Cutting Edge: Human 2B4, an Activating NK Cell Receptor, recruits the Protein Tyrosine Phosphatase SHP-2 and the Adaptor Signaling Protein SAP

Stuart G. Tangye, Sasha Lazetic, Erica Woollatt, Grant R. Sutherland, Lewis L. Lanier and Joseph H. Phillips

*J Immunol* 1999; 162:6981-6985; http://www.jimmunol.org/content/162/12/6981

**Why The *JI***?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

---

**References**  This article cites 21 articles, 9 of which you can access for free at: http://www.jimmunol.org/content/162/12/6981.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Cutting Edge: Human 2B4, an Activating NK Cell Receptor, Recruits the Protein Tyrosine Phosphatase SHP-2 and the Adaptor Signaling Protein SAP

Stuart G. Tangye,* Sasha Lazetic,* Erica Woollatt,† Grant R. Sutherland,‡ Lewis W. Lanier,* and Joseph H. Phillips3,*

The genetic defect in X-linked lymphoproliferative syndrome (XLP) is the Src homology 2 domain-containing protein SAP. SAP constitutively associates with the cell surface molecule, signaling lymphocytic activation molecule (SLAM), and competes with SH2-domain containing protein tyrosine phosphatase-2 (SHP-2) for recruitment to SLAM. SLAM exhibits homology with the mouse cell surface receptor 2B4. The human homologue of 2B4 has now been identified. It is recognized by the c1.7 mAb, a mAb capable of activating human NK cells. Human 2B4 became tyrosine phosphorylated following pervanadate-treatment of transfected cells and recruited SHP-2. SAP was also recruited to 2B4 in activated cells. Importantly, the 2B4-SAP interaction prevented the association between 2B4 and SHP-2. These results suggest that the phenotype of XLP may result from perturbed signaling not only through SLAM, but also other cell surface molecules that utilize SAP as a signaling adaptor protein. The Journal of Immunology, 1999, 162: 6981–6985.

Cell surface receptors regulate activation via the recruitment of different signaling molecules. Activating receptors (TCR, surface Ig, CD16, killer cell Ig-like receptors (KIR)) noncovalently associate with signal-transducing proteins (CD3ζ, Igαβ, FcεRIγ, DAP12) that possess immunoreceptor tyrosine-based activation motifs (D/ExxYxxL/I-X-6–8-YxxL/I) that, once phosphorylated, can recruit Syk and ZAP-70 (1–3). The cytoplasmic domains of inhibitory receptors (KIR, CD22, FcεRIβ) contain immunoreceptor tyrosine-based inhibitory motifs (I/VxYxxL/I-VxYxxL/I-VxYxxL/I) that recruit SH2-domain containing protein tyrosine phosphatase (SHP)-1, SHP-2, and SHIP phosphatases (3–6).

Mouse (m) 2B4 is an Ig superfamily (IgSF) molecule capable of activating T and NK cells (7, 8). m2B4 exhibits homology to the adhesion molecules Ly9, CD48, CD58, and signaling lymphocytic activation molecule (SLAM; 8–10). A curious feature of m2B4 is the presence of four tyrosine-based motifs (TxYxxL/I) in its cytoplasmic domain (8). This motif is also present in SLAM (9) and may be involved in the recruitment of SHP-2, as well as the association between SLAM and SLAM-associated protein (SAP) (11), the defective protein in the inherited immunodeficiency XLP (11–13). We have cloned human (h) 2B4 and investigated its role as a signaling molecule.

Materials and Methods

Cloning Human 2B4 and Human SAP

Based on sequence information obtained from a partial cDNA with homology to m2B4 (Human Genome Sciences, Rockville, MD), a human spleen cDNA library (OriGene Technologies, Rockville, MD) was screened by PCR using as primers: 5′, GGTGATCATCGTGATTCTAGCCTGAGTCGACAGGC; and 3′, AGAACCTGCCAGCCAGGGATC. The open reading frame of h2B4, minus the leader sequence, was amplified using: 5′, GCATCGATGCGCAAAGGATGCAAGGGATC (Clal site in italics); 3′, GCATGCGCGGCGGCCGGAATGTGCGACAATAGG (NotI). The amplified product was digested with Clal/NotI and cloned into pMX-neo downstream of the Flag epitope and the CD8 leader sequence. hSAP was amplified from NK cell cDNA using: 5′, GAAAGAGGATCCGCCCATGGAGCAGTGCCG (BamHI); 3′, GCATTAGATATTCTGGGCGTTCTCGAC; (EcoRI).

The amplified product was digested with BamHI/EcoRI and subcloned into pMX-puro upstream of an in-frame sequence encoding the c-myc epitope. PCR conditions were 30 cycles of 1 min denaturation (94°C), 1 min annealing (55°C), and 45 s (SAP) or 1 min (2B4) extension (72°C).

Transfection

pMX-based constructs were packaged using the Phoenix cell line (14) and virus used to infect the mouse pre-B cell line, BaF3 (2). Infected BaF3 cells were drug-selected; h2B4 transfectants were isolated by cell sorting.
Expression of h2B4 on the transfected cells was assessed by flow cytometry using anti-Flag (M2; Sigma, St. Louis, MO) or c1.7 mAb (Coulter, Hialeah, FL) (2).

Biochemical characterization of h2B4
Transfected BaF3 cells expressing Flag-h2B4 or Flag-h2B4 and SAP-myc were untreated or treated with 100 μM sodium pervanadate for 5 min at room temperature and then solubilized in lysis buffer (10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, and enzyme inhibitors) (2). Flag-h2B4, SAP-myc, and SHP-2 were precipitated from lysates using anti-Flag, anti-myc (9E10; Upstate Biotechnology, Lake Placid, NY), or anti-SHP-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) absorbed onto protein G or A beads (Pharmacia, Hercules, CA), electrophoresed under reducing conditions through SDS-polyacrylamide gels (Bio-Rad, Richmond, CA), and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed with HRP-anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-Flag (for Flag-h2B4), anti-myc (for SAP-myc), or rabbit anti-SHP-2 Abs. Bound Abs were detected with HRP-donkey anti-rabbit IgG and anti-mouse IgG antiserum (Amersham, Arlington Heights, IL). For biochemical characterization, cells were biotinylated or I125-labeled, lysed, and precipitated with anti-Flag or c1.7 mAb (2). Precipitates were untreated or digested with neuraminidase, O-glycosidase and/or N-glycanase. Precipitated proteins were analyzed by SDS-PAGE and Western blotting using HRP-streptavidin (Amersham), and membranes were developed using enhanced chemiluminescence (Pierce, Rockford, IL) or autoradiography.

Results and Discussion
Cloning h2B4
Human 2B4 cDNA is 2308 bp, containing a 1098-bp open-reading frame encoding a type 1 transmembrane protein, and a 1210-bp 3'-untranslated region. The mature peptide consists of a 20-aa leader sequence, 201-aa extracellular domain, 24-aa transmembrane region, and 120-aa cytoplasmic domain (Fig. 1). Like m2B4, h2B4 is an IgSF molecule, comprised of an N-terminal V-set Ig domain and a membrane-proximal C2-set Ig domain. h2B4 exhibits ~66% identity to m2B4 (Fig. 1). The cytoplasmic domain of h2B4 contains four TxYxxV/I motifs that aligned with identical motifs present in m2B4 (Fig. 1). h2B4 also exhibits homology with SLAM, hCD48, hLy9, and CD84 (20–35%; data not shown). The h2B4 gene was localized to chromosome 1q22 (data not shown). Several other IgSF molecules also map to human chromosome 1q22–24, including SLAM (15), CD84 (16), CD48 (17), and Ly9 (18).

Biochemical characterization of h2B4
Immunoprecipitation revealed that Flag-h2B4 was ~86 kDa (Fig. 2). There are eight potential N-linked glycosylation sites in the extracellular domain of h2B4 (Fig. 1) and the predicted core size is ~40 kDa. Treatment with neuraminidase and O-glycosidase resulted in a slight reduction in migration (~80 kDa). Following treatment with N-glycanase, the m.w. of Flag-h2B4 was reduced to ~50 kDa. Removal of both N- and O-linked sugars resulted in the protein migrating as ~48 kDa (Fig. 2). m2B4 is 66 kDa, significantly less that h2B4. This difference is likely due to differential glycosylation because the core sizes of m2B4 and h2B4 are similar (8).

Human 2B4 is recognized by the c1.7 mAb
m2B4 is expressed on all NK and some T cells, and 2B4+ cells mediate non-MHC-restricted cytotoxicity (7). The c1.7 mAb reacts some 1q22–24, including SLAM (15), CD84 (16), CD48 (17), and Ly9 (18).

FIGURE 1. Alignment of human and mouse 2B4. Leader and transmembrane domains are underlined. N-Linked glycosylation sites are indicated (●). Tyrosine-based signaling motifs are boxed. Conserved cysteine residues are shown in bold type. Spaces (-) have been included in the alignment to allow optimal comparison between m2B4 and h2B4. The nucleotide sequence of h2B4 has been deposited as GenBank accession number AF 145782.

FIGURE 2. Biochemical characterization of human 2B4. Flag-h2B4 was precipitated with anti-Flag mAb from biotinylated Flag-h2B4 BaF3 cells. Precipitates were untreated, or treated as indicated and analyzed as described in Materials and Methods.
Human 2B4 is phosphorylated following activation and recruits SHP-2

A tyrosine-phosphorylated protein of ~86 kDa could be detected in anti-Flag mAb precipitates of pervanadate-treated, but not untreated, Flag-h2B4 BaF3 cells lysates. Reprobing these blots with anti-Flag mAb revealed that this phosphoprotein is Flag-h2B4 (Fig. 4, a and b). An unknown phosphoprotein of ~25 kDa (pp25) was also present in anti-Flag mAb precipitates of stimulated cells (Fig. 4a). The tyrosine-based motifs in the cytoplasmic domain of h2B4 are similar to motifs that recruit SH2 domain-containing phosphatases (3). Therefore, the anti-Flag mAb precipitates were assessed for the presence of SHP-1 and SHP-2. Phosphorylated, but not unphosphorylated, Flag-h2B4 recruited SHP-2 (Fig. 4c). This association was specific for SHP-2 because SHP-1 was not recruited to phosphorylated Flag-h2B4 (data not shown).

Phosphorylated h2B4 also recruits SAP

The cytoplasmic domain of SLAM constitutively associates with the adaptor protein SAP, and SAP competes with SHP-2 for binding to phosphorylated SLAM (11). Due to the homology between SLAM and h2B4, we generated BaF3 cells expressing Flag-h2B4 and SAP-myc to test whether h2B4 also interacts with SAP. Flag-h2B4 was phosphorylated in the absence or presence of SAP-myc following pervanadate treatment (Fig. 4a). In Flag-h2B4/SAP-myc transfectants, phosphorylated Flag-h2B4 recruited not only SHP-2 (Fig. 4c) but also SAP-myc, as evidenced by an ~20-kDa protein reactive with anti-myc mAb (Fig. 4d). In contrast to SLAM (11), SAP-myc did not appear to constitutively associate with Flag-h2B4, as shown by the absence of SAP-myc in anti-Flag mAb precipitates of unstimulated transfectants (Fig. 4d). Thus, the h2B4-SAP interaction was phosphorylation-dependent. To determine whether both SHP-2 and SAP-myc could simultaneously bind the same Flag-h2B4 molecule, Flag-h2B4/SAP-myc BaF3 cell lysates were precipitated with anti-Flag, anti-myc, or anti-SHP-2 Ab. Anti-Flag mAb precipitates from stimulated cells contained phosphorylated Flag-h2B4, SHP-2, and SAP-myc (Fig. 5). In contrast, SHP-2 was absent from anti-myc mAb precipitates (Figs. 5c). Similarly, SAP-myc was not present in anti-SHP-2 precipitates (Fig. 5d). Thus, Flag-h2B4, SAP-myc, and SHP-2 do not form a trimolecular complex. Rather, following phosphorylation, Flag-h2B4 can recruit either SAP-myc or SHP-2. The presence of both SHP-2 and SAP-myc in anti-Flag mAb precipitates is likely due to limiting amounts of SAP-myc that cannot completely prevent binding of SHP-2 to overexpressed Flag-h2B4. The absence of SHP-2 and SAP-myc from anti-myc and anti-SHP-2 precipitates, respectively, suggests that, similar to SLAM, SAP-2, and SAP-myc may interact with the same tyrosine-based motif present in Flag-h2B4.

h2B4 is a novel glycoprotein recognized by a mAb that activates human NK and CD8+ T cells. Following phosphorylation, h2B4 recruited SHP-2 and SAP. Importantly, binding of SAP to h2B4 prevented its association with SHP-2. If the signals delivered by h2B4 and SLAM are mediated via SHP-2 (21) the recruitment of SAP could regulate this response by displacing SHP-2 from h2B4 and SLAM. In XLP, such regulation is absent. It is possible that NK and CD8+ T cells from XLP patients would exhibit altered signaling through h2B4. This may contribute to the dysregulated activation of cytotoxic lymphocytes that is characteristic of this immunodeficiency (22). Although SAP was identified by its ability to associate with SLAM (11), XLP may result from an inability to efficiently regulate signals delivered through other cell surface molecules that also utilize SAP as a signaling adaptor protein.
Acknowledgments

We thank Dr. Jim Cupp, Eleni Callas, and Dixie Pollakoff for cell sorting; Debbie Liggett for oligonucleotide synthesis; Dan Gorman and the DNAX sequencing facility for DNA sequencing; Garry Nolan (Stanford University) for providing Phoenix cells; and Maribel Andonian and Gary Burget for graphics.

References


pathways of the killer cell inhibitory receptor and FcRIIB1 require distinct phosphatases. J. Exp. Med. 186:473.