Binding of the Cytoplasmic Domain of Intercellular Adhesion Molecule-2 (ICAM-2) to \(\alpha\)-Actinin

Leena Heiska‡§, Carmela Kantor*, Timothy Parr†, David R. Critchley‡, Pekka Vilja**, Carl G. Gahrmbør‡, and Olli Carpen‡

From the Departments of *Pathology and **Biosciences, Division of Biochemistry, University of Helsinki, FIN-00014 Helsinki, Finland, and the ‡Medical School, University of Tampere, FIN-33101 Tampere, Finland

Intercellular adhesion molecule-2 (ICAM-2) functions as a ligand for lymphocyte function-associated antigen-1 (LFA-1) and is involved in leukocyte adhesion. We studied intracellular associations of ICAM-2 using a peptide encompassing the cytoplasmic amino acids 231–254 as an affinity matrix. Among the proteins from plasmalemmal lysates that bound to the peptide was \(\alpha\)-actinin as demonstrated by immunoblotting. Purified, \(125^I\)-labeled \(\alpha\)-actinin also bound to the peptide. Confocal microscopic analysis of Eahy926 cells demonstrated a colocalization of ICAM-2 and \(\alpha\)-actinin. Of overlapping octapeptides covering the entire ICAM-2 cytoplasmic amino acids, ICAM-2\(_{241-248}\) bound \(\alpha\)-actinin most avidly and effectively competed with the longer cytoplasmic peptide for binding. The site of interaction in \(\alpha\)-actinin was studied using bacterially expressed \(\alpha\)-actinin fusion proteins. Several constructs covering nonoverlapping regions of \(\alpha\)-actinin bound to the ICAM-2 cytoplasmic peptide suggesting that multiple regions in \(\alpha\)-actinin can mediate the interaction. These results, together with previously demonstrated interactions between \(\alpha\)-actinin and the adhesion proteins ICAM-1, L-selectin, \(\beta\)_1- and \(\beta\)_2-integrins emphasize the role of \(\alpha\)-actinin as a linker between cell surface adhesion molecules and the actin-containing cytoskeleton.

Linkages between cell surface adhesion molecules and the cytoskeleton are important for many cellular functions. Such interactions regulate cell growth, morphology, and migration as well as contacts with the extracellular matrix and other cells (reviewed in Refs. 1 and 2). Adhesion molecules located at focal contacts and different cell-to-cell junctions are known to have closely connected with changes in cytoskeletal organization and cell shape with concomitant phosphorylation of many cytoskeleton-associated proteins. So far, the exact molecular mechanisms of adhesion and its regulation are poorly understood.

Several immune functions are dependent on adhesion, e.g. cellular cytotoxicity, antigen presentation, and leukocyte migration to inflammatory and neoplastic sites. The intercellular adhesion molecules (ICAMs) belong to the immunoglobulin superfamily of proteins and mediate leukocyte binding to \(\beta\)_2-integrins (CD11/18). ICAM-2 (CD102) is expressed constitutively on lymphocytes, monocytes, platelets, and most endothelial cells (3–6), but is strongly increased on endothelial cells in lymphomas (7). It is highly glycosylated and has an apparent molecular weight of 55,000 (5). It has two extracellular Ig-like domains, one membrane-spanning region and a short cytoplasmic domain (8). ICAM-2 plays a role in lymphocyte extravasation and may be important for the leukocyte recirculation in normal uninflamed tissues (9).

\(\alpha\)-Actinin is a versatile actin-binding and cross-linking protein which mediates linkages between plasma membrane and the cytoskeleton by several mechanisms. It has been shown to associate with ICAM-1, L-selectin and \(\beta\)_1- and \(\beta\)_2-integrins (10–14) and the intracellular focal contact-associated proteins talin, vinculin, and zyxin (15–19). \(\alpha\)-Actinin is linked to the phospholipid signal pathways by its interactions with phosphatidylinositol 4,5-biphosphate and phosphoinositide 3-kinase (20–22). In this study we describe an interaction between the cytoplasmic domain of ICAM-2 and \(\alpha\)-actinin and map the binding sites on both proteins.

**MATERIALS AND METHODS**

Cell Lines, Antibodies, and GST Fusion Protein Constructs—The cell line Eahy926 is a hybrid between vascular endothelial and carcinoma cells and expresses endothelial markers (23). It was grown in Dulbecco's modified Eagle's medium containing 100 \(\mu\)mol of hypoxanthine, 500 \(\mu\)mol aminopterin and 16 \(\mu\)mol thymidine, and 10% fetal calf serum. The mAb used to detect human ICAM-2 was from MedSystems, Vienna, Austria. X63 IgG (ATCC, Rockville, MD) was used as control mAb. \(\alpha\)-Actinin was detected with three polyclonal antibodies. 592 and 1642 were a kind gift of F. M. Pavalko, Indiana University School of Medicine, Indianapolis, IN (14) and 902-T was raised by immunizing rabbits with purified chicken gizzard \(\alpha\)-actinin. Nonimmune control sera were obtained before immunization. Talin was detected with rabbit antisera kindly provided by K. Burridge, University of North Carolina, Chapel Hill, NC (12). The mAbs used to detect spectrin and vinculin are described in (24). \(\alpha\)-Actinin/GST fusion proteins have been described previously (25).

Peptide Synthesis and Immobilization—The ICAM-2 peptides encompassing amino acids 20–42, 74–96, and 231–254 and amino acids 21–42, 231–254, and 241–248 in random order (Fig. 1) were synthesized by \(t\)-butoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer and purified to >98% purity by high performance liquid chromatography. The purified peptides were controlled by amino acid sequence analysis and mass spectrometry. An additional lysine residue was synthesized to the NH2-terminus of ICAM-2\(_{241-254}\) peptide. The overlapping octapeptides encompassing amino acids 229–236, 232–239, 235–242, 238–245, 241–248, 244–251, and 247–253 (Fig. 1) were synthesized by Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry on a
SMPS 350 multiple peptide synthesizer (Zinsser Analytic, Frankfurt, Germany). An additional cysteine residue was added to the COOH terminus of the octapeptides.

The ICAM-2 peptides lacking an NH₂-terminal cysteine extension were coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech Inc., Uppsala, Sweden) at a concentration of 2 mg/ml according to the protocol provided by the manufacturer. The overlapping ICAM-2 octapeptides were coupled to thiopropyl-Sepharose 6B (Pharmacia). The coupling efficiency for the octapeptides was 86–91% and for the other peptides 90–97%.

Affinity Chromatography—100 g of placental tissue was homogenized with a tissue grinder and lysed in 100 ml of lysis buffer (50 mM octyl-β-D-glucopyranoside, 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM EDTA) and proteinase inhibitors as above. The lysate was first centrifuged at 10 000 x g for 15 min at 4 °C, and the supernatant further centrifuged at 100 000 x g for 1 h at 4 °C. This supernatant was filtered through a sterile gauze. The lysate was applied to a Sepharose CL-4B precolumn followed by the ICAM-2 peptide Sepharose column equilibrated in the lysis buffer. The peptide column (2 ml) was washed with 15 column volumes of lysis buffer for proteins interacting with ICAM-2. Placental lysate was passed through the peptide column and bound material was eluted under three different conditions: first with soluble ICAM-2231–254 peptide (1 mg/ml), washed, and further eluted with lysis buffer containing 1 M NaCl, washed, and finally eluted with fresh 8 ml urea, 50 mM Tris-Cl, pH 7.4. One-ml fractions were collected. The proteins were separated in 8% SDS-PAGE under reducing conditions and visualized by silver staining.

Immunoblot Analysis—10 µl of eluted fractions from the ICAM-2231–254 peptide affinity chromatography were separated by SDS-PAGE, blotted onto nitrocellulose sheets, and blocked overnight using 5% milk powder in TBS (20 mM Tris-Cl, pH 7.6, 137 mM NaCl). Primary and secondary antibodies were applied in appropriate concentrations in 1% milk powder-TBS for 1 h each, followed by extensive washes with 1% Tween 20-TBS. The bound antibodies were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom (UK)).

Indirect Immunofluorescence Microscopy—The Eahy926 cells grown on glass coverslips were double-stained for ICAM-2 and α-actinin. The coverslips were reacted with anti-ICAM-2 mAb, rinsed, fixed in 3.5% paraformaldehyde in phosphate-buffered saline, pH 7.4, and reacted with fluorescein isothiocyanate-conjugated goat F(ab')₂ anti-mouse IgG (Immuno-Research, West Grove, Pennsylvania, USA). The coverslips were then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, stained with rabbit anti-α-actinin or normal rabbit serum followed by staining with tetramethylrhodamine isothiocyanate-conjugated goat F(ab')₂ anti-rabbit IgG (Cappel, Durham, NC). The coverslips were viewed with a confocal microscope (Laser Scan Microscope, Carl Zeiss, Germany).

RESULTS

Isolation of Proteins Interacting with the ICAM-2 231–254 Peptide—To characterize the proteins interacting intracellularly with ICAM-2, we synthesized a 25-amino acid peptide encompassing the cytoplasmic amino acids 231–254 of ICAM-2 (ICAM-2231–254, see Fig. 1). This peptide was coupled to Sepharose CL-4B and used as an affinity matrix. Placental endothe-elial cells express ICAM-2 and were used as a probable source of proteins interacting with ICAM-2. Placental lysate was passed through the peptide column and bound material was eluted under three different conditions: first with soluble ICAM-2231–254 peptide, followed by a high ionic strength (1 M NaCl) buffer and finally with 8 M urea. The eluted fractions contained a variety of proteins visualized by silver staining of SDS-polyacrylamide gels (Fig. 2A). To find the protein(s) possibly linking ICAM-2 to the cytoskeleton, eluted fractions were immunoblotted with antisera against various cytoskeletal proteins. Antisera against α-actinin reacted with a 100-kDa protein, which eluted with all three different elution solutions (Fig. 2B). No reactivity was seen with preimmune sera or with antibodies against vinculin, talin, or spectrin. The strongest α-actinin reactivity was seen in fractions eluted with 1 M NaCl, whereas the soluble peptide eluted significantly less α-actinin. It is possible that the concentration of soluble peptide used for elution (1 mg/ml) was not high enough to efficiently compete with the immobilized peptide on Sepharose beads. Also in silver staining, a band of 100 kDa corresponding to the size of α-actinin was most prominent in the 1 M NaCl eluate.

Interaction of α-Actinin with the ICAM-2 231–254 Peptide—As α-actinin was among the placental proteins binding to ICAM-2 peptide, we investigated if purified α-actinin binds directly to this peptide. Chicken gizzard α-actinin was purified and labeled with 125I. 125I-α-Actinin was incubated with Sepharose-coupled ICAM-2231–254 peptide and control peptides, which included ICAM-2231–254 residues in random order and peptides derived from the extracellular domain of ICAM-2. The results show that α-actinin binds specifically to the cytoplasmic peptide (Fig. 3A). ICAM-2231–254 Peptide-α-actinin binding could be competed with excess unlabeled α-actinin in a dose-dependent manner (Fig. 3B).

To further characterize the interaction we examined the effect of ionic strength, divalent cations and protein folding on the binding (Fig. 4). The binding of labeled α-actinin to ICAM-2231–254 peptide was measured using different NaCl concentrations. The interaction was relatively insensitive to an increase in the ionic strength, and even at a concentration of 1 M NaCl, 45% of the binding was still retained when compared with the binding in 0.15 M NaCl. Chelation of divalent cations with 10 mM EDTA did not affect binding, indicating that Ca²⁺ or Mg²⁺

FIG. 1. Schematic picture of the ICAM-2 molecule with the amino acid sequences of the cytoplasmic domain and the peptides used in studies on ICAM-2-α-actinin interaction. The short octapeptides contain an additional COOH-terminal cysteine for coupling purposes.
ions do not play a role in the interaction between α-actinin and ICAM-2231–254. When α-actinin was denatured by heat treatment in the presence of 2% SDS, the binding was totally abolished.

Identification of the Cytoplasmic Region of ICAM-2 Interacting with α-Actinin—To define the region in the cytoplasmic domain of ICAM-2 that mediates binding to α-actinin, short, overlapping octapeptides were synthesized. The peptides were coupled to thiopropyl-Sepharose through their cysteine extensions at their NH₂ terminus (see Fig. 1). Labeled α-actinin was reacted with these peptides. The results (Fig. 5A) show that the seven peptides bound to α-actinin at variable levels. The peptide VRAAWRRL-C representing amino acids 241–248 bound α-actinin at a degree comparable with the longer cytoplasmic ICAM-2231–254 peptide. A scrambled version of the ICAM-2241–248 peptide showed little specific binding. The overlapping octapeptides (ICAM-2238–245 and ICAM-2244–251), reached both approximately 40% binding compared to ICAM-2231–254. The ICAM-2241–248 peptide efficiently competed for α-actinin binding with the longer ICAM-2 cytoplasmic peptide, whereas only slight competition was seen with the octapeptide ICAM-2232–240 (Fig. 5B).

Identification of the Interacting Region in α-Actinin—a-Actinin consists of an NH₂-terminal globular actin-binding domain, four individual spectrin-like repeats and a COOH-terminal EF-hand-like Ca²⁺-binding motif. To define the region of α-ac-
were extending. α-Actinin reactivity in Eahy926 cells was typically concentrated in the same regions, where ICAM-2 staining was most pronounced (Fig. 8C). Three other cytoskeletal proteins, spectrin, vinculin, and talin, showed a distribution distinctive from α-actinin and were not localized in the areas where ICAM-2 was concentrated (not shown).

**DISCUSSION**

In this study we report an interaction between the cell adhesion molecule ICAM-2 and α-actinin. We enriched α-actinin from placental lysates using an immobilized peptide representing the ICAM-2 cytoplasmic amino acids 231–254 as an affinity matrix. Purified, labeled α-actinin bound to this peptide in a specific manner. As a control we used peptides, whose sequences were derived from the extracellular part of ICAM-2 and two scrambled versions of the cytoplasmic residues. As expected, these peptides did not bind α-actinin, although they resembled the cytoplasmic ICAM-2231–254 peptide in length and charge. We could further map the highest binding activity in the cytoplasmic domain to a stretch of 8 amino acids. Several regions in α-actinin appeared to be responsible for the interaction. Finally, we could show a cellular colocalization of ICAM-2 and α-actinin in a cultured endothelial cell line.

In addition to α-actinin, several other proteins from placental lysates were recovered from the ICAM-2 cytoplasmic peptide column. These proteins still need to be characterized. Three other cytoskeleton-associated proteins, spectrin, talin, and vinculin, were not detected in immunoblot analysis. When visualized with silver staining after SDS-PAGE, the eluted fractions appeared different from analogous fractions eluted from an ICAM-1 cytoplasmic peptide column (10). This is not surprising, since the cytoplasmic domains of these proteins share only limited homology (see Fig. 9). The cytoplasmic domains are short in both molecules and probably do not have any stable conformation. The differences in sequence make interactions with different cytoplasmic proteins possible. On the other hand, α-actinin has been shown to bind also to ICAM-1 (10). This suggests an important function for α-actinin in the linkage between cytoskeleton and adhesion molecules of the immune system, in particular, as the integrin subunits β1, β2, and β3, and L-selectin also display a similar linkage (11–14). The α-actinin binding site in ICAM-2 was primarily located to amino acids 241–248. The sequence VRAAWRRL includes both hydrophobic and positively charged amino acids, but contains no known protein motif. This peptide could compete with the longer ICAM-2231–254 peptide for α-actinin binding, confirming that it contains residues sufficient for the interaction. Mouse and human cytoplasmic ICAM-2 sequences are overall highly conserved, but especially this region is almost identical, with only one amino acid difference (see Fig. 9). Thus, this region could have been conserved because of its functional
ICAM-2 Interaction with α-Actinin

Fig. 8. Immunofluorescence localization of ICAM-2 and α-actinin on endothelial cell line Eahy926. The cells were double-stained for ICAM-2 (A) and α-actinin (B) as described under “Materials and Methods” and analyzed with a confocal microscope. C shows a composite image of ICAM-2 (green) and α-actinin (red) staining. Regions, where the two proteins codistribute appear in yellow.

228 QHLRQRMGT YGVAAWRRL PQAFRP ICAM-2cyt human 228 QWHRRRTGT YGVLAARWL PRAFRAPV ICAM-2cyt mouse

478 RQRKIKYRL QQAKGTPMK PNTQATPP ICAM-1cyt human

Fig. 9. Sequence comparison of the cytoplasmic domains of human and mouse ICAM-2 and human ICAM-1. Identical amino acids are indicated by vertical lines. The regions in ICAM-2 and ICAM-1 involved in α-actinin binding are underlined.

It is interesting to compare our findings to other known binding sites of transmembrane proteins interacting with α-actinin. The binding site of ICAM-1 or α-actinin was mapped to a short RIKIK sequence (10) with a similar kind of approach as used in this study. Although there is no direct sequence homology, the cytoplasmic binding sites of ICAM-1 and ICAM-2 resemble each other by having a highly positive charge and one hydrophobic residue. In a mimotope assay with 10-residue peptides (13), two different binding regions of β2-integrin were detected. The NH2-terminal binding site was highly charged with alternating lysines and glutamic acid residues, while the COOH-terminal binding region was relatively hydrophobic, containing one lysine. There is no direct sequence similarity in either of these binding regions with the binding region of ICAM-2 defined here. Another adhesion molecule, recently shown to bind to α-actinin, is L-selectin. It mediates leukocyte rolling and adhesion to endothelium, and its ligand binding ability depends on its linkage to cytoskeleton. When 11 COOH-terminal cytoplasmatic amino acids are deleted from L-selectin, it fails to coprecipitate with α-actinin (11). The deleted sequence is KKSKRSMNDPY, which contains charged and hydrophobic amino acids. The presence of cationic residues seems to be the only common factor in the α-actinin binding sites of cell surface proteins.

The ionic strength or the absence of Mg2+ or Ca2+ ions do not have a major effect on ICAM-2/α-actinin binding. On the other hand, denaturation of α-actinin blocks binding totally. This indicates the importance of a native conformation of α-actinin for binding. It might be possible that denaturation affects the dimerization of α-actinin. The results with GST fusion protein constructs suggest also that multiple regions of the α-actinin molecule are involved in binding to ICAM-2, and dimerization of α-actinin could be one reason for this phenomenon. In comparison, the binding of α-actinin to β2-integrin is mediated by the spectrin-like repeats (12), but identification of any particular binding site in the α-actinin molecule has been unsuccessful. Rather, all of the GST fusion proteins expressing separate repeats mediated binding. In microinjection of REF-52 cells with GST/α-actinin fusion proteins, only the one with all the repeats in tandem was adequately localized to focal contacts (36). These results also suggest that the dimerization of the α-actinin molecule could be meaningful for its interactions with adhesion receptors.

There are four other ICAM-like molecules presently known, ICAM-1, ICAM-3, LW blood group glycoprotein, and tenecephalin (28–33). All ICAMs are relatively different in their cytoplasmic domains and show variable expression patterns. Recent reports suggest that ICAM-3 regulates the ICAM-1/LFA-1 pathway of intercellular adhesion (34, 35). In these studies a certain activating antibody promoted ICAM-3 redistribution to an uropod-like structure. This change of morphology was accompanied with linear arrays of myosin within the uropod, while actin-based cytoskeleton and α-actinin showed much wider distribution. This result would indicate that at least under these circumstances ICAM-3 is not interacting with α-actinin, although it does not rule out the possibility this linkage could exist in some adhesive event.

Many adhesion molecules seem to interact with cytoskeletal components, and this connection is evidently essential during cellular migration, adhesion, and during different stages of extravasation. For instance, the membrane-spanning and immobilized form of LFA-3 can support CD2-mediated motility better than the GPI-anchored form of LFA-3, which is laterally diffusible in the lipid bilayer (37). Similarly, for migratory leukocytes, endothelial ICAM-1 and ICAM-2, immobilized through a linkage of adhesion receptors to cytoskeleton would give a better foothold for movement, as the actin network can maintain and resist tension during migration. For P-selectin, high tensile strength has been shown to be important for rolling (38). It is apparent that also other adhesion molecules operating in the circulation are subjected to high tensile forces. Anchoring of the receptor and ligand to the cytoskeleton could allow them to resist tensile stress.

The regulation of the cytoskeleton/adhesion receptor interactions is still poorly understood at the molecular level. All the characterized transmembrane binding partners for α-actinin are adhesion molecules, which suggests a key role for α-actinin in the adhesive events. Its multiple associations to other cytoskeletal proteins and connections to the phospholipid signal pathways are probably important in this context. The interaction of α-actinin with ICAM-2 described in this study should give some insight on how cells regulate their complicated adhesive functions.

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