Functional Mapping of the Cytoplasmic Region of Intercellular Adhesion Molecule-3 Reveals Important Roles for Serine Residues*

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The abbreviations used are: ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function antigen-1; IL2, interleukin 2; TCR, T cell receptor; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PKC, protein kinase C; APC, antigen-presenting cell; PCR, polymerase chain reaction; HA, hemagglutinin; MHC, major histocompatibility complex; PMSF, phenylmethylsulfonyl fluoride; PMA, phorbol 12-myristate 13-acetate.

ICAM-3* (CD50) is a member of the Ig superfamily sharing sequence and functional attributes with ICAM-1, -2, -4 (Landsteiner-Weiner blood group glycoprotein), and -5 (telencephalin). ICAM-3 binds to LFA-1 (CD11a/CD18) and the newly described integrin, ICAM-1, -2, -4 (Landsteiner-Weiner blood group glycoprotein), and -5 (telencephalin). ICAM-3 binds to LFA-1 (CD11a/CD18) and the newly described integrin, ICAM-1, -2, -4 (Landsteiner-Weiner blood group glycoprotein), and -5 (telencephalin). ICAM-3 has been functionally characterized with respect to ICAM-3 engagement initiates several distinct aspects of lymphocyte function, which involve the 37-amino acid cytoplasmic portion. Numerous intracellular signaling events have also been observed to be affected by ICAM-3 engagement. Specifically, activation of intracellular calcium flux and stimulation of tyrosine kinase activity possibly via non-receptor tyrosine kinases p56 lck and p59 fyn were seen (8, 9). ICAM-3 engagement has also been observed to up-regulate β1 and β2 integrin function, and to trigger phosphorylation of the cyclin-dependent kinase cdc2 (10–12). Little information, however, is available regarding the molecular mechanisms of these phenomena.

Here we report that ICAM-3 engagement initiates several distinct aspects of lymphocyte function, which involve the 37-amino acid cytoplasmic portion. For these analyses, we developed an ICAM-3-deficient, human T-leukemic Jurkat cell line. Using these cells and gene transfer techniques, a functional map of the cytoplasmic region of ICAM-3 with respect to TCR accessory molecule function, homotypic aggregation, and cell spreading was generated. These data pinpoint serine residues, particularly serine 489, as critical for ICAM-3 function.

MATERIALS AND METHODS

Cells and Cell Culture—Jurkat 77 (J77, a gift from Dr. S. Burakoff, Dana Farber Cancer Research Institute, Boston, MA) and the ICAM-3-deficient J77.50.3 cells were maintained in RPMI complete medium (RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μm L-glutamine, and 1 mM sodium pyruvate) in humidified 5% CO2 at 37 °C.

Monoclonal Antibodies—Murine mAb used in this study were as follows. Anti-ICAM-3 (CD50) mAb ICR1.1 (IgG2a), ICR2.1 (IgG1), ICR9.2 (IgG2a), and anti-ICAM-1 (CD54) mAb 18E3D (IgG1) were generated by the ICOS hybridoma facility. Hybridoma lines secreting anti-ICAM-3 (CD50) mAb were obtained from American Type Culture Collection, Rockville, MD. These mAb were purified by protein A column chromatography of mouse ascites fluid. Purified mAb were dialyzed against and stored in PBS. Isotype-matched control mAb used were UPC10 (IgG1) and MOPC 21 (IgG2a) (Sigma). Anti-ICAM-1 mAb 2A5 (IgG1) and anti-ICAM-3 mAb ICR9.2 (IgG2a) were obtained from American Type Culture Collection, Rockville, MD. These mAb were purified by protein A column chromatography of mouse ascites fluid. Purified mAb were dialyzed against and stored in PBS. Isotype-matched control mAb used were UPC10 (IgG1) and MOPC 21 (IgG2a) (Sigma). Anti-HA mAb 12CA5 (IgG1) was from Boehringer Mannheim. Fluorescein isothiocyanate-conjugated sheep anti-mouse IgG Fab', was purchased from Sigma.

Development and Characterization of ICAM-3-deficient Jurkat Cells—A variant of Jurkat 77 cells deficient in the expression of ICAM-3 (J77.50.3) was generated by two rounds of indirect staining and cell sorting using a mixture of ICR1.1 and 9.2 mAb (4). J77.50.3 cells were generated by the ICOS hybridoma facility. Hybridoma lines secreting anti-ICAM-3 (CD50) mAb were obtained from American Type Culture Collection, Rockville, MD. These mAb were purified by protein A column chromatography of mouse ascites fluid. Purified mAb were dialyzed against and stored in PBS. Isotype-matched control mAb used were UPC10 (IgG1) and MOPC 21 (IgG2a) (Sigma). Anti-HA mAb 12CA5 (IgG1) was from Boehringer Mannheim. Fluorescein isothiocyanate-conjugated sheep anti-mouse IgG Fab', was purchased from Sigma.

Development and Characterization of ICAM-3-deficient Jurkat Cells—A variant of Jurkat 77 cells deficient in the expression of ICAM-3 (J77.50.3) was generated by two rounds of indirect staining and cell sorting using a mixture of ICR1.1 and 9.2 mAb (4). J77.50.3 cells were compared with the parental line for surface expression of numerous membrane proteins by indirect immunofluorescence (FACSCAN, Becton-Dickinson, Mountain View, CA) and found to exhibit similar levels for all except ICAM-3.

Ten micrograms of total RNA isolated from parental J77 and J77.50.3 cells was subjected to blotting, hybridization, and washing as described (13). Labeled probes were generated by random priming of cDNA (14).

ICAM-3 Deletion and Point Mutation Constructs—Coding sequences for HA epitope-tagged ICAM-3 proteins were generated as described (15). To engineer epitope-tagged full-length ICAM-3 construct, three separate PCR fragments that encoded 1) the signal sequence (preceded
ICAM-3 Cytoplasmic Region Functional Map

by a unique HindIII site and Kozak sequence), 2) Ig domains (IgD I-III) of ICAM-3, and 3) a triple (3′) influenza hemagglutinin (HA) epitope tag sequence were synthesized and gel-purified (16). The fragments were combined using PCR in the following order: ICAM-3 signal sequence, HA tag, and ICAM-3 IgD I-III. This product was ligated as a HindIII/StuI fragment with a SacI fragment containing the remainder of the ICAM-3 coding sequence into the HindIII/EcoRI sites of expression vector pMH-neo and all PCR products sequenced (17).

Cytoplasmic region deletions were generated as follows. The region of coding sequence for the extracellular domains described above contained a HindIII/StuI fragment, which was ligated to constructs containing a SacI fragment encoding cytoplasmic domain truncations and ligated to the HindIII/EcoRI sites of pMH-neo. The PCR fragments were synthesized using the following primers: 1) 5′ common anchoring primer CATAAT-GGTACCTATCACTGTC, and 2) 3′ primers D505 (−1/3CT), ATAT-GCCGGCAGGCTCAGTGTCTCCTGAAGACGTACAT. Primer D484 contained a change at codon 483 to increase the membrane anchor region of the maximal cytoplasmic region truncation. Amino acid numbering uses the mature amino terminus for the first residue.

To generate point mutations, the ICAM-3 cDNA was subcloned as a NotI/EcoRI fragment into M13 BM21 replicative form DNA (Boehringer Mannheim). The primers used for mutagenesis were designed to introduce alanine changes at the following codons: serine 457, serine 469, leucine 499, serine 503, and serine 515 (18). Leucine 499 was chosen as a control for mutational effect, since it is not a potential phosphorylation site and a conservative change to alanine is expected to maintain similar overall charge. All mutants were sequenced, and each was subcloned as a SacI/EcoRI fragment along with the HindIII/StuI fragment described above into pMH-neo.

Expression and Selection in J77.50.3 Cells—

Plasmid DNA was transfected into J77.50.3 cells (American Type Culture Collection, Rockville, MD) by the calcium phosphate precipitation method (19). Transfected cells were selected in medium containing 100 μg/ml G418 for 7 days. Cell clones were pooled, diluted serially, and assayed for ICAM-3 expression by the ELISA screening method (20). A population (3%) was ICAM-3-negative. Enzyme-linked immunosorbent assay (Biosource International, Camarillo, CA) for ICAM-3 expression showed that, in J77.50.3 cells, synthesis of the 2.2-kb cell pellet was suspended in 1 ml of cold lysis buffer (PBS containing 1% Triton X-100, 1 mM PMSF, 1 mM Na3VO4, 1 mM Na2MoO4) and incubated on ice for 20 min with occasional rocking. The insoluble fraction was pelleted by centrifugation in a table top microcentrifuge. Soluble proteins were transferred to a fresh tube and 0.1 ml of Sepha-rose 4CL beads added (50% slurry equilibrated in lysis buffer containing PMSF; Pharmacia Biotech Inc., Uppsala). The tube was rocked for 16 h, after which the beads were briefly spun down. The clarified supernatant was transferred to a fresh tube and antibody added to 10 μg/ml final concentration. Immune complexes were formed by incubation on ice for 1 h and harvested by incubation with protein A beads. The immune complexes were pelleted and washed twice with 1 ml of cold 1% Triton X-100, 1 mM NaCl, 1 mM PMSF, 1 mM Na2MoO4, and once with 1 ml of cold lysis buffer. The remaining proteins were eluted by addition of reducing SDS-PAGE loading buffer, boiled for 5 min, and separated by gel electrophoresis.

Phosphoamino Acid Analysis and Peptide Mapping—

Gels containing 32P-labeled proteins was conducted using X-Omat film (Eastman Kodak Corp.) with a single intensifying screen at −70 °C. Gels of 32P-labeled proteins were impregnated with flour and exposed to film at −70 °C.

Phosphorylation sites were determined by trypptic peptide mapping using in vivo 32P-labeled proteins. Labeled protein bands were excised from the polyvinylidene difluoride sheet and the proteins partially acid hydrolyzed and separated as described in (20). Briefly, the samples were dried in vacuo and resuspended in 6 μl of pH 1.9 buffer containing unlabeled phosphoamino acid standards (Sigma). A portion of each sample, representing equal Cerenkov counts, was spotted on cellulose TLC plates and separated by ascending thin layer electrophoresis (HTLE-7000; CBS Scientific, Del Mar, CA). After ninhydrin staining of the standards, the plates were exposed to film for autoradiography.

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Protein Kinase Assay—Human protein kinase Cδ was amplified by PCR with a primer that contained a 6-histidine tag at the carboxyl terminus of the protein coding sequence as described in (21). The cDNA was subcloned into a BACMID vector (Life Technologies Inc.), and recombinant virus was generated as described by the manufacturer. Infected S9 cells were lysed in hypotonic buffer (20 mM Tris, pH 7.5, 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, 100 μg/ml each aprotinin and leupeptin, 1 mM aminothiolbenzenesulfonyl fluoride) by Dounce homogenization. Active soluble protein kinase was separated from insoluble material by centrifugation (16,000 g × g) for 0.4-ml assays performed using 50 μg/ml total protein and 100 μM substrate peptide as described in (21). The following substrates were synthesized: ICAM-3 CT, amino acids 482–518 (REHQRSGSYHVREESTYLPTISMQPTEAM-GEPEESRAE), SCR CT (ARSTEQQGMYAESEELELPGYPREHST- HTMLPRSV), SGS (biotin-FREHQRSGSYHVRE), and SGS-P (biotin-FREHQRSGS/PO4/TVHVREE).

RESULTS

Development and Characterization of ICAM-3-deficient Jurkat Cells—Jurkat cells (J77) examined by indirect fluorescence cytochemistry with antibodies to ICAM-3 routinely display two populations of cells (Fig. 1A). The bulk (97%) of these are ICAM-3-positive, while a small population (3%) is ICAM-3-negative. Enrichment of the ICAM-3-negative population by sequential rounds of cell sorting generated a population displaying >97% ICAM-3-negative cells that we have termed J77.50.3. RNA analysis showed that, in J77.50.3 cells, synthesis of the 2.2-kb ICAM-3 message was below detectable levels (Fig. 1B).

Surface expression of numerous proteins was assessed in
J77.50.3 and the parental J77 line. Both populations exhibited similar fluorescence profiles for all mAb studied including CD3e, CD11a, CD18, and CD45 (Fig. 1A), indicating that J77.50.3 cells were similar to J77 except for the ICAM-3 deficiency.

Expression of ICAM-3 in J77.50.3 Cells Restores Function—Optimal T cell activation is thought to require two signals: one from the antigen receptor and the other from one or more of a large number of accessory molecules including ICAM-3 (22). To confirm that ICAM-3/TCR engagement was costimulatory, Jurkat T cells were seeded onto ICR1.1 coimmobilized with increasing concentrations of OKT3. A dose-dependent increase in IL2 production was observed (Fig. 2A). Cells exposed to either immobilized mAb alone showed no induction of IL2 secretion.

To determine if loss of ICAM-3 expression would impair costimulation, J77.50.3 cells were seeded into mAb-coated wells under conditions that stimulated the parental cells. ICAM-3-deficient J77.50.3 did not secrete IL2 (Fig. 2B). Both cell lines responded to a greater concentration of OKT3 by secreting IL2. Neither cell line responded to a combination of anti-ICAM-1 mAb (18E3D) and OKT3. These data reveal that TCR signaling and the synthetic machinery for IL2 production in J77.50.3 cells was intact. Further, co-engagement of ICAM-1 and CD3 was insufficient to produce IL2.

To evaluate whether ICAM-3 expression in J77.50.3 cells would complement the phenotypic defect, cells were transfected with either a control vector or HA-tagged ICAM-3 (ICAM-3FL). Cells were selected, and several independent lines that maintained stable surface expression were identified (Fig. 3A). Inclusion of the HA tag allowed for validation that the expressed form of ICAM-3 in the transfected cells was from the introduced DNA construct rather than re-expression of the endogenous gene. Indeed, surface staining for either ICAM-3 or HA tag epitopes showed similar levels of fluorescence in the populations (Fig. 3A). While the control-transfected lines lacked the ability to be stimulated by the co-immobilized mAb, J77.50.3 cells expressing ICAM-3FL responded to the costimuli by secreting IL2 into the medium as did the parental J77 cells (Fig. 3B). Therefore, expression of ICAM-3 by J77.50.3 cells restored their ability to respond to ICAM-3/TCR costimulation.

ICAM-3 Cytoplasmic Region Functional Map

FIG. 1. Comparison of parental Jurkat 77 and J77.50.3 cells. A, J77 and J77.50.3 were stained indirectly for flow cytometry with anti-ICAM-3 ICR1.1, anti-CD3, anti-CD11a, anti-CD18, IgG1 control, or IgG2a control. B, total RNA blot from parental J77 and J77.50.3 cells for ICAM-3 (upper) versus control glyceraldehyde-3-phosphate dehydrogenase (lower) message. The band detected with the ICAM-3 probe in the J77.50.3 RNA (lane 2) may have represented a low level of ICAM-3 message (less than 0.5% of normalized parental level), and it appeared to have a smaller molecular weight than the band in the parental lane.

FIG. 2. ICR1.1 costimulates with the TCR of J77 but not J77.50.3 cells. A, dose-response graph of IL2 secretion from J77 cells seeded on no mAb, immobilized ICR1.1 mAb alone (500 ng/well), or with an indicated concentration of OKT3. B, J77.50.3 cells are deficient in ICAM-3 mediated costimulation. Cells plated onto ICR1.1 with the suboptimal (low) OKT3 dose (25 ng/well) or a concentrated (high) dose of OKT3 (500 ng/well) alone as described under "Materials and Methods." Control wells were treated with anti-ICAM-1 mAb 18E3D and OKT3. After 16 h, media were removed and IL2 quantitated by enzyme-linked immunosorbent assay. Data are representative results from one experiment that was repeated four times.
erated to grossly map the cytoplasmic region of ICAM-3: \(-1/3\)CT (Gln 505 terminus), \(-2/3\)CT (Arg 493 terminus), and \(-3\)CT (His 484 terminus) (Fig. 4). Characterization of cells expressing each deletion included monitoring surface expression (Fig. 5A and Table I) and immunoprecipitation from lysates of cells that had been metabolically labeled with \(^{35}\)S (Fig. 5B). SDS-PAGE analysis showed that the relative migration of the truncated proteins was of the expected sizes of \(~120–140\) kDa.

The truncations were tested for their ability to trigger J77.50.3 cells to secrete IL2 when costimulated with anti-ICAM-3 mAb ICR1.1 and anti-CD3 mAb OKT3. Conditioned media from cells expressing either ICAM-3FL or \(-1/3\)CT forms showed 5.4- and 4.8-fold induction, respectively, when normalized for IL2 secretion in negative control-treated wells (Table I). Cells expressing either \(-2/3\)CT or \(-3\)CT forms secreted about 60% less IL2 (2.2-fold each). All of the cell lines tested responded to a more concentrated dose of OKT3 alone by secreting similar levels of IL2 (data not shown).

Immunoregulation of leukocytes has been hypothesized to occur via aggregate formation in which paracrine effects of cytokines (both positive and negative) regulate progression of a cellular immune response (23). J77.50.3 cells expressing ICAM-3FL rapidly flattened and spread on ICR1.1, but remained rounded on the isotype control coating (Fig. 6, A and B, and Table I). Cells expressing either \(-1/3\)CT or \(-2/3\)CT also spread (Fig. 6, C and D), while cells expressing \(-3\)CT were incapable of spreading (Fig. 6E).

Phosphoamino Acid Composition of ICAM-3—Intracellular protein phosphorylation regulates many signaling cascades that lead to activation or morphological changes. Consequently, we examined the phosphorylation of ICAM-3 under conditions that triggered Jurkat cell activation. ICAM-3FL immunoprecipitated from unstimulated J77.50.3 cells had a basal level of \(^{32}\)P incorporation (Fig. 7A, lane 2). Cells expressing either OKT3 cross-linking or PMA had an increase of \(^{32}\)P uptake onto ICAM-3 (Fig. 7A, lanes 3 and 4). Basal and inducible phosphorylation was also observed with peripheral blood leukocytes. Phosphoamino acid analysis revealed that PMA and OKT3 cross-linking elicited phosphorylation of ICAM-3 only on serine residues (Fig. 7B).

Ser^{489} Is Phosphorylated in Vivo—Truncation analysis indicated that residues 485–515 were important for costimulation, trigger aggregation as well. Expression of \(-1/3\)CT resulted in 29% aggregated cells. Truncation to residue Arg^{493} (\(-2/3\)CT) resulted in no further reduction, while cells that expressed \(-3\)CT aggregated to the same level as vector control cells.

The interface between a T cell and antigen-presenting cell (APC) is an area of intercellular adhesion that is both dynamic and highly intimate (24). T cells spread over a large region of the APC surface during the early phase of contact that coincides with a transient calcium flux (25, 26). Since ICAM-3 is present prior to activation, it is plausible that it would be involved in spreading. J77.50.3 cells expressing ICAM-3FL rapidly flattened and spread on ICR1.1, but remained rounded on the isotype control coating (Fig. 6, A and B, and Table I). Cells expressing either \(-1/3\)CT or \(-2/3\)CT also spread (Fig. 6, C and D), while cells expressing \(-3\)CT were incapable of spreading (Fig. 6E).

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while aggregation and cell spreading depended on the presence of residues 506–515 and 485–493. To investigate whether these ICAM-3-triggered functions correlated with phosphorylation of specific serine residues in these functionally important regions, phosphorylation site analysis was undertaken by point mutation and peptide mapping. 32P-Labeled ICAM-3-FL from PMA-treated cells was subjected to tryptic peptide mapping, which revealed several major phosphopeptides (Fig. 8, A and B). The pattern of separated phosphopeptides was similar whether ICAM-3 was derived from cells stimulated with PMA or CD3 cross-linking (data not shown). The tryptic phosphopeptide map of 32P-labeled ICAM-3 Ser489 → Ala resulted in the wild type pattern of labeled phosphopeptides (Fig. 8, compare B and C). The Ser489 → Ala mutation resulted in complete loss of signal for one of the major phosphopeptides (Fig. 8D), indicating that Ser489 is a bona fide phosphorylation site in vivo. Alanine substitution mutants of serine residues 496, 503, and 515 were also subjected to in vivo labeling and endoproteinolytic peptide mapping analyses; however, the results were inconclusive (data not shown).

**Mutation of Ser489 Blocks ICAM-3 Function—Phenotypic effects of the alanine point mutants were tested in homotypic aggregation, cell spreading and TCR costimulation assays. Aggregation of Ser489 → Ala was about 20% of ICAM-3-FL levels, similar to the vector control transfectants (12, 58, and 16%, respectively; Table I). Ser489 → Ala also had a deleterious effect on cell spreading. Conversely, Ser487 → Ala and Leu499 → Ala had no detectable inhibition of aggregation or spreading. Induction of IL2 secretion by Ser489 → Ala in the TCR costimulation assay was also reduced about 50%, compared with Leu499 → Ala and ICAM-3-FL (2.9-, 6.0-, and 5.4-fold, respectively; Table I). The effect of Ser489 → Ala on costimulation was comparable to the −CT truncation. Cells expressing Ser487 → Ala were equally impaired when assayed for costimulation but not for aggregation or spreading. Ser496 → Ala reduced spreading to 33%, while costimulation and aggregation remained intact. Ser515 → Ala blocked costimulation completely and spreading partially, yet left aggregation intact. Ser515 → Ala abrogated costimulation, aggregation, and spreading.

**In Vitro Phosphorylation of Ser489 by PKCθ—**Numerous pro-
protein kinases have been characterized as to the sequence specificity of their substrates. For the family of protein kinase C (PKC) isoforms, a consensus substrate sequence is RXXS/T (27). Examination of the ICAM-3 Ser489 sequence context suggested that it might be a PKC substrate. In addition, TCR cross-linking and PMA treatments, both of which activate multiple PKC isoforms, resulted in the induction of serine phosphorylation of ICAM-3 (Fig. 7). PKCα was chosen for in vitro kinase assay since 1) it is found in abundance in cells of the hematopoietic lineage, 2) it can activate the AP-1 element of the IL2 promoter when overexpressed, and 3) it selectively translocates to the T cell/APC contact region in an antigen-dependent manner (28–31). A 37-residue peptide representing the entire cytoplasmic region (amino acids 482–518) was phosphorylated in vitro by recombinant human PKCα (Fig. 9A). In contrast, a scrambled ICAM-3 peptide had little detectable incorporation. To address whether Ser489 was a PKC phosphorylation site in vitro, the phosphorylation of shorter substrate peptides (amino acids 481–495) containing Ser487 (SGS) or phospho-Ser489 (SGS-P) was also evaluated. Incorporation of 32P was found only with the SGS peptide and not with the scrambled peptide (Fig. 9B). This result suggests that PKC activity is required for ICAM-3-dependent cell spreading.

We investigated the functional requirements of the cytoplasmic region of ICAM-3 by expression of truncated or point mutated proteins in a variant of the human T leukemic cell line Jurkat. A Jurkat cell line deficient in ICAM-3 expression (J77.50.3) was developed and characterized with regard to surface protein expression, message synthesis, and costimulatory phenotype (Figs. 1 and 2). Expression of surface ICAM-3 restored accessory molecule function as measured by secretion of the T cell activation marker IL2 (Fig. 3). Therefore, the functional deficit in the cell line can be complemented by expression of a single protein, ICAM-3.

The natural occurrence of a subpopulation of ICAM-3-deficient cells in unselected Jurkat cultures is curious. Others have reported that the Jurkat cell line was mixed for the expression of ICAM-3, suggesting that this phenotype is inherent to the cell line itself (32). The ICAM-3 deficiency was stable in continuous culture, and cells with wild type levels of ICAM-3 expression do not arise.

This model cell line was quite useful for investigating the structure/function relationship of the cytoplasmic region of ICAM-3. The 37 residues of the cytoplasmic region are grouped as alternating hydrophilic-hydrophobic-hydrophilic segments, which were used to divide the region for mapping studies (33). The 1/3CT truncation, which lacked five charged residues that gave the distal portion of the native tail its hydrophilic nature, terminated with Glu, and contained the hydrophobic core residues 498–501 (Fig. 4). The 2/3CT truncation removed the hydrophobic core residues and terminated with...
Arg482–Glu518, a scrambled CT sequence (truncations of residues 485–505) was used. Abrogation of aggregation or cell spreading requires Ser489, and strong phosphorylation of Ser489 is required for the isolation of hyper-phosphorylated ICAM-3 from aggregated or spread cells. Alternatively, ICAM-3-triggered events like TCR stimulation or PMA treatment may be abrogated all ICAM-3-triggered events tested (Table I). Ser489 function was demonstrated by mutation of this site, which also abrogated all ICAM-3-triggered events tested in the absence of the rest of the cytoplasmic tail. Whether this activity is via associated transmembrane proteins or through simple ICAM-3 mAb-mediated enhancement of cell binding to the OKT3-coated surface is unclear. Aggregation and spreading were partially (50–70%) inhibited with deletion of residues 506–518 and an additional 50% with further deletion of amino acids 485–493. Loss of the hydrophobic core residues 494–505 did not result in incrementally greater deficits on aggregation or spreading. In agreement, the point mutant analysis showed that Ser489 → Ala and Ser515 → Ala blocked, while Ser496 → Ala, Leu499 → Ala, and Ser503 → Ala had no deleterious effect on aggregation. Effects of the point mutants on spreading were also in agreement with those of the truncations. In particular, mutation of serine residues in the membrane distal or proximal regions either partially or completely inhibited spreading. These results suggest that two distinct and well separated regions (amino acids 506–518 and 485–493) contribute to ICAM-3-triggered aggregation and spreading. Interestingly, mutational effects on costimulation, aggregation, and cell spreading were not absolutely coincidental. This suggests that these three functions are separable and not necessarily interdependent. In general, however, these ICAM-3 triggered functions were effected by mutations within the hydrophilic regions proximal and distal to the membrane-spanning segment.

Since many protein functions are regulated by phosphorylation status, the phosphoamino acid composition of ICAM-3 was determined under basal and stimulated conditions. In vitro labeling of ICAM-3, via the intracellular ATP pool, resulted in phosphorylation predominantly on tyrosine. This discrepancy may be attributed to differences in cell isolation procedures or the manner in which the cells were labeled.

We determined that Ser489 is a major phosphorylation site in vitro by tryptic peptide mapping of serine to alanine point mutants and that recombinant PKCθ phosphorylated this site in vitro (Figs. 8 and 9). The requirement for Ser489 in ICAM-3 function was demonstrated by mutation of this site, which abrogated all ICAM-3-triggered events tested (Table I). Ser489 is closely apposed to the cytoplasmic face of the plasma membrane, which could facilitate its phosphorylation by PKC upon recruitment to the lipid microenvironment of the membrane by events like TCR stimulation or phorbol ester treatment. In fact, only under conditions of TCR stimulation or PMA treatment have increased levels of ICAM-3 phosphorylation been found by us. Since phosphorylation is a dynamic event, in which rapid dephosphorylation can occur, specialized conditions may be required for the isolation of hyper-phosphorylated ICAM-3 from aggregated or spread cells. Alternatively, ICAM-3-triggered aggregation and spreading require Ser489 for structural integrity independent of phosphorylation state. In this case, an alanine mutant blocks function by altering important structural features. Whether other serine residues (496, 503, and 515) function, at least in part, by their phosphorylation status remains to be determined.

The studies described here delineate portions of the cytoplasmic region of ICAM-3 required for the TCR accessory function of ICAM-3. These regions (amino acids 506–518 and 485–493) contribute to ICAM-3-triggered aggregation and spreading. Interestingly, mutational effects on costimulation, aggregation, and cell spreading were not absolutely coincidental. This suggests that these three functions are separable and not necessarily interdependent. In general, however, these ICAM-3 triggered functions were effected by mutations within the hydrophilic regions proximal and distal to the membrane-spanning segment.

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The regions implicated in TCR costimulation partially encompass regions linked to aggregation and cell spreading, our data suggest that these functions might share underlying mechanisms, perhaps reorganization of the cytoskeleton. Links between cytoskeleton dynamics and activation have been observed in T cells during the process of APC/T adhesion and contact (35–38). At initial contact, engagement of TCR by antigen-MHC and allows other co-stimulatory molecular interactions to occur, such as CD28-B7. For sustained T cell signaling, actin-based cytoskeletal changes are required and, as the present work suggests, could be induced by engagement of ICAM-3 (37). Preliminary studies suggested that ICAM-3 engagement induced actin polymerization. Later, dephosphorylation of ICAM-3 (37). Preliminary studies suggested that ICAM-3 could contribute to cell dissociation and rounding, as observed with Ser489 mutation, contributing to clonal expansion. Further biochemical studies are required to delineate the mechanisms by which T cell behavior is regulated by the functionally important amino acids of the ICAM-3 cytoplasmic region identified here.

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