Cell Spreading on Extracellular Matrix Proteins Induces Tyrosine Phosphorylation of Tensin*

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A small number of proteins becomes tyrosine-phosphorylated in response to integrin-mediated cell adhesion to extracellular matrix proteins. Previous work has identified two of these tyrosine-phosphorylated proteins as the focal adhesion kinase and paxillin. Here we identify a third focal adhesion protein, tensin, that becomes tyrosine-phosphorylated during cell adhesion to extracellular matrix proteins. The tyrosine phosphorylation of tensin does not occur when cells adhere to plastic or polylysine and is blocked when microfilament assembly and cell spreading are inhibited with cytochalasin D. In addition, we show that other focal adhesion proteins such as talin and vinculin do not become tyrosine-phosphorylated under the same conditions of cell spreading on extracellular matrix proteins.

The extracellular matrix (ECM) can influence the migration of cells, their differentiation, and growth. Recent work has focused on the role of integrins, cell-ECM receptors, in transmitting signals from the ECM. In particular, attention has been directed toward the tyrosine phosphorylation of a small set of proteins that is induced when cells adhere to ECM in an integrin-dependent manner (1-3). Cross-linking of integrins with antibodies has also been found to stimulate tyrosine phosphorylation of what appears to be the same set of proteins (4). With cells in culture, integrins are often concentrated in focal adhesions, the sites where cells adhere most tightly to the underlying ECM substratum and regions of elevated phosphotyrosine (5).

One of the major tyrosine-phosphorylated proteins induced by integrin-mediated adhesion is p125FAK (FAK) (2, 3, 6), which is a tyrosine kinase concentrated in focal adhesions (7, 8). The elevated tyrosine phosphorylation of FAK has been shown to activate its tyrosine kinase activity (6), but the link between integrin occupancy or aggregation and this activation of FAK has not been established. To understand the significance of the tyrosine phosphorylation induced by integrin-mediated cell adhesion, it will be important to identify all the proteins that become tyrosine-phosphorylated in response to adhesion. Besides FAK, one other protein, paxillin, has been shown to contain elevated phosphotyrosine in response to adhesion (2). Paxillin is a focal adhesion protein (9) that shows elevated phosphotyrosine during embryonic development (10) and, like FAK, during transformation of cells by Rous sarcoma virus (RSV) (11).

In this paper we have examined the tyrosine phosphorylation of other focal adhesion proteins in response to cell adhesion to ECM substrata. We show that tensin, like paxillin, but not vinculin or talia, becomes tyrosine-phosphorylated following adhesion and spreading on fibronectin or laminin. If cell spreading is prevented by treatment with cytochalasin D, this phosphorylation is inhibited.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-FAK monoclonal antibody, 2A7, was a generous gift from Dr. I. Parsons (Cambridge, England). Other monoclonal antibodies used include the following: anti-paxillin (9), 84d anti-talin (12), anti-tensi (13), and vin 11-5 anti-vinculin (Sigma).

Cell Culture and Drug Treatment—Rat embryo fibroblasts 52 (REFs) were grown in Dulbecco's modified Eagle's medium (Life Technologies Inc.) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). 50 units/ml penicillin, and 50 μg/ml streptomycin. Tissue culture dishes were coated with substrates overnight at 4 °C, except for polylysine, which was incubated for an hour at 37 °C prior to being used. The substrates included 100 μg/ml human plasma fibronectin (New York Blood Center), 100 μg/ml laminin (ICN Biochemicals, Costa Mesa, CA), 20 μg/ml polylysine (higher concentrations appeared to induce cell lysis) (14, 15), 70,000-150,000 (Sigma). In one experiment tissue culture dishes were coated with serum to generate a surface coated predominantly with vitronectin. REFs were trypsinized with 0.05% trypsin, 0.53 mM EDTA (Life Technologies Inc.), followed by washing with 1 mg/ml turkey trypsin inhibitor (Sigma), or when cells were going to be plated as fibronectin, growth medium containing serum. The cells were washed twice following neutralization of the trypsin with serum-free media. Cells plated onto the various substrates were first incubated for 2 h in 25 μg/ml cycloheximide (Sigma) to inhibit synthesis of endogenous ECM proteins. Cells were maintained in cycloheximide throughout the length of the expeiment. In some experiments, cycloheximide (1.5 μg/ml) was added just prior to plating the cells onto fibronectin.

Immunoprecipitation and Immunoblotting—Cells were lysed on ice by scraping in cold lysis buffer, which was Tris-buffered saline (TBS) with 0.1% sodium deoxycholate (100 mM NaCl, 50 mM Tris-Cl, pH 7.6) containing 1% Triton X-100, 1% deoxycholate, 1 mM EDTA, 1 mM EGTA, 25 μg/ml leupeptin, and 25 μg/ml aprotonin, 200 μg/ml soybean trypsin inhibitor, and 1 mM NaN3. The lysates were clarified for 10 min in a microcentrifuge. Antibodies to immunoprecipitate-specific antigens were added and rotated at 4 °C for 1 h. To precipitate the antibody-antigen complexes, 100 μl of 10% protein A-Sepharose conjugated to rabbit-antimouse IgG was added to the samples, and rotation continued for 1 h. The immunoprecipitates, pelleted by microcentrifuging for 30 s, were washed 2 to 3 times in wash buffer (0.1% Triton X-100, 0.1% deoxycholate, 0.1 mM EDTA, 0.1 mM EGTA, 25 μg/ml leupeptin, and 25 μg/ml aprotonin, 200 μg/ml soybean trypsin inhibitor, and 1 mM NaN3 in TBS). The pellets were boiled in sample buffer and electrophoresed on either 6.0 or 7.5% polyacrylamide gels as described by Laemmli (14), except that the bisacrylamide concentration was 0.1%. After electrophoresis the proteins were transferred to nitrocellulose by the method of Towbin (15). The blots were blocked for 20-30 min using blocking buffer (2% cold water fish gelatin (Sigma) in TBSTB (0.1% bovine serum albumin and 0.05% Tween 20 in TBS). To blot for phosphotyrosine, blots were probed with the PY20 antibody conjugated to horseradish peroxidase (PY20-HRP) (ICN Biochemicals) and used at a dilution of 1:10,000 in TBSTB. Other unrelated primary antibodies were diluted 1:1,000 in TBSTB followed by a 1:1,000 dilution of goat-antimouse antibody conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). Blots were incubated for 30-45 min in primary antibodies and were washed.

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1 The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; RSV, Rous sarcoma virus; REF, REF embryo fibroblast 52; TBS, Tris-buffered saline.

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with several changes of TBSTB. Following incubation with the secondary antibody, the blots were washed as above with TBSTB, and the final washes were in TBS. The blots were then developed using enhanced chemiluminescence (Amersham, UK), according to the manufacturer's protocol. The immunoblots were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% sodium dodecyl sulfate, 100 mM 2-mercaptoethanol at 55 °C, as recommended by the manufacturer, except longer incubations of 1-2 h instead of 30 min were used to ensure complete removal of all antibodies. Before reprobing, the blots were re-equilibrated in TBS.

RESULTS

To investigate whether tensin becomes tyrosine-phosphorylated in response to cell spreading on fibronectin, REFs were either held in suspension for 30 min or plated onto fibronectin for 30 min and 1 h. Following immunoprecipitation with an anti-tensin monoclonal antibody, the samples were run on a 7.5% gel and then immunoblotted with an anti-phosphotyrosine antibody (Fig. 1, left panel). Tensin immunoprecipitated from cells held in suspension (lanes 1 and 3) exhibited very low levels of tyrosine phosphorylation. However, an increase in tyrosine phosphorylation was seen with time when the cells were plated on fibronectin (lanes 2 and 3). The amount of tensin present was essentially the same in all samples as shown when the same blot was stripped and reprobed with anti-tensin (Fig. 1, right panel). Several phosphotyrosine-containing proteins of approximately 60, 65, and 116 kDa were occasionally seen with tensin and remain to be characterized. Tensin has been reported to bind vinculin, raising the possibility that the 116-kDa protein might be vinculin. Vinculin was, indeed, found to be present in the immunoprecipitations by immunoblotting (data not shown), but the 116-kDa phosphotyrosine protein is unlikely to be vinculin, because it is not tyrosine-phosphorylated under these conditions (see Fig. 4).

To characterize further the effect of cell adhesion on tyrosine phosphorylation of tensin, cells were plated onto other substrates. REFs were treated with 25 µg/ml cycloheximide 2 h prior to trypsinizing to prevent endogenous synthesis of ECM proteins. The cells were plated in serum-free media onto naked bacterial plastic and tissue culture plastic and onto dishes coated with either polylysine, fibronectin, laminin, or serum (Fig. 2, lanes 1-6, respectively). Serum was used as a source of vitronectin, which is the dominant adhesive ECM protein in serum, although fibronectin is also present and probably contributes to the adhesion of these cells to serum-coated dishes. The cells plated on bacterial plastic did not attach and remained suspended. The cells plated on tissue culture plastic attached but did not spread, whereas those on polylysine spread, but presumably in an integrin-independent fashion. Photo taken from the image. The immunoblots were probed with anti-phosphotyrosine (PY, left panel) then stripped and reprobed with anti-tensin (right panel). The molecular masses of marker proteins are indicated in kilodaltons, and Ig indicates immunoglobulins.

Fig. 2. Tyrosine phosphorylation of tensin in cells plated on different extracellular matrices. REFs were plated onto various substrates for 1.5 h and tensin was immunoprecipitated from lysates containing equal amounts of protein. An immunoblot with anti-phosphotyrosine is shown of the immunoprecipitates. The cells were plated onto naked bacterial plastic and tissue culture plastic (lanes 1 and 2) or onto plates coated with either polylysine, fibronectin, laminin, or serum (vitronectin) (lanes 3-6, respectively). The blot was stripped and reprobed with anti-tensin to confirm equal loadings (data not shown).

Fig. 3. Inhibition of tyrosine phosphorylation of tensin and FAK by cytochalasin D. REFs treated with cytochalasin D were either held in suspension (lanes 1 and 4), or plated onto fibronectin (lanes 2 and 5), or plated on fibronectin in the absence of cytochalasin D for 1.5 h (lanes 3 and 6). The cell lysates were immunoprecipitated with anti-tensin (lanes 1-3) or anti-FAK (lanes 4-6). Panel A is immunoblotted with anti-phosphotyrosine. In panel B the same blot was stripped and reprobed with either anti-tensin (lanes 1-3) or anti-FAK (lanes 4-6).

Although REFs spread on fibronectin, laminin, serum (vitronectin), and polylysine, a rise in the level of tyrosine phosphorylation on tensin was only seen in the cells plated onto fibronectin, laminin, and serum (vitronectin). Similar results were obtained when FAK was immunoprecipitated from the same cell lysates and reprobed with anti-phosphotyrosine (data not shown). In addition to REFs, other cells including kidney (MDCK) and liver (WB) cell lines also exhibited elevated phosphotyrosine levels in tensin when the cells were plated on fibronectin (data not shown).

To determine whether integrin-mediated attachment or cell spreading was necessary to stimulate tyrosine phosphorylation in tensin, cell spreading on a fibronectin substrate was inhibited with cytochalasin D (Fig. 3). Immunoprecipitations of tensin (lanes 1-3) and FAK (lanes 4-6) from cells treated with cytochalasin D and held in suspension are shown in Fig. 3 (lanes 1 and 4, respectively). Cells that were plated on fibronectin, either in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of cytochalasin D, were immunoblotted with anti-phosphotyrosine (Fig. 3A) or with anti-tensin and anti-FAK (Fig. 3B, lanes 1-3 and 4-6, respectively). In the presence of cytochalasin D and absence of cell spreading on an ECM substrate, neither tensin nor FAK became tyrosine-phosphorylated.

Other focal adhesion proteins were examined for increases in tyrosine phosphorylation in response to adhesion and spreading on fibronectin. REFs were either held in suspension for 30 min and 2 h (Fig. 4, lanes 1 and 3) or plated on 30 min, 1 h, and...
2 h (lanes 2–4). The lysates for each time point were divided for immunoprecipitation of the focal adhesion proteins tensin, vinculin, talin, and paxillin. The immunoprecipitated proteins were then immunoblotted using anti-PY20-HRP. It was observed that of these cytoskeletal proteins, only tensin and paxillin contained elevated levels of phosphotyrosine. Neither talin nor vinculin demonstrated detectable tyrosine phosphorylation under any of these conditions. To confirm that equal amounts of immunoprecipitates were immunoblotted, each panel was stripped and reprobed with the corresponding antibodies used to immunoprecipitate the proteins (data not shown).

DISCUSSION

Several proteins are tyrosine-phosphorylated in response to cell adhesion to and spreading on fibronectin. Two of these proteins have previously been identified as FAK and paxillin (2, 3, 6, 7). In this paper we have detected elevated phosphotyrosine on a third protein, tensin, as cells adhere to ECM substrates. The combination of a relatively low abundance of tensin in cells and the frequently poor transfer of high molecular weight proteins to nitrocellulose during blotting has likely prevented detection of this phosphorylation in earlier experiments. Tensin is a focal adhesion protein with multiple interactions. It binds to vinculin and actin (16, 17), and it contains an SH2 domain (18), which suggests that it may bind specific phosphotyrosine residues in other proteins. In future work it will be important to determine whether the tyrosine phosphorylation of tensin induced by adhesion affects any of these interactions and thereby contributes to the formation of focal adhesions.

Evidence has been provided that the phosphotyrosine response associated with cell adhesion is integrin-dependent (1, 2, 4, 19). Our finding that tensin becomes tyrosine-phosphorylated following cell adhesion to ECM substrates, but not to naked plastic or polylysine, is consistent with this also being mediated by integrins. Cell adhesion to these various ECM proteins involves different integrins, but the same tyrosine phosphorylation response was observed with adhesion to each integrin, suggesting that ligand binding to different integrins can activate the same tyrosine kinase. In addition, we found that the mere attachment of cells to an ECM substrate, where spreading was inhibited by the presence of cytochalasin D, did not elicit the same elevated phosphotyrosine in tensin and FAK as in cells that were allowed to spread. Previous work demonstrated that chelation of intracellular calcium inhibited spreading on an ECM substrate and similarly inhibited the tyrosine phosphorylation response (20). Cell spreading appears to be necessary; however, it is not sufficient on its own to trigger tyrosine phosphorylation, as evidenced by the lack of phosphotyrosine in tensin and other proteins in cells spread on polylysine (data not shown). One possibility is that spreading on an ECM maximizes the number of integrin-ECM interactions that induce this phosphotyrosine. An alternative explanation, suggested previously for platelets (19), is that an intact, functioning cytoskeleton is required to bring together the various components (integrins, FAK, and its substrates) of this signaling complex.

Tyrosine phosphorylation of focal adhesion proteins was originally studied in the context of transformation by RSV. Because transformation by this virus results in a loss of focal adhesions, work on RSV-transformed cells led to the idea that tyrosine phosphorylation of focal adhesion proteins might contribute to focal adhesion disassembly (21, 22). Recent data, however, have indicated that elevated phosphotyrosine in normal cells is associated with focal adhesion assembly rather than disassembly (2). The first focal adhesion proteins found to contain phosphotyrosine were vinculin and talin, again in cells transformed by RSV (21, 22). Subsequent investigations, however, with non-transforming RSV mutants revealed that the phosphotyrosine content of vinculin and talin did not correlate with the transformed phenotype (23, 24). In this paper, we report that vinculin and talin do not contain detectable levels of phosphotyrosine during cell adhesion and focal adhesion assembly, under conditions where proteins such as tensin, paxillin, and FAK become tyrosine-phosphorylated. From this work and the previous studies, it seems unlikely that the tyrosine phosphorylation of vinculin and talin is important in either the assembly or disassembly of focal adhesions.

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REFERENCES

1. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) Cell Regul. 2, 951–964
2. Berridge, L. J., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) J. Biol. Chem. 267, 23439–23442
3. Kornberg, L. J., Earp, H. S., and Juliano, R. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8392–8396
4. Mahler, P. A., Pasquale, E. B., Wang, Y. J., and Singer, S. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6576–6580
5. Guan, J. L. and Shalloway, D. (1992) Nature 358, 690–692
6. Hanks, S. K., Calabiz, B. M., Barker, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8487–8491
7. Schaller, M. D., Borkman, T. C., Cobb, S. B., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196
8. Turner, C. E., Glenney, J. R., and Burridge, K. (1990) J. Cell Biol. 111, 1059–1068
9. Turner, C. E. (1991) J. Cell Biol. 115, 201–207
10. Glenney, J. R., and Zokas, L. (1989) J. Cell Biol. 108, 2401–2408
11. Ottery, C., Gribbles, W., and Burridge, K. (1990) Hybridomas 9, 57–62
12. Bockhoff, S. M., Ottery, C., Glenney, J. R., and Burridge, K. (1992) Exp. Cell Res. 203, 39–46
13. Laemmli, U. K. (1970) Nature 227, 680–685
14. Townsh, W., Baruhel, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
15. Wilkins, J., Raisinger, M. A., and Lin, S. (1986) J. Cell Biol. 103, 1483–1494
16. Wilkins, J., Raisinger, M. A., Coffey, E., and Lin, S. (1987) J. Cell Biol. 105, 102a
17. Davis, S., Lu, M. L., Lo, S. H., Lin, S., Butler, J. A., Drucker, B. J., Roberts, T. M., An, Q., and Chen, L. B. (1991) Science 252, 712–715
18. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) J. Cell Biol. 118, 905–912
19. Pelletier, A. J., Badyer, C. S., and Levinson, A. D. (1992) Mol. Cell. Biol. 3, 989–998
20. Selfon, B. M., and Hunter, T. (1981) Cell 24, 165–174
21. Pasquale, E. B., Mahler, P. E., and Singer, S. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5507–5511
22. Selfon, B. M. (1986) Cell 45, 105–112
23. DeClue, J. E., and Martin, G. S. (1987) Mol. Cell. Biol. 7, 371–378