Transmembrane Signal Transduction by Integrin Cytoplasmic Domains Expressed in Single-subunit Chimeras*

(Received for publication, February 24, 1994, and in revised form, April 1, 1994)

Steven K. Akiyama, Susan S. Yamada, Kenneth M. Yamada, and Susan E. LaFlamme†
From the Laboratory of Developmental Biology, NIDR, National Institutes of Health, Bethesda, Maryland 20892

Integrins are heterodimeric, transmembrane cell adhesion receptors that have recently been shown to function in transmembrane signal transduction. To examine the specific role of integrin intracellular domains in signal transduction, chimeric receptors containing various integrin intracellular domains coupled to a reporter consisting of the transmembrane and extracellular domains of the small, non-signaling subunit of the interleukin-2 receptor were expressed in cultured human fibroblasts and assayed for their ability to trigger tyrosine phosphorylation of the 125-kDa cytoplasmic tyrosine kinase, pp125FAK. Tyrosine phosphorylation of pp125FAK was induced in cultured fibroblasts that transiently expressed chimeric receptors containing either the β1, β2, or β3 integrin intracellular domain and were selected by magnetic bead sorting. However, expression of chimeric receptors containing either the αi or an alternatively spliced form of the βi intracellular domain (βi), as well as those lacking an intracellular domain, failed to induce tyrosine phosphorylation of pp125FAK. These results indicate that information contained in the β1, β2, or β3 integrin intracellular domain is sufficient to stimulate integrin-mediated tyrosine phosphorylation of specific intracellular proteins and that integrin extracellular and transmembrane domains are not required for inducing tyrosine phosphorylation. Our results also indicate that alternative splicing can regulate the ability of β integrin intracellular domains to participate in signal transduction, and they further suggest that the carboxyl-terminal region of specific β integrins may play a role in the signal transduction pathway involving extracellular matrix molecules.

Integrins are members of a large family of more than 20 receptors that function in cell-cell and cell-substrate adhesion and in cell migration (1–6). All integrins consist of noncovalently linked α and β subunits, both of which are transmembrane. None of the integrin intracellular domains is known to have any enzymatic activity. Except for the β3 integrin, all integrins have relatively short cytoplasmic domains of 60 amino acids or less. The integrins that function in cell-substrate adhesion are often localized to specialized structures on the ventral surface of cells termed focal contacts or focal adhesions, where they link extracellular matrix proteins such as fibronectin, laminin, vitronectin, and collagen with intracellular structures such as the cytoskeleton (7).

The hypothesis that integrins form the transmembrane link through which adhesive proteins can modulate cellular behavior is supported by recent studies demonstrating the involvement of integrins in several signal transduction pathways, including those leading to changes in protein tyrosine phosphorylation, intracellular pH, intracellular free calcium concentration, and inositol lipid turnover (8–13). Adhesion of cultured cells to extracellular matrix proteins results in the tyrosine phosphorylation of a number of intracellular proteins, including pp125FAK, paxillin, and tensin (14–18), some of which can be localized in focal contacts.

pp125FAK (also known as focal adhesion kinase, FAK,1 or FadK) is a 125-kDa cytoplasmic protein tyrosine kinase that is localized in focal contacts of cultured cells (19, 20) that can also be tyrosine-phosphorylated in response to the clustering of integrins (21) through an as yet unknown mechanism. Similarly, the adhesion of platelets to adhesive ligands and the binding of fibrinogen to platelets via the αiβi integrin results in tyrosine phosphorylation of pp125FAK in a process that also appears to require either platelet adhesion or aggregation (22–24). pp125FAK may represent a common intermediate in multiple signal transduction pathways, suggesting that processes such as cell adhesion that lead to tyrosine phosphorylation of pp125FAK can modulate diverse cellular functions through multiple pathways. Tyrosine phosphorylation of pp125FAK is also modulated by cellular transformation by pp60src (19, 25, 26). Furthermore, treatment of mouse 3T3 cells with the neuroptide bombesin and aggregation of the IgE receptor of basophilic leukemia cells can each stimulate tyrosine phosphorylation of pp125FAK (27–29).

The cytoplasmic tails of β integrins are required for their localization in focal contacts (30–32). Expression of chimeric receptors derived by fusing the intracellular domains of either the αi or βi subunit of the fibronectin receptor integrin to a reporter domain consisting of extracellular and transmembrane regions of the small, low affinity, non-signaling subunit of the human interleukin-2 (IL-2) receptor (33, 34) demonstrated that the βi intracellular domain alone was sufficient to target the reporter domain to focal contacts (35). Similar observations were made using integrin-cadherin chimeras (36). Because the βi chimeric receptors could not bind an extracellular matrix ligand, these experiments suggested that an intracellular mechanism is also involved in receptor distribution. Such an accumulation of integrins in focal contacts occurs in endogenous receptors as a response to ligand occupancy (35). In this study we have examined the ability of chimeric receptors containing different integrin β intracellular domains to participate in the signal transduction pathway that results in tyrosine phosphorylation of FAK. We have found that only specific intracellular domains can participate in signal transduction and that this participation may be regulated by alternative splicing of β integrin intracellular domains.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Physiology and Cell Biology, Albany Medical College, 47 New Scotland Ave., Albany, NY 12208.

1 The abbreviations used are: FAK, focal adhesion kinase; IL-2, interleukin-2.
MATERIALS AND METHODS

Cells—Normal human foreskin fibroblasts were obtained and cultured as described previously (37). Cells were transfected by electroporation at 170 V and 960 microfarads with a Gene Pulser (Bio-Rad) as described (35, 38). The cell line 7G7B6 secreting anti-IL-2 receptor monoclonal antibody was obtained from the American Type Culture Collection (Rockville, MD) and cultured as described (39).

Chimeric Receptors—Chimeric receptors were constructed using standard molecular biological techniques as described (35, 40). Cells transiently expressing chimeric receptors were isolated by magnetic bead sorting after 48 h of culture as described (41).

Protein Reagents—Fibronectin was purified from outdated human plasma obtained from the Department of Transfusion Medicine, National Institutes of Health using citric acid elution from a gelatin-Sepharose affinity column (42). Anti-IL-2 receptor antibody 7G7B6 was purified from ascites generated in BALB/c mice using sequential ammonium sulfate precipitation, DE52 (Whatman) ion exchange chromatography, Inc., 125I-anti-mouse IgG from Amersham Corp., anti-mouse monoclonal anti-IL-2-receptor antibody was obtained from Boehringer Mannheim, anti-tyrosine phosphate monoclonal antibody PY20 from ICN, anti-pp125FAK monoclonal antibody from Upstate Biotechnology, Inc., 125I-anti-mouse IgG from Amersham Corp., anti-mouse IgG coupled to agarose from Sigma, and BioMag anti-mouse IgG-coupled magnetic beads from Advanced Magnetics, Inc.

Assays—Tyrosine phosphorylation was determined by lysing cells in RIPA buffer and Western immunoblotting as described by Guan et al. (14). Protein concentrations were estimated using the Micro-BCA assay (Pierce Chemical Co.) using bovine serum albumin as a standard. pp125FAK was immunoprecipitated as described (29) and detected by Western immunoblotting using anti-phosphotyrosine monoclonal antibodies.

Fluorescence-activated Cell Sorting—Human fibroblasts transiently expressing the β1 chimeric receptor were harvested after 1 day of culture, treated with fluorescein-labeled anti-IL-2 receptor antibody (Boehringer Mannheim) diluted 1/100 in 1% crystalline bovine serum albumin (Calbiochem) in Dulbecco’s phosphate-buffered saline, and sorted by fluorescence-activated cell sorting under sterile conditions using a Becton Dickinson FACStar Plus fluorescence-activated cell sorter. Cells selected for expression of cell surface IL-2 receptor reporter domain were cultured for an additional day, harvested, and either analyzed immediately for tyrosine phosphorylation or collected by binding to magnetic beads prior to analysis.

RESULTS AND DISCUSSION

In the present study, we have used a series of chimeric human integrin-IL-2 receptor proteins transiently expressed in cultured human foreskin fibroblasts to probe the mechanism of integrin-mediated signal transduction. As shown in Fig. 1, there is enhanced tyrosine phosphorylation of pp125FAK in cultured human foreskin fibroblasts expressing the human β1 chimeric receptor collected by binding to magnetic beads using an anti-IL-2 receptor antibody (lane 4). In contrast, there is a relatively low background level of tyrosine phosphorylation of pp125FAK in control cells expressing the IL-2 extracellular and transmembrane domains with no integrin intracellular domain (lane 3) and in cells expressing the α5 intracellular domain (lane 5). For reference, the background level of protein tyrosine phosphorylation in nontransfected human foreskin fibroblasts allowed to attach for 20 min on poly-l-lysine (lane 1) and the relatively high level of tyrosine phosphorylation of pp125FAK resulting from adhesion of nontransfected cells on fibronectin (lane 2) are also shown. We confirmed that the major tyrosine-phosphorylated protein present in cells expressing the β1 chimeric receptor was pp125FAK by immunoprecipitation (not shown). These results indicate that the single-subunit chimeric integrin containing the β1, but not the α5, intracellular domain is sufficient to stimulate transmembrane signal transduction in the form of tyrosine phosphorylation. Thus, neither integrin extracellular nor transmembrane domains are needed for signal transduction, suggesting that the only integrin-specific interactions required involve the β1 intracellular domain.

Cells transiently expressing the β1 chimeric receptor were also purified by fluorescence-activated cell sorting and assayed for signal transduction. In this purified population of transfected cells expressing chimeric receptors, there was no induction of tyrosine phosphorylation of pp125FAK unless they were subsequently treated with magnetic beads containing monoclonal anti-IL-2 receptor antibodies (Fig. 2, compare lanes 3 and 4). This result is consistent with previous studies showing that some form of extracellular interaction is required for inducing the tyrosine phosphorylation of pp125FAK (14–18, 21).

The participation of other human β integrin intracellular domains in the signal transduction pathway was also examined. As shown in Fig. 3A, chimeric receptors containing the β1 (lane 2), β3 (lane 3), and β5 (lane 5) integrin intracellular domains also have the ability to induce tyrosine phosphorylation of pp125FAK when compared to the chimeric lacking an intracellular domain (lane 1). The ability to trigger tyrosine phosphorylation of pp125FAK appears to be specific to the β1, β3, and β5 intracellular domains. When the alternatively spliced form of the β3 intracellular domain, designated β3Δ (43), was assayed, it did not induce tyrosine phosphorylation of pp125FAK (Fig. 3A, lane 4). The lanes in Fig. 3A were loaded with equal amounts of total protein solubilized from cells bound to magnetic beads containing anti-IL-2 antibodies and, therefore, expressing chimeric receptors on their surfaces. Furthermore, in separate control experiments, flow cytometry analysis indicated that the α5, β1, and β3, chimeric receptors, as well as the control receptor lacking an intracellular domain, were all expressed at similar levels on the cell surface (not shown), making it unlikely that the results shown were due to artificially low expression of the β3Δ receptor. The sequences of the human β1, β3, and β5 intracellular domains are compared in Fig. 3B (43–48). The inability of the β3 intracellular domain to induce tyrosine phosphorylitation of pp125FAK suggests that alternative splicing may be a mechanism to regulate the ability of integrins to participate in the transmembrane signaling process.

Our findings indicate that all the integrin-specific structural information required for transmembrane signaling in the form

2 S. E. LaFlamme, L. A. Thomas, S. S. Yamada, and K. M. Yamada, submitted for publication.

![Fig. 1. Tyrosine phosphorylation of pp125FAK in cultured cells expressing the β1 chimeric receptor. Cultured human foreskin fibroblasts transiently expressing chimeric receptors consisting of the IL-2 receptor extracellular and transmembrane domains coupled to no intracellular domain (lane 3), the β1 intracellular domain (lane 4), or the α5 intracellular domain (lane 5) were transfected, cultured for 2 days, isolated by magnetic bead sorting, lysed, and analyzed for tyrosine phosphorylation by Western blotting. The relative mobilities of molecular size markers are shown on the left, and the position of pp125FAK is indicated by the arrowhead.](image-url)
Signal Transduction by Integrin Cytoplasmic Domains

Fig. 2. Signal transduction by the β1 chimeric receptor in cells enriched by fluorescence-activated cell sorting. Transfected human fibroblasts were harvested after 1 day of culture, stained with fluorescein-labeled anti-IL-2 receptor antibody, and sorted by fluorescence-activated cell sorting under sterile conditions. Cells selected for expression of cell surface IL-2 receptor reporter domain were cultured for an additional day, harvested, and either lysed immediately or stained with anti-IL-2 receptor antibodies (lane 4) before lysing and analyzing for tyrosine phosphorylation of pp125FAK. For reference, tyrosine phosphorylation of pp125FAK resulting from adhesion of non-transfected cells on poly-L-lysine (lane 1) and fibronectin (lane 2) is also shown. The position of pp125FAK is indicated by the arrowhead. All lanes were loaded with lysates derived from an equivalent number of cells (5 x 10⁶/well).

Fig. 3. Signal transduction is triggered by specific integrin β1 intracellular domains. Panel A, cells transiently expressing the indicated integrin intracellular domains were analyzed for tyrosine phosphorylation. Tyrosine phosphorylation of pp125FAK in cells transiently expressing the IL-2 receptor extracellular and transmembrane domains with no integrin intracellular domain or with β1, β2, ββ, or β3 intracellular domains are shown in lanes 1-5, respectively. The position of pp125FAK is indicated by the arrowhead. The upper tyrosine-phosphorylated band is probably that of pp130, which has been observed to be phosphorylated along with pp125FAK (25). All lanes were loaded with equal amounts of total protein, representing approximately 5 x 10⁶ cells/lane. Panel B, comparison of the amino acid sequences of the intracellular domains of β1, β2, ββ, and β3 integrins. The dotted boxes indicate the positions of the cyto-1, cyto-2, and cyto-3 domains. The solid box indicates the portion of the alternatively spliced sequence for the ββ integrin that is conserved with respect to the intracellular domains of those integrins capable of participating in transmembrane signaling.

of tyrosine phosphorylation of pp125FAK is entirely contained within the β1, β2, and β3 intracellular domains, which are as small as 47 amino acid residues. The cytoplasmic domains of these β integrins can mediate signaling even in the absence of their transmembrane and extracellular domains, which can be replaced by the non-integrin transmembrane and extracellular domains from the low affinity subunit of the IL-2 receptor. These domains of the IL-2 receptor by themselves cannot mediate pp125FAK phosphorylation (Fig. 1). Several laboratories have demonstrated by deletion analysis that the presence or absence of particular intracellular domains can alter integrin function (14, 30, 49-55), including observations that the β1 intracellular domain is required for signal transduction (14), whereas the αc intracellular domain is not (55). Our results represent the first direct evidence that an integrin intracellular domain by itself contains sufficient structural information to transfer information to a tyrosine phosphorylated intracellular domain pathway.

The inability of the β3 integrin to trigger tyrosine phosphorylation suggests that integrin-mediated signal transduction can be regulated by alternative splicing that generates different isoforms of integrin intracellular domains. Our results suggest a function for the carboxy-terminal end of the β2, β1, and β3 intracellular domains in the coupling of integrins into the tyrosine phosphate signal transduction cascade. This region may function either by binding or by regulating the binding of pp125FAK or an as yet unidentified protein. Alternatively, the carboxy-terminal end of the β3 may have a negative effect on interactions required for the induction of the pathway involving tyrosine phosphorylation of pp125FAK.

Three separate regions of the β1 intracellular domain, designated cyto-1, cyto-2, and cyto-3 (Fig. 3B), have already been identified as important for cell surface distribution (56). It is not yet known which, if any, of these regions are involved in signal transduction, although the cyto-2 and cyto-3 regions are spliced out of the β3 integrin. Since both integrins and pp125FAK can be localized in focal contacts, perhaps interactions required for integrin clustering in these specialized structures may also be important for tyrosine phosphorylation of pp125FAK. This notion is consistent with the observation that the β1, β2, and β3 chimeric receptors concentrate in focal contacts but the β2 chimeras does not.

The exact sequence of interactions that results in tyrosine phosphorylation of pp125FAK is currently unclear. Recently published studies indicate that pp125FAK is either autophosphorylated or is phosphorylated by pp60src or another 60-kDa phosphoprotein (19, 22-25, 57). Whatever the mechanism, interactions involving integrin β1 intracellular domains appear to be of central importance.

Acknowledgments—We thank Dr. Bruce H. Howard (Laboratory of Molecular Cell Biology, NCI/NIH, National Institutes of Health (NIH)) for providing valuable help and advice with the magnetic bead sorting, Neil J. Hargreaves (Laboratory of Immunology, NIDR, NIH) for valuable expertise and technical assistance with fluorescence-activated cell sorting, Michael Tran and Christopher Yang for valuable technical assistance in the preparation of plasmids, and our colleagues in the Laboratory of Developmental Biology for their helpful comments during the preparation of this manuscript.

REFERENCES

1. Hynes, R. O. (1987) Cell 48, 549-554
2. Akivama, S. K., Nagata, K., and Yamada, K. M. (1990) Biochem. Biophys. Acta 1031, 91-109
3. Albrecht, S. M., and Buck, C. A. (1990) FASEB J. 4, 288-2880
4. Hemler, M. E. (1990) Annu. Rev. Immunol. 8, 385-400
5. Rouslahti, E. (1991) Clin. Invest. 87, 1-5
6. Yamada, K. M., Aota, S., Akivama, S. K., and LaFlamme, S. E. (1991) Cold Spring Harbor Symp. Quant. Biol. 57, 203-212
7. Burridge, K. F., Keth, K., Nuckolls, G., Turner, C. (1988) Annu. Rev. Cell Biol. 4, 487-520
8. Hynes, R. O. (1992) Cell 69, 11-25
9. Schwartz, M. A. (1992) Trends Cell Biol. 2, 304-308
10. Damsky, C. H., and Werb, Z. (1992) Curr. Opin. Cell Biol. 4, 772-781
11. Juliano, R. L., and Haskill, S. (1993) J. Cell Biol. 120, 577-585
12. Ginsberg, M. H., Du, X., and Flow, E. F. (1993) Curr. Opin. Cell Biol. 4, 766-771
13. Sastry, S. K., and Horwitz, A. F. (1993) Curr. Opin. Cell Biol. 5, 619-831
14. Guan, J.-L., Brebrick, J. E., and Hynes, R. O. (1991) Cell Regul. 2, 951-964
15. Burrige, K., Turner, C. E., and Romer, L. H. (1992) J. Cell Biol. 119, 893-903
16. Hanks, S. K., Calabro, M. B., Harper, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 2847-2851
17. Kornberg, L. A., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) J. Biol. Chem. 267, 23439-23442
18. Boekholt, S. M., and Burrige, K. (1993) J. Biol. Chem. 268, 14655-14657
19. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, T. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 5192-5196
20. Zachary, L., and Rosenberg, E. H. (1992) Cell 71, 891-894
21. Kornberg, L. A., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 8382-8386
22. Lipfert, L., Hainovich, M., Clarke, M. D., Cobb, B. S., Parsons, J. T., and Burridge, J. S. (1992) J. Cell Biol. 119, 905-912
23. Hainovich, B., Lipfert, L., Brugge, J. S., and Shaltt, S. J. (1993) J. Biol. Chem. 268, 15868-15877
24. Huang, M.-H., Lipfert, L., Brugge, J. S., and Shaltt, S. J. (1993) J. Biol. Chem. 268, 15868-15877
25. Hailson, M.-H., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H., and Shaltt, S. J. (1993) J. Cell Biol. 122, 473-483
26. Kaner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, T. J. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7928-7932
27. Guan, J.-L., and Shalloway, D. (1997) Nature 388, 690-692
28. Hamawy, M. M., Mergenhagen, S. E., and Stragaman, R. P. (1993) J. Biol. Chem. 268, 6851-6854
29. Leeb-Lundberg, L. M. F., and Xong, X.-H. (1993) J. Biol. Chem. 268, 8151-8157
Signal Transduction by Integrin Cytoplasmic Domains

29. Sinnett-Smith, J., Zachary, I., Valverde, A. M., and Rozengurt, E. (1993) J. Biol. Chem. 268, 14291–14298
30. Solow ska, J., Guan, J.-L., Marcantonio, E. E., Trevithick, J. E., Burk, C. A., and Hynes, R. O. (1990) J. Cell Biol. 109, 863–861
31. Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., and Horwitz, A. (1990) J. Cell Biol. 110, 175–184
32. Marcantonio, E. E., Guan, J.-L., Trevithick, J. E., Hynes, R. O. (1990) J. Biol. Chem. 265, 18427–18430
33. Akiyama, S. K., Hasegawa, Z., Hasegawa, T., and Yamada, K. M. (1991) J. Cell Biol. 117, 1321–1330
34. Padmanabhan, R., Corsico, C., Holter, W., Howard, T., and Howard, B. H. (1988) Anal. Biochem. 170, 341–348
35. Miekkla, S. I., Ingham, K. C., and Menache, D. (1982) Thromb. Res. 27, 1–14
36. van Kuppeveld, T. H., Langmead, L. R., Gaillit, J. O., Suzuki, S., and Ruoslahti, E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5415–5418
37. Geiger, B., Salomon, D., Takeichi, M., and Hynes, R. O. (1992) J. Cell Biol. 103, 437–447
38. Akiyama, S. K., Yamada, S. S., Chen, W.-T., and Yamada, K. M. (1989) J. Cell Biol. 109, 645–650
39. Giordano, T., Howard, T., Coleman, J., Sakamoto, K., and Howard, B. H. (1991) Exp. Cell Res. 192, 193–197
40. Akiyama, S. K., Hasegawa, Z., Hasegawa, T., and Yamada, K. M. (1991) J. Biol. Chem. 266, 12525–12530
41. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
42. Turner, C. E., Schaller, M. D., and Parsons, J. T. (1993) J. Cell Sci. 106, 637–645
43. van Kuppeveld, T. H., Langmead, L. R., Gaillit, J. O., Suzuki, S., and Ruoslahti, E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5415–5418
44. Argraves, W. S., Suzuki, S., Aral, H., Thompson, K., Pietschbacher, M. D., and Ruoslahti, E. (1987) J. Cell Biol. 105, 1183–1190
45. Fitzgerald, L. A., Steiner, R., Kall, S. C. J., Lo, S. S., and Phillips, D. R. (1987) J. Biol. Chem. 262, 3936–3939
46. McLean, J. W., Vestal, D. J., Chen, D. A., and Bodary, S. C. (1990) J. Biol. Chem. 265, 17126–17131
47. Miekkla, S. I., Ingham, K. C., and Menache, D. (1982) Thromb. Res. 27, 1–14
48. Geiger, B., Salomon, D., Takeichi, M., and Hynes, R. O. (1992) J. Cell Biol. 103, 437–447
49. Akiyama, S. K., Hasegawa, Z., Hasegawa, T., and Yamada, K. M. (1991) J. Cell Biol. 109, 645–650
50. Giordano, T., Howard, T., Coleman, J., Sakamoto, K., and Howard, B. H. (1991) Exp. Cell Res. 192, 193–197
51. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
52. Turner, C. E., Schaller, M. D., and Parsons, J. T. (1993) J. Cell Sci. 106, 637–645