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 α_5 or β_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 α_5 chimera was expressed diffusely on the plasma membrane. The β_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 β_1 chimera colocalized both with the fibronectin receptor at sites of extracellular fibronectin fibrils and with the vitronectin receptor at focal contacts. The β_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 β_1 chimeric protein behaves like a ligand-occupied receptor, even though the β_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 β_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 α and β 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–substratum adhesion. Integrin heterodimers can be classified into subfamilies based on the different β subunits. Different combinations of α and β 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–substratum 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 α-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 β_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 β_1 subunits and endogenous α subunits, it has been shown that the β intracellular domain is required for the localization of β_1 integrin receptors in focal contacts (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990), as well as its function in cell adhesion (Hayashi et al., 1990). However, it is still not known whether the β_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 α and β 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, $\alpha_2\beta_1$, 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, $\alpha_v\beta_5$, does not localize to focal contacts (Wayner 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)[1] receptor (gp55 subunit) as the extracellular and transmembrane domains combined with either the α_5 or β_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 α_5 and β_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 β_1 chimera colocalizes with ligand-occupied receptors, suggesting that a β_1 intracellular domain expressed in the absence of a corresponding α_5 intracellular domain functions as if it were a ligand-occupied receptor. Since our β_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 β_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 β_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 α 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 NaeI/XbaI fragment of the IL-2 receptor cDNA was then replaced with complementary oligonucleotides 1A/1B. Oligonucleotides encoding the β_1 and α_5 intracellular domains were then inserted between the new HindIII and XhoI sites.

Oligonucleotides 1A/1B:

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5′-GGCTGTGTTTTCCTGCTGATCAGCGTCCTCCTCCTGAGTGGGCTCACCT
3′-CCGACACAAAAGGACGACTAGTCGCAGGAGGAGGACTCACCCGAGTGGA
   GGAAGCTTTAACCCTGCCTCGAGT-3′
   CCTTCGAAATTGGGACGGAGCTCAGATC-5′
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Oligonucleotides 2A/2B and 3A/3B encode the β_1 intracellular domain.

Oligonucleotides 2A/2B:

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5′-AGCTTTTAATGATAATTCATGACAGAAGGGAGTTTGCTAAATTTGAAAA
     3′-AAATTACTATTAAGTACTGTCTTCCCTCAAACGATTTAAACTTTT
   GGAGAAAATGAATGCCAAATGGGACACGGGTGAAAATCC-3′
   CCTCTTTTACTTACGGTTTACCCTGT-5′
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Oligonucleotides 3A/3B:

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                  5′-TATTTATAAGAGTGCCGTAACAACTGTGGTCAATCC
3′-GCCCACTTTTAGGATAAATATTCTCACGGCATTGTTGACACCAGTTAGG
   GAAGTATGAGGGAAAATGACC-3′
   CTTCATACTCCCTTTTACTGGAGCT-5′
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Oligonucleotides 4A/4B and 5A/5B, encoding the α_5 intracellular domain lacking its HindIII site were inserted in plasmid SP72 (Promega, Madison, WI).

Oligonucleotides 4A/4B:

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5′-CCTCCTGCTCCTAGGTCTACTCATCTACATCCTCTACAAGCTGGGATTC
                GATCCAGATGAGTAGATGTAGGAGATGTTCGACCCTAAG
   TTCAAACGCTCCCTCCCATATGGCACCGCCATGGAAAAAGCTCAG
   AAGTTTGCGAGGGAGGGTATACCGTGGCGGTACCTTTTTCGAGTC
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Oligonucleotides 5A/5B:

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5′-CTGAAGCCTCCAGCCACCTCTGATGCCTGACC
3′-GACTTCGGAGGTCGGTGGAGACTACGGACTGGAGCT
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The α_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′-CTCTACAAGCTTGGATTCTTCAACG-3′
Reverse primer: 5′-CGTACCTCGAGGTCAGGCATCAGAGG-3′

Cells

Normal human foreskin fibroblasts were a generous gift from Dr. Steve Alexander (Bethesda Research Laboratories, Gaithersburg, MD) and were cultured in DME supplemented with 1 mM glutamine, 50 μg/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

1. *Abbreviation used in this paper*: IL-2, interleukin 2.

(Hayashi and Yamada, 1983; Zardi et al., 1985). The synthetic peptides GRGDS and GRGES were synthesized by Peninsula Laboratories, Inc. (Belmont, CA) and were purified by molecular sieve chromatography in 1 M HPLC-grade acetic acid on Bio-Gel P2 columns (Bio-Rad Laboratories, Richmond, CA). The peptide CAQLKPPATSDA was synthesized with an Applied Biosystems model 431A peptide synthesizer, purified using a C18 column, and conjugated to keyhole limpet haemocyanin (Pierce Chemical Co., Rockford, IL) with *m*-maleimidobenzoic acid-N-hydroxysuccinimide ester as described by Green et al. (1987).

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 β_1 intracellular domain has been described previously (Larjava et al., 1990). Polyclonal antibody 4318 to the α_5 intracellular domain was produced after immunization of rabbits with the peptide CAQLKPPATSDA as described previously for polyclonal antibody 4080 (Larjava et al., 1990). Rat mAb 11 to the α_5 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 α_5 subunit was further demonstrated by immunostaining mouse 3T3 cells (ATCC, Rockville, MD) transiently expressing a transfected full-length human α_5 cDNA (not shown). In addition, after preclearing ^{35}S-labeled cell lysates with polyclonal antibody 4318, no additional proteins were immunoprecipitated with mAb 11, and conversely, after preclearing cell lysates with mAb 11, no additional proteins were immunoprecipitated with polyclonal antibody 4318. Polyclonal antibody to the vitronectin receptor, $\alpha_v\beta_3$, 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 coverglasses 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 Nogueira 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 (Fuller and d'Albert, Inc., Fairfax, VA).

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 α_5 and β_1 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 × 10^5 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 $\alpha_5\beta_1$ 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.

(Method B) Cells were plated on laminin-coated coverslips at 1 × 10^5 cells per well and allowed to spread overnight in medium prepared with fibronectin-depleted serum, in the presence of 50 μg/ml mAb 333. Cycloheximide was added, and the cells were then preincubated for 2 h. The cells were then washed several times, fresh serum-free medium alone or containing 200 μg/ml of the 75-kD fragment of fibronectin was added, and the cells were incubated 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 α_5 intracellular domain or polyclonal antibody to $\alpha_v\beta_3$. 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 α_1 or β_1 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 α_5 and β_1 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

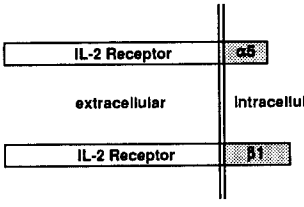
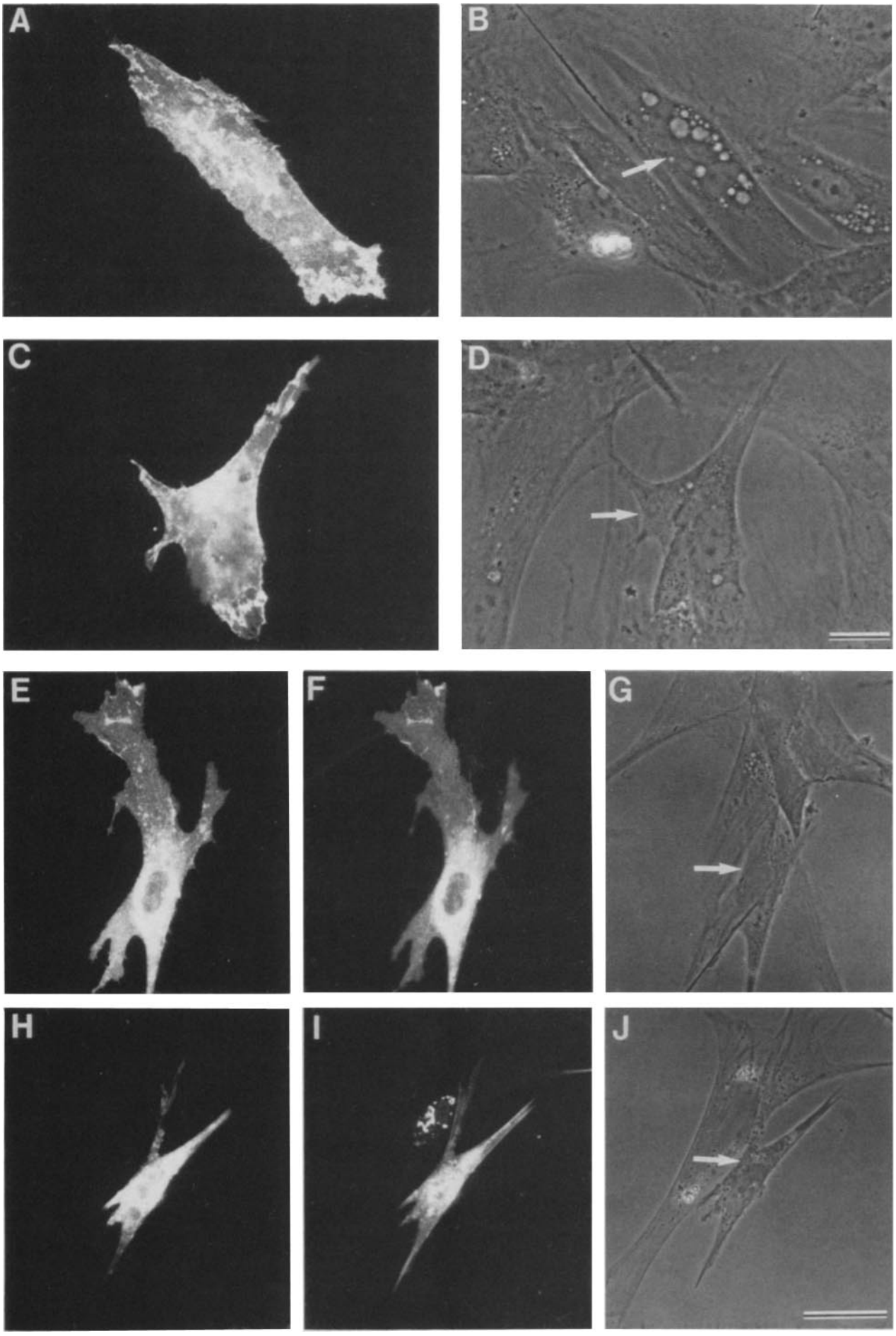


Figure 1. The α_5 and β_1 chimeras. Each chimera contained cDNA sequences of the IL-2 receptor gp55 subunit from the NH_2-terminus through Trp 259 at the end of the transmembrane domain (Leonard et al., 1984; Nikaido et al., 1984), and either the entire α_5 intracellular domain from Lys 1022 to the translational stop codon (Argraves et al., 1987) or the entire β_1 intracellular domain from Lys 753 to the translational stop codon (Argraves et al., 1987).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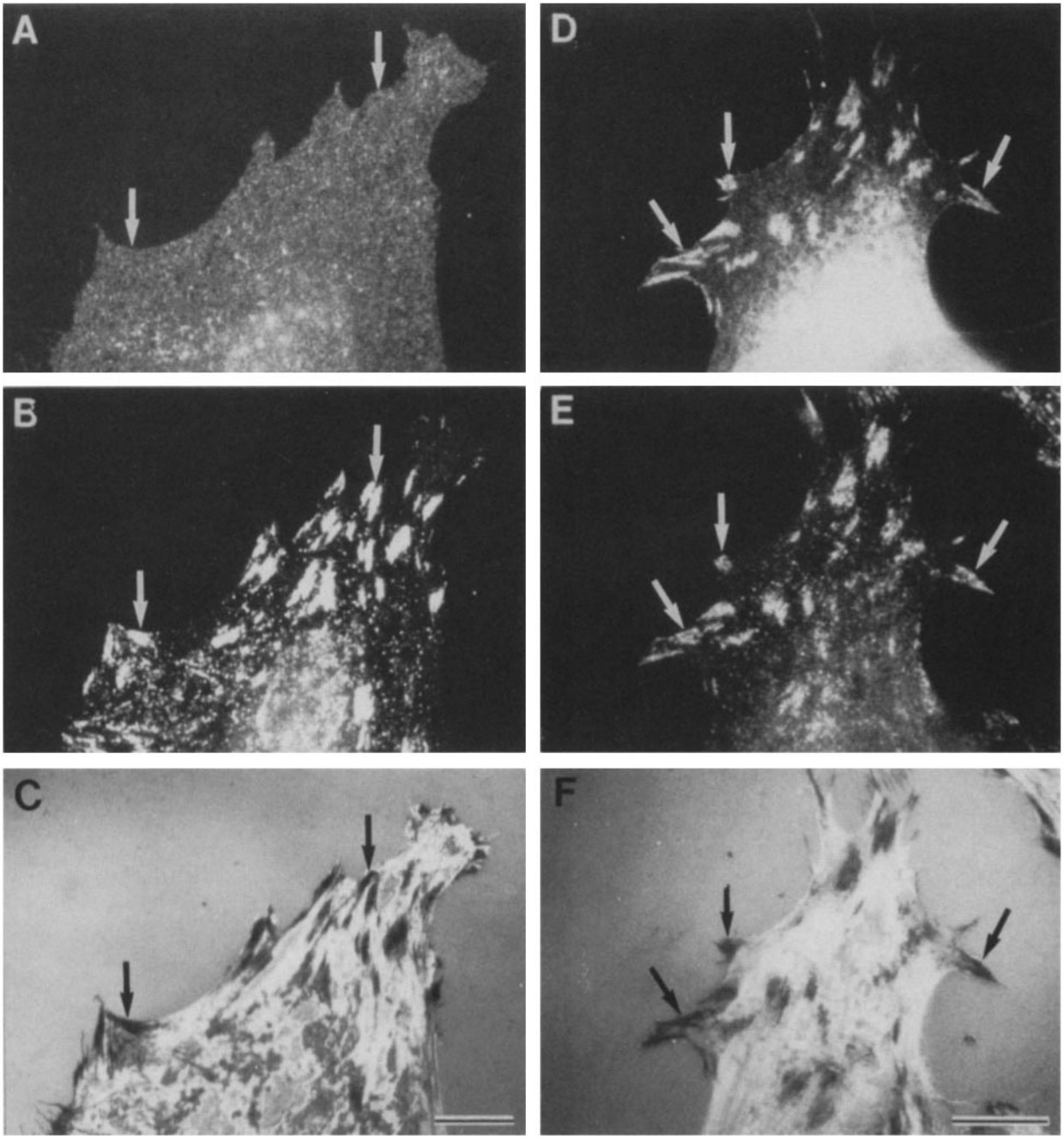

Figure 3. Codistribution of the β_1 chimera with the endogenous $\alpha_5\beta_1$ fibronectin receptor at focal contacts in normal human fibroblasts. 36 h after transfection with either the α_5 (*A–C*) or the β_1 (*D–F*) chimera, cells were pretreated with cycloheximide for 2 h, harvested, and then plated for 2 h on coverslips coated with 10 μg/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 μg/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 μm.

Figure 2. Expression of the transfected chimeras. Cell surface expression of the α_5 chimera (*A* and *B*) or the β_1 chimera (*C* and *D*) in normal human fibroblasts. Transfected nonpermeabilized cells stained with mAb RPN.512 to the IL-2 receptor at 7 μg/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 α_5 chimera, double immunostained with mAb to the IL-2 receptor (*E*) and polyclonal antibody 4318 to the α_5 intracellular domain at 1:100 dilution (*F*), or the β_1 chimera, double immunostained with mAb to the IL-2 receptor (*H*) and polyclonal antibody 4080 to the β_1 intracellular domain at 1:100 dilution (*I*), are shown by immunofluorescence, or indicated by arrows in phase contrast (*G* and *J*). Bars, 20 μm.

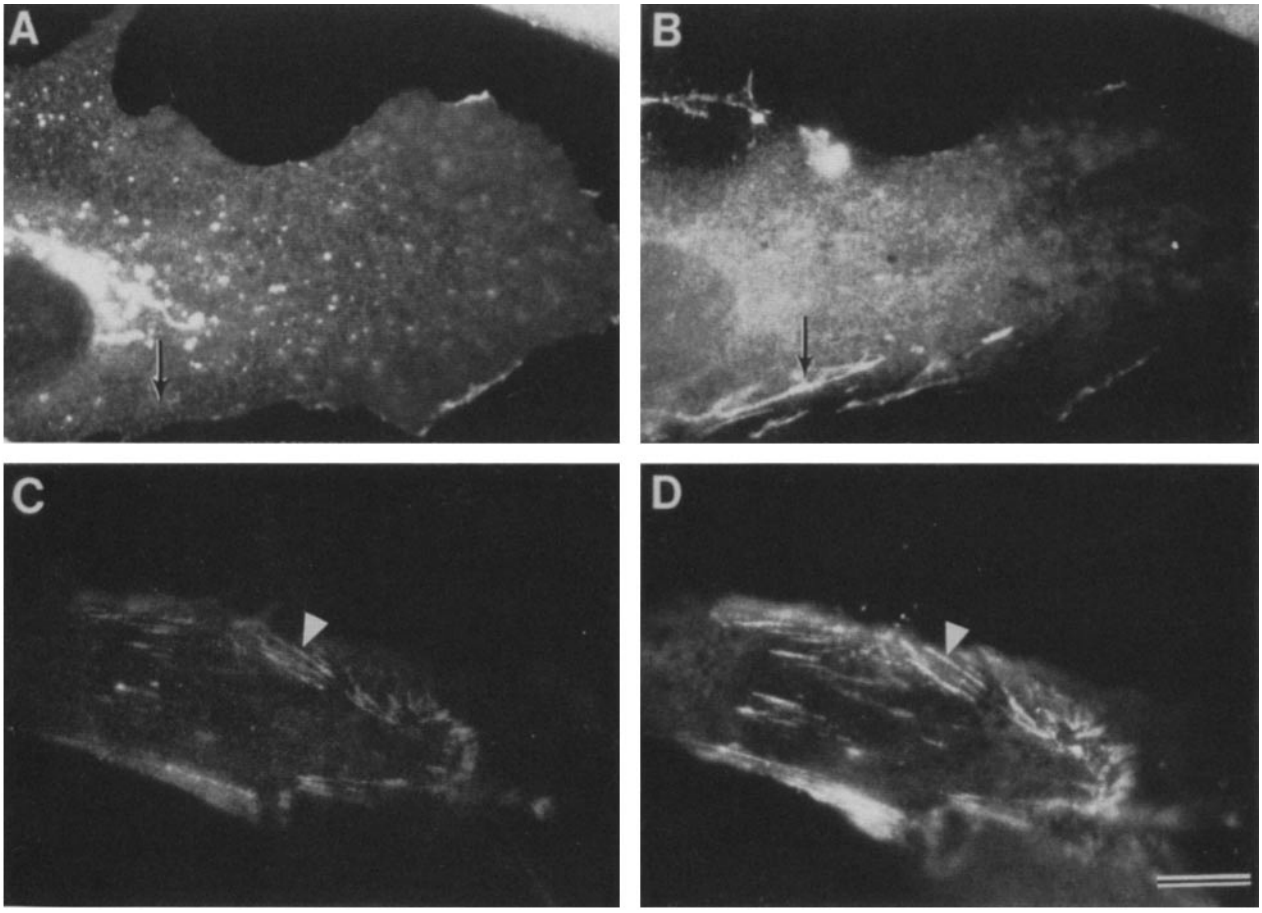

Figure 4. Codistribution of the β_1 chimera with the endogenous $\alpha_5\beta_1$ fibronectin receptor at sites of plasma membrane-fibronectin fibril association. Normal human fibroblasts transfected with either the α_5 (*A* and *B*) or the β_1 (*C* and *D*) chimera were plated on coverslips in serum 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 β_1 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 α_5 chimera with the endogenous fibronectin receptor at these sites. Bar, 10 μm.

the α_5 and β_1 intracellular domains were correctly translated. Polyclonal antibodies 4080 and 4318 recognize the human β_1 and α_5 intracellular domains respectively. The polyclonal antibody to the human α_5 intracellular domain showed only weak staining of endogenous avian receptors (untransfected cells in Fig. 2 *F* and our unpublished observations). The human α_5 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 α_5 intracellular domain (Fig. 2 *F*). Although the human and avian β_1 intracellular domains are identical, cells transiently expressing the β_1 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 β_1 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 $\alpha_5\beta_1$ fibronectin receptors concentrate in focal contacts (Singer et al., 1988). To establish whether either the α_2 or β_1 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 α_5 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 β_1 chimera, however, localized to focal contacts in patterns similar to those of endogenous receptors (Fig. 3, *D–F*), indicating that the β_1 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 $\alpha_5\beta_1$ 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 α_5 or β_1 intracellular domain alone was sufficient to target the chimeric receptors to sites of cell contact with fibronectin

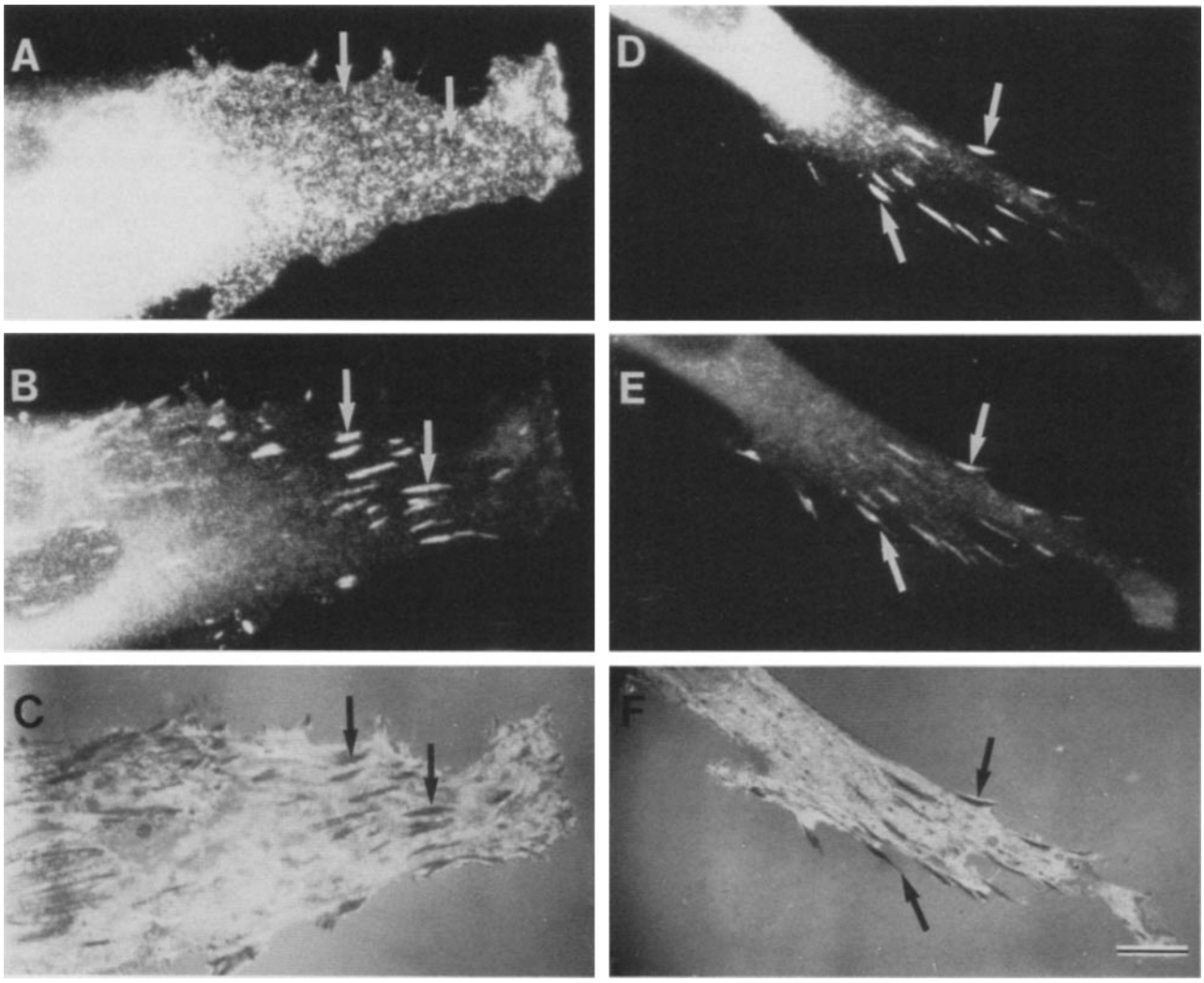

Figure 5. Codistribution of the β_1 chimera with the vitronectin receptor at focal contacts in normal human fibroblasts spread in serum. Cells transfected with either the α_5 (*A–C*) or the β_1 (*D–F*) chimera were plated in serum for 36 h and then double immunostained with mAb to the IL-2 receptor (*A* and *D*) and polyclonal antibodies to the vitronectin receptor at 1:50 dilution (*B* and *E*), with the same regions of the cell shown by interference reflection microscopy (*C* and *F*). Focal contacts are indicated by arrows. Bar, 10 μm.

fibrils, human fibroblasts transiently transfected with the chimeric receptors were plated on coverslips in serum-containing medium for 36 h. The β_1 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 β_1 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 α_5 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 β_1 chimera, it did not colocalize with the vitronectin receptor at focal contacts (Fig. 5, *A–C*). This diffuse expression pattern of the α_5 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 α_5 chimera, it also showed diffuse membrane staining, indicating that the α_5 chimera lacked sufficient information for receptor localization (not shown). However, the α_5 intracellular domain may act as a negative element in the heterodimeric unoccupied receptor suppressing the propensity for receptor redistribution to focal contacts driven by β_1 intracellular domain. Ligand occupancy may release this constraint.

Redistribution of the $\alpha_5\beta_1$ Fibronectin Receptor by Ligand Occupancy

Since our β_1 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 β_1 intracellular domain expressed in the absence of an α_5 intracellular domain may

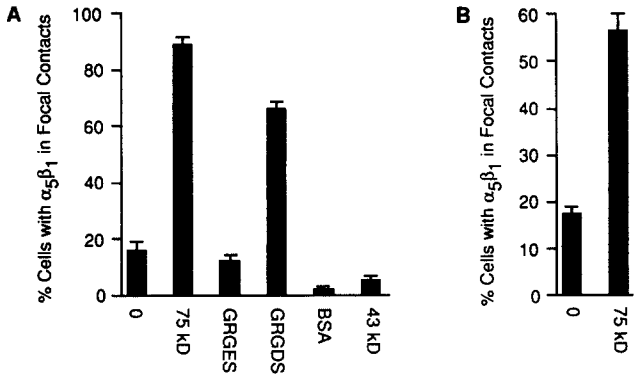

Figure 6. Redistribution of the $\alpha_5\beta_1$ fibronectin receptor by ligand occupancy. (*A*) Normal human fibroblasts pretreated as described in Materials and Methods were incubated on laminin-coated coverslips for 90 min. The cells were incubated an additional 60 min with fresh medium alone or 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 $\alpha_5\beta_1$ 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.

interact with the cytoskeleton in a manner similar to an occupied receptor, suggesting that occupied receptors may concentrate at focal contacts not only because they bind ligand absorbed to the substrate, but also because they have an enhanced ability to interact with cytoskeletal proteins at these sites. If receptor distribution is regulated by the receptor's ability to interact with the cytoskeleton rather than by the availability of substrate-bound ligand, then one would predict that receptor distribution should change by the addition of even a soluble ligand. This prediction was tested by allowing cells to spread on laminin in serum-free medium for 90 min, after which most fibronectin receptors were found diffusely distributed in the plasma membrane. The 75-kD cell-binding fragment of fibronectin was then added and the cells were incubated for an additional 1 h. Redistribution of diffuse fibronectin receptors to focal contacts was evaluated by immunofluorescence and interference reflection microscopy. As shown in Fig. 6 *A*, occupancy with the 75-kD fragment resulted in a redistribution of diffuse $\alpha_5\beta_1$ fibronectin receptors to focal contacts. Incubation with the small peptide GRGDS containing the fibronectin RGD adhesion sequence (Ruoslahti and Pierschbacher, 1987; Yamada, 1991) also resulted in the redistribution of diffuse fibronectin receptors to focal contacts, whereas the control peptide GRGES, BSA, and the 43-kD collagen-binding fragment of fibronectin did not.

To determine whether the redistribution of $\alpha_5\beta_1$ occurs in cells plated for longer periods of time with more mature focal contacts, we plated cells on laminin substrates for 15 h in fibronectin-depleted medium in the presence of sufficient amounts of mAb 333 to block the interaction of endogenous fibronectin with the fibronectin receptor. This pretreatment resulted in a diffuse distribution of the fibronectin receptor 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. 6 *B* 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 $\alpha_v\beta_3$ 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 α_5 and β_1 intracellular domains of the fibronectin receptor can function as separate domains, we constructed chimeric receptors containing either the α_5 or the β_1 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 β_1 intracellular domain, and (*b*) analyze the role of ligand occupancy in the regulation of integrin receptor distribution.

We found that the β_1 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 β_1 intracellular domain in receptor localization, but they also establish that the β_1 intracellular domain is not only required, but is in fact sufficient by itself to target receptor localization to focal contacts. In addition, since the α_5 chimera was expressed relatively diffusely on the cell surface, we can further conclude that the α_5 intracellular domain is not sufficient to determine receptor localization. However, we cannot rule out the possibility that the α_5 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 α_{IIb} intracellular domain of the platelet receptor, $\alpha_{IIb}\beta_3$, functions in the regulation of ligand binding affinity, and the α_5 intracellular domain cannot substitute for the α_{IIb} intracellular domain in this regulation (O'Toole et al., 1991).

The localization of the β_1 chimera to focal contacts on cells plated on a fibronectin substrate suggests that it binds

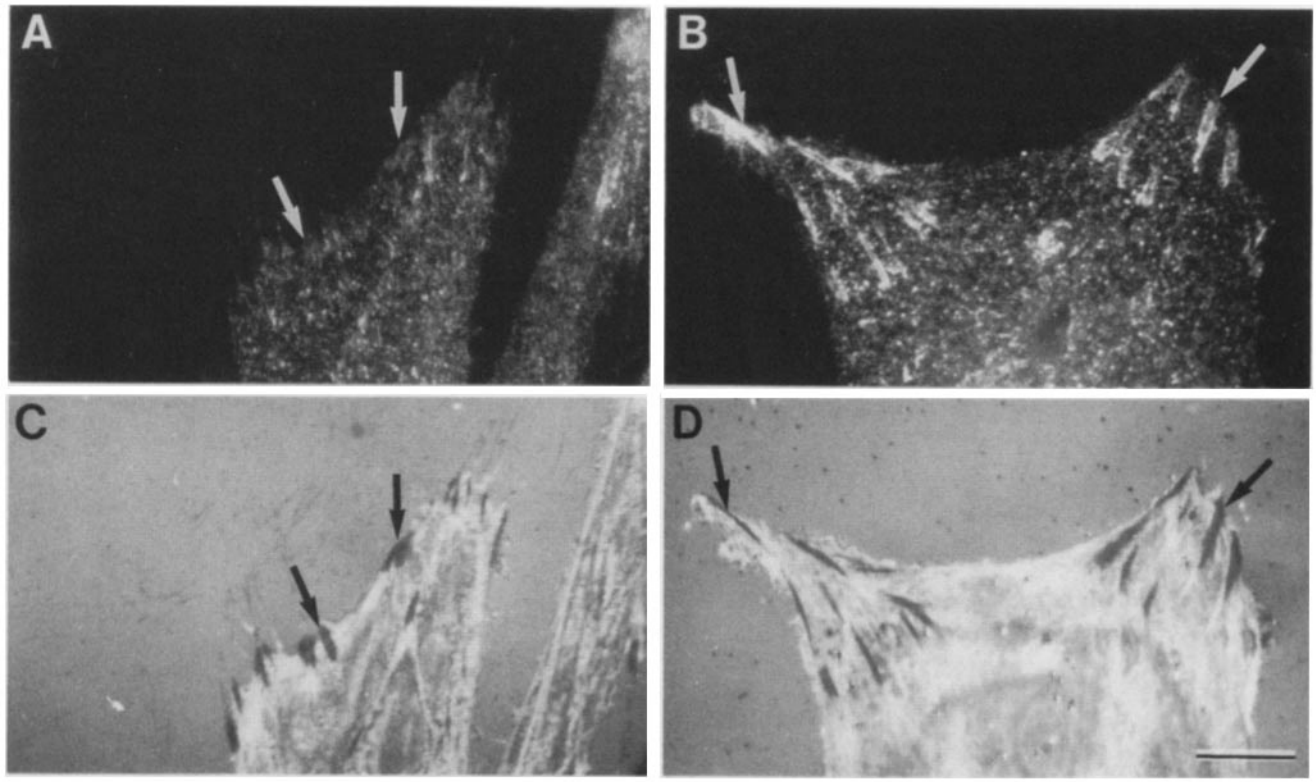

Figure 7. Redistribution of $\alpha_5\beta_1$ by ligand occupancy as shown by immunofluorescence. Normal human fibroblasts were incubated for 15 h on laminin-coated coverslips, and then incubated an additional 1 h with fresh medium alone (*A* and *C*) or with 200 μg/ml 75-kD cell binding fragment (*B* and *D*). Cells stained with polyclonal antibody 4318 to the α_5 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 β_1 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 β_3 and β_1 intracellular domains may bind to the same cytoskeletal protein, since the β_3 and β_1 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 β_3 intracellular domain can substitute for the β_1 intracellular domain in heterodimeric fibronectin receptor localization and function (Solowska et al., 1991).

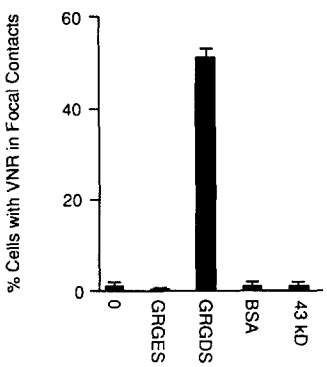

Figure 8. Redistribution of vitronectin receptors by ligand occupancy. Normal human fibroblasts pretreated as described in Materials and Methods were incubated on laminin-coated coverslips for 90 min and then with either fresh medium alone or with either 500 μg/ml GRGES or GRGDS, or either 100 μg/ml BSA or the 43-kD collagen-binding fragment of fibronectin for an additional 60 min. The percent cells with vitronectin receptors in focal contacts was determined as described in Materials and Methods.

The major cytoplasmic difference between the β_1 chimera and the endogenous fibronectin receptor is the absence of the α_5 intracellular domain in the β_1 chimera. This fact taken together with colocalization of the β_1 chimera with endogenous ligand-occupied receptors, suggests that the α_5 intracellular domain may inhibit the ability of the β_1 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 β_1 intracellular domain or regions of the intracellular domain that interact with specific cytoskeletal proteins. The idea that the context of the β_1 intracellular domain may affect its affinity for specific cytoskeletal proteins is supported by in vitro binding studies in which the β_1 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 β_1 intracellular domain may be affected by the α_5 intracellular domain. However, an alternative possibility is that ligand binding, instead of unmasking the β intracellular domain, changes the conformation of the β intracellular domain into a higher affinity or "active" conformation capable of interacting with the cytoskeleton, and that our β_1 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 $\alpha_5\beta_1$ 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; Roman 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.

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