The X-linked lymphoproliferative syndrome gene product SH2D1A associates with p62^{dok} (Dok1) and activates NF-κB

Bakary S. Sylla*†, Kerry Murphy*, Ellen Cahir-McFarland*, William S. Lane‡, George Mosialos*§, and Elliott Kieff*¶

*Departments of Medicine and Microbiology and Molecular Genetics, Brigham and Women's Hospital and Harvard University, 181 Longwood Avenue, Boston, MA 02115; and ‡Microchemistry Facility, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138

Contributed by Elliott Kieff, April 28, 2000

The X-linked lymphoproliferative syndrome (XLP) is a genetic disorder in which affected males have a morbid or fatal response to Epstein–Barr virus infection. The XLP deficiency has been mapped to a gene encoding a 128-residue protein, SH2D1A, which is comprised principally of a Src homology 2 (SH2) domain. We now report that SH2D1A associates with Dok1, a protein that interacts with Ras-GAP, Csk, and Nck. An SH2D1A SH2 domain mutant that has been identified in XLP does not associate with Dok1, in accord with the hypothesis that this interaction is linked to XLP. The association of SH2D1A with Dok1 also depends on phosphorylation of Dok1 Y_{449} in the sequence ALYSQVQK. Further, overexpression of SH2D1A is found to activate NF-κB in 293T cells. NF-κB activation by SH2D1A does not depend on the wild-type SH2 domain and is inhibited by a dominant-negative IκB kinase β. Thus, SH2D1A can affect multiple intracellular signaling pathways that are potentially important in the normal effective host response to Epstein–Barr virus infection.

Epstein–Barr virus | genetic disease

Epstein–Barr virus (EBV) is a herpes virus that infects most people early in life and usually causes mild illness. Infection after early childhood frequently results in infectious mononucleosis, a systemic illness that is caused by the proliferation of EBV-infected B lymphocytes and unusually strong natural killer (NK) and virus-specific T lymphocyte responses (1). The high prevalence of infected B lymphocytes in primary infection is caused by EBV-encoded proteins that cause proliferation of latently infected B lymphocytes. These EBV-encoded proteins engender the strong T lymphocyte responses. In individuals with severe inherited or acquired deficiencies in T lymphocyte responses, EBV-infected B lymphocytes can proliferate without immune control and cause a fatal lymphoproliferative disease.

The X-linked lymphoproliferative syndrome (XLP) is a disease of rare kindreds in which affected males are susceptible to morbid or fatal outcomes after EBV infection. Those individuals who survive the fulminate hepatitis or lymphoproliferative disease that may accompany primary EBV infection frequently develop agammaglobulinemia or B cell lymphoma (2). Importantly, the affected males are not unusual in their response to other infections, including other herpes virus infections. Further, B lymphocytes from these patients do not seem to be unusual in their response to EBV infection *in vitro* and NK or T lymphocyte functional abnormalities have not been demonstrable except after EBV infection.

The XLP gene recently was identified by the finding of mutations in an ORF in the sequence of a DNA segment mapped through sequential genetic analyses of affected kindreds (3, 4). A yeast two-hybrid screen for proteins that can interact with the cytoplasmic domain of the surface lymphocyte adhesion molecule (SLAM) receptor identified the same ORF (5). In normal lymphoid tissues, SH2D1A is expressed in both B and T cell areas, including the thymus (4). SH2D1A RNA is expressed at high levels in T cell lines (3–5) and at low levels in EBV-transformed B cell lines (4). Expression also has been detected in Hodgkin's and non-Hodgkin's lymphomas (4). The ORF is predicted to encode a protein of 128 amino acids (SH2D1A/SAP/DSHP), most of which comprises a Src homology 2 (SH2) domain (3–5). The SH2D1A SH2 domain binds to nonphosphorylated SLAM and binding is increased by tyrosine phosphorylation (5). Alterations in the DNA sequence that are predicted to result in an abnormal protein or abnormal protein expression have been found in $\approx$70% of XLP families (3–6). SH2D1A also has been found to be mutated in isolated cases of non-Hodgkin's lymphoma (7).

Given the unique responses of XLP patients to EBV infection, resulting in exaggerated proliferation of EBV-infected B lymphocytes or severe hepatic inflammation and necrosis, XLP is likely to affect a critical aspect of the NK or T lymphocyte response to EBV-infected B lymphocytes. The SH2D1A SH2 domain is similar to the SH2 domains containing protein tyrosine phosphatase 2 (SHP-2) and inositol phosphatase (SHIP). SH2D1A competes with SHP-2 in binding to the SLAM receptor and overexpression of SH2DIA in Jurkat cells slightly upregulates the activity of a cotransfected IL-2 promoter, consistent with the notion that SH2D1A can have a positive effect on phosphotyrosine (p-Y)-mediated signaling in T lymphocytes (5). Similarly, SH2D1A competes with SHP-2 for binding to h2B4, another member of the SLAM family expressed in NK and CD8+ T cells (8). Inhibition of SHIP would be expected to have a positive effect on inositol 5-phosphate-mediated signaling (9). Thus, the failure of XLP patients to contain the proliferation of EBV-infected B lymphocytes in primary EBV infection without significant morbidity or mortality could be the result of impaired signal transduction from SLAM, h2B4 (5, 8), or other cell receptors with abnormal cell responses and cytokine release. To gain further insight into the role of SH2D1A in the XLP syndrome, we sought to identify additional pathways that can be affected by SH2D1A.

Materials and Methods

Plasmid Construction. The SH2D1A expressed sequence tag (GenBank accession no. N89899) was obtained from Genome Sys-

Abbreviations: XLP, X-linked lymphoproliferative syndrome; SH2, Src homology 2; EBV, Epstein–Barr virus; NK, natural killer; SLAM, surface lymphocyte adhesion molecule; SHP, SH2 domain containing protein tyrosine phosphatase; GST, glutathione *S*-transferase; PH, pleckstrin homology; GAP, GTPase-activating protein; p-Y, phosphotyrosine; F, phenylalanine; IKKβ, IκB kinase β.

†Present address: International Agency for Research on Cancer, F-69372 Lyon, France.

§Present address: Biomedical Sciences Research Center ''Al. Fleming,'' 14–16 Al. Fleming Street, Vari 16672, Greece.

¶To whom reprint requests should be addressed. E-mail: ekieff@rics.bwh.harvard.edu.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.130193097. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.130193097

tems (St. Louis). A prokaryotic expression plasmid for the glutathione *S*-transferase (GST)-SH2D1A fusion protein was made by subcloning the entire ORF from the SH2D1A cDNA into pGEX-2TK (Amersham Pharmacia) (10). The GST-SH2D1A-R32T mutant was created by PCR and subcloned into pGEX-5X2. GST and GST fusion proteins were expressed in *Escherichia coli* and purified on glutathione-agarose beads. pEBG-GST-SH2D1A and pEBG-GST-SH2D1A-R32T are eukaryotic pEBG-based expression vectors (11). pcDNA3-FLAG-SH2D1A is the SH2D1A ORF with a FLAG epitope replacing the SH2D1A initiation codon in pcDNA3-FLAG (12). FLAG-tagged mutants of SH2D1A, pcDNA3-FLAG-SH2D1A-R32T, pcDNA3-FLAG-SH2D1A-P101L, and pcDNA3-FLAG-SH2D1A-C12 with an extension of 12 amino acids at the C terminus (3) also are in pcDNA3-FLAG. The wild-type human cDNA of $p62^{dok}$ (referred to hereafter as Dok1) (13) was obtained from N. Carpino and R. Kobayashi (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Epitope-tagged Dok1 and Dok1 variants bearing specific mutations or deletions were subcloned into pcDNA3-FLAG. pcDNA3-FLAG-Dok1 encodes wild-type Dok1 with a FLAG epitope at the N terminus of Dok1. pcDNA3-FLAG-Dok1Y449F encodes F instead of Y at residue 449. pcDNA3-Dok1-ΔPH lacks the N-terminal 123 amino acids of Dok1, including the pleckstrin homology (PH) domain. pcDNA3-FLAG-Dok1-ΔC35 has a deletion of the C-terminal 35 amino acids. All mutations and deletions were confirmed by DNA sequencing. The human HA-$p56^{dok-2}$ (14) (referred to hereafter as Dok2) was obtained from M. D. Resh (Sloan–Kettering Cancer Center, New York). The human cDNA of FLAG-Dock180 (15) was obtained from M. Matsuda (National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Japan). The cDNA of human Dock180 similar (KIAA0209; referred to hereafter as Dock2) (16) was obtained from T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). pcDNA3-FLAG-Dock2 is Dock2 in pcDNA3-FLAG.

Cell Lines, Transfections, and NF-κB Reporter Gene Assays. BJAB (a Burkitt's lymphoma cell line) stably transfected with pcDNA3-FLAG-SH2D1A, and Jurkat SVT3 (a human T cell line expressing simian virus 40 large T antigen) were maintained in RPMI medium 1640 supplemented with 10% FBS (R10). Cos7 (simian fibroblast) and HEK 293T (adenovirus E1a- and E1b-transformed human embryonic kidney) cell lines expressing simian virus 40 large T antigen were maintained in DMEM containing 10% FBS (D10). Plasmid DNA was introduced into cells by electroporation or by Superfect (Qiagen, Chatsworth, CA) (12). NF-κB luciferase reporter gene assays were performed as described previously (12).

Antibodies. Anti-FLAG M5 mouse monoclonal antibodies and anti-FLAG M2 beads were obtained from Sigma. Polyclonal rabbit anti-Dok1 (13) was a gift of N. Carpino and R. Kobayashi. Polyclonal rabbit antibodies against ZAP70, SAM 68, Ras-GAP; mouse monoclonal antibodies against Lck, Syk, Fyn, and Lyn; and goat antibodies against Ku70 and Ku86 were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-SHP-2 was from Transduction Laboratories (Lexington, KY). Monoclonal mouse anti-STAM was a gift of M. Higuchi (Harvard University), and 4G10 anti-p-Y monoclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-Dock180 was obtained from M. Matsuda. Polyclonal rabbit anti-Eps15R (17) was obtained from P. P. Di Fiore (European Institute of Oncology, Milan, Italy).

Immunoprecipitations, GST Pull-Down Assays, and Western Blotting. For pervanadate stimulation, 1×10^7 or 2×10^9 cells were washed twice with warm PBS and the cells were treated with 200 μM sodium pervanadate for 10 min. Cells were lysed in cell lysis buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/2 mM EDTA/1% Nonidet P-40/3% glycerol/1 mM PMSF/5 μg/ml leupeptin/2 μg/ml aprotinin/50 μg/ml soybean trypsin inhibitor/1 mM NaF/1 mM sodium orthovanadate) for 30 min on ice. Insoluble material was removed by centrifugation at $14{,}000 \times g$ for 15 min. Lysates were precleared with either Sepharose CL-6B or protein G-Sepharose for 30 min. The cleared cell lysates were immunoprecipitated with the indicated antibodies and either protein A- or G-Sepharose or were subjected to pull-down assays with glutathione-agarose-immobilized GST or GST fusion proteins at 4°C for 2 h. The precipitates were washed five times with lysis buffer and analyzed as described previously (12).

Preparative Protein Isolation and Mass Spectrometric Peptide Sequencing. Lysates from 2×10^9 Jurkat T cells were subjected to a pull-down with glutathione-agarose-immobilized GST or GST fusion proteins as described above, and protein precipitates were resolved by preparative SDS/PAGE. Protein adherent to GST-SH2D1A but not to GST were visualized by Coomassie brilliant blue, excised, and subjected to in-gel reduction, carboxyamidomethylation, and tryptic digestion. Multiple peptide sequences were determined by microcapillary reverse-phase chromatography coupled to a LCQ quadrapole ion mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with a nanoelectrospray source. The ion trap was programmed to successively acquire three scan modes: full scan MS over alternate ranges of 395–800 m/z or 800–1,300 m/z, followed by two data-dependent scans on the most abundant ion in those full scans. The data-dependent scans allowed the automatic acquisition of a high-resolution scan to determine charge and exact mass and MS/MS spectra for low femtomole sequence information. MS/MS spectra were with a relative collision energy of 30%, an isolation width of 2.5 Da, and a recurring ion dynamic exclusion. Interpretation was done by using programs of the Microchemistry facility and SEQUEST developed by J. K. Eng, A. L. McCormick, and J. R. Yates, III (University of Washington, Seattle).

Results

Multiple p-Y-Containing Proteins Bind to SH2D1A. To identify p-Y proteins that specifically bind to SH2D1A (9, 18) a bacterially expressed GST-SH2D1A fusion protein was used to affinity-purify proteins from Jurkat or BJAB cell lysates. Cells were treated with pervanadate to achieve a higher level of steady-state p-Y, and the 4G10 p-Y-specific monoclonal antibody was used to detect the proteins on immune blots. After pervanadate treatment, several p-Y-containing Jurkat proteins bound to GST-SH2D1A, but not to GST (Fig. 1*A*, compare lanes 5 and 6). The most prominent p-Y-containing protein that interacted specifically with GST-SH2D1A was a protein at about 65 kDa; other specific p-Y-containing proteins ranged from 15–180 kDa. These GST-SH2D1A-interacting p-Y proteins were not apparent in extracts from cells that were not treated with pervanadate (Fig. 1*A*). The protein slightly larger than the 62-kDa marker was the most abundant p-Y protein in BJAB and Jurkat cell lysates that specifically bound to SH2D1A (Fig. 1*B*, lanes 2 and 6). Furthermore, this and several other p-Y-containing proteins bound much less efficiently to bacterially expressed SH2D1A-R32T, which has T in place of the key R at position 32 in the SH2D1A "FLVRES" sequence (refs. 3 and 5 and Fig. 1*B*, lanes 3 and 7).

Dok1 Binds to SH2D1A *in Vitro*. We attempted to identify the p-Y protein slightly larger than 62 kDa that binds to SH2D1A by immunoblot with antibodies against similarly sized p-Y proteins that are involved in lymphocyte signaling. Antibodies to SLP76, Lck, ZAP70, STAM, SHP-2, or c-Src tyrosine kinase family members failed to identify the protein that was specifically enriched in the wild-type SH2D1A pull-down (data not shown). However, an antibody against Dok1 reacted specifically with the

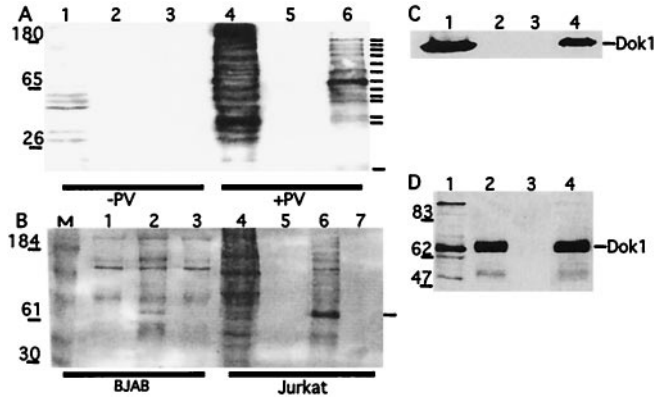

Fig. 1. Multiple p-Y proteins including Dok 1 bind specifically to wild-type SH2D1A *in vitro*. (*A*) Lysates from Jurkat T cells treated or not treated with pervanadate were incubated with GST (lanes 2 and 5) or with GST-SH2D1A fusion protein (lanes 3 and 6) bound to glutathione-Sepharose. Bound proteins were subjected to Western blot analysis with a p-Y-specific antibody. Arrows indicate major p-Y-containing proteins. The cytoplasmic cell lysates (1%) are in lanes 1 and 4. (*B*) Lysates from BJAB B and Jurkat T cells treated with pervanadate were incubated with glutathione-Sepharose-bound GST (lanes 1 and 5), -GST-SH2D1A (lanes 2 and 6), or -GST-SH2D1A-R32T (lanes 3 and 7). Lane 4 is 1% of a cytoplasmic lysate. Protein complexes were separated by SDS/PAGE and subjected to Western blot analysis with a p-Y-specific antibody. The position of a protein of ≈65 kDa precipitated by GST-SH2D1A is indicated by an arrow. All GST fusion proteins were expressed equally (data not shown). (*C*) Lysates (1% in lane 1) from pervanadate-treated BJAB cells were incubated with glutathione-Sepharose-bound GST (lane 2), -GST-SH2D1A (lane 4), or -GST-SH2D1AR32T (lane 3), and bound proteins were subjected to Western blot analysis with a Dok1-specific antibody. (*D*) Lysates (1% in lane 1) from pervanadate-treated BJAB cells were adsorbed onto GST-SH2D1A (lane 2), GST-SH2D1AR32T (lane 3), or p-Y antibody (lane 4). After gel electrophoresis and protein transfer, the membrane was probed with antibody to Dok1.

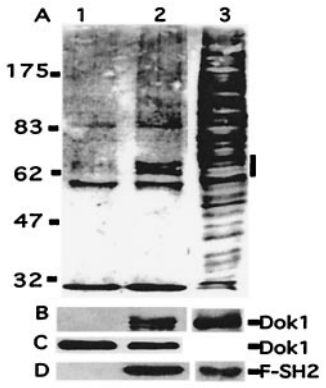

Fig. 2. Dok1 associates with SH2D1A *in vivo*. Lysates from BJAB or FLAG-SH2D1A1-converted BJAB cells treated with pervanadate were incubated with M2-FLAG-antibody beads (*A*, *B*, and *D*) or p-Y-specific antibody complexed to protein G-Sepharose (*C*), and immune-precipitated proteins (BJAB in lane 1 and BJAB/F-SH2D1A in lane 2) were subjected to Western blot analysis with p-Y (*A*), Dok1 (*B* and *C*), or FLAG (*D*) specific antibodies. The position of p-Y proteins migrating as a doublet and associated with F-SH2D1A is indicated by a small vertical bar to the right of lane 3 in *A*. The positions of Dok1 (*B* and *C*) and F-SH2D1A (*D*) also are shown. Lane 3 is 1% of the FLAG-SH2D1A-expressing BJAB cell lysate.

protein bands slightly larger than 62 kDa in blots of proteins bound to wild-type GST-SH2D1A and did not react with any protein that bound to the R to T mutant of GST-SH2D1A (Fig. 1, compare lanes 4 and 3 in *C* and lanes 2 and 3 in *D*). Dok1 is a 481-aa adaptor protein downstream of multiple tyrosine kinases that binds to the Ras-GTPase-activating protein, Ras-GAP (13, 19); the Src C-terminal kinase, Csk (20); and the adaptor protein Nck (21). Dok1 was identical in size to the protein bands slightly larger than 62 kDa identified with p-Y-specific antibody in immunoblots of GST-SH2D1A-bound proteins or p-Y-immune precipitates (Fig. 1*C*, lanes 2 and 4), indicating that these bands are Dok1. Thus, p-Y Dok1 binds specifically to SH2D1A *in vitro* and this association depends on an intact "FLVRES" motif.

Dok1 Associates with SH2D1A *in Vivo*. BJAB cell lines expressing FLAG-SH2D1A were used to evaluate the association of SH2D1A with Dok1 *in vivo*. In these cell lines, FLAG-SH2D1A is expressed at levels similar to SH2D1A in Jurkat T cells, as determined by immune blot with SH2D1A-specific antibody (data not shown). When FLAG-SH2D1A was immune precipitated with FLAG antibody from lysates of BJAB cells expressing FLAG-SH2D1A and probed with FLAG antibody, the efficiency of the FLAG immune precipitation was about 10% (Fig. 2*D*, compare the FLAG antibody immune precipitate in lane 2 with the 1% lysate in lane 3). Immune blotting of the FLAG antibody immune precipitate with Dok1-specific antibody revealed that Dok1 is heterogeneous in size and specifically associated with FLAG-SH2D1A (Fig. 2*B*, compare the FLAG antibody immune precipitate in lane 2 with the 1% lysate in lane 3). Dok1 did not precipitate with FLAG antibody from BJAB cells lacking FLAG-SH2D1A (Fig. 2*B*, lane 1). After correction for the FLAG-SH2D1A immune-precipitation efficiency, about 2% of Dok1 was stably associated with FLAG-SH2D1A. Comparison of the p-Y-specific antibody blot of the FLAG immune precipitate and the cell lysate indicates that p-Y protein bands similar in size to those identified with the Dok1-specific antibody are substantially enriched in the FLAG-SH2D1A immune precipitate in comparison with the lysate (Fig. 2*A*, compare the protein bands indicated by the vertical bar to the right of the figure in the FLAG antibody immune precipitate in lane 2 with the 1% lysate in lane 3). After correction for the FLAG-SH2D1A immune-precipitation efficiency, these data show that substantially >10% of p-Y proteins identical in size to Dok1 are stably associated with SH2D1A. Assuming that most of this Dok1-sized p-Y protein is Dok1, the data suggest that p-Y Dok1 preferentially associates with SH2D1A. The preferential association of p-Y Dok1 with SH2D1A is consistent with the dependence of Dok1 binding to SH2D1A on the wild-type SH2D1A "FLVRES" sequence (Fig. 1) and the dependence of Dok1 association with SH2D1A on the Dok1 Y_{449} and PH domain (Fig. 3).

Anti p-Y 4G10 antibody precipitates approximately equal amounts of p-Y Dok1 from both BJAB and FLAG-SH2D1A-expressing BJAB cells, indicating that FLAG-SH2D1A expression does not affect the amount of Dok1 that is tyrosine phosphorylated in BJAB cells (Fig. 2*C*). Larger p-Y proteins, similar in size to proteins identified in Fig. 1, varied in their coprecipitation with FLAG-SH2D1A.

Dok1 Y_{449} and PH Domain Are Required for SH2D1A Association. Dok1 contains several potential Y phosphorylation sites (refs. 13 and 19 and Fig. 3). Y_{449} is within the sequence ALYSQVQK, which is very similar to the SH2D1A-binding site (TIY_{281}AQVQK) in SLAM (3). To evaluate the importance of Y_{449} of Dok1 for interaction with SH2D1A, Cos7 cells were transiently transfected with an eukaryotic expression plasmid for GST-SH2D1A in combination with expression plasmids for wild-type Dok1, Dok1 Y_{449}F, Dok1 with a deletion of 35 amino acids around Y_{449} (Dok1ΔC35), or Dok1 lacking the PH domain (Dok1ΔPH). Wild-type Dok1 associated with SH2D1A, but Dok1 Y_{449}F (Fig. 3*A*), Dok1ΔPH (Fig. 3*B*), or Dok1ΔC35 (data not shown) associated less well with SH2D1A. Similarly, immune precipi-

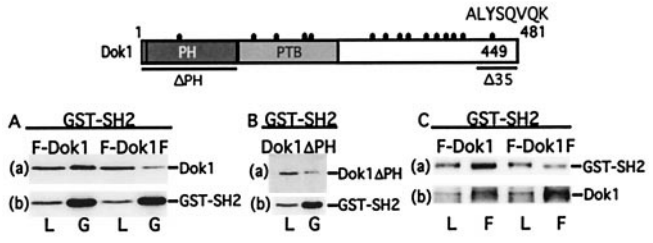

Fig. 3. Dok1 association with SH2D1A *in vivo* requires the Dok1 PH domain and Y_{449}. A schematic diagram of Dok1 and the Dok1 mutations used in these experiments is shown above *A–C*. Dok1, Dok1 with a Y to F mutation at position 449 (Dok1F), Dok1 with C-terminal 35 amino acids deleted (Dok1ΔC35), and Dok1 with deletion of the PH domain (Dok1ΔPH) are represented. The relative positions of the PH domain and the phosphotyrosine binding (PTB) domain are indicated. Tyrosine residues are indicated by a filled circle. Y_{449} is indicated in bold in the context of the potential binding motif for SH2D1A. (*A–C*) Cos 7 cells were transfected with eukaryotic GST pEBG-base expression plasmid for SH2D1A (GST-SH2) in combination with pcDNA3-FLAG-Dok1 (F-Dok1), with pcDNA3-FLAG-Dok1Y449F (F-Dok1F) (*A*), or with pcDNA3-Dok1ΔPH (Dok1ΔPH) (*B*). At 48 h after transfection, cell lysates were incubated with glutathione beads and adsorbed proteins were analyzed by Western blotting with Dok1 (a) or GST-specific antibody (b). The positions of Dok1 and GST-SH2 are indicated. (*C*) The reciprocal experiment is shown with FLAG antibody immune precipitation followed by Western blotting with GST- (a) or αp-Y- (b) specific antibodies. The positions of Dok1 and GST-SH2 are indicated. L, 1% total lysate; G, glutathione bead precipitation; F, FLAG antibody immune precipitation.

tation of FLAG-Dok1 from transfected cells resulted in coprecipitation of similar amounts of SH2D1A from cotransfected cells, whereas FLAG-Dok1 $Y_{449}F$ immune precipitation resulted in much less efficient coprecipitation of SH2D1A (Fig. 3*C*). These results indicate that much of the SH2D1A can associate with Dok1 and that Dok1 Y_{449} and PH domain are required for high-level Dok1 association with SH2D1A. Because phenylalanine (F) is a nonphosphorylatable analog of tyrosine, these data suggest that phosphorylation of Y_{449} is important for strong association of Dok1 with SH2D1A. Further, because the Dok1 PH domain is required for plasma-membrane association and much of the tyrosine phosphorylation occurs at the plasma membrane (21), the PH domain requirement probably is linked to the tyrosine phosphorylation requirement.

Other Proteins Can Interact with SH2D1A. To identify additional proteins that may interact with SH2D1A, extracts from pervanadate-treated Jurkat cells were incubated with GST or GST-SH2D1A immobilized on glutathione beads, and the adsorbed proteins were identified on a preparative gel. Several proteins referred to as p300, p200, p160, p130, p115, p100, and p70 specifically adsorbed to GST-SH2D1A. Each of the proteins was degraded by trypsin, peptides were separated by microcapillary HPLC, and multiple peptides were sequenced by ion trap mass spectroscopy. The major bands were p100 and p70 and these yielded sequences of Ku86 and Ku70, respectively. The p300 band was less evident in stained gels and yielded sequences of DNA-PK (DNA-dependent protein kinase), a Ku86- and Ku70-associated protein. Western blot analysis confirmed the specific binding of Jurkat and BJAB cell Ku86 and Ku70 to GST-SH2D1A *in vitro*, but not to GST or GST-SH2D1A-R32T (see Fig. 4*A*, lanes 3 and 7, versus *B*, lanes 2 and 6 or 4 and 8). The binding was observed with or without pervanadate treatment. However, Ku86 and Ku70 did not coimmune precipitate with FLAG-SH2D1A, either with transient overexpression of FLAG-SH2D1A in 293T cells or with stable expression of FLAG-SH2D1A in BJAB cells (data not shown). Thus, the significance of the affinity of Ku86, Ku70, and DNA-PK for SH2D1A *in vitro* is uncertain.

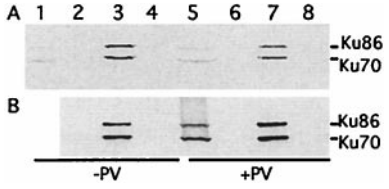

Fig. 4. Ku70 and Ku86 interact with SH2D1A *in vitro*. Cell extracts from Jurkat (*A*) or BJAB (*B*) cells treated (+PV) or without (−PV) pervanadate were incubated with bacterially expressed GST (lanes 2 and 6), GST-SH2D1A (GST-SH2) (lanes 3 and 7), or GST-SH2D1A-R32T (GST-SH2T) (lanes 4 and 8) adsorbed on glutathione beads, and the precipitates were analyzed by SDS/PAGE and Western blotting analyses. Lanes 1 and 5 are 1% total lysate. Filters were probed with a mixture of goat anti-Ku70 and anti-Ku86. The positions of Ku70 and Ku86 are indicated.

The p180 interacting protein was identified as Dock2. Dock2 (16, 22) and Dock180 (15) are related proteins. Whereas Dock180 is expressed in adherent cells, binds to Crk, and activates Rac (23), Dock2 is expressed in cells of hematopoietic lineage, does not bind to CrkII, and activates Rac1 (22). To evaluate whether Dock2 and SH2D1A associate *in vivo*, FLAG-tagged Dock2 was coexpressed with GST-SH2D1A or GST in 293T cells. After pervanadate treatment, cell lysis, and nuclear clearance, GST-SH2D1A or GST was captured on glutathione-beads. Anti-FLAG and anti-p-Y antibody identified Dock2 to be associated with GST-SH2D1A and not with GST. However, the level of association was <1% (data not shown). In similar experiments, Dock180 did not specifically associate with GST-SH2D1A.

The *in vitro* GST-SH2D1A binding proteins p160, p130, and p115 were identified as α-coatomer (24), hnRNPU (25) or GAPII, and nucleolin C23 (26) or Eps15R (17), respectively. The association of FLAG-SH2D1A with Eps15R could not be detected with anti-Eps15R antibodies. The interaction of SH2D1A with the other proteins was not further characterized because of difficulty in obtaining suitable reagents.

Expression of SH2D1A Activates Transcription Factor NF-κB. NF-κB is important for lymphocyte activation, cytokine synthesis, proliferation, and survival (27, 28). NF-κB can be activated by serine/threonine and tyrosine kinase signal transduction pathways (29, 30). To determine whether SH2D1A can affect NF-κB activation, 293T cells were cotransfected with an SH2D1A expression plasmid and an NF-κB-dependent luciferase reporter plasmid (31). Expression of SH2D1A resulted in a 10- to 30-fold increase in reporter gene activity as compared with the levels seen after transfection with expression vector control DNA (Fig. 5*A* and multiple experiments not shown). A dominant-negative mutant of the IκB kinase β (IKKβΔ34) (32, 33) substantially blocked SH2D1A-induced NF-κB activation (Fig. 5*B*).

In an attempt to relate the SH2D1A effect on NF-κB activation to the pathogenesis of XLP, three SH2D1A mutants (SH2D1A-R32T, SH2D1A-P101L, and SH2D1A-C12) encountered in XLP patients were tested for their ability to activate NF-κB. All three SH2D1A mutants activated NF-κB, albeit to lower levels than found in wild-type SH2D1A (Fig. 5*C*). Despite the fact that the mutant and wild-type expression vectors were identical except for the specific mutations, the mutant proteins were consistently expressed at low levels relative to wild-type SH2D1A (data not shown) and the level of expression correlated with the level of NF-κB activation. The lower expression of the mutant SH2D1As is consistent with the hypothesis that these mutations result in less stable proteins. Given the lower level expression of the mutant SH2D1As, these proteins seem to be intrinsically as competent for NF-κB activation as is wild-type

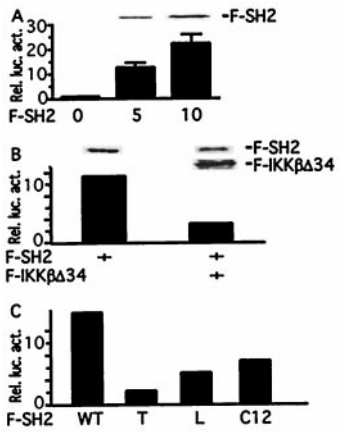

Fig. 5. SH2D1A activates NF-κB. (*A*) Wild-type SH2D1A activates NF-κB. An NF-κB-dependent luciferase reporter plasmid with three upstream NF-κB binding sites was transfected into 293T cells with a control β-galactosidase expression construct (12) and pcDNA3 or increasing amounts of pcDNA3-based expression plasmid for FLAG-SH2D1A (F-SH2). Luciferase activities were normalized for cotransfected β-galactosidase activity and pcDNA3 vector control/luciferase reporter activity. Mean relative luciferase activities (±SD) from at least four different experiments are shown. (*B*) A dominant-negative IKKβ mutant inhibits SH2D1A-induced NF-κB activation. Cells were transfected with 5 μg of pcDNA3-based expression vector for FLAG-SH2D1A (F-SH2) alone or in combination with 1 μg of pcDNA3-IKKβΔ34. Luciferase reporter assays are as in *A*. (*C*). SH2D1A mutants also activate NF-κB. Ten micrograms of pcDNA3-based expression vector for FLAG-SH2D1A (WT), FLAG-SH2D1A-R32T (T), FLAG-SH2D1A-P101L (L), or FLAG-SH2D1A-C12 (C12) were transfected into 293T cells. Western blots for SH2D1A protein expression using the M5-FLAG antibody are shown above the respective experiments in *A* and *B*.

SH2D1A. These data indicate that the wild-type SH2 domain is not required for NF-κB activation.

Discussion

Our results indicate that SH2D1A has multiple effects on intracellular signaling that are likely to be important in the abnormal response of XLP patients to EBV infection. SH2D1A interacts and associates not only with the SLAM and h2B4 receptors (3, 8) but also with cytoplasmic proteins, especially Dok1.

Dok1 is likely to be a physiologically significant target of SH2D1A. First, Dok1 is a major p-Y protein downstream of v-Abl, v-Src, v-Fps, v-Fms, $p210^{bcr\text{-}abl}$, and epidermal growth factor, platelet-derived growth factor, colony-stimulating factor, insulin, insulin-like growth factor, vascular endothelial growth factor, antigen, Fcγ, or CD28 receptor signaling pathways (13, 34–39). Dok1 is an abundant tyrosine phosphorylated protein in pervanadate-treated B lymphoma cells, and p-Y Dok1 is an abundant p-Y protein that binds *in vitro* to SH2D1A. Second, Dok1 binding to SH2D1A depends on the wild-type SH2D1A "FLVRES" sequence; Dok1 does not bind to an SH2D1A with an R to T mutation in the "FLVRES" sequence. Because the R to T mutation occurs in XLP (3), Dok1 is a candidate for a physiologically significant partner of SH2D1A. Third, endogenous phosphorylated Dok1 specifically and significantly immune precipitates by using antibody to epitope-tagged SH2D1A that was stably expressed in B lymphoma cells at a level at which SH2D1A is normally expressed in Jurkat T lymphocytes. The stable association of SH2D1A with p-Y Dok1 could significantly affect Dok1 activity. Fourth, the immune precipitation of Dok1 by using antibody to epitope-tagged SH2D1A largely depends on the Dok1 PH domain and on the "ALYSQVQK" sequence that is similar to the SLAM binding site for SH2D1A and SHP-2. SH2D1A therefore seems to associate with Dok1 through a site that is likely to be critical for SHP-2 or SHIP binding, dephosphorylation, and modulation of Dok1 activity. Fifth, activated Dok1 is implicated in the regulation of Ras-GAP (13, 19, 35, 37, 38), of NCK (21), and of Csk (20, 36). SH2D1A binding to Dok1 is anticipated to alter Dok1's effects on Ras-GAP, NCK, or Csk.

Because Dok1 Y_{449} is not required for Ras-GAP association (40), Dok1 with SH2D1A bound to p-Y_{449} could continue to interact with Ras-GAP and to influence Ras signaling (38). SH2D1A binding to Dok1 p-Y $_{449}$ would prevent SHP-2 phosphatase binding and dephosphorylation of Dok1 and would prolong Dok1's effects. In colony-stimulating factor-1-treated bone marrow macrophages, Dok1 is the predominant tyrosine-phosphorylated protein that is hyperphosphorylated in the absence of SHP-1, which correlates with increased cell survival (41). Dok1 p-Y_{449} is also the potential binding site for Csk, an inhibitor of Src kinase (13, 20). SH2D1A binding to Dok1 would block Csk recruitment to the membrane, prevent Src inhibition, and prolong Src signaling (20). Overall, B cell receptor crosslinking-induced mitogen activated protein (MAP) kinase activation is increased and FcγRIIB-mediated negative regulation is blocked in B cells in which Dok1 has been knocked out by homologous recombination (35). Consistent with these data and a model in which SH2D1A blocks a negative effect of Dok1 on MAP kinase activation, SH2D1A expression in serum-starved BJAB B lymphoma cells was associated with increased p42/p44 MAP kinase activity relative to controls (B.S.S., preliminary results). Thus, through association with Dok1, SH2D1A could have pleiotropic roles in lymphoid or macrophage activation, proliferation, survival, differentiation, or motility, and these effects could be relevant to the failure of XLP patients to mount an effective and nonhepatotoxic response to EBV infection.

A Dok1-related protein, $p56^{dok\text{-}2}$ (also called Dok2, FRIP, or Dok-R) recently has been reported to be similar to Dok1 in being downstream of Lyn or epidermal growth factor receptor activation and in activating Ras-GAP or associating with Nck (14, 40, 42). We have detected SH2D1A association with Dok2 in transfected 293T cells (B.S.S., unpublished results). However, the level of association seems to be less than that with Dok1.

In addition to Dok1, we have identified Ku70/Ku86, Dock2, α-coatomer, hnRNPU, and nucleolin C23 as proteins in Jurkat T cell lysates that bind specifically to an SH2D1A affinity matrix. The most abundant proteins, Ku70/Ku86, are a heterodimeric regulatory subunit of the DNA-PK complex involved in DNA repair and B and T cell receptor recombination (reviewed in ref. 43). Ku70/Ku86 is mostly in the cytoplasm in B cells and in the nucleus in T cells (44). In B cells, tyrosine-phosphorylated Ku can associate with the cytoplasmic domain of the CD40 receptor, and CD40 ligand causes Ku to move to the nucleus and DNA-PK activation (44). Although Ku binds specifically to wild-type SH2D1A and not to SH2D1A with R32T mutation in the SH2 domain, no significant stable association was detected *in vivo*, even with overexpression of SH2D1A.

Although Dock2 only marginally associated with SH2D1A *in vivo* transfected 293T cells, the SH2D1A-associated Dock2 was tyrosine-phosphorylated (data not shown), and the binding also could be of physiological significance. Dock2 appears to be the Dock180 of hematopoietic lineage cells. Dock180 associates with Crk and p130Cas and is an important regulator of Rac, cell migration, and phagocytosis of apoptotic bodies (22, 23, 45–47). Higher-level association of Dock2 with SH2D1A may require factors exclusively expressed in lymphoid tissues.

Surprisingly, overexpression of wild type or putative null mutants of SH2D1A in 293T cells resulted in up to 25-fold NF-κB activation. This SH2D1A effect is not mediated by the SH2D1A SH2 domain, because the SH2-R32T and P101L mutants of SH2D1A also activated NF-κB. SH2D1A-induced NF-κB activation also was inhibited by a dominant-negative IKKβ, an enzymatic activity not required for direct tyrosine kinase-mediated IκB inactivation. An

XLP-associated SH2D1A, SH2D1AC12, that has an additional 12 residues also was able to activate NF-κB. These data implicate a putative non-SH2 domain activity of SH2D1A in NF-κB activation. Further, the observation that three XLP-associated SH2D1A mutants could still activate NF-κB seems to indicate that NF-κB activation is not linked to the XLP phenotype. Nevertheless, SH2D1A-mediated NF-κB activation could still have a role in the XLP phenotype. NF-κB can be critical for hematopoietic and hepatocyte cell growth or survival, and these cells are implicated in the morbid outcome of EBV infection in XLP (27, 30, 48, 49). SH2D1A expression is induced by T cell activation, and SH2D1A may be important for T or NK cell control of EBV-infected B cells (4). The SH2D1A-C12 mutation is of uncertain significance in SH2D1A SH2 activity. Moreover, all three SH2D1A mutations that we studied yielded lower SH2D1A protein levels in cells, which correlated with less than wild-type NF-κB activation. If these XLP-associated mutations result in lower SH2D1A levels and less NF-κB activation *in vivo*, NF-κB activation could be linked to XLP.

The experiments reported in this paper reveal a distinct activity of SH2D1A in NF-κB activation and extend the spectrum of significant SH2D1A SH2 interactions beyond receptor cytoplasmic domains (5, 8) by identifying Dok1 as a major SH2D1A-associated protein. SH2D1A can associate with a significant fraction of p-Y Dok1, and Dok1 can associate with a significant fraction of SH2D1A. The Dok1-interacting sequence ($ALY_{449}SQVQK$) is quite similar to the SLAM sequence ($TIY_{281}AQVQK$). Based on their primary sequence similarity, vascular endothelial cell cadherin CD144 (50), platelet-endothelial cell adhesion molecule-1 CD31 (51), and the organic cation transporter (52) also may be affected by SH2D1A. Indeed, *in vitro*, SH2D1A peptide binding studies indicate that SH2D1A and the related protein EAT-2 may interact with a broad range of proteins that have a TIpYXX(V/I)-like primary sequence (53–55).

Masaya Higuchi, Siu Chun Hung, Myung Soo Kang, and other members of our laboratory contributed helpful advice, as did Professor Tom Gilmore. We thank Drs. N. Carpino and R. Kobayashi for p62 Dok1 reagents, Dr. M. D. Resh for p56 Dok2, Dr. M. Matsuda for Dock180 reagents, Dr. T. Nagase for the Dock180 similar cDNA, Dr. M. Higuchi for STAM antibody, Dr. P. P. Di Fiore for Eps15R reagents, and Kerry Pierce and Daniel Kirby for ion trap mass spectrometry. This work was supported by Public Health Service Grants CA47006 (to E.K.) and CA71705 (to G.M.). B.S.S. was a visiting fellow from the International Agency for Research on Cancer, Lyon, France.

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