X-linked lymphoproliferative disease (XLP) is a rare immune disorder commonly triggered by infection with Epstein-Barr virus. Major disease manifestations include fatal acute infectious mononucleosis, B-cell lymphoma, and progressive dys-gammaglobulinemia. SAP/SH2D1A, the product of the gene mutated in XLP, is a small protein that comprises a single SH2 domain and a short tail of 26 amino acids. SAP binds to a specific motif in the cytoplasmic tails of the cell surface receptors SLAM and 2B4, where it blocks recruitment of the phosphatase SHP-2. Here it is reported that Ly-9 and CD84, 2 related glycoproteins differentially expressed on hematopoietic cells, also recruit SAP. Interactions between SAP and Ly-9 or CD84 were analyzed using a novel yeast 2-hybrid system, by COS cell transfections and in lymphoid cells. Recruitment of SAP is most efficient when the specific tyrosine residues in the cytoplasmic tails of Ly-9 or CD84 are phosphorylated. It is concluded that in activated T cells, the SAP protein binds to and regulates signal transduction events initiated through the engagement of SLAM, 2B4, CD84, and Ly-9. This suggests that combinations of dysfunctional signaling pathways initiated by these 4 cell surface receptors may cause the complex phenotypes of XLP. (Blood. 2001;97:3867-3874)

Introduction

After infection with Epstein-Barr virus (EBV), patients with X-linked lymphoproliferative disease (XLP) mount a vigorous, uncontrolled polyclonal expansion of both T and B cells. The primary cause of death is hepatic necrosis and bone marrow failure, which appears to stem from uncontrolled T-cell responses. However, 2 major manifestations of the XLP syndrome, B-cell lymphomas of the gastrointestinal tract and dys-gammaglobulinemia, can develop in the absence of EBV infection.1 Collectively, genetic and functional studies of patients with XLP suggest that a mutation in SAP causes an intrinsic T or natural killer (NK) cell defect that becomes particularly life threatening with EBV infection.

The XLP gene SAP (or SH2D1A) encodes a 15-kd single SH2 domain that can function as a natural inhibitor of signal transduction events initiated by the cell surface receptors SLAM (CD150) and 2B4.1,2,4 In fact, on phosphorylation, both receptors recruit the tyrosine phosphatase SHP-2, which is blocked by SAP. SAP binds to the cytoplasmic tail of SLAM in the absence of phosphorylation in yeast, COS cells, and T lymphocytes.5

SLAM (CD150), a member of the immunoglobulin superfamily, is a glycosylated transmembrane protein expressed on all activated T, B, and dendritic cells.3,15 Engagement of SLAM by specific monoclonal antibodies induces interferon-γ (IFN-γ) production and redirects Th2 responses of antigen-specific T-cell clones to a Th1 or a Th0 phenotype.14 In B cells, SLAM triggering with anti-CD40 antibodies or by the soluble SLAM ectodomain leads to B-cell proliferation and production of IgM, IgG, and IgA.15 Because SLAM appears to be a self-ligand, it is likely that it signals bidirectionally.15,16 However, because SLAM associates with the natural inhibitor SAP in T cells and not in B cells, a SLAM-induced signal transduction pathway could be different in T and B cells.

SAP also associates with 2B4 (CD244), a membrane protein that has sequence homologies with SLAM in its ectodomain and its cytoplasmic tail and that is expressed constitutively on the surfaces of NK cells.17 In addition, 2B4 is expressed on the surfaces of a subset of human and mouse CD8+ T cells and human monocytes. Engagement of 2B4 induces cytokine secretion (IFN-γ) and enhances non-major histocompatibility complex–restricted killing by NK cells.18-21 The mechanism by which SAP controls 2B4 function in NK cells may be different than in CD8+ T cells, because regulation of expression of the SAP gene is different in NK cells than in T lymphocytes.8 SAP mRNA levels fall rapidly on triggering of the T-cell receptor for antigen. By contrast, the level of SAP expression in very low in resting mouse NK cells but increases after virus infection.3 Interestingly, recent studies show that 2B4 inhibits cytolysis induced by its ligand CD48 or by anti-CD3 in NK cells from patients with XLP. It appears, therefore, that SAP blocks a negative signal induced by the engagement of 2B4.7,9 This is of particular interest for the dissection of the complex phenotypes of XLP because the 2B4 ligand, CD48, is expressed on the surfaces of all EBV-induced B-cell blasts.

To identify other proteins that interact with SAP, a novel yeast 2-hybrid system was used in which tyrosine residues of the...
bait-protein could be phosphorylated. This altered yeast 2-hybrid system uses a mutated form of the src-family kinase c-fyn to phosphorylate proteins in the yeast cell. The mutations are designed to eliminate toxicity for the yeast cell. Using this method, 2 cell surface proteins, Ly-9 (CD299) and CD84, were found to interact with phosphorylated SAP. Further analyses indicate that the binding properties of SAP and the cytoplasmic tails of Ly-9, CD84, and 2B4 are slightly different from those of SAP and SLAM. Nevertheless, the current study shows that SAP can be recruted to the cytoplasmic tail of at least 4 cell surface molecules on the surfaces of hematopoietic cells.

Materials and methods

Plasmid construction

For expression in yeast, a sequence encoding human SAP was amplified by polymerase chain reaction (PCR) from the original yeast-expression plasmid pGAD424 and cloned into the EcoRI/BamHI sites in the multiple cloning site of a vector termed pBRIDGE. The SAP 5' sense primer was 5'-ATGGAATCTCATGACACGTGTTGCTTCTTCCAA-3' and the second cdNA fragment using primers Ly-9 581 F 5' sense, 5'-GAGAACACCGCTTTGGCAAAAGTGTCAAC-3' and Ly-9 3' antisense primer, 5'-ATCGGATCCCTAGGTTGTCTTGCTTGCGGAC-3'. Mutant Ly-9 581 Y-F was generated in a similar way using the primers Ly-9 581 F 5' sense, 5'-TCACGACAAAATTCCTTGGCCTACAGGAAACCT-3' and Ly-9 581 F 3' antisense primer, 5'-CCTGTATGAGAAGATGGTTGCTGAGCCTTC-3'. Mutant Ly-9 581 Y-F was obtained in the same way as Ly-9 581 F but using Ly-9 581 Y as a template for the PCR reactions.

An altered yeast 2-hybrid system

Human SAP cloned in pBRIDGE and transformed in the yeast strain CG1945 was used as bait to screen a human T-cell cdNA library in pGAD424 (KT3). The principle of the altered 2-hybrid system was that SAP and mutated c-fyn were inserted in one bi-cistronic vector pBRIDGE as described above. In mutant c-fyn, the regulatory tyrosines 420 and 531 were substituted by phenylalanine (Fyn 531 Y-F, Fyn 420, 531 Y-F). Conditional expression of the resultant fusion proteins was driven by the MET25 promoter in response to methionine levels in the medium. In the presence of 1 mM methionine, the expression of the protein was repressed, whereas the absence of methionine in the medium induced protein expression. Mutated c-fyn still phosphorylated endogenous yeast proteins, and its capability to become autophosphorylated was maintained (data not shown).

For the 2-hybrid screen in the presence of Fyn 420, 531 Y-F, the yeast strain CG1945 was cotransformed with the vector (pBRIDGE), containing both SAP and the c-fyn mutant. These transformants were selected on SD media lacking tryptophan for 5 days. Next, these transformants were transformed for a second time with 1 mg cDNA library derived from the human T-cell line KT3 in pGAD424. Double transformants were then plated in SD media lacking Trp, Leu, His, and Met in the presence of 5 mM 3-amino triazole.

Yeast clones that grew under these restrictive conditions were then tested by the β-galactosidase assay. Plasmid DNA was extracted from clones that were positive by both criteria. These plasmid DNAs were then expanded after transformation of HB101 bacteria and selection in M9 medium lacking Leu to isolate the GAL4 activation domain plasmid pGAD424. Purified plasmids were analyzed by restriction analysis and sequenced.

The β-galactosidase colony-lift filter assay and liquid culture assay using ONPG as a substrate were carried out as described in the Clontech (Palo Alto, CA) protocols handbook. The vector pBRIDGE containing SAP in the absence of mutated c-fyn was used as an alternative to repressing c-fyn expression with methionine because in the high-density cultures necessary for methionine can be depleted, thus activating expression of c-fyn.

Cells and antibodies

EL-4/SLAM4, Jurkat cells, Jurkat stably transfected with human CD84 cells, and Raji cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. COS cell transfections were carried out as previously described.2,3

An antihuman 2B4 monoclonal antibody (C1.7) was purchased from Immunotech, and monoclonal antirat Ly-9 (clone 30C7) hybridoma from ATCC (Manassas, VA).21 Antihuman Ly-9 (clone H3 Ly-9.1.84) and CD84 (clone CD84.1.21 and CD84.1.7) were produced by immunizing BALB/c mice with 300.19 murine cells stably transfected with full-length cdNA. The antihuman SLAM antibody (A12) was a gift from DNA-X (Palo Alto, CA).

Monoclonal antihuman SAP was obtained by standard procedures by immunizing BALB/c mice with the synthetic peptide CQGTTGIREDPDVG coupled to KLH (Pierce, Rockford, IL). Phosphotyrosine monoclonal antibody cocktail horseradish peroxidase–conjugated (PY-7E1, PY-1B2, PY-20) and horseradish peroxidase–conjugated streptavidin were from Zymed (San Francisco, CA).
Cell activation, immunoprecipitation, and immunoblotting

Mouse thymocytes (BALB/c), Jurkat, Jurkat/CD84, Raji, and EL-4/SLAM4 cells (10^6/mL) were activated with 1 mM pervanadate for 20 minutes at 37°C. Lysis was carried out with 2% Triton X-100 as described before. Cell lysates were centrifuged at 14,000 g for 15 minutes at 4°C, and the crude lysate was preclariﬁed for 1 hour with 50 µL protein G-agarose beads (Gibco BRL, Rockville, MD) and 5 µL normal mouse serum. Immunoprecipitations used 1µg indicated antibody and 30 µL protein G-agarose beads for 3 hours at 4°C. Beads were then washed as described. Crude lysates and immunoprecipitates were subjected to SDS-PAGE and transferred onto nitrocellulose filters (Millipore, Bedford, MA). Filters were blocked for 1 hour with 5% skim milk (or 3% bovine serum albumin) and then probed with the indicated antibodies. Bound antibody was detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Supersignal; Pierce).

Immunofluorescence microscopy

COS-7 cells were transfected with human Ly-9 or human CD84 cDNA using the lipofectamine method (Roche, Pleasanton, CA). After 48 hours, cells were labeled with biotinylated antihuman mAb Ly-9 or (HLy-9.1.84), biotinylated antihuman mAb CD84 (CD84.1.7), or biotinylated anti-SLAM (A12) (5 µg/mL) at 4°C for 30 minutes. After 2 washes with ice-cold PBS, cells were incubated with streptavidin-ﬂuorescein isothiocyanate (FITC) (Dako, Carpinteria, CA) or streptavidin-Cy3 (Dako). Cells were then washed twice with ice-cold PBS, immobilized in poly-L-lysine-treated coverslips at 4°C for 15 minutes, and ﬁxed in 20°C methanol for 15 minutes. After 2 washes, cells were incubated for 30 minutes at room temperature with blocking buffer (PBS containing 0.2% skim milk, 2% fetal bovine serum, 1% bovine serum albumin, 0.1 mM Glic) and then with Cy3-conjugated anti-SAP antibody (10C4.2) or FITC-conjugated anti-SAP antibody for 30 minutes at room temperature. Controls used an isotopic IgG1 conjugated with Cy3 or FITC. Cells were washed twice with PBS and mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL). Fluorescence images were obtained using a confocal microscope (TCS NT; Leica, Heidelberg, Germany).

Jurkat cells were stained with biotinylated antihuman CD84 (CD84.1.7), biotinylated antihuman Ly9 (HLy-9.1.84), or mouse IgG1 isotype control (5 µg/mL) at 4°C for 30 minutes as described above.

Results

A novel yeast 2-hybrid system for binding studies between SAP and other proteins

To study binding of the SAP SH2 domain to phosphorylated and nonphosphorylated proteins, a novel yeast 2-hybrid system was set up using c-fyn. Efﬁciency of the method was ﬁrst tested using the cytoplasmic tail of 2B4 and full-length SAP (see “Materials and methods”). Only when a yeast cell coexpressed SAP, 2B4, and FYN_420, 531_Y-F was an interaction detected in the β-galactosidase assay (Figure 1A, hatched bars). Cotransfection of SAP with 2B4 in the presence of FYN_420, 531_Y-F resulted in higher values in the β-galactosidase assay (Figure 1A, dotted bars), implying a stronger interaction between SAP and 2B4 than that detected in the presence of FYN_420, 531_Y-F, which had an intact SH2 domain. This demonstrated that 2B4 and SAP interact in yeast, but only when 2B4 is phosphorylated. The experiment suggests that the c-fyn SH2 domain might interfere with the binding of SAP to the cytoplasmic tail of 2B4 (see below).

In contrast to 2B4, SLAM interacted with SAP in a phosphorysine-independent fashion. β-Galactosidase activity was detected

Figure 1. The cell surface molecule 2B4 recruits the XLP gene product SAP after phosphorylation of the tyrosine residues in its cytoplasmic tail. (A) Comparison of the interactions between 2B4/SAP and SLAM/SAP in yeast cells. To detect binding between the cytoplasmic tail of 2B4 and SAP, a yeast 2-hybrid system was adapted to measure interactions with phospho-proteins. To this end, mutations of c-fyn were cotransfected with SAP into the yeast cell. Binding between 2 proteins in yeast cell extracts was detected by a β-galactosidase assay, as described in “Materials and methods.” For each construct, at least 3 independent colonies were tested in the β-galactosidase assay. Open bars: cells transfected with empty pBRIDGE vector and pGAD424 encoding the cytoplasmic tail of 2B4 or SLAM; solid bars: cells transfected with pBRIDGE-SAP and pGAD424 encoding the cytoplasmic tail of 2B4 or SLAM; hatched bars: cells transfected with pBRIDGE-SAP and FYn_420, 531_Y-F and with pGAD424 encoding the cytoplasmic tail of 2B4 or SLAM; dotted bars: cells transfected with pBRIDGE-SAP and Fyn_420, 531_Y-F, 176_R-Q and with pGAD424 encoding the cytoplasmic tail of 2B4 or SLAM. Control, empty pGAD424 with pBRIDGE encoding the indicated DNA sequences. (B) SAP binds to phosphorylated 2B4 in NK cell line. Interactions between SAP and 2B4 in the NK cell line were studied by immunoprecipitation of 2B4 followed by Western blot analysis with anti-SAP. YT cells (50,000/mL) were lysed in detergent, and 2B4 was immunoprecipitated with the 2B4 speciﬁc monoclonal antibody C1.7 (α-2B4). After SDS-PAGE, proteins were transferred to a PVDF membrane and identiﬁed with streptavidin, antiphosphotyrosine (α-pY), or antihuman SAP (α-SAP). (C) SAP binds to nonphosphorylated and phosphorylated SLAM in a T-cell line. Interactions between SAP and SLAM were studied in the T-cell transfectant cell line EL-4/SLAM4 by immunoprecipitation of SLAM, followed by Western blot analysis with anti-SAP. EL-4/SLAM4 cells (20 × 10^6 cells/mL) expressing human SLAM were treated with pervanadate, as described in panel B, and SLAM was immunoprecipitated by an antihuman SLAM monoclonal antibody (α-SLAM). After SDS-PAGE, proteins were transferred to a PVDF membrane and identiﬁed by Western blot analysis with a rabbit antihuman SLAM antibody, antiphosphotyrosine (α-pY), or antihuman SAP (α-SAP) 10C4.2 CONTROL, immunoprecipitation with an irrelevant monoclonal antibody.

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when SLAM was coexpressed with either SAP alone or with SAP and Fyn420, 531 Y-F (Figure 1A). In yeast cells cotransformed with SLAM, SAP, and Fyn420, 531 Y-F (Figure 1A, hatched bars), β-galactosidase activity was lower than in yeast cells that express SAP and SLAM (Figure 1A, solid bars). One possible reason for this was that c-fyn’s own SH2 domain would bind to a SLAM phosphotyrosine, thus competing with SAP for the same docking site. To test this possibility, arginine 176 in the c-fyn sequence was substituted by a glutamine Fyn420, 531 Y-F. This mutation disabled the binding properties of the SH2 domain of c-fyn. Indeed, the β-galactosidase assay showed a stronger response when the triple mutant of c-fyn, Fyn420, 531 Y-F, 558 R-Q, was used (Figure 1A, dotted bars). This suggests that c-fyn binds to phosphorylated SLAM through its SH2 domain. Taken together, the data show that in yeast, SAP interacts with phosphorylated 2B4 only and with both nonphosphorylated and phosphorylated SLAM.

To confirm that SAP bound to phospho-2B4 preferentially, cells of the NK line YT were used for communoprecipitation of SAP and 2B4. Only when 2B4 was phosphorylated by pervanadate treatment of the cells did this surface receptor bind to SAP (Figure 1B). By contrast, SAP binds to both phospho- and nonphospho-SLAM in the T-cell line EL4 (Figure 1C). Thus, the binding experiments in yeast truthfully represented interactions found in hematopoietic cells.

**SAP binds specifically to the cytoplasmic tail of the hematopoietic cell surface receptor Ly-9**

When $1 \times 10^6$ clones of a yeast T-cell library (KT3) were screened using SAP as bait in the presence of Fyn420, 531 Y-F, 5 clones were isolated. These were all positive, as judged by their ability to grow in media lacking histidine and by their β-galactosidase activity. Each clone encoded a fragment with the exact nucleotide sequence of the cytoplasmic tail of human Ly-9. Ly-9 is a glycoprotein whose extracellular domain belongs to the same subfamily of the immunoglobulin superfamily of proteins as SLAM and 2B4. When Ly-9 was subsequently cotransfected in yeast with SAP alone, no β-galactosidase activity was detected. However, in the presence of Fyn420, 531 Y-F, the interaction between phospho-Ly-9 and SAP resulted in detectable β-galactosidase activity (Figure 2A).

To refine the specificity of the interaction and to determine which of the 3 phosphotyrosines in the cytoplasmic tail of Ly-9 were involved in SAP binding, Ly-9 mutants were tested in the yeast 2-hybrid system. Because the $2 (TV/I pY x x V/I)$-motifs (see Figure 4), in the cytoplasmic tail of Ly-9 were the most logical candidates for SAP binding, 3 mutants were made. Tyrosine 558 (Ly9-558-YF), tyrosine 581 (Ly9-581-YF), or both tyrosine residues (Ly9-558581-YF) were substituted by phenylalanine. The Ly-9 mutants were then subcloned in the GAL4-binding domain of pGAD424, and these constructs were cotransfected in yeast with SAP alone or with SAP and Fyn420, 531 Y-F. As shown in Figure 2B, the phosphorylated form of each single mutant (Ly9-558-YF or Ly9-581-YF) binds SAP, in fact better than the wt Ly9 segment. However, no interaction was detected when both tyrosine residues were absent in the cytoplasmic tail of Ly-9 (Ly9-558581-YF). We conclude that each of the 2 phosphotyrosine motifs binds SAP specifically and that no other binding sites are involved.

To verify the interaction between SAP and Ly-9, mouse thymocytes, which express large amounts of both Ly9 and SAP, were activated with pervanadate and subjected to immunoprecipitation using an anti-Ly-9 antibody. As predicted by the yeast data, SAP interacted with Ly-9, but only after the cells were treated with pervanadate and Ly-9 became tyrosine-phosphorylated (Figure 2C). No interaction was detected in untreated cells. We conclude that Ly-9, like 2B4 and SLAM, recruits SAP but that, unlike SLAM, this binding appears to be dependent on the phosphorylation status of the tyrosine motif in the cytoplasmic tail of Ly-9.

**Figure 2. SAP interacts with the phosphorylated cytoplasmic tail of Ly-9.** A human cDNA library made from poly A RNA of the human T-cell line KT3 in pGAD424 was screened with the altered yeast 2-hybrid system. Thus, 5 cDNA clones encoding the cytoplasmic tail of Ly-9 were isolated; an example is shown in panel A. To map the binding sites in the cytoplasmic tail of human Ly-9, 3 mutations of Ly-9 were analyzed in panel B. SAP binding to phospho-2B4 was shown in murine thymocytes in panel C. (A) Interactions of SAP and the cytoplasmic tail of Ly-9 in yeast are dependent on the presence of Fyn420, 531 Y-F. The interaction of SAP with the cytoplasmic tail of Ly-9 in the presence or absence of Fyn420, 531 Y-F took place in the yeast cell and was measured in a β-galactosidase assay. For each construct, at least 3 independent colonies were tested in the galactosidase assay. Open bars: cells transfected with empty pBRIDGE vector and pGAD424 encoding the cytoplasmic tail of Ly-9; solid bars: cells transfected with pBRIDGE-SAP and Fyn420, 531 Y-F and with pGAD424 encoding the cytoplasmic tail of Ly-9. Control, empty pGAD424 with pBRIDGE encoding the indicated DNA sequences. (B) SAP interacts with 2 phosphotyrosine motifs in the cytoplasmic tail of Ly-9, and 2 phosphotyrosine motifs in the cytoplasmic tail of human Ly-9. Ly-9 is a glycoprotein whose extracellular domain belongs to the same subfamily of the immunoglobulin superfamily of proteins as SLAM and 2B4. When Ly-9 was subsequently cotransfected in yeast with SAP alone, no β-galactosidase activity was detected. However, in the presence of Fyn420, 531 Y-F, the interaction between phospho-Ly-9 and SAP resulted in detectable β-galactosidase activity (Figure 2A).
SAP binds to the cytoplasmic tail of the cell surface receptor CD84

CD84, a member of the SLAM family expressed on the surface of B and T lymphocytes and monocytes, contains 3 potential SAP-binding motifs in its cytoplasmic tail (Figure 4). That prompted us to examine the interaction of CD84 with SAP in yeast and in lymphocytes. CD84 was transfected into yeast together with SAP or with SAP and Fyn 420, 531 Y-F. As judged by the β-galactosidase assay, CD84 interacted with SAP (Figure 3A). Again this interaction could be detected only when c-fyn was cotransfected and no binding was detected in the absence of the tyrosine kinase.

SAP and the cytoplasmic tail of CD84 interact in a variant of the human B-cell line Raji, but only when the cells were treated with pervanadate (Figure 3B). Pervanadate treatment results in a strong phosphorylation of CD84, which permits association with SAP. No SAP binding was observed on untreated cells. Because SAP is primarily a T-cell protein, the CD84-SAP interaction was also examined in JURKAT cells, which had been stably transfected with human CD84. Once again, SAP coprecipitated with the phosphorylated form of CD84 (Figure 3C).

Taken together, the observations demonstrate that the cell surface receptor CD84 interacts with SAP. The results also indicate that the mode of interaction between SAP and SLAM differs from interactions between SAP and Ly-9 or CD84. This suggests that the apparent affinity between the SH2 domain of SAP and its recognition sites in the cytoplasmic tail of Ly-9 or CD84 must be lower. Because the minimal binding motifs (Figure 4), however, are similar if not identical, interactions with other segments might play a role.

SAP inhibits the association of SHP-2 with phosphorylated Ly-9 and CD84

The cytoplasmic tail of SLAM contains 2 binding sites for SAP—one is in a peptide segment that includes Y281, and one includes Y327. Optimal binding of SAP occurs when each site is phosphorylated, but SAP also binds strongly to the Y281 site in the absence of phosphorylation. For activation of the tyrosine phosphatase SHP-2, both of its SH2-domains are required to bind to their docking sites. In the cytoplasmic tail of SLAM, the SHP-2 docking sites are the same as the SAP docking sites (the pY281 and the pY327 motif) (D.H., unpublished data, February 2000). As expected, SHP-2 does not bind to nonphosphorylated SLAM.2,10

As reported previously, SAP blocks recruitment of SHP-2 to the phosphorylated cytoplasmic tail of SLAM.2 The structure of SAP is consistent with this role as a natural inhibitor, and the affinity of SAP for a phosphorylated tyrosine pY281-peptide (binding constant, approximately 120 nM) is higher than the affinity of other SH2 domains for their phosphotyrosine-binding motifs.10

Because Ly-9 and CD84 each contain 2 SAP-binding motifs (Figure 4), the ability of SAP to block recruitment of SHP-2 to these receptors was tested in COS cells.2 As expected, SHP-2 binds to phosphorylated Ly-9 only (Figure 5A), and SAP interferes with that binding. Similarly, SHP-2 binds to CD84, if c-fyn is cotransfected into the same COS cells (Figure 5B), and this binding is blocked by the presence of SAP.

In the COS cell experiments, SAP was found to bind frequently to phosphorylated Ly-9 and CD84, which prompted us to examine the interaction of CD84 with SAP in yeast and in lymphocytes.
model in which SAP acts as a natural inhibitor of the docking sites for SH2-containing enzymes and adapters in the cytoplasmic tail of Ly-9 or CD84.

**SAP is recruited to the cell surface on phosphorylation of Ly-9 and CD84**

To examine whether the associations between Ly-9 or CD84 and SAP took place on the cell surfaces, COS cell transfectants were analyzed by immunofluorescence techniques. As shown in Figure 6, SAP is evenly distributed throughout the cytoplasm of COS cells. By contrast, most Ly-9 and CD84 molecules are expressed in the plasma membrane. In double transfectants, a large proportion of the immunofluorescence developed with α-SAP colocalizes with α-Ly-9 or α-CD84 staining (Figure 6). The selected pictures are representative of observations made in 3 independent COS cell experiments.

To test whether SAP was recruited to the plasma membrane in T cells, cocapping experiments were done in Jurkat cells with α-SAP and α-CD84 or α-Ly-9 antibodies. First, Jurkat cells were stained with α-CD84 for 10 minutes at 4°C. Then SAP was visualized with a directly labeled monoclonal antibody using standard cytoplasmic staining techniques. CD84 was homogeneously distributed on the membrane of nonactivated Jurkat cells, whereas SAP was mostly expressed in the cytosolic compartment (Figure 7A-B). When cells were treated with biotinylated anti-CD84 antibody followed by streptavidin-FITC for 30 minutes at 4°C and were incubated for 10 minutes at 37°C, the CD84 molecules capped (Figure 7C). No capping was detected when cells were incubated under the same conditions with an isotypic control (IgG1) instead of anti-CD84 (data not shown). Jurkat cells that had been treated with anti-CD84 under capping condition were then stained with an CY3 conjugated anti-SAP antibody (10C4).2 Under those conditions, the distribution of SAP and CD84 on the plasma membrane of capped cells overlapped (Figure 7D). No cocapping was detected in CD84 capped cells stained with CY3-conjugated mouse IgG1 isotypic control (data not shown).

Identical results were obtained when Jurkat cells were treated with an anti-Ly-9 monoclonal antibody in a similar set of experiments. Under capping conditions, SAP colocalized with Ly-9 (Figure 7E-H). We conclude from the immunofluorescence studies that on triggering of Ly-9 and CD84 with their respective monoclonal antibodies, SAP colocalizes with the receptors at the plasma membrane.

**Discussion**

Ly-9 and CD84 are both members of the immunoglobulin superfamily, and their ectodomains are related to the other 2 SAP-binding proteins SLAM and 2B4 (Figure 8).3,5,10,23,24 All 4 molecules comprise 2 immunoglobulin-like domains (V and C2 domains) with the exception of Ly-9, which contains a tandem repeat of V-C2 domains (Figure 8). By contrast, CD48 does not have a cytoplasmic tail and has been shown to be attached to the plasma membrane by a conventional phosphoinositide lipid tail.25 CD48 serves as the ligand for 2B4,19 and a weak but measurable homophilic interaction between SLAM has been reported.26 The interaction between SAP and the Y281 motif of the cytoplasmic tail of SLAM appears to be unique in that it does not require tyrosine phosphorylation. Understanding the physicochemical parameters of the interactions between SAP and SLAM has been facilitated by the 3-dimensional structure of SAP associated...
with a peptide that included the binding motif in the cytoplasmic tail of SLAM. This peptide binds to SAP regardless of whether its essential tyrosine residue Y281 is phosphorylated. However, the apparent binding constant, as determined by fluorescence polarization, was different with the phosphophorylated peptide (120 nM) than with the nonphosphorylated peptide (830 nM), indicating a higher affinity for the phosphorylated peptide. Thus, the structure of SAP and physicochemical studies support the notion that SAP can act as a natural inhibitor.

The physicochemical parameters, which determine the differences in affinity of binding between SAP and the 4 members of the SLAM family, are unknown. However, it is likely that segments of their cytoplasmic tails located outside the binding motif areas could affect the binding affinity. It is an attractive speculation that the observed dependence on phosphorylation of the interaction of SAP and 2B4, Ly9, or CD84 may indicate differences in the way SAP governs signal transduction pathways initiated by these receptors.

Although the function of Ly-9 and CD84 is unknown, it has been suggested that they may participate in adhesion between T and B cells.
lymphocytes and antigen-presenting cells by homophilic interactions.\(^{24}\) CD84 is expressed on macrophages and T and B lymphocytes, whereas Ly-9 expression is restricted to T and B cells.\(^{22}\) Thus, 3 known SAP-binding cell surface structures—namely, SLAM, Ly-9, and CD84—are found on the surfaces of activated T cells, whereas 2B4 is detected on the surfaces of CD8 cells and NK cells. Because the SAP gene is expressed in activated NK cells, SAP controls the signal transduction pathways initiated by 2B4 in this cell type.\(^{5,9}\) Given that SLAM expression was induced on the surfaces of mouse NK cells after infection with LCMV, but not after MCMV infection,\(^{8}\) SLAM-SLAM interactions might play a role in NK cells as well.

As expansion of CD8 and CD4 cells has been observed in XLP patients infected with EBV and because NK cell functions are impaired in a subset of XLP patients, the 4 SAP-binding cell surface molecules are likely to have a cumulative effect on the pathogenesis of the disease.

Based on their tissue distribution, we speculate that all 4 receptors are engaged on the surface of a T cell (CD8 cell in the case of 2B4) that recognizes an EBV-infected B cell. Moreover, in the absence of SAP, the CD48/2B4 pair induces abnormal signaling in NK cells. This might happen in concordance with aberrant SLAM signaling in NK cells.

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## References

Cell surface receptors Ly-9 and CD84 recruit the X-linked lymphoproliferative disease gene product SAP

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