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Distinct Interactions of the X-Linked Lymphoproliferative Syndrome Gene Product SAP with Cytoplasmic Domains of Members of the CD2 Receptor Family

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X-linked lymphoproliferative syndrome (XLP; Duncan's disease) is a primary immunodeficiency disease that manifests as an inability to regulate the immune response to Epstein–Barr virus (EBV) infection. Here we examine the ability of the product of the gene defective in XLP, SAP (DSHP/SH2D1A), to associate with the cytoplasmic domains of several members of the CD2 subfamily of cell surface receptors, including SLAM, 2B4, and CD84. While recruitment of SAP to SLAM occurred in a phosphorylation-independent manner, SAP was found to bind preferentially to tyrosine-phosphorylated cytoplasmic domains within 2B4 and CD84. Missense or nonsense mutations in the SAP open reading frame were identified in five of seven clinically diagnosed XLP patients from different kindreds. Four of these variants retained the ability to bind to the cytoplasmic tails of SLAM and CD84. While ectopic expression of wild-type SAP was observed to block the binding of SHP-2 to SLAM, mutant SAP derivatives that retained the ability to bind SLAM did not inhibit recruitment of SHP-2 to SLAM. In contrast, SAP binding to CD84 had no effect on the ability of CD84 to recruit SHP-2, but instead displaced SHP-1 from the cytoplasmic tail of CD84. These results suggest that mutations in the gene encoding the XLP protein SAP lead to functional defects in the protein that include receptor binding and SHP-1 and SHP-2 displacement and that SAP utilizes different mechanisms to regulate signaling through the CD2 family of receptors.

Key Words: **X-linked lymphoproliferative syndrome; Duncan's disease; SAP; SLAM; CD84.**

INTRODUCTION

X-linked lymphoproliferative syndrome (Duncan's disease; XLP; OMIM 308240) (1) is a rare immunodeficiency disorder in which affected patients develop inappropriate responses to Epstein–Barr virus (EBV) infection, which include severe or fatal infectious mononucleosis, acquired hypogammaglobulinemia, and malignant lymphoma (2, 3). Patients with XLP typically exhibit sustained polyclonal B-cell proliferation, prolonged cytotoxic T-lymphocyte (CTL) response, and defective natural killer (NK) cell cytotoxicity, suggesting a generalized inability to downregulate the immune response to EBV infection (4–6).

The gene defective in XLP encodes a 128-amino-acid protein containing a single SH2 domain termed SLAM-associated protein (SAP; SH2D1A; DSHP), which was recently identified by genetic analysis of affected kindreds and independently in a genetic screen for proteins that bind to the cytoplasmic tail of the cell surface receptor SLAM (signaling lymphocytic activation molecule; CDw150) (7–9). SLAM is a costimulatory receptor that has recently been reported to function as a receptor for lymphotrophic strains of measles virus (10) and is expressed on subsets of B and T cells (11). SLAM is a member of the CD2 family of receptors that includes 2B4, which is primarily expressed on NK cells but has also been detected on cytotoxic T cells (12), and CD84, which is expressed predominantly on monocytes, and platelets, and at low levels on $CD3^+$ T cells (13, 14). SLAM and 2B4 have been shown to contain multiple copies of the consensus SAP-binding motif TIYxxV/I.

A number of reports have revealed a defect in 2B4 receptor-mediated NK cell cytotoxicity in patients with XLP, implicating a role for SAP in the regulation of signaling through these molecules (15–17). This lack of NK cell activation may affect the killing of EBV-infected B cells, thus contributing to the pathogenesis of

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the disease. Previous studies have shown that SAP regulates SLAM signaling by preventing recruitment of the protein phosphatase SHP-2 to the receptor (7). Since SAP contains only a single SH2 domain, it has been proposed to function as a regulator of signaling through receptors such as SLAM and 2B4 by binding to and blocking the association of other SH2 domain-containing molecules.

Here we examine the ability of wild-type SAP, as well as a panel of SAP variants identified from XLP patients, to bind to SLAM, 2B4, and CD84. As reported previously, SAP was found to bind to the cytoplasmic tail of SLAM without prior phosphorylation of the receptor (7). In contrast, binding of SAP to the cytoplasmic domains of CD84 and 2B4 was dependent on tyrosine phosphorylation of these domains. An analysis of the SAP coding sequence from seven kindreds of XLP patients revealed that five of the seven contained missense or nonsense mutations in the SAP gene, and the abilities of these SAP mutants to bind to the cytoplasmic tails of SLAM and CD84 were determined. Based on receptor binding, these mutants could be subdivided into two distinct groups: those incapable of binding to the cytoplasmic tails of these receptors and those retaining the ability to bind. Interestingly, several SAP mutants still capable of receptor binding were unable to displace SHP-2. Finally, the binding of SHP-2 to the cytoplasmic domain of CD84 was unaffected by SAP, although the binding of SHP-1 to CD84 was potently inhibited by SAP. These data imply that SHP-1 and SHP-2 bind to distinct sites in the cytoplasmic tail of CD84. Collectively, these findings suggest, first, that an important consequence of SAP mutation is to render the altered molecule incapable of displacing SHP-2 and, second, that despite the general process of receptor binding, SAP may regulate different signal transduction pathways in a manner that depends on the specific signaling intermediate displaced from the receptor. Mutations in SAP that disrupt the normal signal transduction cascade downstream of receptor–ligand interaction may contribute to the clinical syndrome seen in XLP patients.

MATERIALS AND METHODS

Cells and Transfections

Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 2 mM glutamine. All cell lines were grown at 37°C with 5% CO_2.

Generation and Characterization of XLP Cell Lines

Blood was obtained from patients with the clinical diagnosis of XLP as part of an Institutional Review Board-approved clinical research protocol at the Warren Magnuson Clinical Center at the National Institutes of Health. Peripheral blood mononuclear cells from XLP patients were isolated on Ficoll–Hypaque gradients (Amersham Pharmacia, Piscataway, NJ) and were infected with EBV strain B95-8. EBV-transformed B-lymphoid cell lines were isolated 21 days postinfection. The cells were cultured in RPMI 1640 containing 25 mM Hepes, 2 mM glutamine, and 10% FBS. Total genomic DNA was isolated from cell lines derived from XLP patients using exon-specific primers exactly as previously described (15).

Plasmid Construction

The pEBG mammalian glutathione *S*-transferase (GST) fusion vector (18) was kindly provided by Dr. B. J. Mayer. The pEBB-FLAG mammalian expression vector has been described previously (19). The pSX Lck, pCMV5 SHP-1, and pCMV5 SHP-2 mammalian expression vectors were kindly provided by Dr. J. S. Gutkind. The human SAP full-length cDNA and the SLAM and CD84 cytoplasmic domains were all amplified by PCR from a human leukocyte cDNA library (Clontech, Palo Alto, CA) using Advantage cDNA polymerase (Clontech) under standard conditions. SAP was amplified using the two primers 5′-ATAGGATCCATGGACGCAGTGGCTGTGTATCATG-3′and 5′-AATATCGATTCATGGGGCTTTCAGGCAGACATC-3′ and was cloned into the *Bam*HI/*Cla*I sites of pEBB-FLAG. The 77 amino acid cytoplasmic tail of SLAM was amplified using the two primers 5′-ATAGGATCCCAGTTGAGAAGAAGAGGTAAAACG-3′ and 3′ primer 5′-AATGCGGCCGCAGAAAGTCCCTTTGTTGGTCTCTGGTGTC-3′ and was cloned into the *Bam*HI/*Not*I sites of the mammalian GST expression vector pEBG. The 88-amino-acid cytoplasmic domain of human CD84 was amplified using 5′ primer 5′-ATAGGATCCTTGTTCAAGAGAAGACAAGATGCTG-3′ and 3′ primer 5′-AATATCGATTGTAACTCAGTTTCCAGAGGAG-3′ and was cloned into the *Bam*HI/*Cla*I sites of pEBG. The cytoplasmic domain of human 2B4 was amplified from a pCR2.1 clone (Invitrogen, Carlsbad, CA) containing full-length human 2B4, kindly provided by Dr. C. Watzl, using the two primers 5′-ATAAGATCTTGGACTAAGAAGAGGAAGCAGTTAC-3′ and 3′ primer 5′-AATATCGATAGTCAATGATGCTGCATGACACAG-3′. The resulting PCR product was then cloned into the *Bam*HI/*Cla*I sites of pEBG.

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Strat-

agene, La Jolla, CA) following the manufacturer's instructions.

GST Precipitation and Immunoblotting

Subconfluent 10-cm^2 dishes of human 293 cells were transfected with 4 μg of the indicated plasmids (unless indicated) using the calcium phosphate precipitation procedure as previously described (20). Cells were lysed and coprecipitations performed in Triton X-100 buffer as previously described (19). Samples were resolved on 4–12% gradient SDS–PAGE gels (Invitrogen), transferred to nitrocellulose membranes (Invitrogen), and blocked with 5% milk solution in TBS + 0.2% Tween 20 (TBS-T). Due to the small size of the FLAG-tagged SAP protein, those lysates were run on Tricine gels (Invitrogen) and transferred to PVDF membranes (Invitrogen). The membranes were incubated with the indicated primary antibody (diluted 1:1000 in TBS-T + 2.5% milk), incubated with secondary antibody, either horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit (Amersham Pharmacia) (diluted 1:2000 in TBS-T + 2.5% milk), and visualized using the enhanced chemiluminesence Western blot detection system (Amersham Pharmacia). Primary antibodies used in these studies include monoclonal anti-FLAG antibody (Sigma, St. Louis, MO), monoclonal anti-SHP-1/PTP1C (Transduction Labs, Lexington, KY), monoclonal anti-SHP-2/PTP1D (Transduction Labs), and rabbit polyclonal anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

SAP Binds to the Cytoplasmic Tail of SLAM, 2B4, and CD84

SAP has previously been demonstrated to bind to the cytoplasmic tail of the cell surface receptor SLAM and has also been shown to associate with another member of the CD2 family, 2B4. The cytoplasmic tails of these molecules each contain multiple copies of a TIYxxV/I motif that is predicted to be the consensus SAP-binding motif. In a search for other potential SAP-interacting proteins, we identified two putative SAP-binding domains in the cytoplasmic tail of another member of the CD2 family, CD84. To determine whether SAP could associate with CD84, mammalian expression vectors were generated in which GST was fused to the cytoplasmic domains of SLAM, 2B4, or CD84. These constructs were coexpressed in 293 cells with an epitope-tagged SAP expression vector. To examine the requirement of tyrosine phosphorylation of the receptor cytoplasmic domains, an expression vector encoding Lck tyrosine kinase was also cotransfected. Lysates from transfected cells were precipitated with glutathione–Sepharose beads, and coprecipitating proteins were detected by immunoblot analysis. SAP was found to coprecipitate with the GST-SLAM and GST-2B4 proteins, and the association of SAP with GST-SLAM was independent of the phosphorylation state of the receptor (Fig. 1A). In contrast, the SAP–GST-2B4 interaction was markedly enhanced in the presence of Lck. SAP was also capable of binding to the cytoplasmic domain of CD84, and similar to 2B4, the association was greatly enhanced in the presence of Lck. Immunoblot analysis of lysates using a GST antibody revealed the presence of an additional higher molecular weight species in the sample expressing CD84 and Lck (Fig. 1A), presumably representing a phosphorylated form of the GST-CD84 protein, suggesting that enhancement of binding is due to tyrosine phosphorylation of the receptor rather than tyrosine phosphorylation of SAP. SAP did not bind to the control mammalian GST (Fig. 1A).

SAP Can Bind Independently to Two SAP-Binding Sites in the Cytoplasmic Tail of CD84

Sequence analysis of the cytoplasmic domain of CD84 revealed the presence of two elements, TIYTVI (residues 260–265) and TVYSEV (residues 296–301), that resemble the canonical SAP-binding motif (21). To examine the ability of SAP to bind to these elements, derivatives of the GST-CD84 expression vector were generated in which either the TIY or TVY residues of the two different elements were mutated to AAA. These mutants were coexpressed with SAP and tested for their ability to bind SAP by coprecipitation and immunoblot analysis. Both mutants were found to coprecipitate SAP to a degree that was indistinguishable from that of wild-type GST-CD84 (Fig. 1B), suggesting that each of these sites can function as a SAP-binding site in the context of the CD84 cytoplasmic tail.

Identification of Mutations in the SAP Gene from XLP Patients

Seven unrelated individuals each having the clinical diagnosis of XLP were analyzed for mutations in their SAP coding sequences. Analysis of the four exons of the SAP gene from these seven patients revealed missense or nonsense mutations in five of the seven individuals. These sequences were compared to previously reported mutations (9, 22), as shown in Table 1. In our patient group, three had the same single A to C mutation that resulted in a change at amino acid 99 from Gln to Pro (mutant 5). One patient was found to have a C to T mutation that resulted in the insertion of a premature termination codon at amino acid 55 (mutant 6). Another patient had a complete deletion of exon 2 (mutant

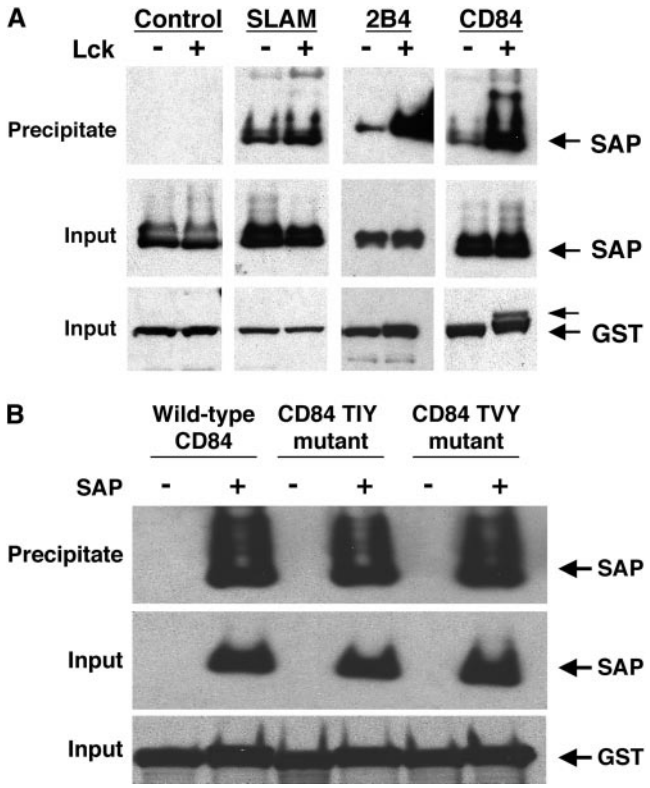

FIG. 1. SAP binds to the cytoplasmic domain of members of the CD2 family of receptors, including SLAM, 2B4, and CD84. (A) Human 293 cells were transfected with a FLAG-tagged SAP expression vector along with the indicated GST receptor tail constructs or GST alone (control). The effect of tyrosine phosphorylation on binding of SAP was assessed by the addition of a plasmid encoding the Src tyrosine kinase, Lck. Coprecipitations were performed with glutathione–Sepharose beads, and bead-associated proteins were analyzed using SDS–PAGE and Western blot. The top two rows were probed with a monoclonal anti-FLAG antibody and the bottom row with a polyclonal anti-GST antibody. (B) The ability of SAP to bind to the indicated GST-CD84 cytoplasmic domain mutants was determined using coprecipitation experiments as described for (A).

7). The two remaining patients had no mutations in their SAP coding sequences.

SAP Mutations Found in XLP Patients Differ in Their Abilities to Bind SLAM and CD84

To evaluate the receptor-binding properties of the variants of SAP identified from XLP patients, site-directed mutagenesis was performed to generate the point mutants in the SAP mammalian expression vector. The abilities of these mutants to associate with the cytoplasmic tail of SLAM and CD84 were assessed. Four of the five mutants tested showed impairment in their abilities to bind SLAM (Fig. 2A). Mutant 1 (R32T) was greatly compromised in its ability to bind to the receptor, whereas the SLAM-binding properties of mutants 2 (T68I), 4 (G93D), and 5 (Q99P) were less affected. Analysis of the input lanes consistently revealed differences in the expression levels of several variants (for example, Fig. 2A, mutant 5), possibly indicating differences in stability, as has previously been proposed (21). Interestingly, the ability of mutant 3 (P101L) to bind SLAM was actually enhanced.

To determine whether the receptor-binding properties of the SAP variants was dependent on the tyrosine phosphorylation status of the receptor, the ability of the SAP mutants to bind to CD84, in the presence of Lck, was also examined. The pattern of binding of the SAP mutants to GST-CD84 was similar to that observed with binding to GST-SLAM (Fig. 2B). The mutants that were impaired in their ability to bind SLAM (mutants 1, 2, and 4) were also reduced in their ability to associate with CD84. Mutant 3, whose binding to SLAM was enhanced, also bound strongly to CD84.

SAP Displaces SHP-2 from the Cytoplasmic Domain of SLAM

The observation that most of the mutants tested retained the ability to bind to the cytoplasmic tail of SLAM and CD84 raised the possibility that properties other than receptor binding might be impaired. Previous reports have revealed that SAP can prevent association of the tyrosine phosphatase SHP-2 with the

TABLE 1

1 MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGV 40

41 YCLCVLYHGYIYTYRVSQTETGSWSAETAPGVHKRYFRKI 80

81 KNLISAFQKPDQGIVIPLQYPVEKKSSARSTQGTTGIRED 120

121 PDVCLKAP 128

Patient mutation	Change	Result	Exon
mutant 1	C→G	Arg32→Thr	1
mutant 2	C→T	Thr68→Iso	3
mutant 3	C→T	Pro101→Leu	3
mutant 4	G→A	Gly93→Asp	3
mutant 5	A→C	Gln99→Pro	3
mutant 6	C→T	Arg55→stop	2
mutant 7	---	Δ exon 2	2

Note. SAP mutations used in this study. Mutations 1–4 have been reported previously (9, 22), and mutations 5–7 were identified from XLP patients as described under Materials and Methods. Mutation 5 was identified from three unrelated patients; mutations 6 and 7 were each identified once. Two patients were found to have no mutations in their coding sequences. The intron/exon boundaries are denoted with arrows. The amino acid changes found in XLP patients are indicated with asterisks.

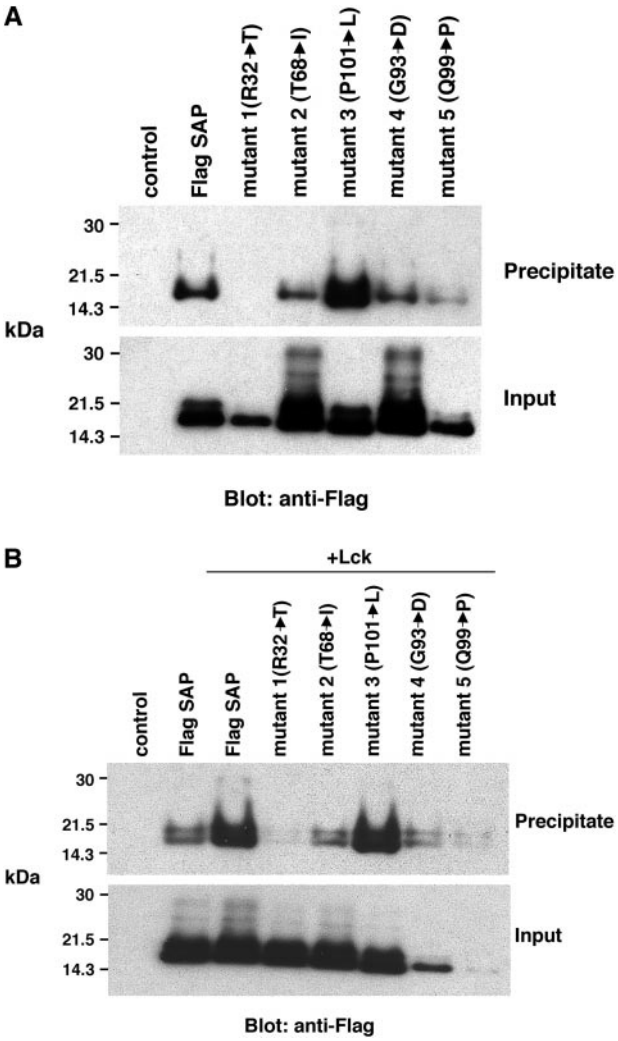

FIG. 2. Binding of the SAP mutants to SLAM and CD84. Human 293 cells were transfected with wild-type or mutant epitope-tagged SAP constructs or the empty pEBB-FLAG vector (control) together with the expression vector encoding (A) the GST-SLAM cytoplasmic tail or (B) that encoding the GST-CD84 cytoplasmic tail. An expression vector for the tyrosine kinase Lck was included where indicated in (B). The ability of these mutations to bind was assessed as described in the legend to Fig. 1. Expression of the GST fusion proteins was confirmed by probing with an anti-GST polyclonal antibody (data not shown).

cytoplasmic domain of either SLAM or 2B4 . Therefore, SHP-2 was examined for its ability to bind to the cytoplasmic domain of SLAM when ectopically coexpressed with SAP. While SAP was found to coprecipitate with SLAM in a phosphorylation-independent manner (Fig. 1A), the binding of SHP-2 to SLAM was found to require phosphorylation of the cytoplasmic tail of SLAM, which was provided by the presence of Lck (Fig. 3A). Consistent with previous reports, titration of SAP resulted in displacement of SHP-2 from the cytoplasmic tail of SLAM (7).

SAP Mutants Differ in Their Abilities to Displace SHP-2 from the Cytoplasmic Domain of SLAM

The data presented in Fig. 2A suggest that the SAP mutants differ in their abilities to bind to the cytoplasmic tail of SLAM. To determine whether the mutants identified from XLP patients retain the ability to displace SHP-2, mutant SAP expression vectors were transfected into 293 cells together with SHP-2 and GST-SLAM, and the abilities of the mutants to block association of SHP-2 with SLAM were determined.

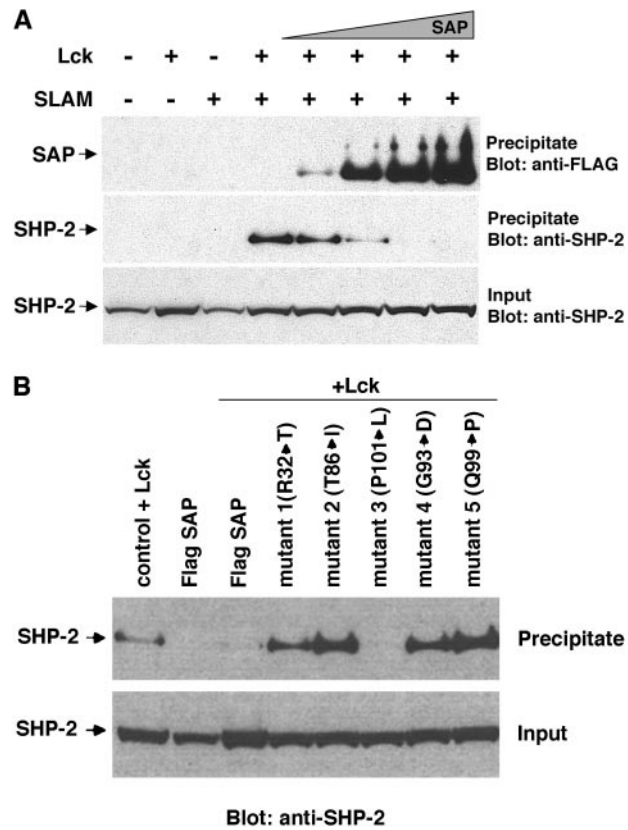

FIG. 3. (A) SAP displaces SHP-2 from the cytoplasmic tail of SLAM. Human 293 cells were transfected with a GST-SLAM tail expression vector along with a SHP-2 expression vector. The amount of SHP-2 was kept constant while increasing amounts of SAP (0.1, 0.5, 1, and 4 μg/well) were titrated into the transfection mixture. The expression vector encoding Lck was included where indicated. Binding studies were performed as described in the legend to Fig. 1. The top row was probed with a monoclonal anti-FLAG antibody and the lower two with monoclonal anti-SHP-2 antibody. (B) SAP mutants differ in their abilities to displace SHP-2. Human 293 cells were transfected with the GST-SLAM tail along with an expression vector for SHP-2 as well as the indicated SAP expression constructs or empty vector (control). Lck was included in the transfection where indicated. Coprecipitation experiments and Western blot were performed as described in the legend to Fig. 1. Both rows were probed with monoclonal anti-SHP-2 antibody. Expression of the GST fusion proteins and SAP expression were confirmed by Western blot with anti-GST polyclonal antisera and monoclonal anti-FLAG antibody, respectively (data not shown).

Several mutants that retained at least partial receptor-binding properties did not displace SHP-2 (Fig. 3B), while the mutant that had been shown to exhibit enhanced binding to SLAM (mutant 3) effectively blocked SHP-2 recruitment to the receptor. Therefore a number of mutations that retain SLAM binding properties may have altered abilities to displace SHP-2 and thereby regulate signaling through this receptor. Similar experiments were performed with CD84 (Fig. 4A). SHP-2 was also observed to bind to CD84 in a phosphorylation-dependent manner. However, titration of increasing amounts of SAP had no effect on the ability of SHP-2 to bind the receptor, even at levels that completely abolished binding of SHP-2 to SLAM (Fig. 4A). Therefore, SAP does not displace SHP-2 from the CD84 receptor cytoplasmic tail.

SHP-1, But Not SHP-2, Is Displaced by SAP from the Cytoplasmic Tail of CD84

SHP-1 is a protein tyrosine phosphatase with a very similar substrate specificity to SHP-2 (23), although these proteins are thought to play opposing roles in signal transduction. SHP-2 is postulated as positively regulating activation signals through a variety of growth factor receptors (24, 25). In contrast, SHP-1 has been implicated in inhibition of cytokine signaling (26–28). Because the consensus binding sites for these two molecules are very similar and closely resemble the TIYxxV/I binding site that has been defined for SAP, SHP-1 was examined for its ability to associate with the cytoplasmic tails of SLAM and CD84. In the case of SLAM, SHP-1 was able to weakly bind the phosphorylated receptor and SAP had no effect on its ability to bind SLAM (Fig. 4B). In contrast, SHP-1 was found to bind strongly to the cytoplasmic domain of phosphorylated CD84. Interestingly, SAP was found to efficiently displace SHP-1 from the CD84 cytoplasmic tail. Taken together with the SHP-2 binding studies (Figs. 4A and 4B), these data demonstrate that SAP can differentially regulate signal transduction events initiated by these two receptors.

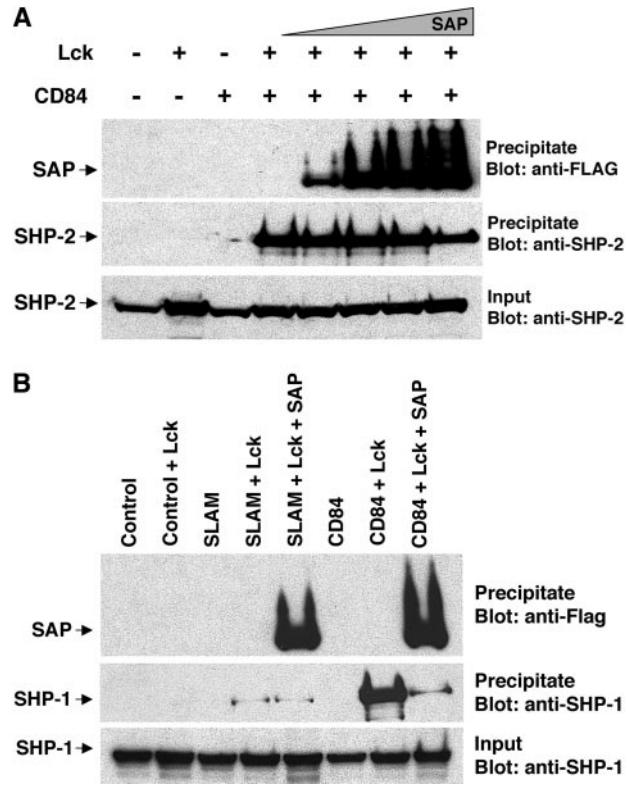

FIG. 4. SAP prevents association of SHP-1, but not SHP-2, with the cytoplasmic tail of CD84. (A) SAP binding to the cytoplasmic tail of CD84 does not inhibit SHP-2 recruitment to the receptor. Binding studies were performed as described in the legend to Fig. 3A, except that a GST-CD84 tail expression vector was transfected instead of GST-SLAM. (B) SAP prevents SHP-1 association with the cytoplasmic tail of CD84 but not SLAM. Human 293 cells were transfected with GST alone (control) or the GST-SLAM tail or the GST-CD84 tail along with Lck and FLAG-tagged SAP expression constructs where indicated. An expression vector for human SHP-1 was included in all samples. Binding studies were performed as described in the legend to Fig. 1. The top row was probed with a monoclonal anti-FLAG antibody and the bottom two rows were immunoblotted with a monoclonal antibody to SHP-1. Expression of GST fusion proteins was confirmed by immunoblotting with anti-GST antisera (data not shown).

DISCUSSION

The gene defective in XLP, termed SAP (DSHP/SH2D1A), encodes a 128-residue protein comprised of an SH2 domain and a short carboxy-terminus. Previous reports have revealed the ability of SAP to interact with a distinct element in the cytoplasmic tail of SLAM, a member of the CD2 subfamily of cell surface receptors that is expressed on the surface of B cells and T cells and is thought to play a role as an activation molecule in these cell types (7). SAP has also been reported to associate with 2B4, another member of the CD2 family, which is expressed on NK cells and cytotoxic T-lymphocytes (12). Although the mechanisms by which mutations in SAP result in the clinical manifestations of XLP are not entirely understood, recent studies have suggested that SAP might function normally as a negative regulator of signaling that may function, at least in part, by the competitive displacement of SH2 domain-containing proteins, such as SHP-1 and SHP-2, from their cognate motifs in the cytoplasmic domains of these receptors.

SLAM, 2B4, and CD84 are members of the CD2 subset of the immunoglobulin superfamily the other members of which include CD2, CD48, CD58, and human Ly-9 (29, 30). The genes encoding these proteins are clustered in a region on chromosome 1, suggesting

that these molecules may have arisen as a result of a gene duplication event (31). Although the members of this family are most homologous in their extracellular immunoglobulin repeats, SLAM, 2B4, and CD84 also contain short elements within their cytoplasmic domains that conform to the consensus SAP-binding motif TIYxxV/I. CD84, which is expressed predominantly on monocytes and platelets and at low levels on $CD3^+$ T cells, contains two potential SAP-binding domains (13). Therefore, SAP was examined for its ability to bind the cytoplasmic tail of CD84 in cells (Fig. 1A). In contrast to the interaction of SAP with SLAM, SAP was observed to bind to the cytoplasmic domain of CD84 in a manner that greatly increased upon phosphorylation of the receptor. Mutagenesis of the two candidate domains in CD84 suggested that SAP can bind independently to either domain (Fig. 1B), although the possibility that SAP binds elsewhere in the CD84 cytoplasmic tail cannot be formally excluded.

An unusual feature of the interaction between SLAM and the SH2 domain of SAP is that phosphorylation of the tyrosine residue in the consensus motif of the receptor is not required (7). Recent structural analysis of the SLAM–SAP interaction has revealed the importance of residues in SLAM amino terminal to the phosphotyrosine pocket that interact with SAP and are thought to stabilize the interaction in the absence of tyrosine phosphorylation (21, 22, 32). In this regard, an essential residue in SLAM is a leucine at the -3 position relative to the central tyrosine. This residue is not conserved among other members of the CD2 family and this may explain why SAP was observed to bind to SLAM in the absence of phosphorylation, but bound strongly to 2B4 and CD84 in the presence of the Lck tyrosine kinase (Fig. 1A).

Sequence analysis of the SAP coding sequence of seven unrelated patients revealed missense or nonsense mutations in five cases (Table 1). These data are consistent with other studies in the literature that have shown that 3 to 40% of XLP patients have no detectable SAP mutations (8, 9, 33, 34), which may be due to a number of possibilities, ranging from inaccurate clinical diagnosis to the potential presence of mutations in regulatory elements outside the open reading frame. Interestingly, four of the five mutations identified in the SAP coding sequence resulted in proteins that retained receptor binding ability (Figs. 2A and 2B), indicating that the mutations found in patients do not simply abrogate receptor binding, but may affect other functions of SAP. Since SAP has been shown to competitively displace SHP-2 from the cytoplasmic tail of SLAM, the patient-derived SAP variants were tested for an ability to displace SHP-2 from the cytoplasmic tail of SLAM. Interestingly, four mutants were unable to displace SHP-2 from SLAM, suggesting that in these cases the defect in SAP function is in its SHP-2 displacement property, rather than in its receptor binding capacity.

Examination of the recently described structure of SAP (21, 32) suggests that the mutations can be divided into two categories. One group, which includes the R32T mutation described here, is located within the central tyrosine binding pocket, and mutations in this category would therefore be predicted to disrupt association with the tyrosine residue. The second group, which includes P101L, Q99P, and T68I, is removed from the binding cleft. The nature of the defect in this group of XLP mutations is less clear, but one possible explanation is that these variants are compromised in their ability to displace SHP-1 or SHP-2. This might be due either to an altered affinity for the competing site on the receptor or to a reduced stability of the SAP protein, as has been previously postulated (21). Another possibility is that the mutations may disrupt interactions with other as yet unknown proteins. Consistent with this idea, SAP has recently been reported to bind $p62^{dok}$ (35).

Interestingly, overexpression of SAP did not result in the displacement of SHP-2 from the cytoplasmic domain of CD84. This observation might be explained either by a reversal of the relative affinities of SHP-2 and SAP, if they share the binding sites on CD84, or by the presence of a cognate site for SHP-2 that is distinct from the SAP-binding sites. In contrast to SHP-2, SHP-1 was effectively displaced from the CD84 receptor tail by SAP, which suggests that SAP may function to regulate different members of the CD2 receptor family by displacing distinct signaling components in a receptor-specific manner.

Recent studies have suggested a role for SAP in the control of EBV infection. Infection with EBV typically elicits a strong immunological response that includes a massive polyclonal expansion of peripheral B cells. In some cases, EBV^+ B-cell blasts can comprise up to 10% of circulating peripheral blood B cells (36). Control of this huge number of cells requires the concerted effort of cytotoxic T cells and natural killer cells. A defect in either of these arms of the immune system could lead to an imbalance resulting in disease. NK cell lines have been derived from two of the patients with defined mutations in their SAP gene presented in this study (15). In that report, the patients with mutation 6 (Arg55 to stop) and mutation 7 (delExon2) were studied. Despite the observation that NK cells from both of the XLP patients expressed normal levels of cell surface NK cell receptors, including 2B4, functional studies indicate that patients with XLP were found to have a defect in 2B4 receptor-mediated NK cell cytotoxicity. These results suggest that the defect in the cell signaling pathway occurs downstream of receptor–ligand binding.

The clinical presentation of XLP is complex and it is unclear how this defect in cell signaling contributes to the pathogenesis of disease. The XLP disease phenotype is thought to result primarily from a defect in NK and T cell function. Since SAP has been shown to bind to and regulate signaling differently through multiple members of the CD2 receptor family, it is possible that deregulation of signaling through each of these receptors may contribute in some way to disease progression. The development of infectious mononucleosis, the most common phenotype of XLP, as well as malignant lymphoma, may be explained by the defect in NK cell-mediated cytotoxicity resulting from aberrant signaling through the receptor 2B4. SLAM is expressed on both B and T cells and is thought to be an important mediator of B–T cell interactions. Signaling through SLAM promotes T-cell activation and results in the production of IFN-γ (37); however, the role of cytokine misregulation in XLP is not well understood. The data presented in this paper suggest that mutations in the SAP coding sequence that result in the expression of aberrant SAP protein may disrupt signaling events downstream of receptor–ligand binding. This may have a profound impact on cytotoxic T cell as well as T helper cell function and may contribute at least in part to the clinical syndrome of XLP. The function of CD84 is currently unknown. CD84 is expressed primarily by B cells, platelets, and monocytes; it is also expressed at low levels in certain subsets of T cells (13, 14). The observation that other members of the CD2 receptor superfamily, including CD2, SLAM, and 2B4, have been shown to function as costimulatory molecules in NK and T cells (38) suggests that CD84 may be playing a similar role in these cells.

In summary, these findings suggest that the defect in SAP function in a large proportion of XLP patients may not be in the absolute binding properties of the variant proteins to SAP cognate sites, but rather in the relative abilities of these variants to displace signaling molecules such as SHP-1 and SHP-2. Changes in displacement ability may be due either to altered affinity of the SAP protein for its cognate sites or to reduced stability of SAP, leading to a lower intracellular concentration. These results have implications for the understanding of the molecular basis of XLP and provide insight into the distinct roles played by SAP in homeostatic control of the immune repertoire.

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