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The Epstein-Barr Virus Nuclear Antigen Leader Protein Associates with hsp72/hsc73

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Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) is important for primary B-lymphocyte growth transformation. We now demonstrate that the W repeat-encoded domain of EBNA-LP significantly associates with proteins of the heat shock protein 70 family (hsp72/hsc73). hsp72/hsc73 may mediate the previously observed interaction between EBNA-LP and the retinoblastoma protein or p53.

The Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) plays a critical role in EBV-induced transformation (4, 9). EBNA-LP mRNA is composed of repeating 22- and 44-amino-acid-encoding exons (the W repeats) and two unique exons (Y1 and Y2) encoding the C-terminal 45 amino acids (15, 21). EBV recombinants with a 45-amino-acid C-terminal truncated EBNA-LP due to a deletion of the Y1 and Y2 exons yielded 10- to 20-fold-fewer cell colonies in a fibroblast feeder layer soft agar assay than virus stocks derived from recombinant EBVs expressing an intact EBNA-LP (4). Recombinant viruses with a 45-amino-acid C-terminal deletion or stop codon truncation of EBNA-LP were even more markedly impaired in experiments using an endpoint dilution clonal transformation assay (9).

The mechanism by which EBNA-LP affects transformation remains unknown although in vitro biochemical data have indicated a possible interaction with the retinoblastoma protein (RB) or p53. Glutathione-*S*-transferase (GST)-RB and GST-p53 bacterial fusion proteins can specifically bind a small fraction of EBNA-LP from cell lysates. Conversely, GST–EBNA-LP can bind approximately 1% of an RB bacterial fusion protein or an unknown fraction of p53 (19). To further elucidate EBNA-LP molecular interactions, we have immunoprecipitated EBNA-LP from cell extracts, sequenced an EBNA-LP-associated protein, and found that EBNA-LP associates with the heat shock protein 70 family (hsc73 and hsp72).

The IB4 EBV-infected lymphoblastoid cell line (LCL) was labeled overnight with [^{35}S]methionine, and cellular proteins were extracted with an isotonic salt buffer (50 mM Tris [pH 8], 170 mM NaCl, 0.5% Nonidet P-40, 10 mg of apoprotinen per ml, 1 mM phenylmethylsulfonyl fluoride). The cellular proteins were then immunoprecipitated with an anti-EBNA-LP monoclonal antibody (JF186) (2) or an irrelevant anti-CD39 monoclonal antibody (AC2) (14), separated on an 8% polyacrylamide gel, and visualized with an autoradiograph. A 70-kDa protein was consistently present in the EBNA-LP monoclonal antibody immunoprecipitates but not in the irrelevant control monoclonal antibody immunoprecipitates, suggesting that this protein specifically associates with EBNA-LP (Fig. 1A). As can be seen in Fig. 1A, several other proteins were also immunoprecipitated by the EBNA-LP monoclonal antibody. However, these proteins were not evaluated because they were not consistently immunoprecipitated from EBNA-LP-positive cell lines (e.g., 22 and 56 kDa), were immunoprecipitated from EBNA-LP-negative cell lines (e.g., 160 kDa), or were immunoprecipitated with irrelevant antibodies (e.g., 42 and 100 kDa). EBNA-LP is not detected by the autoradiograph because it contains only one methionine and therefore is not labeled efficiently with [^{35}S]methionine. When polyacrylamide gels containing EBNA-LP immunoprecipitates were silver stained, both EBNA-LP and the 70-kDa protein were detected at approximately equal intensities relative to their molecular masses, suggesting that a large fraction of EBNA-LP is associated with the 70-kDa protein (Fig. 1B). The 70-kDa protein was not immunoprecipitated with the anti-EBNA-LP monoclonal antibody from an LCL expressing an EBNA-LP variant not recognized by the monoclonal antibody, indicating that precipitation of the 70-kDa protein is EBNA-LP dependent (Fig. 1B). These data suggest that the 70-kDa protein is specifically and significantly associated with EBNA-LP.

In order to identify the 70-kDa protein, a large-scale immunoprecipitation was done with 4×10^9 IB4 cells. Immunoprecipitated proteins were again separated on an 8% polyacrylamide gel. A gel lane containing a fraction of the immunoprecipitated proteins was silver stained to confirm that the 70-kDa protein as well as EBNA-LP had been efficiently precipitated. Proteins in the remaining gel lanes were transferred to nitrocellulose and stained with Ponceau S. Three discreet bands were detectable by Ponceau S staining: a band with a size of approximately 40 kDa corresponding to EBNA-LP, a 55-kDa band corresponding to immunoglobulin heavy chain, and a 70-kDa band (data not shown). The 70-kDa Ponceau S-stained nitrocellulose band was cut from the filter. At the Harvard Microchemistry Facility, oligopeptides were digested from the band with endopeptidase Lys-C. The resultant oligopeptides were separated on a high-performance liquid chromatography column. Several similar narrow peaks in the elution profile were subjected to matrix-assisted laser desorption mass spectrometry. One peak consisted of a homogeneous 2,846-Da peptide. Sequencing of the peptide yielded 28 residues. The sequence, DAGTIAGLNVLRIINEPTAAAIAYGLDK, was compared with the protein sequence database, and a perfect match to an internal sequence of the human heat shock cognate protein hsc73 was found. The peptide sequence also matched heat shock proteins from several other species.

To confirm that hsp70 family members interact with EBNA-LP, EBNA-LP was immunoprecipitated from the IB-4 or X9 LCLs with the JF186 anti-EBNA-LP monoclonal antibody or control antiserum. X9 is an LCL infected with a wild-type recombinant EBV. As a negative control, the anti-EBNA-LP monoclonal antibody was used to immunoprecipitate proteins

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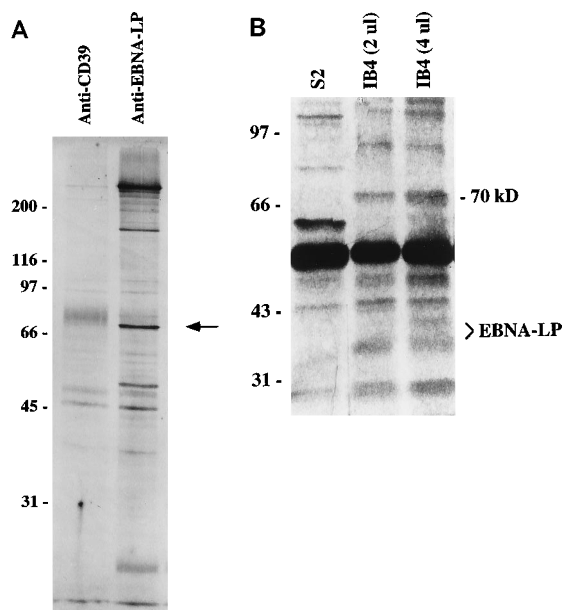

FIG. 1. A 70-kDa protein is coimmunoprecipitated with EBNA-LP. (A) [^{35}S]methionine-labeled IB4 cells (20×10^6) were immunoprecipitated with an anti-EBNA-LP monoclonal antibody (Anti-EBNA-LP) or a control anti-CD39 monoclonal antibody (Anti-CD39). The arrow indicates a 70-kDa protein which was consistently present in EBNA-LP immunoprecipitates but not in CD39 immunoprecipitates. Molecular size markers (in kilodaltons) are indicated on the left. (B) EBNA-LP immunoprecipitations were performed as described for panel A, with the IB4 or S2 LCL. S2 is a cell line which expresses an EBNA-LP not recognized by the anti-EBNA-LP monoclonal antibody. The S2 lysate was immunoprecipitated with 2 μl of the anti-EBNA-LP monoclonal antibody, and the IB4 lysate was immunoprecipitated with 2 μl [IB4 (2 μl)] or 4 μl [IB4 (4 μl)] of the monoclonal antibody. The bands corresponding to EBNA-LP and the 70-kDa protein are indicated. The size of EBNA-LP varies as a result of heterogeneous splicing that generates transcripts with different numbers of W repeats in each cell (2).

from an EBV-negative cell line (BJAB) or from the S2 LCL which expresses an EBNA-LP which is not recognized by the monoclonal antibody. The immunoprecipitated proteins were analyzed for hsp72 and hsc73 expression by Western blot (immunoblot), using a rabbit polyclonal antiserum which recognizes both human hsp72 and hsc73 (18). hsp72 and hsc73 are the two major members of the hsp70 family in the cytosol and nuclei of human cells and share considerable homology. hsc73 is constitutively expressed whereas hsp72 synthesis is induced as a result of cellular stress and during early S phase (10, 11). The EBNA-LP immunoprecipitates from the IB4 or X9 LCL contained hsp72/hsc73 while immunoprecipitates with a control monoclonal antibody did not (Fig. 2). The amount of coimmunoprecipitated protein was estimated by visually comparing the intensity of the band on Western blot with 10-fold serial dilutions of whole-cell lysates (data not shown). In three separate experiments, approximately 0.5 to 1% of the total cellular hsp72/hsc73 was immunoprecipitated from IB-4 or X9 cells by the EBNA-LP monoclonal antibody. The monoclonal antibody precipitated the majority of EBNA-LP in these experiments (data not shown). hsp72/hsc73 was not detected in immunoprecipitates of the negative control cell lines BJAB and S2 (Fig. 2A). To confirm that hsp72/hsc73 did not associate with all EBV nuclear proteins, immunoprecipitates of EBV nuclear antigen 2 (EBNA2) were blotted for hsp72/hsc73. Immunoprecipitation of the majority of EBNA2 yielded no detectable hsp72/hsc73 (data not shown).

Since the amino acid sequence of the 70-kDa EBNA-LP-

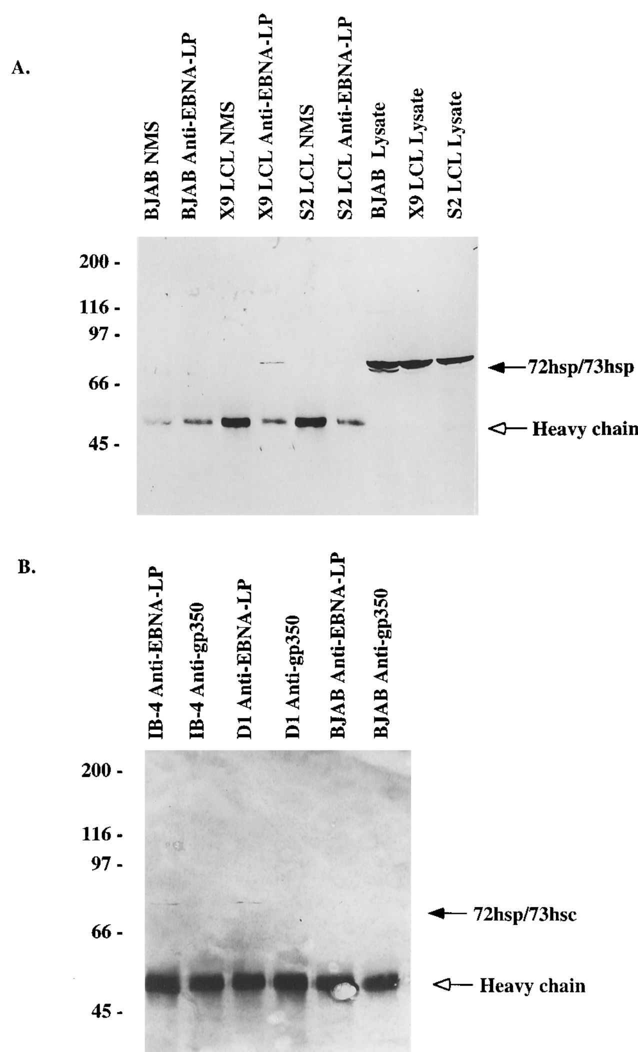

FIG. 2. EBNA-LP immunoprecipitates contain hsp72/hsc73. (A) Cells (20×10^6) were immunoprecipitated with normal mouse serum (NMS) or with an anti-EBNA-LP monoclonal antibody (Anti-EBNA-LP). The immunoprecipitated proteins were then analyzed by Western blot for hsp72/hsc73 expression (72hsp/73hsc) by using a rabbit polyclonal antiserum which recognizes both hsp72 and hsc73. BJAB is an EBV-negative B-cell line. X9 is an EBV-infected LCL which expresses an EBNA-LP recognized by the EBNA-LP monoclonal antibody. S2 is an EBV-infected LCL which expresses an EBNA-LP variant not recognized by the monoclonal antibody. hsp72/hsc73 levels in whole-cell lysates from 2×10^6 cells are shown (BJAB lysate, X9 LCL lysate, and S2 LCL lysate). The Western blots were developed by using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Promega) which detects the immunoglobulin heavy chain used in the immunoprecipitations (Heavy chain). Molecular size markers (in kilodaltons) are indicated on the left. (B) The experiment was performed as described for panel A except cells were immunoprecipitated with a monoclonal antibody directed against the EBV gp350 protein (20) as a negative control (Anti-gp350). IB4 is an LCL infected with a wild-type EBV. D1 is an LCL infected with a recombinant EBV which expresses a carboxy-truncated EBNA-LP.

associated protein was identical to that of hsc73, at least part of the interaction of EBNA-LP with hsp72/hsc73 is due to an interaction with hsc73. To determine whether EBNA-LP also associated with hsp72, EBNA-LP immunoprecipitates from IB-4 cells were analyzed by Western blot by using a rabbit polyclonal antiserum which detects only hsp72. hsp72 was detected in the immunoprecipitates (data not shown), indicating that EBNA-LP associates with both hsp72 and hsc73. Recip-

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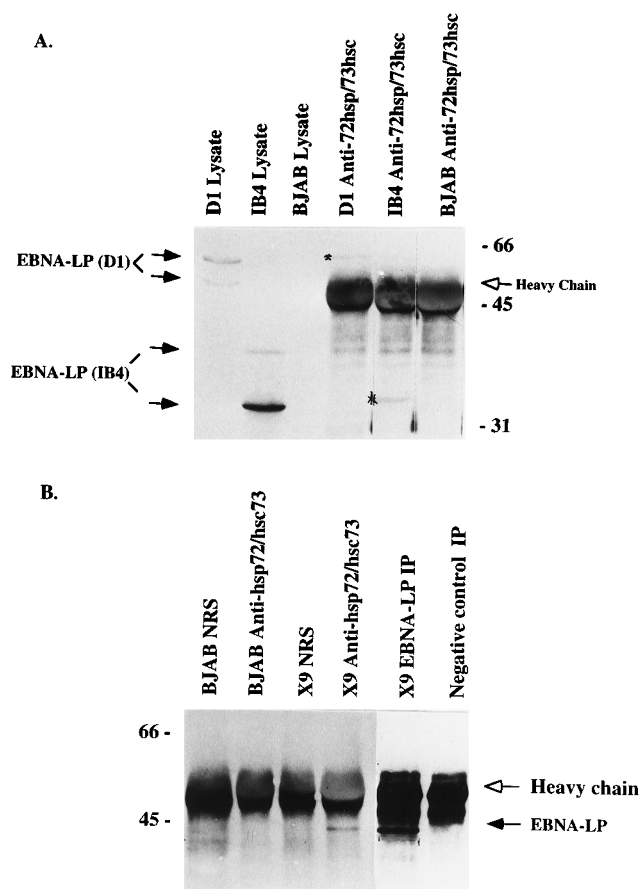

FIG. 3. Hsp72/hsc73 immunoprecipitates contain EBNA-LP. (A) Cells (20×10^6) were immunoprecipitated with a rabbit polyclonal antiserum directed against hsp72/hsc73 (Anti-72hsp/73hsc) or with normal rabbit serum (NRS). EBNA-LP was detected in immunoprecipitates by Western blot analysis using an anti-EBNA-LP monoclonal antibody. The cell lines are described in the legend to Fig. 2. EBNA-LP levels in whole-cell lysates from 2×10^6 cells are shown (D1, IB4, and BJAB lysates). The size of EBNA-LP varies between cell lines and within a given cell line as a result of heterogeneous splicing that generates transcripts with different numbers of W repeats in each cell (2). The sizes of EBNA-LP in the D1 [EBNA-LP (D1)] or IB4 cell lysates [EBNA-LP (IB4)] are indicated. EBNA-LP in each hsp72/hsc73 immunoprecipitate is indicated by an asterisk. The Western blots were developed by using an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Promega) which detects the immunoglobulin heavy chain (Heavy chain) used in the immunoprecipitations. Molecular size markers (in kilodaltons) are indicated on the right. (B) The same experiment was performed as described for panel A, with the X9 LCL described in the legend to Fig. 2A. The size of EBNA-LP in the X9 cell line was determined by Western blot analysis of an EBNA-LP immunoprecipitate of 20×10^6 X9 cells (X9 EBNA-LP IP) because the level of EBNA-LP expressed in X9 cells is low. As a negative control immunoprecipitation, the EBNA-LP monoclonal antibody was used to immunoprecipitate proteins from the S2 cell line which expresses an EBNA-LP not recognized by the monoclonal antibody (Negative control IP).

rocal immunoprecipitations were then performed with the polyclonal rabbit antiserum specific for hsp72/hsc73. The hsp72/hsc73-specific antiserum yielded between 1 and 5% of the total cellular EBNA-LP from the IB-4 or X9 LCL but not cross-reactive proteins from control BJAB cells (Fig. 3). In the same experiments, the hsp72/hsc73 antiserum precipitated less than 1% of the Nonidet P-40-extractable hsp72/hsc73 (data not shown), suggesting that a much larger fraction of EBNA-LP is associated with hsp72/hsc73 in this extract.

To determine whether hsp72/hsc73 interacts with the repeating or unique carboxy-terminal residues of EBNA-LP, EBNA-LP was immunoprecipitated from an LCL which expresses a carboxy-truncated EBNA-LP due to a deletion of the Y1 and Y2 exons (9). Immunoprecipitation of this carboxy-truncated EBNA-LP yielded hsp72/hsc73 (Fig. 2B), and hsp72/hsc73 immunoprecipitates yielded this carboxy-truncated EBNA-LP (Fig. 3A). These results indicate that hsp72/hsc73 associates with the repeating component of EBNA-LP.

In retrospect, the association of EBNA-LP and hsp70 might be anticipated from the finding that peptides composed of at least seven residues that include large hydrophobic and basic amino acids, but few or no acidic residues, bind with high affinity to at least three hsp70 molecular chaperones (3). The EBNA-LP W2 exon encodes an oligopeptide, Leu-Gly-Gln-Leu-Leu-Arg-Arg-His-Arg, which meets these criteria.

These studies add EBNA-LP to a growing list of transformation-associated proteins which interact with the hsp70 family. Other members of this group include p53 (5, 13), RB (12), c-*myc* (7), v-*rel* (8), the adenovirus protein E1A (22), and the simian virus 40 large T antigen (T) (16). The hsp70 family is involved in protein folding, nuclear translocation (17), and assembly of multimeric protein complexes. hsc73/hsp72 may be serving one or more of these functions during its association with EBNA-LP. The repeating component of EBNA-LP is responsible for the association with hsp72/hsc73, and its role in EBV-induced transformation has not been evaluated.

The repeating component of EBNA-LP is also the domain which associates with RB or p53 in vitro (19). RB and p53 appear to bind to the same EBNA-LP site. RB association with EBNA-LP is different from its association with simian virus 40 T, human papillomavirus E7, or E1A since these other proteins have an LXCXE RB interaction motif which EBNA-LP lacks. In addition, a mutant GST-RB fusion protein which cannot bind to T and E1A can still bind to EBNA-LP (6, 19). Moreover, both mutant and wild-type GST-RBs bind only a small fraction of EBNA-LP, and GST–EBNA-LP binds to only a small fraction of trpE-RB. Since RB and p53 associate with the hsp70 family, hsp72/hsc73 may mediate the interaction between RB or p53 and EBNA-LP. The interactions between RB and EBNA-LP, which have been detected by using isolated bacterial fusion proteins (19), may also be due to an interaction with hsp70 family members because heat shock proteins are highly evolutionarily conserved (23), and the bacterial hsp70 homolog (DnaK) can interact with murine p53 (1). In our study, RB could not be detected in EBNA-LP immunoprecipitates, with the limits of detection being 1% of the total cellular RB. Further, EBNA-LP could not be detected in RB or p53 immunoprecipitates, with the limits of detection being 0.5% of the total cellular EBNA-LP (data not shown). The binding of EBNA-LP to hsp72/hsc73 and the binding of hsp72/hsc73 to p53 or RB may account for the association of EBNA-LP with p53 or RB.

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