Src Homology Domain 2-containing Tyrosine Phosphatase 2 Associates with Intercellular Adhesion Molecule 1 to Regulate Cell Survival*

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Intercellular adhesion molecule-1 (ICAM-1) binds to the plasma protein fibrinogen (Fg) to mediate leukocyte/endothelial cell interactions. In our studies, the ligation of Fg to ICAM-1 on tumor necrosis factor-α-stimulated endothelial cells resulted in the tyrosine phosphorylation of Src homology domain 2 (SH2)-containing phosphatase-2 (SHP-2). The ICAM-1 cytoplasmic sequence IKKYRLQ conforms poorly to the consensus immunoreceptor tyrosine-based inhibition motifs found in receptors that bind SHP-2. Nevertheless, the tyrosine phosphorylated sequence (IKKYRLQ) bound specifically to the SH2 domain proximal to the NHL-terminal of SHP-2 (SHP-2-N) but not to the SH2 domain proximal on the COOH-terminal side (SHP-2-C). Phosphorylated ICAM-1 bound SHP-2-N. In immunoprecipitation experiments, SHP-2 associated with phosphorylated ICAM-1. Cells expressing truncated ICAM-1 that lacked the cytoplasmic sequence (ICAM-1(TR)) failed to associate with SHP-2. ICAM-1 containing the tyrosine to alanine substitution at position 485 (ICAM-1(Y485A)) associated weakly with SHP-2. Cells expressing ICAM-1(TR) and ICAM-1(Y485A) underwent apoptosis upon adhesion to Fg, whereas the wild type ICAM-1 maintained cell survival. These results indicate that ICAM-1 interactions with SHP-2 allow better cellular survival mediated through Fg-ICAM-1 ligation.

Intracellular adhesion molecule-1 (ICAM-1, also termed CD54) is a receptor expressed on diverse cell types and belongs to the Ig-like family of proteins. Endothelial cells (ECs) express very low amounts of ICAM-1 and require stimulation with cytokines tumor necrosis factor-α (TNFα) or interleukin-1 to upregulate ICAM-1 levels (1–3). ICAM-1 functions as a costimulatory molecule on antigen-presenting cells to activate major histocompatibility complex class II restricted T-cells and on other cell types in association with major histocompatibility complex class I to activate cytotoxic T-cells. The recognition of ICAM-1 by β2-integrins results in the adhesion of leukocytes to the endothelium and in the extravasation of leukocytes to sites of inflammation (3–5). The extravasation of leukocytes also occurs through a process involving ICAM-1 and the plasma protein fibrinogen (Fg). In this process, the integrin-bound Fg interacts with ICAM-1, mediating the bridging between blood cells and ECs (6–9). TNFα-stimulated ECs interact with Fg primarily through ICAM-1 (2, 10). A region within the first Ig-like motif of ICAM-1, ICAM-1-(8–21), and a segment within the γ-chain of Fg, Fg γ(117–133), participate in Fg-ICAM-1-mediated cellular bridging, cell survival, and proliferation (10–15).

The 28-amino acid cytoplasmic tail of ICAM-1 lacks the consensus sequence required for intrinsic kinase activity. Moreover, ICAM-1 lacks the motifs resembling the Src homology domains (SH) that can recruit phosphorylated proteins at the cytoplasmic, membrane-proximal site (16). Nevertheless, Fg-ICAM-1 ligation in Raji B-cells results in proliferative signals that causes 2–3-fold increase in the phosphorylation of pp60c-src and of the extracellular signal-regulated kinase (ERK) (13, 14). However, the ligation of TNFα-stimulated ECs to Fg results in a dramatic increase (8–10-fold) in ERK phosphorylation, which is implicated in EC survival and in preventing TNFα-mediated apoptosis (15). In other studies, the ligation of ICAM-1 from EC derived from rat brain microvessels with β2-integrins from activated T-cells resulted in phosphorylation of a Src kinase substrate, cortactin (17). The activation of the small molecular weight GTPase Rho, following cross-linking of ECs with ICAM-1 antibodies, has been implicated in leukocyte transmigration (18, 19). ICAM-1 cross-linking in B-lymphoma and in T-cells activated the Src family kinase Lyn and inactivated Cdc2 kinase, respectively (20, 21).

The cytoplasmic sequence of several Ig-like receptors such as CD22 (22–24), CD33 (25), platelet endothelial cell adhesion molecule-1 (PECAM-1) (26, 27), FcγRIIB (28, 29), and the killer cell inhibitory receptor (30, 31) contain module(s) termed immune receptor tyrosine-binding inhibition motifs (ITIM). The ITIM consensus sequence (I/V/L)XX(I/L)V, when phosphorylated, associates with the Src homology 2 (SH2) domain-containing phosphatases SHP-1, SHP-2, and SHIP-1 (SH2-containing inositol polyphosphate 5-phosphatase). These cytosolic phosphatases down-regulate tyrosine kinase activity and cellular functions induced through immune receptor tyrosine-binding activation motifs (ITAM). SHP-2 (previously called...
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SH-PTP2, PTP2C, PTP1D, and Syp) is a widely expressed phosphatase and contains two tandem SH2 domains at the amino-terminal third of the protein, followed by a catalytic phosphatase domain and a carboxyl region that becomes tyrosine phosphorylated (32). The SH2 domains of SHP-2 physically interact with ligand-activated receptors that either elicit or lack tyrosine kinase activity, as well as other cytoplasmic signaling molecules (32–36). This protein-protein interaction enhances the tyrosine phosphatase activity of SHP-2 by relieving the inhibitory intramolecular interactions between the amino-terminal SH2 domain and the catalytic phosphatase domain (37). SHP-2 is the mammalian homolog of the gene product of Drosophila corkscrew (Csw) (38, 39). In several instances, SHP-2 has been reported to act as a positive regulator to promote mitogenic signals, whereas SHP-1 acts as a negative regulator of cellular functions (32, 34, 36).

ITIM have been reported to occur in pairs that are spaced by >16 amino acids. Single ITIM sequences have been reported to bind SHP-1 and/or SHP-2 in mast cell function-associated antigen (40), in the human and mouse FcγRIIB (28, 29), the mouse homolog of the killer cell inhibitory receptor, Ly 49a (41), and CTLA-4 (42). ICAM-1 contains a single sequence (IKKYRLQ) that poorly conforms to the consensus ITIM in that it has glutamate at the Y + 3 position instead of the invariant L/V; and lysine is at Y – 2 instead of the obligatory hydrophobic residue I/V/L. However, these invariant residues occur at Y + 2 and Y – 3 positions in ICAM-1. Our results demonstrate that the tyrosine phosphorylated peptide IKKTYRLQ binds to the SH2 domain proximal to the NH₂ terminus of SHP-2 (SHP-2-N). SHP-2 associates with phosphorylated ICAM-1 under cellular conditions. Cells expressing ICAM-1 mutation Tyr → Ala at position 485, ICAM-1(Y485A), associate with SHP-2 at greatly diminished levels. The failure of ICAM-1 to associate with SHP-2 results in cells undergoing apoptosis despite extracellular Fg-ICAM-1 ligation.

EXPERIMENTAL PROCEDURES

Reagents, Synthetic Peptides, and Antibodies—TNFa was purchased from R&D Systems (Minneapolis, MN). Recombinant protein G-Sepharose was from Zymed Laboratories Inc. (South San Francisco, CA). The apoptosis assay kit utilizing annexin V binding was purchased from R & D Systems, Inc. (Minneapolis, MN). Prestained SDS-PAGE standards were purchased from Bio-Rad. Enhanced chemiluminescence Western blotting detection kit and UltraLink-Immobilized-Streptavidin-agarose were obtained from Pierce. Bulk GST Purification from Pierce. Purified human recombinant c-Src kinase came from Upstate Biotechnology, Inc. (Lake Placid, NY). The apoptosis assay kit and anti-mouse IgG were from Bio-Rad. Biotinylated goat anti-rabbit IgG (Becton Dickinson, San Jose, CA), and clone P2A4 (Chemicon International Inc., Temecula, CA). The peroxidase-linked goat anti-rabbit IgG and anti-mouse IgG were from Bio-Rad.

Cell Culture—ECs were obtained from umbilical cord veins as described previously (10, 45). Cells were plated on tissue culture-treated polystyrene plates (Costar Corp., Cambridge, MA) precoated with 1.0 μg/cm² human fibronectin (Roche Molecular Biochemicals) and grown in Dulbecco’s modified Eagle’s medium/ Ham’s F-12 medium (DMEM/F-12) with 10% FCS, 90 μg/ml heparin (Sigma), 250 μg/ml penicillin, and 150 μg/ml streptomycin (Clonetics, San Diego, CA). Cells were grown in T75 culture flasks; and from passages 2–4 were used for this study. 293 cells of human kidney fibroblast origin and ICAM-1-expressing lymphoblastoid Raji cells were obtained from the American Type Culture Collection (Rockville, MD). Raji were grown in RPMI 1640 (BioWhittaker, Inc.) containing 10% FCS and 1.0 mM glutamine. 293 cells were maintained in DMEM/F-12 containing 10% FCS and 1.0 mM glutamine.

Preparation of ICAM-1 cDNA Constructs for Transfection of 293 Cells—Wild-type (WT) ICAM-1 DNA was recloned from a pcDNA3 vector (10, 13) into pcDNA 3.1 (+) (+) using Xbal-XbaI restriction sites. The 28 amino acids of ICAM-1(WT) were truncated, with the sequence remaining at residue 478 (ICAM-1/TR) using polymerase chain reaction with the following primers: lower primer, which introduced a stop codon, 5’-GTC TGA ATT CCT TGA TCT TCC GCT AAC GGT T-3’; upper primer, 5’-CTA AGC TCC CCT ATG GTC CCC AGC-3’, containing HindIII and EcoRI restriction sites, respectively. The polymerase chain reaction product was cloned into the pcDNA3.1 (+) vector. To generate full-length ICAM-1 mutant Y485A, a single point mutation was introduced using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the primer pair: the upper ICAM-1(Y485A), 5’-GCT GTT GTA TCC TGG CTT CTG TGA TCC TCT TCC-3’; lower ICAM-1(Y485A), 5’-GGA AGA TCA AAG CCA GAC TAC AAC AGG-3’. The sequences of the DNA constructs were verified by sequence analysis.

Next, 293 cells were stably transfected in the absence of serum using LipofectAMINE Plus reagent (Life Technologies, Inc.) with 1–5 μg of pcDNA 3.1 containing cDNA for ICAM-1(WT), ICAM-1 (TR), or ICAM-1 (Y485A) or pcDNA3.1 vector alone as control. Transfected cells were selected using G418 (Inovitro, Carlsbad, CA) in DMEM/F-12. Cells expressing ICAM-1 were detected by incubation with mAb anti-ICAM-1 (LB2) antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and isolated using the fluorescence-activated cell sorter (FACS). Levels of ICAM-1 expression were monitored by FACS analysis and by immunoblotting as described previously.

FACS—Resting and TNFα-stimulated ECs and 293 cells were removed by brief trypsin treatment and washed in Dulbecco’s PBS. Cells were resuspended in a staining medium of Hanks’ balanced salt solution containing 2.0 μM CaCl₂, 2.0 μM MgCl₂, 10 mM HEPES (pH 7.4), 10% FCS, 90 μg/ml EC growth supplements, and 0.1% BSA and incubated at 4 °C for 30 min with 1 μg/ml of either control mouse IgG or the anti-ICAM-1 mAb LB-2. Cells were centrifuged through a cushion of FCS and resuspended in staining medium containing 50 μg/ml FITC-conjugated goat anti-mouse IgG antibodies (Zymed Laboratories Inc.) for 30 min at 4 °C. Cell-bound antibodies were detected using a FACSScan and analyzed on the LYSIS program (Becton Dickinson).

Adhesion Assays—Petri dishes (Corning, NY) were coated with human Fn (200 μg in PBS) for 16 h at 4 °C and then blocked with 1% heat-inactivated BSA in PBS for 1 h at room temperature. Prior to use the dishes were rinsed three times with PBS. ECs, Raji cells, and 293 cells were maintained in DMEM/F-12 containing 1% FCS for 18 h prior to the commencement of an experiment. In addition to serum deprivation, some ECs were stimulated with TNFα (10 ng/ml) for 18 h. ECs and 293 cells were briefly trypanopiosed (BioWhittaker, Inc.), harvested by low speed centrifugation (800 rpm for 5 min), and resuspended in the medium. Cells were seeded onto Petri dishes coated with proteins at 1–2 × 10⁵ cells/dish and incubated at 37 °C for 15–120 min. Cells were then processed for immunoprecipitation, Western blot analysis, or annexin V binding assay.

For quantitative cellular adhesion, 293 cells (1 × 10⁶/well) expressing ICAM-1 were allowed to adhere to Fg-coated tissue culture plates (Costar Corp., Cambridge, MA) for 15 min at 37 °C. The plates were washed three times with PBS, and the number of adherent cells in each well was quantitated using the Cyquant Cell Proliferation Assay Kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer’s instructions. Briefly, after washing the plates were frozen at −70 °C for 2 min, thawed, and the green fluorescent dye, incorporating into DNA, was added. After 5 min of incubation at room temperature, fluorescence was measured using a microplate reader with excitation at 480 nm and emission detection at 530 nm.

Annexin V Binding Assay—Adherent 293 cells were detached by gentle pipetting. Cells were washed and resuspended in calcium- and magnesium-free binding buffer (1 × 10⁵ cells in 0.1 ml) and incubated with
FITC-labeled annexin V for 15 min at room temperature; then 100 μl of binding buffer was added. Annexin V-FITC-stained cells were detected by FACS analysis.

**Immunoprecipitation and Western Blot Analysis**—ICAM-1, SHP-2, and tyrosine phosphorylated proteins were purified from 10 well plates of a human umbilical vein endothelial cells (HUVECs) which were cultured with PBS and lysed in 500 μl of ice-cold Triton X-100 buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 1.0% Triton X-100, 0.1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride). Lysate were clarified by centrifugation at 14,000 × g for 15 min at 4 °C. Supernatants were precleared with 20 μl of protein G-Sepharose and then assayed for proteins by Western blot analysis. After washing with PBS and lysed with 500 μl of PBS, ICAM-1 were captured by addition 20 μl of biotinylated GST-SHP-2(N) (2 μg/ml) and then the bound proteins were eluted by boiling in SDS gel loading buffer.

**Peptide Precipitation Analysis**—Biotinylated ICAM-1 (480–489) phosphopeptides (5 μg), both phosphorylated and nonphosphorylated, were incubated with GST-SHP-2-N or GST-SHP-2-C or GST alone as a control in 1 ml of ice-cold Triton X-100 lysis buffer for 16 h. The biotinylated peptides were captured by addition 20 μl of U-labeled immobilized-Streptavidin beads for 3 h at 4 °C. In competition experiments, nonbiotinylated ICAM-1(480–489) peptides, both phosphorylated or nonphosphorylated, at 0.5–4 μg were preincubated with GST-SHP-2-N (2 μg) for 3 h at room temperature and then incubated with 5 μg of biotinylated ICAM-1 peptide for 16 h at 4 °C. The beads were washed four times with ice-cold Triton X-100 lysis buffer and twice with PBS containing 0.1% Triton X-100. The bound proteins were eluted by boiling in SDS sample buffer and subjected to Western blotting using goat anti-GST antibody.

**Preparation and Expression of GST Fusion Proteins**—SHP-2 DNA was amplified by polymerase chain reaction utilizing the human placental cDNA library and the following primers: upper, 5′-CGA AGA CGG GGG ATT CAT GAC ATC GGC G-3′; lower, 5′-CTG CCT GTG CTC GAG TAC-3′. Immunoammals were developed using enhanced chemiluminescence. Some blots were stripped using the stripping buffer and subjected to Western blotting using goat anti-GST antibody.

**Solid Phase Radioimmunoassay**—Flexible 96-well Falcon plastic plates (BD Labware, Franklin Lakes, NJ) were coated with 100 μl (20 μg/ml) of PBS, pH 7.4, nonphosphorylated ICAM-1(480–489) phosphopeptides immobilized in ice-cold PBS. Wells were then postcoated with 3% gelatin in PBS for 3 h at 37 °C and washed four times with 1 ml of PBS containing 1% gelatin at 4 °C. After washing the blots were incubated with 50 μl of 10,000 cpm/well for 3 h at room temperature. This mixture was incubated with the phosphorylated ICAM-1(480–489) peptide and attached to microtiter wells at 2 h for 16°C. Bound proteins were detected as described previously.

**RESULTS**

The Adhesion of TNFα-stimulated ECs to Fg Results in the Tyrosine Phosphorylation of ICAM-1 and SHP-2—The ligation of Fg with TNFα-stimulated ECs occurs predominantly through ICAM-1 (2, 10, 15). The reported cytoplasmic sequence of ICAM-1 contains a single tyrosine residue at position 485 (16). To establish whether ICAM-1 becomes tyrosine phosphorylated upon Fgstimulated binding, TNFα-stimulated ECs were allowed to adhere to Fg or BSA for 15 and 30 min. Adherent cells were lysed and immunoprecipitated with agarose-conjugated anti-ICAM-1 mAb. Immunoprecipitates were analyzed on gels and Western blots probed with anti-phosphotyrosine mAb. Fig. 1A (upper panel) shows that ICAM-1 is strongly phosphorylated when cells were ligated to Fg but not to BSA. Equal amounts of ICAM-1 were immunoprecipitated from cells that ligated to either Fg or BSA, as indicated in the blots that were probed with anti-ICAM-1 antibody (Fig. 1A, lower panel). Immunoprecipitation carried out in the presence of normal mouse IgG, instead of anti-ICAM-1 mAb, indicated the absence of the protein band migrating in the region of ICAM-1. Therefore, phosphorylation of ICAM-1 is a specific and an early event following Fg-ICAM-1 ligation.

We were interested in identifying molecules that associated with phosphorylated ICAM-1. We had earlier observed the tyrosine phosphorylation of proteins migrating at 70 kDa, upon Fg-ICAM-1 ligation in TNFα-stimulated ECs and in B-lymphoid Raji cells (14, 15). By immunoprecipitation of TNFα-stimulated ECs, one of the proteins was identified as SHP-2 (Fig. 1B). The SHP-2 phosphorylation levels in resting and TNFα-stimulated cells upon adhesion to Fg or BSA (as control) for 0–120 min at 37 °C was evaluated. Following adhesion, equivalent amounts of cell protein were immunoprecipitated with anti-SHP-2. Fig. 1B shows the Western blots of the immunoprecipitates probed with the anti-phosphotyrosine and anti-SHP-2 mAbs. SHP-2 was highly phosphorylated at 5–30 min upon adhesion of TNFα-stimulated ECs to Fg. At 60 min, the SHP-2 phosphorylation levels were only 20% of those observed at 15 min and by 120 min SHP-2 was almost completely dephosphorylated. In contrast, in resting ECs the SHP-2 phosphorylation was at least 6-fold lower than those observed with stimulated cells at 15 min. SHP-2 was dephosphorylated at 60 and 120 min in TNFα-stimulated ECs, but in resting ECs SHP-2 phosphorylation levels were increased. As ICAM-1 levels are low on nonstimulated ECs, Fg ligation in

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Fig. 1. Tyrosine phosphorylation of ICAM-1 (A) and SHP-2 (B and C) upon adhesion of TNFα-stimulated EC to Fg. Nonstimulated or TNFα-stimulated endothelial cells (3 × 10⁶) were allowed to adhere to Fg or BSA for 5–120 min at 37 °C. Adherent cells were lysed and antibodies against either ICAM-1 (A) or SHP-2 (B) were used to immunoprecipitate (IP) these proteins from lysates containing equivalent amounts of protein. Normal mouse or rabbit IgG and lysates from the cells adhered to Fg for 15 min were used as IP controls for ICAM-1 and SHP-2, respectively (lanes C). Immunocomplexes were captured using protein G-Sepharose and eluted with 1× SDS sample buffer. The captured proteins were separated on SDS gels and transferred to nitrocellulose membranes. Western blots (WB) were probed using an antiphosphotyrosine (anti-PY) mAb. Membranes were then stripped and reprobed with anti-ICAM-1 or anti-SHP-2 mAb to determine equal loading of ICAM-1 and SHP-2 (lower panels, A and B, respectively). C. TNFα-stimulated EC were preincubated in the presence of 20 μg/ml anti-ICAM-1 function blocking mAb (clone P2A4) or normal mouse IgG (NM) for 30 min at 37 °C. The cells were allowed to adhere to BSA or Fg for 30 min at 37 °C. Adherent cells were processed as described.

these cells occurs predominantly through the RGD-sensitive integrins, most likely αvβ3 and αvβ5 (46, 47). The presence of anti-ICAM-1 mAb (P2A4), in the reaction mixture of stimulated ECs that were allowed to ligate to Fg, specifically blocked SHP-2 phosphorylation, indicating that this system is ICAM-1-dependent (Fig. 1C).

SHP-2 Interacts with Phosphorylated ICAM-1 (1480–489)—A sequence within the cytoplasmic tail of ICAM-1 resembles, albeit poorly, the ITIM found in immunoregulatory receptors that bind SHP-1 and SHP-2 (22–27). Given that SHP-2 became specifically phosphorylated upon Fg-ICAM-1 ligation, it was of interest to determine whether SHP-2 became associated with ICAM-1 through the “putative” ITIM sequence. Biotinylated-peptides corresponding to the ICAM-1 cytoplasmic sequence RKIKKYRLQ (ICAM-1(1480–489)) were synthesized with the tyrosine residue at position 485, either phosphorylated or nonphosphorylated. In addition, a scrambled phosphorylated ICAM-1(1480–489) peptide was synthesized and biotinylated. These peptides were also prepared without the biotin group for use in certain experiments. The biotinylated peptides were incubated with the SHP-2 fragments SHP-2-N and SHP-2-C that were expressed as GST fusion proteins. The mixture was allowed to bind streptavidin-conjugated agarose beads. Following extensive washing, proteins bound to the agarose beads were eluted by boiling in SDS-PAGE sample buffer. Samples were separated on gels and probed with anti-GST antibody (Fig. 2A). The nonphosphorylated ICAM-1(1480–489) and the scrambled phosphorylated ICAM-1(1480–489) peptides failed to bind either SHP-2-N or SHP-2-C. The phosphorylated peptide bound to SHP-2-N but not to SHP-2-C. In further experiments, we noted that only the phosphorylated ICAM-1(1480–489), but not the corresponding nonphosphorylated peptide, competed in a dose-dependent manner for the binding of phosphorylated ICAM-1(1480–489) to SHP-2-N (Fig. 2B). In these experiments the nonbiotinylated peptides were used as competitors. The competition with the phosphopeptide at 2.0 μg was >95%, whereas with the nonphosphopeptide at 4.0 μg, competition was virtually nonexistent.

In another independent assay, we established the binding of SHP-2-N to the phosphorylated ICAM-1(1480–489). In this assay, phosphorylated and nonphosphorylated ICAM-1(1480–489) peptides without biotin were attached to plastic microtiter wells. SHP-2-N and SHP-2-C expressed as GST fusion proteins were allowed to bind the immobilized peptides. After extensive washing, the bound proteins were incubated with an anti-GST mAb followed by 125I-labeled goat anti-mouse IgG (Fig. 2C). SHP-2-N bound to phosphorylated ICAM-1(1480–489) in a dose-dependent manner, whereas SHP-2-C binding was negligible. At the highest concentration applied (2.0 μg), SHP-2-N binding was 7-fold greater than SHP-2-C. In this assay also soluble phosphorylated ICAM-1(1480–489) competed for the binding of SHP-2-N to the immobilized phosphorylated ICAM-1(1480–489), whereas the corresponding nonphosphorylated peptide was ineffective (Fig. 2D). With 2.0 μg of the input phosphorylated peptide, approximately 90% competition was achieved. These results indicate that the SH2 domain, proximal to the amino-terminal of SHP-2, binds specifically to the ITIM-like sequence within the cytoplasmic tail of ICAM-1.

SHP-2-N Interacts with Phosphorylated Intact ICAM-1—Having established that a short sequence within ICAM-1 bound directly to SHP-2-N, it was of importance to determine whether intact ICAM-1 could also bind SHP-2-N. ICAM-1 from lysates of 293 cells, transfected with ICAM-1, was captured on microtiter wells precoated with anti-ICAM-1 IgG directed against the extracellular first two Ig-like domains. Following washing, the plates were incubated with pp60 src and γ-ATP to phosphorylate the immobilized ICAM-1. SHP-2-N and SHP-2-C expressed as GST fusion proteins were then added. The bound proteins were detected as described in Fig. 2C. The results in Fig. 3A shows that SHP-2-N bound predominantly to immobilized intact ICAM-1(1WT) incubated in the presence of pp60 src. In the absence of pp60 src, the binding was considerably lower. There was no difference in SHP-2-N binding either in the presence or absence of pp60 src by lysates from cells expressing the single Tyr → Ala mutation at position 485 ICAM-1(Y485A), as well as from the truncated ICAM-1 expressed without the cytoplasmic sequence ICAM-1(1TR) and the mock transfected cells. The binding of GST alone to intact ICAM-1 was negligible (data not shown). To verify whether ICAM-1 was indeed phosphorylated under these assay conditions, the bound proteins were extracted from the protein-coated wells with SDS-PAGE sample buffer and analyzed on gels and probed with antiphosphotyrosine mAb. ICAM-1(1WT) was specifically phosphorylated in the presence of pp60 src, whereas in the absence of pp60 src, ICAM-1 remained nonphosphorylated (Fig. 3A, inset). The binding of SHP-2-C, both in the
presence and absence of pp60\textsuperscript{Src}, to lysates from either intact ICAM-1, ICAM-1(Y485A) or ICAM-1(TR) cells was considerably low (Fig. 3B). In the presence of pp60\textsuperscript{Src}, the binding of SHP-2-C was about 3-fold lower than those observed with SHP-2-N. Although there were negligible differences in the binding of SHP-2-N to ICAM-1(Y485A) or ICAM-1(TR) compared with mock, there was in fact a small but significant difference in the binding of SHP-2-C (Fig. 3). These results suggest that SHP-2-N associates with intact phosphorylated ICAM-1 and that Tyr\textsuperscript{485} in ICAM-1 mediates this interaction. Intact ICAM-1, ICAM-1(Y485A), and ICAM-1(TR) appear to support a low level of SHP-2-C binding.

**SHP-2 Associates with Cellular ICAM-1**—To verify the binding of SHP-2 to ICAM-1 under cellular conditions, TNF\textalpha-stimulated ECs were allowed to ligate Fg, poly-L-lysine, or BSA for 30 min, as described in Fig. 1. Cell lysates were immunoprecipitated with anti-SHP-2 mAb and Western blots of immunoprecipitates were probed with anti-ICAM-1 mAb. Conversely, lysates were immunoprecipitated with anti-ICAM-1 mAb, and blots were probed with anti-SHP-2 mAb. Fig. 4A shows that using either of the above procedures, EC ligation to Fg but not to poly-L-lysine resulted in the co-immunoprecipitation of SHP-2 and ICAM-1. The ligation of Fg with ICAM-1 on B-lymphoid Raji cells also resulted in the co-immunoprecipitation of SHP-2 and ICAM-1 (Fig. 4B). The presence of an anti-ICAM-1 mAb P2A4 during ligation of these cells to Fg blocked SHP-2 association with ICAM-1. These results demonstrate for the first time that SHP-2 directly associates with ICAM-1 in both lymphoid cells and ECs following Fg-ICAM-1 ligation.

**Tyrosine at Position 485 in ICAM-1 Mediates the Interaction with SHP-2**—To further establish the role of the cytoplasmic sequence in ICAM-1 to associate with SHP-2, we utilized 293 cell lines expressing wild type and mutant forms of ICAM-1. Three stable cell lines were developed: (a) wild type ICAM-1 (ICAM-1(WT)), (b) truncated ICAM-1 (ICAM-1(TR)), wherein the cytoplasmic sequence was deleted from residues 478–505, and (c) with the single amino acid substitution (Tyr\textsuperscript{485}Ala) at position 485 (ICAM-1(Y485A)). ICAM-1 expression in these transfected cells was verified by immunoprecipitation and by FACS analysis and was found to be equivalent in each of the cell lines (Fig. 5A). These cells, including a mock 293 cell line transfected with an empty vector, were allowed to ligate with Fg. The levels of cells adherent to Fg was also comparable in each of the ICAM-1 expressing cell (Fig. 5A). Cell lysates were immunoprecipitated with anti-SHP-2 mAb, and Western blots of the immune complexes were probed with anti-ICAM-1 mAb. In cells expressing ICAM-1(WT), SHP-2 became associated with ICAM-1 (Fig. 5B). However, in mock cells and in cells expressing ICAM-1(TR),...
SHP-2 was not associated with ICAM-1 in the immunoprecipitates. In cells expressing ICAM-1(Y485A), less than 10% of ICAM-1 was bound to SHP-2. Reprobing the blots with anti-SHP-2 mAb indicated that equal amounts of SHP-2 were immunoprecipitated from each of the cell lines (Fig. 5B, lower panel). In experiments similar to those in Fig. 5B, lysates were immunoprecipitated with anti-ICAM-1, and Western blots of these immunoprecipitates were probed with an antiphosphotyrosine mAb. The results in Fig. 5C indicate that ICAM-1 was highly phosphorylated but the phosphorylation of ICAM-1(Y485A) and ICAM-1(TR) was grossly diminished.

SHP-2 Association with ICAM-1 Mediates Cell Survival upon Fg-ICAM-1 Ligation—The annexin V binding assay was utilized to assess the survival levels of ICAM-1-transfected cells. As shown in Fig. 6, cells expressing ICAM-1(WT) that adhered to Fg maintained cell viability, whereas those that adhered to BSA underwent apoptosis. Similarly, ICAM-1(WT) cells upon ligation to the specific ICAM-1 recognition peptide Fg-(117–133), maintained cell survival, which was comparable with that observed with Fg, whereas cells expressing either ICAM-1(TR) or ICAM-1(Y485A) failed to survive even upon ligation to Fg or Fg-(117–133). These results provide the compelling evidence that the binding of SHP-2 to ICAM-1 through tyrosine 485 promotes cellular survival mediated through the Fg-ICAM-1 pathway.

DISCUSSION

Tyrosine kinases and phosphatases regulate the phosphorylation of tyrosine residues within the cytoplasmic sequence of membrane-bound receptors and provide a control mechanism for processes that affect cell adhesion, growth and differentiation, and metabolism (48–50). Our results demonstrate that upon ICAM-1 ligation on TNFα-stimulated ECs, ICAM-1 becomes tyrosine phosphorylated (Fig. 1A). SHP-2 binds to phosphorylated ICAM-1 resulting in the phosphorylation of SHP-2 (Fig. 4). SHP-2/ICAM-1 interaction provides a mechanism for cell survival mediated through the Fg-ICAM-1 pathway.
very early stages of ICAM-1 ligation (1–30 min), SHP-2 becomes highly phosphorylated on TNFα-stimulated ECs (Fig. 1B), because of the phosphorylation of multiple tyrosine residues within SHP-2 (32, 36, 51). However, at 60 min TNFα was dephosphorylated by >70% (Fig. 1B). The adhesion of nonstimulated ECs to Fg resulted in the SHP-2 phosphorylation at 30–60 min. At 15 min in unstimulated ECs, the levels of SHP-2 phosphorylation were weak and about 5-fold less than those on TNFα-stimulated ECs. The activation of SHP-2 in nonstimulated ECs at later time points is likely due to cell spreading, whereas

the immediate SHP-2 activation in TNFα-stimulated ECs is a direct consequence of ICAM-1 ligation with Fg.

The ITIMs bind to the SH2 domain containing phosphatases SHP-1 and SHP-2 (32, 36). These phosphatases contain two SH2 domains at the amino-terminal half of the protein, with a catalytic phosphatase domain at the carboxyl end. The cytoplasmic sequence IKKY465RLQ from ICAM-1 poorly resembles the ITIMs found in other receptors. ICAM-1(480–489) peptide that was phosphorylated at Tyr485 associated with the SH2 domain of the amino-proximal region of SHP-2 (SHP-2-N). This phosphopeptide failed to interact with the SH2 domain at the carboxyl side of SHP-2 (SHP-2-C). Nonphosphorylated ICAM-1(480–489) peptide failed to bind either SHP-2-N or SHP-2-C (Fig. 2A). Only the phosphopeptide, but not the native nonphosphorylated peptide, competed for the binding the phosphopeptide ICAM-1(480–489) to SHP-2-N (Fig. 3). Therefore, ICAM-1 now can specifically binds to SHP-2-N (Fig. 3). ICAM-1(480–489) peptide failed to bind either SHP-2-N or SHP-2-C (Fig. 2A). Only the phosphopeptide, but not the native nonphosphorylated peptide, competed for the binding the phosphopeptide ICAM-1(480–489) to SHP-2-N (Fig. 3). These results demonstrate the specificity in the interaction of phosphopeptide ICAM-1(480–489) with SHP-2-N. The apparent dissociation constant ($K_D$) was calculated to be about 57 nM for this interaction (Fig. 2, B and D). The $K_D$ compares favorably with those recently reported for PECAM-1 (27). More importantly, our results demonstrate that phosphorylated purified ICAM-1 specifically binds to SHP-2-N (Fig. 3). Therefore, ICAM-1 now can be included in the class of other Ig-like receptors (such as CD22, CD33, and PECAM-1) that bind to SHP-2 (22–27). The SHP-2 binding ITIM-like sequence of ICAM-1 is highly unique in that it lacks the invariant residues at positions Y + 3 and Y – 2. To our knowledge, this is the only SHP-2 binding sequence that lacks both of the essential residues that have been reported to form the core for the binding of SH2 containing phosphatases. The sequence in CTLA-4 also lacks both invariant residues and has methionine at Y + 3 and glycine at Y – 2. However, the direct binding of this sequence to the phosphatases has not been determined, and, therefore, it is questionable whether this sequence in CTLA-4 is involved in SHP-2 binding (42). Moreover, ITIM sequences occur in pairs that are spaced at least 16 amino acids apart. Both human and mouse CD22 have three ITIMs. Presently, it appears that ICAM-1 has only one ITIM as does the mast cell function-associated antigen (40) and the mouse killer cell inhibitory receptor (30). However, because ICAM-1-Y485A was still phosphorylated, albeit weakly, upon Fg ligation (Fig. 5C), there is a possibility that Tyr476 and Tyr474 within ICAM-1 could poten-
tially become phosphorylated. However, these residues, according to the reported ICAM-1 sequence setting the boundaries for the transmembrane segment, are located within the membrane. Either this portion of ICAM-1 comprising Tyr$^{474}$ and Tyr$^{476}$ may in fact be within the cytosol or, upon ICAM-1 ligation and activation, these residues may move downwards within the planar membrane. We are currently investigating this aspect of ICAM-1 and the possibility of Tyr$^{474}$ and Tyr$^{476}$ residues being phosphorylated. However, this segment of ICAM-1 also does not conform to an ITIM.

The binding of SHP-2 to the cytoplasmic sequence within ICAM-1 allows a better understanding of the signals generated through ICAM-1 ligation and provides a framework for defining ICAM-1-mediated cellular functions. The association between ICAM-1 and SHP-2 occurs in several cell types such as ECs, Raji cells, and 293 cells (Figs. 4 and 5). ECs express only SHP-2 and lack SHP-1. B-lymphoid, T-cells, and NK cells express ICAM-1, ICAM-1, and SHP-2. It remains to be verified whether ICAM-1 could also interact with SHP-1 in addition to SHP-2 in these cells. In platelets, PECAM-1 can associate with both SHP-1 and -2 (27), and the stoichiometry for each of the phosphatases is different for the same ITIM in PECAM-1. We have, however, established the importance of Tyr$^{485}$ for the SHP-2 and lack SHP-1. B-lymphoid, T-cells, and NK cells express ICAM-1, ICAM-1, and SHP-2. It remains to be verified whether ICAM-1 could also interact with SHP-1 in addition to SHP-2 in these cells. In platelets, PECAM-1 can associate with both SHP-1 and -2 (27), and the stoichiometry for each of the phosphatases is different for the same ITIM in PECAM-1. We have, however, established the importance of Tyr$^{485}$ for the SHP-2 and lack SHP-1. B-lymphoid, T-cells, and NK cells express ICAM-1, ICAM-1, and SHP-2. It remains to be verified whether ICAM-1 could also interact with SHP-1 in addition to SHP-2 in these cells. In platelets, PECAM-1 can associate with both SHP-1 and -2 (27), and the stoichiometry for each of the phosphatases is different for the same ITIM in PECAM-1. We have, however, established the importance of Tyr$^{485}$ for the ICAM-1 also does not conform to an ITIM.

The identification of SHP-2 substrates in our system is an important regulatory substrate of SHP-2, and this interaction was highly compromised (Fig. 6). Similar results were also noted with ICAM-1(Tr). Therefore, ICAM-1/SHP-2 association is a vital component in the Fg-ICAM-1-mediated cell survival process. The reduced association with SHP-2 in ICAM-1(Y485A) expressing cells results in the dampened activation of ERK-1/2, which is likely to compromise the ability of the cell to survive. In this respect, we have noted the activation of ERK-1/2 as an important component in Fg-mediated Raji mitogenesis (14).

The ITIM-bearing molecules such as those found in killer cell inhibitory receptor inhibit cell-mediated cytoxicity when they bind to major histocompatibility complex class I molecules on target cells (30, 31, 52). In myeloid cells, the SHP-substrate-1 (SHPS-1 and SIRP-1) upon interaction with SHP-1 and -2 retards cell proliferation (53). Jak2 has been identified as an important regulatory substrate of SHP-2, and this interaction affects the activation of STATs (54). SHP-2 also serves as a scaffolding protein mediating the assembly of Grb-2, which is bound to SOS and promotes the activation of Ras, initiating the Raf-1/Mek/ERK pathway. More recently, Gab2, a pleckstrin homology domain-containing adapter protein has been shown to associate with SHP-2 and regulate cytoplasmic-nuclear signal transduction (55). The ITIM-like domain in ICAM-1, through its ability to bind SHP-2, mediates EC survival upon Fg-ICAM-1 ligation. This cellular function is most likely regulated through downstream effectors and substrates of SHP-2. The identification of SHP-2 substrates in our system is an avenue for further investigation.

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