-treated cells. Longer exposures of autoradiographs showed that cyclin E-associated kinase activity was unchanged by RGD treatment. Kinase activity was detected in cyclin A immunoprecipitates from control cells. Modest but consistent increases in cyclin A-associated kinase activity were observed in RGD-treated compared with control-treated cells in each of three experiments. These findings are compatible with a number of interpretations. Immunoprecipitation followed by Western blotting was performed to determine whether RGD treatment caused an association between cyclin A and cdc2 kinase. Cyclin A or control immunoprecipitates from control or RGD-treated cells were probed with antisera to cdc2 or cdk2. The reverse of this experiment, where cdc2 and irrelevant antibody immunoprecipitates were probed with anti-cyclin A, was also performed. These experiments demonstrated that the RGD-induced increase in cdc2 kinase activity was not due to cyclin A-associated cdc2 (data not shown).

These results clearly demonstrate that an FNR ligand activates cdc2. p34/cdc2 controls both the G1/S and G2/M transitions in yeast (11), although the situation in mammalian cells is more complicated. Since increased DNA synthesis was observed in less time than would be expected for cells to complete mitosis and enter another G1/S transition, it appeared that GRGDs was affecting a kinase acting at the G1/S transition. To investigate this further, the SDS-PAGE mobility of the retinoblastoma protein immunoprecipitated from control and GRGDs-treated cells was examined. I reasoned that if retinoblastoma protein mobility was altered coincident with cdc2 kinase activation, then GRGDs was indeed affecting a G1/S kinase. The retinoblastoma protein is an endogenous cyclin-dependent kinase substrate whose growth-suppressing function is inhibited by phosphorylation (15). Phosphorylation inhibits the ability of the retinoblastoma protein to bind cyclins and triggers the G1/S transition (16–18). The retinoblastoma protein does not appear to participate in the G2/M transition. Several studies have shown that retinoblastoma protein phosphorylation reduces its SDS-PAGE mobility (18–20). Cells were biosynthetically labeled with [35S]methionine so that both phosphorylated and non-phosphorylated retinoblastoma protein isoforms would be de-
Fig. 2. GRGDS induces cell cycle progression of FA-K562 cells. FA-K562 and K562 cells were incubated with GRGDS or GRGES or with no peptide for up to 24 h. Cells were sampled at 4 and 24 h, and their DNA content was measured by propidium iodide (PI) staining and flow microfluorometry. Shown here are the DNA fluorescence histograms for FA-K562 and K562 cells incubated for 24 h in the presence of GRGDS (dashed lines) or GRGES (solid lines) as indicated (left panel). Staining in the absence of peptide was similar to staining in the presence of GRGES. No differences were observed in the various treatment groups at the 4-h time point. The two major peaks observed in GRGES-treated cells represent, from left to right, G1 and G2/M. GRGDS reduces the number of G1 and increases the number of S phase FA-K562 (but not K562) cells.

Fig. 3. GRGDS induces p34/cdc2- and cyclin A-associated kinase activities in FA-K562 cells. FA-K562 cells were incubated for 2 h with GRGDS (+) or GRGES (−). The p34/cdc2, cdk2, and cyclins A and E immunoprecipitated from FA-K562 cells were assayed for kinase activity by using exogenous histone H1 as a substrate. Shown here are the results obtained using rabbit antiserum to cyclin E (CY-E), rabbit antiserum to the unique carboxyl terminus of cdc2 or cdk2, normal rabbit serum (NRS), mouse monoclonal antibody to cyclin A (CY-A), or an irrelevant mouse antibody (IR), as indicated. The migration position of histone H1 is indicated to the left. Phosphorylation was not observed in the absence of added histone. Kinase activity quantitated in two manners (densitometry of developed films and Cerenkov counting of excised bands) showed 15-fold (cdc2) and 3-fold (cyclin A) increased kinase activity in GRGDS-treated compared with GRGES-treated cells.

Fig. 4. Treatment of FA-K562 cells with GRGDS induces accumulation of high molecular mass forms of retinoblastoma protein. FA-K562 or K562 cells were incubated with GRGDS (+) or GRGES (−) for various lengths of time. The 3-h time point is shown. Upper and lower arrows indicate the positions of the 110- and 105-kDa forms of the retinoblastoma protein, respectively. GRGDS (but not GRGES) treatment led to the appearance of the slower 110-kDa form of the retinoblastoma protein in FA-K562 cells. Neither peptide had this effect on K562 cells.

tectable on the same gel. Results of one experiment (representative of three performed) are shown in Fig. 4. Control FA-K562 and K562 cells expressed only the ~105-kDa underphosphorylated form of the retinoblastoma protein. The K562 cell line, which shows no growth response to GRGDS, expressed only the 105-kDa form of the retinoblastoma protein after the addition of GRGDS. In contrast, GRGDS-treated FA-K562 cells accumulated the 110-kDa form of the retinoblastoma protein within 3 h. This suggests that the FNR/GRGDS interaction induces retinoblastoma protein phosphorylation and cdc2 kinase activation with similar kinetics. These data provide additional indirect evidence that cdc2 activated by GRGDS treatment is acting at the G1/S transition.

In summary, FNR perturbation by GRGDS leads to early activation of a normal cdc2-dependent proliferation pathway in FA-K562 cells. Increased DNA synthesis and soft agar colony formation are subsequently observed. The commitment of FA-K562 cells to proliferate occurs within 2–3 h of the addition of peptide, arguing against a requirement for de novo production of growth factors. Peptide effects on cdc2 activation, retinoblastoma protein phosphorylation, cell cycle progression, and cell proliferation are limited to the FNR-binding peptide, GRGDS. This suggests that FNR perturbation is involved in each of these events. Furthermore, the close temporal association of FNR perturbation, cdc2 activation, and retinoblastoma protein phosphorylation suggests that some type of interaction occurs. Future studies will investigate more proximal signals transduced by FNR occupancy and seek to link these signals to retinoblastoma protein activity.

The data presented here demonstrate that integrin perturbation alone can affect the cell cycle. This is interesting in light of a recent report that cyclin-cyclin-dependent kinase interactions may be affected by suspension versus adherent culture of HeLa cells (21). In suspension cells, cyclin A was associated with cdc2, whereas in adherent HeLa cells, cyclin A was associated with cdk2. This “switching” of cyclin-dependent kinase partners could result from perturbation of integrins that are known to mediate cell-substrate adhesion. Ongoing studies using normal human keratinocytes suggest that these effects are not restricted to cultured or transformed cell lines. It is unclear why only the FNR-overexpressing hematopoietic cell line was growth-stimulated by GRGDS. One potential explanation is that GRGDS displaces a fibro-
nectin-associated growth inhibitor, such as fibronectin-transforming growth factor-$
\beta$ (22). In this model, the more FNR expressed by the cell, the higher the local concentration of growth inhibitor. Another possibility is that the parental K562 cell line expresses a mutant form of the retinoblastoma protein whose function is corrected by FNR overexpression. The latter explanation is more likely in view of results obtained using normal human keratinocytes, which express low levels of the $\alpha_\beta_1$ FNR. It is intriguing, in this context, that FNR overexpression and retinoblastoma protein re-expression similarly inhibit tumor formation in nude mice, colony formation in soft agar, and growth in culture (5, 6, 23).

Regardless of the mode of action of GRGDS, this study demonstrates that FNR perturbation can result in mitogenesis. This may explain earlier observations that neoplastic transformation of cells affects cell-surface fibronectin and FNR expression or function (1, 3, 4, 24). It also suggests that one way to control tumor cell growth may be to manipulate FNR ligands presented to these cells.

Acknowledgments—I thank Dr. Andrew Koff for generously providing reagents and advice, Dr. Eduardo Firpo for help with DNA staining, Dr. William Carter for encouragement, and Dr. Frank Symington for helpful discussions and critical manuscript review.

REFERENCES

1. Hemler, M. E. (1990) Annu. Rev. Immunol. 8, 365-400
2. Hynes, R. O. (1987) Cell 48, 549-564
3. Planteibe, L. C., and Hynes, R. O. (1989) Cell 56, 281-290
4. Symington, B. E., Symington, F. W., and Rohrschneider, L. R. (1989) J. Biol. Chem. 264, 13258-13266
5. Symington, B. E. (1990) Cell Regul. 1, 637-648
6. Giancotti, F. G., and Ruoslahti, E. (1990) Cell 60, 849-859
7. Matsuyama, T., Yamada, A., Kay, J., Yamada, K. M., Akiyama, S. K., Schlossman, S. F., and Morimoto, C. (1989) J. Exp. Med. 170, 1133-1148
8. Nojima, Y., Humphries, M. J., Mould, A. P., Komoriya, A., Yamada, K. M., Schlossman, S. F., and Morimoto, C. (1990) J. Exp. Med. 172, 1185-1192
9. Koff, A., Cross, F., Fisher, A., Schumacher, J., Legaelle, K., Phillipe, M., and Roberts, J. M. (1991) Cell 66, 1217-1228
10. Fang, F., and Newport, J. W. (1991) Cell 66, 731-742
11. Nasmyth, K. (1991) New Biol. 3, 955-958
12. Draetta, G., and Beach, D. (1988) Cell 54, 17-26
13. Hunter, T., and Pines, J. (1991) Cell 66, 1071-1074
14. Norbury, C., Blow, J., and Nurse, P. (1991) EMBO J. 10, 321-329
15. Yin, B. T. Y., Gruenewald, S., Morla, A. O., Lee, W.-H., and Wang, Y. Y. J. (1991) EMBO J. 10, 857-864
16. Bandara, L. R., Adamczewski, J. P., Hunt, T., and LaThangue, N. B. (1991) Nature 352, 249-252
17. Goodrich, D. W., Wang, N. P., Qian, Y.-W., Lee, E. Y.-H. P., and Lee, W.-H. (1991) Cell 67, 293-302
18. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J., and Lee, W.-H. (1989) Cell 58, 1193-1196
19. Leib, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massague, J. (1990) Cell 62, 173-185
20. Ludlow, J. W., Shon, J., Pipas, J. M., Livingston, D. M., and DeCaprio, J. A. (1990) Cell 69, 387-396
21. Elledge, S. J., Richman, R., Hall, F. L., Williams, R. T., Lodgeon, N., and Harper, J. W. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 2907-2911
22. Fava, R. A., and McCabe, D. B. (1987) J. Cell. Physiol. 131, 184-189
23. Huang, H.-J. S., Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookstein, R., Friedmann, T., Lee, E. Y.-H. P., and Lee, W.-H. (1988) Science 242, 1563-1569
24. Olden, K., and Yamada, K. M. (1977) Cell 11, 957-969