Activation of the p21 Pathway of Growth Arrest and Apoptosis by the β4 Integrin Cytoplasmic Domain*

(Received for publication, May 26, 1995, and in revised form, July 18, 1995)

Astrid S. Clarke, Margaret M. Lotz, Celia Chao, and Arthur M. Mercurio†

From the Laboratory of Cancer Biology, Deaconess Hospital and Harvard Medical School, Boston, Massachusetts 02115

The integrin αβ4 is a receptor for members of the laminin family of basement membrane components, contributes to the function of epithelial cells and their oncogenically transformed derivatives. In our efforts to study αβ4-mediated functions in more detail and to assess the contribution of the β4 cytoplasmic domain in such functions, we identified a rectal carcinoma cell line that lacks expression of the β4 integrin subunit. This cell line, termed RKO, expresses αβ4, but not αβ6 and it interacts with laminin-1 less avidly than similar cell lines that express αβ6. We expressed a full-length β4 cDNA, as well as a mutant cDNA that lacks the β4 cytoplasmic domain, in RKO cells and isolated stable subclones of these transfectants. In this study, we report that subclones that expressed the full-length β4 cDNA in association with endogenous α6 exhibited partial G1 arrest and apoptosis, properties that were not evident in RKO cells transfected with either a β4 cytoplasmic domain mutant or the expression vector alone. In an effort to define a mechanism for these observed changes in growth, we observed that expression of the αβ4 integrin induced expression of the p21 (WAF1; Cip1) protein, an inhibitor of G1 cyclin-dependent kinases (10–12). These data suggest that the β4 integrin cytoplasmic domain is linked to a signaling pathway involved in cell cycle regulation in the β4-transfected RKO cells.

The integrin αβ6 is a receptor for members of the laminin family of basement membrane components. Initial studies established that αβ6 is a receptor for laminin-1 (1–3), and subsequent work has shown that it also functions as a receptor for other laminin isoforms (4, 5). In its capacity as a laminin receptor, αβ6 is involved in the formation and maintenance of hemidesmosomes (6–8) and in the dynamic adhesion and migration of carcinoma cells (2, 3). Most likely, other functions of epithelial and carcinoma cells are dependent upon αβ6 because it plays such a pivotal role in mediating their interac-

tions with laminin matrices. It is widely assumed that the unusually large and structurally unique cytoplasmic domain of the β4 integrin subunit associates with cytoskeletal and signaling molecules and that such associations provide the basis for the distinct functions associated with αβ4 (5, 8, 9).

In our efforts to study αβ4-mediated functions in more detail and to assess the contribution of the β4 cytoplasmic domain in such functions, we identified a rectal carcinoma cell line that lacks expression of the β4 integrin subunit. This cell line, termed RKO, expresses αβ4, but not αβ6 (2, 3). In this study, we report that RKO transfectants, which expressed the full-length β4 cDNA in association with endogenous α6, exhibited G1 arrest and a basal rate of apoptosis, properties that were not evident in RKO cells transfected with either a β4 cytoplasmic domain mutant or the expression vector alone. In an effort to define a mechanism for these observed changes in growth, we observed that expression of the αβ4 integrin induced expression of the p21 (WAF1; Cip1) protein, an inhibitor of G1 cyclin-dependent kinases (10–12). These data suggest that the β4 integrin cytoplasmic domain is linked to a signaling pathway involved in cell cycle regulation in the β4-transfected RKO cells.

MATERIALS AND METHODS

Cloning of the β4 Integrin Subunit—A full-length β4 cDNA was isolated from the Clone A colon carcinoma cDNA library (Clontech). This library was screened using a polymerase chain reaction product that was obtained from a partial sequence of the β4 subunit that we previously published (13). Multiple clones that encompassed the full-length β4 cDNA sequence were isolated and subcloned into PBluescript (Stratagen). The full-length β4 cDNA was ligated into the pcDNA3 expression vector (Invitrogen) using the β4 restriction sites BglII and BssHII and the unique EcoRI vector site. This cDNA harbored the 70- and 7-amino acid splice variants and the 5’ upstream 49-base pair region (13, 14). In addition, a β4 cDNA with a truncated cytoplasmic tail termed β4ΔCYT was constructed by polymerase chain reaction of a 400-base pair region from base pairs 2010–2398 (14), which introduced a stop codon and an XbaI site after the first 4 amino acids of the tail. This product was digested with Smal and XbaI and ligated together with the 5’ end of β4 (digested with EcoRI and Smal) into the mammary expression pcDNA3 (Invitrogen) via the unique EcoRI and XbaI sites in this vector.

Transfections and Flow Cytometry—The rectal carcinoma cell line RKO (15) was obtained from M. Brattain (Medical College of Ohio) and maintained in RPMI 1640 medium supplemented with 25 mM HEPES buffer, 10% FCS, 1% penicillin/streptomycin, and L-glutamine (Life Technologies, Inc.). RKO cells were transfected with either the full-length β4 construct, the β4ΔCYT construct, or vector alone using 20 μg of plasmid DNA. Geneticin (G418; Life Technologies, Inc.) was added to the growth medium at a concentration of 0.5 mg/ml for selection purposes. The transfected cells were sorted by FACS using the β4-specific mAb UM-A9 (16) (provided by T. Carey, Michigan). FACS sorting was repeated twice to obtain a population of cells that exhibited significant surface expression of β4. These populations were then subcloned by single cell sorting into 96-well plates (Costar).

Growth Assays—The transfected cells were trypsinized, washed in divalent cation-free PBS (CMF-PBS), and resuspended in 5% FCS growth medium containing G418. Cells (1 × 10⁴) were plated in triplicate 12-well plates (Costar) and allowed to grow for 3–6 days. Each well was harvested by trypsinization and the number of cells was counted using a Coulter counter.

For propidium iodide staining, cells at approximately 50% confluency

1 The abbreviations used are: FCS, fetal calf serum; EHS, Engelbreth-Holm-Swarm; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody.
were plated overnight on either tissue culture plastic, EHS-laminin (4.2 μg/cm²), or laminin-5 (0.2 μg/cm²; provided by R. Burgeson) and then harvested by trypsinization. The cells were stained with propidium iodide (Sigma; 2 mg/ml in 4 mM sodium citrate containing 3% (w/v) Triton X-100 and RNase A (0.1 mg/ml)). The stained cells were analyzed by FACS.

DNA Fragmentation—Samples were prepared as described in Green and co-workers (17) for analysis of DNA fragmentation and analyzed by agarose gel electrophoresis (1.2%).

Apoptag Staining—Cells (1 x 10⁶) were plated overnight on either plastic, EHS-laminin, or laminin-5 in normal culture medium (RPMI-I containing 10% FCS and G418), fixed in 1% paraformaldehyde in PBS, permeabilized in 70% ethanol, and then stained with the Apoptag reagent (apoptosis in situ detection kit, Oncor) and propidium iodide (as above). The stained cells were analyzed by FACS.

p21 Expression—For immunoblot analysis, cells (2 x 10⁶) were plated for 18 h in 60-mm² dishes containing 5% FCS growth medium and G418. Some of the dishes were precocciated with EHS-laminin (4.2 μg/cm²) before the addition of cells. Cells were extracted for 10 min on ice in 10 ml Tris (pH 7.4) containing 10 mM NaCl, 3 mM MgCl₂, 1% (w/v) Tween 40, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and pepstatin (Boehringer Mannheim), and 50 μg/ml leupeptin (Boehringer Mannheim). Samples (10 μg of total protein) were resolved by SDS-polyacrylamide gel electrophoresis (12%), transferred to nitrocellulose, and probed for p21 using the mouse anti-human Sdi1 (p21) monoclonal antibody 6B6 (Pharmingen; 1 μg/ml) and a peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry; 0.5 μg/ml). Bound protein was detected by enhanced chemiluminescence (Amersham Corp.).

Cells for immunohistochemistry were plated for 18 h in complete growth medium on coverslips precoated with either poly-l-lysine (10 μg/cm²) or EHS-laminin (5 μg/cm²). Cells were fixed for 8 min in 4% paraformaldehyde, permeabilized for 2 min in 0.2% Triton X-100, and stained with the p21 (10 μg/ml) and a fluorescein-conjugated donkey anti-mouse IgG (Jackson Laboratories; 1:30). The cells were examined using a confocal microscope (Bio-Rad MRC 600, Bio-Rad Microsciences, Cambridge, MA) attached to a Zeiss Axiovert 35 equipped with a ×63 Plan-Neofluar objective.

RESULTS

Representative RKO subclones from the full-length β₄ cDNA and the β₄ΔCYT transfections that expressed varying levels of β₄ surface expression were chosen for functional studies (Fig. 1A). No β₄ expression was evident in subclones obtained from transfection of the expression vector alone (Neo). The association of the transfected β₂ subunits with endogenous α₉ was confirmed by immunoprecipitation of surface-biotinylated cells with the A9 mAb (data not shown). Also, the full-length β₄ transfectants exhibited increased adhesion, spreading, and migration on laminin-1 providing evidence that the expressed α₉β₄ integrin is functional (18). Initially, we observed that bulk sorts of the full-length β₄ transfectants did not maintain high levels of β₄ surface expression for more than 3–4 days after sorting. To gain insight into the behavior of these transfectants, we assessed their DNA content using propidium iodide. A significant Aₚₙ peak, characteristic of cells undergoing apoptosis (19), was evident in the bulk transfectants (Fig. 1B). Stable subclones that expressed full-length β₄ cDNA maintained β₄ surface expression, although the level of expression was less than that seen in the initial bulk sorts (Fig. 1A). These subclones grew noticeably more slowly than the mock transfectants. Propidium iodide staining revealed that the number of cells in G₁ was significantly greater in these transfectants than in the mock transfectants (Fig. 1C). The D4 subclone, for example, exhibited twice the percentage of total cells in G₁ compared with the mock transfectants. However, subclones that expressed the β₄ΔCYT subunit on the surface at levels comparable with that of full-length β₄ exhibited no increase in the number of cells in G₁. To extend these observations, the doubling time for each of the subclones was determined (Table I). The doubling time for RKO cells, as well as for the mock transfectants, is approxi-
Mock transfected subclone (Neo 22, 26, 28), β4-ΔCYT subclone (3C1, 5A4), and full-length β4 subclone (A7, B8, D4, F10) cells were plated in triplicate 12-well plates (Costar) and allowed to grow for 3–6 days as described under “Materials and Method.” Each well was harvested by trypsinization, and the number of cells was counted using a Coulter counter. Doubling times were determined by the formula: \[ \frac{(t_2 - t_0) \log(N - N_0)}{\log(N_0)} \], where \( t \) is end time (hours), \( t_0 \) is initial time (hours), \( N \) is final cell number, and \( N_0 \) is initial cell number. The results shown represent the average multiple experiments (±S.D.). The p values shown reflect the significance of the difference between a particular subclone and the mock transfectants.

### Table I

| Cell line | Doubling time in hours | Significance (p < 0.020) |
|-----------|------------------------|--------------------------|
| Neo 22    | 21 (±2.0)              | −                        |
| Neo 26    | 21 (±5.1)              | −                        |
| Neo 28    | 21 (±2.1)              | −                        |
| ΔC31      | 21 (±1.8)              | −                        |
| ΔA4       | 20 (±0.7)              | −                        |
| A7        | 29 (±0.0)              | +                        |
| B8        | 25 (±1.5)              | +                        |
| D4        | 39 (±11.0)             | +                        |
| F10       | 27 (±0.0)              | +                        |

A possible mechanism for the observed changes in growth that correlate with \( \alpha_6 \beta_4 \) expression was suggested by the report that RKO cells express relatively low levels of wild-type p53 (21). Moreover, the growth-suppressive function of p53 can result from its ability to induce expression of p21 (WAF1; Cip1), an inhibitor of \( G_1 \) cyclin-dependent kinases (10–12). Based on these observations, we hypothesized that expression of the \( \alpha_6 \beta_4 \) integrin in RKO cells increases p21 expression. This hypothesis was assessed initially by immunostaining using a p21-specific mAb. The results obtained revealed little p21 expression in either the Neo or \( \beta_4-\Delta CYT \) subclones (Fig. 3, A and B). In these subclones, fewer than 5% of the cells exhibited p21 staining. However, nuclear staining of p21 was much more evident in both the B8 and D4 subclones (Fig. 3, C and D). The frequency of nuclear staining was greater in the D4 subclone (30–40% cells stained) than it was in the B8 subclone (15–20% cells stained), an observation that correlates with the relative level of \( \alpha_6 \beta_4 \) expression in these two subclones. The expression of p21 in the subclones was also assessed by immunoblotting detergent extracts prepared from the \( \beta_4 \) subclones. Relatively little p21 expression was evident in either the Neo or \( \beta_4-\Delta CYT \) subclones based on these immunoblots (Fig. 3E). The level of p21 expression in these subclones is consistent with other reports of cells that express low levels of wild-type p53 (12). In contrast, a substantial increase in p21 expression was seen in both the B8 and D4 \( \beta_4 \) subclones. Attachment to EHS laminin-1 did not alter this pattern of p21 expression (Fig. 3E).

### Table II

| Apoptag reactivity of RKO transfectants |
|----------------------------------------|
| A mock transfected subclone (Neo27)    |
| a β4-ΔCYT subclone (3C1)               |
| and two full-length β4 subclones (B8 and D4) were plated on either tissue culture plastic or laminin-5 for 18 h as described under “Materials and Method.” The cells were then analyzed using the Apoptag in situ apoptosis detection kit ( Oncor, Inc.). Positively stained cells were detected by FACS. The values shown above from a representative experiment are the percent of positive cells in each population. |

| Cell line | Substrate | Apoptag positive |
|-----------|-----------|-----------------|
| Neo 27    | Plastic   | 1.63            |
|           | Laminin-5 | 1.57            |
| ΔC31      | Plastic   | 1.34            |
|           | Laminin-5 | 1.53            |
| B8        | Plastic   | 3.10            |
|           | Laminin-5 | 2.35            |
| D4        | Plastic   | 8.08            |
|           | Laminin-5 | 8.35            |

FIG. 2. DNA fragmentation in the RKO transfectants. DNA was extracted from the \( \beta_4 \) transfectants as described under “Materials and Methods” and analyzed by agarose gel electrophoresis (1.2%). Lane 1, 1-kilobase pair DNA ladder; lane 2, 3C1, a \( \beta_4-\Delta CYT \) subclone; lane 3, B8, a full-length \( \beta_4 \) subclone; lane 4, D4, a full-length \( \beta_4 \) subclone.

FIG. 3. Expression of p21 in the RKO transfectants. A–D, detection of p21 by immunohistochemistry. A mock transfected subclone (A), a \( \beta_4-\Delta CYT \) subclone (B), and two full-length \( \beta_4 \) subclones B8 and D4 (C and D) were plated on 1% poly-L-lysine and stained with a p21-specific mAb as described under “Materials and Methods.” Bar in A equals 50 \( \mu \)M. E, immunoblot detection of p21. RKO transfectants were plated on either tissue culture plastic (lanes 1, 3, 5, 7, and 9) or EHS laminin-1 (lanes 2, 4, 6, 8, and 10) for 18 h. After detergent extraction, the samples were normalized for protein content, resolved by SDS-polyacrylamide gel electrophoresis (12%), transferred to nitrocellulose, and blotted with the p21-specific mAb. Bound protein was detected by enhanced chemiluminescence. Lanes 1 and 2, Neo-24, a mock transfected subclone; lanes 3 and 4, Neo-26, a mock transfected subclone; lanes 5 and 6, 3C1, a \( \beta_4-\Delta CYT \) subclone; lanes 7 and 8, B8, a full-length \( \beta_4 \) subclone; lanes 9 and 10, D4, a full-length \( \beta_4 \) subclone.

DISCUSSION

We have shown that expression of the \( \alpha_6 \beta_4 \) integrin in RKO cells, a \( \beta_4 \)-deficient carcinoma cell line, results in partial \( G_1 \) arrest as well as the apoptotic death of some transfectants. A possible mechanism for these observed changes in growth is provided by our finding that \( \alpha_6 \beta_4 \) also induces expression of the \( G_1 \) cyclin-dependent kinase inhibitor p21. The specificity of the observed induction of p21 expression is provided by the finding that the \( \alpha_6 \beta_4-\Delta CYT \) integrin failed to affect p21 expression or growth even though it was expressed on the cell surface at levels comparable with the full-length integrin. In addition, we had previously observed that expression of either the E-cadherin (22) or galectin-3 cDNAs\(^2\) in RKO cells had no effect on their growth.

Our finding that \( \alpha_6 \beta_4 \) can trigger apoptosis in a population of

\(^2\) M. M. Lotz and A. M. Mercurio, unpublished observation.
transfectants is intriguing because our efforts to isolate transfectants with \( \beta_4 \) surface expression greater than that observed in the D4 subclone, for example, were not successful. These transfectants did not survive for more than a few days after their selection by FACS. A reasonable interpretation for these data is that lower levels of \( \alpha_6 \beta_4 \) surface expression induce expression of p21 sufficient to induce partial G1 arrest and some apoptosis and that higher levels of \( \alpha_6 \beta_4 \) expression result in more p21 expression and more widespread apoptotic death.

The \( \beta_4 \)-dependent increase in growth arrest and p21 expression does not appear to depend on attachment to either a laminin-1 or laminin-5 matrix. For this reason, we suggest that the \( \beta_4 \) cytoplasmic domain is linked constitutively to the p21 pathway of growth arrest and apoptosis in RKO cells. This possibility of constitutive activation of adhesion-dependent signaling pathways in transformed cells is supported, for example, by the recent report that the binding of focal adhesion kinase to SH2-containing proteins in v-src-transformed 3T3 cells is independent of integrin engagement and cell attachment (23).

The results of this study raise the issue of the role of \( \alpha_6 \beta_4 \) in the regulation of normal cell growth and apoptosis. The intestinal epithelium may provide a superb model for examining such a relationship. In this epithelium, undifferentiated cells at the base of the crypts proliferate rapidly and give rise to differentiated enterocytes that migrate to the tips where they are sloughed off into the intestinal lumen. Apoptosis occurs at the tips and may provide the mechanism for cell loss in this structure (24).

Interestingly, p21 is expressed in mature enterocytes but not in undifferentiated crypt cells (25), and there is evidence that \( \alpha_6 \beta_4 \) is not expressed in the undifferentiated crypt cells. For these reasons, the possibility of a functional relationship between \( \alpha_6 \beta_4 \) expression and p21 induction merits investigation.

Several reports have implicated cell adhesion as well as specific adhesion receptors in growth control and apoptosis (26–30). The significance of our findings is that they establish a link between the cytoplasmic domain of a specific integrin subunit and the expression of a molecule known to be critical for growth suppression, apoptosis, and tumorigenesis. These findings should facilitate the elucidation of the signaling pathways that result in the induction of p21 expression by cell surface receptors.

\* W. G. Carter, unpublished observation.

Acknowledgments—We thank Dr. M. Brattain for providing the RKO cell line, Dr. T. Carey for the UM-A9 mAb, and Dr. R. Burgeson for purified laminin-5.

REFERENCES
1. Lotz, M., Korzelius, C., and Mercurio, A. M. (1990) Cell Regul. 1, 249–257
2. van der Geer, P., Schlaepfer, D. D., Hunter, T., and Hanks, S. K. (1994) Cell 78, 311–316
3. Tozeren, A., Kleinman, H. K., Mercurio, A. M., and Byers, S. W. (1994) J. Cell Biol. 127, 457–464
4. Meredith, J. E., Fazeli, B., and Schwartz, M. A. (1995) Mol. Biol. Cell 6, 2111–2118
5. Spinardi, L., Einheber, S., Cullen, T., Milner, T. A., and Giancotti, F. G. (1995) EMBO J. 14, 671–678
6. Stepp, M. A., Thrall, S. C., and Mercurio, A. M. (1994) J. Cell Biol. 127, 457–464
7. Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, M. H., Faldoni, R., Kennel, S. J., Aplin, A. D., Baker, J., Loizidou, M., and Garrod, D. (1991) J. Cell Biol. 113, 907–917
8. Quaranta, V., and Jones, J. C. R. (1993) Trends Cell Biol. 3, 4–6
9. Spinardi, L., Ren, Y. L., Sanders, R., and Giancotti, F. G. (1993) Mol. Biol. Cell 4, 871–884
10. El-Dayer, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
11. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) Cell 75, 805–816
12. El-Dayer, W. S., Wade Harper, J., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jakobson, J., Piette, E., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W., and Vogelstein, B. (1994) Cancer Res. 54, 1169–1174
13. Clarke, A. S., and Mercurio, A. M. (1994) Cell Adhesion Commun. 2, 1–6
14. Takaishi, R. N., Rozzo, C., Stahl, S., Chambers, J., Reichard, L. F., Cooper, H. M., and Quaranta, V. (1990) J. Cell Biol. 111, 1593–1604
15. Boyd, D., Florent, G., Kim, P., and Brattain, M. (1988) Cancer Res. 48, 3112–3116
16. Kimmel, K. A., and Carey, T. E. (1986) Cancer Res. 46, 3614–3623
17. Bisscheroux, R. P., Echeverri, F., Mahboubi, A., and Green, D. R. (1992) Nature 356, 552–554
18. Clarke, A. S., and Mercurio, A. M. (1994) Mol. Biol. Cell 5, 424a
19. Telford, W. G., King, L. E., and Fraker, P. J. (1992) Cytometry 13, 137–143
20. Gavrieliev, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) J. Cell Biol. 119, 501
21. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. V., and Vogelstein, B. (1990) Science 249, 912–915
22. Bren, E., Steege, G. J., and Mercurio, A. M. (1995) Ann. Surg. Oncol., in press
23. Schlapeper, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
24. Hall, P. A., Coates, P. J., Ansari, B., and Hopwood, D. (1994) J. Cell Biol. 127, 2655–2663
25. Marx, J. (1995) Science 267, 963–964
26. Meredith, J. E., Fazeli, B., and Schwartz, M. A. (1993) Mol. Biol. Cell 4, 953–961
27. Montgomery, A. M. P., Reisfeld, R. A., and Cheresh, D. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8856–8860
28. Ager, M., Chen, A., Cone, R. I., Pytel, R., and Shepard, D. (1994) J. Cell Biol. 127, 547–556
29. Guadagno, T. M., Ohtsubo, M., Roberts, J. M., and Assoian, R. K. (1993) Science 262, 1572–1575
30. Ruuslahti, E., and Reed, J. C. (1994) Cell 77, 477–478
Activation of the p21 Pathway of Growth Arrest and Apoptosis by the β4 Integrin Cytoplasmic Domain
Astrid S. Clarke, Margaret M. Lotz, Celia Chao and Arthur M. Mercurio

J. Biol. Chem. 1995, 270:22673-22676.
doi: 10.1074/jbc.270.39.22673

Access the most updated version of this article at http://www.jbc.org/content/270/39/22673

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 29 references, 18 of which can be accessed free at http://www.jbc.org/content/270/39/22673.full.html#ref-list-1