Regulation of Fibronectin Receptor Distribution

Susan E. LaFlamme, Steven K. Akiyama, and Kenneth M. Yamada

Laboratory of Developmental Biology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

Abstract. To determine the role of each intracellular domain of the fibronectin receptor in receptor distribution, chimeric receptors were constructed containing the human interleukin-2 receptor (gp55 subunit) as the extracellular and transmembrane domains, in combination with either the \( \alpha_5 \) or \( \beta_1 \) intracellular domain of the fibronectin receptor as the cytoplasmic domain. These chimeric receptors were transiently expressed in normal fibroblasts, and their localization on the cell surface was determined by immunofluorescence using antibodies to the human interleukin-2 receptor. The \( \alpha_5 \) chimera was expressed diffusely on the plasma membrane. The \( \beta_1 \) chimera, however, colocalized with the endogenous fibronectin receptor at focal contacts of cells spread on fibronectin. On cells spread in the presence of serum, the \( \beta_1 \) chimera colocalized both with the fibronectin receptor at sites of extracellular fibronectin fibrils and with the vitronectin receptor at focal contacts. The \( \beta_1 \) intracellular domain alone, therefore, contains sufficient information to target the chimeric receptor to regions of the cell where ligand-occupied integrin receptors are concentrated. The finding that the \( \beta_1 \) chimeric protein behaves like a ligand-occupied receptor, even though the \( \beta_1 \) chimera cannot itself bind extracellular ligand, suggests an intracellular difference between occupied and unoccupied receptors, and predicts that the distribution of integrin receptors can be regulated by ligand occupancy. We tested this prediction by providing a soluble cell-binding fragment of fibronectin to cells spread on laminin. Under conditions preventing further ligand adsorption to the substrate, this treatment nevertheless resulted in the relocation of diffuse fibronectin receptors to focal contacts. Similarly, a redistribution of diffuse vitronectin receptors to focal contacts occurred on cells spread on laminin after the addition of the small soluble peptide GRGDS. We conclude that the propensity for receptor redistribution to focal contacts driven by the \( \beta_1 \) cytoplasmic domain alone is suppressed in heterodimeric unoccupied fibronectin receptors, and that ligand occupancy can release this constraint. This redistribution of integrin receptors after the binding of a soluble substrate molecule may provide a direct means of assembling adhesion sites.

The integrins, a family of transmembrane heterodimeric receptors consisting of \( \alpha \) and \( \beta \) subunits, play a central role in cell adhesion and migration. These processes are important in development, wound healing, metastasis, and other biological events. Integrins function in both cell–cell and cell–substrate adhesion. Integrin heterodimers can be classified into subfamilies based on the different \( \beta \) subunits. Different combinations of \( \alpha \) and \( \beta \) subunits give rise to receptors with different ligand specificities, including receptors for fibronectin, vitronectin, collagen, and laminin. Although some integrins are cell-type specific, most function in many cell types, and most cell types express a variety of integrin receptors, allowing them to interact with many extracellular matrix components (recent reviews include Akiyama et al., 1990; Albelda and Buck, 1990; Hemler, 1990; Hynes, 1990; Mosher, 1989; Ruoslahti, 1991).

Integrin receptors involved in cell–substrate adhesion are generally believed to function as transmembrane links between the extracellular matrix and the cytoskeleton. When cells form stable adhesions, integrin receptors concentrate in adhesion sites termed focal contacts. Via their intracellular domains, integrins are thought to interact directly with some of the cytoskeletal proteins that colocalize with them at these sites, such as talin and \( \alpha \)-actinin (Singer, 1982; Chen et al., 1985, Damsky et al., 1985; Horwitz et al., 1986; Burridge et al., 1988; Tapley et al., 1989; Otey et al., 1990).

The integrin \( \beta_1 \) intracellular domain is particularly well conserved in vertebrates from humans to amphibians, suggesting its importance in receptor function (DeSimone and Hynes, 1988). This role has been supported by transfection experiments. By assaying the function of heterodimers consisting of transfected normal and mutant \( \beta_1 \) subunits and endogenous \( \alpha \) subunits, it has been shown that the \( \beta \) intracellular domain is required for the localization of \( \beta_1 \) integrin receptors in focal contacts (Solowska et al., 1989; Hayashi et al., 1990; Marconato et al., 1990), as well as its function in cell adhesion (Hayashi et al., 1990). However, it is still not known whether the \( \beta_1 \) intracellular domain alone is sufficient for receptor interaction with the cytoskeleton, or whether in analogy to the requirements for ligand–receptor interaction, both the \( \alpha \) and \( \beta \) subunits are required for receptor interaction with the cytoskeleton (Buck et al., 1986).
The distribution of specific integrin receptors at focal contacts is regulated at least in part by the availability of ligand on the substrate. For example, the fibronectin receptor, $\alpha_5\beta_1$, concentrates in focal contacts in cells spread on a substrate of fibronectin; the vitronectin receptor, $\alpha_v\beta_3$, is in focal contacts in cells spread on vitronectin; and on a collagen substrate, the collagen receptor, $\alpha_\beta_1$, is in focal contacts (Singer et al., 1988; Dejana et al., 1988; Fath et al., 1989; Carter et al., 1990). However, it is puzzling that the vitronectin receptor, $\alpha_v\beta_3$, does not localize to focal contacts (Weyner et al., 1991), even though it contains sequence motifs previously demonstrated to be important in receptor localization to these adhesion sites (McLean et al., 1990; Ramaswamy and Hemler, 1990; Marcantonio et al., 1990). Thus, the molecular mechanisms of the regulation of integrin receptor distribution and the specific contributions of the various aspects of receptor function to this process are still unknown.

To determine the specific roles of each intracellular domain of the $\alpha_5\beta_1$ integrin fibronectin receptor in receptor distribution, we have constructed chimeric receptors containing the interleukin-2 (IL-2) receptor (gp55 subunit) as the extracellular and transmembrane domains combined with either the $\alpha_5$ or $\beta_1$ intracellular domain of the fibronectin receptor. These chimeric receptors were transiently expressed in normal human and chick embryo fibroblasts, and their expression was analyzed using antibodies to the IL-2 receptor. We then analyzed the individual functions of the $\alpha_5$ and $\beta_1$ intracellular domains in receptor localization by using the IL-2 receptor as a reporter domain. We have been able to dissect the function of each intracellular domain from the function of ligand binding.

We find that our $\beta_1$ chimera colocalizes with ligand-occupied receptors, suggesting that a $\beta_1$ intracellular domain expressed in the absence of a corresponding $\alpha_5$ intracellular domain functions as if it were a ligand-occupied receptor. Since our $\beta_1$ chimera cannot itself bind extracellular matrix ligands, and since endogenous unoccupied receptors are expressed diffusely on the cell surface, our results suggest that there may be an intracellular difference between occupied and unoccupied receptors. This notion predicts that one should be able to change the distribution of diffuse integrin receptors, via an intracellular mechanism, by the addition of a soluble ligand. This concept is in contrast to previous studies of $\beta_1$ integrins which involved binding to insoluble, immobile, substrate-attached or fibrillar ligands. We tested the ability of soluble ligands and even soluble synthetic peptide ligands, under conditions preventing their adsorption to substrates, to cause the redistribution of diffuse integrin receptors to focal contacts. Our results suggest that specific ligand occupancy and the $\beta_1$ integrin cytoplasmic domain play central roles in the regulation of fibronectin receptor distribution.

### Materials and Methods

#### Construction of Chimeric Receptors

Chimeric receptors were constructed using standard molecular biological techniques (Sambrook et al., 1989). Each construct was confirmed by nucleotide sequence analysis. DNA oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer; complementary oligonucleotides were annealed before ligation. The plasmid CMV-IL2R (Giordano et al., 1991) was a generous gift of Dr. Anthony Giordano and contains the cytomegalovirus LTR directing the transcription of the IL-2 receptor gp55 subunit (also known as the $\alpha$ subunit) cDNA sequences (Leonard et al., 1984; Nikaido et al., 1984). To construct the chimeric receptors, a plasmid containing IL-2 receptor cDNA sequences through the membrane domain followed by a Lys residue and the stop codon TGA was constructed. Specifically, the intracellular domain of the IL-2 receptor was replaced with a small fragment containing a HindIII site immediately adjacent to the membrane domain and 3' XhoI site. First, CMV-IL2R's unique HindIII site was eliminated by digestion with HindIII, extension of the resulting 3' ends with the Klenow fragment of E. coli DNA polymerase I, and the recircularization of the plasmid with T4 DNA ligase. The Ncol/Xbal fragment of the IL-2 receptor cDNA was then replaced with complementary oligonucleotides 1A/1B. Oligonucleotides encoding the $\alpha_5$ and $\beta_1$ intracellular domains were then inserted between the new HindIII and XhoI sites.

Oligonucleotides 1A/1B:

- 5'-GGCTGGTTTTTCTCTGCAGTCCTCCTGCTAGTTGGCTCAACCT
- 3'-CGGACACCAAAAGGGACCTAGTGCACCAAGGGACTCCACCGAGT

Oligonucleotides 2A/2B and 3A/3B encode the $\beta_1$ intracellular domain.

Oligonucleotides 2A/2B:

- 5'-AGCTTTTAATGACTGACAGCAAGGAGGTTGTCATAATTTTGAAAA
- 3'-AAATTACATTAAAGTACTGCTCTTCTCCAAGCAATATTTTTTTTTTTT

Oligonucleotides 3A/3B:

- 5'-TTTATATAAGGTTGCGATAAACCAGTGCTCAACCT
- 3'-GCCACCTTAGGATAAAAAATTTCTCAAGCAGCTTTGAGATTG

Oligonucleotides 4A/4B and 5A/5B, encoding the $\alpha_5$ intracellular domain lacking its HindIII site were inserted in plasmid SP27 (Promega, Madison, WI).

Oligonucleotides 4A/4B:

- 5'-CCCTGTGCTCTAGGTCTACCTTCTACATCACTCTCTAACGCTT
- 3'-GCCACCTTAGGATAAAAAATTTCTCAAGCAGCTTTGAGATTG

Oligonucleotides 5A/5B:

- 5'-CTGAAAGTCACCACCATCTGATGCTGAGC
- 3'-GAGCTGAGGTGGGAGTAGTATCTATGTGGAGAGTCTGAGCT

The $\alpha_5$ intracellular domain containing a HindIII site at its membrane intracellular junction was amplified from the above plasmid by the polymerase chain reaction using a PCR kit from Perkin-Elmer Cetus (Norwalk, CT) using as primers:

Forward primer: 5'-CTTACAAAGCTTGGATTTCTGCAAG-3'
Reverse primer: 5'-CTTACCTGGAGATCTACGGAGA-3'

### Cells

Normal human foreskin fibroblasts were a generous gift from Dr. Steve Alexander (Bethesda Research Laboratories, Gaithersburg, MD) and were cultured in DMEM supplemented with 1 mM glutamine, 50 $\mu$g/ml streptomycin, 50 U/ml penicillin, and 10% heat-inactivated FCS. Cell passages 9-18 were used. Chick embryo fibroblasts were prepared as described by Vogt (1969), and were cultured as above with the addition of 1% heat-inactivated chicken serum. Cell passages three to five were used.

### Purified Proteins, Fragments, and Peptides

Laminin was purchased from Bethesda Research Laboratories and was also generously provided by Dr. Hynda Kleinman (National Institutes of Health, Bethesda, MD). The 75-kD cell-binding fragment and the 43-kD collagen-binding fragment of fibronectin were purified as previously described.
LaFlamme et al. Regulation of Integrin Distribution

LaFlamme et al.

Immunological Reagents

The polyclonal antibody R3134 to the IL-2 receptor (Sharon et al., 1986) was a generous gift of Dr. Warren Leonard (National Institutes of Health), and the mouse mAb RPN.512 to the IL-2 receptor's gp55 subunit was purchased from Amersham Chemical Corp. (Arlington Heights, IL). Polyclonal antibody 4080 to the β1 intracellular domain has been described previously (Larjava et al., 1990). Polyclonal antibody 4318 to the α0 intracellular domain was produced after immunization of rabbits with the peptide CAQLKPATSDA as described previously for polyclonal antibody 4080 (Larjava et al., 1990). Rat mAb 11 to the α0 subunit was generated, characterized, and purified as described for mAb 16 previously (Akiyama et al., 1989). Because mAb 11 was noninhibitory, its specificity for the α0 subunit was further demonstrated by immunostaining mouse 3T3 cells (ATCC, Rockville, MD) transiently expressing a transfected full-length human α0 cDNA (not shown). In addition, after preincubation with 35S-labeled cell lysates, no additional proteins were immunoprecipitated with mAb 11, and conversely, after preincubating cell lysates with mAb 11, no additional proteins were immunoprecipitated with polyclonal antibody 4318. Polyclonal antibody to the vitronectin receptor, α0β5, was purchased from Telios (San Diego, CA). mAb 333 has been previously described (Akiyama et al., 1985). FITC and rhodamine-labeled second antibodies were purchased from Rockland, Inc. (Gilbertsville, PA).

Immunofluorescence and Interference Reflection Microscopy

Cells were cultured on glass coverslips as indicated, fixed for 30–60 min with 4% formaldehyde, 5% sucrose in Dulbecco’s PBS, then washed several times in PBS, permeabilized for 5 min in 0.2% Triton X-100 in PBS, washed several times with PBS, incubated for 30 min in 3% BSA, 0.1% glycine (pH 7.5) to block nonspecific binding, washed once in PBS, then incubated for 1 h at ambient temperature with primary antibody in PBS with 1 mg/ml BSA, then washed for 30 min in PBS with gentle agitation with several changes of PBS, then incubated with the appropriate FITC- or rhodamine-conjugated goat antibody and washed as above. The coverslips were then mounted on microscope slides with 10% glycerol in PBS containing 1 mg/ml 1,4-phenylenediamine (Fluka Chemical Corp., Ronkonkoma, NY) to inhibit photobleaching (Johnson and Noguetra Araujo, 1981). Adhesions on the ventral cell surface were characterized by interference reflection microscopy (Izzard and Lochner, 1976) by standard methods using an Antiflex Neofluar 63x/NA 1.25 objective on a Zeiss photomicroscope III and photographed with Kodak technical pan film processed with Diafine developer (Izzard and Lochner, 1976) by standard methods using an Antiflex Neofluar 63x/NA 1.25 objective on a Zeiss photomicroscope III and photographed with Kodak technical pan film processed with Diafine developer.

Transfections

Electroporation of cells was performed as described by Giordano et al. (1991) at 170 V and 960 µF with a Gene Pulser (Bio-Rad Laboratories). The expression patterns observed for the α0 and β1 chimeras were the same without incubation in sodium butyrate, although the number of transfected cells and the level of expression of the transfected receptor were significantly lower.

Redistribution of Receptors by Ligand Occupancy

Acid-washed glass coverslips were coated with 25 µg/ml laminin overnight at 4°C and then blocked for 1 h at ambient temperature with 10 mg/ml heat-denatured BSA (Calbiochem-Behring Corp., San Diego, CA). To prevent fibronectin synthesis during the course of these experiments, cells were preincubated for 2 h with 10 µg/ml cycloheximide (Calbiochem-Behring Corp.), and cycloheximide was then included in all subsequent solutions. Redistribution studies of the fibronectin receptor were performed by two protocols.

(Method A) Subconfluent cultures were harvested with trypsin EDTA, preincubated at 37°C in suspension in medium prepared with fibronectin-depleted serum in the presence of cycloheximide and 50 µg/ml antifibronectin mAb 333, and then 1–2 x 10^6 cells were plated on laminin-coated coverslips in 24-well clusters in serum-free medium + mAb 333 for 90 min. To reduce the background of α0β1 fibronectin receptors in focal contacts in cells spread for short periods of time on laminin (see also Singer et al., 1988), we preincubated the cells with mAb 333, which binds near the RGD site on fibronectin and inhibits the interaction of fibronectin with the fibronectin receptor (data not shown). The cells were then washed several times, fresh serum-free medium containing fragment or peptide was added as indicated in the figure legends, and the cells were incubated at 37°C for an additional 60 min.

To examine the redistribution of vitronectin receptors, subconfluent cultures were preincubated for 2 h with cycloheximide. The cells were then harvested with trypsin-EDTA, washed, and preincubated in suspension for 30 min at 37°C in serum-free medium. Cells were then plated and treated as described in Method A with mAb 333 omitted.

To determine the number of cells with redistributed fibronectin and vitronectin receptors, cells were fixed and stained with either polyclonal antibody 4318 to the α0 intracellular domain or polyclonal antibody to α0β5. Cells with focal contacts were located by interference reflection microscopy, and the presence of fibronectin or vitronectin receptors was determined by immunofluorescence. All experiments were analyzed blind, with 100 cells analyzed for each condition.

Results

Expression of the Chimeric Receptors

Chimeric receptors were constructed containing the IL-2 receptor (gp 55 subunit) as the extracellular and transmembrane domains, and either the α0 or β1, intracellular domain of the fibronectin receptor as the cytoplasmic domain (Fig. 1). These chimeric receptors were transiently expressed in normal human fibroblasts or in chick embryo fibroblasts. Since fibroblasts do not normally express the IL-2 receptor, expression and localization of the transfected receptors could be analyzed by immunofluorescence using antibodies to the IL-2 receptor. Generally 25–50% of the transfected cells expressed the transfected receptor (data not shown; Goldstein et al., 1989). Successful cell surface expression of the α0 and β1 chimeras was demonstrated by immunofluorescence staining of non-permeabilized transfected normal human fibroblasts (Fig. 2, A–D). Although the integrity of these chimeras was confirmed by nucleotide sequence analysis, we were also able to demonstrate by immunofluorescence that...
Figure 3. Codistribution of the $\beta_1$ chimera with the endogenous $\alpha_5\beta_1$ fibronectin receptor at focal contacts in normal human fibroblasts. 36 h after transfection with either the $\alpha_5$ (A–C) or the $\beta_1$ (D–F) chimera, cells were pretreated with cycloheximide for 2 h, harvested, and then plated for 2 h on coverslips coated with 10 $\mu$g/ml fibronectin. The cells were then double immunostained for the transfected receptor with polyclonal antibodies to the IL-2 receptor at 1:300 dilution (A and D) and for the endogenous fibronectin receptor with mAb 11 at 25 $\mu$g/ml (B and E) with the same regions of the cells shown by interference reflection microscopy (C and F). Arrows indicate focal contacts. Bars, 10 $\mu$m.

Figure 2. Expression of the transfected chimeras. Cell surface expression of the $\alpha_5$ chimera (A and B) or the $\beta_1$ chimera (C and D) in normal human fibroblasts. Transfected nonpermeabilized cells stained with mAb RPN.512 to the IL-2 receptor at 7 $\mu$g/ml (A and C) are shown by immunofluorescence or indicated by the arrows in the phase-contrast micrographs (B and D). Immunostaining of the intracellular domains of the chimeric receptors (E–J). Chick embryo fibroblasts transfected with either the $\alpha_5$ chimera, double immunostained with mAb to the IL-2 receptor (E) and polyclonal antibody 4318 to the $\alpha_5$ intracellular domain at 1:100 dilution (F), or the $\beta_1$ chimera, double immunostained with mAb to the IL-2 receptor (H) and polyclonal antibody 4080 to the $\beta_1$ intracellular domain at 1:100 dilution (I), are shown by immunofluorescence, or indicated by arrows in phase contrast (G and J). Bars, 20 $\mu$m.
the α5 and β1 intracellular domains were correctly translated. Polyclonal antibodies 4080 and 4318 recognize the human β1 and α5 intracellular domains respectively. The polyclonal antibody to the human α5 intracellular domain showed only weak staining of endogenous avian receptors (untransfected cells in Fig. 2 F and our unpublished observations). The human α5 chimera transiently expressed in chick embryo fibroblasts could be detected with either the mAb to the IL-2 receptor (Fig. 2 E) or the polyclonal antibody to human α5 intracellular domain (Fig. 2 F). Although the human and avian β1 intracellular domains are identical, cells transiently expressing the β1 chimera at high levels could be detected with either the mAb to the IL-2 receptor (Fig. 2 H) or the polyclonal antibody to the β1 intracellular domain (Fig. 2 I), whereas the nontransfected cells could not (Fig. 2 J and our unpublished observations). These results established that the intracellular domains were correctly expressed at the protein level as well.

**Localization of the Chimeric Receptors**

When fibroblasts spread on a fibronectin substrate, their α5β1 fibronectin receptors concentrate in focal contacts (Singer et al., 1988). To establish whether either the α5 or β1 intracellular domain alone can determine where the chimeric receptors localize, normal human fibroblasts transiently expressing these chimeras were plated for 2 h on fibronectin-coated coverslips, and the expression of the transfected and endogenous receptors in focal contacts was analyzed by immunofluorescence and interference reflection microscopy. The α5 chimera was expressed diffusely on the plasma membrane even in cells that had their endogenous fibronectin receptors concentrated in focal contacts (Fig. 3, A–C). The β1 chimera, however, localized to focal contacts in patterns similar to those of endogenous receptors (Fig. 3, D–F), indicating that the β1 intracellular domain itself is sufficient to target the chimeric receptor to focal contacts.

When fibroblasts are plated in serum for 24 h or more on uncoated coverslips, their α5β1 fibronectin receptors become concentrated at sites where extracellular fibronectin fibrils are associated with the plasma membrane, and their vitronectin receptors become concentrated at focal contacts (Singer et al., 1988). To determine whether either the α5 or β1 intracellular domain alone was sufficient to target the chimeric receptors to sites of cell contact with fibronectin fibrils, normal human fibroblasts transfected with either the α5 (A and B) or the β1 (C and D) chimera were plated on coverslips in serum for 36 h and stained for the transfected receptor with polyclonal antibodies to the IL-2 receptor (A and C) and for the endogenous fibronectin receptor with mAb 11 (B and D). Arrowheads indicate regions where the β1 chimera colocalizes with the endogenous fibronectin receptor at sites where extracellular fibronectin fibrils are associated with the plasma membrane, whereas arrows indicate the lack of colocalization of the α5 chimera with the endogenous fibronectin receptor at these sites. Bar, 10 μm.
fibrils, human fibroblasts transiently transfected with the chimeric receptors were plated on coverslips in serum-containing medium for 36 h. The β1 chimera was found to be colocalized with the fibronectin receptor at sites of plasma membrane-fibronectin fibril association (Fig. 4, C and D). Therefore, the β1 intracellular domain was also sufficient to target receptors to other regions of the cell where ligand-occupied receptors were concentrated. However, its most striking localization was still at focal contacts, where vitronectin receptors also localized (Fig. 5, D-F).

The α5 chimera was again found to be expressed diffusely in the plasma membrane even in cells where endogenous fibronectin receptor was concentrated at sites of fibronectin fibril formation (Fig. 4, A and B), and in contrast to the β1 chimera, it did not colocalize with the vitronectin receptor at focal contacts (Fig. 5, A-C). This diffuse expression pattern of the α5 chimera suggested that it was either insufficient for receptor localization or that it encoded a dominant negative element for receptor localization. To distinguish between these two possibilities, we examined the expression of a transfected IL-2 receptor lacking an intracellular domain (see Materials and Methods). We found that like the α5 chimera, it also showed diffuse membrane staining, indicating that the α5 chimera lacked sufficient information for receptor localization (not shown). However, the α5 intracellular domain may act as a negative element in the heterodimeric unoccupied receptor suppressing the propensity for receptor redistribution to focal contacts driven by β1 intracellular domain. Ligand occupancy may release this constraint.

Redistribution of the α5β1 Fibronectin Receptor by Ligand Occupancy

Since our β1 chimera does not contain an extracellular domain that can bind extracellular matrix ligands, its distribution at focal contacts is most likely determined by its interaction with the cytoskeleton. The β1 intracellular domain expressed in the absence of an α5 intracellular domain may
The Journal of Cell Biology, Volume 117, 1992

Redistribution of Other Integrins by Ligand Occupancy

The localization of the fibronectin receptor to focal contacts in the cell membrane. Treatment with the 75-kD fragment under these conditions resulted in a similar redistribution of diffuse fibronectin receptors to focal contacts. This redistribution of the fibronectin receptor is shown quantitatively in Fig. 6B and by immunofluorescence in Fig. 7.

Redistribution of Other Integrins by Ligand Occupancy

The relocation of receptors by ligand binding may be a general characteristic of integrin receptors. The vitronectin receptor α5β1 binds readily to the small peptide GRGDS (Pytela et al., 1985). When this small soluble peptide was added to cells spread on a laminin substrate, redistribution of vitronectin receptors to focal contacts was observed, similar to what was seen for fibronectin receptors (Fig. 8). The redistribution of the vitronectin receptor did not occur with the addition of the control peptide GRGES, BSA, or the 43-kD collagen binding fragment of fibronectin.

Discussion

Understanding the biology of the fibronectin receptor and other integrin receptors requires the identification of the functional domains of these receptors. To determine whether the α5 and β1 intracellular domains of the fibronectin receptor can function as separate domains, we constructed chimeric receptors containing either the α5 or the β1 intracellular domain with the IL-2 receptor gp55 subunit as the common extracellular and transmembrane domains. The determination of protein domain function by deletion analysis is always difficult because of the possibility that a particular deletion or mutation may affect the conformation of the protein. Therefore, we have used the alternative approach of testing for gain-of-function by the addition of a specific domain. Using the IL-2 receptor portion as a reporter domain, we were able to characterize the function of each intracellular domain individually and independently of the receptor's ability to bind to ligand. We were able to (a) define function for the β1 intracellular domain, and (b) analyze the role of ligand occupancy in the regulation of integrin receptor distribution.

We found that the β1 intracellular domain alone was sufficient to target the reporter domain to regions of the cell where ligand-occupied receptors were concentrated, such as focal contacts and sites of association between the cell and extracellular fibronectin fibrils. Our results confirm a central role for the β1 intracellular domain in receptor localization, but they also establish that the β1 intracellular domain is not only required, but is in fact sufficient by itself to target receptor localization to focal contacts. In addition, since the α5 chimera was expressed relatively diffusely on the cell surface, we can further conclude that the α5 intracellular domain is not sufficient to determine receptor localization. However, we cannot rule out the possibility that the α5 intracellular domain plays a supportive or regulatory role in receptor distribution and function, or that it plays a more active role in receptor localization in other cell types. Interestingly, the α5 intracellular domain of the platelet receptor, c5mβ3, functions in the regulation of ligand binding affinity, and the α5 intracellular domain cannot substitute for the α5 intracellular domain in this regulation (O'Toole et al., 1991).

The localization of the β1 chimera to focal contacts on cells plated on a fibronectin substrate suggests that it binds

Figure 6. Redistribution of the α5β1 fibronectin receptor by ligand occupancy. (A) Normal human fibroblasts pretreated as described in Materials and Methods were incubated on laminin-coated coverslips for 90 min. The cells were incubated an additional 60 min with fresh medium alone or with either 100 μg/ml of the 75-kD cell-binding fragment of fibronectin, or BSA, or the 43-kD collagen-binding fragment of fibronectin, or either 500 μg/ml GRGES or GRGDS. The percent cells with α5β1 in focal contacts was determined as described in Materials and Methods. (B) Cells pretreated as described in Materials and Methods were incubated on laminin-coated coverslips for 15 h and were then incubated for an additional 60 min after the addition of fresh medium alone or containing 200 μg/ml of the 75-kD cell-binding fragment of fibronectin.
Figure 7. Redistribution of α5β1 by ligand occupancy as shown by immunofluorescence. Normal human fibroblasts were incubated for 15 h on laminin-coated coverslips, and then incubated an additional 1 h with fresh medium alone (A and C) or with 200 μg/ml 75-kD cell binding fragment (B and D). Cells stained with polyclonal antibody 4318 to the α5 intracellular domain at 1:100 dilution are shown by immunofluorescence (A and B) or by interference reflection microscopy (C and D). Bar, 10 μm.

the same cytoskeletal proteins that bind the endogenous fibronectin receptors concentrated at these sites. The β1 chimera also localized to focal contacts in cells plated in serum, suggesting that it also binds to proteins in focal contacts formed by vitronectin receptors. The β1 and β3, intracellular domains may bind to the same cytoskeletal protein, since the β3 and β1 intracellular domains have considerable homology (Argraves et al., 1987; Fitzgerald et al., 1987) and colocalize with the same cytoskeletal proteins at adhesion sites (Fath et al., 1989). Interestingly, the β1 intracellular domain can substitute for the β3 intracellular domain in heterodimeric fibronectin receptor localization and function (Solowska et al., 1991).

The major cytoplasmic difference between the β1 chimera and the endogenous fibronectin receptor is the absence of the α5 intracellular domain in the β1 chimera. This fact taken together with colocalization of the β1 chimera with endogenous ligand-occupied receptors, suggests that the α5 intracellular domain may inhibit the ability of the β1 intracellular domain of unoccupied endogenous receptors to interact with cytoskeletal proteins, and further suggests that a change in the endogenous receptor occurs upon ligand binding that results in an unmasking of the β1 intracellular domain or regions of the intracellular domain that interact with specific cytoskeletal proteins. The idea that the context of the β1 intracellular domain may affect its affinity for specific cytoskeletal proteins is supported by in vitro binding studies in which the β1 intracellular domain expressed as a peptide had a higher affinity for α-actinin than the purified intact heterodimeric receptor (Otey et al., 1990). On the other hand, receptor interaction with talin requires an intact, heterodimeric receptor for binding (Buck et al., 1986). These results support the notion that the availability of binding sites on the β1 intracellular domain may be affected by the α5 intracellular domain. However, an alternative possibility is that ligand binding, instead of unmasking the β1 intracellular domain, changes the conformation of the β1 intracellular domain into a higher affinity or “active” conformation capable of interacting with the cytoskeleton, and that our β1 chimera has an intracellular domain in this “active” conformation.
The hypothesis that occupied receptors have an enhanced ability to interact with the cytoskeleton suggests a mechanism by which ligand could determine the localization of receptors, not only by the availability of substrate-bound ligand, but in addition by the receptor's ability to interact with specific cytoskeletal proteins. We tested this possibility by the addition of soluble ligands for either the \( \alpha\beta_1 \) fibronectin receptor or the vitronectin receptor to cells spread on laminin and found that ligand occupancy targeted diffuse receptors to heterologous focal contacts. This redistribution of receptors occurred even with small peptide ligands, ensuring that the striking redistribution observed was not due to receptors binding to ligands absorbing to the substrate, since the use of GRGDS as a substrate requires covalent linkage to a carrier protein (Singer et al., 1987). Furthermore, redistribution of receptors occurred both in cells spread for 90 min with newly formed focal contacts and in cells spread for 15 h with more stable and mature focal contacts. Additionally, when the experiment was performed with cells spread on vitronectin instead of laminin, both the vitronectin and fibronectin receptors were in the same focal contacts (data not shown). In some cell types, both cell-binding and heparin-binding domains of fibronectin are required for focal contact formation (Izzard et al., 1986; Woods et al., 1986). In the redistribution experiments with normal human fibroblasts, the cell-binding domain was sufficient for the redistribution of the fibronectin receptor to focal contacts.

Generally, integrin receptors do not concentrate at focal contacts unless their ligand is absorbed to the substrate (Singer et al., 1988; Dejana et al., 1988; Fath et al., 1989; Carter et al., 1990). Similarly the distribution of the fibronectin receptor with plasma membrane-associated fibronectin fibrils requires the interaction of the fibronectin receptor with extracellular fibrils (Chen et al., 1986; Giancotti et al., 1986; Akiyama et al., 1989; Romani et al., 1989). These previous experiments demonstrated that the regulation of integrin receptor distribution requires the interaction of the receptor with its ligand and that the distribution of ligand determines the distribution of its receptor. However, these experiments implied that the regulation of integrin distribution by ligand occupancy is largely extracellular. The results of our chimeric receptor experiments taken together with our receptor redistribution experiments suggest a major internal control of receptor distribution regulated by ligand occupancy. Our results support the hypothesis that a conformational change occurs in some integrin receptors that enhance their ability to interact with particular cytoskeletal proteins and that this enhanced ability to interact with the cytoskeleton can determine receptor distribution. This intracellular control of receptor distribution provides a simple mechanism for recruitment of integrin receptors with their bound ligand to adhesion sites and sites of extracellular fibril formation.

We thank Susan Yamada for valuable assistance in the preparation of antibodies; Bernadette Hickson for technical assistance; and Drs. Anthony Giordano, Hynda Kleinman, and Warren Leonard for providing necessary materials.

Received for publication 14 November 1991 and in revised form 23 January 1992.

References

Akiyama, S. K., E. Hasegawa, T. Hasegawa, and K. M. Yamada. 1985. The interaction of fibronectin fragments with fibroblastic cells. J. Biol. Chem. 250:33256–33260.

Akiyama, S. K., S. S. Yamada, W. -T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monocular antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J. Cell Biol. 109:863–875.

Akiyama, S. K., K. Nagata, and K. M. Yamada. 1990. Cell surface receptors for extracellular matrix components. Biochim. Biophys. Acta. 1031:91–109.

Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. FASEB (Fed. Am. Soc. Exp. Biol.) J. 4:2868–2880.

Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Fierschbacher, and E. Rossolashi. 1987. Amino acid sequence of the human fibronectin receptor. J. Cell Biol. 105:1183–1190.

Buck, C. A., E. Shea, K. Duggan, and A. F. Horwitz. 1986. Integrin (the CSAT antigen): Functionality requires oligomeric integrity. J. Cell Biol. 103:2421–2429.

Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. 4:487–525.

Carter, W. G., E. A. Wayner, T. S. Bonehard, and P. Kaur. 1990. The role of integrins \( \alpha\beta_1 \) and \( \alpha\beta_5 \) in cell-cell and cell-substrate adhesion of human epidermal cells. J. Cell Biol. 110:1387–1404.

Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultivated fibroblasts. J. Cell Biol. 100:1103–1114.

Chen, W.-T., J. Wang, T. Hasegawa, S. S. Yamada, and K. M. Yamada. 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. J. Cell Biol. 105:1649–1661.

Damsky, C. H., K. A. Knudsen, D. Bradley, C. A. Buck, and A. F. Horwitz. 1985. Distribution of the CSAT cell-matrix antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. 100:1528–1539.

Dejana, E., M. C. Coletta, G. Conforti, M. Abbadini, M. Gaboli, and P. C. Marchisio. 1986. Fibronectin and vitronectin regulate the organization of their respective arg-gly-aspa adhesion receptors in cultured human endothelial cells. J. Cell Biol. 107:1215–1223.

DeLemos, D. W., and R. O. Hynes. 1988. Xenopus laevis integrins. Structural conservation and evolutionary divergence of integrin \( \beta \) subunits. J. Biol. Chem. 263:5533–5540.

Fath, K. R., C. J. S. Edgel, and K. Burridge. 1989. The distribution of distinct integrins in focal contacts is determined by the substratum composition. J. Cell Sci. 92:67–75.

Fitzgerald, L. A., B. Steiner, S. C. Rall Jr., S.-S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. J. Biol. Chem. 262:3936–3939.

Giancotti, F. G., P. M. Comoglio, and G. Taronne. 1986. A 135,000 molecular weight plasma membrane glycoprotein involved in fibronectin mediated cell adhesion. Immunofluorescence localization in normal and RSV-transformed fibroblasts. Exp. Cell Res. 163:47–62.

Giordano, T., T. H. Howard, J. Coleman, K. Sakamoto, and B. H. Howard. 1991. Isolation of a population of transiently transfected quiescent and senescent cells by magnetic affinity cell sorting. Exp. Cell Res. 192:193–197.

Goldman, S., C. M. Ford, and B. H. Howard. 1989. Enhanced transfection efficiency and improved cell survival after electroporation of G2/M-synchronized cells and treatment with sodium butyrate. Nucleic Acids Res. 17:3959–3971.

Green, N., H. Alexander, A. Olson, S. Alexander, T. M. Shinnick, J. G. Sulchtle, and R. A. Lerner. 1982. Immunogenic structure of the influenza virus hemagglutinin. Cell. 28:477–487.

Hayashi, M., and K. M. Yamada. 1983. Domain structure of the carboxy-terminal half of human plasma fibronectin. J. Biol. Chem. 258:3332–3340.

Hayashi, Y., B. Haimovich, A. Retska, D. Boettiger, and A. Horwitz. 1990. Expression and function of chicken integrin \( \beta \) subunit and its cytoplasmic domain mutants in mouse 3T3 cells. J. Cell Biol. 110:175–184.

Hernler, M. E. 1990. VLA proteins in the integrin family: Structures, functions, and their roles on leukocytes. Annu. Rev. Immunol. 8:365–400.

Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature (Lond.). 320:531–533.

Hynes, R. O. 1990. Fibronectins. Springer-Verlag, NY. 546 pp.

Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflection study with an evaluation of the technique. J. Cell Sci. 21:129–159.

Izzard, C. S., R. Radinsky, and L. A. Culp. 1986. Substratum contacts and cytoskeletal reorganization of BALB/c 3T3 cells on a cell-binding fragment and heparin-binding fragments of plasma fibronectin. Exp. Cell Res. 165:320–336.

Johnson, G. D., and G. M. Nogueira Araujo. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods. 43:349–350.

Jarjavai, H., J. Peltjone, S. K. Akiyama, S. S. Yamada, H. R. Gralnick, J.
Uitto, and K. M. Yamada. 1990. Novel function for β integrins in keratinocyte cell-cell interactions. J. Cell Biol. 110:803-815.

Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphrey, R. J. Robb, M. Krones, P. B. Svetlik, N. J. Peffer, T. A. Waldmann, and W. C. Greene. 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. Nature (Lond.). 311:626-631.

Marcantonio, E. E., J.-L. Guan, J. E. Trevithick, and R. O. Hynes. 1990. Mapping of the functional determinants of the integrin βi cytoplasmic domain by site-directed mutagenesis. Cell Reg. 1:597-604.

McLean, J. W., D. J. Vestal, D. A. Chereshe, and S. C. Bodary. 1990. cDNA sequence of the human integrin βi subunit. J. Biol. Chem. 265:17126-17131.

Mosher, D. F., editor. 1989. Fibronectin. Academic Press, NY. 474 pp.

Nikaido, T., A. Shintzu, N. Ishida, H. Sabe, K. Teshigawara, M. Maeda, T. Uchiyama, J. Yodó, and T. Honjo. 1984. Molecular cloning of cDNA encoding human interleukin-2 receptor. Nature (Lond.). 311:626-631.

Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the βi subunit in vitro. J. Cell Biol. 111:721-729.

O'Toole, T. E., D. Mandelman, J. Forsyth, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991. Modulation of the affinity of integrin αIIbβ3 (GPIIb-IIIa) by the cytoplasmic domain of αIIb. Science (Wash. DC). 254:845-847.

Pytel, R., M. D. Pierschbacher, and E. Ruoslabhi. 1985. A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic-acid adhesion sequence derived from fibronectin. Proc. Natl Acad. Sci. USA. 82:5766-5770.

Ramaswamy, H., and M. E. Hemler. 1990. Cloning, primary structure and properties of a novel human integrin β3 subunit. EMBO (Eur. Mol. Biol. Organ.) J. 9:1561-1568.

Roman, J., R. M. LaChance, T. J. Broekelmann, C. J. R. Kennedy, E. A. Wayner, W. G. Carter, and J. A. McDonald. 1989. The fibronectin receptor is organized by extracellular matrix fibronectin: implications for oncogenic transformation and for recognition of fibronectin matrices. J. Cell Biol. 108:2529-2543.

Ruoslabhi, E. 1991. Integrins. J. Clin. Invest. 87:1-5.

Ruoslabhi, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. Science (Wash. DC). 238:491-497.

Sambrook, J., E. F. Flisch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 545 pp.

Sharon, M., R. D. Klausner, B. R. Cullen, R. Chizzonite, and W. J. Leonard. 1986. Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. Science (Wash. DC). 234:859-863.

Singer, I. J. 1982. Association of fibronectin and vinculin with focal contacts and stress fibers in stationary hamster fibroblasts. J. Cell Biol. 92:398-408.

Singer, I. I., D. W. Kwakka, S. Scott, R. A. Mumford, and M. W. Lark. 1987. The fibronectin cell attachment sequence Arg-Gly-Asp-Ser promotes focal contact formation during early fibroblastic attachment and spreading. J. Cell Biol. 104:573-584.

Singer, I. I., S. Scott, D. W. Kwakka, D. M. Kazazia, J. Gailit, and E. Ruoslabhi. 1988. Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. J. Cell Biol. 106:2171-2182.

Solowska, J., J.-L. Guan, E. M. Marcantonio, J. E. Trevithick, C. A. Buck, and R. O. Hynes. 1989. Expression of normal and mutant avian integrin subunits in rodent cells. J. Cell Biol. 109:853-861.

Solowska, J., J. M. Edelman, S. M. Albelda, and C. A. Buck. 1991. Cytoplasmic and transmembrane domains of integrin αi and βi subunits are functionally interchangeable. J. Cell Biol. 114:1079-1088.

Tapley, P., A. Horwitz, C. Buck, K. Duggan, and L. Rohrschneider. 1989. Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. Oncogene. 4:325-333.

Vogt, P. K. 1969. Focus assay of Rous sarcoma virus. In Fundamental Techniques in Virology. K. Habel and N. Salzman, editors. Academic Press, NY. 198-211.

Wayner, E. A., R. A. Orlando, and D. A. Chereshe. 1991. Integrins αiβi and αiβ3 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J. Cell Biol. 113:919-929.

Woods, A., J. R. Couchman, S. Johansson, and M. H. H. 1986. Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. EMBO (Eur. Mol. Biol. Organ.) J. 5:665-670.

Yamada, K. M. 1991. Adhesive recognition sequences. J. Biol. Chem. 266:12809-12812.

Zardi, L., B. Carmelotta, E. Balza, L. Borsí, F. Castellani, M. Rocco, and A. Sirti. 1985. Elution of fibronectin proteolytic fragments from a hydroxyapatite chromatography column. A simple procedure for the purification of fibronectin domains. Eur. J. Biochem. 146:571-579.