Association of Intercellular Adhesion Molecule-1 (ICAM-1) with Actin-containing Cytoskeleton and α-actinin

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Abstract. We have studied the cytoskeletal association of intercellular adhesion molecule-1 (ICAM-1, CD54), an integral membrane protein that functions as a counterreceptor for leukocyte integrins (CD11/CD18). A linkage between ICAM-1 and cytoskeletal elements was suggested by studies showing a different ICAM-1 staining pattern for COS cells transfected with wild-type ICAM-1 or with an ICAM-1 construct that replaces the cytoplasmic and transmembrane domains of ICAM-1 with a glycophosphatidylinositol (GPI) anchor. Wild-type ICAM-1 appeared to localize most prominently in microvilli whereas GPI–ICAM-1 demonstrated a uniform cell surface distribution. Disruption of microfilaments with cytochalasin B (CCB) changed the localization of wild-type ICAM-1 but had no effect on GPI–ICAM-1. Some B-cell lines demonstrated a prominent accumulation of ICAM-1 into the uropod region whereas other cell surface proteins examined were not preferentially localized. CCB also induced redistribution of ICAM-1 in these cells. For characterization of cytoskeletal proteins interacting with ICAM-1, a 28-residue peptide that encompasses the entire predicted cytoplasmic domain (ICAM-1$_{478-505}$) was synthesized, coupled to Sepharose-4B, and used as an affinity matrix. One of the most predominant proteins eluted either with soluble ICAM-1$_{478-505}$-peptide or EDTA, was 100 kD, had a pI of 5.5, and in Western blots reacted with α-actinin antibodies. A direct association between α-actinin and ICAM-1 was demonstrated by binding of purified α-actinin to ICAM-1$_{478-505}$-peptide and to immunoaffinity purified ICAM-1 and by a strict colocalization of ICAM-1 with α-actinin, but not with the cytoskeletal proteins talin, tensin, and vinculin. The region of ICAM-1$_{478-505}$ interacting with α-actinin was mapped to the area close to the membrane spanning region. This region contains several positively charged residues and appears to mediate a charged interaction with α-actinin which is not highly dependent on the order of the residues.

LINKAGES between transmembrane proteins and cytoskeletal elements are crucial for a variety of cellular functions. Such functions include cell–cell and cell–matrix interactions, cell motility, maintenance of cell polarity, and directed exocytosis and endocytosis. Most of the information regarding the transmembrane and cytoskeletal molecules that interact and support these functions originate from observations of their spatial arrangements by immunostaining methods. These studies have suggested, for instance, that membrane-cytoskeleton interactions take place in specialized contacts such as focal adhesions (12) or desmosomes (54) and during internalization of receptors through coated pits (23).

In spite of the valuable information that morphological analysis has provided, alternative methods are required to study associations between transmembrane and cytoskeletal proteins at a molecular level. Increasing information using various techniques has accumulated recently about such linkages but few studies have described specific interactions between characterized proteins. Using equilibrium chromatography, a cytoskeletal protein talin, was shown to interact with fibronectin receptor (26), with the aid of a synthetic peptide, the β_1 subunit of the fibronectin receptor was shown to associate with another cytoskeletal protein, α-actinin (40). Using fusion proteins as an affinity matrix, an interaction between low density lipoprotein (LDL)[1] and mannose-6-phosphate receptors and clathrin adaptor proteins was characterized (21, 42).

In this work we have studied that cytoskeletal associations of intercellular adhesion molecule-1 (ICAM-1, CD54), an adhesion molecule that serves as a ligand for the leukocyte-integrins LFA-1 and Mac-1 and as a receptor for the major group of human rhinoviruses and strains of *Plasmodium falciparum* (5, 16, 24, 33, 46, 53). We show that ICAM-1 is associated with the actin-containing cytoskeleton and that this interaction leads to a specialized cell surface distribution of ICAM-1 both on adherent COS cells and on Epstein-Barr virus (EBV)-transformed B-cells. We further show evidence

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1. *Abbreviations used in this paper*: CCB, cytochalasin B; EBV, Epstein-Barr virus; GPI, glycophosphatidylinositol; TSA, Tris-Saline-Azide.

that α-actinin, an actin binding cytosolic protein, associates with a stretch of charged residues in the ICAM-1 cytoplasmic domain synthetic peptide and in cells codistributes with ICAM-1 suggesting that it may serve as an anchorage between ICAM-1 and actin-containing cytoskeleton.

Materials and Methods

Cell Lines, cDNAs, and Antibodies

JY, an EBV-transfected B-cell line, obtained from Dr. J. Strominger (Dana Farber Cancer Institute, Boston, MA), KOS (29) and SLA (2), EBV-transfected B-cell lines established from patients with leukocyte adhesion deficiency (LAD) (1), Raji, a Burkitt's lymphoma line (43) and COS-7, a monkey kidney epithelial cell line (22) were used in the studies.

COS cells were transfected with ICAM-1 cDNA subcloned into CDM8 expression vector (47) or with a construct, which replaces the transmembrane and cytoplasmic domains with a glycophosphatidylinositol (GPI) anchor. The construct was generated by replacing ICAM-1 cDNA encoding transmembrane and cytoplasmic domains with GPI signal sequence from LFA-3 (47). CDM8 vector alone was used as a control for transfections. Transfections were performed with the DEAE-dextran method as described (27). The cells were replated after 48 h and fixed for analysis 24–36 h later.

A mixture of two mouse mAb, RR1/1 (46) and R6.5 (49) or CL203 (31), was used to detect ICAM-1. LFA-1 was stained with a combination of three mAb N217, N225, and N226 obtained from IVth Leukocyte Typing Workshop (28). CD43 was detected with mAb N23, CD44 with mAb N85, and CD45 with mAb N88 all obtained from IVth Leukocyte Typing Workshop (Vienna). LFA-3 was detected with TS2/9 mAb (30), and class I MHC with mAb W6/32 (3). X63 IgG was used as a control mAb. Cytoskeletal proteins were detected with rabbit antisera against α-actinin and talin (kindly provided by K. Burridge, University of North Carolina, Chapel Hill, NC) (11, 40), tensin (62) (kindly provided by L. B. Chen, Dana Farber Cancer Institute, Boston, MA), and vinculin (kindly provided by I. Virtanen, University of Helsinki, Helsinki, Finland) (13).

Indirect Immunofluorescence Microscopy

Before fixation lymphoid cells were spun onto cytocentrifuge slides. The slides and transfected COS cells grown on Permanex slides (Nunc, Roskilde, Denmark) were fixed in 3.5% paraformaldehyde in PBS, pH 7.4, at 4°C for 10 min. For double staining of ICAM-1 and α-actinin the cells were fixed in −20°C methanol. mAb were used as 10 μg/ml of purified IgG (R6.5) diluted in PBS, as undiluted hybridoma culture supernatants (RR1/1, TS2/9, W6/32, X63) or as 1:500 dilution of ascites (workshop antibodies). The slides were rinsed and reacted with FITC-conjugated goat F(ab')2 anti-mouse Ig (Tago Inc., Burlingame, CA). For double stainings the cells were further permeabilized in 0.1% Triton X-100/PBS, stained with rabbit antisera or normal rabbit serum (1:100 dilution) and with rhodamine-conjugated goat F(ab')2 anti-rabbit Ig (Tago Inc.). Double staining of ICAM-1 and α-actinin in methanol fixed slides was done according to a similar protocol except that 0.1% Triton X-100 was omitted. F-actin was detected with rhodamine-phallacidin (Molecular Probes Inc., Eugene, OR). The slides were mounted in glycerol/PBS and viewed with a Zeiss epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed with Kodak T-Max P3200 black and white film (Eastman Kodak Co., Rochester, NY).

Modification of Cell Surface Distribution of ICAM-1

The following agents were tested for alteration of ICAM-1 distribution: NaN_3 (Sigma Chemical Co., St. Louis, MO), final concentration 10^{-3} M, colchicine (Sigma Chemical Co.) 10 μg/ml, cytochalasin B (CCB) (Calbiochem Corp., La Jolla, CA) 10 μg/ml, EDTA (Sigma Chemical Co.) 10 mM and PMA (Calbiochem Corp.) 50 ng/ml. JY cells were treated with PMA for 20 min and with other agents for 60–120 min before preparation of the slides and fixation. After staining the distribution of ICAM-1 was analyzed from coded slides and at least 200 cells/slide were counted.

Coupling of Synthetic Peptides

A synthetic peptide corresponding to the 28-amino acid cytoplasmic domain of ICAM-1 (ICAM-$1_{478-505}$) (48, 51), two shorter peptides of the NH_2-terminal region of ICAM-1 cytoplasmic domain, a peptide of ICAM-1 cytoplasmic amino acids in reverse order (ICAM-$1_{505-478}$) and control peptide 4 were synthesized with the solid phase method (34). Peptides ICAM-$1_{478-505}$ and ICAM-$1_{505-478}$ were synthesized with an Applied Biosystems synthesizer (model 430; Applied Biosystems Inc., Foster City, CA) using small scale rapid cycle-tBOC chemistry option and were cleaved from the resin by HF. Peptides ICAM-$1_{478-486}$ and ICAM-$1_{480-484}$ were synthesized with an Applied Biosystems synthesizer (model 431) using the FMOC chemistry option and were cleaved by trifluoroacetic acid. All peptides were purified by reversed phase HPLC on a C18 column using aqueous trifluoroacetic acid (0.1%)/acetonitrile-based mobile phases. Purified peptides were characterized by HPLC and fast atom bombardment mass spectrometry. Three lysine-rich control peptides (nos. 1–3) were a kind gift from Dr. H. Rauvala (Department of Biochemistry, University of Helsinki). All peptides were coupled to CnBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) at a concentration of 2 mg/ml except for ICAM-$1_{478-505}$ when used for affinity chromatography, in which case it was coupled at 4 mg/ml. The efficiency of coupling was verified spectrophotometrically by absorption at 214 nm before and after coupling. Coupling efficiency always exceeded 95%. After coupling, Sepharose was blocked with 1 M ethanolamine, pH 9.0.

Peptide Affinity Chromatography

Placental tissue was homogenized with a tissue grinder and solubilized in lysis buffer (50 mM octyl-β-D-glucopyranoside, 50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM PMSF, 5 μg/ml leupeptin). Insoluble material was removed by centrifuging the lysate at 100,000 *g* for 60 min at 4°C and the lysate supernatant was filtered through a Whatman 1 filter paper (Whatman Chemical Separation Inc., Clifton, NJ). The lysate was passed through an ethanolamine-Sepharose precolumn and ICAM-$1_{478-505}$-peptide–Sepharose column. The column was washed with 50 column volumes of lysis buffer. Bound material was eluted with 2 mg/ml of soluble peptide in lysis buffer or with lysis buffer in which divalent cations were replaced with 10 mM EDTA, pH 8.0. 1.5-ml fractions were collected and analyzed by SDS-PAGE and silver staining (37).

Western Blotting and Two-dimensional Gel Electrophoresis

Material that eluted from the ICAM-$1_{478-505}$-peptide–Sepharose column was separated in 8% SDS-PAGE minigels (Bio-Rad Laboratories, Richmond, CA) and transferred to nitrocellulose paper (200 mA, 45 min). The sheets were blocked in 2% BSA in Tris-Saline-Azide (TSA) (50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 0.1% NaN_3). The blots were reacted with rabbit antisera against cytoskeletal proteins or with normal rabbit serum as a 1:500 dilution in blocking buffer. The blots were washed twice with washing buffer (BSA/TSA/0.1% NP-40), incubated in ^{125}I-protein A (Amersham Corp., Arlington Heights, IL) in BSA/TSA, washed twice, and autoradiographed.

For two-dimensional (2-D) IEF SDS-PAGE eluted material was concentrated with Centricon-30 microconcentrators (Amicon). The 2-D gel analysis was done using Mini-Protean II 2-D gel system (Bio-Rad Laboratories). Isoelectric focusing was performed according to the method by O'Farrell (39). Separated proteins were visualized by staining with Coomassie blue R-250.

Iodination of Cytoskeletal Proteins

Chicken gizzard α-actinin purified as described (20) was kindly provided by M. Beckerle (University of Utah, Salt Lake City, UT) and vinculin by I. Virtanen. The proteins were iodinated using Iodogen. Iodogen (Pierce Chemical Co., Rockford, IL) was dissolved in chloroform (1 mg/ml) and 60 μl was dried to the walls of an eppendorf tube under argon stream. The protein in 200 μl of TSA was mixed with 0.5 mCi of ^{125}I (Amersham Corp.) and the solution was transferred to the Iodogen-coated tube. The reaction was terminated by transferring the solution to a tube containing 20 μl of tyrosine (0.4 mg/ml) in borate-saline. Free ^{125}I was separated from iodinated protein in a PD10 Sephadex G-25 M column (Pharmacia Fine Chemicals) equilibrated with 20 mM NaCl, 0.1 mM EDTA, 20 mM Tris Acetate, pH 7.6, 0.1% β-mercaptoethanol, 0.02% NaN_3.

Interaction of α-actinin with Peptides

^{125}I–α-actinin or ^{125}I-vinculin (60,000 cpm) was reacted in an affinity precipitation assay with 10 μl of 1:1 slurry of peptide-Sepharose/TSA in 200 μl of binding buffer (50 mM Tris/HCl, pH 7.4, 0.15 M NaCl, 0.5% Tween

20, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The mixture was shaken for 5 h at 4°C. Sepharose was washed five times with binding buffer containing 0.1% BSA. Material bound to Sepharose was counted for radioactivity or eluted with 0.1 M NaOH, 2% SDS and analyzed in SDS-PAGE and autoradiography. Tranexamic acid and ε-aminocaproic acid added to some experiments were purchased from Sigma Chemical Co.

Purification of ICAM-1

ICAM-1 was purified from placental lysate by immunoaffinity chromatography using Sepharose coupled-RR1/1 mAb as described (33) except that the lysis buffer and washing buffers contained 10 mM EDTA instead of $MgCl_2$. The purity of the material was verified by SDS-PAGE and silver staining.

Solid-Phase Assay of ICAM-1–α-actinin Interaction

Purified ICAM-1 or purified α-actinin was allowed to bind to 96-well flat-bottomed polystyrene plates (Linbro, Flow Laboratories, Hamden, CT) overnight at 4°C. The following day the plates were washed and subsequently blocked with 1% heat-denatured BSA in TSA overnight at 4°C. Dilutions of α-actinin were added to ICAM-1 or BSA-coated wells and dilutions of ICAM-1 to α-actinin or BSA-coated wells. Binding buffer was used in all dilutions and washes. After overnight incubation at 4°C the wells were washed five times by flicking the plates. Bound α-actinin was detected using rabbit α-actinin serum and ^{125}I-protein A (Amersham Corp.). Bound ICAM-1 was detected by CL203 mAb labeled with ^{125}I using Iodogen.

Results

The Distribution of Wild-Type ICAM-1 and Glycophosphatidylinositol-anchored ICAM-1 on Transfected COS Cells

COS cells were transfected with cDNA encoding either ICAM-1 or an engineered form of ICAM-1 which is anchored to the cell membrane through a glycophosphatidylinositol anchor. The cell surface distribution of the two forms of ICAM-1 was examined by indirect immunofluorescence microscopy. Wild-type ICAM-1 staining was most prominent in microvillar projections on the surface of the cells (Fig. 1 *A*). The distribution colocalized with F-actin staining present in these areas but not with actin-containing stress fibers (Fig. 1 *B*). The majority of GPI–ICAM-1, on the other hand, was uniformly distributed on the cell membrane (Fig. 1 *C*). COS cells expressing GPI–ICAM-1 occasionally contained brightly stained round-shaped areas on the membrane or in the space between cells (Fig. 1 *G*). These apparently represented membrane components shed by the cells and were not seen in cells expressing wild-type ICAM-1. Treatment of the cells with a microfilament disrupting agent, CCB, resulted in a loss of stress fibers and microvilli and formation of large F-actin clusters (Fig. 1, *F* and *H*). Concommitantly the distribution of wild-type ICAM-1 in these cells was altered. Most of the ICAM-1 in the CCB-treated cells colocalized with the areas containing clustered actin, although uniformly distributed ICAM-1 was somewhat increased in comparison with untreated cells (Fig. 1 *E*). In contrast, GPI–ICAM-1 distribution was not affected by CCB treatment (Fig. 1 *G*). These results suggested that in transfected COS cells wild-type ICAM-1 is associated with the actin-containing microfilament network and that the interaction is mediated by the cytoplasmic or transmembrane domains of ICAM-1.

The Distribution of ICAM-1 in Lymphoid Cells

EBV-transformed B-cells demonstrated an ICAM-1 distribution pattern that differed from transfected COS cells. In a fraction of the cells (usually between 20 and 50%), ICAM-1 was concentrated into one side of the cell membrane, often into a small area in the uropod region (Fig. 2 *A*). Such a pattern was not typical for all cell surface components since several other B-cell membrane proteins that we examined were uniformly distributed (Fig. 2, *B–G*). The accumulation of ICAM-1 in the uropod was detected in three EBV-transformed B-cell lines studied, JY, KOS, and SLA. Two of the EBV-transformed lines were derived from patients with defective expression of leukocyte-integrins LFA-1, Mac-1, and p150/95 indicating that the expression of receptor molecules for ICAM-1 does not regulate its distribution (not shown).

Regulation of ICAM-1 Distribution in B-cell Lines

To investigate how ICAM-1 localization is regulated in B-cell lines its distribution was determined after treatment with agents that affect cellular functions (Fig. 3). Treatment of the cells with the energy-depleting agent NaN_3 or with the divalent cation chelator EDTA decreased the number of cells in which ICAM-1 concentrated in the uropod region. Disruption of microfilaments with CCB led to even more drastic change; virtually all cells had a uniform distribution of ICAM-1. Similarly, activation of protein kinase C with PMA caused ICAM-1 to dislocate from the uropod and become uniform. In contrast to the CCB effect, disruption of microtubules with colchicine did not lead to a uniform distribution of ICAM-1 but increased the number of cells with polarized ICAM-1.

Codistribution of ICAM-1 with α-actinin

The results described above suggested that a link exists between ICAM-1 and the actin-containing cytoskeleton in B-cells. To further analyze which cytoskeletal proteins might anchor ICAM-1 to microfilaments the cells were double stained to localize ICAM-1 and various cytoskeletal elements. The staining of JY cells revealed a striking colocalization between ICAM-1 and α-actinin (Fig. 4, *A* and *E*). All cells that had polarized ICAM-1 also showed polarized α-actinin. On the other hand, talin, tensin (Fig. 4, *F* and *G*), and vinculin (not shown), three other cytoskeletal proteins, demonstrated no preferential distribution in the areas where ICAM-1 was concentrated. PMA treatment, which dissociated ICAM-1 from concentrated areas, also resulted in a loss of polarized α-actinin (not shown). The distribution of ICAM-1 on a Burkitt's lymphoma line Raji was not polar (Fig. 4 *D*). On typical Raji cells ICAM-1 was most prominently localized on microvilli-like hairy projections present on the cell surface. Accordingly, α-actinin was also more evenly distributed in Raji cells than in JY cells (Fig. 4, *D* and *H*). A slightly punctate α-actinin staining pattern gave an impression of concentration of the protein to microvilli. However, colocalization with ICAM-1 was not as evident as in JY cells.

Isolation of ICAM-1 Cytoplasmic Peptide Binding Proteins

To characterize molecules that associate with the cytoplasmic domain of ICAM-1, a 28-amino acid peptide corresponding to the cytoplasmic sequence of ICAM-1 (ICAM-$1_{478-505}$) (Fig. 5) was synthesized. The peptide was coupled to CNBr-activated Sepharose CL-4B and used as an affinity matrix. We have found that placental tissue can serve as a

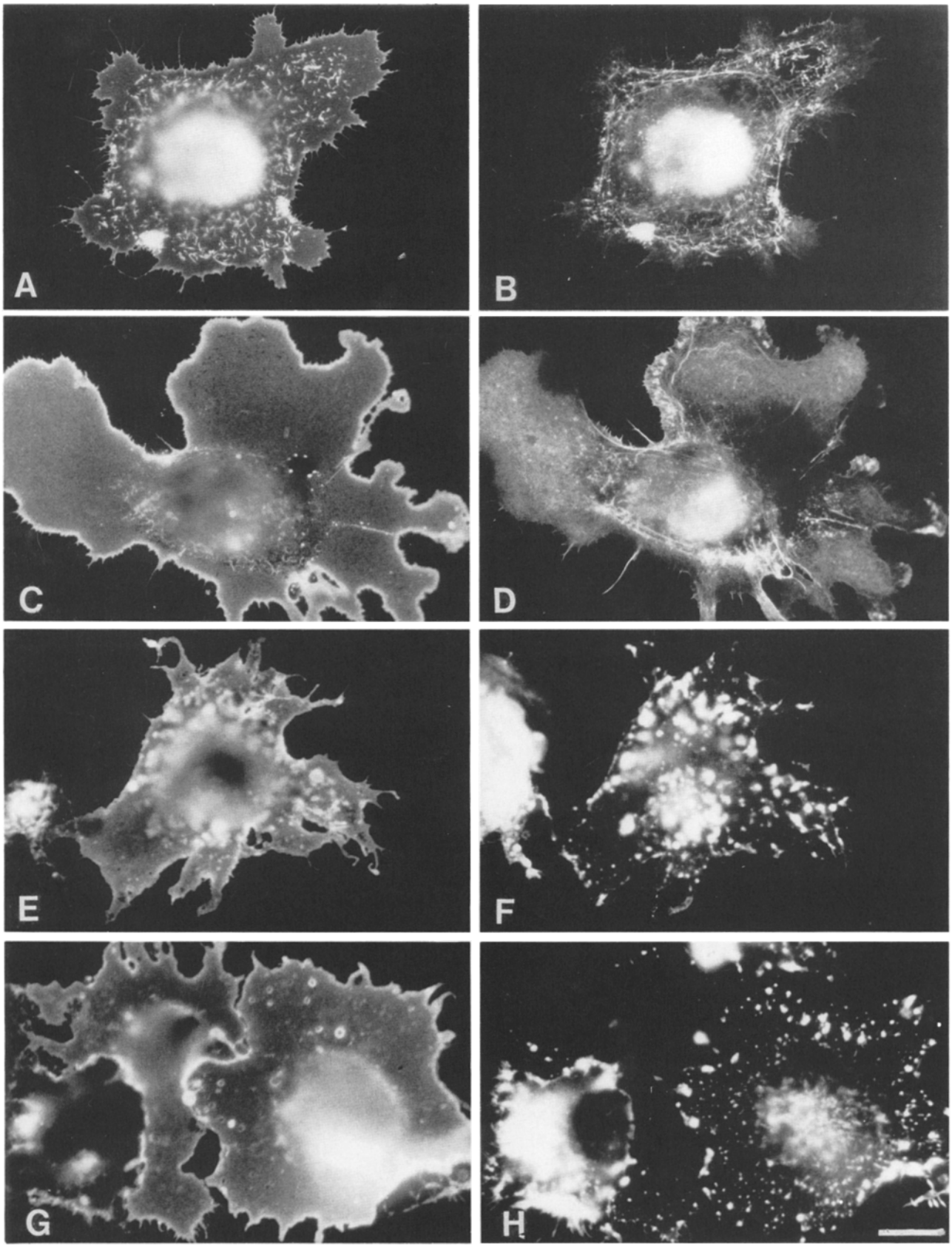


Figure 1. The distribution of wild-type ICAM-1 and GPI–ICAM-1 on COS cells and the effect of cytochalasin B on the distribution. COS cells transfected with cDNA for wild-type ICAM-1 (*A*, *B*, *E*, and *F*) or GPI–ICAM-1 (*C*, *D*, *G*, and *H*) were grown on plastic slides. The cells were fixed and ICAM-1 was visualized by mAb and FITC-conjugated goat F(ab')2 anti–mouse Ig (*A*, *C*, *E*, and *G*). F-actin was visualized by rhodamine–phallacidin (*B*, *D*, *F*, and *H*). In *E–H*, the cells were treated with 10 μg/ml of CCB for 60 min before fixation. Bar, 10 μm.

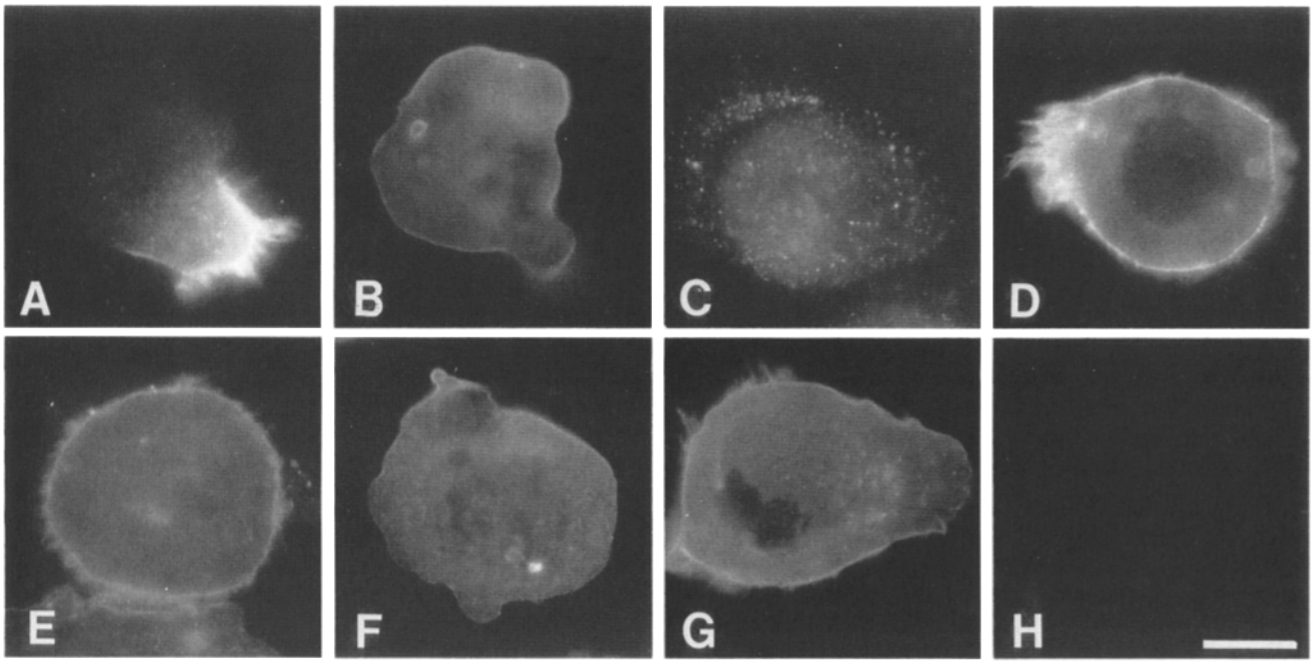


Figure 2. Immunofluorescence localization of membrane proteins on EBV-transformed B-cell line JY. The cells were cytocentrifuged onto slides, fixed, and stained with mAb against ICAM-1 (*A*), LFA-1 (*B*), CD43 (*C*), CD44 (*D*), CD45 (*E*), LFA-3 (*F*), HLA (*G*), or a control mAb X63 (*H*) followed by FITC-conjugated goat F(ab')2 anti-mouse Ig. Bar, 10 μm.

source for immunoaffinity purification of ICAM-1 (see Materials and Methods). Thus, it was predicted that it would also contain ICAM-1-associated proteins. Solubilized placental proteins were passed through an ICAM-$1_{478-505}$-peptide–Sepharose column and after extensive washing the bound material was eluted either with soluble ICAM-$1_{478-505}$ peptide or with EDTA (Fig. 6 *A*). Both soluble ICAM-$1_{478-505}$ peptide and EDTA eluted a similar profile of polypeptides from the column. No major protein bands could be detected in material eluted from the ethanolamine precolumn using either elution condition. In control experiments, elution of the ICAM-$1_{478-505}$-peptide–Sepharose column with an irrelevant peptide (a cytoplasmic domain peptide of β_2-integrins, CD18) did not release any bound material from the column. To analyze whether any of the proteins bound to ICAM-$1_{478-505}$-peptide–Sepharose correlated to known cytoskeletal structures, Western blot analysis was performed using different antisera (Fig. 6 *B*). Antiserum against α-actinin reacted with a 100-kD band eluted from the column whereas talin, vinculin (Fig. 6 *B*), or tensin (not shown) antisera did not react with the eluted material. In 2-D-gel electrophoresis the 100-kD protein had a pI of 5.3–5.5 (not shown) which correlates with the published pI of 5.46 for α-actinin (9).

control	15.7 %	30.4 %	53.9 %
NaN3	4.8 %	18.9 %	76.3 %
EDTA	4.8 %	22.6 %	72.6 %
CCB	0.0 %	1.9 %	98.1 %
Colchicine	26.4 %	34.1 %	39.5 %
PMA	2.8 %	11.8 %	85.4 %

Figure 3. The effect of various treatments on ICAM-1 distribution on JY cells. JY cells were incubated in the presence of the indicated drugs, cytocentrifuged on slides, fixed, and stained for ICAM-1. The distribution of ICAM-1 on the cell surface was determined from 200 cells. The values represent an average of two experiments with virtually similar results.

Association of α-actinin with ICAM-1 Cytoplasmic Domain

α-actinin was among the proteins that bound to an ICAM-$1_{478-505}$-peptide column. However, the result did not prove a direct interaction with ICAM-1. Therefore, further experiments were performed to determine if α-actinin can bind directly to ICAM-$1_{478-505}$-peptide or immunoaffinity-purified ICAM-1. Purified α-actinin and another cytoskeletal protein, vinculin, were iodinated and incubated with ICAM-$1_{478-505}$-peptide Sepharose. α-actinin but not vinculin bound directly to the cytoplasmic domain of ICAM-1 (Fig. 7 *A*). The interaction between Sepharose-bound ICAM-$1_{478-505}$ and α-actinin could be competed with soluble ICAM-$1_{478-505}$ peptide (Fig. 7 *B*).

An interaction between purified ICAM-1 and α-actinin was studied in two reciprocal solid-phase assays. Immunoaffinity-purified ICAM-1 or purified α-actinin was absorbed to polystyrene plates and the plates were blocked with BSA. Soluble α-actinin or ICAM-1, respectively, were added to the wells, unbound proteins were washed away, and the association with the immobilized proteins was measured

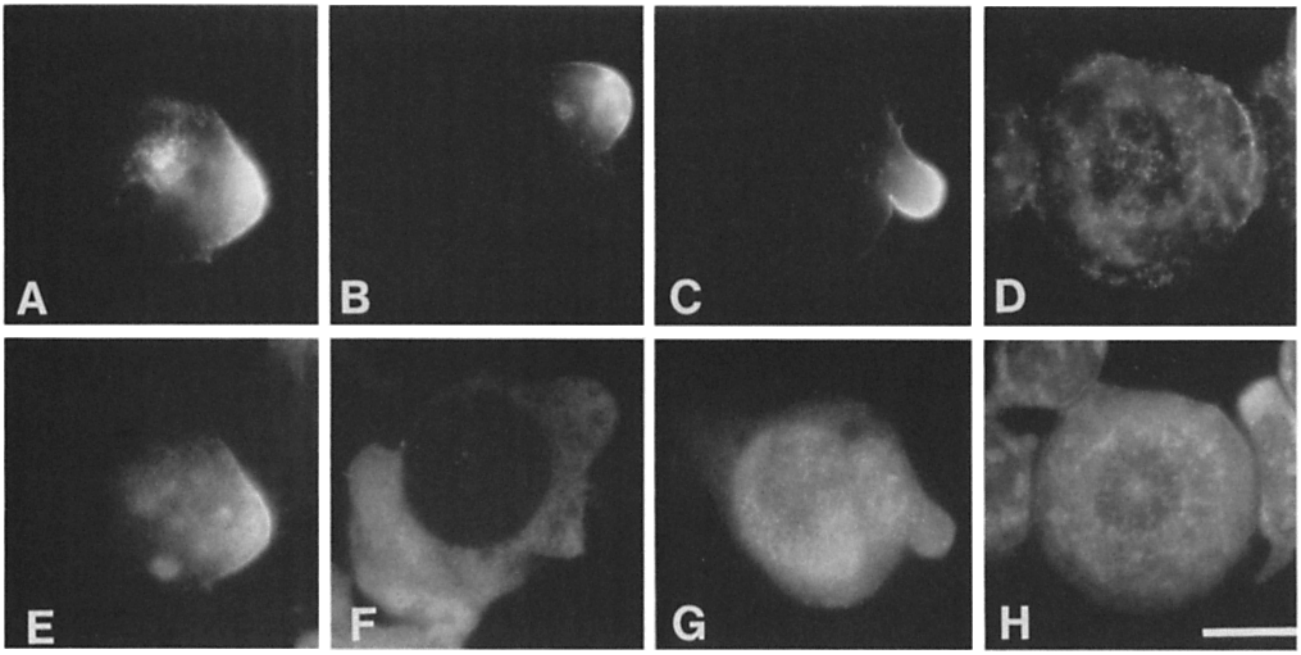


Figure 4. Double immunofluorescence staining of ICAM-1 and cytoskeletal proteins on EBV-transformed B-cell line JY and on Burkitt's lymphoma line Raji. JY (*A–C*, and *E–G*) or Raji (*D* and *H*) cells were cytocentrifuged onto slides and fixed. ICAM-1 was detected with mAb and FITC-conjugated goat F(ab')2 anti-mouse Ig (*A–D*). Cytoskeletal proteins in the same cells were detected with rabbit antisera against α-actinin (*E* and *H*), talin (*F*), or tensin (*G*) and rhodamine-conjugated goat F(ab')2 anti-rabbit Ig. Bar, 10 μm.

using α-actinin antiserum and radiolabeled protein A or anti-ICAM-1 mAb. Bound α-actinin was detected in wells containing immobilized ICAM-1 but not in wells coated with BSA only (Fig. 8 *A*). In a complementary assay, soluble ICAM-1 bound to wells with immobilized α-actinin, but not to control wells (Fig. 8 *B*). No radioactivity was detected in wells containing immobilized molecules if soluble ICAM-1 or α-actinin were not added (Fig. 8, *A* and *B*) demonstrating the specificity of the detection method.

Analysis of the Region in ICAM-1 Cytoplasmic Domain that Interacts with α-actinin

To localize the region in the ICAM-1 cytoplasmic domain that mediates interaction with α-actinin, affinity precipitation experiments using several peptides and radiolabeled α-actinin were performed. The different peptides are listed in Fig. 5. One of the peptides contained the 28-ICAM-1 cytoplasmic domain amino acids in reverse order. This peptide (ICAM-$1_{505-478}$) bound to α-actinin in a quantitatively similar way as ICAM-$1_{478-505}$ (Fig. 9 *A*). This result demonstrated that the interaction is not specific for a specific amino acid sequence. The ICAM-1 cytoplasmic sequence has a stretch of positively charged amino acids (three lys and three arg in nine residues) close to the transmembrane region. Two shorter peptides, one with all nine amino acids (ICAM-$1_{478-486}$) and another with five (ICAM-$1_{480-484}$) were also tested for binding to α-actinin. Both peptides were able to interact with α-actinin at levels comparable to ICAM-$1_{478-505}$ peptide. Moreover, the nine-amino acid peptide effectively competed for α-actinin binding with the ICAM-$1_{478-505}$ peptide (Fig. 9 *A*). The residues 478–486 appeared to solely be responsible for the interaction, since a peptide (ICAM-$1_{486-505}$) containing the cytoplasmic amino acids except those residues did not bind to α-actinin.

Since positive charge may be important for the interaction we tested non-ICAM-1-derived peptides for α-actinin binding. We used three lysine-rich peptides and a peptide with several adjacent arginine residues; all the peptides having a net positive charge. None of these control peptides showed significant association with α-actinin (Fig. 9 *A*) indicating that net positive charge alone is not sufficient to provide interaction with α-actinin.

Peptide	Sequence
ICAM-$1_{478-505}$	RQRKIKKYRLQQAQKGTPMKPNTQATPP
ICAM-$1_{478-486}$	RQRKIKKYR
ICAM-$1_{480-484}$	RKIKK
ICAM-$1_{486-505}$	RLQQAQKGTPMKPNTQATPP
ICAM-$1_{505-478}$	PPTAQTNPKMPTGKQAQQLRYKKIKRQR
Control 1	GKKEKPEKKVKKSDC
2	GKGDPKKPRGK
3	KPDAAKKGVVC-amide
4	YNIMIEEDRRRRQ

Figure 5. Peptides used for studies on ICAM-1–α-actinin interaction.

Nature of ICAM-1–α-actinin Interaction

In further experiments the nature of α-actinin binding to ICAM-$1_{478-505}$ was characterized. The interaction appeared to be sensitive to an increase of ionic strength. When the

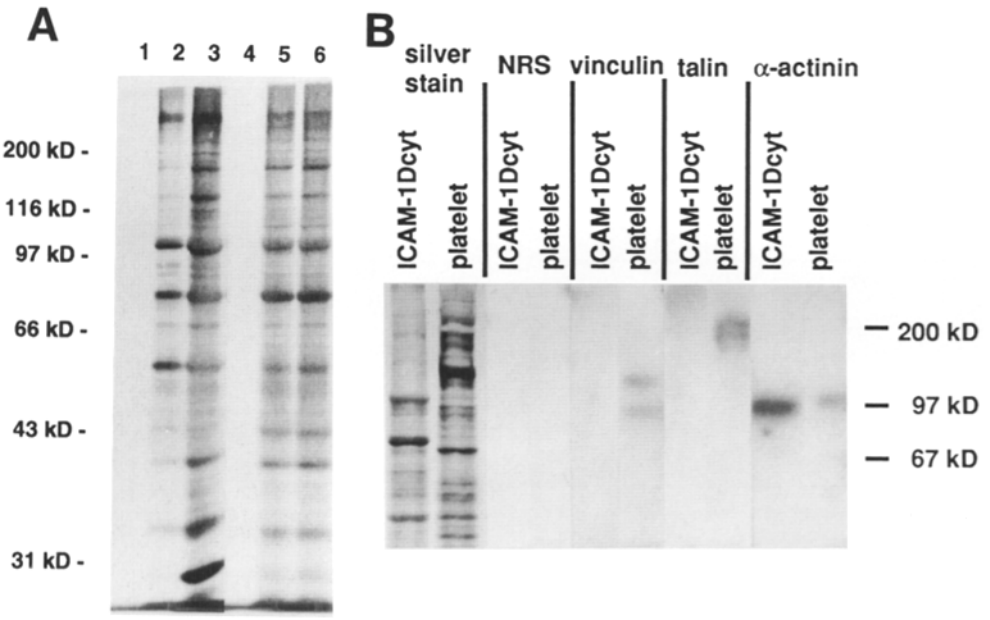
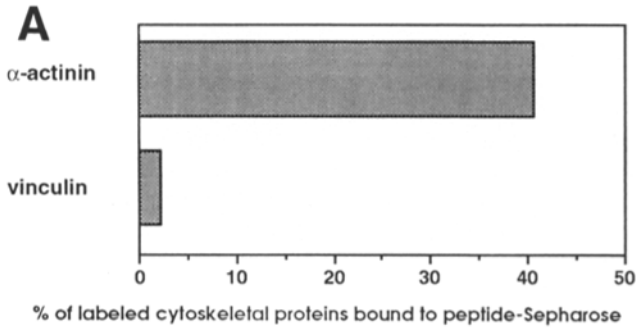


Figure 6. SDS-PAGE and immunoblot analysis of proteins eluted from ICAM-1 cytoplasmic domain peptide column. (*A*) Placental lysate was passed through an ICAM-$1_{478-505}$-peptide–Sepharose column and the column was washed extensively (lane *1*). The following elution conditions were applied sequentially: soluble ICAM-$1_{478-505}$-peptide (0.1 mg/ml) (lanes *2* and *3*), lysis buffer (lane *4*), and lysis buffer with 10 mM EDTA and no divalent cations (lanes *5* and *6*). Eluted material was analyzed in SDS-PAGE and the proteins were visualized by silver staining. (*B*) Proteins from ICAM-$1_{478-505}$-peptide–Sepharose column elution and from a control platelet lysate were separated in SDS-PAGE and detected by silver staining or blotted to nitrocellulose filters. Cytoskeletal proteins were probed with the indicated rabbit antisera or normal rabbit serum (*NRS*) and ^{125}I-protein A.

molarity of NaCl was raised from 0.15 to 0.3 M or above the interaction was markedly reduced or totally abolished (Fig. 9 *B*). Chelation of divalent cations with EDTA also decreased the binding of α-actinin and ICAM-$1_{478-505}$ (Fig. 9 *B*) as could be expected, since α-actinin was eluted from the ICAM-$1_{478-505}$-peptide–Sepharose column with EDTA. The requirement of a native form of α-actinin was tested by denaturation with heat or SDS before binding. Although both treatments, either alone (not shown) or in combination (Fig. 9 *B*), reduced the level of interaction, a significant amount of binding remained between denatured α-actinin and ICAM-$1_{478-505}$.

The presence of lysine residues in the region of the ICAM-1 cytoplasmic domain associating with α-actinin raised the possibility that the interaction might resemble plasminogen activator binding to plasminogen. This interaction is mediated by a lysine binding region in plasminogen and is effectively blocked with ω-aminocarboxylic acids (35). We tested the effect of two ω-aminocarboxylic acids, tranexamic acid, and ε-aminocaproic acid on ICAM-$1_{478-505}$-peptide–α-actinin binding. Neither compound inhibited binding, even at concentrations 100-fold greater than those reported to block the lysine binding sites (Fig. 9 *B*), suggesting no similarity between the two types of interactions.



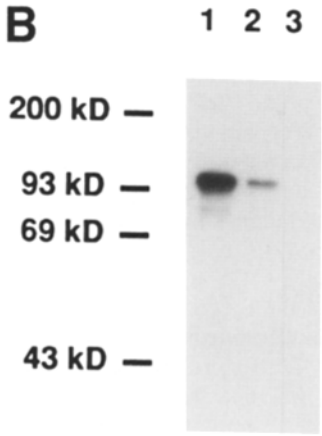


Figure 7. Association of purified α-actinin but not vinculin with ICAM-$1_{478-505}$-peptide. (*A*) 60,000 cpm of ^{125}I–α-actinin or ^{125}I-vinculin was incubated with ICAM-$1_{478-505}$-peptide–Sepharose. Unbound material was removed by washing and the Sepharose-bound radioactivity was counted. (*B*) 60,000 cpm of ^{125}I–α-actinin was incubated with ICAM-$1_{478-505}$-peptide–Sepharose (lane *1*), with ICAM-$1_{478-505}$-peptide–Sepharose in presence of fivefold excess of soluble ICAM-$1_{478-505}$-peptide (lane *2*) or with control ethanolamine–Sepharose (lane *3*). Bound material was eluted, separated in SDS-PAGE, and autoradiographed.

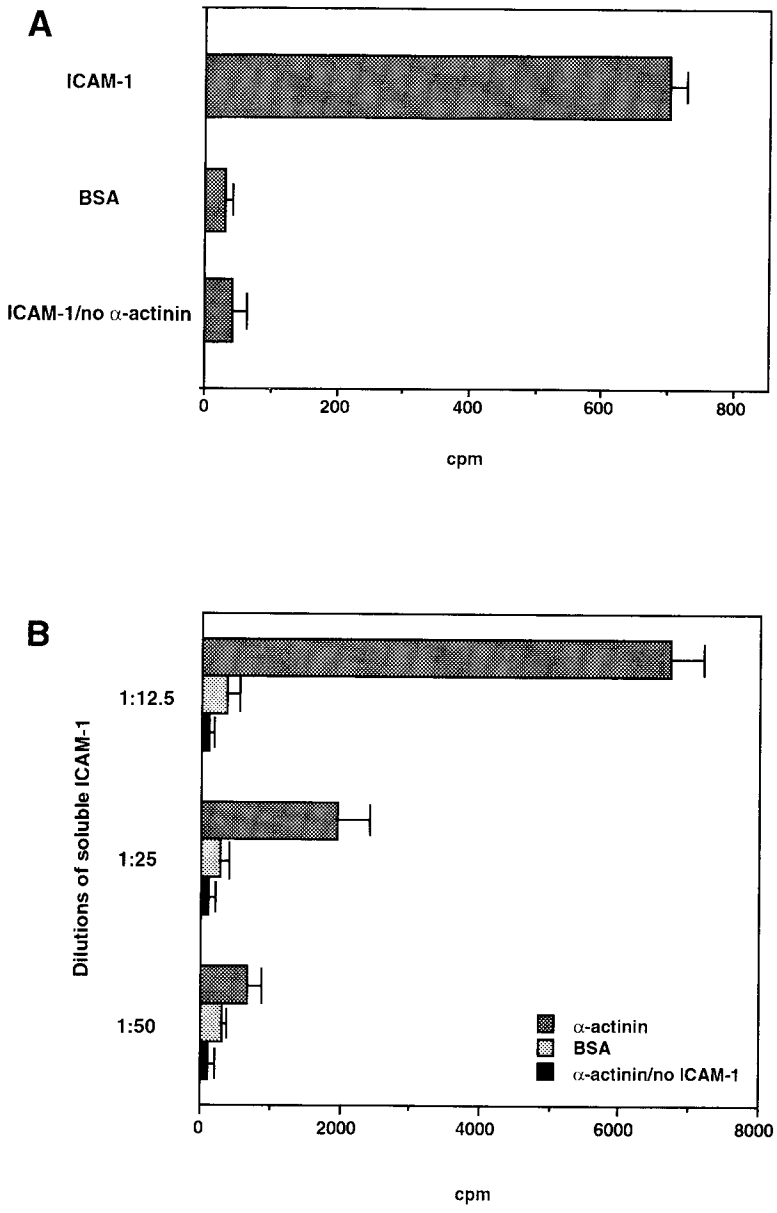


Figure 8. Radioimmunoassay showing association of α-actinin with purified ICAM-1. Soluble α-actinin (*A*) or ICAM-1 (*B*) was added to wells coated with ICAM-1 (*A*) or α-actinin (*B*), respectively, and blocked with BSA. In *A*, bound α-actinin was detected with α-actinin antiserum and ^{125}I-labeled protein A. In *B*, bound ICAM-1 was detected by ^{125}I-labeled ICAM-1 mAb. Dilutions in *B* were made from ICAM-1 stock obtained from immunoaffinity chromatography purification. Mean ± SD of triplicate wells.

Discussion

In this study we show that ICAM-1, a cell surface protein involved in cell adhesion, is associated with the actin-containing cytoskeleton. This was first suggested by our findings that the distribution of ICAM-1 in both adherent COS cell transfectants and in nonadherent B-cell lines is modified by agents that dissociate the actin-containing microfilament network but not by disruption of microtubules. The different distribution of wild-type ICAM-1 and GPI–ICAM-1 indicates that such an interaction is mediated through the transmembrane or cytoplasmic domains of the molecule.

The localization of wild-type ICAM-1 in transfected COS cells was different from most of the B-cell lines studied. In the B-cells ICAM-1 was often concentrated to the uropod region of the cells whereas in COS cells preferential staining of the microvillar projections was detected. Whether the more intense ICAM-1 staining in the microvilli truly reflects higher concentation of the molecules per surface area or is merely a result of additive effects of membrane projections is not clear but comparison to the uniform cell surface staining pattern of GPI–ICAM-1 suggests that ICAM-1 actually is more prominent on the microvilli.

A polarized distribution of ICAM-1 was evident in a large fraction of cells in all three EBV-transformed B-cell lines studied but not in the Burkitt's lymphoma line Raji, in which ICAM-1 was most prominent in microvilli. Polarized distribution of ICAM-1 was originally reported for the T cell line HSB-2 (18). We were unable to confirm this finding on two samples of the cell line, including the one previously studied, and found little expression of ICAM-1 on this line. In the EBV-transformed B-cell lines accumulation of ICAM-1 in the uropod does not appear to be due to interaction with LFA-1 on the cell surface, because a similar staining pattern was seen in cells derived from leukocyte adhesion deficiency (LAD) patients, which do not express LFA-1 (1).

Several lines of evidence indicated α-actinin as a cytoskel-

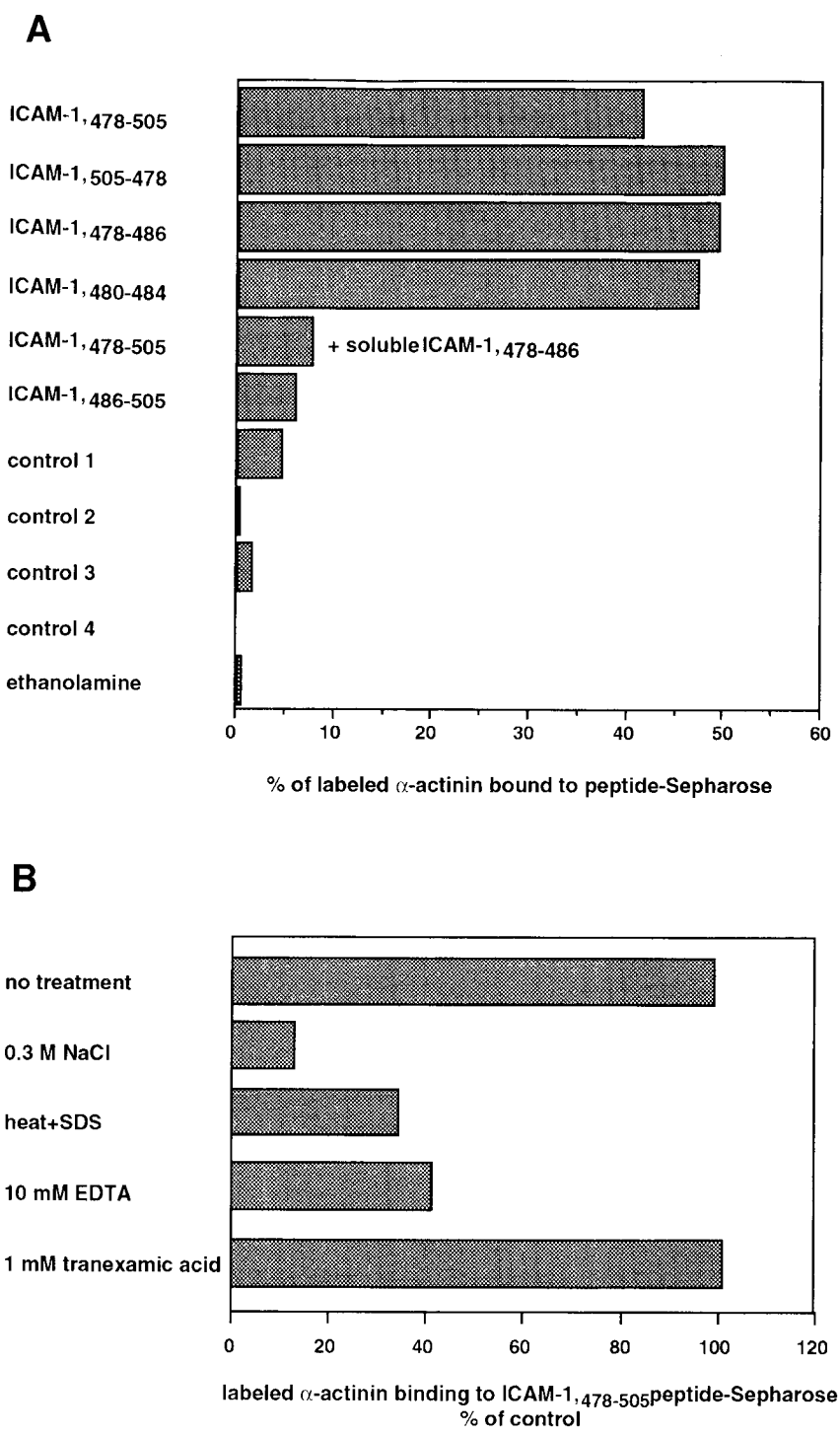


Figure 9. Binding of purified α-actinin to ICAM-1–derived and control peptides and the nature of the interaction between α-actinin and ICAM-$1_{478-486}$. (*A*) ^{125}I–α-actinin was incubated with the indicated Sepharose-coupled peptides listed in Fig. 5. In one of the reactions 10-fold molar excess of soluble ICAM-$1_{478-486}$ was added. Unbound material was washed away and the radioactivity remaining in Sepharose beads was counted. (*B*) ICAM-$1_{478-505}$-peptide Sepharose was incubated with 60,000 cpm of ^{125}I–α-actinin in binding buffer (no treatment) or in binding buffer containing 0.3 M NaCl, with ^{125}I–α-actinin denatured by heating at 100°C in 2% SDS for 15 min, with ^{125}I–α-actinin in presence of 10 mM EDTA and no divalent cations, or with ^{125}I–α-actinin in presence of tranexamic acid. After washes the ICAM-$1_{478-505}$-peptide-Sepharose bound radioactivity was counted. The results are expressed as percentage of cpm compared with control.

etal protein that can associate to ICAM-1. α-actinin could be enriched from placental lysates using ICAM-1 cytoplasmic peptide Sepharose affinity chromatography. Also, a direct interaction was demonstrated between ICAM-$1_{478-505}$ peptide and purified α-actinin as well as between native purified ICAM-1 and α-actinin. Finally, a strict colocalization and comodulation was shown between ICAM-1 and α-actinin but not between ICAM-1 and other B-cell cytoskeletal proteins examined. The immunofluorescence analysis revealed a polarized distribution of α-actinin. The distribution in EBV-transformed B-cell lines is thus different from other cells in which α-actinin is localized along actin fibers, in focal adhesions, in podosomes, and in myotendinous junctions (12). Most of these studies, however, were performed with adherent cells which frequently differ from nonadherent cells in cytoskeletal organization. Nonerythroid spectrin, a protein sharing sequence homology with α-actinin (4, 60), for instance, in lymphoid cells often shows a polarized distribution which resembles that of α-actinin (6, 13, 44), whereas in most other cell types it is organized as a subplasmalemmal network (57).

The function of α-actinin has remained in many respects unclear. The protein has actin-binding properties (36) and has been shown to interact with another cytoskeletal protein, vinculin and with certain lipids (10, 41, 59). Based on these

properties and the subcellular localization, α-actinin has been suggested to participate in anchoring of actin filaments to the cell membrane (36), possibly by acting as a distal link in a chain of several interacting molecules (7). The present results and a study showing an interaction between the cytoplasmic domain of the β_1 subunit of the fibronectin receptor and α-actinin (40) suggest a more direct role for α-actinin as a link between some cell membrane proteins and the microfilament network. A brief study recently described an interaction between ICAM-1 and actin but also in that paper the possibility that a linking protein would mediate the association, was raised (58). α-actinin is not the only major protein from placental lysates that binds to ICAM-$1_{478-505}$ peptide. The nature of the other, unidentified proteins that also elute from the ICAM-1 cytoplasmic domain peptide column needs to be resolved before the possibility of alternative or additional linkages between ICAM-1 and cytoskeleton can be addressed.

The experiments with various ICAM-1 cytoplasmic domain peptides indicate that the region of the cytoplasmic tail near the transmembrane domain that contains several positively charged residues is responsible for the association between ICAM-1 and α-actinin. Two shorter peptides containing this charged region bound α-actinin at levels comparable with the ICAM-$1_{478-505}$ peptide and the ICAM-$1_{478-486}$ peptide effectively competed for α-actinin binding with ICAM-$1_{478-505}$ peptide. Moreover, the ICAM-1 cytoplasmic peptide which lacked the eight residues was unable to interact with α-actinin. A peptide containing the cytoplasmic amino acids in reverse order also bound to α-actinin and competed for the binding with specific peptide (not shown) suggesting that the overall configuration of charge is more important to binding than stereoselective interactions. The electrostatic nature of the interaction was supported by other observations. Although binding occurred at physiologic ionic strength (0.15 M), increasing ionic strength (0.3–1.0 M NaCl) rapidly decreased binding; the binding could be decreased, but not totally abolished by denaturation of α-actinin; and finally, an interaction was detected between α-actinin and poly-L-lysine–Sepharose (not shown).

The presence of charged residues in the cytoplasmic domain is characteristic of transmembrane proteins. These residues are believed to form an anchor, arresting translocation across the bilayer during biosynthesis and assuring the correct topological orientation (8). It was of interest to test whether any positively charged peptide would associate with α-actinin. We tested four control peptides derived from biological sequences unrelated to ICAM-1. Although all peptides had a net positive charge they did not demonstrate significant interaction with α-actinin, indicating some degree of sequence specificity for the charged residues. Comparison of the ICAM-1 cytoplasmic domain sequence to integrin-β_1 cytoplasmic domain and membrane immunoglobulin (mIg) cytoplasmic domain, another membrane protein potentially associating with α-actinin (25), reveals that the only common denominator is KXK where X is E in integrin-β_1 subunit (55), I in ICAM-1, and V in mIg (45). The control peptide no. 1, however, also contains a KVK sequence but mediated little interaction with α-actinin indicating a more complex requirement for binding. The ICAM-1 cytoplasmic domain contains no acidic residues, whereas such residues are present in most cytoplasmic domains and in our control peptides.

Binding domains for lysine-rich sequences are present in some neural cell receptors and in the kringles of plasminogen (32, 50). Occupation of the lysine binding sites induces conformational changes in plasminogen and affects its enzymatic activity (15, 56). The association between ICAM-1 and α-actinin, however, is not identical to the plasminogen pathway since ω-aminocarboxylic acids which compete for the lysine binding sites in plasminogen (35) did not affect ICAM-1–α-actinin interaction. The interaction was partially dependent on divalent cation concentration which is of interest since the COOH-terminal region of α-actinin contains two EF hand-like calcium binding motifs (38).

Association with α-actinin may be important to the function of several types of adhesion molecules. Otey et al. (40) recently demonstrated that α-actinin binds to a β_1 integrin cytoplasmic domain peptide and to purified chicken smooth muscle (β_1) integrin and to the platelet integrin gp IIb/IIIa; here we show an interaction between α-actinin and ICAM-1, a member of the immunoglobulin superfamily of adhesion receptors. We do not currently know whether ICAM-1 and the integrins bind to the same region in α-actinin. However, some similarity in the nature of the interactions is suggested by the comparable sensitivity of both interactions to changes in ionic strength. In adherent cells a colocalization has been demonstrated between β_1 integrins and α-actinin in focal adhesions. However, our immunofluorescence localization studies in the B-cell lines do not suggest a similar association between α-actinin and LFA-1, a member of the leukocyte (β_2) integrins.

The role of a cytoskeletal interaction in ICAM-1 functions remains elusive. The GPI form of ICAM-1, which lacks cytoskeletal associations, mediates adhesion to LFA-1 at levels comparable with the wild-type ICAM-1 and both forms support binding and internalization of human rhinoviruses equally (52, 53). Internalization of a major group human rhinovirus does not require cytoplasmic or transmembrane domains of ICAM-1 (53). We have recently shown that purified ICAM-1 incorporated in artificial membranes can support lymphocyte motility (14, 19), which may be of importance during lymphocyte emigration to inflammatory foci (17, 61). Another adhesion ligand, LFA-3, also provided a matrix for locomotion but an immobile transmembrane form of the molecule was severalfold more efficient in supporting cell movement than a freely mobile GPI-anchored form (14). In analogy, a cytoskeletal anchorage which immobilizes ICAM-1 on endothelial cells and cells within tissues could be crucial for providing a firm foundation or foothold for leukocytes undergoing transendothelial emigration and subsequent migration within tissues.

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