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Mechanistic Studies of the Degradation of Juvenile Hormone Esterase in *Manduca sexta*

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The mechanisms of degradation of juvenile hormone esterase (JHE) were investigated in larvae of the tobacco hornworm, *Manduca sexta*. JHE is removed from the hemolymph by the pericardial cells by receptor-mediated endocytosis and is ultimately degraded in the lysosomes. Immunoprecipitation experiments and native PAGE followed by Western blotting showed that JHE associates with a putative heat shock cognate protein (Hsp). Approximately 25% of the active JHE in the pericardial cell complex is associated with the putative Hsp 1 h postinjection of affinity purified JHE. Electron microscope analysis revealed that the putative Hsp is located in the *trans*-Golgi network of pericardial cells, where it is hypothesized to be involved in sorting of proteins destined for the lysosomes, from those destined for the cell membrane. Data acquired from immunoprecipitation and Western blotting experiments argue against the involvement of ubiquitin in the degradation of JHE. Injection of radiolabeled JHE into larvae of *M. sexta* followed by SDS-PAGE of pericardial cell homogenates revealed covalent binding of an unidentified protein to JHE in the pericardial cell complex. Arch. Insect Biochem. Physiol. 34:275–286, 1997.

Key words: ***Manduca sexta*; juvenile hormone esterase; protein degradation; heat shock protein**

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INTRODUCTION

Production and Degradation of JHE

Juvenile hormone esterase (JHE) (EC 3.1.1.1) is produced by the fat body and epidermal tissues (Wroblewski et al., 1990) at certain times during the development of lepidopteran larvae. JHE hydrolyzes juvenile hormone (JH) in the hemolymph and is partially responsible for regulation of the titer of JH (Hammock, 1985; Touhara et al., 1995). Regulation of the titer of JH is critical for larval development in the Lepidoptera (Jones, 1995).

On the basis that overexpression of JHE at an inappropriate time would disrupt larval development, baculoviruses were genetically engineered to express recombinant JHE (Hammock et al., 1990). The cDNA sequence used for JHE expression was derived from *Heliothis virescens* (Hanzlik et al., 1989). Despite high level expression of the recombinant JHE in vivo, no enhancement of insecticidal activity of the virus was seen compared to the wild-type, nonengineered virus (Bonning et al., 1992). Pharmacokinetic analysis of injected JHE in the hemolymph of both *M. sexta* and *H. virescens* demonstrated that removal is a saturable process and was hypothesized to be receptor-mediated (Ichinose et al., 1992a). Electron microscope analysis demonstrated that recombinant JHE is taken up by the pericardial cell complex and becomes concentrated in the lysosomes, where it is presumed to be degraded (Booth et al., 1992; Ichinose et al., 1992b).

We have further tested the hypothesis that JHE is removed from the hemolymph by receptor-mediated endocytosis and transported to lysosomes for degradation. Our studies support the preliminary data for this hypothesis, as opposed to removal by passive filtration and ubiquitin-dependent degradation in the cytosol. In addition, we demonstrate binding of JHE to two proteins during the endocytosis-lysosomal degradation pathway; the first is a putative heat shock cognate protein (Hsp) hypothesized to be essential for lysosomal targeting of JHE, and the second protein, which binds covalently to JHE, is of unknown identity.

MATERIALS AND METHODS

Electrophoresis and Immunoblotting

In order to test the hypothesis of degradation by selective uptake by receptor-mediated endocytosis vs. passive uptake followed by ubiquitin-based degradation in the cytosol, larvae were injected with affinity purified recombinant JHE and pericardial cell homogenates analyzed by Western blotting. Recombinant JHE was produced by infection of Sf21 cells (Vaughn et al., 1977) with the virus AcJHE (Bonning et al., 1992) using standard techniques (King and Possee, 1992). Cells were cultured in 50 ml spinner flasks in ExCell 401 medium (JRH Biosciences, Woodland, CA) containing 3% fetal calf serum (Intergen, Neshanic Station, NJ) and 100 U penicillin and 100 μg streptomycin per milliliter. The medium containing the recombinant JHE was diluted 1:2 with purification buffer (Shiotsuki et al., 1994), and the recombinant JHE was affinity purified by using a trifluoroketone column (3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone [MBTFP]) (Shiotsuki et al., 1994). JHE was eluted from the column by using 3-*n*-pentylthio-1,1,1-trifluoro-2-

propanone (PTFP) with 0.1% *n*-octyl β-D-glucopyranoside (OG). Following concentration with Centricon-30 microconcentrators (Amicon, Beverly, MA), the detergent and inhibitor were removed by dialysis as described previously by Shiotsuki et al. (1994).

Fourth stadium larvae of *M. sexta* were injected into a proleg with affinity purified recombinant JHE (25–50 μl of 1.5–10 μmoles [^{3}H]JH III hydrolyzed / min / ml or bovine serum albumin (BSA) (0.5 mg / ml) by using a fine glass capillary. Fat body samples and pericardial cell complexes were dissected out 1 h postinjection as described previously (Ichinose et al., 1992b) and homogenized in 20 μl sample buffer (20 mM Tris HCl, pH 6.8, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) on ice. Samples from test and control (uninjected) larvae were either immunoprecipitated with anti-JHE antiserum or loaded directly onto polyacrylamide gels. For immunoprecipitation, 5–15 μl of the homogenate (in a total volume of 15 μl) was immunoprecipitated with 1 μl anti-JHE antiserum and Immunobead second antibody reagent (BioRad, Hercules, CA) (200 μl). The immunoprecipitates or homogenized tissue samples were loaded onto 7.5% native polyacrylamide or 7.5% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose and detected using the Enhanced ChemiLuminescence technique (Amersham Corp., Arlington Heights, IL) with the anti-JHE, anti-Hsp, or anti-ubiquitin (Haas and Bright, 1985) antisera. The anti-JHE antiserum which was raised to JHE derived from *H. virescens* did not recognize the JHE of *M. sexta* under the conditions employed. The anti-Hsp antiserum (universal anti-Hsp 70 antiserum) was raised to the peptide sequence TVPAYFNDSQ-RQATDA, which is highly conserved in the Hsp70 family from multiple taxa. Gels and Western blots were repeated for analysis of tissues from at least four larvae for each test condition.

Immunoprecipitation of JHE

Fourth stadium larvae of *M. sexta* were injected with JHE or BSA as described above. Pericardial cells were homogenized, and immunoprecipitation was carried out as described above with 1 μl of anti-Hsp antiserum or 1 μl of anti-JHE antiserum. Crude recombinant rabbit carboxylesterase (CBE) (20 μl; 47 mmol α-naphthyl acetate hydrolyzed / min / ml) from insect cell culture was also injected and immunoprecipitated as a control enzyme using the same procedure. The rabbit CBE was cloned from rabbits based on the published protein sequence (Korza and Ozols, 1988). It was expressed in baculovirus and produced as described above. CBE was selected as a control for JHE since it is also a serine esterase and is in the same family of α / β-fold enzymes. CBE is of similar size to JHE and is expressed well in the baculovirus expression system. The amount of catalytic activity in the original homogenate, in the precipitated pellet, and in the supernatant was determined by radiochemical assay for JHE (Hammock and Sparks, 1977) for JHE and BSA injections or by using α-naphthyl acetate for CBE precipitations.

Electron Microscope Analysis of JHE and Hsp

For analysis of JHE uptake into the pericardial cell complex, fourth stadium larvae of *M. sexta* were injected with affinity purified JHE (10–20 μl of

37.5 nmoles [^{3}H]JH III hydrolyzed / min / ml). Pericardial cells were dissected out 1 h postinjection and fixed in 1% gluteraldehyde (EM grade). Sections were labeled with immunogold using anti-JHE antiserum as described previously (Booth et al., 1992). Sections were either labeled before embedding to label the basal surface of the pericardial cells or on-section immunogold labeling was carried out for detection of JHE within the cytoplasm of the pericardial cells.

For analysis of the intracellular location of the putative Hsp, tissue samples from uninjected larvae were fixed and stored in 2.3 M sucrose in PBS for cryosectioning. On-section immunogold labeling was carried out by using the universal anti-Hsp70 antiserum or preimmune serum.

Analysis of Radiolabeled JHE

Recombinant radiolabeled JHE was produced in insect cell culture as follows: Two 175 ml flat cultures were seeded with 10^{7}Sf21 cells (Vaughn et al., 1977) in cell culture medium as described above. Cells were allowed to settle for 2 h prior to infection at 5 pfu / cell with the recombinant baculovirus AcJHE (Bonning et al., 1992). Eight hours postinfection, the medium was replaced with fresh medium, and 500 μCi [^{35}S]methionine (0.5 nM) (tissue culture grade; Amersham Corp.) was added to each flask. The medium was harvested from the flasks 5 days postinfection and assayed for JHE activity using the colorimetric substrate *S*-(methyl) thiohexylthioethanoate (HEXTAT) (McCutchen et al., 1993). The [^{35}S]JHE was affinity purified from the medium using a trifluoroketone column as described above.

Purified samples were assayed for JHE and radioactivity. Fifth stadium larvae of *M. sexta* were injected with 50 μl radiolabeled JHE. The pericardial cell complex, fat body tissue, and epidermal tissue were dissected out 1 h postinjection. Samples were transferred directly to Eppendorf tubes containing cracking buffer and were run on a 7.5% SDS polyacrylamide gel along with molecular weight markers (Amersham Corp., high range). The gel was stained with Coomassie brilliant blue R-250, photographed, and dried down. Radiolabeled bands were visualized by exposing a Fuji phosphoimager screen for 3 days within a cassette encased in lead sheeting to reduce background noise. Images were analyzed using a Fuji BAS2000 phosphoimager.

RESULTS

Electrophoresis and Immunoblotting

When the anti-JHE antiserum immunoprecipitate was analyzed by SDS-PAGE followed by Western blot, there was no evidence of a ubiquitin ladder above the JHE band in the pericardial cell sample when screened with either the anti-JHE antiserum (Fig. 1A) or the anti-ubiquitin antiserum (Fig. 1B). Four proteins above 90 kDa were detected by both antisera in both fat body and pericardial cell tissues (Fig. 1A,B). The identity of these proteins is not known. SDS-PAGE followed by Western blot analysis with the anti-Hsp antiserum showed proteins with relative molecular masses of 100,000 and 140,000 daltons in the pericardial cells but not in fat body samples (Fig. 1C). The

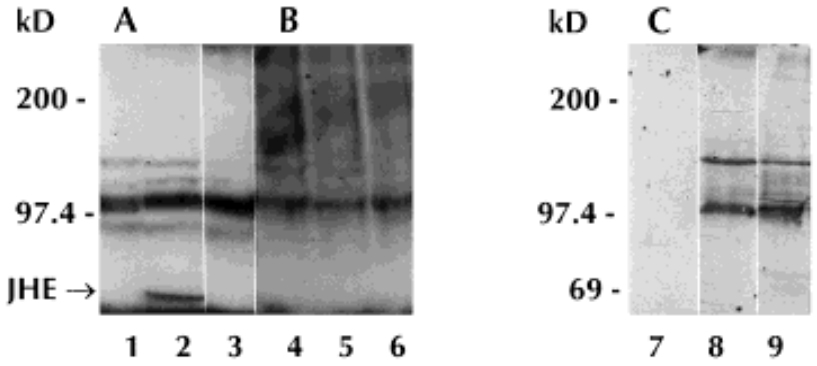

Fig. 1. Immunoprecipitation of proteins with the anti-JHE antiserum followed by SDS-PAGE and Western blot analysis with (**A**) anti-JHE antiserum and (**B**) anti-ubiquitin antiserum. **C**: SDS-PAGE of tissues followed by Western blot analysis with the anti-Hsp antiserum. Lanes 1,4,7: Fat body samples from JHE-injected larvae, Lanes 2,5,8: Pericardial cell samples from JHE injected larvae. Lanes 3,6,9: Pericardial cell samples from BSA-injected larvae. The position of molecular weight markers and of JHE (lane 2) corresponding to the sample of affinity purified JHE injected are shown. The immunoreactivity at the base of the blots shown in A and B is due to the IgG heavy chain used for immunoprecipitation.

proteins detected in fat body samples from BSA-injected larvae were the same as those seen in fat body samples from JHE-injected larvae shown in Figure 1, lanes 1, 4 and 7 (data not shown).

In a series of native polyacrylamide gels, only the pericardial cells showed immunoreactive material at Rf 0.7 with anti-Hsp antiserum (Fig. 2). No cross-reactivity was detected for fat body samples with the anti-Hsp antiserum. Upon injection of JHE, a diffuse immunoreactive band appears at Rf 0.2 which cross-reacts with both anti-Hsp and anti-JHE antisera. No immunoreactivity was observed at this Rf with either antisera in any tissue other than the pericardial cells. Free JHE has an Rf of 0.4. There was no evidence of JHE uptake by the fat body based on Western blot analysis for bands with Rf of 0.4 (free JHE) or 0.2 (putative JHE-Hsp complex). Although a difference in the degree of immunocrossreactivity is evident in the replicates shown in lanes 3–5 of Figure 2, no difference was apparent on examination of the Coomassie blue–stained native gel prior to Western blotting.

Immunoprecipitation of JHE

JHE activity recovered following immunoprecipitation was greater than 80% of the initial activity of the pericardial cell homogenate prior to immunoprecipitation. Data shown (Fig. 3) are for mean % catalytic activity precipitated for precipitations from three or four pericardial cell homogenates. Neither antiserum nor immunobeads affected the catalytic activity of JHE. JHE activity detected following immunoprecipitation with the anti-Hsp antiserum from pericardial cells from larvae injected with BSA was not above background levels. Only 0.5% of the total CBE activity (935 μmol α-naphthyl acetate hydrolyzed / min / 20 μl) was precipitated with the anti-Hsp antiserum. Approximately 25% of the active recombinant JHE in the homogenate of pericardial cells was immunoprecipitated with anti-Hsp antiserum 1 h postinjection (Fig. 3).

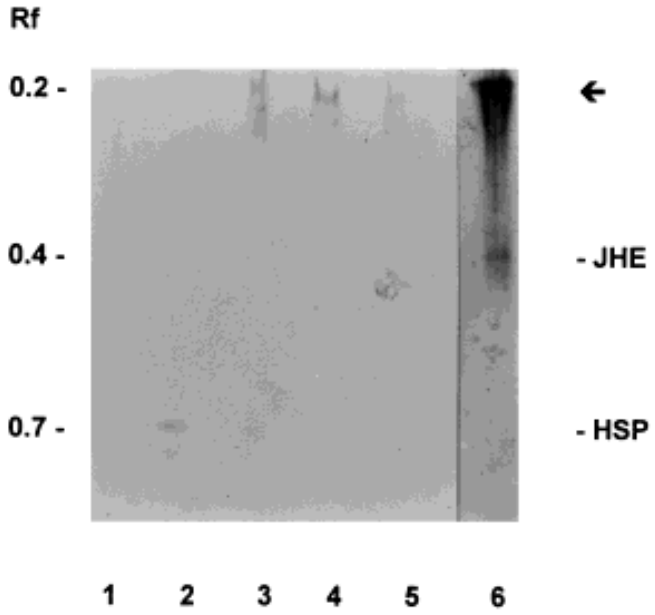

Fig. 2. Binding of Hsp to JHE in the pericardial cell complex of *M. sexta*. Western blot of native polyacrylamide gel with anti-Hsp antiserum (lanes 1–5) and anti-JHE antiserum (lane 6). The anti-JHE antiserum did not cross-react with the JHE from *M. sexta* under the conditions used. **Lane 1:** Fat body from larva injected with recombinant JHE. **Lane 2:** Pericardial cell complex from an untreated larva. **Lanes 3–5:** Pericardial cells from individual larvae of *M. sexta* injected with recombinant JHE. **Lane 6:** Western blot of pericardial cells from larva injected with JHE using anti-JHE antiserum. The appearance of the Hsp in pericardial cells from untreated larvae (lane 2) suggests that this putative Hsp is constitutively expressed. For these experiments, tissue samples were removed 1 h postinjection and homogenized prior to loading onto a 7.5% native polyacrylamide gel. Proteins were transferred to nitrocellulose and detected using the Enhanced ChemiLuminescence technique.

Electron Microscope Analysis

Electron microscope analysis with anti-JHE antiserum showed apparent association of JHE with coated pits (Fig. 4a) and vesicle-dependent transport (Fig. 4b–d). The appearance of these labeled vesicles was dependent upon the presence of JHE and time postinjection. Labeled JHE accumulated in the lysosomes as described previously (Booth et al., 1992).

Immunogold-labeled Hsp was detected in the *trans*-Golgi network of the pericardial cells (Fig. 5A). Control micrographs using preimmune serum showed little or no labeling in this region (Fig. 5B).

Radiolabeling of JHE

Following affinity purification of [^{35}S]JHE, samples of 3,500–4,000 cpm / 50 µl (500 nmol HEXTAT hydrolyzed / min-ml) were injected into fifth stadium larvae. Radiolabeled proteins were separated by SDS-PAGE and visualized by using a phosphoimager screen and the Fuji BAS2000 system. Radiolabeled proteins were detected only in the pericardial cell tissue (Fig. 6). Relative molecular masses (M_r) were calculated with reference to molecular weight

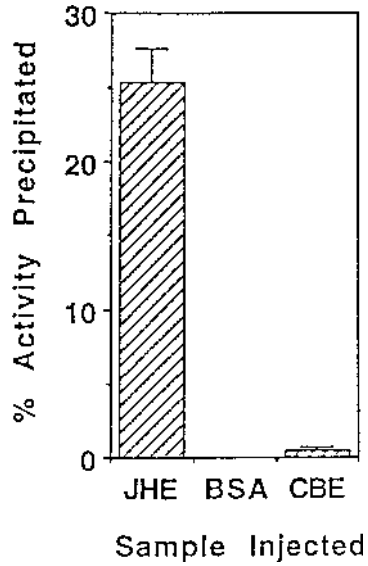

Fig. 3. Immunoprecipitations of JHE with anti-Hsp antiserum to quantify the extent of Hsp binding to JHE. Larvae of *M. sexta* were injected with affinity purified JHE or the control samples BSA or a related mammalian carboxylesterase (CBE). Pericardial cells were removed 1 h postinjection and homogenized in sample buffer. Aliquots of the homogenate were immunoprecipitated with anti-Hsp antiserum and Immunobead second antibody reagent. Data shown are for mean % catalytic activity precipitated for precipitations from three or four pericardial cell homogenates.

markers visualized by protein staining of the gel. In addition to a 66,000 dalton band of radiolabeled JHE corresponding to the injected protein, several bands with molecular masses of approximately 36,000, 40,000 and 48,000 daltons and a high molecular weight band (approximately 200,000) were detected (Fig. 6).

DISCUSSION

Several lines of evidence support the hypothesis of receptor-mediated endocytosis of JHE followed by lysosomal degradation in the pericardial cells. The first order, saturable, apparently active uptake of JHE by the pericardial cells (Ichinose et al., 1992a) and electron micrographs of JHE associated with pits at the apical surface of pericardial cells and within vesicles (Fig. 4) support this hypothesis. Immunoblotting data using the anti-ubiquitin antiserum (Fig. 1B) and analysis of radiolabeled JHE in the pericardial cells (Fig. 6) argue against a ubiquitin-dependent process playing a dominant role in the degradation of JHE. Ubiquitin conjugation, which is a cystolic event, is characterized by appearance of a ladder at 8,000 dalton intervals of the conjugated protein following SDS-PAGE.

When immunoprecipitation was carried out with anti-Hsp antiserum, there was a clear association of catalytic activity for JHE with an Hsp in pericardial cells (Fig. 3). By 1 h post injection of larvae, approximately 25% of the JHE in the pericardial cell complex was associated with the putative Hsp,

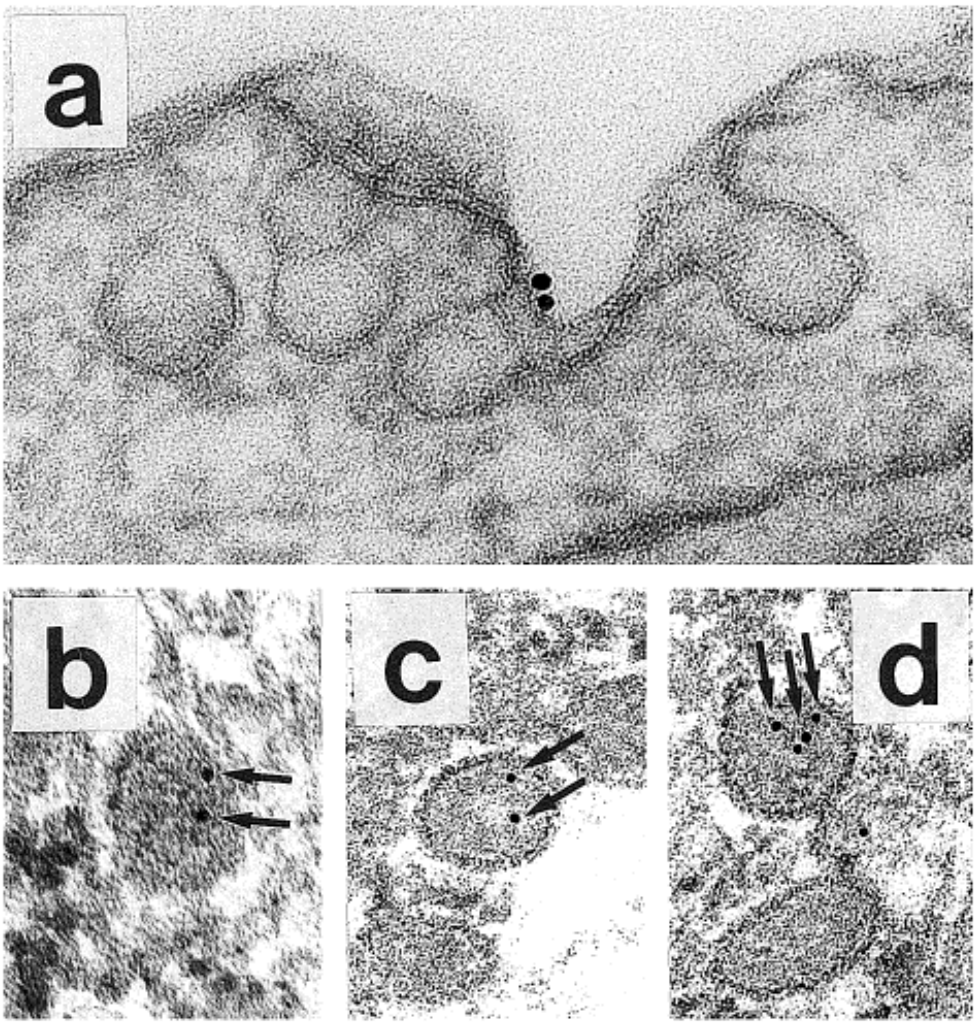

Fig. 4. **a:** Electron micrograph of immunogold-labeled JHE on the surface of endocytosing vesicles at the basal surface of pericardial cells. **b–d:** Apparent clathrin-coated vesicles containing immunogold-labeled JHE within the cytoplasm of pericardial cells. Larvae of *M. sexta* were injected with affinity purified JHE (Shiotsuki et al., 1994). Pericardial cells were dissected out 1 h postinjection and fixed in 1% gluteraldehyde (EM grade). Sections were labeled with immunogold using anti-JHE antiserum. Sections were labeled before embedding to label the basal surface of the pericardial cell for panel a, and on-section immunogold labeling was carried out for detection of JHE within the cytoplasm of the pericardial cells for panels b–d. Arrows 10 nm gold particles.

with the remaining 75% presumed to be at different stages in the endocytosis-lysosome pathway. The association of JHE with the putative Hsp was also shown by using native polyacrylamide electrophoresis followed by Western blotting of pericardial cell samples by using both anti-Hsp and anti-JHE antisera (Fig. 2). The JHE injected was shown to bind to an apparent Hsp in pericardial cells but not in the fat body.

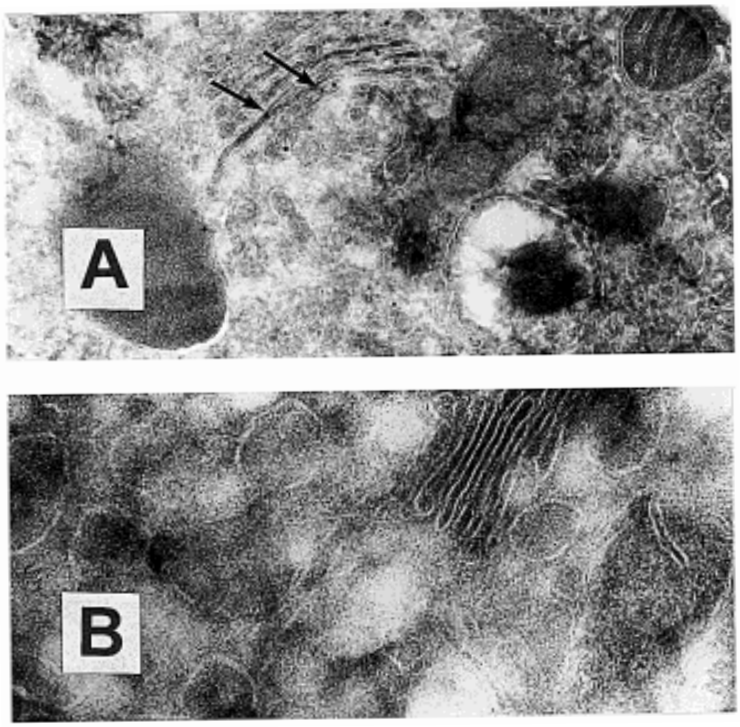

Fig. 5. Electron micrograph of a pericardial cell immunogold-labeled with the anti-Hsp antiserum. Labeling (10 nm gold particles indicated by arrows) was detected in the *trans*-Golgi network (**A**) which sorts proteins destined for the lysosomes form those destined for the plasma membrane. There was little or no background labeling in this area when sections were labeled by using the preimmune serum (**B**).

The putative Hsp was detected by using the universal anti-Hsp70 antiserum and therefore is considered to be related to the heat shock proteins. Since the putative Hsp is expressed constitutively, as opposed to being heat- or stress-induced, it is classified as a putative heat shock cognate protein. However, it is surprising that no other constitutively expressed Hsp70 proteins were detected by the universal anti-Hsp70 antiserum. This Hsp70 of *M. sexta* was detected, however, by using the *Drosophila* anti-Hsp70 mAb 7.10 (Affinity Bioreagents Inc., Purchase, NY) (data not shown). For the Hsp70 protein family, the highly conserved N-terminal amino acids (approximately the first 450) are associated with ATP-binding and hydrolytic activity. The C-terminal domain is more variable and probably the "specificity" domain that couples binding of target proteins to the ATPase activity of the conserved N-terminal domain. The universal anti-Hsp70 antiserum used for detection was raised to a peptide sequence correlating to amino acids 145–160 of the human Hsp70. This sequence is clearly within the highly conserved region of the N-terminal domain. Epitope mapping suggests that the epitope recognized by mAb 7.10 is located between amino acids 437 and 479 of human Hsp70 (Kurtz et al., 1986). It is also of note that the proteins detected by the universal anti-

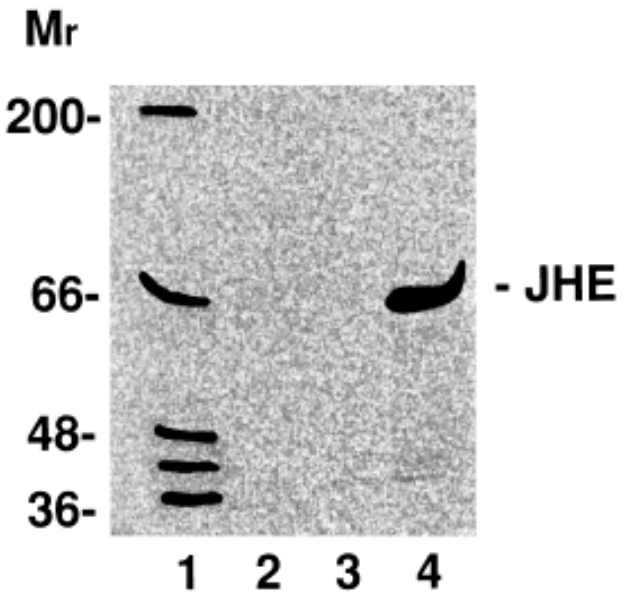

Fig. 6. Injection of *M. sexta* with affinity purified [^{35}S]JHE followed by SDS-PAGE analysis of radiolabeled proteins in different tissues. **Lane 1:** Pericardial cells. **Lane 2:** Fat body. **Lane 3:** Epidermis. **Lane 4:** Affinity purified [^{35}S]JHE injection sample (15 μl). In addition to putative degradation products and the 66,000 dalton band corresponding to the injected sample, a protein of about 200,000 molecular mass is also seen. No radiolabeling was detected in any tissues other than the pericardial cells using this technique.

Hsp70 antiserum in the pericardial cells of *M. sexta* have molecular weights of 100,000 and 140,000 rather than 70,000. A search for peptide sequences similar to that used to raise the universal anti-Hsp70 antiserum by using the computer program BLAST (Altschul et al., 1990) did not reveal similar sequences in anything other than proteins in the Hsp70 family. These results suggest that the proteins detected in *M. sexta* pericardial cells are related to but different from other Hsp70.

We have shown that JHE associates with an Hsp-related protein at some point in the receptor-mediated endocytosis-lysosomal degradation pathway. In mammalian cells, some cytosolic proteins are bound at the lysosomal targeting sequence by a chaperonin-type Hsp (Chiang et al., 1989) prior to lysosomal degradation. The putative Hsp in the pericardial cells of *M. sexta* appear to be located in the *trans*-Golgi network of the pericardial cells which sorts proteins destined for the lysosomes from those destined for the plasma membrane. We hypothesize that the Hsp functions as a chaperone of JHE during this sorting process. This hypothesis, along with determination of which of the two anti-Hsp cross-reactive proteins binds JHE, is under investigation.

Following injection of [^{35}S]JHE into larvae and SDS-PAGE analysis of radiolabeled proteins in different tissues, several radiolabeled proteins appeared in the pericardial cells. The radiolabeled proteins with lower molecular mass than JHE are assumed to be degradation products. A radiolabeled, high molecular weight band was also detected in the pericardial cells. JHE or a deg-

radation product of JHE appears to be binding covalently to some protein other than the Hsp in the pericardial cell complex. Hsp binding is discounted since the protein binding of Hsp does not typically withstand denaturing electrophoresis. The absence of a 200,000 dalton band following injection of larvae with JHE, SDS-PAGE, and Western blotting with the anti-Hsp antiserum (Fig. 1C) also suggests that this is not binding to the putative Hsp. The identity of the protein that covalently binds JHE in the pericardial cell complex remains to be determined.

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