A Novel Serine-rich Motif in the Intercellular Adhesion
Molecule 3 Is Critical for Its Ezrin/Radixin/Moesin-directed
Subcellular Targeting

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Intercellular adhesion molecule 3 (ICAM-3) is a leukocyte-specific receptor involved in primary immune responses. We have investigated the interaction between ICAM-3 and ezrin/radixin/moesin (ERM) proteins and its role in LFA-1-induced cell-cell interactions and membrane positioning of ICAM-3 in polarized migrating lymphocytes. Protein-protein binding assays demonstrated a phosphorylatedinositol 4,5-bisphosphate-induced association between ICAM-3 and the amino-terminal domain of ERM proteins. This interaction was not essential for the binding of ICAM-3 to LFA-1. Dynamic fluorescence videomicroscopy studies of cells demonstrated that moesin and ICAM-3 coordinately redistribute on the plasma membrane during lymphocyte migration. Furthermore, overexpression of the amino-terminal domain of moesin, which lacks the consensus moesin action-binding site, caused the subcellular mislocalization of ICAM-3. A CD4 chimerical protein containing the cytoplasmic tail of ICAM-3 was targeted to the trailing edge. Point mutation of Ser487, Ser489, and Ser496 to alanine in the juxtamembrane region of ICAM-3 significantly impaired both ERM binding and polarization of ICAM-3. ERM-directed polarization of ICAM-3 was also impaired by phosphorylation-like mutation of Ser487 and Ser489, but not of Ser496. Our results underscore the key role of specific serine residues within the cytoplasmic region of ICAM-3 for its ERM-directed positioning at the trailing edge of motile lymphocytes.

The relationship between adhesion receptors and the cytoskeleton is crucial for leukocyte migration and cell-cell interactions (1). In migrating leukocytes, ICAMs† are redistributed to the cellular uropod, a membrane protrusion at the rear of the cell (2). ICAMs act as both adhesion and signaling receptors and have partially overlapping functions (3). The β2 integrin LFA-1 specifically binds to the intercellular adhesion molecules ICAM-1, -2, and -3 (4–6). ICAM-3 is highly expressed in naive T cells and interacts preferentially with two additional ligands, the integrin αMβ2 (7) and DC-SIGN, a novel C-type lectin expressed in dendritic cells that binds with high affinity to ICAM-3 (8). ICAM-3-DC-SIGN interaction seems to play a key role during the initiation of the immune response.

ICAMs are co-localized with members of the ezrin, moesin, and radixin family of proteins in several cell types (9, 10). The latter proteins are thought to connect constituents of the plasma membrane with the cytoskeleton and are commonly associated with protrusive formations such as microvilli, microspikes, and filopodia (11, 12). In leukocytes, ERM proteins are concentrated at the tips of microvilli (13, 14), facilitating macrophage phagocytosis (15) and CD95-mediated apoptosis of T cells (16). Interactions between ICAM-1, ICAM-2, and moesin/ezrin/radixin have been studied previously in vitro (10, 17). The functional relevance of such interactions has been illustrated for natural killer cell-mediated killing of target cells (18) and for microwillar organization during cortical morphogenesis (19).

We have investigated here the dynamics of the interaction of ERM proteins with ICAM-3 and the critical residues in its cytoplasmic region, accounting both for binding to moesin and ezrin and for targeting of this adhesion receptor to the cellular uropod.

** Experimental Procedures

Antibodies, Cells, and Reagents—The anti-ICAM-3 HP2/19, TP1/24 and TP1/25; anti-CD4 HP2/6; anti-ICAM-1 RR1.1; anti-LFA-1a TS1/11; anti-CD45 D3; and anti-moesin/radixin 39/87 mAb have been described (2, 9, 20). The blocking anti-ICAM-3 140.11 mAb was generously provided by Dr. R. Villela (Hospital Clinic, Barcelona, Spain). The anti-LFA-1a NKI-L16 mAb was generously provided by Dr. C. G. Figdor (University Hospital, Nijmegen, The Netherlands). The anti-ICAM-3 polyclonal antiserum (pAb) was generously provided by Dr. D. Simmons (Imperial Cancer Research Fund Laboratories, Oxford, United Kingdom). The anti-moesin 95/2 and the anti-ezrin 90/3 pAbs were raised in rabbits by immunization with recombinant human moesin and purified human ezrin, respectively, and the former was purified by affinity chromatography (13). The affinity-purified polyclonal antibody 454 raised against a unique peptide from mouse moesin was generously provided by Dr. F. Solomon (Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA). The rat mAb 297S specific for mouse phosphorylated threonine at the COOH terminus of each ERM was generously given by Dr. S. Tsukita (Department of Cell Biology, Kyoto University, Japan). P3 × 63 myeloma protein (IgG1, Kappa) was used as negative control. The
extension PCR (22) using pSr
ers, respectively. The CD4/ICAM-3 chimera was obtained by overlap
NH2-terminal portion (residues 1–381) of rat moesin (moesin-GFP and
the carboxyl-terminal end of the full-length (residues 1–577), or at the
constructs.

Vector. Sequence analysis confirmed identity and orientation of all DNA
R3I in a second PCR. All PCR products were cloned into pCDNA3.1
ment, whereas primers D4Q3I were used to amplify the ICAM-3 frag-
TGAAGGCTTTATTGG), antisense to the distal cytoplasmic tail of
transmembrane domain of CD4 with a region of homology to the cyto-
GCCTAGCCCAATGAAAAGC), antisense corresponding to the distal
coding ICAM-3 cytoplasmic truncations were obtained by PCR using
PCR3 vector (Invitrogen) as a
ICAM-3 expression, the ICAM-3 cDNA was subcloned from pCDM8 to
ICAM-3 expression, the ICAM-3 cDNA was subcloned from pCDM8 to
ICAM-3 cDNA as template, AATGGCCACCATGGTACCATCC as 5`

primers. Point muta-

previously described (9). Fluorescence histograms are represented in a
logarithmic scale.

Time-lapse Fluorescence Videomicroscopy—36 h after transfection, NS1 cells were adhered to FN-80-coated glass cover slips and coverslips

were mounted on Artodfluor open chambers (Molecular Probes, Eugene,
OR) and placed on the microscope stage. Cells were maintained at 37 °C
in a 5% CO2 atmosphere using an incubation system (La-con (GBr)
and 100

g of these proteins with

immunoprecipitation and Western Blot— Transfected K562 cells (1 ×
10^7) were labeled overnight with a mixture of [35S]methionine/cysteine
in methionine/cysteine-free RPMI 1640 medium supple-
mented with 10% FCS. [35S]-Labeled cells were processed for immuno-
precipitation. Western blot analysis of unlabeled proteins was carried
out as described (9).

Contraction, Expression, and Purification of GST Fusion Proteins, Recombinant Moesin and Ezrin, and in Vitro Binding Assay—Construc-
tion of the GST fusion protein with the cytoplasmic regions of human
ICAM-3 has been reported elsewhere (9). Constructs coding for deletion
and point mutation cytoplasmic variants of both proteins were per-
formed by oligonucleotide site-directed mutagenesis either by overlap
extension or site-directed mutagenesis. Recombinant full-length moesin,
full-length ezrin, and their respective NH2-terminal regions contain-
ing amino acid residues 1–310 were expressed in Escherichia coli, isolated,
and purified as GST fusion proteins (25). Recombinant ezrin containing moesin assays for binding activity to ICAM-3 incubating 2
μg of these proteins with ~10 μg of GST-ICAM-3 mutants linked up glutathione-Sepharose beads, in 0.8 ml of binding
buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.5, 0.2% Triton X-100) at
4 °C for 1.5 h. Beads were washed six times in binding buffer. GST
fusion proteins and their associated proteins were eluted from glutathione-Sepharose beads using 50 mM Tris buffer containing 20 mM

Cell Adhesion and Cell Aggregation Assays—For cell adhesion,
mock-, ICAM-3WT-, or ICAM-3-transfected K562 cells were labeled in complete medium with the fluorescent dye BCECF-AM (Molecular
Probes) and incubated in RPMI medium containing 1% FCS, 2 mM MgCl2,
and 2 mM CaCl2 to 96-well microtiter plates (Costar Corp., Cambridge,
MA) coated with parental or LFA-1-transfected t-cells (1 × 10^5/well)
that were grown overnight at 37 °C. After incubation for 30 min at
37 °C, unbound cells were removed by three washes and with RPMI
medium, and adhered cells were quantified using a fluorescence ana-
lyzer (CytoFluor 2300, Millipore Co.). Heterotypic aggregation between
LFA-1-transfected and ICAM-3-transfected K562 cells was performed
as previously described (20).

Immunofluorescence Microscopy and Flow Cytometry Analysis—
LFA-1-transfected and ICAM-3-transfected K562 cells (2 × 10^5/well)
were mixed and incubated in flat-bottomed, 24-well plates (Costar
Corporation). Cells were fixed in 4% PFA in 0.1 M sodium cacodylate
buffer (pH 7.4) for 10 min. The concentration of the activating anti-LFA-1 NKI-L162 mAb was added and cells were incubated at 37 °C and 5% CO2 atmosphere for 10 min at room temperature and rinsed in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl. To directly visualize the LFA-1 molecule, cells were stained
with the anti-LFA-1 mAb 28.9. After incubation with the anti-LFA-1 mAb,
cells were washed three times with PBS and incubated with the biotinylated anti-ICAM-3 TP1/24, and co-staining with LFA-1 and moesin was carried out using rhodamine X- or Alexa 488-streptavidin (Molecular Probes), respec-
tively. For double immunofluorescence of ICAM-3 and LFA-1, an addi-
tional step with saturating amounts of normal mouse serum was car-
ried out prior to the addition of biotinylated anti-ICAM-3 mAb. 24 h
after transfection, NS1 cells were allowed to adhere on FN-80-coated
glass cover slips for 30 min, fixed in 4% paraformaldehyde in phosphate-
buffered saline for 10 min at room temperature, rinsed in 50 mM
Tris-HCl, 150 mM NaCl, pH 7.6, and visualized by staining the cells
with the anti-ICAM-3 TP1/24 mAb plus a 1:50 dilution of an FITC-
labeled goat F(ab')2 anti-mouse Ig (Dako). Cells were observed using a
Leica DMR photomicroscope (Leica, Heidelberg, Germany) with 40, 63,
and 100 × oil immersion objectives. Images were acquired using the
Leica QFISH 1.0 software. The proportion of ICAM-3-redistributed cells
was calculated by random choice of 10 different fields (80% objective)
of each condition and counting of at least 700 cells.

Flow cytometry analysis of transfected K562 cells was performed as
previously described (9). Fluorescence histograms are represented in a
logarithmic scale.

Immunoprecipitation and Western Blot— Transfected K562 cells (1 ×
10^7) were labeled overnight with a mixture of [35S]methionine/cysteine
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80-kDa filamentous fragment (FN80) was a generous gift of Dr. A.
García-Pardo (Centro de Investigaciones Biológicas, Madrid, Spain).
The LFA-1-transfected mouse t-cells and human K562 cells were kindly
provided by Dr. Y. van Kooyk (University Hospital, Nijmegen, The
Netherlands) and Dr. A. L. Corbi (Centro de Investigaciones Biológicas,
Madrid, Spain). Human leukocytes, primary peritoneal mononuclear cells,
and EL-4 cells were grown in modified Dulbecco's medium with Glutamax-I (Invitrogen) supplemented with 10% fetal calf serum (FCS), and the human erythroleukemic cell line K562, the HSB-2 T cell line, and the mouse myeloma cell line NS1 were grown in RPMI 1640 culture medium (Invitrogen)
supplemented with 10% FCS. PIP2 was purchased from Sigma.

Recombinant DNA Constructs and Transfection of Cells

Generation of GFP fusion constructs with the GFP cDNA inserted at
the carboxy-terminal end of the full-length (residues 1–577), or at the
NH2-terminal portion (residues 1–381) of rat moesin (moesin-GFP and
N-moesin-GFP, respectively), and the GFP cDNA inserted at the amino-
terminal end of full-length rat moesin (GFP-moesin) were described
previously (23). Stably and transiently transfected K562 and NS1 cells,
respectively, were generated by electroporation, basically as has been
described (23). After electroporation burst, NS1 cells were transferred
to coverslips in flat-bottomed 24-well plates (Costar, Cambridge, MA)
in a final volume of 1 ml of complete medium + 50 μM β-mercaptoethanol
(Sigma). 24 h after transfection, cells were fixed in 3.7% formaldehyde
in phosphate-buffered saline for 10 min at room temperature and rinsed
in 50 mM Tris-HCl, 150 mM NaCl, pH 7.6.

ERM-directed Adhesion Receptor Localization
glutathione. Moesin binding assays to ICAM-3 in the presence of PIP3 were carried out preincubating recombinant full-length moesin with liposomes of PIP3, at 50 μg/ml in 20 mM HEPES buffer, pH 7.4, containing 130 mM KCl and 0.2 mM EGTA. Bound moesin and ezrin were determined by SDS-PAGE and immunoblotting using moesin 952 or ezrin 903 pAbs.

In Vitro Translation and Binding Assays—The pc3R plasmids carrying the inserts of untagged moesin and ezrin amino-terminal regions containing amino acid residues 1–310 (MSNα/pC3R and E3R/pC3R, respectively) were described previously (24). The plasmids were transcribed and translated in vitro using a TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of T7 RNA polymerase and [35S]methionine, and binding assays carried out as previously described (24).

RESULTS

Interaction of the Cytoplasmic Region of ICAM-3 with Moesin and Its Possible Role in the Binding of ICAM-3 to LFA-1—Wild type ICAM-3 (ICAM-3WT) and an ICAM-3 protein lacking the cytoplasmic domain by truncation at Arg 482 (ICAM3α) were stably expressed in K562 cells, which as determined by flow cytometry show ICAM-1, but negligible amounts of ICAM-3 and LFA-1 (Fig. 1, A and B). Similar levels of ICAM-3WT and ICAM-3α were expressed by K562 transfectants (Fig. 1B, b and c). As expected, the molecular mass of ICAM-3WT was ~120 kDa, whereas ICAM-3α was somewhat lower (Fig. 1C, a). Moesin and ezrin co-precipitated with ICAM-3WT but not with ICAM-3α (Fig. 1C, b and c). Neither moesin nor ezrin was detected in precipitates from mock K562 cells.

To study the role of the interaction of ICAM-3 with moesin in the binding of ICAM-3 to LFA-1, we analyzed the adhesion of ICAM-3WT and ICAM-3α K562 cells to mouse L-cells stably transfected with recombinant human LFA-1; these cells reacted strongly with anti-LFA-1 mAb, whereas parental L-cell did not (Fig. 1D, a, and data not shown). In this assay, ICAM-3WT and ICAM-3Δ K562 cells bound to LFA-1-L-cells similarly to mock K562 cells, because parental and transfected K562 cells significantly express ICAM-1 (Fig. 1B, a, and data not shown). The adhesion of mock, ICAM-3WT, and ICAM-3α K562 cells to LFA-1-L cells was blocked by anti-LFA-1 TS1/11 mAb, which induces the high affinity state (Fig. 1C, a, and data not shown). In contrast, anti-ICAM-3 140.11 mAb significantly reduced the adhesion of ICAM-3WT and ICAM-3α cells, without affecting the adhesion of mock cells (Fig. 1E). Interestingly, the combination of anti-ICAM-1 and anti-ICAM-3 mAbs blocked the adhesion of all transfectants to LFA-1-L cells, whereas the anti-CD45 D93/9 mAb control had no effect (Fig. 1E).

To confirm that the interaction of ICAM-3 with moesin was not required for the binding of ICAM-3 to LFA-1, the localization of moesin in ICAM-3/LFA-1 cell-cell contacts was studied by two-color immunofluorescence staining. K562 cells stably transfected with LFA-1 (Fig. 1D, b) were conjugated with ICAM-3WT or ICAM-3Δ K562 cells. Because LFA-1 is inactive in LFA-1-transfected K562 cells (25), the heterotypic intercellular adhesion of transfectants was triggered using the activator N-moesin-GFP, were expressed in HSB-2 T cells. GFP-moesin showed a cytoplasmatic pattern, as has been observed previously in NIH3T3 cells (23). This fusion protein, GFP is linked to the NH2-terminal domain of full-length moesin and this prevents binding to membrane sites. This fusion protein did not affect the localization of ICAM-3 (Fig. 3, a and b). On the other hand, moesin-GFP co-localized with endogenous ICAM-3 at the uropod, as shown in Fig. 3 (c and d), and this localization is identical to that of the endogenous moesin (data not shown). In contrast, when N-moesin-GFP was highly expressed in these cells, ICAM-3 was not concentrated in the uropod and remained evenly distributed throughout the plasma membrane (Fig. 3, e and f).

Dynamics of Moesin and ICAM-3 Redistribution in Migrating Lymphocytes—Motile N12 myeloma cells showed the characteristic “hand mirror” shape of polarized cells (Fig. 4A, a). Two poles were distinguished in these cells, a leading edge, enriched in β-actin, and the uropod, where α-tubulin, moesin, and carboxyl-terminal phosphorylated ERM proteins are concentrated (Fig. 4A, b, c, and d). To examine the subcellular distribution of activated endogenous ERM proteins, we stained NS1 cells with rat 297S mAb, which recognizes carboxyl-terminal phosphorylated ERM proteins (27). Activated ERM proteins were found polarized at the uropod area (Fig. 4A, e and f). To assess the role of ICAM-3/moesin interaction in the redistribution of ICAM-3 to the uropod of polarized migrating lymphocytes, we first examined the membrane localization of ICAM-3WT and ICAM-3Δ in transiently transfected mouse NS1 cells. Although wild type and ICAM-3 truncated proteins were expressed on the plasma membrane, solely ICAM-3WT was concentrated at the cellular uropod (Fig. 4A, g and h). In contrast, ICAM-3Δ was evenly distributed on cell membrane (Fig. 4A, i and j). No expression of endogenous ICAM-3 was detected in mock-transfected NS1 cells (data not shown).

To assess whether the cytoplasmic domain of ICAM-3 is sufficient to target other transmembrane proteins to the cellular uropod, the cytoplasmic domain of CD4 was replaced by that of ICAM-3. CD4 was uniformly distributed on the plasma membrane of both migrating polarized T lymphoblasts and NS1 cells transfected with either wild type CD4 or a CD4 truncated form lacking the cytoplasmal tail (CD4Δ) (Fig. 4B). Interestingly, the chimeral CD4 protein containing the cytoplasmic tail of ICAM-3 was redistributed to the uropod (Fig. 4B).

To study the role of moesin in the redistribution of ICAM-3 during cell motility, we used COOH-terminal fusions of full-length moesin with green fluorescent protein (moesin-GFP)
and ICAM-3 with red fluorescent protein (ICAM-3-RFP). The two fusion proteins were co-expressed and their distribution recorded by time lapse fluorescence videomicroscopy in live and moving NS1 cells. Initially, moesin-GFP (green) and ICAM-3-RFP (red) accumulated in the cellular uropod of such migrating cells as described above (Fig. 5a). The polarity of NS1 cells often reversed spontaneously when cells moved on substratum. When such cells were analyzed, both proteins changed their distribution within minutes to a more diffuse localization (Fig. 5b and c); finally, both were concentrated again in a newly formed cellular uropod (Fig. 5e and f). The changes in distribution continue with every change in the direction of movement (Fig. 5g–j). They are thus coordinated and closely associated with the changes in shape and polarity.

FIG. 1. Characterization of transfected cells and adhesion of ICAM-3WT and ICAM-3Δ K562 cells to LFA-1 transfectants. A, schematic representation of native ICAM-3 and its cytoplasmic truncated form. The plasma membrane is represented by a vertical bar; the cytosol is on the right and the five external immunoglobulin-type domains of ICAM-3 on the left. Numbers indicate the residues deleted from ICAM-3 (45). The underlined residues are predicted as part of the transmembrane domain. B, flow cytometry analysis with the anti-ICAM-3 TP1/24 mAb (thick line) of mock (a), ICAM-3WT (b), and ICAM-3Δ (c) K562 cells. The staining of LFA-1 (TS1/11 mAb) (dashed line) and ICAM-1 (RR1.1 mAb) (thin line) on mock K562 cells is shown in a. The staining of control P3 × 63 (thin line) are also shown in b and c. C, co-immunoprecipitation of moesin with ICAM-3 in ICAM-3-transfected K562 cells. K562 cells stably transfected with empty PCR3 vector, ICAM-3WT, or ICAM-3Δ were lysed and immunoprecipitated with anti-ICAM-3 TP1/25 mAb. Immunoprecipitates were then resolved on SDS-PAGE and immunoblotted with rabbit anti-ICAM-3 pAb (a), anti-moesin pAb 95/2 (b), and anti-ezrin pAb 90/3 (c). D, LFA-1 expression of transfectants used in cell adhesion and aggregation assays. Flow cytometry analysis with the anti-LFA-1 mAb TS1/11 (thick line) of L-cells (a) and K562 cells (b) stably transfected with LFA-1. Thin lines represent P3 × 63 control staining. E, adhesion of ICAM-3WT and ICAM-3Δ-transfected K562 cells to LFA-1-transfected L-cells. Mock, ICAM-3WT, and ICAM-3Δ K562 cells were allowed to adhere on LFA-1-t-cell-coated plastic wells pretreated or not for 15 min with saturating concentrations of the indicated mAbs against LFA-1 (TS1/11), ICAM-1 (RR1.1), ICAM-3 (140.11), and CD45 (D3/9). The results are expressed as the percentage of cells bound to LFA-1-t-cells minus the percentage of cells bound to parental t-cells, where 100% is calculated as the total number of cells added to a t-cell-coated well. The mean ± S.E. from three experiments is shown.

F, subcellular localization of LFA-1, ICAM-3WT, ICAM-3Δ, and moesin in LFA-1-induced cell conjugates. LFA-1 K562 cells and ICAM-3WT- or ICAM-3Δ-K562 cells were allowed to adhere on FN-80-coated glass coverslips, and cell conjugates were induced by heterotypic cell aggregation at 37 °C, incubating cells with the activating anti-LFA-1 NKI-L16 mAb for 30 min. Double immunofluorescence of LFA-1 (a and b, left) with ICAM-3WT (a, right) or ICAM-3Δ (b, right); and of moesin (c and d, right) with ICAM-3WT (c, left) or ICAM-3Δ (d, left) was carried out as described under “Experimental Procedures.”
Serine Residues in the Cytoplasmic Tail of ICAM-3 Are Critical for Moesin and Ezrin Binding—To identify the region(s) within the amino acid sequence of the cytoplasmic domain of ICAM-3 with potential interaction with ERM proteins, the deletion mutants of 16 (Y498stop), and 8 (Y490stop) cytoplasmic residues and several mutants within the amino acid segment of residues 484–503 were expressed in bacteria and purified as GST fusion proteins (Figs. 6A and 7A). To determine the amino acid segment required for the interaction with moesin and ezrin, N-moesin and N-ezrin were translated and isotope-labeled in vitro, and added to Sepharose beads containing amino acid deletions and double mutations of the ICAM-3 cytoplasmic region. Whereas binding of N-moesin and N-ezrin to Y498stop and L501A/S503A mutants was similar to the wild type protein, binding to Y490stop, S496G/Y498A, and H491A/S503A or T497A point mutations was reduced, whereas R493A and S503A mutants bound both ERM proteins at levels comparable with those of the wild type protein (Fig. 7, A–C). Remarkably, mutation of Ser487 to Ala caused the highest loss of binding activity.

A phosphorylation-like change of these residues in the binding of moesin was assessed by generating ICAM-3 mutations, in which Ser487, Ser489, and Ser496 have been replaced by aspartic acid. The binding of N-moesin to S497D and S498D was significantly reduced (Fig. 7D), in accordance with results corresponding to alanine mutations (Fig. 7B). In contrast, the binding of S496D mutant to N-moesin was slightly higher than the wild type protein (Fig. 7D), suggesting a role for Ser496 phosphorylation in regulating the interaction of ICAM-3 with moesin in vivo. Similar results were obtained with N-ezrin (data not shown). These results demonstrate that Ser487, Ser489, and Ser496 in the cytoplasmic region of ICAM-3 are key residues for the binding of ERM proteins.

Figure 2. Interaction of recombinant moesin and ezrin with the cytoplasmic region of ICAM-3. A, Coomassie Blue-stained 12% SDS-PAGE of recombinant full-length moesin, ezrin, and their amino-terminal proteins purified from bacteria as described under “Experimental Procedures.” Small amounts of proteolytic peptides are present. Glutathione-Sepharose beads alone (Glut-Seph) or coated either to GST or to GST fused to the cytoplasmic region of ICAM-3 (Cy3WT) were incubated with identical amounts of purified recombinant full-length moesin (B and E), N-moesin (C), and N-ezrin (D). GST, Cy3WT, and bound proteins were eluted together from the beads with 50 mM Tris-buffer containing 20 mM glutathione. Purified recombinant proteins were loaded in the first lane of each panel. In D, Cy3WT not incubated with ezrin was loaded as control. Eluted proteins were resolved by 12% SDS-PAGE followed by immunoblotting with the anti-moesin 95/2 pAb in B and C and with the anti-ezrin 90/3 pAb in D. Coomassie staining of the amounts of GST and Cy3WT GST fusion protein used in these experiments are shown down in C, D, and E. Similar amounts of GST fusion proteins were used in B, E, PIP2-induced binding of moesin to ICAM-3. Full-length moesin was preincubated for 30 min with liposomes of PIP2 at 10 µg/ml and then glutathione-Sepharose beads alone (Glut-Seph) or coated either to GST or to GST fused to the cytoplasmic region of ICAM-3 (Cy3WT) were added to interact with full-length moesin in the presence or the absence of PIP2 liposomes, and bound moesin detected by Western blot. Partially degraded purified recombinant full-length moesin was loaded in the first lane. Molecular mass values (in kDa) are indicated on the left.

and one (Ser496) within the amino acid segment lost from Y498stop to Y490stop mutant, which have been shown to be involved in ICAM-3-mediated activation and spreading of T cells (28). A detailed mutational analysis of this region (residues 487–503) showed that binding of N-moesin and N-ezrin to S487A, S498A, S496A, and T497A point mutations was reduced, whereas R493A and S503A mutants bound both ERM proteins at levels comparable with those of the wild type protein (Fig. 7, A–C). Remarkably, mutation of Ser487 to Ala caused the highest loss of binding activity.

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Mutation of Ser487, Ser489, or Ser496 Significantly Impairs the Targeting of ICAM-3 to the Uropod of Polarized Lymphocytes—We next studied whether these and other mutations in ICAM-3 altered its membrane localization in motile cells. Upon transfection of NS1 mouse lymphocytes with Y498stop, R493A, L501A/S503A, E494A/E495A, H491A, S503A, or T497A, ICAM-3 mutated proteins were concentrated at cellular uropod (Table I), similar to ICAM-3 WT. In contrast, ICAM-3Δ, Y490stop, H484A/R486A, S496A/Y498A, S487A, S489A, and S496A mutations were uniformly distributed throughout the plasma membrane, showing a significant lower concentration at the uropod, espe-
pecially for the H484A/R486A and S487A mutations (Table I). When serine phosphorylated-like mutations of ICAM-3 were analyzed, S487D and S489D did not polarize in NS1 cells, similar to the corresponding alanine mutations. In contrast, S496D was concentrated at the cellular uropod, whereas the polarization of S496A protein was significantly reduced (Table I).

To investigate this region further, NS1 cells were co-transfected with full-length moesin-GFP and ICAM-3 mutants. The additional mutant S503A co-localized with moesin-GFP in the cellular uropod, but S487A and H484A/R486A clearly showed a diffuse localization (Fig. 8). Taken together, we conclude that in addition to Ser487, Ser489, and Ser496 the positively charged residues His484 and Arg486 are critical for the ERM-directed redistribution of ICAM-3 toward the trailing edge of polarized migrating lymphocytes.

**DISCUSSION**

ICAMs were initially considered “passive” receptors that solely supported leukocyte intercellular adhesion through their interaction with β2 integrins. However, it has become increasingly evident that ICAM function is more dynamic and complex, because these molecules form clusters in the plasma membrane, act as signal transducing receptors, and associate to cytoskeletal proteins (3). During leukocyte polarization, the cellular localization of ICAM-3 changes in characteristic ways (2, 29). ICAM-3 plays a rather specific role in mediating intercellular interactions during T lymphocyte-APC conjugate formation (30) and homotypic cell aggregation (20). In this regard, it has been described the involvement of ICAM-3 in the initial interaction of naïve T lymphocytes with dendritic cells (DC) by recognition of a novel ligand from the C-type lectin superfamily in DC (8).
In motile lymphocytes, ICAM-3 is localized at the cellular uropod, and this localization depends on an intact cytoplasmic domain as revealed by immunofluorescence microscopy, despite the fact that formation of the uropod was independent of ICAM-3 expression. Although a fraction of ICAM-3 and other adhesion molecules might be concentrated in this cell structure by membrane folding, a mechanism that would not require a cytoplasmic domain (31), our data indicate that, at least in motile leukocytes, the concentration of ICAM-3 in the uropod is regulated by interactions with ERM proteins. Members of this family of proteins are concentrated in the cellular uropod (9), and earlier studies showed that the uropod of motile lymphocytes is enriched in microvilli and microspikes (32), structures in which certain adhesion receptors and moesin-like proteins are enriched in many other cell types.

It has been described that the phospholipid PIP$_2$ enhances the binding of moesin to CD44 in vitro (33). Moreover, it has been postulated that by binding to ERM proteins, PIP$_2$ abrogates the intramolecular interaction between the NH$_2$- and COOH-terminal domains, unmasking the membrane binding site and activating the molecule (34, 35). Our data also demonstrate a role for PIP$_2$ in regulating the binding ability of moesin to ICAM-3. Recently, studies carried out in ezrin-transfected COS1 cells have demonstrated that specific PIP$_2$ binding in the NH$_2$-terminal domain of ERM proteins are essential for mediating appropriate targeting to plasma membrane actin-rich structures (36). However, a dominant negative role for the NH$_2$-terminal domain of ezrin has been proposed for the abnormal distribution of microvilli in N-ezrin-expressing LLC-PK1 cells (37). Our findings demonstrating the mislocalization of N-moesin and ICAM-3 in HSB-2 cells transfected with the NH$_2$-terminal domain of moesin are consistent with this idea and suggest that the COOH-terminal region of moesin is required as well. This region contains a high affinity F-actin binding site (38) suggesting that, although the PIP$_2$ and the ICAM-3 membrane binding sites in the NH$_2$-terminal domain preserve their integrity, the COOH-terminal domain plays a critical role in the moesin-mediated redistribution of ICAM-3. Moesin clearly co-localized with ICAM-3 in the uropod of NS1 cells, as demonstrated for both endogenous and full-length moesin-GFP fusion proteins, and, therefore, they potentially interact at such sites. However, mechanisms that govern interactions between ERM and transmembrane proteins need further exploration and clarification. Observations from other groups have pointed to a role for PKC-θ (39) and several components of the Rho pathway, such as phosphatidylinositol 4-phosphatase 5-kinase (33, 34, 40), but not ROCK kinases (34) in the regulation of moesin activation in vivo. In this regard, our data indicate that carboxy-phosphorylated ERM proteins are polarized in the uropod region. Despite the direct interaction between ICAM-3 and moesin and ezrin that we have observed in vitro, our data do not exclude involvement of other linker proteins in cells (12).

A sequence within ICAM-3 corresponding to residues 484–497 appears to be critical for ERM binding. In vitro binding assays showed that the deletion mutant Y498stop conserved most of the ERM binding activity and the amino acid residues Ser$^{489}$ within the sequence played a role in this interaction. Ser$^{489}$ is also required for redistribution of ICAM-3 to the uropod of polarized lymphocytes as we have demonstrated here. Additionally, the juxtamembrane cluster containing the positively charged residues His$^{483}$/Arg$^{486}$ and serines Ser$^{487}$/Ser$^{489}$ appears to be critical for both moesin binding and ICAM-3 localization. Hence, our data indicate the existence of two regions in the cytoplasmic tail of ICAM-3 responsible for the subcellular targeting mediated by linkages with ERM proteins. ICAM-3, like human ICAM-2, lacks the positively charged juxtamembrane clusters required for ERM binding to
CD44 and CD43 (17, 41). Instead, two basic amino acids and specific serines within juxtamembrane regions appear to be critical for this binding (Fig. 9). Juxtamembrane clusters of positively charged amino acids are characteristics of most of the transmembrane proteins. However, few proteins have been described capable to link ERM proteins. How is this specific binding regulated? It has been proposed that, by distal membrane regions, the transmembrane proteins themselves could regulate negatively their binding to ERM proteins (17). Another possibility is that serine residues near these regions play a role to provide a particular conformation for ERM binding; this binding could be regulated by phosphorylation. In this regard, it has been described the PKC-mediated phosphorylation of serine residues in the ICAM-3 cytoplasmic tail (28, 42). Phosphorylation of intracellular Ser489 of ICAM-3 plays a key role for IL-2 secretion, aggregation, and spreading of Jurkat cells co-stimulated with anti-ICAM-3 mAb (28), and substitution of Ser487 and Ser496 by Ala significantly decreased IL-2 secretion and cell spreading, respectively, induced by anti-ICAM-3 mAb (28). In addition, PMA-induced phosphorylation of ICAM-3 redistributes it uniformly on the plasma membrane of lymphocytes (29). Taken together, these results concur with our findings, in which interaction of moesin with ICAM-3 and uropod targeting of ICAM-3 were significantly impaired by mutation of Ser487 or Ser489 to Ala or Asp. Recently, it has been demonstrated that a negatively charged peptide is able to bind the inositol site in the FERM (4.1, ezrin/radixin/moesin homology domain) of moesin (43). In this context, although still undetermined, it could be proposed that the phosphorylation of Ser496 of ICAM-3 may generate an strongly negatively charged cluster (494EESp) that would bind to moesin by displacing PIP2 from the PIP2-moesin complex. Although elusive, this possibility would explain a stron-

**FIG. 7. Interaction of in vitro translated amino-terminal moesin and ezrin with ICAM-3 cytoplasmic point mutations.** A, amino acid sequence of the wild type (WT) and several selected point mutations of the ICAM-3 cytoplasmic domain fused to GST analyzed for moesin binding. B and C, GST or the GST fusion proteins containing wild type cytoplasmic domain of ICAM-3 (Cy3WT) or the point mutants S487A, S489A, R493A, S496A, T497A, and S503A within this domain bound to glutathione-Sepharose beads were incubated with the [S35]Met-labeled in vitro translated N-moesin (B) and N-ezrin (C). GST or the GST fusion proteins of the cytoplasmic domain of ICAM-3 (Cy3WT) or the serine phosphorylated-like point mutants S487D, S489D, and S496D bound to glutathione-Sepharose beads were incubated with [S35]Met-labeled N-moesin for pull-down experiments. Bound moesin and ezrin were detected as in Fig. 6. Coomassie staining of GST fusion proteins used in B–D is shown. Relative binding of N-moesin and N-ezrin to GST fusion proteins was estimated by densitometric analysis comparing the amount of moesin (B and D) and ezrin (C) bound to equal amounts of each mutant with those eluted from Cy3WT, as described previously by Yonemura et al. (17). Values represent relative binding capability averaged from three experiments ± S.D.
Interestingly, in T lymphocytes, activation of PKC-θ is a T cell receptor-induced event, and PKC-θ activity has been implicated in the phosphorylation of both the cytoplasmic region of ICAM-3 and the COOH-terminal domain of moesin (28, 39).

It has been proposed that, during immune interactions, the redistribution of ICAM-1 toward the contact zone of T cell/APC conjugates requires the role of actin cytoskeleton in the T cell but not in the APC (44). As it occurs during the transient initial cell-to-cell contacts in leukocyte intercellular interactions (20), it has been recently found that, during the early phase of the immune response, ICAM-3 could be redistributed in the T cell to bind more avidly dendritic cell- or other APC-bearing ligands (30). In this regard, our data strongly suggest that ICAM-3 does not require to interact with moesin to bind LFA-1. However, it could not be excluded that in other cellular context ERM proteins facilitate the ligand accessibility of ICAMs by driving their redistribution toward defined plasma membrane cell compartments. For ICAM-3, this redistribution seems to be a phenomenon selectively regulated by serine residues in its cytoplasmic domain. Elucidation of mechanisms that control rear to front motility of adhesion molecules when T cells contact with APCs is a crucial aspect of T-lymphocyte biology that deserves further research.

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**Table I**

Quantitative analysis of the redistribution to the uropod of ICAM-3 mutant proteins

| Mutant   | Cell no. | ICAM-3 redistributed | Polarization |
|----------|----------|---------------------|-------------|
| ICAM-3 WT | 1040     | 815                 | 78.4%       |
| ICAM-3ΔA | 1101     | 237                 | 21.5%       |
| Y490stop | 1861     | 364                 | 19.5%       |
| Y498stop | 2002     | 1415                | 70.7%       |
| H484A/F486A | 1320 | 438                 | 33.2%       |
| S496A/F498A | 1541 | 631                 | 40.9%       |
| E494A/F495A | 987   | 754                 | 76.4%       |
| S501A/S503A | 984   | 752                 | 76.4%       |
| S487A    | 778      | 220                 | 28.3%       |
| S487D    | 870      | 306                 | 35.2%       |
| S489A    | 867      | 330                 | 38.1%       |
| S489D    | 927      | 237                 | 25.6%       |
| H491A    | 920      | 648                 | 70.4%       |
| R493A    | 879      | 603                 | 68.6%       |
| S496A    | 1120     | 571                 | 51.0%       |
| S496D    | 913      | 691                 | 75.7%       |
| T497A    | 954      | 585                 | 61.3%       |
| S503A    | 1003     | 703                 | 70.1%       |
| S489D    | 927      | 237                 | 25.6%       |
| L501A/S503A | 984   | 752                 | 76.4%       |
| E494A/E495A | 987   | 754                 | 76.4%       |
| S496A/Y498A | 1541 | 631                 | 40.9%       |
| H491A    | 920      | 648                 | 70.4%       |
| R493A    | 879      | 603                 | 68.6%       |
| S496A    | 1120     | 571                 | 51.0%       |
| S496D    | 913      | 691                 | 75.7%       |
| T497A    | 954      | 585                 | 61.3%       |
| S503A    | 1003     | 703                 | 70.1%       |

**Fig. 8.** Substitution of Ser487 by Ala caused subcellular mislocalization of ICAM-3. Schematic representation of ICAM-3 point mutations transiently transfected in NS1 mouse myeloma cells. The position of amino acid substitutions are indicated on the right. NS1 cells were transiently co-transfected with full-length moesin-GFP (green) and the ICAM-3 mutants, S503A, S487A, and H484A/F486A (red) and cells were grown at 37 °C for 24 h. Then, cells were allowed to adhere on FN-80-coated glass coverslips for 30 min, fixed, and stained with the anti-ICAM-3 TP 1/24 mAb. Bar, 10 μm.

**Fig. 9.** Amino acid sequence of the juxtamembrane cytoplasmic regions of ICAM-3 involved in their subcellular targeting and ERM binding. Important residues of ICAM-3 analyzed for polarized experiments are shown.
ERM-directed Adhesion Receptor Localization

10409

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