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A Novel Interleukin-12 p40-Related Protein Induced by Latent Epstein-Barr Virus Infection in B Lymphocytes

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We have isolated a cDNA encoding a novel hematopoietin receptor family member related to the p40 subunit of interleukin-12 and to the ciliary neurotrophic factor receptor, whose expression is induced in B lymphocytes by Epstein-Barr virus (EBV) infection. This gene, which we have designated EBV-induced gene 3 (*EBI3*), encodes a 34-kDa glycoprotein which lacks a membrane-anchoring motif and is secreted. Despite the absence of a membrane-anchoring motif and of cysteines likely to mediate covalent linkage to an integral membrane protein, EBI3 is also present on the plasma membrane of EBV-transformed B lymphocytes and of transfected cells. Most newly synthesized EBI3 is retained in the endoplasmic reticulum in an endoglycosidase H-sensitive form associated with the molecular chaperone calnexin and with a novel 60-kDa protein. EBI3 is expressed in vivo by scattered cells in interfollicular zones of tonsil tissue, by cells associated with sinusoids in perifollicular areas of spleen tissue, and at very high levels by placental syncytiotrophoblasts. *EBI3* expression in vitro is induced in EBV-negative cell lines by expression of the EBV latent infection membrane protein-1 and in peripheral blood mononuclear cells by pokeweed mitogen stimulation. *EBI3* maps to chromosome 19p13.2/3, near genes encoding the erythropoietin receptor and the cytokine receptor-associated kinase, Tyk2. EBI3 synthesis by trophoblasts and by EBV-transformed cells and similarities to interleukin-12 p40 are compatible with a role for EBI3 in regulating cell-mediated immune responses.

Epstein-Barr virus (EBV) causes infectious mononucleosis (33), a benign proliferation of infected B lymphocytes, and is etiologically associated with lymphoid malignancies (reviewed in reference 44) and Hodgkin's disease (34). Infection of normal human B lymphocytes in vitro results in latent infection, expression of a restricted set of viral genes, and changes in cell phenotype and growth (49; reviewed in reference 38). These changes include continuous proliferation in vitro to form immortalized lymphoblastoid cell lines (LCLs) (49), increased expression of surface antigens associated with B-lymphocyte activation (8, 48, 56, 59), and elaboration of soluble B-cell growth factors (2, 7, 10, 11, 24, 28, 57, 60). The phenotypic similarities with mitogen- or antigen-stimulated B cells suggest that EBV may transform B cells through specific interactions between viral proteins and normal mediators of B-lymphocyte activation and proliferation (12, 48, 58, 59).

Investigation of the effects of latent EBV infection on the expression of B-lymphocyte genes has been facilitated by the availability of EBV-infected [EBV(+)] Burkitt's lymphoma (BL) cell lines that were obtained by in vitro infection of EBV-negative [EBV(−)] BL cells (12, 22, 41, 52). EBV(−) BL cells typically express low levels of B-lymphocyte activation markers, while EBV(+) BL cells express high levels of CD23, CD39, Bac-1, and CD21 and resemble EBV-LCLs(12, 13, 22, 29, 52). These phenotypic changes depend on expression of virus-encoded nuclear antigens and latent membrane proteins (LMPs) (29). EBV effects have been further delineated by using subtractive hybridization to identify genes expressed at higher levels in EBV-infected BL41/B95-8 cells than in the parental EBV(−) BL41 line (5, 6, 46, 47). Two novel genes which are predicted to encode lymphocyte-specific G-protein-coupled peptide receptors were identified (5). One of these putative receptors is also induced in T lymphocytes by infection with human herpesvirus 6 or 7 (32). This report describes a third novel EBV-regulated cell gene, *EBI3*, which encodes a soluble hematopoietin receptor related to the p40 subunit of interleukin-12 (IL-12) and the ciliary neurotrophic factor receptor (CNTFR).

MATERIALS AND METHODS

Cell culture. BL30, BL41, Louckes, and BJAB are EBV(−) BL lines. The BL41/B95-8 cell line was derived from BL41 by infection with the transforming B95-8 EBV (41). SLA (a gift from T. Springer, Dana-Farber Cancer Institute, Boston, Mass.), IB4, and LCL-W91 are LCLs generated by EBV transformation of primary human B lymphocytes with EBV B95-8 (SLA and IB4) and W91 (LCL-W91; a gift from S. Swaminathan, Thomas Jefferson University, Philadelphia, Pa.). P3HR1 is an EBV(+) BL cell line infected with the nontransforming P3HR1 EBV. RHEK-1 (a gift from J. Rhim, National Cancer Institute, Bethesda, Md.) is an adenovirus 12-simian virus 40 hybrid virus-transformed human keratinocyte line. K562, U937, and HL60 are human myeloid leukemia cell lines with erythroid, monocytic, and myeloblastic features, respectively. Jurkat and MOLT-4 are human T-cell leukemia lines. TK143 is a human osteosarcoma line. HeLa is a cervical carcinoma cell line. COS7 is a simian virus 40-transformed monkey kidney cell line. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll separation of blood (Ficoll-Hypaque; Pharmacia, Vineland, N.J.). The cells were resuspended at 10^6 cells per ml in RPMI 1640 medium supplemented with 20% fetal bovine serum and were divided into parallel cultures grown for 72 h with or without 2.5 μg of pokeweed mitogen (Sigma, St. Louis, Mo.) per ml.

Subtractive hybridization and sequence analysis. A BL41/B95-8 cDNA library was screened with subtracted BL41/B95-8 cDNA probes as previously described (5). Nucleotide and predicted amino acid sequences were compared by the BLAST algorithm (1) with known sequences of the National Center for Biotechnology Information databases, using the Experimental GENINFO BLAST Network Service through the Molecular Biology Computer Research Resource of

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FIG. 1. Complete nucleotide and deduced amino acid sequences of *EBI3* cDNA. The 1,161-nucleotide *EBI3* cDNA contains a 687-nucleotide open reading frame predicted to encode a 26-kDa polypeptide. A hydrophobic amino-terminal segment (double underline) is predicted to form a signal peptide for membrane translocation. Two potential asparagine-linked glycosylation sites are indicated (CHO######). The nucleotide sequence of the 3′ untranslated region bears significant homology with the human Alu repeat element (single underline). Nucleotide sequences in boldface type correspond to the *Eco*RI site of the cloning linker.

the Dana-Farber Cancer Institute. Alignment of multiple sequences was aided by using the CLUSTAL program (PCGene; IntelliGenetics, Mountain View, Calif.) (36).

Chromosome mapping. Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. Fluorescence in situ hybridization was performed as described previously (53). Biotin-labeled *EBI3* probe was generated by nick translation with Bio-11-dUTP (Enzo Diagnostics), using a 1.09-kb truncated cDNA template from which AT-rich Alu repeat sequences were deleted. Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, Calif.), and chromosomes were identified by staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI).

RNA isolation and analysis. Cytoplasmic RNA was isolated from exponentially growing cells by acid phenol-guanidinium isothiocyanate extraction (14). Total cellular RNA was purified from excised human spleen and tonsil and from postmortem bone marrow by acid phenol-guanidinium isothiocyanate extraction followed by reprecipitation in 4 M LiCl. Polyadenylated IB4, BL41, and BL41/B95-8 RNAs were purified by chromatography on oligo(dT)-cellulose. RNA samples (4 to 12 μg per lane) were denatured, size fractionated on 0.66 M formaldehyde–1% agarose gels, and transferred to charged nylon membranes (GeneScreen Plus; New England Nuclear, Billerica, Mass.). Expression in other human tissues was analyzed by using a commercially prepared blot (multiple-tissue Northern; Clontech, Palo Alto, Calif.) containing 2 μg of polyadenylated heart, brain, placenta, lung, liver, kidney, skeletal muscle, and pancreas RNA. RNA blots were hybridized to [^{32}P]cDNA probes as previously described (5).

Antibody preparation. A DNA fragment encoding EBI3 codons 25 to 229 was synthesized by PCR amplification of *EBI3* cDNA with oligonucleotide primers 10.7A (5′-ACTGAATTCGAGCTCCTGGCCTCAAG-3′) and 10.7B (5′-AAG GATCCCCAGCAGCTCTGACACTG-3′), which add *Eco*RI and *Bam*HI restriction sites. The DNA fragment was ligated in frame to the 3′ end of the glutathione-*S*-transferase open reading frame of the pGEX-2TK vector (Pharmacia). The GST-EBI3 fusion protein was purified from bacterial lysates by affinity chromatography on glutathione-Sepharose or by preparative electrophoresis from "inclusion body" preparations and was used to immunize rabbits (Pocono Rabbit Farms, Canadensis, Pa.). Immune rabbit serum was preabsorbed against purified glutathione-*S*-transferase-coupled agarose beads. Anti-EBI3 antibody was affinity purified by affinity chromatography with GST-EBI3 fusion protein coupled to agarose beads.

Transfections. The protein-coding region of the *EBI3* cDNA was cloned into the *Eco*RI site of the eukaryotic expression vector, pSG5 (Stratagene, La Jolla, Calif.). An EBI3-FLAG expression plasmid was constructed in pSG5 by joining DNA encoding a cyclic AMP-dependent protein kinase site (RRASVG) and FLAG epitope (DYKDDDDK) in frame to the 3′ end of the *EBI3* open reading frame. The structure of the resulting open reading frame was verified by nucleotide sequencing. Plasmid DNA was purified by isopycnic banding on CsCl gradients. Approximately 10^7 target cells were transfected with 40 to 50 μg of purified plasmid DNA by electroporation at 200 V and 960 μF in 0.4-cm cuvettes (Bio-Rad, Hercules, Calif.). BJAB cells stably expressing EBI3-FLAG protein were established by cotransfection of the pSG5 EBI3-FLAG construct together with a vector expressing the hygromycin resistance gene. Transfectants were selected in 400 μg of hygromycin (Calbiochem, La Jolla, Calif.) per ml and cloned by limiting dilution.

Immunofluorescence. Tissue samples of placenta or tonsil were frozen in dry ice-isopentane. Tissue sections or cultured cells were air dried on slides and fixed for 10 min in acetone-methanol (50:50) at −20°C. After rehydration, slides were incubated with affinity-purified rabbit EBI3 antiserum diluted in phosphate-buffered saline (PBS)–20% goat serum (1:25 dilution) or nonimmune normal rabbit serum (NRS, Sigma) (1:1,250 dilution). Antibody binding was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Southern Biotechnology, Birmingham, Ala.) (1:500 dilution) and was visualized in a Reichert Microstar IV fluorescence microscope. Frozen tonsil sections were double stained with rabbit EBI3 antiserum (diluted 1:25) and anti-CD22 mouse monoclonal antibody (diluted 1:50; Dako, Carpenteria, Calif.). Primary antibody binding was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse secondary antibodies. For live-cell staining, IB4 or transfected BJAB cells were washed with PBS and incubated with rabbit EBI3 antiserum diluted 1:25 or NRS diluted 1:1,250 in PBS–20% goat serum. Transfected COS7 cells were dissociated from culture flasks with 1 mM EDTA–PBS and stained in suspension under identical conditions.

Immunoprecipitation and Western blotting. Cells were metabolically labeled for 18 to 24 h with 50 μCi of [^{35}S]methionine (Trans ^{35}S-label; ICN, Cleveland,

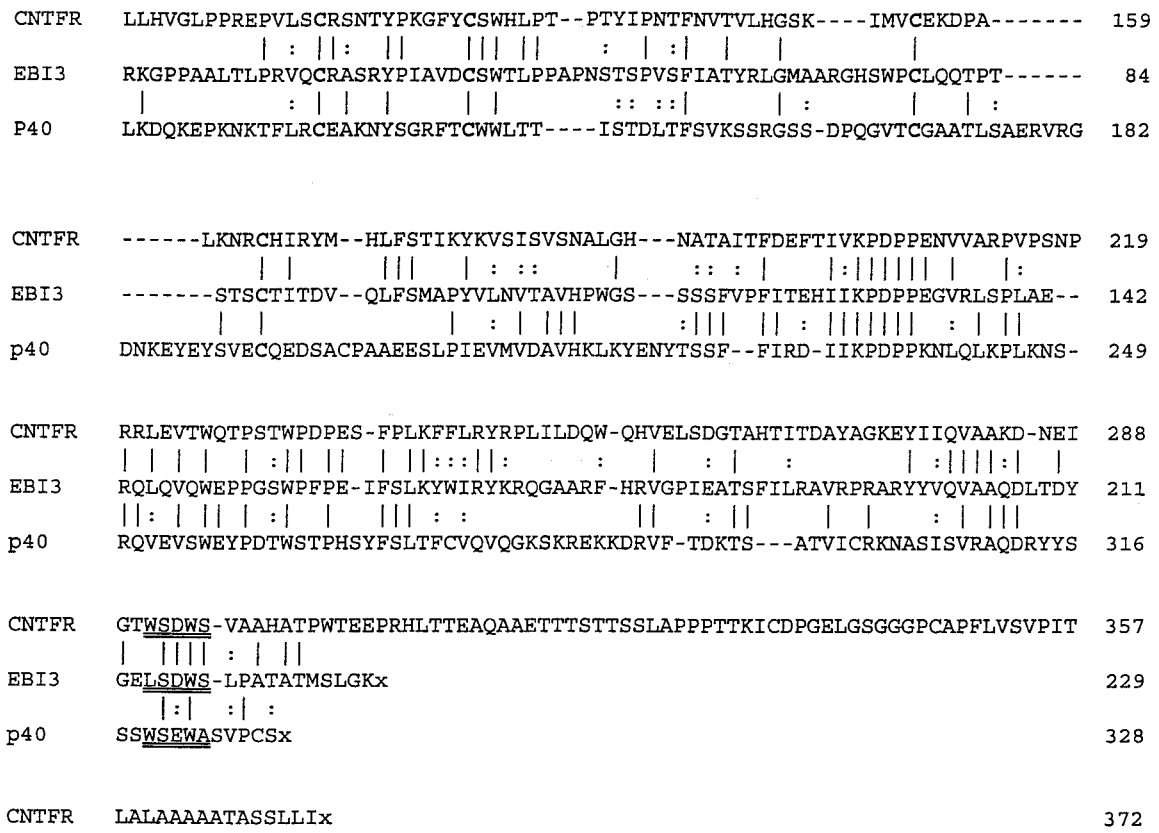

FIG. 2. Comparison of EBI3, CNTFR, and IL-12 p40 subunit amino acid sequences. The complete sequence of the EBI3 protein following signal peptidase cleavage was aligned with carboxyl-terminal sequences of CNTFR and p40. Identical residues are indicated by a vertical line, and similar residues are indicated by double dots. Cysteines believed to be involved in intramolecular disulfide linkage are shown in boldface type. The conserved WSXWS motif is indicated by double underlines. EBI3 is 30% (63 of 209) identical to CNTFR and 27% (56 of 209) identical to IL-12 p40.

Ohio) per ml in methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum. Transfected BJAB or COS7 cells were labeled 24 h after electroporation. Labeled cells were washed in ice-cold PBS and lysed in digitonin lysis buffer (1% digitonin, 10 mM triethanolamine [pH 7.5], 150 mM NaCl) or Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM Tris [pH 7.4], 150 mM NaCl, 3% glycerol, 1.5 mM EDTA) containing 1 mg of bovine serum albumin per ml and protease inhibitors (1 mM phenylmethylsulfonyl chloride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml). Lysates were centrifuged for 30 min at 14,000 × *g* and precleared with NRS bound to protein A-Sepharose (Pharmacia) or normal mouse serum bound to protein G-Sepharose. Cleared extracts were incubated at 0°C for 1 h with primary antibodies. Immune complexes were bound to Sepharose beads and washed with lysis buffer.

In pulse-chase experiments, 15×10^7 BJAB cells stably transfected with *EBI3-FLAG* were preincubated for 1.5 h at 37°C in 20 ml of Met- and Cys-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum (ICN) and pulsed for 10 min at 37°C in the same medium containing 2 mCi of [^{35}S]Met-[^{35}S]Cys (ICN). Labeled cells were centrifuged and resuspended at a concentration of 10^7/ml in complete RPMI 1640 medium supplemented with 10% fetal bovine serum. At various times, aliquots of 3×10^6 cells were harvested, washed in cold PBS, and lysed in 1% NP-40 lysis buffer. Cell lysates were immunoprecipitated with anti-FLAG M2 antibody (International Biotechnologies, Inc., New Haven, Conn.) as described above.

For *N*-glycanase digestion, immunoprecipitates were washed once in digitonin buffer containing 1 mg of bovine serum albumin per ml, four times in digitonin buffer, twice in 0.5 M LiCl–0.1 M Tris (pH 7.4), and once in 10 mM Tris (pH 7.4). Immune complexes were eluted from protein A-Sepharose by boiling for 5 min in 100 μl of 50 mM Tris (pH 7.4)–0.5% sodium dodecyl sulfate (SDS)–50 mM β-mercaptoethanol. Aliquots (20 μl) of immunoprecipitates were adjusted to 1.25% NP-40 and incubated with or without 0.3 U of *N*-glycanase (Genzyme, Cambridge, Mass.) for 18 h at 37°C. Samples were boiled in SDS-protein sample buffer and analyzed on 10% polyacrylamide gels. EBI3 was detected in lysates of cultured cells or fresh placenta by standard immunoblotting techniques with rabbit polyclonal anti-EBI3 and ^{125}I-protein A (Amersham, Arlington Heights, Ill.).

Purification and amino acid sequencing of EBI3-associated proteins. BJAB cells stably transfected with an *EBI3-FLAG* expression vector were washed in cold PBS and lysed at 4°C for 30 min in 1% NP-40 lysis buffer (1 ml/10^8 cells) containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1.5 mM EDTA, 3% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, and 1 μg of leupeptin per ml. After centrifugation at 14,000 × *g* for 30 min, the supernatant was incubated with anti-FLAG M2 beads for 1.5 h at 4°C. The beads were washed first with 1% NP-40 lysis buffer and subsequently with TBS (10 mM Tris [pH 7.4], 150 mM NaCl), and bound proteins were eluted by addition of FLAG peptide (250 nM in TBS). Proteins were concentrated by ultrafiltration (Centricon 10; Amicon, Beverly, Mass.), separated by SDS-polyacrylamide gel electrophoresis (PAGE) (6.5% polyacrylamide) under reducing conditions, and transferred to polyvinylidene difluoride sequencing membrane (ProBlott; Applied Biosystems, Foster City, Calif.). Proteins were stained with Ponceau S, and bands corresponding to p95 and p60 were excised. In situ digestion with trypsin, peptide analysis by high-performance liquid chromatography, laser desorption mass spectroscopy, and Edman microsequencing were performed at the Harvard Microsequencing Facility, Boston, Mass. On the basis of one of the peptide sequences, the oligonucleotide p60.1R (5′-CCAGCCGCCCTCGTCNGAGAAGCCCAT NGACAGCATCTG-3′) was radioactively end labeled with [γ-^{32}P]ATP and used for screening the BL41/B95-8 cDNA library.

Nucleotide sequence accession number. The *EBI3* sequence and *p60* sequences have been deposited with the GenBank database and given accession numbers L08187 and U41086, respectively.

RESULTS

Cloning of cDNA for the novel EBV-induced cytokine receptor, EBI3. Of 25 cDNA clones identified by subtractive hybridization, 2 were derived from mRNA of a novel gene designated *EBI3*. The complete sequence of the 1,161-nucleotide *EBI3* cDNA is shown in Fig. 1. A unique AUG codon at nucleotides 14 to 16 conforms to a Kozak consensus sequence for translational initiation (40) and precedes a 687-nucleotide open reading frame predicted to encode a 25,391-Da polypeptide. The

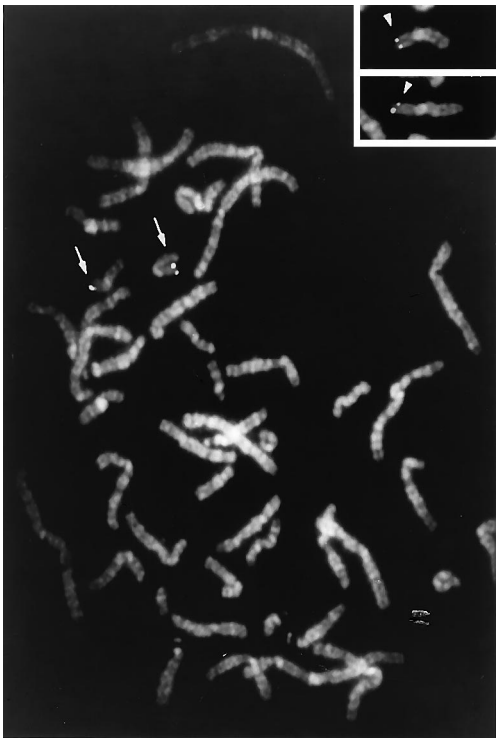

FIG. 3. In situ hybridization of *EBI3* probe to human metaphase chromosomes. Biotin-labeled *EBI3* probe was hybridized to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. The chromosome 19 homologs are identified by arrows; specific labeling was observed at 19p13.3. The inset shows partial karyotypes of two chromosome 19 homologs illustrating specific labeling at 19p13.3 (arrowheads). Images were obtained with a Zeiss Axiophot microscope coupled to a cooled charge-coupled device camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged by using image analysis software (NU200 and Image 1.52b).

first 20 amino acids are highly hydrophobic and are predicted to be a cleavable signal peptide. Two potential N-linked glycosylation sites are also present. The predicted protein shows structural features characteristic of members of the type I cytokine receptor (hematopoietin receptor) family (3, 16). These include two pairs of conserved cysteines at positions 35, 46, 79, and 89 and an LSDWS motif at residues 215 to 219, similar to the WSXWS consensus sequence. Among members of the hematopoietin receptor family, EBI3 is most closely homologous to the CNTFR (30% identity [20]) and to the IL-12 p40 subunit (27% identity [30, 66]), with conservative amino acid substitutions at many of the nonidentical residues (Fig. 2). *EBI3* also resembles IL-12 p40 in that both genes have a 3′ untranslated Alu repeat sequence (30, 66). Similarly to IL-12 p40 and in contrast to all other members of this family, EBI3 lacks obvious membrane-anchoring sequences, such as a hydrophobic or amphipathic transmembrane segment or a glycosyl-phosphatidylinositol linkage consensus site (23). Sequencing of five additional independent clones isolated from the unamplified cDNA library failed to reveal alternative carboxyl-terminal domains which could mediate membrane association. Several clones of the murine homolog of *EBI3* have been isolated, and they all similarly lack a transmembrane domain or potential glycosyl-phosphatidylinositol addition signal (39).

***EBI3* localizes to chromosome 19, band p13.3.** To map the chromosomal location of *EBI3*, biotin-labeled *EBI3* cDNA was hybridized to normal human metaphase chromosomes. The *EBI3* probe resulted in specific labeling only of chromosome 19 (Fig. 3). Specific labeling of 19p13 was observed on one (1 cell), two (8 cells), three (3 cells), or all four (6 cells) chromatids of the chromosome 19 homologs in 18 cells. Of 53 signals, 29 (55%) were at 19p13.3, 17 (32%) were at the junction of 19p13.2 and p13.3, and 4 (8%) were at 19p13.2. One signal was at 4p16, 3cen, or 3q26.2. Similar results were obtained in another hybridization experiment. These results localize the *EBI3* gene to chromosome 19, band p13.3. Several other proteins involved in hematopoietic cell growth and differentiation are encoded in this region, including the erythropoietin receptor (EpoR [65]), the nonreceptor tyrosine kinases Hy1 (54) and Tyk2 (26), and the B-lymphocyte activation antigen CD23 (64). EpoR is also a member of the hematopoietin receptor family, while Tyk2 is involved in signal transduction from several cytokine receptors (55).

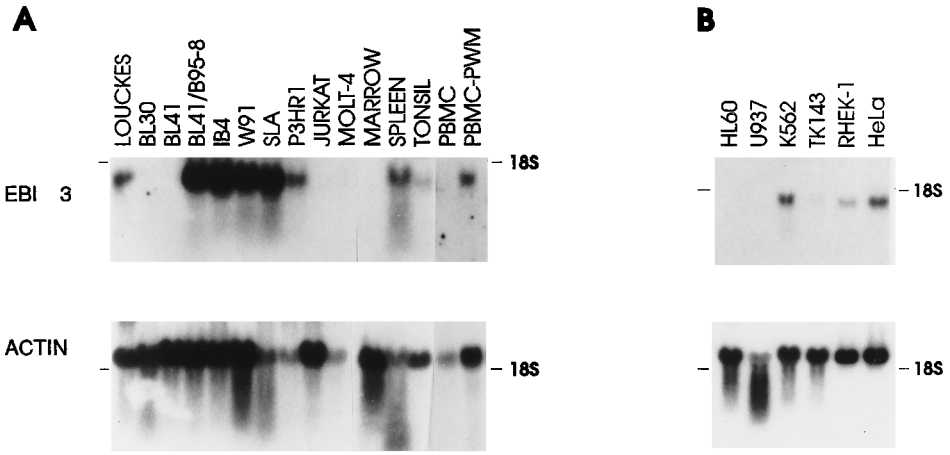

FIG. 4. Expression of *EBI3* RNA in human hematopoietic tissues and cell lines. *EBI3* or β-actin probes were hybridized to blots containing RNA from the cell lines or tissues indicated at the top of each lane. Dashes indicate positions of rRNA bands (18S, 28S). (A) The 1.4-kb *EBI3* RNA is undetectable in the EBV(−) BL control cell lines BL41 and BL30 but is abundant in the BL41/B95-8 cell line, derived by in vitro infection of BL41 cells with the transforming B95-8 EBV strain (41). *EBI3* RNA is also present at high levels in the EBV-transformed LCLs IB4, LCL-W91 (W91), and SLA. A parallel blot hybridized with β-actin probe demonstrates that the BL41 lane contains at least as much RNA as the EBV-infected cell lanes do (lower panel). Jurkat and MOLT-4 are human T-cell leukemia lines; MARROW, SPLEEN, and TONSIL are from human tissue samples; PBMC are unfractionated peripheral blood mononuclear cells; PBMC-PWM are PBMC stimulated for 72 h with pokeweed mitogen (2.5 μg/ml). (B) HL60, U937, and K562 are human myeloid leukemia cell lines; TK143 is an osteosarcoma line; RHEK-1 and HeLa are epithelial cell lines.

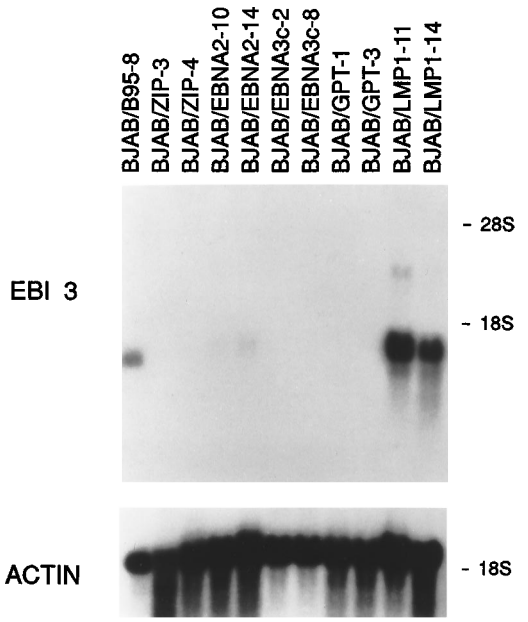

FIG. 5. *EBI3* RNA induction by EBV LMP-1. RNA from stably transfected EBV(−) BJAB B lymphocytes was hybridized with *EBI3* probe. *EBI3* RNA is undetectable in two independent control cell lines transfected with pZIPneo (BJAB/ZIP-3, BJAB/ZIP-4) or pSVgpt (BJAB/GPT-1, BJAB/GPT-3) expression plasmids and in cells transfected with EBNA2 (BJAB/EBNA2-10, BJAB/EBNA2-14) or EBNA3C (BJAB/EBNA3c-2, BJAB/EBNA3c-8) genes. *EBI3* RNA is abundant in two independent clones transfected with LMP-1 gene (BJAB/LMP1-11, BJAB/LMP1-14).

***EBI3* is expressed at high levels in EBV-transformed B lymphocytes, EBV-infected or LMP-1-expressing BL cells, and human placenta.** Hybridization of ^{32}P-*EBI3* probe to RNA blots detected a 1.4-kb mRNA in the EBV-transformed LCLs IB4, LCL-W91, and SLA and in the EBV-converted BL-cell line BL41/B95-8 (Fig. 4A, upper panel). *EBI3* RNA was undetectable in the EBV(−) control cell lines BL41 and BL30. Parallel blots hybridized with probes for β-actin (Fig. 4A, lower panel) or glyceraldehyde phosphate dehydrogenase (data not shown) demonstrated that comparable amounts of RNA were loaded in the EBV(−) and EBV(+) cell lanes. Densitometric quantitation of autoradiograph band intensities from similar blots indicated that BL41/B95-8 and IB4 cells contained at least 200-fold more *EBI3* RNA, relative to actin RNA levels, than did BL41 cells. *EBI3* RNA was undetectable in two T-cell leukemia lines, MOLT-4 and Jurkat, and in the myelocytic leukemia lines U937 and HL60 (Fig. 4B).

EBI3 RNA was detected at low levels in the nontransforming EBV mutant BL cell line P3HR1, in the EBV(−) BL line Louckes (Fig. 4A), and in several nonlymphoid cell lines, including K562, TK143, RHEK-1, and HeLa (Fig. 4B). The small amount of *EBI3* RNA detected in Louckes cells is consistent with the low level of expression by this cell line of several other proteins typically up-regulated by EBV, including vimentin, CD23, and CD21 (6, 62).

In human lymphoid tissues, *EBI3* RNA was present at low levels in normal unfractionated tonsil cells and at significantly higher levels in spleen cells but was undetectable in bone marrow and in resting PBMC. However, *EBI3* mRNA was induced in PBMC by stimulation with the B- and T-lymphocyte activating agent pokeweed mitogen (Fig. 4A).

EBI3 expression was analyzed in a series of EBV(−) BJAB cell lines stably transfected with different EBV latent genes. Expression of EBNA-2 or EBNA-3C failed to induce higher *EBI3* mRNA expression relative to that in parental BJAB or vector-transfected BJAB/neo or BJAB/gpt control cell lines (Fig. 5). However, cells converted to expression of the EBV latent membrane protein 1 (LMP-1) had high levels of *EBI3* mRNA, comparable to levels in EBV-transformed LCLs or EBV-infected BL41/B95-8 cells (Fig. 5). Similar analyses of transfectants in EBV(−) BL41 and Louckes cells also showed induction of *EBI3* gene expression by LMP-1 (data not shown).

The 1.4-kb *EBI3* RNA was detected in placenta at significantly higher levels than in lymphoid cells (Fig. 6) (data not shown). The placental *EBI3* mRNA was identical in size to *EBI3* RNA detected in lymphoid tissues and cell lines. *EBI3* RNA was also faintly detectable in liver, but immunoglobulin M heavy-chain RNA could also be detected in this tissue, indicating infiltration of the liver with B lymphocytes (5). *EBI3* RNA was not detectable in any other nonhematopoietic tissue.

Tissue expression of EBI3 was further analyzed by immunostaining with polyclonal antiserum. Staining of frozen sections of human placenta demonstrated EBI3 in trophoblast cells lining placental villi in a diffuse cytoplasmic staining pattern with perinuclear accentuation (Fig. 7A). Expression of *EBI3* by trophoblasts has been confirmed by in situ hybridization with an *EBI3* antisense RNA probe (data not shown). In human tonsil tissue, EBI3 was detected in scattered mononuclear cells of interfollicular zones (Fig. 7B). The EBI3-positive cells showed a reticular cytoplasmic staining pattern and had abundant cytoplasm and indented nuclei that were generally larger than nuclei of neighboring lymphocytes. Two-color immunofluorescence with rabbit EBI3 antiserum (Fig. 7C) and the mouse monoclonal antibody anti-CD22 (Fig. 7D) indicated that EBI3-positive cells failed to express the B-cell marker, CD22. The morphology and location of EBI3-producing cells suggest that these may be macrophages. In human spleen tissue EBI3-positive cells were identified in perifollicular zones surrounding periarteriolar sheaths and lymphoid follicles (Fig. 7E) and appeared to be associated with vascular spaces. Overall, EBI3-positive cells were significantly more abundant in spleen than in tonsil tissue, consistent with RNA blot analysis. No cell staining was observed in spleen or tonsil sections when either nonimmune normal rabbit serum or affinity-purified rabbit polyclonal anti-glutathione-*S*-transferase serum was used (data not shown).

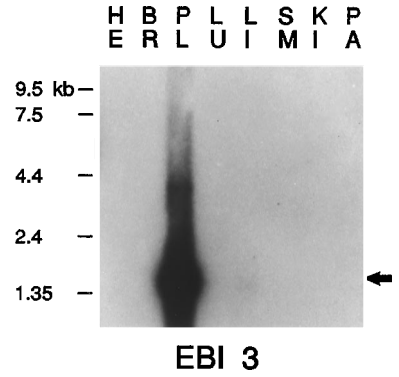

FIG. 6. *EBI3* RNA expression in human tissues. *EBI3* probe was hybridized to a blot (multiple-tissue Northern) containing polyadenylated RNA (2 μg per lane) from human heart (HE), brain (BR), placenta (PL), lung (LU), liver (LI), kidney (KI), skeletal muscle (SM), and pancreas (PA). The *EBI3* probe specifically detects an abundant 1.4-kb RNA in the placenta (arrow). A faint band of similar size is also observed in liver RNA but is not detectable in RNA from any other tissue. Numbers at the left indicate positions and sizes (in kilobases) of RNA markers.

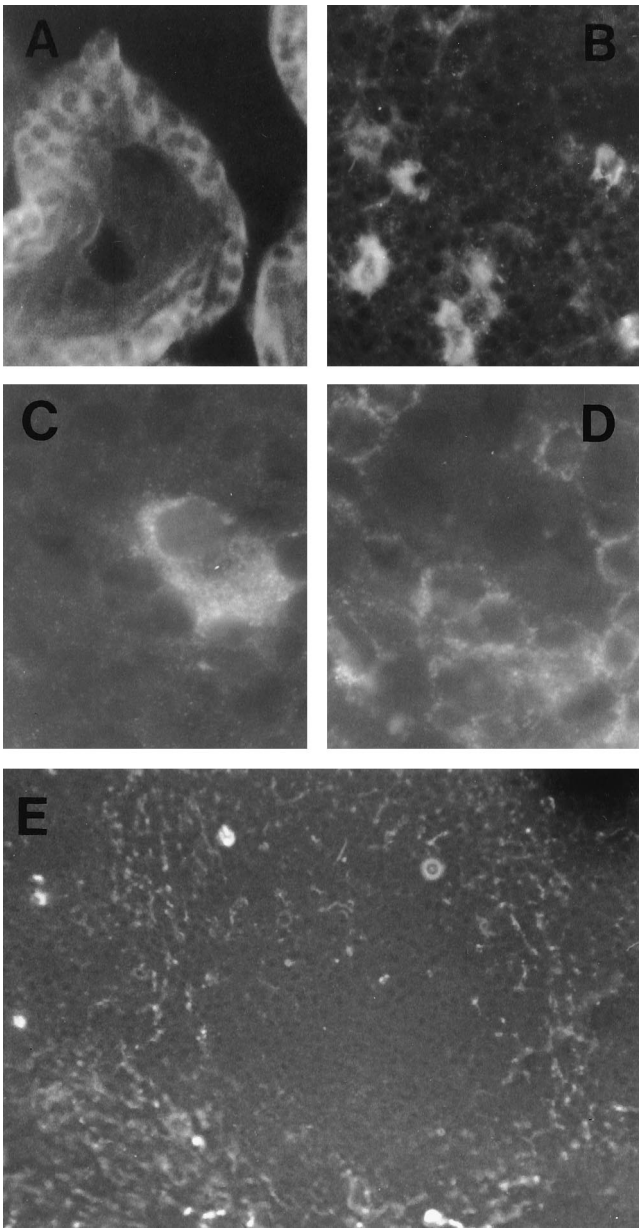

FIG. 7. EBI3 protein expression in human tissues. (A) Staining of postpartum placenta with rabbit EBI3 antiserum detects EBI3 only in syncytiotrophoblast cells lining chorionic villi. (B) Frozen sections of human tonsil stained with anti-EBI3 antibody demonstrate EBI3 expression in a minor population of mononuclear cells of interfollicular zones. (C) On high power, EBI3-producing tonsil cells have abundant cytoplasm and nuclei which are larger than most surrounding lymphocyte nuclei. (D) The same field shown in panel C was simultaneously stained with a monoclonal antibody specific for the B-cell marker CD22, which was detected with Texas red-conjugated goat anti-mouse antibody. The EBI3-positive cell fails to express CD22 antigen. (E) Staining of human spleen with EBI3 antiserum reveals a population of cells restricted to perifollicular zones. Magnification, ×400 (panels A, B, and E) and ×1,000 (panels C and D).

EBI3 is present in the cytoplasm and on the plasma membranes of producing cells. Live IB4 cells, or BJAB lymphocytes and COS7 cells transfected with *EBI3* cDNA cloned into a simian virus 40 promoter expression vector, were stained in suspension with rabbit polyclonal EBI3 antiserum. In IB4 lymphocytes, faint membrane fluorescence was observed (data not shown). More intense plasma membrane staining was observed in both BJAB B lymphocytes and COS7 cells transfected with *EBI3* cDNA (Fig. 8A and C). An identical pattern was observed in *EBI3-FLAG*-transfected BJAB or COS7 cells stained with the anti-FLAG M2 monoclonal antibody (data not shown). Fluorescence staining of live pSG5 vector-transfected control cells was not detected with EBI3 antiserum or anti-FLAG M2 antibody (data not shown). Staining of IB4 or of *EBI3*-transfected BJAB or COS7 cells was not observed when NRS was used.

Immunostaining of fixed IB4 cells (Fig. 8D) or *EBI3*-transfected BJAB (Fig. 8B) or COS7 cells (data not shown) revealed cytoplasmic fluorescence in a reticular pattern and weaker plasma membrane staining. No staining was observed in vector-transfected control cells or with NRS. The more intense cytoplasmic staining indicates that most cell-associated EBI3 protein is present in intracytoplasmic compartments, with the pattern of staining suggesting retention in the endoplasmic reticulum (ER).

EBI3 is a secreted glycoprotein. EBI3 antiserum identified a 33-kDa protein in immunoblots of placenta and of EBV(+) BL41/B95-8 and IB4 cells, but not in EBV(−) BL41 cells (Fig. 9). A protein of identical size was detected in lysates of BJAB or COS7 cells transfected with *EBI3* cDNA pSG5 expression plasmid but was not present in control COS7 or BJAB cells transfected with the pSG5 expression vector alone. These results confirm that the *EBI3* cDNA contains the complete reading frame. In addition to the predominant 33-kDa protein, 30- and 24- to 25-kDa proteins were detected in *EBI3*-transfected BJAB and COS7 cells. These are probably stable degradation products, because they are not observed after short periods of pulse-labeling and immunoprecipitation (see below; data not shown).

To determine how much of the apparent 33-kDa molecular mass of EBI3 is due to N-glycosylation, ^{35}S-EBI3 was immunoprecipitated from IB4 cell lysates and incubated in vitro with *N*-glycanase. This enzyme, which removes unprocessed as well as Golgi-processed N-linked sugars, reduced the apparent EBI3 molecular mass from 33 to 28 kDa (Fig. 10). Endoglycosidase H digestion, which removes exclusively high-mannose oligosaccharide, resulted in a quantitatively similar reduction, indicating that most of the cell-associated EBI3 has not been processed in the Golgi apparatus (data not shown).

In immunoblots of filtered supernatants from transiently transfected COS7 or BJAB cells, EBI3 was detected as a 34-kDa molecule that is slightly larger than cell-associated EBI3 (Fig. 9). No reactivity was detected in supernatants of pSG5 vector-transfected control cells. Similarly, a 34-kDa protein was specifically precipitated with polyclonal EBI3 antiserum from supernatants of [^{35}S]methionine-labeled, *EBI3*-transfected BJAB or COS7 cells (Fig. 11) but was not detected in immunoprecipitates of pSG5 vector-transfected control BJAB or COS7 cell supernatants or in supernatants precipitated with serum from nonimmunized rabbits (Fig. 11; data not shown). As observed in immunoblots (Fig. 9), the secreted protein was slightly larger than the protein from cell lysates.

EBI3 associates with calnexin and a 60-kDa protein. Proteins of 95 and 60 kDa coimmunoprecipitated with EBI3 from digitonin or NP-40 lysates of [^{35}S]methionine-labeled BJAB cells transfected with *EBI3-FLAG* (Fig. 12) or *EBI3* (data not shown). Proteins of identical size were detected in anti-FLAG M2 immunoprecipitates from *EBI3-FLAG*-transfected COS7 cells and in EBI3 immunoprecipitates from *EBI3*-transfected COS7 cells (data not shown) but were not precipitated with the M2 monoclonal antibody or EBI3 antibody from vector-transfected control BJAB or COS7 cells (Fig. 12; data not shown).

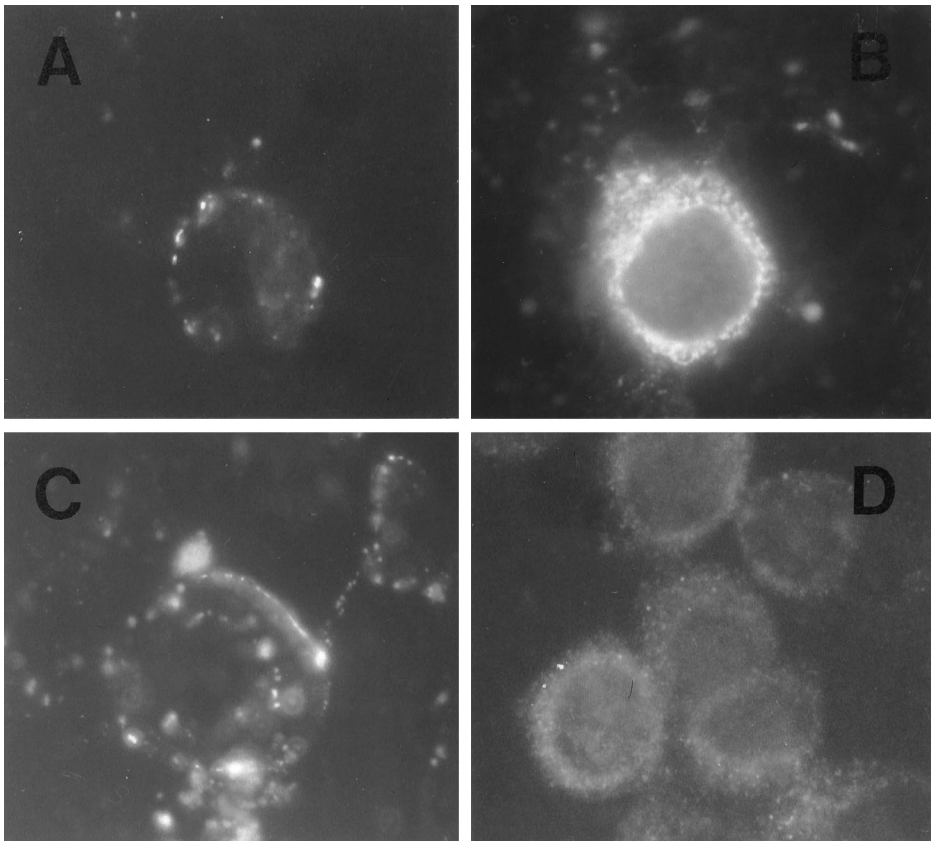

FIG. 8. Immunofluorescence detection of EBI3 in cells. (A) Staining of live *EBI3*-transfected BJAB cells in suspension results in detection of EBI3 protein on cytoplasmic membranes. (B and D) Staining of fixed *EBI3*-transfected BJAB cells (B) and IB4 cells (D) reveals EBI3 in a reticular cytoplasmic distribution. (C) Plasma membrane staining was also observed in COS7 cells transfected with *EBI3* cDNA and stained in suspension. No staining was observed in pSG5 vector-transfected control BJAB cells stained with EBI3 antibody or in EBI3-transfected cells stained with NRS (data not shown). Magnification, ×1,000.

The 95-kDa protein was also observed in immunoprecipitates from IB4 cells with EBI3-specific antiserum (data not shown). Migration of the 95-kDa protein was not substantially altered when immunoprecipitates were resolved under nonreducing conditions, indicating that this protein is not covalently associated with EBI3 by disulfide linkage (Fig. 12, lanes 5 and 6).

To characterize p95 and p60, both proteins were affinity purified by coimmunoprecipitation from BJAB cells stably transfected with *EBI3-FLAG*, separated by SDS-PAGE, and protease digested. Sequences for two peptides were obtained for each protein. The two p95 peptides, APVPTGEVYFADS FDRGTL and TPYTIMFGPDK, precisely matched peptide sequences from human calnexin, amino acids 42 to 60 and 163 to 173, respectively (19). Calnexin is a molecular chaperone resident in the ER and is known to associate transiently with many glycoproteins during their transport through the ER (19, 50; reviewed in reference 4). Staining of fixed, transiently *EBI3*-transfected BJAB cells with anti-calnexin antibody (monoclonal antibody AF8 [37]) confirmed pancytoplasmic expression (data not shown). Although live-cell immunostaining of these cells demonstrated membrane EBI3 expression, no membrane staining with calnexin antibody was detected, indicating that calnexin could account for EBI3 localization in the ER but does not account for EBI3 association with the plasma membrane.

The two p60 peptide sequences, LIESLSQMLSMGFSDE GG and EVDPSTGELQSLQMPES, were not identical to any sequences in the databases. A degenerate oligonucleotide probe complementary to the RNA sequence predicted to encode a portion of the first peptide was used to isolate p60 cDNA clones from the BL41/B95-8 cDNA library. The predicted p60 protein contains sequences which match identically both peptide fragments and has limited homology to the *Drosophila* Ref(2)P protein (21). However, no potential signal peptide for membrane translocation or transmembrane domain was identified. These results indicate that p60 is probably a cytoplasmic protein which may associate with EBI3 via the cytosolic domain of calnexin or some other, as yet unidentified EBI3-associated integral membrane protein.

In pulse-chase experiments, p95 calnexin and p60 showed similar kinetics of association with EBI3 (Fig. 13). This association was detectable within 10 min. The rapid kinetics of EBI3 association with p95 are similar to those previously shown for calnexin binding to T-cell receptor components (19). A protein of 78 kDa also associated with nascent EBI3. However, this association was transient.

DISCUSSION

This report describes a novel gene, *EBI3*, that is expressed in EBV-transformed lymphocytes and encodes a member of the hematopoietin receptor family related to IL-12 p40 and CNTFR. While most other members of this family have both integral membrane and soluble forms (reviewed in references 16 and 25), EBI3, IL-12 p40 (30, 66), and CNTFR (20) encode only isoforms which lack an integral membrane domain.

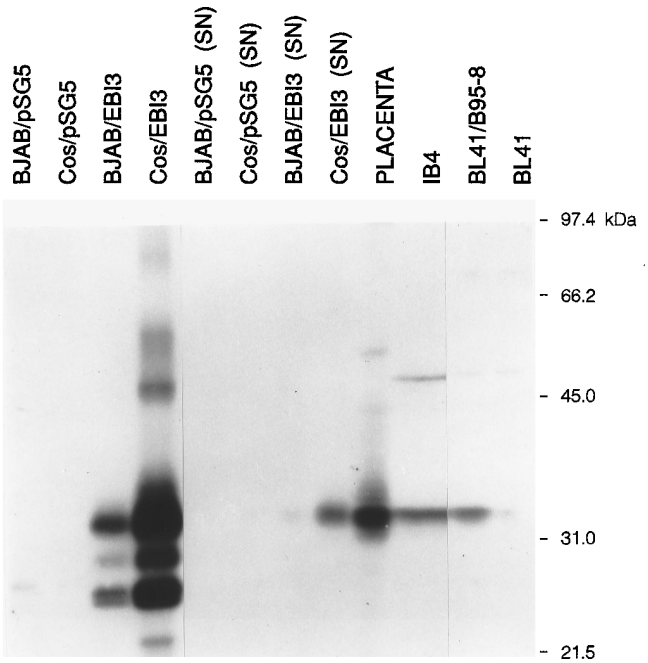

FIG. 9. Immunoblot detection of EBI3 in cell lysates and culture supernatants. EBI3 antiserum recognized a 33-kDa protein in extracts from fresh postpartum placenta and from EBV(+) BL41/B95-8 or IB4 cells which is not detected in extracts from EBV(−) BL41 cells. A protein of identical size was detected in lysates of BJAB or COS7 cells transfected with *EBI3* cDNA cloned into the expression vector, pSG5 (BJAB/pSG-*EBI3*, Cos/pSG-*EBI3*) but was not present in control COS7 cells transfected with the pSG5 expression vector alone (BJAB/pSG5, Cos/pSG5). Immunoblots of *EBI3*-transfected BJAB and COS7 cells also detected a protein of 30 kDa and a 24- to 25-kDa doublet. A 34-kDa protein was detected in filtered supernatants from COS7 cells transfected with *EBI3* cDNA [Cos/pSG-*EBI3* (SN)] but was undetectable in supernatants of pSG5 vector-transfected control cells [BJAB pSG5 (SN), Cos pSG5 (SN)]. With longer autoradiographic exposures, the 34-kDa EBI3 could also be detected in *EBI3*-transfected BJAB supernatants [BJAB/pSG-*EBI3* (SN)] but at lower levels than in COS7 supernatants.

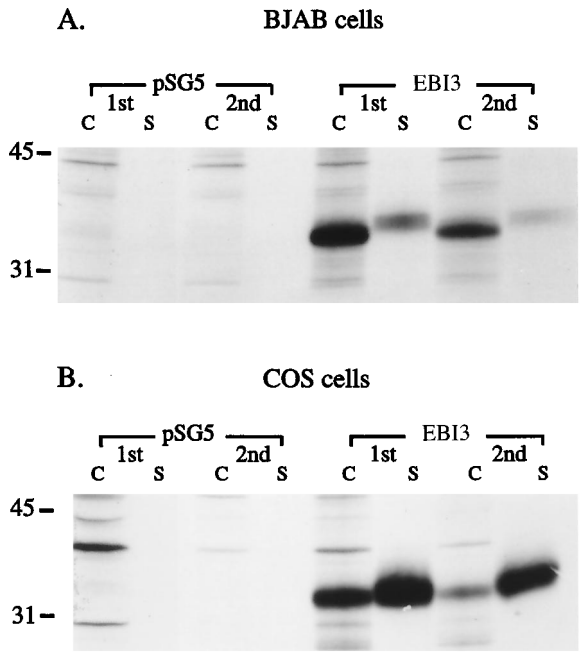

FIG. 11. Immunoprecipitation of EBI3 from cell lysates and supernatants. BJAB (A) or COS (B) cells were transfected with pSG5 vector alone or with *EBI3*-expressing pSG5 vector and labeled for 24 h with [^{35}S]methionine. Digitonin-solubilized cell lysates (lanes C) or cell culture supernatants (lanes S) were subjected to an initial immunoprecipitation with EBI3 antiserum (1st). Supernatants remaining after the initial precipitation were reprecipitated with EBI3 antiserum (2nd). As observed in the immunoblot analyses, the secreted protein is slightly larger than the protein from cell lysates. Numbers indicate positions of standard molecular weight proteins (in thousands).

CNTFR can be anchored to membranes via glycosyl-phosphatidylinositol linkage (20), whereas both EBI3 and IL-12 p40 lack a glycosyl-phosphatidylinositol addition signal.

On the basis of similarities to IL-12 p40, EBI3 function may relate to IL-12 activity. IL-12 is critical to the development of helper T-cell type 1 (T_H1), cytotoxic T-cell, and natural killer cell responses (reviewed in reference 61). EBV-transformed lymphocytes and placental syncytiotrophoblasts could secrete soluble EBI3 to modulate or antagonize IL-12 activity and alter natural killer or cytotoxic T-cell function. Early in the establishment of latent infection in B lymphocytes, EBV expresses nuclear and integral membrane proteins that induce proliferation of virus-infected cells. Since virus-encoded nuclear and membrane proteins are highly immunogenic for cytotoxic T cells, modulation of T-cell activity by elaboration of EBI3 from proliferating cells could facilitate initial expansion of the latently infected cell pool. The importance of modulating host immune responses during this critical step in infection is supported by observations that EBV has incorporated a close homolog of human IL-10 into its genome (45) and that EBV infection induces expression of cellular IL-10 by B lymphocytes (11). IL-10 is a potent regulator of cell-mediated immune responses and inhibits IL-12 synthesis by accessory cells (17). EBI3 is expressed at its highest levels in the placenta, where it may play a similar role. Since embryonic tissues express paternal alloantigens, defense against maternal immune attack is critical for maintenance of fetal implantation. It has been previously shown that cytokines promoting T_H2 activity are synthesized at the maternal-fetal interface (42; reviewed in reference 63); however, their significance in suppressing maternal alloresponses toward fetal antigens is uncertain.

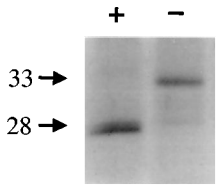

FIG. 10. EBI3 is N-glycosylated. ^{35}S-labeled extracts from IB4 cells were immunoprecipitated with EBI3 antiserum, treated with (+) or without (−) *N*-glycanase, and separated by SDS-PAGE. Removal of N-linked sugars causes a shift in the apparent molecular mass of EBI3 from 33 to 28 kDa (arrows).

EBI3 also differs from IL-12 p40 in several important aspects. IL-12 p40 is secreted from B lymphocytes and macrophages both as a disulfide-linked heterodimer with the p35 subunit of IL-12 (30, 66) and in monomeric form as excess free p40 (18, 43). While EBI3 has all four cysteines implicated in intramolecular disulfide linkage, it lacks the additional cysteines that could mediate p40 heterodimerization with p35. Moreover, despite the absence of a membrane-anchoring motif, EBI3 can be detected on plasma membranes of EBV-transformed lymphocytes and of EBV(−) cells transfected with *EBI3* cDNA. The latter finding suggests that EBI3 can associate with a membrane protein(s). A similar mechanism for membrane localization has been demonstrated for other

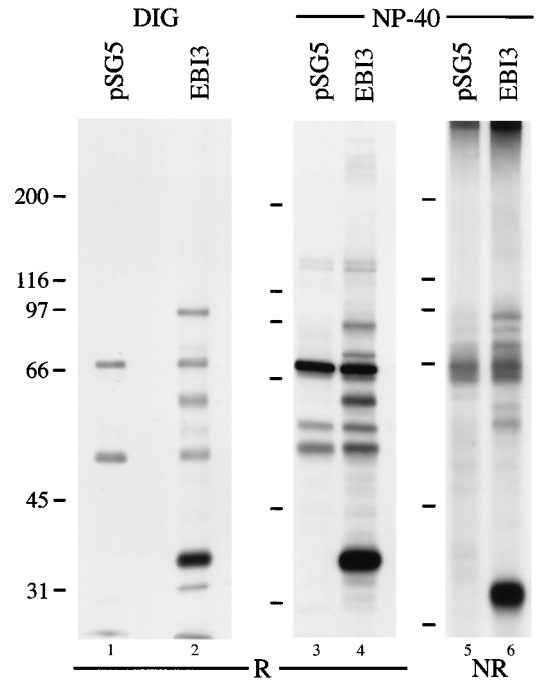

FIG. 12. EBI3 coimmunoprecipitates with 95- and 60-kDa proteins. [^{35}S]methionine-labeled BJAB cells transfected with pSG5 expressing *EBI3-FLAG* or a pSG5 control vector were lysed with 1% digitonin (DIG) or 1% NP-40. Cell extracts were immunoprecipitated with anti-FLAG M2 antibody, and the immunoprecipitates were separated on an SDS–8% polyacrylamide gel under reducing (R) or nonreducing (NR) conditions. Numbers at left indicate positions of standard molecular weight proteins (in thousands).

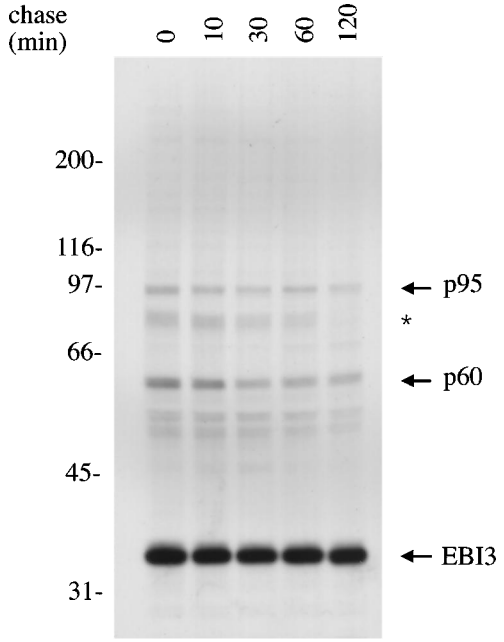

FIG. 13. Kinetics of EBI3 association with p95 and p60. Stably *EBI3-FLAG*-transfected BJAB cells were metabolically labeled with [^{35}S]Met-[^{35}S]Cys for 10 min and chased for the times indicated. After the chase, cells were solubilized with 1% NP-40 and lysates were immunoprecipitated with anti-FLAG M2 antibody. Immunoprecipitates were separated on an SDS–8% polyacrylamide gel under reducing conditions. In addition to p60 and p95, a protein of about 78 kDa was observed (asterisk).

secreted proteins, such as lymphotoxin-α, which can associate noncovalently during its biosynthesis with the integral membrane glycoprotein, lymphotoxin-β, to form a membrane complex (9).

Attempts to characterize a putative EBI3 membrane complex have been hindered by the substantial amount of ER-associated EBI3. In this regard, EBI3 resembles the hematopoietin receptor, EpoR, which is not efficiently processed in normal or transfected hematopoietic cells, with most nascent molecules being retained and degraded in the ER (36). EBI3 retention in the ER is most probably related to its stable association with calnexin, an integral ER membrane molecular chaperone with specificity for glycoproteins (19, 50; reviewed in reference 4). Calnexin binding can retain polypeptide subunits in the ER until assembly of a multimeric protein complex has occurred, and it appears to be important in the formation of the T- and B-cell antigen receptor complexes (19, 37, 51), class I major histocompatibility complex antigens (27, 37), and the vesicular stomatitis virus G glycoprotein trimers (31).

EBI3 transport to the Golgi apparatus may be limited by its need to associate with a less abundant protein. Similar ER retention has been shown for the lutropin β subunit, which requires association with the α subunit for ER exit and secretion (15), and for the p35 subunit of IL-12, which requires the p40 subunit for efficient secretion (30, 66). In light of similarities between EBI3 and p40, one simple hypothesis would be that EBI3 associates noncovalently with p35 or a related molecule to form a secreted heterodimer. Experiments to address this question directly are in progress.

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