The Cytoplasmic Domain of L-Selectin Interacts with Cytoskeletal Proteins Via α-Actinin: Receptor Positioning in Microvilli Does Not Require Interaction with α-Actinin

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Abstract. The leukocyte adhesion molecule L-selectin mediates binding to lymph node high endothelial venules (HEV) and contributes to leukocyte rolling on endothelium at sites of inflammation. Previously, it was shown that truncation of the L-selectin cytoplasmic tail by 11 amino acids abolished binding to lymph node HEV and leukocyte rolling in vivo, but the molecular basis for that observation was not determined. This study examined potential interactions between L-selectin and cytoskeletal proteins. We found that the cytoplasmic domain of L-selectin interacts directly with the cytoplasmic actin-binding protein α-actinin and forms a complex with vinculin and possibly talin. Solid phase binding assays using the full-length L-selectin cytoplasmic domain bound to microtiter wells demonstrated direct, specific, and saturable binding of purified α-actinin to L-selectin (K_d = 550 nM), but no direct binding of purified talin or vinculin. Interestingly, talin potentiated binding of α-actinin to the L-selectin cytoplasmic domain peptide despite the fact that direct binding of talin to L-selectin could not be measured. Vinculin binding to the L-selectin cytoplasmic domain peptide was detectable only in the presence of α-actinin. L-selectin coprecipitated with a complex of cytoskeletal proteins including α-actinin and vinculin from cells transfected with L-selectin, consistent with the possibility that α-actinin binds directly to L-selectin and that vinculin associates by binding to α-actinin in vivo to link actin filaments to the L-selectin cytoplasmic domain. In contrast, a deletion mutant of L-selectin lacking the COOH-terminal 11 amino acids of the cytoplasmic domain failed to coprecipitate with α-actinin or vinculin. Surprisingly, this mutant L-selectin localized normally to the microvillar projections on the cell surface. These data suggest that the COOH-terminal 11 amino acids of the L-selectin cytoplasmic domain are required for mediating interactions with the actin cytoskeleton via a complex of α-actinin and vinculin, but that this portion of the cytoplasmic domain is not necessary for proper localization of L-selectin on the cell surface. Correct L-selectin receptor positioning is therefore insufficient for leukocyte adhesion mediated by L-selectin, suggesting that this adhesion may also require direct interactions with the cytoskeleton.

LYMPHOCYTE migration through lymphoid organs and leukocyte traffic into sites of inflammation are related processes that are both regulated principally at the level of leukocyte interactions with the lumenal surface of postcapillary venules. A number of leukocyte and endothelial cell surface molecules that participate in this interaction have been identified, including members of at least three gene families: the integrins, the immunoglobulin (Ig) superfamily, and the selectins (for reviews see Springer, 1994; Butcher, 1991; Arnaout, 1993). Selectins mediate the initial phase of leukocyte recognition of endothelium, which usually takes the form of rolling along the vessel wall at velocities much slower than freely flowing blood elements (Lawrence and Springer, 1991; Bevilacqua and Nelson, 1993). In contrast, integrins mediate the subsequent firm arrest of the leukocytes to the endothelium, spreading on the endothelial surface, and transendothelial migration into the tissues (Smith, 1992). While the integrin and Ig families consists of many members and are broadly expressed by numerous cell types, there are only three known selectins, each of which

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exhibits a much narrower range of expression. In particular, L-selectin is expressed exclusively on leukocytes (Kansas et al., 1985; Tedder et al., 1990), E-selectin is expressed only on endothelium activated in vivo or in vitro by inflammatory stimuli such as TNF-α, IL-1, or LPS (Bevilacqua et al., 1987), and P-selectin is selectively expressed on both platelets and endothelium which have been activated in vitro by thrombin, histamine, or phorbol esters (Berman et al., 1986; McEver et al., 1989). This pattern of expression of the selectins is consistent with their specialized role in regulating the interaction of circulating elements of the blood with the vascular endothelium.

Although the selectins exhibit considerable homology in their extracellular regions, no homology exists between the cytoplasmic domains of different selectins, suggesting that distinct functions are encoded within these regions. Consistent with this idea, each of the selectin cytoplasmic tails is well conserved between different species (for review see Bevilacqua and Nelson, 1993). In addition, direct functional evidence supports this view. Sorting of newly synthesized P-selectin to Weibel-Palade bodies and α-granules of platelets and endothelial cells and to granules of transfected AtT-20 cells is mediated by the P-selectin cytoplasmic tail, and endocytosis of E-selectin by human endothelial cells may involve the E-selectin cytoplasmic tail (Disdier et al., 1992; Koedam et al., 1992; Eckhardt et al., 1992).

A precise function for the L-selectin cytoplasmic tail has not yet been established. However, truncation of the predicted 17-amino acid cytoplasmic domain of L-selectin by 11 residues abolishes both binding to lymph node high endothelial venules (HEV)[1] and leukocyte rolling in vivo (Kansas et al., 1993). Because the lectin/ligand recognition activity of this mutant was preserved, the molecular basis for this phenotype was unclear. At least two possibilities must be considered. The observation that cytochalasin B, which blocks the function of actin microfilaments, also abolishes L-selectin-mediated adhesion without affecting lectin activity (Kansas et al., 1993), suggests that interactions between one or more cytoskeletal proteins and the L-selectin cytoplasmic tail are necessary for L-selectin function. A second, not mutually exclusive possibility is that the cytoplasmic domain of L-selectin mediates the preferential localization of this receptor to the microvilli and ruffles on the surface of normal leukocytes (Picker et al., 1991; Erlandsen et al., 1993; Bruce and Doerschuk, 1994), analogous to the sorting function of the P-selectin cytoplasmic domain, and that inappropriate subcellular positioning is responsible for the defect exhibited by the L-selectin cytoplasmic domain truncation mutant.

We have investigated whether L-selectin binds to cytoskeletal proteins using solid phase binding assays of purified cytoskeletal proteins to a peptide corresponding to the L-selectin cytoplasmic domain and coimmunoprecipitation experiments in vivo using cells transfected with wild-type and truncated L-selectin. In addition, the distribution of wild-type and truncated L-selectin has been examined by immunoelectronic microscopy. The results indicate that the L-selectin cytoplasmic domain interacts with the cytoskeleton via the actin-binding protein α-actinin, and that this interaction may also involve two other cytoskeletal proteins, vinculin and talin. These interactions, however, are not required for localization of L-selectin to the microvillar projections.

1. *Abbreviation used in this paper*: HEV, high endothelial venule.

Materials and Methods

Cell Culture

The mouse pre-B cell line 300.19 was transfected by electroporation with either full-length (LAM-1) or truncated human L-selectin cDNA (LΔcyto, lacking the amino terminal 11 residues of the cytoplasmic domain) in the pZIPneoSV(X) vector as previously described (Kansas et al., 1993). Stable transfectants and mock-transfected cells (300.19) were maintained in suspension culture in RPMI-1640 medium containing 0.5 mg/ml G418 (geneticin, Sigma Immunochemicals, St. Louis, MO) and supplemented with 10% FBS and antibiotics at 37°C in a humidified 5% CO_2 atmosphere. Cells were passaged approximately every 2 d at 1:10 dilution into fresh media.

Coimmunoprecipitation and Immunoblotting

300.19, 300.19/LAM-1, and 300.19/LΔcyto cells were harvested during log phase of growth and washed in HBSS. Cells at a concentration of 5×10^6/ml were extracted with 1 ml of coimmunoprecipitation lysis buffer (1% TX-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% NaN_3, 20 mM DNaseI, 1 mM aprotinin, 10 mM benzamidine, and 1 mM PMSF) on ice for 5 min. Extracts were clarified by centrifugation at 15,000 *g* for 10 min, and the supernatants transferred to a fresh tube. Cellular proteins which bind to protein A were precleared by incubation with 50 μl of a 10% wt/vol solution of protein-A positive *S. aureus* cells for 30 min at 4°C. The *S. aureus* cells were sedimented, and an excess of primary antibody (monoclonal antibody [mAb] LAM1-14 against the extracellular domain of L-selectin or antibody against talin, vinculin, α-actinin, paxillin, filamin, or tensin) was added to the supernatant, and then incubated for 1 h at 4°C. 100 μl of a 10% vol/vol suspension of protein A-Sepharose CL4B (or a protein A-Sepharose-rabbit anti-mouse Ig conjugate when the primary antibody was a monoclonal) was subsequently added for an additional 1 h. Immunocomplexes were washed 4× in coimmunoprecipitation lysis buffer containing 0.1% TX-100, released from the Sepharose beads by addition of SDS-PAGE sample buffer, and boiled for 5 min. Samples were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose which was blocked with 2% gelatin in Tris-buffered saline-Tween (TBS-T, 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h. Depending upon the experiment, blots were probed with LAM1-14 or with antibody against talin, vinculin, α-actinin, paxillin, tensin, or filamin in TBS-T for 1 h, washed in TBS-T, and then incubated with either goat anti-mouse Ig or goat anti-rabbit Ig conjugated to horseradish peroxidase for 1 h. After a final wash in TBS-T, blots were visualized by chemiluminescence generated upon addition of LumiGLO™ and exposure to X-ray film.

Analysis of L-Selectin Surface Expression

Expression of the full-length and truncated L-selectin was evaluated by indirect immunofluorescence microscopy. Harvested cells were washed in HBSS and suspended in PBS. Cell suspensions were incubated with LAM1-14 (5 μg/ml) for 30 min, washed in PBS, and then incubated in FITC-conjugated anti-mouse Ig (Jackson Immunoresearch, West Grove, PA) for an additional 30 min. Cells were again washed in PBS and analyzed by flow cytometry on a FACStar Plus (Becton Dickinson, San Jose, CA). Surface expression was also measured by surface iodination using lactoperoxidase catalyzed iodination followed by immunoprecipitation with mAb LAM1-14. Iodinated full-length or truncated L-selectin was located by SDS-PAGE and autoradiography of LAM1-14 immunoprecipitates and quantitated by gamma-counting of the excised bands from the gel.

Protein Purification and Solid Phase Binding Assays

α-Actinin, talin, and vinculin were purified from chicken gizzard as previously described (Feramisco and Burridge, 1980). Proteins were judged to be >95% pure by SDS-PAGE. Purified proteins were iodinated using the

Iodogen method (Fraker and Speck, 1978). Iodogen (50 μl of 1 mg/ml in $CHCl_3$; Pierce Chem. Co., Rockford, IL) was dried onto the inside of a microfuge tube under a gentle nitrogen stream. Protein, dialyzed into PBS, was added to the tube followed by 0.5 mCi of Na-^{125}I (Amersham) and the reaction was carried out at 4°C for 3 min. Saturated tyrosine was added to terminate the reaction and labeled protein was separated from free iodine and iodo-tyrosine by chromatography on Sephadex G-50 in 50 mM Tris, 20 mM NaCl, 0.1% NaN_3, and 0.1% β-mercaptoethanol (buffer B) plus 0.2% gelatin as a carrier. The proteins were labeled to a specific activity of 0.5 × 10^7 cpm/μg for α-actinin, 1.0 × 10^7 cpm/μg for vinculin, and 0.3 × 10^7 cpm/μg for talin. Proteolytic fragments of α-actinin were generated using thermolysin as previously described (Pavalko and Burridge, 1991).

The L-selectin cytoplasmic domain peptide (RRLKKGKKSKRSMNDPY) was synthesized by Multiple Peptide Systems, Inc. (San Diego, CA), purified by reverse phase HPLC on a C_{18} column, and subjected to amino acid analysis and partial sequence analysis to confirm that it contained the correct sequence. The scrambled peptide consisted of the sequence KMYPKRSKDNKRLSKGR. A polyclonal antibody against the L-selectin cytoplasmic domain did not recognize this scrambled peptide in solid phase radioimmunoassays (not shown). The β_2-cytoplasmic domain peptide corresponded to residues 726-744. We have previously shown that this $\beta_{2(726\text{-}744)}$ peptide interacts with α-actinin (Pavalko and LaRoche, 1993). The E-selectin cytoplasmic domain peptide corresponded to the complete E-selectin cytoplasmic domain and was shown previously not to interact with α-actinin (Pavalko and LaRoche, 1993). Removable microtiter wells (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 50 μl of cytoplasmic domain peptide (1 mg/ml in PBS) for 2 h at 37°C. The wells were rinsed briefly with wash buffer (0.1% BSA, 0.1% NaN_3, in PBS), blocked with 2% BSA for 30 min at 37°C, and then rinsed again with wash buffer. Control wells were coated with 2% BSA alone. Iodinated protein and unlabeled competitor protein were added to the wells and diluted to a final volume of 110 μl with PBS. The wells were incubated for 2 h at 37°C, and then washed four times with wash buffer. Individual wells were removed and bound radioactivity measured in a Packard-Bell γ-counter. Each data point is the average of duplicate or triplicate wells. Background binding to BSA was not subtracted from the binding to peptide but is plotted separately for each experiment.

Immunoelectron Microscopy

Lymphoblasts expressing either the intact or the truncated L-selectin were cultured at a low density. The cells were suspended at a concentration of 10^7/ml in PBS and incubated with 10 μg/ml anti-L-selectin antibody, Leu-8 (Becton Dickinson) or Laml.14 (gift from Dr. T. Tedder), or with control mouse IgG for 60 min at room temperature. After centrifugation, the cells were suspended in PBS-1% FCS and incubated with goat anti-mouse IgG antibody bound to 10 nm colloidal gold (final concentration 10%, Sigma Chem. Co.) for 30 min at room temperature. After washing, the lymphoblasts were fixed in 0.5% glutaraldehyde in PBS for 10 min. The cell pellet was embedded in 2% agarose. After sectioning into 1 mm^3 cubes, the cells were postfixed in osmium tetroxide, embedded in epoxy resin, sectioned using an ultramicrotome at 90 nm, collected on formvar-coated grids, stained with uranyl acetate, and examined using transmission electron microscopy.

Cell profiles were randomly selected, photographed at 11,500× or 15,500× magnification, and printed at 20,125 or 27,125 magnification. Each gold particle was counted and categorized as on microvillar projections or flat intervening regions. The fraction of gold particles that were expressed on microvillar projections was calculated. The distribution of in-

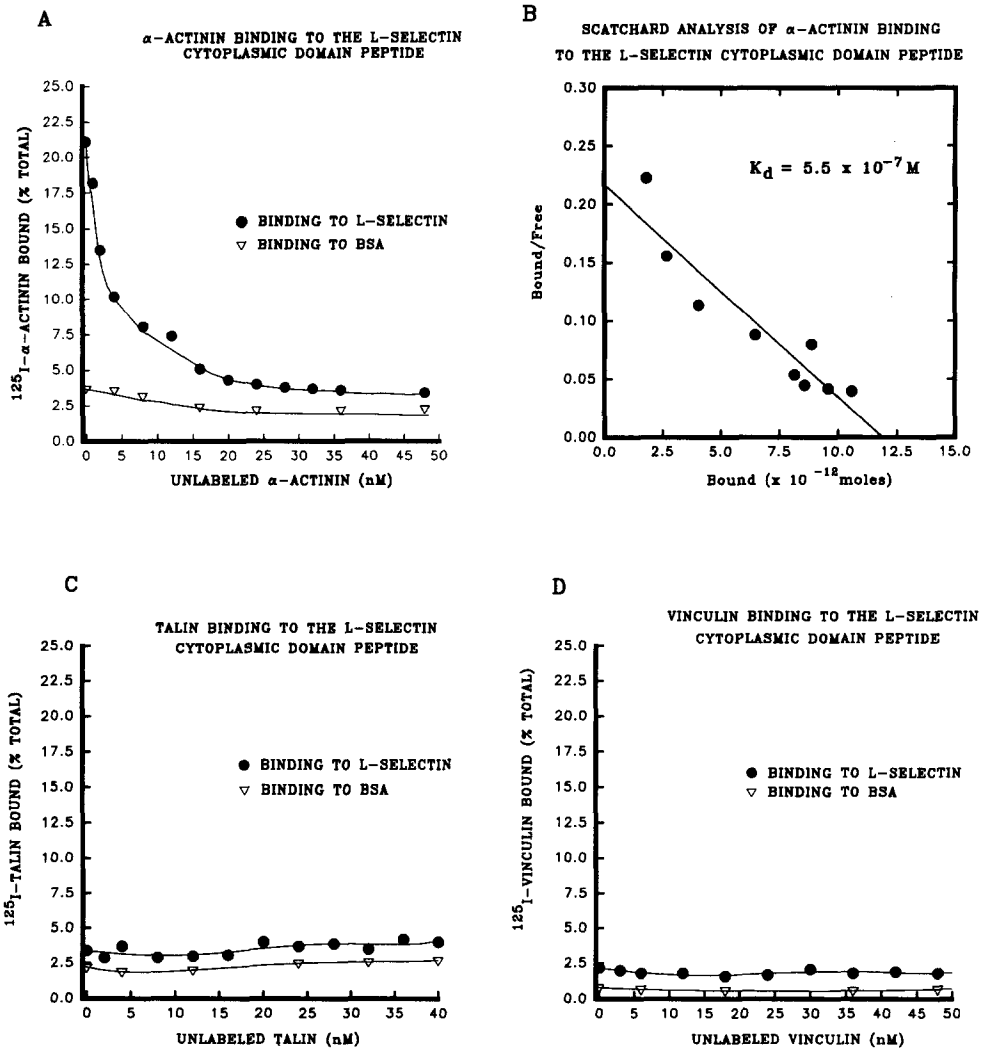

Figure 1. Solid phase binding assays measuring the binding of three purified cytoskeletal-membrane linker proteins to the L-selectin cytoplasmic domain peptide. Binding of iodinated α-actinin (*A*), talin (*C*), and vinculin (*D*) in the presence of increasing concentration of unlabeled competitor protein to microtiter wells coated with the full-length L-selectin cytoplasmic domain peptide (*closed circles*), or to control wells coated with BSA (*open triangles*). (*B*) Scatchard analysis of the binding of α-actinin to the L-selectin cytoplasmic domain peptide. Each point represents the average of triplicate wells.

tact L-selectin was compared to that of the truncated form using a Student's *t* test.

Results

The L-Selectin Cytoplasmic Domain Interacts with the Actin-binding Protein α-Actinin

Solid phase binding assays were performed to determine if talin, vinculin, and α-actinin, which have been shown to participate in linking the actin cytoskeleton to the cytoplasmic tails of integrins, might also participate in linking the cytoskeleton to L-selectin cytoplasmic domains. Talin, vinculin, and α-actinin were purified from smooth muscle for these in vitro studies. The purified proteins were labeled with ^{125}I-Na and assayed for their ability to interact with a peptide corresponding to the cytoplasmic domain of L-selectin that was used to coat plastic microtiter wells (Fig. 1). α-Actinin bound to the L-selectin cytoplasmic domain peptide, and this binding was inhibited by an excess of unlabeled α-actinin (Fig. 1 *A*). These data were subjected to analysis by Scatchard plot, and a dissociation constant (K_d) of 5.5 × 10^{-7} M was calculated (Fig. 1 *B*). Neither talin (Fig. 1 *C*) nor vinculin (Fig. 1 *D*) were able to bind directly to the L-selectin cytoplasmic domain peptide. Binding of ^{125}I-α-actinin to the L-selectin cytoplasmic domain peptide was saturable (Fig. 2 *A*). ^{125}I-α-actinin did not bind to a scrambled 17-amino acid peptide with the same amino acid composition as the L-selectin peptide but arranged in a random sequence (Fig. 2 *A*) further suggesting that the α-actinin–L-selectin interaction was specific. Furthermore, binding of ^{125}I-α-actinin to the L-selectin cytoplasmic domain was competed by excess soluble peptide, but was not competed by the scrambled peptide (Fig. 2 *B*). Peptides corresponding to either the complete E-selectin cytoplasmic domain or an 18-amino acid region within the β_2 integrin cytoplasmic domain that was previously shown to interact with α-actinin (Pavalko and LaRoche, 1993) did not compete for binding of α-actinin to L-selectin (Fig. 2 *B*).

To determine the L-selectin-binding region on α-actinin, 27-kD and 53-kD proteolytic fragments of α-actinin were generated using the enzyme thermolysin and purified on a FPLC Mono Q column. The 27-kD fragment binds to actin but not to integrin β subunit cytoplasmic domains, whereas

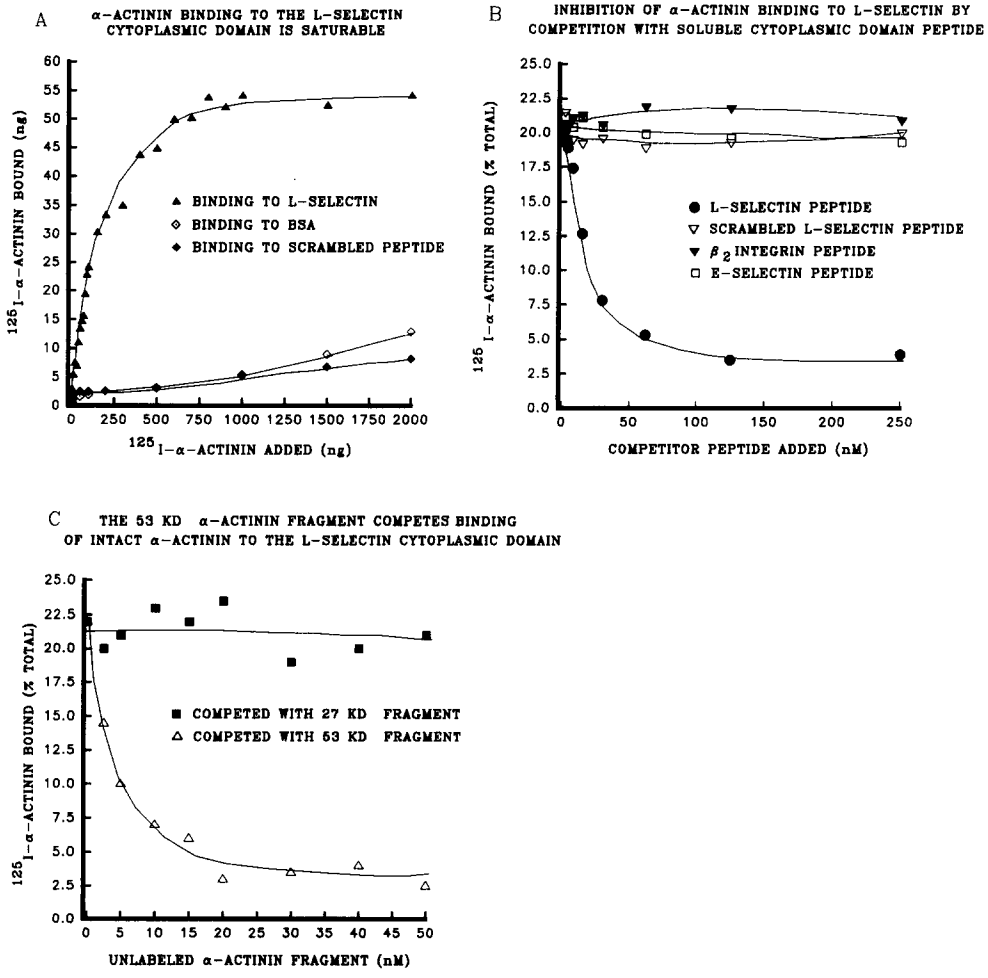

Figure 2. Characterization of the interaction between α-actinin and the L-selectin cytoplasmic domain peptide. (*A*) Binding of purified α-actinin to the L-selectin cytoplasmic domain (*closed triangles*) is saturable. No binding of α-actinin to a scrambled 17-amino acid version of the L-selectin cytoplasmic domain peptide (*closed diamonds*) or to BSA (*open diamonds*) could be measured. (*B*) Addition of soluble L-selectin cytoplasmic domain peptide (*closed circles*) inhibits binding of α-actinin to microtiter wells coated with the L-selectin cytoplasmic domain peptide. Three other soluble peptides: the scrambled L-selectin peptide (*open triangles*), the β_2-integrin cytoplasmic domain peptide (*closed triangles*), or the E-selectin cytoplasmic domain peptide (*open squares*) failed to inhibit α-actinin binding. (*C*) Binding of α-actinin to the L-selectin cytoplasmic domain peptide is competed by the 53-kD proteolytic fragment of α-actinin (*open triangles*), but not by the 27-kD α-actinin fragment (*closed squares*), suggesting that the L-selectin binding region lies within the 53-kD rod domain of α-actinin and not in the 27-kD globular actin-binding region. Each point represents the average of triplicate wells.

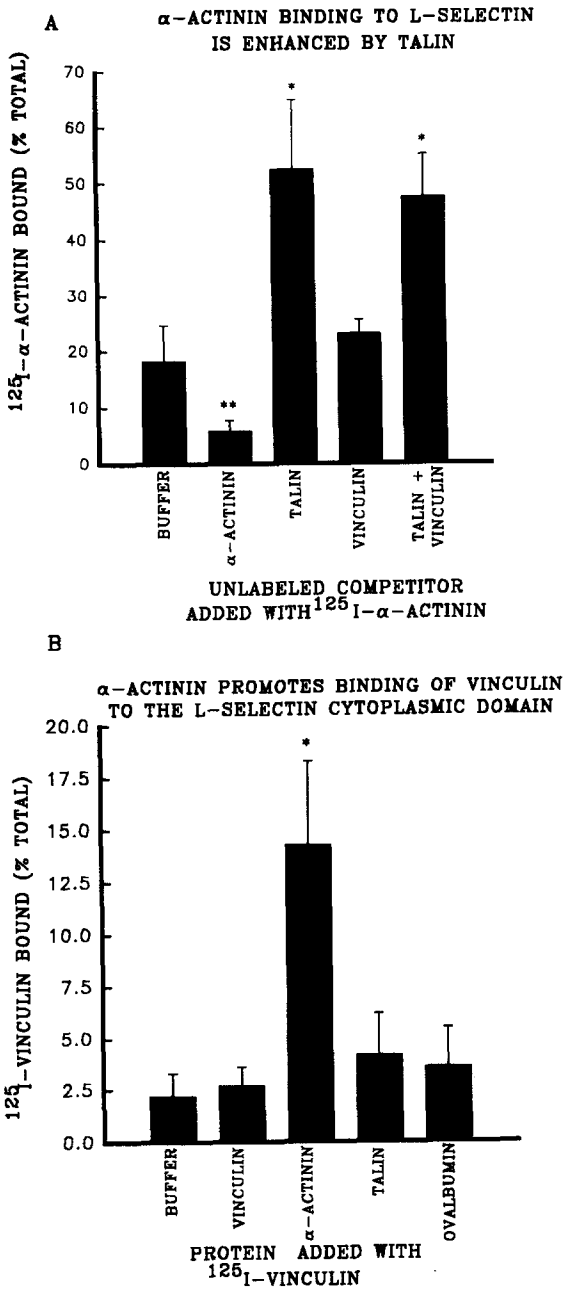

Figure 3. Solid phase assays characterizing the effects of talin and vinculin on the interaction between α-actinin and the L-selectin cytoplasmic domain peptide. (*A*) Binding of iodinated α-actinin to the L-selectin cytoplasmic domain peptide was significantly enhanced in the presence of talin, and talin plus vinculin, compared to buffer only controls,(*) $p > 0.05$. Unlabeled α-actinin significantly inhibited binding compared to buffer only controls, (**) $p > 0.05$. Vinculin alone did not affect binding of iodinated α-actinin to L-selectin. (*B*) Binding of iodinated vinculin to the L-selectin cytoplasmic domain peptide was significantly higher in the presence of excess unlabeled α-actinin, (*) $p > 0.05$, compared to buffer only controls. The addition of excess unlabeled vinculin, talin, or ovalbumin did not elevate vinculin binding to the L-selectin peptide suggesting that α-actinin forms a bridge between vinculin and the L-selectin peptide.

the 53-kD fragment binds to integrin β subunit cytoplasmic domains but not to actin (Mimura and Asano, 1987; Otey et al., 1990; Pavalko and Burridge, 1991). When the 27- and 53-kD α-actinin fragments were used as potential competitors of intact ^{125}I-α-actinin binding to the L-selectin peptide, only the 53-kD fragment was able to inhibit binding (Fig. 2 *C*), suggesting that the L-selectin binding site on α-actinin is located within the 53-kD domain of α-actinin, and that the 27-kD actin-binding domain does not directly bind to L-selectin.

Because α-actinin, vinculin, and talin are each found in sites of actin-membrane interaction, we examined the effect of mixtures of these proteins on binding of α-actinin to the L-selectin cytoplasmic domain. When talin and vinculin were both added to L-selectin-coated microtiter wells in the presence of ^{125}I-α-actinin, α-actinin binding to the peptide increased ~2.5-fold (Fig. 3 *A*). Addition of vinculin alone to labeled α-actinin had no effect on α-actinin binding, while addition of talin alone increased α-actinin binding (Fig. 3 *A*). These results indicated that α-actinin binding to L-selectin was enhanced in the presence of talin, despite the fact that talin does not appear to interact directly with L-selectin. When ^{125}I-vinculin was added to L-selectin-coated wells, α-actinin, but not talin, promoted vinculin binding (Fig. 3 *B*). This result was consistent with the possibility that vinculin may associated indirectly with L-selectin cytoplasmic domains by binding to α-actinin; vinculin binding to α-actinin has previously been characterized (Otto, 1983; Wachsstock et al., 1987).

Coimmunoprecipitation of L-Selectin with Cytoskeletal Proteins

The pre-B lymphocyte cell lines, 300.19, transfected with wild-type L-selectin cDNA or with cDNA lacking a large portion (11 of 17 residues) of the L-selectin cytoplasmic domain were used to assess the ability of L-selectin to interact with cytoskeletal proteins in vivo. The wild-type transfectant has been shown previously to bind HEV (Kansas et al., 1993) and roll along endothelium in vivo (Ley et al., 1993). Cells transfected with the cytoplasmic domain deletion mutant (LΔcyto) failed to bind HEV or roll in vivo (Kansas et al., 1993). Before making a qualitative evaluation of L-selectin- cytoskeletal interactions, the levels of L-selectin surface expression on cells transfected with the wild-type and truncated L-selectin were assessed using both indirect immunofluorescence followed by flow cytometry and surface iodination followed by immunoprecipitation. Flow cytometry of the surface labeled transfectants and nontransfected 300.19 cells revealed 2-3-fold higher levels of L-selectin surface expression on the wild-type transfectants when compared to the truncated protein (Fig. 4 *A*). Immunoprecipitation after surface iodination also indicated approximately threefold higher levels of surface expression by the wild-type transfectant (Fig. 4 *B*). Therefore, the number of cells used in subsequent coimmunoprecipitation experiments was correspondingly adjusted.

Antibodies against the cytoskeletal proteins α-actinin, vinculin, talin, paxillin, tensin, and filamin were used to immunoprecipitate from detergent extracts of 300.19 L-selectin transfectants under nondenaturing conditions to investigate whether L-selectin interacts with these proteins in vivo. Western blots of whole cell extracts confirmed that all of the

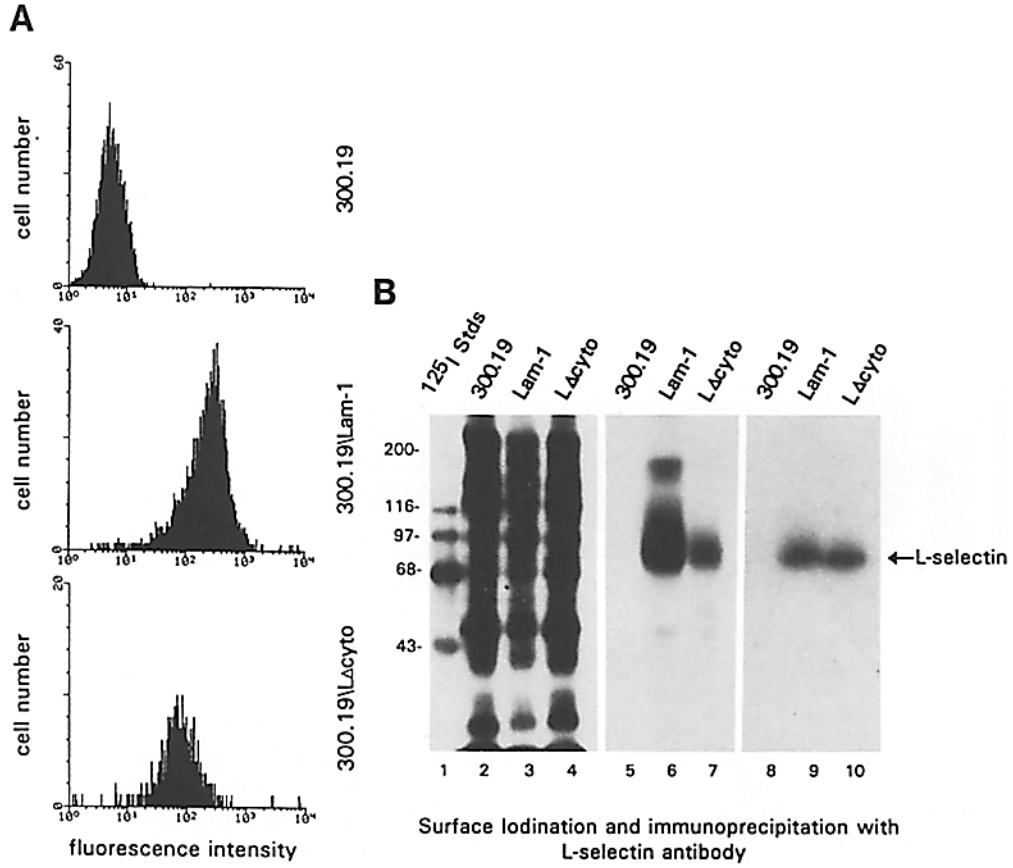

Figure 4. Quantitation of L-selectin surface expression. (*A*) Flow cytometry demonstrated a 2–4-fold greater level of surface expression on the wild-type L-selectin transfected cells as opposed to the truncated L-selectin transfected cells. The top panel established the background fluorescence observed in the 300.19 cells transfected with vector alone and no L-selectin. 300.19/ LAM-1 (*middle panel*) shows that the relative fluorescence intensity of the cell surface of the wild-type transfected cells is ~2–4-fold greater than cells transfected with the cytoplasmic domain truncated L-selectin (*bottom panel*). (*B*) Surface iodination and immunoprecipitation of 300.19 cells with antibody against L-selectin substantiates the 2–4-fold increase in surface expression of wild-type transfected cells over deletion mutant transfected cells. Lanes *2–4* are whole cell extracts before immunoprecipitation with anti–L-selectin antibody. Lanes *5–7* demonstrate the difference in radioactivity observed when equal numbers of cells are iodinated, and then immunoprecipitated with anti–L-selectin. When cell numbers are adjusted so that there are a 3–4 greater number of the LΔcyto cells, expression as quantitated by ^{125}I, appears to be comparable (lanes *8–10*).

antibodies used in these studies recognized the corresponding protein in 300.19 cells (not shown). Coimmunoprecipitates using these antibodies were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for L-selectin that had coprecipitated with cytoskeletal complexes. L-selectin was easily detected in coimmunoprecipitates with both α-actinin and vinculin (Fig. 5 *A*), but not with any of the other cytoskeletal proteins tested including talin, paxillin, tensin, and filamin (not shown). In addition, when the experiment was done in reverse, i.e., by immunoprecipitating with L-selectin antibody and immunoblotting for cytoskeletal proteins, both α-actinin and vinculin were detected in L-selectin immunoprecipitates (Fig. 5 *B*) while talin, paxillin, tensin, and filamin were not detected (not shown). To visualize the total protein precipitated with anti–L-selectin antibodies, cells were labeled with [^{35}S]methionine (Fig. 5 *C*). A band migrating at the appropriate molecular weight for L-selectin was the most prominent band that could be detected. In combination with the in vitro solid phase binding assays, these data suggest that L-selectin forms a complex with both α-actinin and vinculin in vivo and that the interaction of L-selectin with vinculin is indirect, probably through binding to α-actinin.

In contrast to the results obtained with the full-length L-selectin transfectant, no L-selectin was detected in coimmunoprecipitates of α-actinin or vinculin from the LΔcyto transfectant (Fig. 5 *A*). Similarly, these cytoskeletal proteins were not detected in L-selectin coimmunoprecipitates from the LΔcyto transfectant (Fig. 5 *B*). These data indicate that L-selectin interacts with the cytoskeleton through a direct interaction between α-actinin and the 11 carboxy terminal amino acids of the L-selectin cytoplasmic tail. Despite the abundance of talin in 300.19 cells and the ability of talin to potentiate α-actinin binding to the L-selectin peptide in vitro, coprecipitation of L-selectin with talin was never detected from cells transfected with either the full-length or truncated L-selectin.

Cell Surface Localization of Wild-Type and Truncated L-Selectin by Immunoelectron Microscopy

The role of the L-selectin cytoplasmic domain on the localization of L-selectin on the surface of transfected cells was examined by immunoelectron microscopy. L-selectin is normally localized predominantly to the microvillar projections which cover the surface of neutrophils (Picker et al., 1991; Erlandsen et al., 1993; Burns and Doerschuk, 1994). Cells transfected with wild-type or truncated L-selectin were labeled with an antibody against the extracellular domain of L-selectin, followed by a secondary antibody conjugated to a gold particle, and processed for immunoelectron microscopy (Fig. 6). Quantitation of gold particles revealed that ≈90% of the label was localized to the microvillar projections of cells transfected with wild-type L-selectin (Table I). The subcellular localization of L-selectin in these transfected 300.19 cells was therefore quite similar to that in neutrophils. Interestingly, the LΔcyto mutant also localized almost exclu-

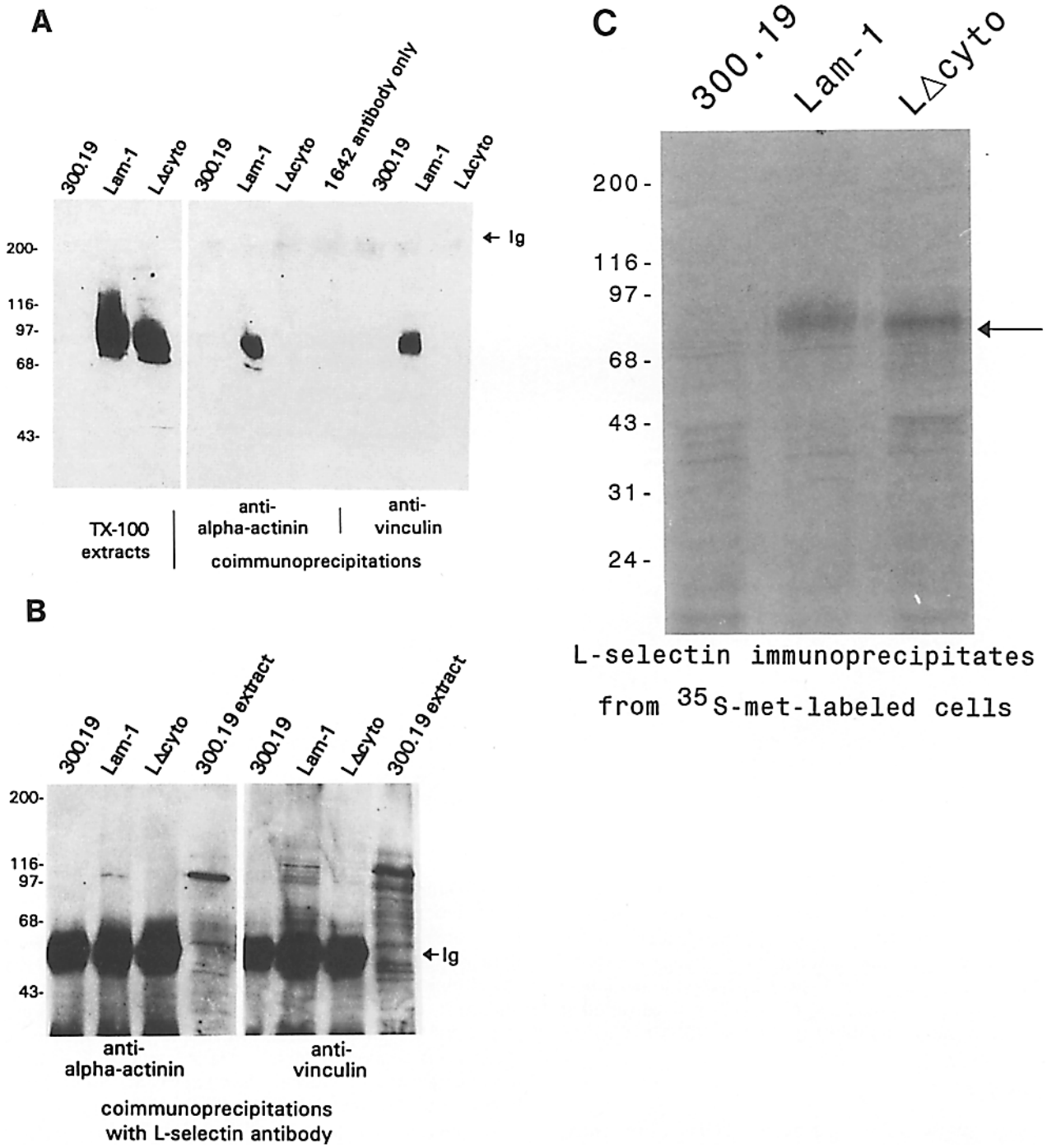

Figure 5. α-Actinin and vinculin form a complex with L-selectin through the cytoplasmic domain that is detected by coimmunoprecipitation. 300.19, 300.19/LAM-1, and 300.19/LΔcyto cells were lysed with detergent and these extracts were used to determine if specific cytoskeletal proteins formed a complex with the cytoplasmic domain of L-selectin. (*A*) The first three lanes are whole cell detergent extracts before immunoprecipitation with cytoskeletal proteins and are immunoblotted for anti–L-selectin. The next lanes, corresponding to the immunoprecipitates with anti–α-actinin and anti-vinculin followed by immunoblotting with anti–L-selectin, demonstrate the in vivo association of the wild-type L-selectin (*Lam-1*), but not the cytoplasmic domain truncation mutant (*L*Δcyto), with the cytoskeletal proteins α-actinin and vinculin. (*B*) Full-length L-selectin (Lam-1), but not the deletion mutant (LΔcyto) coimmunoprecipitates α-actinin and vinculin. Antibody against L-selectin was used to immunoprecipitate from cell extracts and the cytoskeletal proteins that coprecipitated were detected by immunoblotting with antibodies against α-actinin and vinculin. Antibodies against four other cytoskeletal proteins: talin, paxillin, tensin, and filamin, failed to detect the corresponding protein coprecipitating with L-selectin (not shown). (*C*) To visualize the total protein precipitated with L-selectin under coimmunoprecipitation conditions, cells were labeled with [^{35}]methionine before lysis. L-selectin (*arrow*) is the only major band visible from LAM-1 and LΔcyto cells immunoprecipitated with anti–L-selectin when the total protein is visualized by autoradiography of the immunoprecipitates.

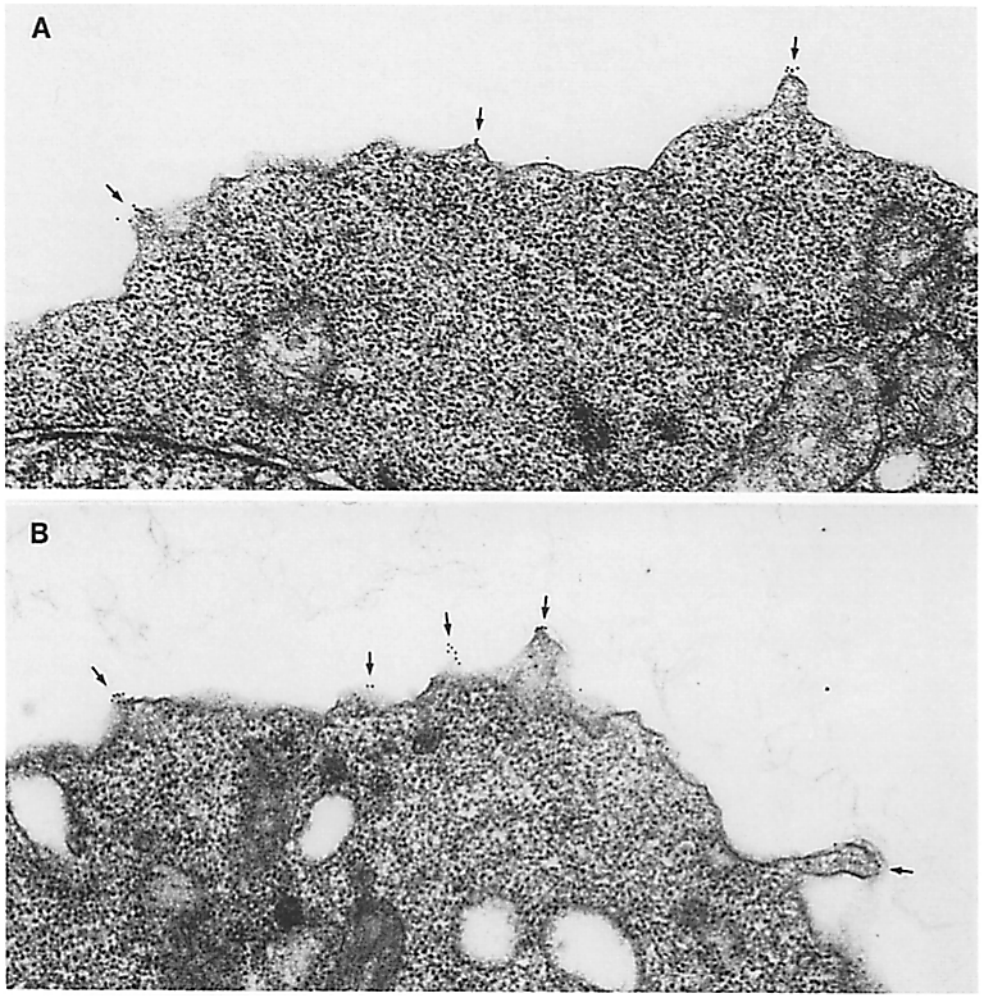

Figure 6. Immunoelectron microscopic localization of L-selectin on the surface of cells transfected with truncated (*A*) or full-length L-selectin cDNA (*B*). Cells were labeled with a monoclonal antibody (Leu8) against the extracellular domain of L-selectin, followed by 10-nm gold particle conjugated goat anti-mouse Ig antibody conjugated to 10-nm gold particles. Labeling is limited to the plasma membrane and occurs predominantly on the small microvillar projections (see also Table I).

sively to the microvilli (Fig. 6; Table I). Lymphoblasts that were exposed to control IgG instead of anti-L-selectin showed no labeling. These results indicated that an association with the cytoskeleton via α-actinin involving the COOH-terminal 11 residues of the L-selectin cytoplasmic domain was not necessary to maintain the selective localization of L-selectin to the microvilli of transfected 300.19 cells.

Discussion

This study represents the first demonstration of a direct interaction between the cytoskeleton and a member of the selectin family of cell adhesion molecules. This interaction is mediated by a region within the carboxy terminal 11 residues of the 17-amino acid cytoplasmic domain and occurs via a direct interaction with the rod domain of the actin-binding protein α-actinin. In contrast to selectins, cytoskeletal interactions with integrins are better defined (for review see Sastry and Horwitz, 1993). Talin was shown to interact with the cytoplasmic domain of integrin heterodimers (Horwitz et al., 1986), and α-actinin mediates cytoskeletal attachment to the cytoplasmic domains of members of at least two other families of cell adhesion molecules: β_1 (Otey et al., 1990, 1993; Pavalko and Burridge, 1991) and β_2 (Pavalko and LaRoche, 1993) integrins, and an Ig family member, ICAM-1 (Carpen et al., 1992). The interaction between α-actinin and integrins occurs through binding of a defined portion of the cytoplasmic domain of integrin β_1 (Otey et al., 1990, 1993) and

Table I. Distribution of L-Selectin on the Plasma Membrane

	Intact L-selectin	Truncated L-selectin
	(n = 18 cells)	(n = 15 cells)
Percent gold particles on membrane of:		
Microvillar projections	87 ± 2	89 ± 2
Flat regions	13 ± 2	11 ± 2

Data expressed as mean ± SEM

β_2 (Pavalko and LaRoche, 1993) subunits in vitro to a site within the 53-kD rod domain of α-actinin. Similarly, binding of α-actinin to L-selectin in vitro was also inhibited by the 53-kD fragment of α-actinin (Fig. 2 *C*). α-Actinin binding to L-selectin and to integrins appears to occur through distinct regions of the α-actinin rod domain, however, since the β_2 cytoplasmic domain peptide failed to inhibit binding of α-actinin to the L-selectin peptide. The association of α-actinin with members of the β_2 integrin subfamily in vivo can be induced upon activation of neutrophils with chemotactic peptides (Pavalko and LaRoche, 1993) or by activation of T cells by cross-linking of the T cell receptor (Pardi et al., 1993). In contrast, the interaction between α-actinin and L-selectin is constitutive in these transfected cell lines. The results of this study indicate that L-selectin can be added to the list of cell adhesion molecules that use α-actinin as a primary linker molecule to the actin cytoskeleton.

Our results also suggest that two additional cytoskeletal proteins, vinculin and talin, might also be involved in the interaction between L-selectin and the cytoskeleton, perhaps as part of a multiprotein complex. Although a direct interaction between either vinculin or talin and the L-selectin cytoplasmic domain peptide could not be detected in the in vitro solid phase binding assays, vinculin was detected in immunoprecipitates from L-selectin transfectants but not from the LΔcyto transfectants. Solid phase binding experiments indicated that vinculin bound to wells coated with L-selectin cytoplasmic domain peptide only in the presence of α-actinin. Coimmunoprecipitation of vinculin with L-selectin is therefore probably indirect, and is likely to be mediated through the high affinity interaction of α-actinin with vinculin (Wachsstock et al., 1987).

Our results with talin are somewhat more puzzling. The ability of talin to potentiate the binding of α-actinin to the L-selectin cytoplasmic domain peptide is difficult to reconcile with a lack of binding of talin to this peptide in vitro and the lack of coimmunoprecipitation of L-selectin and talin in vivo. Furthermore, no evidence exists that talin can directly interact with α-actinin, although talin does bind to vinculin in vitro (Otto, 1983; Burridge and Mangeat, 1984). This potentiation effect appears to be specific for talin, inasmuch as vinculin does not exhibit any ability to modulate the binding of α-actinin to the L-selectin cytoplasmic domain peptide. It is possible that talin does not interact with L-selectin or α-actinin with sufficient affinity to be detected in our assays, but that it can nonetheless facilitate interactions between L-selectin and α-actinin, perhaps by lowering the free energy barrier of the L-selectin/α-actinin interaction. Experiments are in progress to determine the mechanism of action by which talin potentiates the binding of L-selectin to α-actinin.

We found that the eleven carboxy terminal amino acids of the L-selectin cytoplasmic tail were not required for localization of L-selectin to the microvillar projections of the plasma membrane. Interaction of L-selectin with α-actinin or vinculin is therefore not required for correct receptor positioning. The few remaining amino acids of the L-selectin cytoplasmic tail may be sufficient for this function, through alternative interactions with other cytoskeletal or cytoplasmic proteins that are not sufficient for L-selectin's adhesive function. In this regard, it will be interesting to examine the possibility that the membrane proximal region of the L-selectin cytoplasmic domain interacts with members of the ERM (ezrin/radixin/moesin) family of cytoskeletal proteins (Sato et al., 1991), which have recently been shown to be required for the maintenance of microvilli and ruffles (Takeuchi et al., 1994). A less likely explanation for the unique distribution of L-selectin in microvillar projections may involve a role for proteins other than cytoskeletal molecules, such as interactions with molecules that are located within the plasma membrane. In either case, the normal localization of the LΔcyto mutant argues strongly that L-selectin positioning alone is not sufficient to promote L-selectin–mediated adhesion to endothelium, and hence that the unique association between L-selectin and cytoskeletal proteins fulfills a distinct requirement for adhesion by L-selectin.

The results of this study highlight the potential functional importance of a link between L-selectin and the actin cytoskeleton. The LΔcyto mutant exhibits normal carbohydrate ligand recognition (Kansas et al., 1993) and normal receptor positioning (this report), and yet it is unable to mediate leukocyte rolling or adhesion to HEV (Kansas et al., 1993). Ongoing studies are aimed at determining the factors necessary for normal L-selectin function, in addition to carbohydrate ligand recognition and microvillar localization, and the precise role of cytoskeletal interactions with the L-selectin cytoplasmic domain in regulating leukocyte rolling and adhesion to HEV.

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