A PEPTIDE BINDING PROTEIN HAVING A ROLE IN ANTIGEN PRESENTATION IS A MEMBER OF THE HSP70 HEAT SHOCK FAMILY

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Immune responses to globular protein antigens require the interaction of helper T cells with B cells. Helper T cell recognition of such antigens requires their uptake and processing, followed by presentation of antigen fragments by APCs that express class II gene products (Ia) of the MHC (1). The molecular mechanisms underlying antigen presentation are believed to involve the internalization of antigen into acidic vesicles where proteolysis occurs, generating a peptide that is transported to and held on the APC surface. The peptide, together with Ia, interacts with the specific T cell at the T cell receptor. Antigenic peptides have been shown to bind to purified Ia in solution (2, 3). However, it is not known if such peptides bind directly to Ia intracellularly, or whether any accessory structures facilitate peptide-Ia association. We have previously identified a peptide binding protein of 72/74 $\times$ 10^3 M_r (PBP72/74) that plays a role in antigen presentation, as shown by the ability of an antiserum against PBP72/74 to block APC presentation of the antigen cytochrome *c*, to a cytochrome *c*-specific T cell hybrid (4). Here we show that PBP72/74 shares several major characteristics of the HSP70 family of heat shock proteins. HSP70 proteins have been shown to associate with newly synthesized (5–7), unfolded, aberrantly folded, or aberrantly glycosylated proteins (8–11), and to bind to ATP with high affinity (10, 12–14). In addition, many HSP70 proteins are recognized by the rat mAb 7.10 raised against *Drosophila* HSP70, which is specific for an evolutionarily conserved epitope of these proteins (15). Similarly, PBP72/74 has been demonstrated to bind to a peptide fragment of pigeon cytochrome *c* (P*c* 81-104), but not to this peptide region on the surface of the native protein, and thus appears to be specific for some feature of the peptide not available in the native structure (4). Here we show that PBP72/74 binds ATP and is released from binding to P*c* 81-104 by ATP. PBP72/74 is recognized in Western blot by mAb 7.10 as well as by mAb N27 (16) specific for both the constitutively expressed and inducible 72/73 $\times$ 10^3 M_r HSP70 proteins. Thus, it appears that characteristics of heat shock proteins are shared by a protein playing a role in antigen presentation, suggesting similarities in function.

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Materials and Methods

Mice. CBA/J mice, 6–8 wk old, were obtained from The Jackson Laboratory, Bar Harbor, ME.

Antibodies and Cell Lines. mAb 7.10 was a kind gift of Dr. Susan Linquist (University of Chicago, Chicago, IL). mAbs N27 and N15 (17) were generated and kindly provided by Dr. William Welch (University of California, San Francisco, CA). Rabbit antisera to peptide affinity-purified PBP72/74 were generated as previously described (4). Briefly, rabbits were immunized three times at 3-wk intervals with freshly prepared affinity-purified PBP72/74 from detergent solubilized spleen cells (antiserum 12/86) or from hypotonically lysed spleen cells (antiserum 7/88), first in CFA and subsequently in incomplete adjuvant and bled before immunization or 7 d after the last immunization. LK35.2, a H-$2^{d/k}$ B cell hybridoma APC line, was developed by Kappler et al. by somatic cell fusion of the BALB/c lymphoma cell line L10.A2J and T cell–depleted spleen cells of B10.BR mice (18). TP*c* 9.1 is a mouse T cell hybridoma specific for pigeon cytochrome *c* (P*c*) presented in the context of the I-E^k molecule (19). The CTLL line, an IL-2-dependent cell line, was described by Gillis et al. (20).

Preparation and Analysis of PBP72/74. Soluble PBP72/74 was prepared as follows. Approximately 10^9 LK35.2 cells were lysed hypotonically at a concentration of 10^7 cells/ml in a buffer of 10 mM Tris-HCl, pH 8.5, 5 mM EDTA, 1 mM PMSF for 1 h on ice. NaCl was added to a final concentration of 0.15 M, the lysate was centrifuged at 100,000 *g* and 33 ml supernatant was applied to a P*c* 81-104–Sepharose column prepared by coupling 6 mg P*c* 81-104 to 1 g CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to manufacturer's instructions. Flow through material was collected and reserved for repassage over the column. Columns were washed with ~30 column volumes of 10 mM Tris-HCl, pH 8.5, 5 mM EDTA buffer containing 0.5 M NaCl. PBP72/74 was eluted from the column with a 2.8% acetic acid, pH 5, containing 0.5 M NaCl. 1-ml fractions were collected, and the first five fractions with a pH lower than 8.5 were neutralized immediately with 2 M Tris-HCl, pH 9, pooled, and subsequently concentrated to 300–500 μl, and the buffer was changed to 0.15 M NaCl using Centricon concentrators (Amicon Corp., Danvers, MA). The column was then cleaned with 5 column volumes of pH 3 acetic acid containing 0.5 M NaCl and reequilibrated with pH 8.5 wash buffer. Reserved flow through material was reapplied to the column, the column was washed, and PBP72/74 was eluted as described. PBP72/74 fractions from the entire LK35.2 lysate were pooled and ~5% analyzed by SDS-PAGE on a 10% polyacrylamide gel. Detergent solubilized LK35.2 was prepared similarly except that all buffers contained 0.5% NP-40 (LKB Instruments, Gaithersburg, MD) and the lysis buffer contained the 0.15 M NaCl during the initial incubation before centrifugation. Western blotting was carried out as follows. Affinity-purified PBP72/74 obtained from the hypotonic lysate of ~5 × 10^7 LK35.2 cells was subjected to SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose blocked with blotto, incubated with mAbs diluted 1:50 to 1:100 or with rabbit sera diluted 1:16 in 100 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and washed. Bound antibodies were detected using biotinylated, affinity-purified antibodies to mouse or rabbit antibodies and developed with peroxidase-avidin and diaminobenzidene plus nickel chloride as a substrate (Vectastain; Vector Laboratories, Burlingame, CA) following the manufacturer's instructions.

Measurement of T Cell Activation. B cells used as APC were prepared from spleen cells of CBA/J mice by depletion of red blood cells by centrifugation on Ficoll-Hypaque gradients and T cells by treatment with mAbs antibodies directed toward Lyt-2, Thy-1, and L3T4 and rabbit serum as a source of complement, as described (19). The resulting cell population was >95% Ig^+ and unresponsive to Con A. B cells (2 × 10^5) were incubated with the P*c*-specific T cell hybrid TP*c*9.1 (5 × 10^4 cells) in 0.2 ml cultures in the presence of P*c* and dilution of rabbit serum. IL-2 was measured by the incorporation of [^{3}H]thymidine by the IL-2-dependent cell line CTLL.

Detection of Cell Surface PBP72/74. Splenic B cells (8 × 10^6/ml) were cultured with or without LPS (50 μg/ml) for 24 h, nonadherent cells were harvested, and dead cells were removed by Ficoll Hypaque density centrifugation. Cells were washed with cold PBS containing 0.02% NaN_3 (PBS-NaN_3) and 5% FCS (PBS-NaN_3-FCS), incubated at 4°C for 1 h with

preimmune rabbit sera or PBP72/74-specific rabbit sera, washed with PBS-NaN_3-FCS, incubated with 100 μl fluoresceinated goat anti-rabbit Ig (150 μg/ml) at 4°C for 30 min, washed with PBS-NaN_3-FCS, and fixed with 1% formaldehyde. Cells were analyzed using a FAC-Scan microfluorimeter (Becton Dickinson & Co., Mountain View, CA).

Analysis of ATP Binding by PBP72/74. The effect of ATP on PBP72/74 bound to peptide was analyzed as follows. LK35.2 cells (5×10^8) were hypotonically lysed as described, and the lysate was applied to a 1-ml Pc 81-104 Sepharose column and washed with 100 ml of wash buffer-1 (50 mM Tris HCl, pH 8.0, 0.5 M NaCl). Bound material was eluted in succession with 5 ml of wash buffer-1 containing 1 mM $MgCl_2$ and either 1 mM AMP + 1 mM NaPPi, 1 mM ADP + NaPi or 1 mM ATP, followed by 5 ml of wash buffer containing 1 mM $MgCl_2$. The column was then washed with 10 ml of 0.5 M acetic acid, pH 2.5, containing 0.3 M NaCl, and the acid eluate adjusted to neutral pH with Tris base. Each sample was concentrated to 60 μl in Centricon-30 microconcentrators, and 30 μl of each sample was electrophoresed in a 7.5% SDS-polyacrylamide gel. Proteins were either visualized by silver staining or electrophoretically transferred to nitrocellulose and probed with mAb 7.10.

The effect of peptide on PBP72/74 bound to ATP was analyzed as follows. LK35.2 cells (3×10^8) were hypotonically lysed as above, the lysate was adjusted to 20 mM $MgCl_2$ and applied to a 2.5 ml ATP agarose column (Sigma Chemical Co., St. Louis, MO) that had been equilibrated with 50 mM Tris-HCl (pH 8.5) containing 0.15 M NaCl and 20 mM $MgCl_2$. The column was washed with 200 ml of a 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl and 20 mM $MgCl_2$ (wash buffer-2), the resin was divided into two 1-ml columns and eluted with either 2.5-ml aliquots of the wash buffer-2 containing 1, 5, 10, 25, 50, or 100 μM Pc 81-104 followed by 2.5 ml of wash buffer-2 containing 10 mM ATP or with wash buffer-2 containing 10 mM ATP alone. The eluates were concentrated in Centricon-30 microconcentrators to a final volume of 100 μl and 50 μl of each was electrophoresed in a 10% SDS-polyacrylamide gel and proteins were visualized by Western blotting, probing with mAb 7.10.

Results and Discussion

Serological Identity of PBP72/74 as a Member of the HSP70 Family. PBP72/74 has been shown previously to be present in a variety of different mouse tissues and cell lines (4) and for the studies presented here was isolated from the mouse antigen-presenting B cell line LK35.2. PBP 72/74 obtained by affinity chromatography on Pc 81-104-Sepharose of LK35.2 cells that have been solubilized in detergent appears by SDS-PAGE and Coomassie blue staining as two or three discrete bands between 72 and 74 $\times 10^3$ M_r (Fig. 1 *A*), as was previously shown for [^{35}S]methionine-labeled affinity-purified PBP72/74 (4). Similar proteins are obtained by peptide affinity chromatography of LK35.2 cells that have been hypotonically lysed and whose membranes have been removed by centrifugation (Fig. 1 *A*), indicating that at least a portion of PBP72/74 exists in water soluble form. As seen in Fig. 1 the number and intensity of the bands obtained from detergent solubilized and hypotonically lysed cells is not identical. However, such differences are not consistently observed and the relative amount of each band obtained by affinity chromatography varies somewhat from preparation to preparation following either method of purification.

To establish a relationship between PBP72/74 and the HSP70 family of proteins, Western blots of peptide affinity-purified PBP72/74 from LK35.2 cells were probed with rabbit antiserum to PBP72/74 or normal rabbit sera or with mAb 7.10, mAb N27, or mAb N15 (Fig. 1, *B* and *C*). Rabbit antisera raised to affinity-purified PBP72/74 had been shown previously (4) to block the activation of cytochrome *c* to a cytochrome *c*-specific T cell hybridoma and to immunoprecipitate PBP72/74 from whole cell lysates. mAb 7.10 reacts with most members of the HSP70 family,

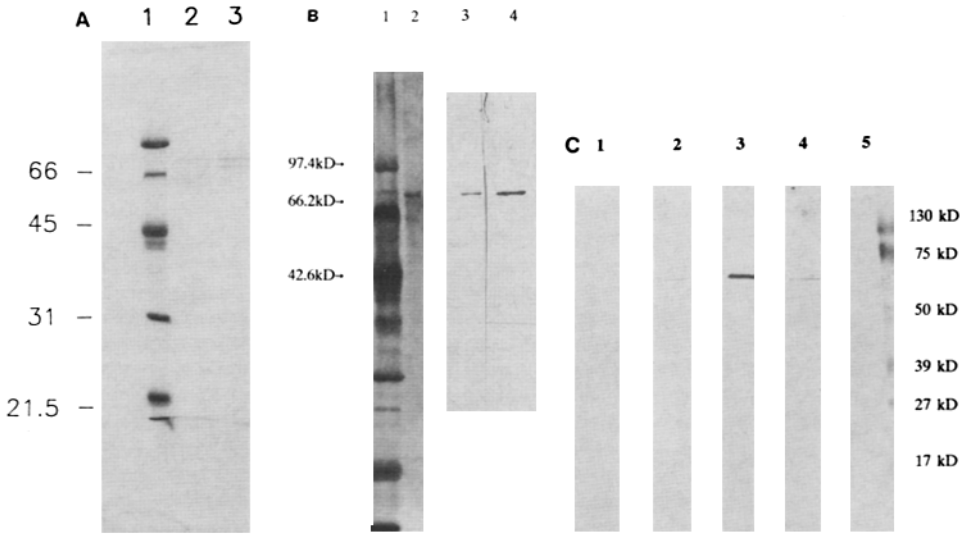

FIGURE 1. Affinity purification and serological identification of PBP72/74 as a member of the HSP70 family. (*A*) SDS-PAGE and Coomassie blue staining of PBP72/74 isolated from hypotonically lysed LK35.2 cells (lane *2*) and from detergent solubilized LK35.2 (lane *3*). Molecular weight standards are shown in lane *1*. (*B*) Western blot analysis of peptide affinity-purified PBP72/74. SDS-PAGE and Coomassie blue staining of PBP72/74 isolated from hypotonically lysed LK35.2 cells and eluted from peptide columes at pH 5 (lane *2*) and the eluted material in Western blot probed with PBP72/74-specific rabbit antiserum 7/88 (lane *3*) or with mAb 7.10 (lane *4*). Molecular weight standards (lane *1*). (*C*) Western blot analysis of affinity-purified LK35.2 PBP72/74 probed with normal rabbit sera (lane *1*), PBP72/74-specific rabbit antiserum 7/88 (lane *2*), mAb 7.10 (lane *3*), mAb N27 (lane *4*), mAb N15 (lane *5*). Similar staining is observed using antiserum 12/86. Molecular weight standards shown on far right.

while mAb N27 (16) and mAb N15 (18) are more restricted. N27 recognizes both the constitutive 73 × 10^3 M_r and stress-induced 72 × 10^3 M_r HSP70 proteins, and N15 is specific for the induced 72 × 10^3 M_r protein. The SDS-PAGE profile of the preparation of PBP72/74 analyzed in Western blot (Fig. 1 *B*) shows a predominant protein of 74 × 10^3 M_r and faintly staining bands of 72 × 10^3 M_r. The rabbit antisera, mAb 7.10 and mAb N27 bind strongly to PBP72/74, while the normal rabbit antiserum and mAb N15 show no binding. In addition, a mAb directed toward the Ig heavy chain binding protein (BIP, GRP78) (5) does not recognize affinity-purified PBP72/74 by immunoprecipitation, even though it can be shown to precipitate a 78 × 10^3 M_r protein from ^{35}S-labeled LK35.2 cell lysates (data not shown).

Identification of PBP72/74 on B Cell Surfaces. As shown above, the rabbit antiserum to PBP72/74 recognizes peptide affinity-purified PBP72/74 as does mAb 7.10. This antiserum had been shown previously to block the activation of a cytochrome *c*–specific T cell hybridoma by cytochrome *c* pulsed paraformaldehyde fixed B cells (4), suggesting that it may function, at least in part, at the cell surface. Antiserum 12/86, prepared against PBP72/74 from detergent solubilized cells and antiserum 7/88 raised in rabbits to hypotonically lysed spleen cells block the presentation of cytochrome *c* by splenic B cells to the cytochrome *c*–specific T cell hybrid TP*c*9.1 (Fig. 2 *A*) and

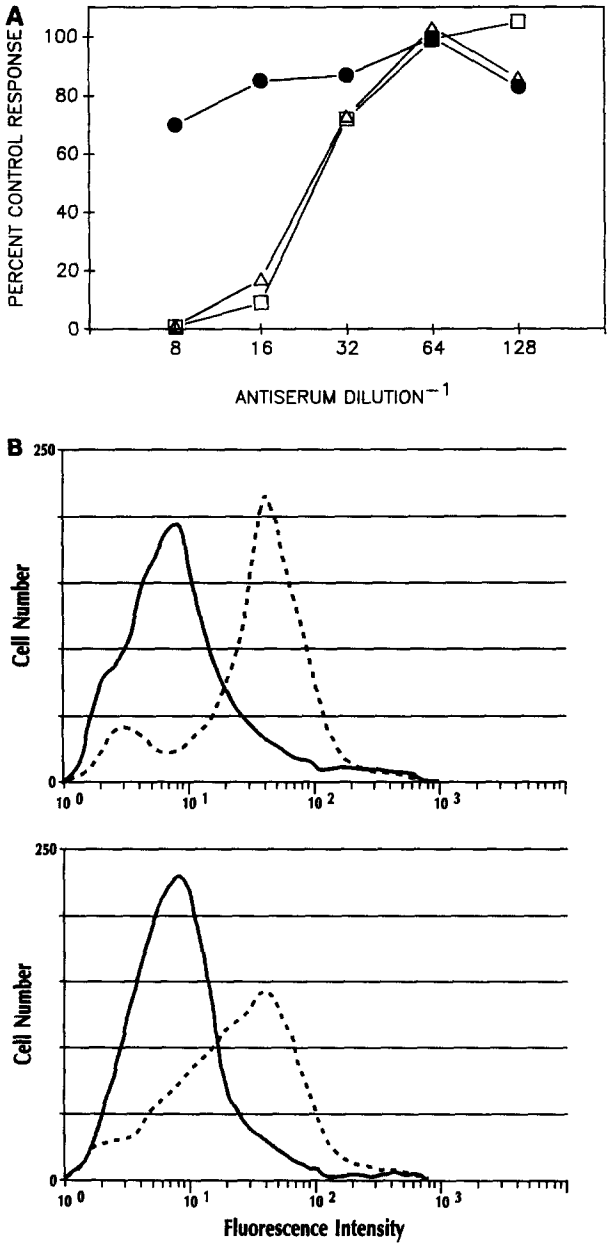

FIGURE 2. Antisera raised in rabbits to affinity-purified PBP72/74 blocks the presentation of Pc to a Pc-specific T cell hybrid and stains resting and activated B cells. (*A*) Splenic B cells as APC were pulsed with Pc, washed and cultured with TPc9.1 cells in the presence of Pc and dilutions of normal rabbit serum (●) or of rabbit sera generated to affinity-purified PBP: Antiserum 12/86 (△) or Antiserum 7/88 (□). Culture supernatants were tested 24 h later for presence of IL-2 and results expressed as a percent of the response where no antiserum was added. Control cultures that contained no serum yielded 24,500 ± 400 cpm/culture. (*B*) B cells prepared from spleens of resting (*top panel*) and LPS-activated (*bottom panel*) B cells stained with preimmune rabbit sera (—) or PBP72/74-specific rabbit serum 7/88 (---) followed by fluoresceinated-goat anti-rabbit antibodies were analyzed by flow cytometry. Shown is the fluorescence intensity versus cell number. Similar staining is observed using Antiserum 12/86.

stain the B cell surface (Fig. 2 *B*). Indeed, B cells incubated with the rabbit antisera and fluoresceinated goat anti-rabbit antibodies show fluorescent staining as analyzed by flow cytometry (Fig. 2 *B*). The staining is detectable on resting splenic B cells and on B cells treated with the mitogen LPS for 24 h (Fig. 2 *B*). Thus, a portion of PBP72/74 appears to be constitutively present on B cell surfaces that may function in antigen presentation.

Analysis of ATP and Peptide Binding by PBP72/74. A common feature of the HSP70 family of proteins is their ability to bind ATP with high affinity. Thus, all members of this family are readily purified by affinity chromatography on ATP-agarose. Furthermore, ATP disrupts complexes formed between BIP and the μ heavy chain (12) and BIP is apparently unable to bind to certain inappropriately folded or glycosylated proteins in the presence of ATP (10). Another member of the HSP70 family catalyzes the ATP-dependent depolymerization of clathrin from coated vesicles in vitro (13) and ATP causes the release of the major heat inducible HSP70 from its binding site in the nucleolus (14). A subset of HSP70 proteins in yeast have been shown to be involved in the ATP-dependent translocation of precursor polypeptides into mitochondria (6) and into the lumen of the endoplasmic reticulum (6, 7). Similarly, PBP72/74 is completely eluted from the Pc 81-104-Sepharose column by solutions containing 1 mM ATP at pH 8.0 and by 1 μM ATP at pH 7.4, but not by solutions containing AMP with NaPPi or ADP with NaPi at the same concentrations (Fig. 3 and data not shown). Material eluted from the peptide column with ATP is recognized by the mAb 7.10 (Fig. 3). Since elutions are performed in 0.5 M NaCl the association of ATP with PBP72/74 is unlikely to be nonspecific.

PBP72/74 can also be isolated on an ATP-agarose column and eluted with ATP (Fig. 4) or with Pc 81-104, in a concentration-dependent fashion (Fig. 4). However, peptide does not elute all ATP binding proteins, as shown with subsequent ATP elution (Fig. 4). Experiments are in progress to further determine the relationship of ATP binding and hydrolysis to the release of PBP72/74 from the peptide substrate.

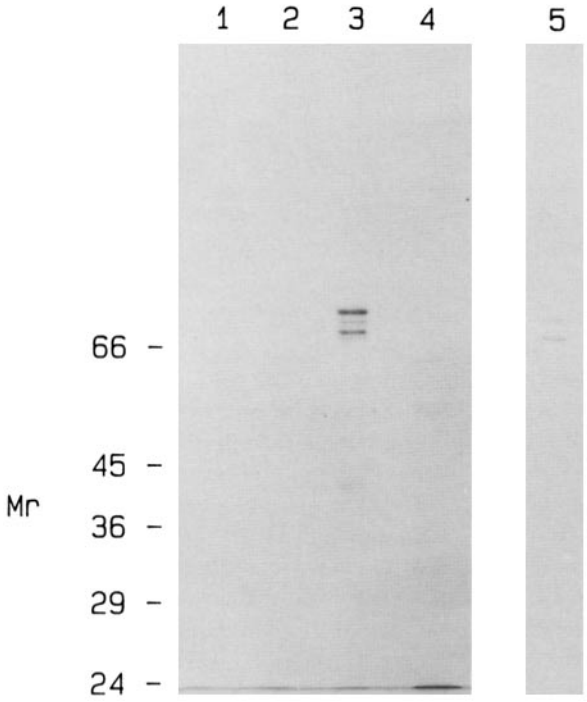

FIGURE 3. Effect of ATP on PBP72/74 peptide binding. PBP72/74 is eluted from peptide columns by ATP (lane *3*) but not by AMP + NaPPi (lane *1*) or by ADP + NaPi (lane *2*). ATP removes all bound PBP72/74 as no material is eluted with subsequent treatment with an acid pH 2.5 (lane *4*). The material eluted with ATP is recognized by the mAb 7.10 in Western blot (lane *5*).

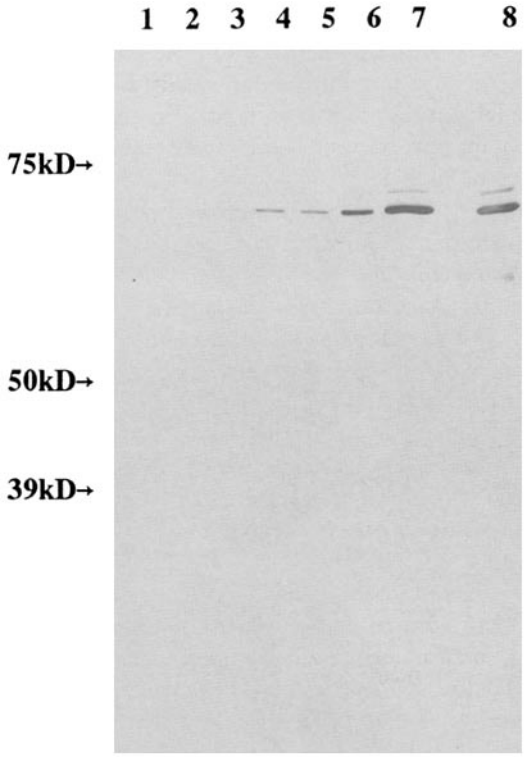

FIGURE 4. PBP72/74 is eluted from ATP-agarose columns by Pc 81-104. Hypotonic lysates of LK35.2 cells were applied to ATP-agarose columes, washed and successively eluted as described in Materials and Methods with Pc 81-104 at: 1 μM (lane *1*), 5 μM (lane *2*), 10 μM (lane *3*), 25 μM (lane *4*), 50 μM (lane *5*), or 100 μM (lane *6*) followed by 10 mM ATP (lane *7*). Alternatively, PBP is eluted by 10 mM ATP (lane *8*). Eluates are shown in Western blot probed with mAb 7.10.

The findings presented here indicate that a protein having the characteristics of the HSP70 family of heat shock proteins, PBP72/74, has a role in the presentation of processed antigen to antigen-specific T cells. However, the precise mechanisms by which PBP72/74 facilitates antigen presentation is not known; neither is it known what relation PBP72/74 bears to the binding of T cell antigenic peptides to MHC molecules. The evidence for peptide-Ia binding is as follows. The crystal structure of a class I MHC protein shows peptide material bound in a groove between two α-helices (21, 22), and because of structural similarities between MHC class I and class II molecules, it has been proposed that a similar peptide binding site occurs in the latter (23). Synthetic peptides bind to detergent solubilized, purified class II molecules (2, 3) and there is a correlation between MHC restriction and peptide binding to specific class II proteins (2, 3). A similar correlation can be shown between low molecular weight material from purified, acid-treated, class II molecules and their MHC specificity (24). On this basis, it has been proposed that the MHC molecules may function as intracellular peptide binding proteins, associating with newly processed antigen in the appropriate intracellular compartments and transporting the peptide to the cell surface for recognition by the specific T cell. A problem with this concept is that the binding of peptide to MHC molecules has unusual properties (3), in that the rate of association is extremely slow and once bound dissociation is nearly impossible, peptide remaining bound during the rather stringent conditions used in the purification of MHC molecules. Such rates suggest that unusual conditions may be necessary for peptide-MHC association in the cell or that addi-

tional structures may be required to facilitate the binding event. These may arise from the association of processed antigen with the plasma membrane (25) or with peptide binding structures such as PBP72/74 (4). Additional evidence for the role of non-Ia accessory structures in the presentation of processed antigen comes from studies in which the ability of nonstimulatory peptides to block the T cell response to cytochrome *c* as processed and presented by B cells was assessed (26, 27). In this case no correlation was found between the ability of a peptide to compete with processed antigen for binding sites on the APC surface and the ability of the nonstimulatory peptide to bind to a particular MHC molecule.

Several recent findings from other laboratories also suggest a role for heat shock proteins in immune responses. The HSP70 proteins have been shown to be major antigenic determinants in *Schistosoma mansoni* infections (28) and in leprosy and tuberculosis (29). Born and co-workers identified a HSP65 protein from *Mycobacterium tuberculosis* as an antigen for γ/δ-expressing T cells (30). In addition, a high degree of spontaneous IL-2 secretion was observed by such T cells, which was attributed to crossreaction with a cell surface–expressed mammalian heat shock protein. Serum from patients with systemic lupus erythematosus have been shown to frequently contain antibodies specific for constitutively expressed 73 kD member of the HSP70 family (31). In addition, HSP70 proteins were shown to be present on stimulated but not on unstimulated human HL60 cells, on a portion of macrophages from joint effusions of individuals with rheumatoid arthritis and on alveolar macrophages lavaged from a patient with interstitial fibrosis (32). Lastly, the human major histocompatibility complex has been shown to contain genes for HSP70 proteins (33). Taken together, these obsevations and the findings presented here are intriguing and suggest a role for members of the heat shock family in T cell responses to soluble protein antigens, bacterial and parasite antigens, and in autoimmune responses to self antigens.

We would propose that PBP72/74 may bind to denatured protein or peptides and subsequently facilitate their association with MHC molecules intracellularly or following transport to the cell surface. Indeed, the HSP proteins would appear to be expressed both intracellularly and on the cell surface as described here and as indicated by recent studies of Ottenhoff et al. (34) and by Jarjour et al. (32). The mechanism by which the HSP proteins associate with the plasma membrane or whether the intracellular form is identical to the cell surface form is not known. The function proposed here for PBP72/74 is analogous to that postulated for HSP70 proteins in the intracellular binding of at least partially denatured or unfolded proteins or peptides so as to allow them to maintain a needed conformation (35). ATP binding and/or hydrolysis would be necessary for either the binding of HSP70 to or release from its substrate. The advantage of such a mechanism may be as simple as transporting and concentrating the peptide at the cell surface before MHC binding, or as complex as aiding in the actual binding of peptide to MHC molecules. The extent to which proteins such as PBP72/74 are involved in the presentation of T cell antigens in general remains to be determined.

The identification of PBP72/74 as a member of the HSP70 family of heat shock proteins and the fact that there are a large number of available natural, recombinant and synthetic, relatively simple substrates for it, may allow a precise definition of the properties required of a heat shock protein binding. The availability of serological reagents for HSP70 proteins may allow for their localization in the antigen pro-

cessing pathway, particularly in those compartments in which antigen degradation occurs.

Summary

The T cell recognition of gobular protein antigens requires the processing and presentation of the antigen by Ia-expressing APCs. Processing is believed to involve the uptake of antigen into an acidic compartment where proteolysis occurs. The resulting peptides containing the T cell antigenic determinant are associated with Ia and presented at the cell surface to the specific T cells. The mechanisms by which antigenic peptides become associated with Ia is not known. We previously described a peptide binding protein of 72/74 × 10^3 M_r (PBP72/74) that plays a role in antigen presentation as shown by the ability of an antiserum raised in rabbits to affinity-purified PBP72/74 to block presentation of cytochrome *c* to a cytochrome *c*–specific T cell hybrid. Here we show that PBP72/74 is recognized by mAbs specific for members of the HSP70 family of proteins. In Western blots PBP72/74 is bound by mAb 7.10, specific for an evolutionarily conserved epitope of HSP proteins and by mAb N27, specific for both the constitutively expressed and inducible 72/73 × 10^3 M_r HSP70 proteins. In addition, PBP72/74 shares a second common feature of the HSP proteins, that of binding to ATP. Indeed, ATP causes the release of PBP72/74 from binding to a peptide fragment of cytochrome *c* (P*c* 81-104) and PBP72/74 can be eluted from ATP columns by P*c* 81-104. Finally, a portion of PBP72/74 is shown to be present on B cell surfaces by immunofluorescence staining. Thus, it appears that characteristics of the heat shock proteins are shared by a protein playing a role in antigen presentation, suggesting some commonality in function.

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