Expression and Functional Analysis of a Cytoplasmic Domain Variant of the β1 Integrin Subunit

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Abstract. We have previously described a variant form of the integrin β1 subunit (β1B)[1] characterized by an altered sequence at the cytoplasmic domain. Using polyclonal antibodies to a synthetic peptide corresponding to the unique sequence of the β1B, we analyzed the expression of this molecule in human tissues and cultured cells. Western blot analysis showed that the β1B is expressed in skin and liver and, in lower amounts, in skeletal and cardiac muscles. The protein was not detectable in brain, kidney, and smooth muscle. In vitro cultured keratinocytes and hepatoma cells are positive, but fibroblasts, endothelial cells, and smooth muscle cells are negative. An astrocytoma cell line derived from immortalized fetal astrocytes was found to express β1B. In these cells β1B represent ~30% of the β1 and form heterodimers with α1 and α5 subunits. To investigate the functional properties of β1B, the full-length cDNA coding for this molecule was transfected into CHO cells. Stable transfectants were selected and the β1B was identified by a mAb that discriminate between the transfected human protein and the endogenous hamster β1A. Immunoprecipitation experiments indicated that the β1B was exported at the cell surface in association with the endogenous hamster α subunits. The α5/β1B complex bound to a fibronectin-affinity matrix and was specifically released by RGD-containing peptides. Thus β1B and β1A are similar as far as the α/β association and fibronectin binding are concerned. The two proteins differ, however, in their subcellular localization. Immunofluorescence studies indicated, in fact, that β1B, in contrast to β1A, does not localize in focal adhesions. The restricted tissue distribution and the distinct subcellular localization, suggest that β1B has unique functional properties.

INTEGRINS are a large family of heterodimeric membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response, and metastatic diffusion of tumor cells (Hynes, 1987; Hemler, 1990; Ruoslahti, 1991). These molecules provide a link between extracellular structures and cytoskeletal components and transduce mechanical forces across the plasma membrane during cell adhesion and migration. Both α and β integrin subunits span the lipid bilayer and possess a large extracellular and a short cytoplasmic domain (Hynes, 1987; Hemler, 1990; Ruoslahti, 1991). The extracellular domain mediates the interaction with matrix proteins. Many of the known integrins bind the Arg-Gly-Asp (RGD in one letter code) sequence present in several extracellular matrix proteins (Ruoslahti and Pierschbacher, 1987) and representing a recognition signal of broad importance in cell adhesion. Binding to the RGD sequence involves the extracellular domains of both α and β subunit of the heterodimer and requires divalent cations. The cytoplasmic domain of integrins interacts with cytoskeletal components and is required for the localization of these membrane proteins at focal contact sites with the substratum (Hayashi, 1990; Marcantonio, 1990). These sites correspond to areas where actin microfilament bundles are anchored to the plasma membrane via cytoskeletal components, such as vinculin, talin, and α-actinin (Burridge et al., 1988). Experimental evidence for binding of the integrin complex to talin has been provided (Horwitz et al., 1986). More recently binding of α-actinin to synthetic peptides reproducing the cytoplasmic domain of the β1A[2] and β3 subunit has also been reported (Otey et al., 1990), suggesting that these two cytoskeletal proteins may be responsible for bridging integrins to actin microfilament bundles. Vari-

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1. This β1 variant was previously indicated as β13'v (Altruda, F., P. Cervella, G. Tarone, C. Botta, F. Balzac, G. Stefanuto, and L. Silengo. 1990. *Gene.* 95:261–266). For the sake of uniformity with other integrin variants this molecule is now indicated as β1B to distinguish it from the classical form B1A (Argraves, S., S. Suzuki, H. Aria, K. Thompson, M. Pierschbacher, and E. Ruoslahti. 1987. *J. Cell Biol.* 105:1183–1190) and from a second variant form β1C (Languino, L. R., and E. Ruoslahti. 1992. *J. Biol. Chem.* 267:7116–7120).

ant forms of integrin β1 and β3 with distinct sequences at the cytoplasmic domain have been described (van Kuppevelt et al., 1989; Altruda et al., 1990; Languino and Ruoslahti, 1992). In addition cytoplasmic domain variants of the β4 and α6 subunits have also been identified (Tamura et al., 1990; Hogervorst et al., 1991; Tamura et al., 1991). The existence of such variants suggests diverse functions of the integrin cytoplasmic domains.

We have previously isolated the full length cDNA for a variant form of the integrin β1 subunit in which a unique 12-amino acid sequence replaces the last 21 COOH-terminal amino acids of the β1A (Altruda et al., 1990). To gain information on the functional significance of the β1B, we investigated its expression in tissues and cultured cells and analyzed its properties in CHO cell transfected with the human β1B full-length cDNA.

Materials and Methods

Cells

CHO cells were grown in HAM F12 medium with 10% FCS. The human astrocytoma cell line Asch-7 was produced by immortalization of human fetal astrocytes as described (Balabanov et al., 1990). Human skin fibroblasts, human umbilical vein endothelial cells, and human keratinocytes were kindly provided by Drs. O. Ornatsky, M. Frid, and M. Lukashev (Institute of Experimental Cardiology, Moscow, Russia). Human aortic medial smooth muscle cells were isolated and cultured as described (Chamley-Campbell et al., 1979). Human hepatoma cell line HepG-2 was from the American Type Culture Collection (Rockville, MD).

Constructs and Transfections

A 3.5-kb EcoRI fragment of the β1B integrin (Altruda et al., 1990) containing the entire coding sequence was inserted into the EcoRI cloning site of the SV-40-based expression vector pECE (Ellis et al., 1986). The full-length cDNA for β1A cloned in the pECE vector was a kind gift of Filippo Giancotti, New York University, New York (Giancotti and Ruoslahti, 1990). CHO cells (10^6 cells/50-cm^2 plate) were co-transfected with 20 μg of the plasmid containing β1B cDNA and 2 μg of pSV2-neo (Southern and Berg, 1982) by the calcium phosphate precipitation method (Chen et al., 1987). After 48 h the cells were split 1:10 and incubated with 800 μg/ml of G418 (GIBCO BRL, Gaithersburg, MD) to select neomycin-resistant clones. 10 to 14 d later colonies of G418-resistant cells were collected by scraping with micropipette tips and transferred to 24-well plates. Colonies were subsequently maintained in complete medium with 400 μg/ml of G418.

FACS Analysis

For FACS analysis, transfected cells were detached from culture plates by incubation in 5 mM EDTA in PBS (10 mM phosphate buffer, pH 7.3, 150 mM sodium chloride) and washed twice at 4°C in PBS with 0.1 mM EDTA and 1 mg/ml of BSA. The cells were then incubated for 1 h at 4°C in the same buffer with the appropriate dilution of the A1A5 mAb to the human β1 integrin. After washing, the cells were incubated 45 min with a fluorescein-labeled affinity-purified secondary antibodies, and analyzed on the flow cytometer Facs-Star (Becton Dickinson & Co., Mountainview, CA) equipped with 5 W argon laser at 488 nm. 5,000 cells per sample were analyzed.

Antibodies and Immunoprecipitation of Integrins

The polyclonal antisera to the β1A and β1B subunits were prepared by immunizing rabbits against synthetic peptides reproducing amino acid sequences from the cytoplasmic domains specific for each subunit. The following peptides, obtained from Multiple Peptide System (San Diego, CA), were used: β1A, CTTVVNPKYEGK; and β1B, CSYKTSKKQSGL. Peptides were coupled to hemocyanin with glutaraldehyde (approximate peptide/carrier molar ratio of 50:1) and rabbits were injected with 500 μg of the conjugate in complete Freund adjuvant. Antibodies to cytoplasmic sequences of the α3 and α5 integrin subunits were previously described and characterized (Defilippi et al., 1991). For Western blotting and immunoprecipitation experiments the β1B antibodies were purified from the serum by affinity chromatography on β1B–albumin conjugate coupled to Sepharose. A polyclonal antibody to β3 was prepared in our laboratory by immunizing rabbits with β3 purified from human platelets as described (Defilippi et al., 1991). Mouse mAbs to human integrin α1 (mAb TS2/7) and to integrin β1 (mAb A1A5) were a kind gift from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA). mAb 102DF5 (Ylanne et al., 1989) to human β1 was a kind gift from Dr. Ismo Virtanen (University of Helsinki, Helsinki, Finland).

The mAb A1A5 reacts with human, but not with hamster β1 and identifies an epitope in the extracellular domain which is common to both β1A and β1B.

Integrins were immunoprecipitated from cells either unlabeled or labeled with ^{125}I. Labeling of membrane proteins with ^{125}I was performed as described previously (Rossino et al., 1990). Briefly, cells were released from culture dishes by 5 mM EDTA treatment in PBS and washed three times by centrifugation with culture medium. Cells were suspended in PBS containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and labeled with 1 mCi of ^{125}I in presence of lactoperoxidase (200 μg/ml) and H_2O_2 (0.002%). For immunoprecipitation labeled or unlabeled cells were extracted for 20 min at 4°C with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, England) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin (all from Sigma Immunochemicals, St. Louis, MO). After centrifugation at 10,000 *g* for 10 min, extracts were incubated with the specific antibodies for 1 h at 4°C with gentle agitation. Soluble immunocomplexes were bound to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) and recovered by centrifugation. After washing, bound material was eluted by boiling beads in 1% SDS (Pierce, Rockford, IL) and analyzed by SDS-PAGE (6%) in the absence of reducing agents (Laemmli, 1970). The radioactive proteins were visualized by fluorography (Chamberlain, 1979). In the experiment shown in Fig. 4 *A* the immunoprecipitation was performed after protein denaturation with SDS. In this case cells were extracted in TBS containing 0.1% SDS and boiled for 3 min. After cooling, Triton X-100 was added to the final concentration of 0.5% and the sample was subjected to immunoprecipitation as described above.

Western Blotting

Human adult tissues were obtained at autopsies taken within 2–4 h from death. The samples were homogenized and immediately boiled in 2% SDS, 100 mM Tris-HCl, pH 6.8, 10 mM EDTA, and 10% glycerol. After protein determination, 5% beta mercaptoethanol and bromophenol blue were added and samples were boiled again. At least three independent samples were analyzed for each tissue. For Western blotting, 150 μg of total tissue or cell proteins (per each lane) were separated by SDS-PAGE and transferred to nitrocellulose sheets by standard procedure. After transfer to nitrocellulose, strips were cut, washed with TBS with 0.1% Tween 20 (TBS/Tween), and incubated in the same buffer with 2% BSA and 0.3% gelatin. Strips were then incubated with affinity-purified antibodies to β1B (1 μg/ml for 15 h at 4°C) in TBS/Tween, washed three times with TBS/Tween, and incubated for 2 h at room temperature with ^{125}I-labeled antibodies to rabbit IgG (Amersham Corp., Arlington Heights, IL). Blots were then extensively washed, dried and exposed to X-ray films for 40–96 h at −70°C.

Affinity Chromatography on Fibronectin-Sepharose

The fibronectin-Sepharose matrix was a generous gift of Dr. R. Pytela (Department of Medicine, University of California San Francisco, San Francisco, CA) and was prepared as described (Pytela et al., 1987).

CHO cells transfected with the β1B subunit were collected in PBS and radiolabeled with ^{125}I as described above. Labeled cells were extracted with 1 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 200 mM β-octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin. After centrifugation at 10,000 *g*, soluble material was chromatographed on the fibronectin-Sepharose column. After washing with 5 Vol of 50 mM β-octylglucoside in TBS with cations, the column was eluted sequentially with: (*a*) TBS, 50 mM β-octylglucoside 1 mg/ml of GRGDSP peptide; (*b*) 10 mM EDTA in TBS octylglucoside. In some experiments GRGESP peptide was used as control. GRGDSP and GRGESP synthetic peptides were synthesized by Drs. L. Lozzi and P. Neri (University of Siena, Siena, Italy). The column flow was kept at 40 ml/h through all steps of the chromatography. 100-μl aliquots from each fraction were analyzed by SDS-PAGE under non reducing conditions.

Immunofluorescence

CHO cells transfected with either β1B or β1A cDNAs were plated on glass coverslips pre-coated with human plasma fibronectin (10 μg/ml). To improve cell spreading cells were starved for 48 h in serum-free HAM F12 medium and treated during the last 12 h with transforming growth factor β 5 ng/ml (TGF-β, Sigma Immunochemicals). Under these conditions virtually all cells showed well organized stress fibers and focal contacts. Cells were fixed for 15 min with 4% paraformaldehyde in PBS at room temperature, washed in 20 mM Tris-HCl, pH 7.3, 150 mM sodium chloride (TBS) and permeabilized for 1 min in TBS containing 0.5% Triton X-100 at 4°C. The β1B and β1A integrins were localized with the A1A5 mAb specific for the human β1 (1/100 dilution of ascitic fluid). The endogenous CHO cell β1A was localized with the mAb 7E2 specific for the hamster β1 (generous gift of Rudy Juliano, University of North Carolina, Chapel Hill, NC). Bound antibodies were then visualized by appropriate rhodamine-labeled secondary antibodies (KPL). In some experiments cells were double labeled with fluorescein-conjugated phalloidin (Sigma Chemie GmbH, Taufkirchen, Germany). Cells were examined with a fluorescence microscope (BH-2; Olympus Corp., Lake Success, NY) and pictures were taken on 400 ASA Kodak black and white film exposed to 1,600 ASA (Eastman Kodak Co., Rochester, NY). Interference reflection microscopy was performed on a Zeiss photomicroscope equipped with the appropriate optical system (Carl Zeiss, Oberkochen, Germany).

Results

Expression of β1B in Human Tissues and in Cultured Cells

To analyze the expression of the β1B protein we developed an antiserum directed to a synthetic peptide reproducing the last 11 COOH-terminal amino acids unique of this molecule (see Materials and Methods and Fig. 1). The specificity of this antiserum was demonstrated by immunoprecipitation of CHO cells transfected with the full length cDNA for the β1B molecule (see below and Fig. 3).

To investigate the expression of the β1B, human tissue samples were extracted with SDS and processed for Western blot analysis. As shown in Fig. 2 *A*, an intensely positive band with a molecular mass of 130 kD (reduced) was detected in extracts from skin and liver, while a barely appreciable signal was present in cardiac and skeletal muscle extracts. At the same time no reaction was observable with extracts from kidney, brain, or aortic media. All samples gave a strong positive reaction with antibodies to the classical β1A (Fig. 2 *B*). The positive reaction of skin and liver with β1B antibody was confirmed by analysis of cultured cells. As shown in Fig. 2 *C*, cultured human keratinocytes were clearly positive, while no reaction was observed with cultures of skin fibroblasts, smooth muscle, or endothelial cells. Thus, keratinocytes are the β1B-positive cell type in the skin. Furthermore, the HepG2 hepatoma cell line was positive indicating that the reaction in the liver is likely to be due to hepatocytes. In addition to these cells, an astrocytoma cell line, produced by immortalization of human fetal astrocytes (Balabanov et al., 1990), was found to express β1B (Fig. 2 *C*).

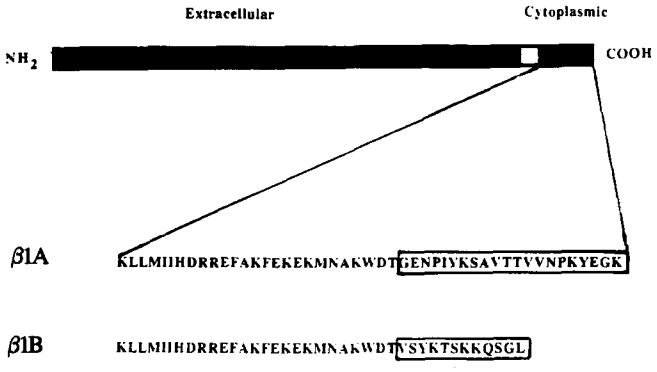

Figure 1. Cytoplasmic domain sequence of β1A and β1B integrins. The β1 molecule is schematically represented with the extracellular and cytoplasmic domains (filled rectangles) and transmembrane (open rectangle) domain. The sequence of the β1A and β1B cytoplasmic domains is indicated with the one-letter code and the boxed area represent the unique regions of the two proteins (Argraves et al., 1987; Altruda et al., 1990).

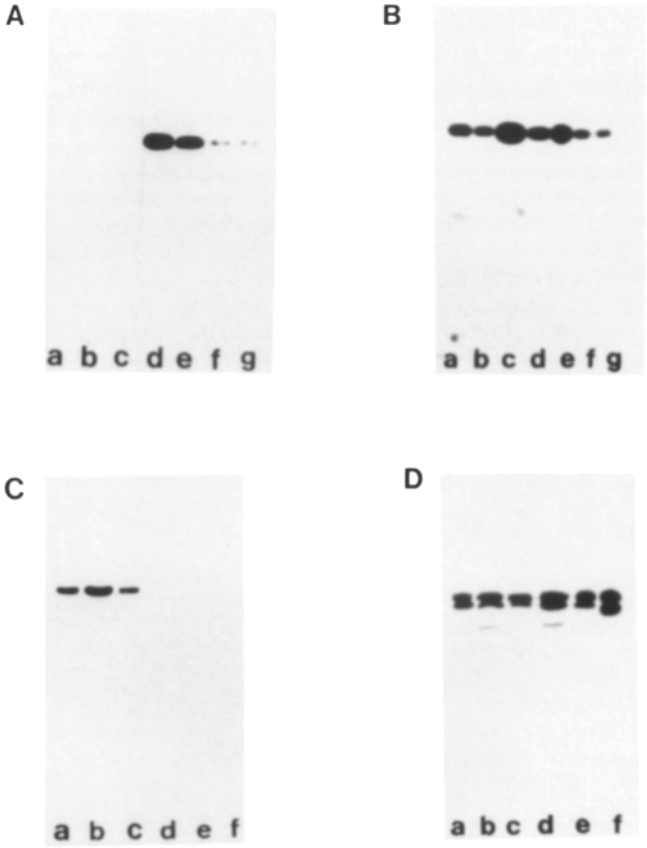

Figure 2. Analysis of β1B expression in human tissues and cultured cells by Western blotting. Human tissue samples or cells were extracted with boiling SDS and 150 μg of protein were separated on a 7.5% polyacrylamide gel under reducing conditions. After transfer to a nitrocellulose filters, β1B and β1A were identified with affinity purified antibodies to the COOH-terminal sequence of the proteins followed by ^{125}I-labeled secondary antibodies. *A* and *B* show human tissue extracts stained for β1B (*A*) and β1A (*B*): (lanes *a*) kidney; (lanes *b*) brain; (lanes *c*) aortic media; (lanes *d*) skin; (lanes *e*) liver; (lanes *f*) cardiac muscle; and (lanes *g*) skeletal muscle. *C* and *D* show cultured cell extracts stained for β1B (*C*) and β1A (*D*): (lanes *a*) astrocytoma cell line; (lanes *b*) keratinocytes; (lanes *c*) HepG-2 hepatoma cell line; (lanes *d*) skin fibroblasts; (lanes *e*) smooth muscle cells; and (lanes *f*) endothelial cells. The doublets of bands in *D* is likely to be due to the presence of a considerable amount of immature β1A in cultured cells (see Rossino et al., 1991).

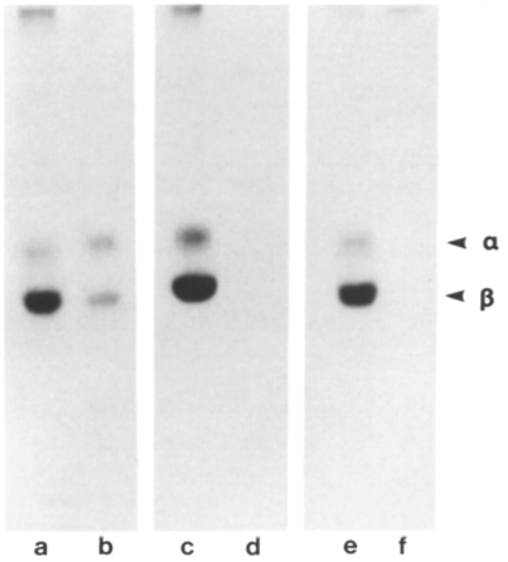

Figure 3. Immunoprecipitation of β1B from transfected CHO cells. Cells were surface labeled with lactoperoxidase catalyzed ^{125}I iodination and after detergent extraction integrin complexes were immunoprecipitated with specific antibodies. Radioactive proteins are visualized by fluorography after separation in 6% polyacrylamide gels under nonreducing conditions. CHO cells transfected with the β1B cDNA (clone 18/24) immunoprecipitated with: (lane *a*) polyclonal antibody for the β1A peptide; and (lane *b*) mAb A1A5 to human β1. Untransfected control CHO cells immunoprecipitated with: (lane *c*) polyclonal antibody specific for the β1A peptide; and (lane *d*) mAb A1A5 to human β1. Immunoprecipitation with polyclonal antibody specific for the β1B peptide from β1B transfected CHO (clone 18.24) (lane *e*) and untransfected CHO (lane *f*). The position of β (120 kD) and α (150 kD) subunits is indicated.

Surface Expression and Binding Properties of β1B in Transfected CHO Cells

To analyze the functional properties of the β1B we transfected CHO cells with the full-length cDNA for the human β1B. These cells do express the classical but not the variant form of β1 (see below and Fig. 3). The transfected protein was identified by means of A1A5 mAb that recognize human-specific epitopes common to β1B and β1A, but does not react with β1 of CHO cells (hamster) (see Fig. 3, lanes *b* and *d*). Conversely the endogenous CHO β1A was identified with polyclonal antibodies to a peptide representing the COOH-terminal sequence specific of the classical β1A and absent in the β1B form (see Materials and Methods).

Transfected CHO cells were selected by neomycin resistance and clones expressing β1B at the cell surface were identified by immunofluorescence and FACS analysis.

Transfected cells were labeled with lactoperoxidase cathalyzed surface radioiodination followed by immunoprecipitation. The human-specific mAb A1A5 reacted only with transfected cells and identified two bands of 150- and 120-kD co-migrating, respectively, with the αs and β1 proteins precipitated with the antibodies to the β1A (Fig. 3, lanes *a* and *b*). Thus the β1B is appropriately exported at the cell surface in association with hamster α subunits. The β1B was also specifically recognized by the polyclonal antibodies to the synthetic peptide specific of the β1B. These antibodies, in fact, reacted only with β1B transfected cells, and failed to recognize the classical β1A subunit (Fig. 3, lanes *e* and *f*).

To test the ability of the β1B to form a fibronectin-binding receptor, ^{125}I-labeled extract of β1B transfected cells were chromatographed through a fibronectin column. Elution of bound material with GRGDSP peptide yielded two bands of 150 and 120 kD (Fig. 4). Further elution of the column with

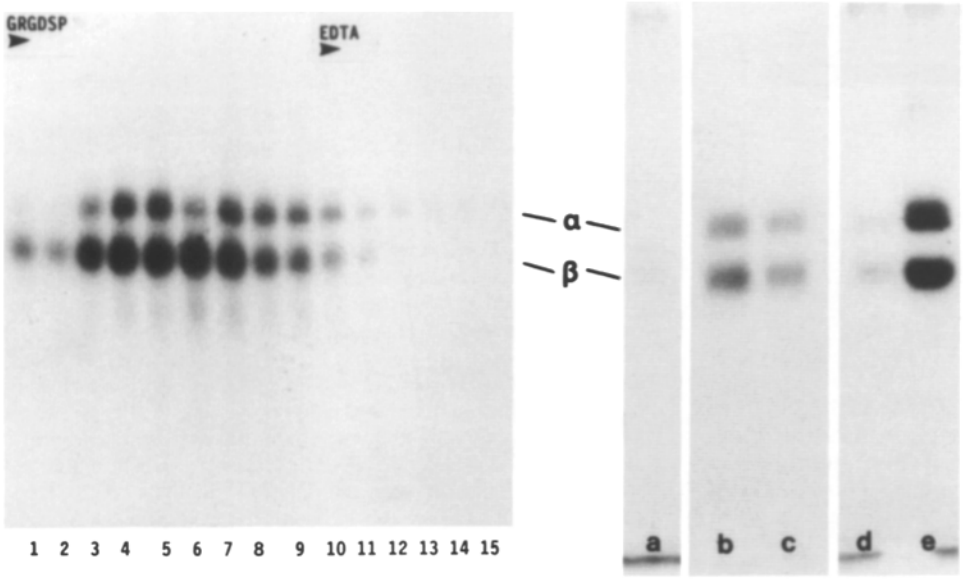

Figure 4. Binding of β1B integrin complexes to fibronectin affinity matrix. Detergent extracts from ^{125}I-labeled CHO cells expressing human β1B molecule (clone 18.24) were chromatographed through a fibronectin-Sepharose column. After extensive washing, bound material was eluted with buffer containing 1 mg/ml of the GRGDSP peptide or 10 mM EDTA. Aliquots of each fractions (1–15) were analyzed by SDS-PAGE under non-reducing conditions. The wash fractions prior to elution with GRGDSP were immunoprecipitated with antibodies to β1 (lane *a*). The bound and eluted fractions (1–9) were immunoprecipitated with: polyclonal antibodies to the classical β1 (lane *b*); mAb A1A5 to human β1 to identify the transfected β1B (lane *c*); polyclonal antibodies to α3 (lane *d*); and α5 (lane *e*) integrin subunits.

EDTA, known to release integrins from their ligands (Pytela et al., 1987), did not yield any further material (Fig. 4). The positive fractions were pooled and probed with antibodies for the two β1 forms and for α subunits. As shown in Fig. 4 both β1A and β1B are present in the eluted material together with α5. A small amount of α3 subunit was also detected consistently with the ability of this molecule to bind the RGD site of fibronectin (Elices et al., 1991). The β1B-containing complex can not be eluted with GRGESP peptide (not shown). These results indicate, thus, that β1B forms heterodimeric complexes with integrin α subunits and it binds fibronectin in an RGD-dependent manner.

The association of β1B with α subunits and expression at the cell surface was further demonstrated in the astrocytoma cell line that normally express this variant. These cells were surface labeled with ^{125}I and immunoprecipitated with β1A or β1B antibodies. As shown in Fig. 5, both antibodies precipitated a band of 130 kD (reduced). The intensity of the β1B band is ~30% than that of the β1A as determined by densitometric analysis. Assuming a comparable efficiency of the two antibodies, the β1B represents about one third of the total β1A in these cells. To test the association with α subunits, integrin complexes were first immunoprecipitated with α-specific antibodies from unlabeled cell extracts, and after SDS-PAGE and Western blotting, were probed with β1B antibodies. As shown in Fig. 5, β1B was found in association with both α1 and α5.

In conclusion these experiments indicate that the β1B molecule associates with α subunits, is correctly processed at the cell surface and binds fibronectin in a RGD-dependent manner.

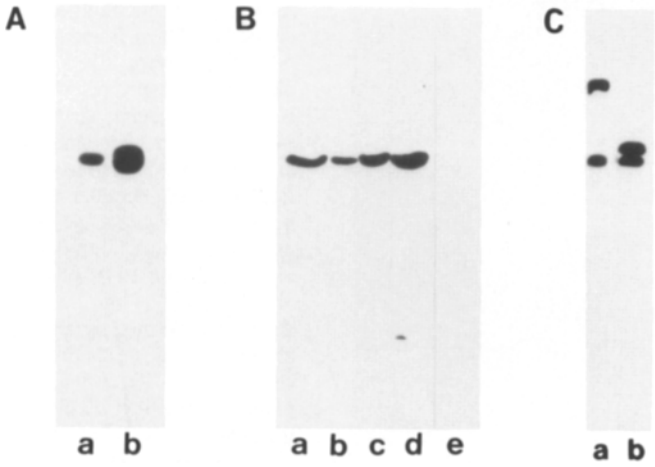

Figure 5. Immunoprecipitation of β1B integrin from human astrocytoma cells and association with α subunits. (*A*) ^{125}I surface-labeled astrocytoma cells were extracted with 0.1% SDS, and after re-naturation with Triton X-100, the sample was divided in two identical aliquots and immunoprecipitated with polyclonal antibodies to β1B (lane *a*) or β1A (lane *b*) cytoplasmic peptides. Radioactive proteins are visualized by fluorography after separation by SDS-PAGE under reducing conditions. (*B*) To analyze the association of β1B with α subunits, integrin complexes were immunoprecipitated from unlabeled astrocytoma cells under standard conditions using: (lane *a*) mAb 102DF7 to β1; (lane *b*) mAb TS2/7 against α1; (lane *c* and *d*) polyclonal antibodies to α5; and (lane *e*) anti-β3 antibody as a negative control. The immunoprecipitated material was separated by SDS-PAGE, transferred to nitrocellulose filters and probed with affinity purified antibodies to β1B cytoplasmic peptide followed by radiolabeled secondary antibodies. The autoradiography of the Western blot is shown. Note that the β1B subunit is precipitated in association with both α1 (lane *b*) and α5 (lane *c* and *d*) subunits under non-dissociating conditions, but it is not precipitated by β3 antibodies (lane *e*). (*C*) To demonstrate the expression of the α1/β1 and α5/β1 integrin complexes on astrocytoma cells, ^{125}I-labeled cells were immunoprecipitated under non dissociating conditions (Triton X-100 0.5%) with the mAb TS2/7 to α1 (lane *a*) and polyclonal antibody to α5 (lane *b*). Radioactive proteins are visualized by fluorography after separation by SDS-PAGE under non-reducing conditions.

Immunofluorescence Localization of β1B in Transfected Cells

To investigate the subcellular localization of the β1B in transfected cells, immunofluorescence experiments were performed. CHO cells plated on fibronectin coated dishes were starved in serum-free medium and treated with TGF-β as described in Materials and Methods. This treatment was required to obtain maximal organization of actin stress fibers and focal adhesions.

Analysis of transfected cells showed that the β1B molecule was uniformly distributed at the cell surface and did not concentrate at focal adhesions nor it co-localized with stress fiber ends (Fig. 6). Even in cells fully spread on the substratum with well organized actin stress fibers β1B was evenly distributed on the cell surface (Fig. 6, *d* and *e*). The uniform distribution of β1B was also observed in cells not treated with TGF-β and grown in the presence of serum and identical results were obtained with three independent clones (18.24, 18.6, 18.2) of β1B transfected CHO cells. Moreover, in β1B transfected cells, the endogenous hamster β1A localized to focal contacts (not shown), indicating that integrins in these cells retain the ability to cluster at cell substratum contacts. To further investigate this point we analyzed CHO cells transfected with human β1A. As shown in Fig. 7 the classical β1A was organized in patches and streaks co-localized with actin stress fibers and corresponding to interference reflection black areas (Fig. 7). The inability of β1B to form clusters at cell–substratum contacts was also demonstrated in the astrocytoma cell line that constitutively express this protein (not shown).

Discussion

We have investigated the expression and functional properties of a previously identified variant of the integrin β1 subunit. The data indicate that the variant protein (β1B) behaves similarly to the classical β1A in terms of association with α subunits and fibronectin binding but it has a restricted tissue expression and does not localize at focal contacts with the substratum.

We have originally identified the β1B at the cDNA level and the data reported here establish the existence of the β1B as protein molecule. The β1B cDNA most likely originate by premature termination of the transcription within the last intron of the β1 gene; in the mature β1B transcript the retained intronic sequence code for the unique COOH-terminal tail of β1B (Altruda et al., 1990). The existence of the β1B protein in a restricted number of cell types, as shown

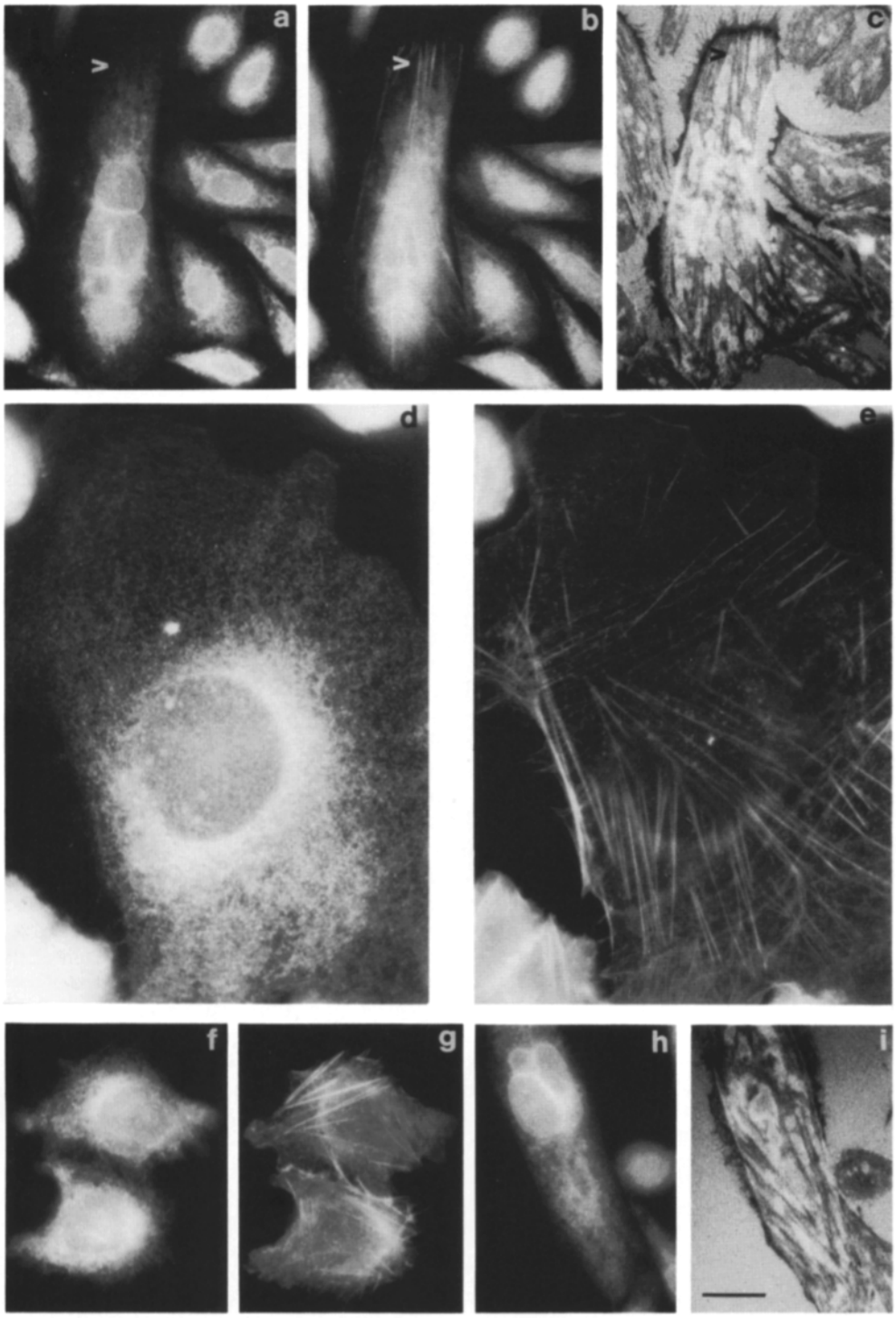

Figure 6. Immunofluorescence localization of human β1B integrin in transfected CHO cells. Cells expressing the transfected human β1B (clone 18.24) were plated on fibronectin-coated glass coverslips and treated with TGF-β in serum-free medium as described in Materials and Methods. Cells were fixed with paraformaldehyde, permeabilized with detergent, and then incubated with the monoclonal antibody A1A5 followed by RITC-labeled secondary antibody to localize β1B (*a*, *d*, *f*, and *h*). Actin fibers were visualized with FITC-phalloidin (*b*, *e*, and *g*) and interference reflection microscopy was used to identify focal adhesion sites (*c* and *i*). Arrowhead in *c* points to dark IRM streaks coincident with actin fibers (*b*) and showing no obvious clustering of β1B (*a*). Note also the diffuse staining for β1B in *d* and *f* showing no codistribution with actin fibers (*e* and *g*). The cell in *d* and *e* is a giant polyploid cell (normal-size cells are visible at the corners). The perinuclear staining with β1B antibodies (*a*, *d*, *f*, and *h*) is probably due to β1B accumulated in the Golgi apparatus. Bar, 5 μm.

in this paper, clearly demonstrates that the message is translated and strongly argues against the possibility that the β1B mRNA is generated by an incorrect transcription mechanism.

The β1B and the β1A are identical except for the COOH-terminal portion corresponding to the cytoplasmic domain. The cytoplasmic domain of the β1A is 47-amino acid (aa)[2] residues long, assuming Lys752 as first residue out of the lipid bilayer (Argraves et al., 1987). The last 21 aa residues of this domain are replaced in the β1B by a new, nonhomologous, sequence of 12 aa (see Fig. 1). Theoretical consideration suggests that this alteration should cause significant functional differences in the protein. In fact, the amino acid sequence of the cytoplasmic domain of the β1A is rigorously conserved during evolution. Comparative analysis of the *Xenopus* and human proteins indicate that the last 47-aa COOH-terminal residues are identical in the two species, while the remaining portion is 80% homologous (DeSimone et al., 1988). This indicates that molecular interactions of this domain pose rigorous structural constraints.

2. *Abbreviation used in this paper*: aa, amino acid.

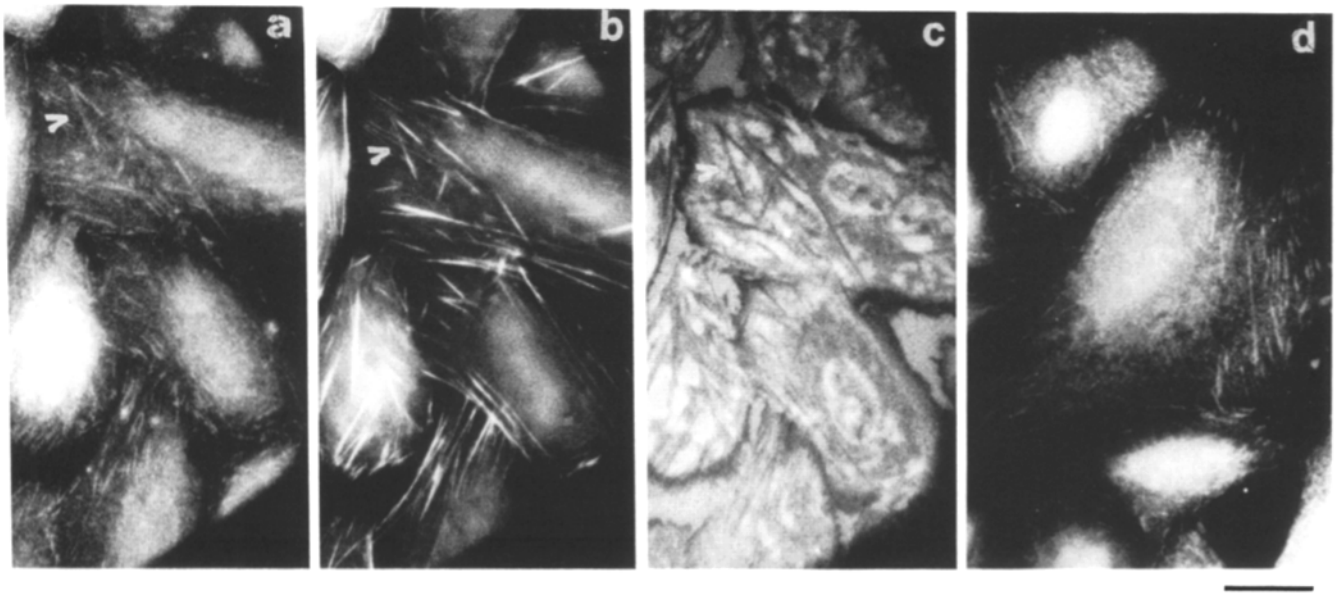

Figure 7. Immunofluorescence localization of human β1A integrin in transfected CHO cells. Cells expressing the transfected human β1A (clone 8.37) were plated on fibronectin-coated glass coverslips and treated with TGF-β in serum-free medium as described in Materials and Methods. Cells were fixed with paraformaldehyde, permeabilized with detergent and incubated with the mAb A1A5 followed by RITC-labeled secondary antibody to localize human β1A (*a* and *d*). Actin fibers were visualized with FITC–phalloidin (*b*) and interference reflection microscopy was used to identify focal adhesion sites (*c*). β1A is clustered in streaks (arrowheads in *a*) coincident with actin fibers (*b*) and with dark IRM areas (*c*). Bar, 5 μm.

The data presented here indicate that the new cytoplasmic sequence does not affect the ability of the β1B to form functional heterodimers with α subunits. In fact, the transfected β1B, as well as that normally expressed in a human astrocytoma cell line, associate with α subunits to form complexes that are expressed at the cell surface. Moreover, in association with α5, β1B form a functional complex capable of binding fibronectin in a RGD-dependent manner. Previous data from other laboratories have shown that an artificial deletion mutant of β1 molecule lacking the entire cytoplasmic domain shows normal capacity to associate with α subunit and to bind fibronectin (Hayashi et al., 1990; Marcantonio et al., 1990). Our data further indicate that the new sequence present in β1B does not confer specific properties to the molecule in these respects.

The β1B molecule, however, is functionally different from the β1A with respect to its subcellular localization. The variant form, in fact, does not localize at focal contacts in adherent cells, but it remains diffuse over the whole cell surface. This suggests that the β1B differ in its ability to interact with cytoplasmic components. The β1A is normally concentrated in focal contacts, specialized sites of cell–matrix adhesion (Burridge et al., 1988). At these sites, β1A co-localizes with vinculin, talin, and α actinin, three cytoskeletal proteins that are part of the molecular machinery connecting integrins with actin microfilaments. The ability to localize at focal contacts is lost if the cytoplasmic domain of β1A is artificially deleted (Hayashi et al., 1990; Marcantonio et al., 1990). In addition, analysis of several different deletions in this region of the molecule, suggests the existence of three sites important for focal contact localization (Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992). One is within residues 764-774 close to the lipid bilayer and the other two are further down-stream at residues 785-788 and 797-800 (Reszka et al., 1992). According to these data the β1B still retain the first site required for focal contact localization (residues 764-774), but is missing the two COOH-terminal ones, which are replaced by the new sequence. The presence of the new sequence abolishes the localization of β1B at focal contacts presumably altering the interaction with cytoskeletal component such as vinculin, talin, and alpha actinin. The existence of a naturally occurring variant of β1 with these properties poses the question of its possible role in cell adhesion. One intriguing possibility is that β1B act as a dominant negative regulator of cell adhesion.

An important finding is that β1B expression is restricted to certain cell types. This conclusion is based on a Western blot analysis of human tissue samples and of cultured cell lines with antibodies to a synthetic peptide specific for the β1B. In particular keratinocytes, hepatoma, and astrocytoma cells are positive, while several other cell types and tissues do not express detectable levels of the protein. The expression of β1B in positive cells reaches considerable levels. In fact, in the astrocytoma cell line the β1B is ~30% of the classical β1A. So far we have not detected cell types expressing only the β1B and lacking the β1A form. Previous analysis indicated expression of the β1B in several cell type including endothelial cells, lymphoma, hepatoma, and neuroblastoma cell lines (Altruda et al., 1990). This conclusion was based on the detection of the β1B transcript with a reverse PCR assay. This technique is very sensitive but is poorly, if at all, quantitative. Using antibodies we now find that among the cell lines previously tested only hepatoma cells express the β1B protein at detectable level, while endothelial, lymphoma, and neuroblastoma cells are negative. This indicates that, while very low levels of the β1B mRNA are present in most cells, the protein is accumulated only in some cell types.

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