The Journal of Immunology

BRIEF REPORT | JUNE 15 1999

Cutting Edge: Human 2B4, an Activating NK Cell Receptor, Recruits the Protein Tyrosine Phosphatase SHP-2 and the Adaptor Signaling Protein SAP[1]

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J Immunol (1999) 162 (12): 6981–6985.
https://doi.org/10.4049/jimmunol.162.12.6981

Cutting Edge: Human 2B4, an Activating NK Cell Receptor, Recruits the Protein Tyrosine Phosphatase SHP-2 and the Adaptor Signaling Protein SAP[1]

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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)[3]) noncovalently associate with signal-transducing proteins (CD3ζ, Ig$\alpha\beta$, 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, FcRγIIb) contain immunoreceptor tyrosine-based inhibitory motifs (I/VxYxxL/V-$_{26-31}$-I/VxYxxL/V) 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 (TxYxxV/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′, GGTGATCATCGTGATTCTA AGCGC; and 3′, AGAACCTGCCAGCCAGTTCAC. The open reading frame of h2B4, minus the leader sequence, was amplified using: 5′, GCAT GC*ATCGAT*GGCAAAGGATGCCAGGGATC (*Cla*I site in italics); 3′, GCATGC*GCGGCC*GCGAGAATTGCTGCAGCAACTAGG (*Not*I).

The amplified product was digested with *Cla*I/*Not*I and cloned into pMX-neo downstream of the Flag epitope and the CD8 leader sequence. hSAP was amplified from NK cell cDNA using: 5′, GAAGAA*GGATC C*GCCATGGACGCAGTGGCTG (*Bam*HI); 3′, GCATTA*GAATTC*T GGGGCTTTCAGGCAGACATC (*Eco*RI).

The amplified product was digested with *Bam*HI/*Eco*RI 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.

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Received for publication March 2, 1999. Accepted for publication April 20, 1999.

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[1] This work is supported by grants from the J.H. & J.D. Gunn Medical Research Foundation, and National Health and Medical Research Council of Australia (to E.W. and G.R.S.). DNAX is supported by the Schering-Plough Corporation.

[2] Address correspondence and reprint requests to Dr. Joseph Phillips, DNAX Research Institute, 901 California Ave, Palo Alto, CA 94304. E-mail address: phillips@dnax.org

[3] Abbreviations used in this paper: KIR, killer cell Ig-like receptors; aa, amino acid; SLAM, signaling lymphocytic activation molecule; SAP, SLAM-associated protein; SHP-2, SH2-domain containing protein tyrosine phosphatase-2; XLP, X-linked lymphoproliferative syndrome; IgSF, Ig superfamily; h, human; m, mouse

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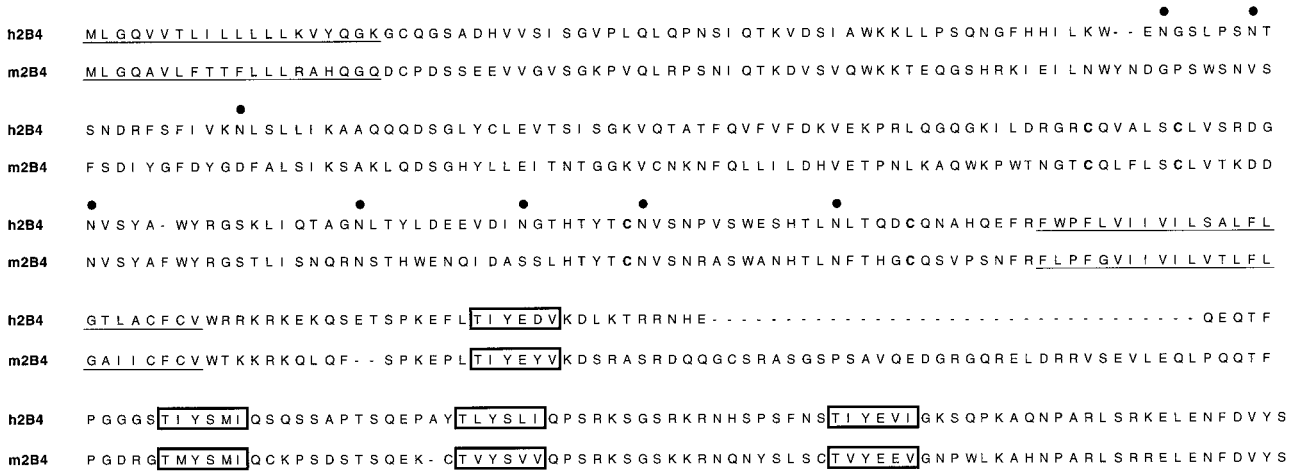

FIGURE 1. Alignment of human and mouse 2B4. Leader and transmembrane domains are underlined. *N*-linked glycosylation sites are indicated (●). Tyrosine-based signaling motifs are boxed. Conserved cysteine residues are shown in bold type. Spaces (-) have been included in the alignment to allow optimal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

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 I^{125}-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

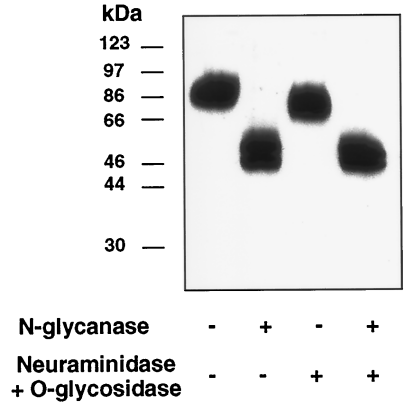

FIGURE 2. Biochemical characterization of human 2B4. Flag-h2B4 was precipitated with anti-Flag mAb from biotinylated Flag-h2B4 BaF3 cells. Precipitates were untreated, or treated as indicated and analyzed as described in *Materials and Methods*.

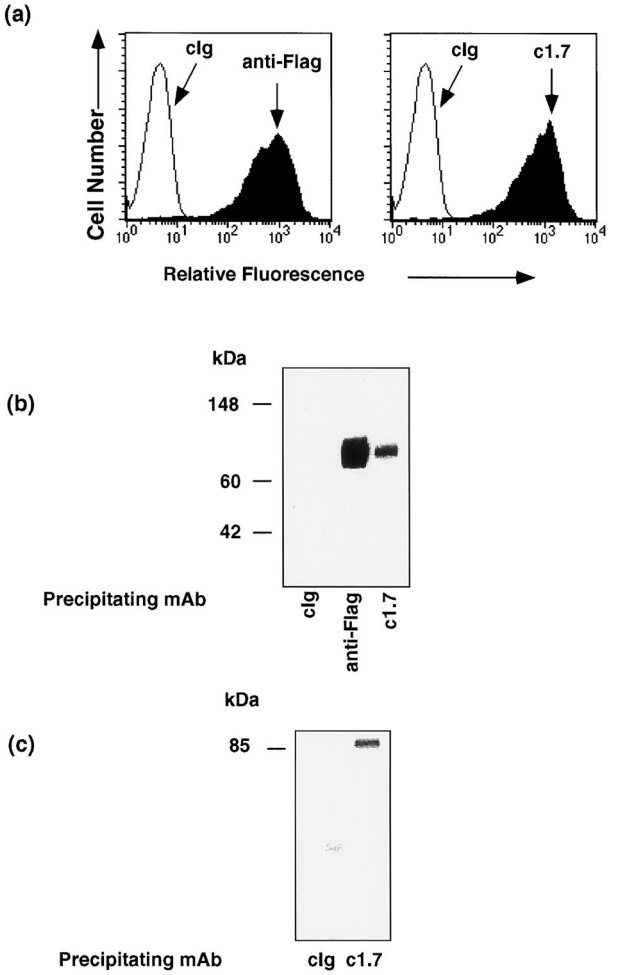

FIGURE 3. Human 2B4 is recognized by the c1.7 mAb. *a*, Flag-h2B4 BaF3 cells were incubated with either anti-Flag mAb or c1.7 mAb or with an isotype control mAb (cIg), followed by PE-anti-mouse Ig antiserum. *b*, Flag-h2B4 BaF3 cells were precipitated with control Ig (cIg), anti-Flag mAb, or c1.7 mAb, and analyzed as described for Fig. 2. *c*, The human NKL cell line was labeled with I^{125}, lysed, and precipitated with control Ig (cIg) or c1.7 mAb.

with all human NK cells and ~50% of $CD8^+$ T cells; $c1.7^+$ cells mediate non-MHC-restricted killing by NK and T cells (19). Therefore, we tested whether c1.7 recognized h2B4. Both anti-Flag and c1.7 mAb reacted with Flag-h2B4 BaF3 transfectants (Fig. 3*a*). The molecule recognized by c1.7 was reported to be 38 kDa (19), while Flag-h2B4 was ~86 kDa. To address this discrepancy, Flag-h2B4 was precipitated from Flag-h2B4 BaF3 cells with anti-Flag or c1.7 mAb. Both mAb precipitated a protein of ~86 kDa (Fig. 3*b*). c1.7 mAb also precipitated an ~86 kDa protein from I^{125}-labeled human NKL cells, confirming that the m.w. of native h2B4 is ~86 kDa (Fig. 3*c*). c1.7 mAb is not efficient at precipitating h2B4 (Fig. 3*b*), precluding extensive biochemical analysis of h2B4 from normal human cells. However, we could confirm that ligating h2B4 on NK cells induced killing of FcR-bearing target cells in a redirected cytotoxicity assay, demonstrating that h2B4 is an activating molecule (data not shown). Thus, identifying h2B4 as the molecule recognized by c1.7 mAb revealed that the biological function of 2B4 is conserved across species. An inhibitory isoform of mouse 2B4 has recently been described (20). It is presently unknown whether such an isoform of h2B4 exists.

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 cell 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. 4*a*). 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. 4*c*). 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. 4*a*). In Flag-h2B4/SAP-*myc* transfectants, phosphorylated Flag-h2B4 recruited not only SHP-2 (Fig. 4*c*) but also SAP-*myc*, as evidenced by an ~20-kDa protein reactive with anti-*myc* mAb (Fig. 4*d*). 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. 4*d*). 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. 5*c*). Similarly, SAP-*myc* was not present in anti-SHP-2 precipitates (Fig. 5*d*). Thus, Flag-h2B4, SHP-2, and SAP-*myc* 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, SHP-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.

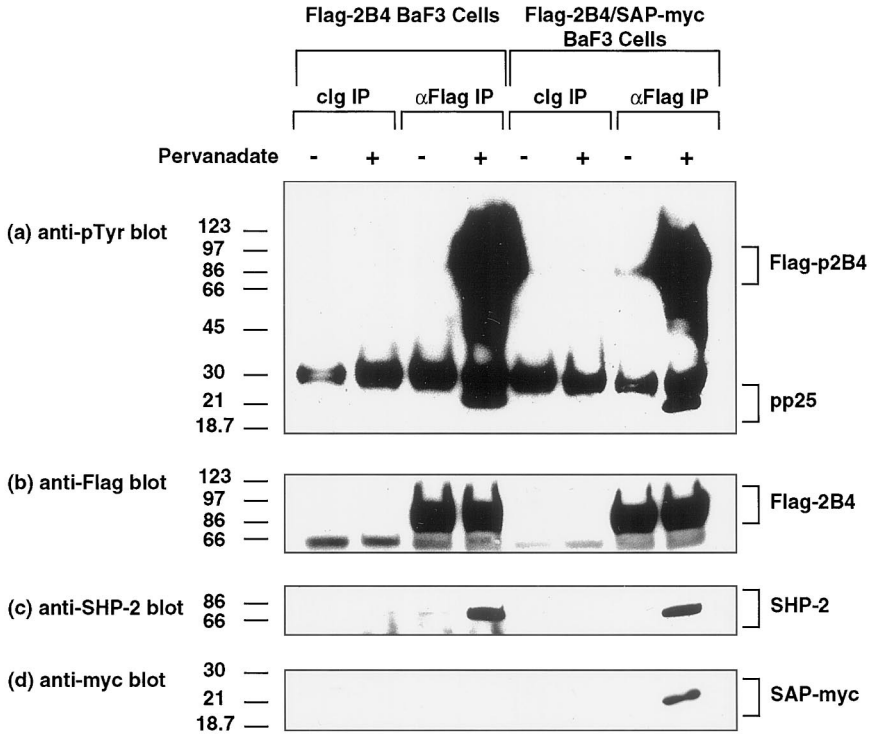

FIGURE 4. Human 2B4 is phosphorylated following activation and recruits SHP-2 and SAP. Flag-h2B4 BaF3 and Flag-h2B4/SAP-*myc* BaF3 cells were either unstimulated (−) or stimulated (+) for 5 min with sodium pervanadate, then lysed in 1% Nonidet P-40 lysis buffer. Lysates were precipitated with control Ig or anti-Flag mAb. Precipitated proteins were analyzed by SDS-PAGE and Western blot analysis using Ab specific for: *a*, proteins phosphorylated on tyrosines (anti-pTyr); *b*, Flag-h2B4 (anti-Flag); *c*, SHP-2 (anti-SHP-2); and *d*, SAP-*myc* (anti-*myc*). p2B4, phosphorylated Flag-h2B4.

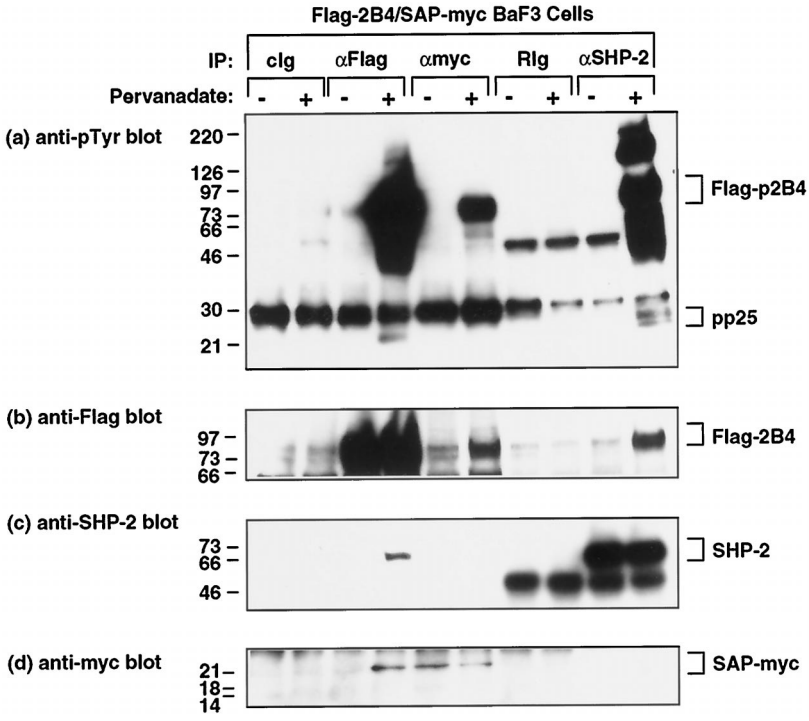

FIGURE 5. Association of SAP with h2B4 prevents recruitment of SHP-2. Flag-h2B4/SAP-*myc* BaF3 cells were either unstimulated (−) or stimulated (+) for 5 min with sodium pervanadate, then lysed in 1% Nonidet P-40 lysis buffer. Flag-h2B4, SAP-*myc*, or SHP-2 were precipitated with specific Ab; precipitated proteins were analyzed as described for Fig. 4.

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.

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