Antibodies to the Conserved Cytoplasmic Domain of the Integrin β_1 Subunit React with Proteins in Vertebrates, Invertebrates, and Fungi

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Abstract. The integrin family of cell surface receptors can be divided into three groups on the basis of their homologous β subunits: β_1, β_2, and β_3. We have raised an antibody against a synthetic peptide corresponding to the COOH-terminal domain of the chicken integrin β_1 subunit that reacts with β subunits from a variety of vertebrates, invertebrates, and fungi, demonstrating strong evolutionary conservation of sequences in this domain. In *Drosophila* cells, the antibody recognizes integrin αβ complexes that appear to be identical with position-specific antigens. Cross-reactive proteins are also detected in *Caenorhabditis elegans* and *Candida albicans*. The antiserum is specific for β_1 subunits and does not recognize other integrin β subunits in humans. In immunofluorescence analyses of cultured cells, the antibody reacts only with permeabilized cells confirming that this highly conserved COOH-terminal segment is a cytoplasmic domain.

INTEGRINS comprise a large family of cell surface receptors which function in cell-cell and cell-matrix adhesion in many cell types (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). These receptors clearly participate in a wide variety of processes, including cellular differentiation and migration in development, as well as thrombosis, wound healing, and leukocyte helper and killer functions.

The structures of integrins have been deduced by cDNA sequence and immunological analysis (Tamkun et al., 1986; Argraves et al., 1987; Suzuki et al., 1987; Kishimoto et al., 1987; Law et al., 1987; Poncz et al., 1987; Fitzgerald et al., 1987; Hemler et al., 1987; DeSimone and Hynes, 1988). These receptors consist of transmembrane, noncovalently linked heterodimers with distinct α and β subunits. There are at least three different integrin β subunits and 10 different α subunits. For simplicity, we have divided the integrins into three classes on the basis of their component β subunits (Hynes, 1987). The β_1 class includes the chicken integrin complex (Horwitz et al., 1985), the human fibronectin receptor (Pytela et al., 1985*a*), and the very late antigens (VLAs).[1] The VLAs, which are present on a wide variety of human cells, were initially discovered on activated human T cells (Hemler et al., 1985) and consist of heterodimers of five distinct α subunits each associated with a common β subunit (Hemler et al., 1987). The β_2 class appears to be leukocyte specific and includes the heterodimers LFA-1, Mac-1, and p150,95 (Anderson and Springer, 1987). Finally the β_3 class includes the human vitronectin receptor and platelet glycoprotein IIb/IIIa (Pytela et al., 1986; Ginsburg et al., 1987).

All three classes of β subunits have a large extracellular domain with a total of 56 cysteine residues including four cysteine-rich repeats. This high cysteine content may account for the shift in electrophoretic mobilty of the β subunit to a higher apparent molecular mass on SDS-PAGE when reducing agent is present. The α subunits of integrins have a large extracellular domain with a series of metal-binding domains (Argraves et al., 1987; Suzuki et al., 1987; Poncz et al., 1987) and, in some cases, consist of two disulfide-linked polypeptides, termed a heavy and a light chain (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). This structure accounts for the decrease in apparent molecular weight of many α subunits on SDS-PAGE when reducing agent is included. Different α/β combinations have different ligand specificities, interacting via their extracellular domains with a wide variety of ligands, including extracellular matrix molecules such as fibronectin (Pytela et al., 1985*a*; Gardner and Hynes, 1985; Akiyama et al., 1986), laminin (Horwitz et al., 1985), collagen types I and III-VI (Wayner and Carter, 1987), vitronectin (Pytela et al., 1985*b*), von Willebrand factor, and fibrinogen (Pytela et al., 1986), as well as the complement component C3bi (Wright et al., 1987).

There is a single, strongly hydrophobic region in each of the α and β subunits of integrin, preceding short, hydrophilic COOH-terminal (putative cytoplasmic) domains. These domains are thought to interact with the cytoskeleton, consis-

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; KLH, keyhole limpet hemocyanin; sulfo-MBS, *m*-maleimidobenzoylsulfosuccinimide ester; VLA, very late antigen.

tent with the coalignment of matrix and cytoskeletal proteins with integrins (Damsky et al., 1985; Chen et al., 1985). Within the COOH-terminal domain of the β_1 subunit, there is a putative site of tyrosine phosphorylation (Tamkun et al., 1986). Indeed, the β_1 subunit of Rous sarcoma virus–transformed chicken cells has been shown to be phosphorylated on tyrosine (Hirst et al., 1986). The COOH-terminal segments of integrin β subunits are remarkably well conserved in sequence among vertebrates (DeSimone and Hynes, 1988).

To study the function of the integrin β_1 COOH-terminal domain, we have used a synthetic peptide comprising this putative cytoplasmic domain to generate a domain-specific antiserum. We have used this antiserum to verify the cytoplasmic location of this domain and we report that the antiserum reacts with proteins from vertebrates, invertebrates, and fungi, suggesting the widespread occurrence of integrin receptors.

Materials and Methods

Cells

Chicken embryo fibroblasts (CEFs) were prepared from day-11 embryos (Spafas, Inc., Norwalk, CT) as described (Rein and Rubin, 1968). Cells were grown in DME plus 5% FCS (Gibco, Grand Island, NY) and used between passages 3 and 6. The Nil8 hamster cell line (Mautner and Hynes, 1977) was cultured in DME with 5% FCS. MG-63 human osteosarcoma cells (Billiau et al., 1977) were grown in DME with 10% FCS. Human platelets were isolated from citrated platelet-rich plasma as described by Lawler et al. (1982). Human peripheral blood monocytes were isolated as described by Wright and Silverstein (1982) and were kindly provided by L. Van De Water (Beth Israel Hospital, Boston, MA). S-2 cells were grown in Schneider medium with 10% FCS and were kindly provided by M. L. Pardue (Massachusetts Institute of Technology [M.I.T.], Cambridge, MA). *Caenorhabditis elegans* adults and embryos were kindly provided by L. Miller and B. Meyer (M.I.T.). Membranes from *Saccharomyces cerevisiae* (strain CHS1) and *Candida albicans* (strain 73/055) were prepared as described (Orlean, 1987) and were generously provided by J. Au-Young (M.I.T.). Extracts of *C. elegans* and day-11 chicken embryos were prepared as described by Knudsen et al. (1985).

Immunoprecipitation

Cells were labeled with ^{125}I and lactoperoxidase (Sigma Chemical Co., St. Louis, MO) either in suspension or as a monolayer as described (Hynes, 1973). 10^7 cells and 1.0 mCi/ml were used per experiment. Cells were washed three times with PBS (phosphate-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$), resuspended in 1 ml of extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM $CaCl_2$, 0.5% NP-40), and incubated for 15 min on ice, then sedimented for 10 min at 10,000 *g*. The supernatant was preincubated with 50 μl of protein A–Sepharose for 5 min and the beads sedimented for 2 min at 10,000 *g*. The resulting supernatant was used for immunoprecipitation. On average 5×10^6 TCA-precipitable counts were used per sample.

In some experiments, the integrin complex was denatured: 1/20 vol of 20% SDS was added, followed by boiling for 3 min. After cooling, a fivefold excess of Triton X-100 was added, and this sample was then treated as the nondenatured ones.

2 μl of primary antiserum was added and incubated for 1 h at 4°C. 30 μl of protein A–Sepharose was added and the samples were rotated overnight at 4°C. After spinning the sample for 2 min at 10,000 *g*, the beads were washed four times with 1 ml extraction buffer, followed by elution with sample buffer.

Production of Anti-peptide Antibodies

100 mg of crude 39-mer (Fig. 1) was synthesized by Peninsula Laboratories, Inc. (Belmont, CA). The synthesis was monitored by HPLC and amino acid analysis of the products. The peptide was coupled to keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., La Jolla, CA) using *m*-maleimidobenzoylsulfosuccinimide ester (sulfo-MBS) (Pierce Chemical Co., Rockford, IL) essentially as described (Kitagawa and Aikawa, 1976). KLH was dialyzed against 10 mM KPO_4, pH 7.0, and sedimented at 10,000 *g* for 10 min. 6.2 mg of KLH in 1 ml KPO_4 was mixed with 0.24 ml of sulfo-MBS (5 mg/ml stock) and stirred for 15 min at 4°C. The reaction mixture was desalted using a 10 × 0.5-cm G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ) with 100 mM KPO_4, pH 6.0. Protein-containing fractions were pooled and incubated with 15 mg of peptide. The reaction mixture was adjusted to pH 7.0 with NaOH and stirred overnight at 4°C, then quenched with 0.5 M ethanolamine, pH 8.0, for 1 h. This mixture was dialyzed against NH_4HCO_3 and lyophilized.

Trace amounts of the peptide ^{125}I labeled with Bolton-Hunter reagent (2,000 Ci/mmol; New England Nuclear, Boston, MA and Dupont Co., Wilmington, DE; Bolton and Hunter, 1973) were added to monitor coupling efficiency, with a 90% recovery of input counts. For injections, 200 μg of peptide conjugate in PBS was mixed with 1 ml of complete Freund's adjuvant (Gibco) by sonication and was injected subcutaneously at multiple sites along the dorsal midline of rabbits. At 3–4-wk intervals, booster injections of 100 μg of protein in incomplete adjuvant were given. Significant antibody titers were observed after two boosts. This response contrasts with the poor one obtained when a 10–amino acid peptide corresponding to the tryptic peptide containing the dotted tyrosine in Fig. 1 was used.

Other Antibodies

Rabbit anti-IIB was prepared as described (Gardner and Hynes, 1985). Rabbit antisera to the β subunit of LFA-1 and to native Mac-1 complex were kindly donated by T. K. Kishimoto and T. Springer (Dana-Farber Cancer Institute, Boston, MA). Monoclonal anti-VLA β ascites was a gift of M. Hemler (Dana-Farber Cancer Institute). CSAT antibody was a gift of C. Buck (Wistar Institute, Philadelphia, PA) and R. Horwitz (University of Pennsylvania, Philadelphia, PA) and monoclonal anti-PS-3 conjugated to agarose was a gift of M. Wilcox (Laboratory of Molecular Biology, Cambridge, England) and N. Brown (Harvard University, Cambridge, MA).

Gel Electrophoresis and Immunoblotting Analysis

SDS-PAGE was performed by the method of Laemmli (1970). Separation gels were 7.0% acrylamide with a 3.0% stacking gel. Samples were prepared in sample buffer (5% SDS, 100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 10% glycerol, and bromphenol blue) and boiled for 3 min. Reduced sample buffer contained 50 mM dithiothreitol (DTT). The following were used as reduced molecular mass markers: myosin (M_r 200,000), beta-galactosidase (M_r 116,000), phosphorylase B (M_r 97,000), and BSA (M_r 66,000).

In immunoblotting experiments, polypeptides were transferred from the gels to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH; BA83) using a Hoeffer transfer apparatus for 1,600 mA/h in 20 mM Tris-HCl, pH 6.8 (Towbin et al., 1979). After transfer, strips were cut and washed three times with buffer A (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20), then blocked with 5% BSA (fraction V) in buffer A for 30 min at 25°C. Strips were then incubated with primary antisera at a 1:100 dilution in buffer A for 1 h at room temperature, washed three times with buffer A, and stained using the Vecta-stain kit (Vector Laboratories, Inc., Burlingame, CA) as per instructions, using 4-chloro-1-naphthol as the enzyme substrate.

Immunofluorescence

Nil8 fibroblasts were stained using minor modifications of the method of Mautner and Hynes (1977) with 0.5% NP-40 used to permeabilize cells. The cells were plated on coverslips and grown in DME with 0.3% FCS to allow maximum spreading.

CEFs were plated for 2 h on coverslips previously coated with human plasma fibronectin, which was purified as described (Engvall and Ruoslahti, 1977). These cells were used either live or after fixation. For fixation, cells were rinsed twice in PBS and fixed for 15 min in a freshly prepared 4% solution of paraformaldehyde (Fluka Chemical Co., Bern, Switzerland) in PBS, rinsed, and permeabilized with 0.5% NP-40 in PBS for 15 min at room temperature. Fixed and live cells were stained as described (Mautner and Hynes, 1977) except for washing and incubation with DME instead of PBS. After the final wash, live cells were fixed with 4% paraformaldehyde for 15 min. Coverslips were examined using a Zeiss Photo-III microscope and photographed using Kodak Tri-X film.

Results

Production of Anti-peptide Antisera

To study the COOH-terminal (putative cytoplasmic) domain

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                                                *
XENOPUS  BETA-1          HDRREFAKFEKEKMNAKWDTGENPIYKSAVaTVVNPKYEGK
XENOPUS  BETA-1*         HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
CHICKEN  BETA-1          HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
HUMAN    BETA-1          HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK

HUMAN    BETA-2          sDIREyrrFEKEKIksqWn-ndNPIfKSAtTTVmNPKfaes
HUMAN    BETA-3          HDRkEFAKFEeErarAKWDTanNPIYKeAtsTftNItYrGt
                                                *
PEPTIDE                    CREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
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Figure 1. Conservation in COOH-terminal sequence of vertebrate integrin β subunits. COOH-terminal amino acid sequences derived from cDNA sequences of a number of integrin β subunits are shown in the single letter code. Uppercase letters indicate residues conserved in all or most β_1 subunits, while lowercase letters indicate differences from the conserved β_1 sequence. The asterisk marks the tyrosine residue proposed to be phosphorylated in chicken integrin β_1. The sequence of the synthetic peptide is shown at the bottom. Sources of the sequence data are as follows: chicken β_1 (Tamkun et al., 1986); *Xenopus* β_1 and β_1* (DeSimone and Hynes, 1988); human β_1 (Argraves et al., 1987); human β_2 (LFA-1 β; Kishimoto et al., 1987); and human β_3 (IIIa; Fitzgerald et al., 1987).

of the integrin β_1 subunit, we generated an antiserum against a peptide comprising the bulk of this domain which was coupled to KLH via an amino-terminal cysteine (Fig. 1). The synthetic peptide sequence is highly conserved among vertebrate integrins and contains the putative site of tyrosine phosphorylation.

When an extract of ^{125}I surface-labeled CEFs was immunoprecipitated using this anti-peptide antiserum, multiple bands corresponding to the chicken integrin complex were recovered (Fig. 2, lane *b*). Unlabeled peptide completely blocked this precipitation, but had no effect on the recovery of the integrin complex when the mAb CSAT was used (Fig. 2, lane *c* and *e*). When the extract was denatured by heating in SDS, only the β subunit was immunoprecipitated using the anti-peptide antiserum (Fig. 2, lane *d*).

Since cDNA sequence analysis has shown that the COOH termini of the β_1 subunits of chicken, *Xenopus*, and humans are virtually identical (DeSimone and Hynes, 1988), we would expect that an antibody to this domain would react with integrin β_1 subunits of these and other vertebrate species. In Fig. 2, extracts of surface-labeled hamster cells (Nil8) and human osteosarcoma cells (MG-63) were immunoprecipitated. The anti-peptide antiserum specifically immunoprecipitated α/β heterodimers from both species (Fig. 2, lanes *g* and *k*). Again, only the β subunit was recovered by immunoprecipitation from denatured extracts (Fig. 2, lanes *i* and *m*) while no integrin bands were observed after immunoprecipitation using either preimmune serum or anti-peptide antiserum with competing peptide added (Fig. 2, lanes *f*, *h*, *j*, and *l*). These results confirm the specific reactivity of this anti-peptide antiserum with β subunits from several vertebrates and demonstrate coprecipitation of several different α subunits with these β subunits. Fig. 2 also shows that the human integrin heterodimers recovered with anti-peptide antiserum comigrate with those imunoprecipitated with monoclonal anti-VLA β (Fig. 2, lanes *k* and *n*), confirming the previous conclusion that the VLA β subunit is similar or identical to integrin β (Takada et al., 1987).

β_1 Specificity

Since the COOH-terminal domains of the three classes of β subunits—β_1, β_2, and β_3—are homologous, with many conservative substitutions (see Fig. 1) we investigated whether the antibodies directed against the β_1 COOH terminus would also react with β_2 or β_3. For the analysis of β_1 vs. β_2, extracts of surface-labeled human monocytes (Fig. 3, lanes *a–i*) were immunoprecipitated either with anti-peptide antiserum or with antisera directed against either the β_2 subunit or native Mac-1. The results show that the anti-peptide antiserum immunoprecipitated only β_1 and associated α subunit (Fig. 3, lanes *a* and *g*), while β_2 and associated α subunits (p150 and Mac-1) were precipitated with the appropriate antisera (Fig. 3, *c*, *d*, *h*, and *i*). The β subunit precipitated with the anti-peptide antiserum (β_1) showed a characteristic shift in electrophoretic mobility upon reduction while the β_2 subunit showed only a minor shift in mobility (cf. Fig. 3, lanes *a*, *c*, and *d* with lanes *g–i*).

For the analysis of β_1 vs. β_3, extracts of surface-labeled human platelets were immunoprecipitated with various antibodies (Fig. 3, lanes *j–n*). Anti-IIb immunoprecipitated IIb complexed with IIIa (β_3) (Fig. 3, lane *j*), while anti-peptide antiserum and anti-VLA β immunoprecipitated β_1 complexes with no β_3 seen (Fig. 3, *k* and *n*). Immunoprecipitation of denatured extracts with the anti-peptide antiserum yielded only β_1 (lane *m*). The specificity of this antiserum is again seen by the absence of integrin in immunoprecipitates recovered with preimmune or immune serum plus com-

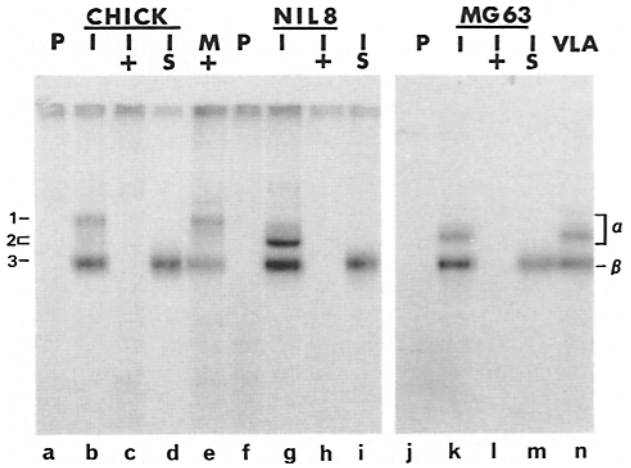

Figure 2. Immunoprecipitation of integrins from chicken, hamster, and human cells. Extracts of ^{125}I surface-labeled CEFs (lanes *a–e*), Nil8 hamster cells (lanes *f–i*), and MG63 human osteosarcoma cells (lanes *j–n*) were incubated with preimmune (*P*) serum (lanes *a*, *f*, and *j*); immune (*I*) anti-peptide antiserum (lanes *b*, *g*, and *k*); anti-peptide antiserum with 10 μg of (+) peptide added (lanes *c*, *h*, and *l*); anti-peptide antiserum after SDS (*S*) denaturation of the extracts (lanes *d*, *i*, and *m*); CSAT mAb (*M*) plus peptide (lane *e*), or anti-VLA beta antibody (lane *n*). The samples were immunoprecipitated using protein A–Sepharose and analyzed by SDS-PAGE (nonreduced) followed by autoradiography of the dried gels. β subunits are specifically precipitated from all three cell lines, whereas α subunits are precipitated only under nondenaturing conditions.

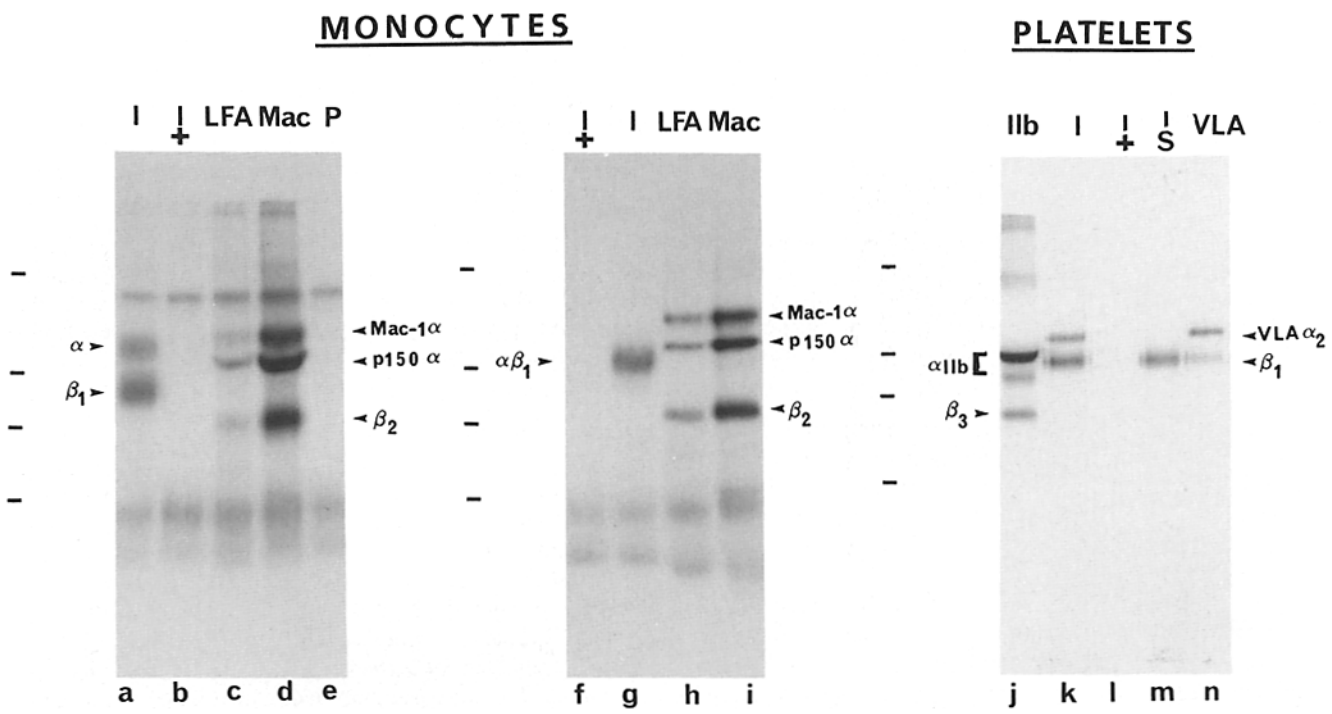

Figure 3. Immunoprecipitation of integrins from human monocytes and platelets. Extracts of ^{125}I surface-labeled monocytes (lanes *a–i*) or platelets (lanes *j–n*) were incubated with (*I*) anti-peptide antiserum (lanes *a*, *g*, and *k*); anti-peptide antiserum with 10 μg of peptide (*I+*) added (lanes *b*, *f*, and *l*); anti-peptide antiserum after SDS denaturation (*S*) of the extracts (lane *m*); preimmune (*P*) serum (lane *e*); anti-LFA beta antiserum (lanes *c* and *h*); anti-native Mac-1 antiserum (lanes *d* and *i*); anti-IIb antiserum (lane *j*); or anti-VLA antibody (lane *n*). The samples were immunoprecipitated using protein A–Sepharose and analyzed by SDS-PAGE under nonreduced (lanes *a–e* and *j–n*) or reduced (lanes *f–i*) conditions followed by autoradiography of the dried gels. β_1 subunits are precipitated by the anti-peptide antiserum from both cell types, together with associated α subunits. β_2 and β_3 subunits and their associated α subunits are precipitated by the relevant antisera but not by the anti-peptide antiserum. The lower band of the pair marked αIIb in lane *j* is probably a degradation product of glycoprotein IIb. Molecular mass markers (200, 116, 97, 66 kD) are shown by bars on the left.

peting peptide (Fig. 3, lanes *b*, *e*, *f*, and *l*). Thus, there was no detectable cross-reactivity of the anti-β_1 peptide antiserum with β_2 or β_3 and this antiserum is specific for the β_1 class in humans and can be used to identify β_1 integrins in many tissues and species.

Invertebrate Integrins

Since we had observed cross-reaction of integrin β_1 cDNA probes with genomic DNAs of several invertebrate species (DeSimone and Hynes, 1988), we investigated whether the anti-β_1 antiserum would react with proteins of invertebrates. In the case of *Drosophila melanogaster* it had also been suggested that the position-specific antigens (PS1, PS2, and PS3) are related to vertebrate integrins (Leptin et al., 1987). Extracts of surface-labeled S-2 *Drosophila* cells were immunoprecipitated using the anti-integrin peptide antiserum and monoclonal anti-PS-3. Both antibodies immunoprecipitated αβ heterodimers, which showed characteristic shifts in mobility upon reduction (Fig. 4, lanes *b*, *d*, *f*, and *h*). Integrins were absent after immunoprecipitation with immune serum plus peptide (Fig. 4, lanes *c* and *e*) and the anti-peptide antiserum precipitated only the smaller (β) subunits from SDS-denatured extracts (data not shown). The two β-related bands seen under reducing conditions (Fig. 4, lanes *f* and *h*) were a consistent finding in repeated experiments with both the anti-peptide antiserum and anti-PS-3 and presumably are due to proteolysis. The immunologic identity of the bands recovered with the anti-peptide antiserum and with the anti-PS-3 antibody was proven by preclearing experiments, in which either antibody could remove all of the antigen (data not shown).

The striking cross-reactivity between the anti-peptide antiserum and *Drosophila* integrin led us to investigate other species as well. Immunoblotting experiments demonstrated cross-reactivity in embryo extracts of the nematode *C. elegans*. Two bands were observed at apparent molecular masses of 120 and 70 kD, respectively, both of which could be completely competed with added peptide (Fig. 5, lanes *a* and *b*). These two bands comigrated with two immunoreactive bands observed in chicken embryo extract (Fig. 5, lane *c*). The lower molecular mass band (M_r 70,000) seen in both cases is probably a degradation product since a similar band is seen in chicken embryo extracts with many anti-chicken β_1 antibodies and it shifts in electrophoretic mobility to a higher apparent molecular mass upon reduction. Furthermore, this band is only seen in blots of embryo extracts or stored extracts of tissue culture cells, but is not seen in freshly prepared cell extracts (Marcantonio and Hynes, unpublished observations).

Finally, we investigated the degree of cross-reactivity in yeasts. Immunoblotting experiments showed no specific bands in membrane extracts of *Saccharomyces cerevisiae* (data not shown). However, immunoblotting experiments

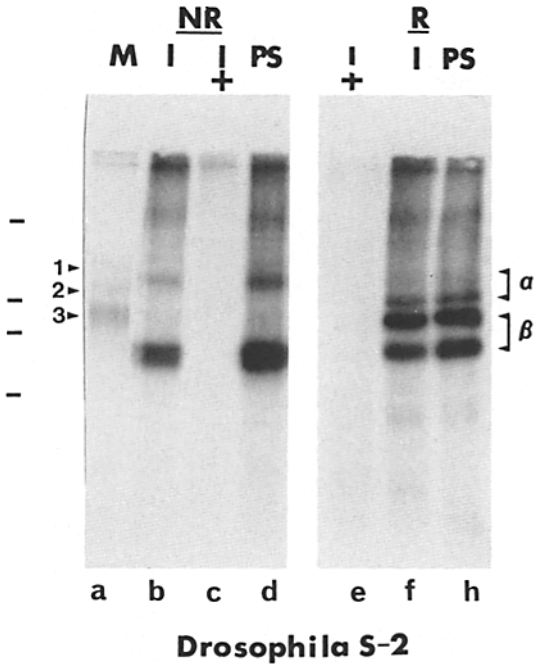

Figure 4. Immunoprecipitation of extracts of *Drosophila* S-2 cells. Extracts of ^{125}I surface-labeled S-2 cells (lanes *b–h*) were incubated with either immune (*I*) anti-peptide antiserum (lanes *b* and *f*), anti-peptide antiserum with 10 μg of peptide (*I+*) added (lanes *c* and *e*), or anti-PS-3 (*PS*) agarose beads (lanes *d* and *h*). After immunoprecipitation, samples were analyzed by SDS-PAGE under nonreduced (lanes *a–d*) or reduced (lanes *e–h*) conditions. An immunoprecipitate of the chicken integrin complex (*M*; lane *a*) using CSAT antibody is shown for comparison; *arrowheads* indicate bands *1*, *2*, and *3* of the complex. Molecular markers are indicated at left.

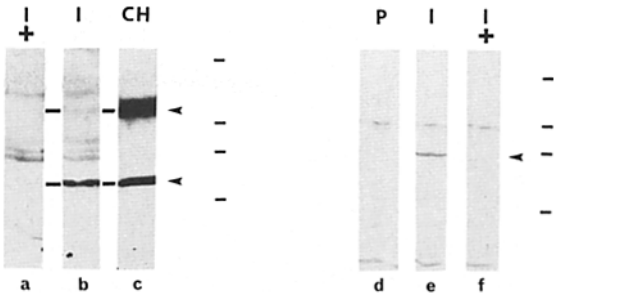

Figure 5. Immunoblots of integrin-related proteins in *C. elegans* and *C. albicans*. Extracts of *C. elegans* embryos (*a* and *b*), chicken embryos (*CH*; lane *c*), or *C. albicans* membranes (lanes *d–f*) were separated by SDS-PAGE (nonreduced, lanes *a–c*; reduced, lanes *d–f*), transferred to nitrocellulose, and probed with either preimmune (*P*) serum (lane *d*), immune (*I*) anti-peptide antiserum (lanes *b*, *c* and *e*), or immune anti-peptide antiserum with (*I+*) 20 μg of peptide added (lanes *a* and *f*). Positions of molecular mass markers are shown.

with another yeast, *C. albicans*, showed a band of M_r 95,000 with anti-peptide antiserum, which was absent when preimmune or immune serum plus peptide were used (Fig. 5, lanes *d–f*).

Topology of the COOH Terminus

Since the COOH-terminal segment of integrin β_1 is preceded in the primary sequence by a stretch of hydrophobic amino acids characteristic of a transmembrane domain, it has been proposed that the COOH terminus is the cytoplasmic domain (Tamkun et al., 1986). Antibodies to the amino-terminal third of the integrin β_1 subunit stain live cells, indicating that this portion of the molecule is extracellular (Tamkun et al., 1986). In contrast, when live CEFs were stained with the anti-peptide antisera, no staining was seen (Fig. 6, *A* and *B*). However, when the cells were fixed and permeabilized, staining characteristic of the integrin distribution in CEFs was seen (Fig. 6, *C* and *D*). These results confirm the proposed topology of the integrin β_1 subunit.

In contrast with the CEFs, where the staining appeared as multiple short contact sites, fixed and permeabilized Nil8 hamster cells stained with the anti-peptide antiserum (Fig. 6, *E* and *F*) exhibited long delicate fibrils corresponding with the longer contact sites typical of these cells (see Discussion). As in the case of CEFs, live Nil8 cells showed no staining (data not shown). In both cell types, the integrin staining pattern coaligned with both the fibronectin and actin staining patterns (data not shown). In all cases, no specific staining was seen with preimmune serum or with immune serum plus competing peptide (data not shown).

Discussion

The antiserum we describe here has allowed demonstration of three main points concerning integrins: proof of the presumed topology of the β_1 subunit, conservation of the COOH-terminal cytoplasmic domain, and wide phylogenetic distribution.

The wide cross-reactivity of the anti-peptide antiserum with proteins of similar sizes in vertebrates, invertebrates, and fungi shows that sequences in the cytoplasmic domain of integrin β subunits have been strongly conserved during long periods of evolution (up to 10^9 yr). Intimations of this high degree of conservation had already been obtained from primary sequence data but the present results considerably extend the known phylogenetic distribution of integrins. We have shown that in human cells the antiserum is specific for the integrin β_1 subunit and does not cross-react with the closely related β_2 and β_3 subunits (Fig. 3). Thus, the widely distributed β subunit which we have detected is presumably the β_1 subunit. It is currently unclear whether β_2 and β_3 subunits, which appear to have diverged from β_1 before the evolution of vertebrates (DeSimone and Hynes, 1988) are as widely distributed as the β_1 subunit.

In immunoblotting experiments or in immunoprecipitates of denatured material, the antiserum recognizes only the β_1 subunit (Figs. 2, 3, and 5). However, when extractions and immunoprecipitations are performed under nondenaturing conditions, larger α subunits are coprecipitated as would be expected given the known αβ structure of integrins. In many cell types, more than one α subunit coprecipitates with the β_1 subunit indicating the presence in the cells of two or more αβ heterodimers as previously reported.

In the human cells analyzed here, we can relate the heterodimers precipitated with the anti-β_1 antiserum to the VLA antigens found on these cells. In both MG-63 osteosarcoma

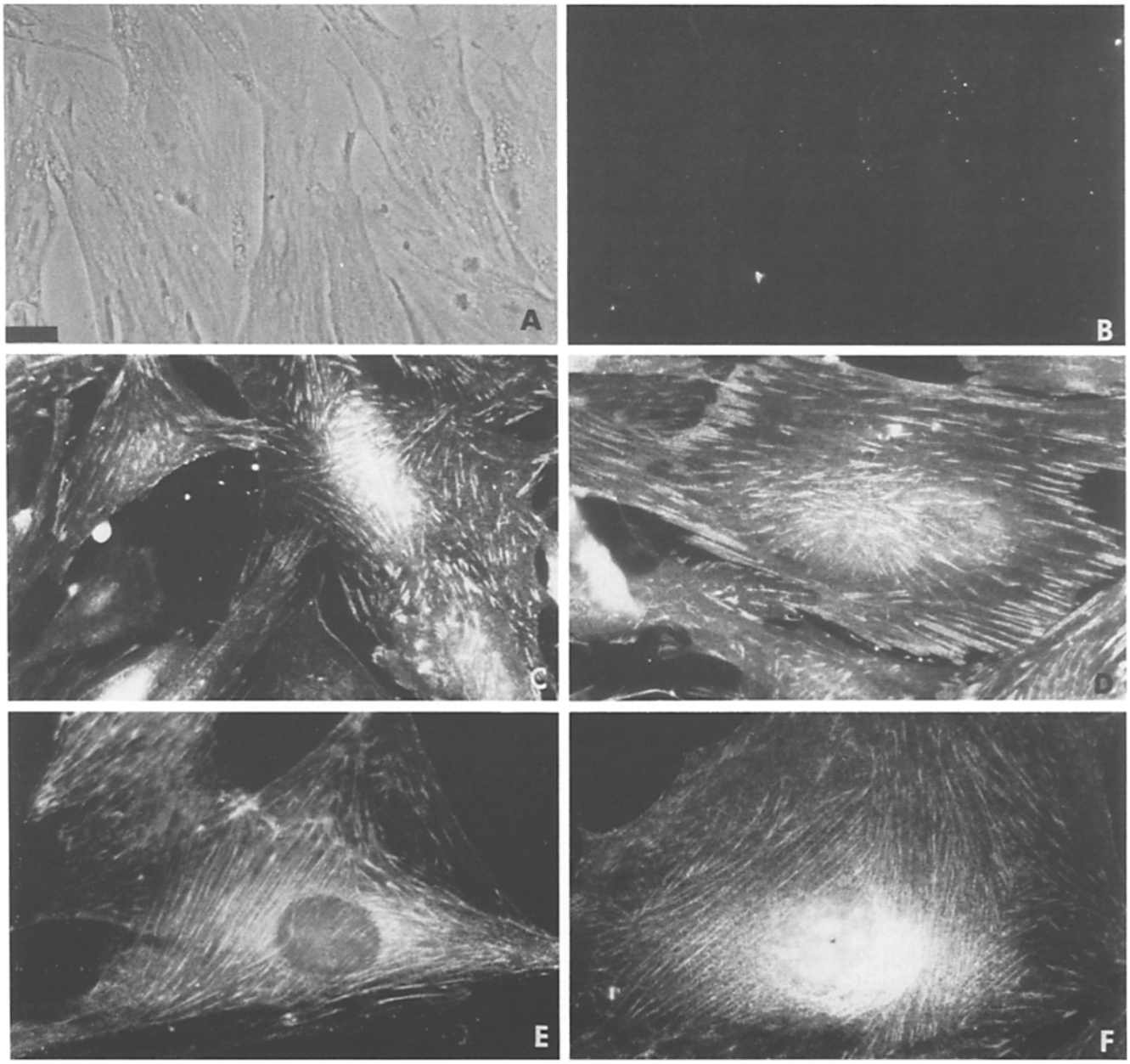

Figure 6. Cytoplasmic localization of the COOH-terminal domain. Immunofluorescence staining of CEFs (*A–D*) and Nil8 (*E–F*) cells under various conditions. *A* and *B* show phase and fluorescence micrographs of CEFs stained while intact and alive. *C* and *D* show fluorescence micrographs of CEFs stained after fixation and permeabilization. Note the pattern characteristic of focal contact localization. *E* and *F* show fluorescence micrographs of permeabilized, fixed Nil8 cells. Note the elongated fibrillar staining pattern characteristic of the extended substrate contacts in these cells. Bar, 10 μm.

cells (Fig. 2) and in platelets (Fig. 3), the αβ complexes precipitated by the anti-β_1 antiserum appear identical to the VLAs, which have been shown previously to be VLA α_3 and α_5 on MG63 osteosarcoma cells, and predominantly VLA α_2 on platelets (Hemler et al., 1987; Takada et al., 1987; Giancotti et al., 1987).

In monocytes (Fig. 3) both subunits of the complex immunoprecipitated with the anti-β_1 antiserum shifted in electrophoretic mobility upon reduction, a property consistent with the presence of VLA-5 (α_5 β_1) on monocytoid cell lines (Hemler et al., 1987) and the binding studies indicating the presence of fibronectin receptors on monocytes (Hosein and Bianco, 1985). Interestingly, β_2 found on monocytes (Fig. 3) had only a small shift in electrophoretic mobility upon reduction, despite the fact that all 56 cysteine residues of integrin β subunits are conserved. The β_2-associated α subunits are probably the Mac-1 α subunit and p150 (Miller et al., 1986). It is worth noting that the results shown in Fig. 3 indicate that each β subunit associates with a specific set of α subunits and that interaction of α subunits with other β subunits is not observed.

The results with the *Drosophila* S-2 cells confirm the identity of the position-specific antigens as integrins (Fig. 4). These antigens are expressed in many insect cell lines and

in embryos, where they occur in characteristic patterns in developing tissues (Brower et al., 1984; Wilcox and Leptin, 1985). Their biochemical structure is typical of integrins, with a common β subunit (PS3) and variable α subunits (PS1 and PS2) associated in noncovalent complexes (Brower et al., 1984). Our anti-β_1 peptide antiserum precipitates αβ complexes which appear identical with those precipitated by anti-PS3 mAb (Fig. 4). Previous data have shown homology in NH_2-terminal sequence between PS-1 and vertebrate integrin α chains (Leptin et al., 1987). Recent cDNA sequence data for PS2 published while this paper was in review confirm that it is an integrin α subunit (Bogaert et al., 1987). The present data show that PS3 is probably a β_1 subunit; it remains unclear whether other β subunits occur in *Drosophila*.

We also detect what appear to be integrin β_1 subunits in the nematode *C. elegans* and the fungus *C. albicans*. In these two species, we have so far been unsuccessful in performing immunoprecipitations because of difficulties with extraction and degradation. Therefore, we cannot demonstrate αβ complexes here. However, the immunoblotting experiments (Fig. 5) indicate the presence of specifically cross-reactive bands of sizes similar to integrin β subunits. It will be interesting to see whether the cross-reacting protein found in *C. albicans* plays a role in the ability of these fungi to attach to and invade blood vessels and tissues. Integrins may play a role in the pathogenesis of infectious diseases; e.g., infection of cells in vitro by the parasite *Trypanosoma cruzi* can be blocked by peptides derived from the cell-binding domain of fibronectin (Ouaissi et al., 1986).

Finally, our immunofluorescence data (Fig. 6) establish that the COOH-terminal segment of the β_1 subunit is indeed the cytoplasmic domain as has been widely assumed. We have previously reported that antisera specific for segments in the NH_2-terminal third of the β_1 subunit stain live cells, placing this part of the molecule outside the membrane (Tamkun et al., 1986). Since only a single strongly hydrophobic stretch is found in the sequence immediately preceding the COOH-terminal cytoplasmic domain, it seems highly likely that this short segment comprises the entire cytoplasmic domain of this subunit.

As shown here and elsewhere, this domain is highly conserved suggesting that it plays an important role in the functions of integrin presumably including interactions with the cytoskeleton. It has been demonstrated that integrins coalign with both fibronectin and cytoskeletal proteins (Chen et al., 1985; Damsky et al., 1985) and that purified chicken integrins bind to talin in vitro (Horwitz et al., 1986). The distribution of staining we observe in CEFs is very similar to that reported by others and corresponds with the distribution of cytoskeletal proteins such as talin and vinculin and with attachment plaques. The staining pattern of Nil8 cells (Fig. 6, *E* and *F*) is somewhat different. The integrin is arrayed in very long fine striae similar to the elongated attachment sites observed in these cells (Hynes et al., 1982; Singer, 1982). The codistributions between fibronectin and cytoskeletal proteins in these cells are also very extended (Hynes and Destree, 1978; Singer, 1982; Singer and Paradiso, 1981; Hynes et al., 1982). Thus, in both cell types the distribution of integrin β_1 subunits corresponds well with the coalignments observed between fibronectin and the cytoskeleton.

The availability of this cytoplasmic domain peptide and of its antibody should facilitate future investigations of the role of the cytoplasmic domain of integrin β_1 in the transmembrane link between the extracellular matrix and the cytoskeleton. In addition, the cross-reactivity of this antiserum with proteins in invertebrate species where genetic analysis is feasible should facilitate investigation of the role of integrins in development.

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References

Akiyama, S. K., S. S. Yamada, and K. M. Yamada. 1986. Characterization of a 140-kD avian cell surface antigen as a fibronectin-binding molecule. *J. Cell Biol.* 102:442–448.

Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu. Rev. Med.* 38:175–194.

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

Billiau, A., V. G. Edy, H. Heremans, J. Van Damme, J. Desmyter, J. A. Georgiades, and P. DeSomer. 1977. Human interferon: mass production in a newly established cell line, MG-63. *Antimicrob. Agents Chemother.* 12: 11–15.

Bogaert, T., N. Brown, and M. Wilcox. 1987. The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell.* 51:929–940.

Bolton, A. E., and W. M. Hunter. 1973. The labeling of proteins to high specific radioactivities by conjugation to a [^{125}I] containing acylating agent. *Biochem. J.* 133:529–539.

Brower, D. L., M. Wilcox, M. Piovant, R. J. Smith, and L. A. Reger. 1984. Related cell-surface antigens expressed with positional specificity in *Drosophila* imaginal discs. *Proc. Natl. Acad. Sci. USA.* 81:7485–7489.

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

Damsky, C. M., 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.

DeSimone, D. W., and R. O. Hynes. 1988. *Xenopus laevis* integrins: structural conservation and evolutionary divergence of integrin β subunits. *J. Biol. Chem.* 263:5333–5340.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:201–205.

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

Gardner, J. M., and R. O. Hynes. 1985. Interaction of fibronectin with its receptor on platelets. *Cell.* 42:439–448.

Giancotti, F. G., L. R. Languino, A. Zanetti, G. Peri, G. Tarone, and E. Dejana. 1987. Platelets express a membrane protein complex immunologically related to the fibroblast fibronectin receptor and distinct from GPIIb/IIIa. *Blood.* 69:1535–1538.

Ginsberg, M. H., J. Loftus, M. D. Pierschbacher, E. Ruoslahti, and E. F. Plow. 1987. Immunochemical and N-terminal sequence comparison of two cytoadhesins indicates they contain similar or identical beta subunits and distinct alpha subunits. *J. Biol. Chem.* 262:5437–5440.

Hemler, M. E., C. Huang, and L. Schwarz. 1987. The VLA protein family: characterization of five distinct cell surface heterodimers each with a common 130,000 M_r subunit. *J. Biol. Chem.* 262:3300–3309.

Hemler, M. E., J. G. Jacobson, and J. L. Strominger. 1985. Biochemical characterization of VLA-1 and VLA-2. *J. Biol. Chem.* 260:15246–15252.

Hirst, R., A. Horwitz, C. Buck, and L. Rohrschneider. 1986. Phosphorylation

of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 83:6470–6474.

Horwitz, A., E. 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.

Horwitz, A., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134–2144.

Hosein, B., and C. Bianco. 1985. Monocyte receptors for fibronectin characterized by a monoclonal antibody that interferes with receptor activity. *J. Exp. Med.* 162:157–170.

Hynes, R. O. 1973. Alteration of cell-surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. USA.* 70:3170–3174.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48: 549–554.

Hynes, R. O., and A. T. Destree. 1978. Relationships between fibronectin (LETS protein) and actin. *Cell.* 15:875–886.

Hynes, R. O., A. T. Destree, and D. D. Wagner. 1982. Relationships between fibronectin, actin, and cell-substratum adhesion. *In* Organization of the Cytoplasm. *Cold Spring Harbor Symp. Quant. Biol.* 46:659–670.

Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell.* 48:681–690.

Kitagawa, T., and T. Aikawa. 1976. Enzyme coupled immunoassay of insulin using a novel coupling reagent. *J. Biochem. (Tokyo).* 79:233–236.

Knudsen, K. A., A. F. Horwitz, and C. A. Buck. 1985. A monoclonal antibody identifies a glycoprotein complex involved in cell-substratum adhesion. *Exp. Cell Res.* 157:218–228.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Law, S. K. A., J. Gagnon, J. E. K. Hildreth, C. E. Wells, A. C. Willis, and A. J. Wong. 1987. The primary structure of the beta-subunit of the cell surface adhesion glycoproteins LFA-1 CR3 and p150,95 and its relationship to the fibronectin receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:915–919.

Lawler, J., F. E. Chao, and C. M. Cohen. 1982. Evidence for calcium-sensitive structure in platelet thrombospondin: isolation and partial characterization of thrombospondin in the presence of calcium. *J. Biol. Chem.* 257:12257–12265.

Leptin, M., R. Aebersold, and M. Wilcox. 1987. *Drosophila* position-specific antigens resemble the vertebrate fibronectin-receptor family. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1037–1043.

Mautner, V. M., and R. O. Hynes. 1977. Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells. *J. Cell Biol.* 75:743–768.

Miller, L. J., R. Schwarting, and T. A. Springer. 1986. Regulated expression of the Mac-1, LFA-1, p150,95 glycoprotein family during leukocyte differentiation. *J. Immunol.* 137:2891–2900.

Orlean, P. 1987. Two chitin synthases in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 262:5732–5739.

Ouaissi, M. A., J. Cornette, D. Afchain, A. Capron, H. Gras-Masse, and A. Tartar. 1986. *Trypansoma cruzi* infection inhibited by peptides modeled from a fibronectin cell attachment domain. *Science (Wash. DC)* 234:603–607.

Poncz, M., R. Eisman, R. Heidenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb: homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. *J. Biol. Chem.* 262:8476–8482.

Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of arg-gly-asp-specific adhesion receptors. *Science (Wash. DC).* 231:1559–1562.

Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985*a*. Identification and isolation of a 140-kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.

Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985*b*. 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.

Rein, A., and H. Rubin. 1968. Effects of local cell concentrations upon the growth of chick embryo cells in tissue culture. *Exp. Cell Res.* 49:666–678.

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

Singer, I. I. 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., and P. R. Paradiso. 1981. A transmembrane relationship between fibronectin and vinculin (130-kd protein): serum modulation in normal and transformed hamster fibroblasts. *Cell.* 24:481–492.

Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor α subunit and comparative expression of adhesion receptor mRNAs. *J. Biol. Chem.* 262:14080–14085.

Takada, Y., C. Huang, and M. E. Hemler. 1987. Fibronectin receptor structures are included within the VLA family of heterodimers. *Nature (Lond.).* 326:607–609.

Tamkun, J. W., D. W. DeSimone, D. Fonda, R. S. Patel, C. Buck, A. F. Horwitz, and R. O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell.* 42:271–282.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Wayner, E. A., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J. Cell Biol.* 105:1873–1884.

Wilcox, M., and M. Leptin. 1985. Tissue-specific modulation of a set of related cell surface antigens in *Drosophila*. *Nature (Lond.).* 316:351–354.

Wilcox, M., N. Brown, M. Piovant, R. J. Smith, and R. A. H. White. 1984. The *Drosophila* position-specific antigens are a family of cell surface glycoprotein complexes. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2307–2313.

Wright, S. D., and S. C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3b′ receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149–1164.

Wright, S. D., P. A. Peddy, M. T. C. Jong, and B. W. Erickson. 1987. C_3bi receptor (complement receptor type 3) recognizes a region of complement protein C3 containing the sequence arg-gly-asp. *Proc. Natl. Acad. Sci. USA.* 84:1965–1968.