α-Crystallin protein cognates in eggs of the moth, *Plodia interpunctella*: possible chaperones for the follicular epithelium yolk protein

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Abstract

α-Crystallin protein cognates were found in germ cells of the Indianmeal moth, *Plodia interpunctella* (Shirk and Zimowska, 1997). A cDNA clone of 674 bp with a single open reading frame was isolated for a 25 000 molecular weight polypeptide member of this family, αCP25, and a single transcript of approximately 700 bp was found in the ovary of vitellogenic females. Both the DNA sequence and predicted amino acid sequence showed considerable homology with the embryonic lethal gene, *l(2)efl*, in *Drosophila melanogaster*. In addition to the sequence for *l(2)efl*, the predicted amino acid sequence for *αcp25* also showed significant sequence similarity with the α-crystallin A chain polypeptides from the lenses of vertebrate eyes. An N-terminal hydrophobic aggregation site and a C-terminal protective binding site common to α-crystallin proteins were present in the predicted *αcp25* and *l(2)efl* amino acid sequences, while only the C-terminal protective binding site was present in the small heat shock protein sequences from *D. melanogaster*. On the other hand, the cDNA sequence for *αcp25* showed more similarity to small heat shock proteins in *D. melanogaster*. This evidence suggests that although the α-crystallin protein cognates in *P. interpunctella* evolved from a gene common with small heat shock protein genes, the amino acid sequence has converged on a structure similar to that of α-crystallin proteins. Native immunoblot analysis showed that the α-crystallin proteins formed high molecular weight complexes with the follicular epithelium yolk protein (FEYP) but not vitellin in yolk. An electroblot binding assay was used to show that the germ-cell α-crystallins of *P. interpunctella* bind specifically with the FEYP and that the binding was reversible in the presence of ATP or low pH. This evidence in conjunction with the evidence that the α-crystallins and FEYP form a stable complex that co-purifies from native egg proteins suggests that the α-crystallin cognates function as chaperones for the follicular epithelium yolk proteins in the embryos of *P. interpunctella*. © Published by Elsevier Science Ltd. All rights reserved.

Keywords: Alpha-crystallins; Chaperones; Small heat shock proteins; Lepidoptera; Germ cells

1. Introduction

The α-crystallin proteins present in the lens of the vertebrate eye and the small heat shock proteins (*hsps*) have major regions of conserved sequences and are considered to have a common ancestral gene (Ingolia and Craig, 1982; for reviews see: Wistow and Piatigorsky, 1988; de Jong et al., 1989, 1993). The conserved regions between these two groups of proteins also carries over into some functional similarities as well (Klemenz et al., 1991; Merck et al., 1993; James et al., 1994).

In the lens, multimeric aggregates of the αA-crystallin and αB-crystallin subunits, in combination with the minor β-, γ-, δ-, ϵ-, τ-, and ρ-crystallin subunits, form a transparent tissue with the refractive index and elasticity necessary for the lens (Wistow and Piatigorsky, 1988; de Jong et al., 1993). The formation of the aggregates is apparently dependent on the presence of a conserved motif in the amino terminal region of the proteins (James et al., 1994). This aggregation site is present in some but

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not all of the heat shock proteins such as the small *hsps* from *Drosophila melanogaster*.

Although the αB-crystallins are a major component of the lenses, they have also been found in non-lenticular tissues: the central nervous system, heart and skeletal muscles, spleen, thymus and kidney (Bhat and Nagineni, 1989; Iwaki et al., 1989; Longoni et al., 1990; Kato et al., 1991a, b). In the non-lenticular tissues, the αB-crystallins are inducible by heat shock (Klemenz et al., 1991), where they function primarily as molecular chaperones (Horowitz, 1992; Merck et al., 1993).

The chaperone and binding function is dependent on a shared highly conserved region in the carboxyl terminal of the α-crystallins and small *hsps* (Ingolia and Craig, 1982; Wistow and Piatigorsky, 1988; de Jong et al., 1993). Functions for the small *hsps* of *Drosophila* have been observed following severe heat stress where they are in association with the nucleoskeleton (Sinibaldi and Morris, 1981) and prosomes (Arrigo et al., 1985). On the other hand, they are only in association with the perinuclier cytoskeleton during mild heat shock (Leicht et al., 1986). In addition to the heat shock aggregation, the small *hsps* have also been found to act as chaperones that influence protein refolding following heat stress (Jakob et al., 1993; Jakob and Buchner, 1994).

We have reported the presence of a potential family of α-crystallin protein cognates that are constitutively present in the germ cells of the Indianmeal moth, *Plodia interpunctella* (Hubner) (Zimowska et al., 1991; Shirk and Zimowska, 1997). The α-crystallin proteins were initially isolated through co-purification with the follicular epithelium yolk protein (FEYP). The FEYP consists of two subunits, YP2 (69 kDa) and YP4 (33 kDa) (Shirk et al., 1984; Bean et al., 1988) and is synthesized and secreted from the follicular epithelial cells during vitellogenesis (Shirk et al., 1984, 1992; Zimowska et al., 1994, 1995). The apparent complex formed between the α-crystallin proteins and FEYP during the purification was of interest, because they are segregated into separate cytoplasmic compartments within the oocyte: FEYP within the yolk spheres and α-crystallins within the cytoplasm (Shirk and Zimowska, 1997). Because it was of interest to clarify the structural relationship of the α-crystallin proteins from *P. interpunctella* with the α-crystallins and small *hsps*, a cDNA clone for one of these proteins was isolated and characterized. In addition, we developed a binding assay to assess potential functionality of these proteins within the germ cells and embryo.

2. Materials and methods

The *Plodia interpunctella* colony was reared according to Silhacek and Miller (1972) in a 16 h light: 8 h dark cycle at 30°C and 70% relative humidity.

2.1. cDNA library construction

Total RNA was extracted from the ovaries of late pharate adult females using an RNA extraction kit (Pharmacia Biotech; Piscataway, NJ). To construct the cDNA library, mRNA was isolated from the total RNA by two cycles of chromatography through an oligo(dT)-cellulose column.

The cDNA library was constructed from poly(A)$^+$ RNA of the ovaries by the Library Construction Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida using the Lambda ZAP II cDNA Synthesis Kit (Stratagene; La Jolla, CA) with an oligo(dT) primer that included an *Xho*I linker. The cDNA was size-fractionated on a Sephacryl S-400 column with a minimum size cutoff of 0.5 kb. An *Eco*RI adaptor was added to the 5′ end of the cDNA and then the cDNA was ligated into Lambda ZAP II arms that were restriction digested with *Eco*RI and *Xho*I. The ligation was packaged into lambda phage according to the manufacturer's protocols.

2.2. Screening of the cDNA library

Affinity purified polyclonal antibodies for αCP25 were prepared as described by Robinson et al. (1988). The cDNA library was screened with the affinity purified antibodies by the plaque method of Huynh et al. (1985). Positives were rescreened 3 times before excision of the recombinant pBluescript II (SK$^+$) plasmid according to the manufacturer's protocols.

2.3. DNA sequencing

The DNA Sequencing Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida performed the DNA sequencing. The sequencing was accomplished by the Taq DyeDeoxy Terminator (#401388) and DyePrimer (#401386) Cycle Sequencing protocols developed by Applied Biosystems (a division of Perkin-Elmer Corp., Foster City, CA) using fluorescent-labeled dideoxynucleotides and primers, respectively. The labeled extension products were analyzed on a Model 373A DNA Sequencer (Applied Biosystems).

The DNA and conceptual translation sequences were used to search peptide and nucleotide sequence databases using the BLAST (Altschul et al., 1990) network service. Sequence alignments were made using MEGA (Kumar et al., 1993). Distance values were calculated for the matrix, and relationship dendograms for the amino acid and DNA sequences were constructed using the Neighbor-Joining associations.

2.4. Northern blot analysis

Total RNA from ovaries and somatic tissues of pharate adults was isolated using TRIZOL reagent as directed by the manufacturer (Life Technologies; Gaithersburg, MD). Northern analysis was carried out using NorthernMax (Ambion; Austin, TX) reagents. Eight micrograms of total RNA of each sample was resolved in a 1% formaldehyde-agarose gel. The gel was run for 4 h at 60 V with buffer recirculation. RNA Millenium marker (Ambion; Austin, TX) was included in the gel to determine the size of the target mRNA. At the end of the run the marker lane was cut out, stained with ethidium bromide, and photographed. The RNA was then transferred onto a Nytran (Schleicher and Schuell; Keene, NH) blotting membrane by downward capillary transfer using the rapid transfer buffer of the NorthernMax reagent system. After 1 h the transfer apparatus was disassembled, the membrane was washed in the transfer buffer briefly and UV crosslinked using a Stratalinker (Strategene; LaJolla, CA).

Pre-hybridization and hybridization of the Northern blot was carried out at 48°C in the NorthernMax hybridization buffer. The labeled DNA probe was synthesized by PCR amplification of the 5′ region of the *αcp25* cDNA clone that did not include the conserved regions shared with the small heat shock proteins. The amplification reaction contained 10 pg of template DNA, 3 μl of alpha ^{32}P-dCTP (10 μCi/mmol), 10 pmol each of the T3 forward primer in the Bluescript vector and the Piac25-327R reverse primer, 100 nmol each of dATP, dGTP, and dTTP, and 2.5 mM MgCl$_2$ in a 15 μl volume. Probe was purified from unincorporated nucleotides and primers using a BioGel P-60 (100–200 mesh) column. Heat denatured probe was added to the hybridization buffer and incubated overnight. At the end of hybridization, the probe was removed and the filter was washed twice with NorthernMax low stringency wash buffer at room temperature and then twice with high stringency wash buffer at 48°C for 15 min each. An autoradiogram of the blot was made by exposing X-OMAT AR film (Eastman Kodak; Rochester, NY) for 6 hours to visualize the signal.

2.5. Native Western blot

Equal quantities of the αCP-FEYP fraction and vitellin fraction from DEAE-Sepharose chromatography (Shirk and Zimowska, 1997) were electrophoresed in native conditions using a 5–20% polyacrylamide gel. Native molecular weight markers (Pharmacia Biotech) were co-electrophoresed to determine molecular size. The proteins were allowed to attain a terminal migration distance that was limited by pore size (Slater, 1969). The proteins were electroblotted to nitrocellulose (Schleicher and Schuell) according to Towbin et al. (1979). The rep-

licate immunoblots were reacted individually with polyclonal rabbit antiserum to vitellin (Shirk et al., 1984), FEYP (Shirk et al., 1984) or αCP25 (Shirk and Zimowska, 1997) as primary antiserum and goat-antirabbit IgG linked alkaline phosphatase (BioRad; Hercules, CA) as the secondary antiserum. The immunoreactive protein bands were identified by color development with the alkaline phosphatase substrate kit (BioRad).

2.6. Immunoblot binding assay

The qualitative binding activities of αCPs were assessed by utilizing a binding assay for protein bound to PVDF-membrane (Transblot Transfer membrane, BioRad). Protein fractions used for electroblotting and the binding assay were the αCP-FEYP fraction and vitellin fraction from DEAE-Sepharose chromatography as described previously (Shirk and Zimowska, 1997). The polyclonal rabbit antiserum for YP3 (Bean et al., 1988), YP4 (Shirk et al., 1992), and αCP25 (Shirk and Zimowska, 1997) were used as primary antisera to identify antigens bound to the electroblots. Equal amounts of FEYP plus αCPs, FEYP, and vitellin were resolved by SDS-PAGE and electroblotted to Transblot membrane according to Towbin et al. (1979). The membranes were blocked with 3% gelatin in 25 mM Tris, pH 7.5, 0.5 M NaCl (TBS) and washed twice with TBS (pH 6.5). Either FEYP or vitellin were dissolved in 50 mM Tris (pH 6.5), 3 mM MgCl$_2$, 0.5 M NaCl, 0.05% Tween-20 and 1% gelatin at 10 μg/ml. The electroblots were then incubated with the FEYP, vitellin or no added protein solutions overnight at room temperature. The blots were washed three times with TBS (pH 7.5) plus 0.05% Tween-20 (TTBS) and then incubated with antiserum for YP4 or YP3, respectively. To test if the FEYP could be washed off the blot after the initial binding, some blots were either washed with TTBS (pH 2.5) or TTBS (pH 7.5) plus 0.1 M ATP. When washed with pH 2.5 TTBS, the blots were incubated at pH 2.5 for 30 min and then neutralized with three washes of TTBS. When washed with TTBS plus 0.1 M ATP, the blots were incubated for 5 min with the ATP solution and then washed three times with TTBS. Following the stripping procedures, the blots were reblocked and then exposed to primary antiserum. To determine the antibody binding patterns, the blots were incubated with goat-antirabbit IgG linked alkaline phosphatase (BioRad) and then developed with the alkaline phosphatase substrate kit (BioRad). The reactions were allowed to go past optimal contrasts in order to assure visualization of minimum binding reactions.

3. Results

3.1. cDNA cloning and the nucleotide and predicted amino acid sequences of αcp25

Five different positive clones of cDNA were isolated through the immunological screening of 5×10^3 recombinant phage from the ovarian cDNA library. The positive clones were considered to comprise the cDNA clones for the four α-crystallin cognates that react with the antiserum to αCP25 (Shirk and Zimowska, 1997). Following the excision of the recombinant Bluescript phagemid, each of the positive phagemids contained an insert of approximately 0.7 kb. The complete nucleotide sequence and a predicted amino acid sequence of the 741 bp insert from the positive cDNA clone, Pi-hs25-8, is shown in Fig. 1. A single open reading frame was identified that encoded for a 185 amino acid polypeptide. The 30 amino acid sequence derived from an internal fragment generated by digestion of αCP25 with endopeptidase Lys-C reported previously (Shirk and Zimowska, 1997) had total identity with amino acids 71 to 100 of the predicted amino acid sequence in the cDNA clones. The 3′ noncoding region of the cDNA clones is 122 bp long and contains two AATAAA poly (A)⁺ addition signals at bp 552, coincident with the last codon, and at bp 650, 24 bp upstream from the start of

the 3′ poly(A)⁺ tail. This cDNA clone was re-designated αcp25. Northern blot analysis of total RNA isolated from ovaries and abdomens without ovaries of vitellogenic females showed that a single transcript of approximately 700 bp was present in the ovaries (Fig. 2). An infinitesimally small amount of cross-hybridizing material was seen in the non-ovarian RNA. Because the probe was for the non-conserved 5′ region of the gene (see below), this signal may be due to the presence of αcp25 transcript in oenocytes, αcp25 immunoreactive material had been observed in this tissue when immuno-fluorescently stained with αcp25 antiserum (Shirk and Zimowska, 1997). Alternatively the transcript signal may have been an artifact from crosshybridization with small hsp transcript.

3.2. Sequence similarity between αcp25 and other proteins

The predicted αcp25 amino acid sequence was BLAST searched against the available gene and protein banks. The greatest sequence similarity was shared with an embryonic lethal, *l(2)efl*, in *D. melanogaster* (Kurzik-Dumke and Lohman, 1995) (Fig. 3). Over the entire length of the sequence, the two proteins had 84 identical amino acids plus 23 conserved substitutions. However, at the region of the endopeptidase Lys-C fragment from

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                    GCTCGCAAGAGAACTAATAACGAGTT      -61

        TCACTCACTGCGTTTACCTATTACGCGTTTACTAGTTTATTTATTATAAGAAAGAAGAAA    -1

        ATGTCCCTACTACCATTTGTGTTCGGCTACGAATCGCCACGCTACTACCATCACAGCTGG     60
      1 M   S   L   L   P   F   V   F   G   Y   E   S   P   R   Y   Y   H   H   S   W
        CCTAGCCGCCTGATCGATCAAAATTTCGGCTTGGCGTTGACACCGGACGAAATGCTCACG    120
     21 P   S   R   L   I   D   Q   N   F   G   L   A   L   T   P   D   E   M   L   T
        GCTGTGGCGTGTCCGTTGCTGTCCACAGACTACTACAGACCCTGGAGACAGCTGGCGGCG    180
     41 A   V   A   C   P   L   L   S   T   D   Y   Y   R   P   W   R   Q   L   A   A
        GCGGCGCGGGACATCGGCTCGAACATCAAGGCCGACAAGGACAAGTTGCAGATCAACTTG    240
     61 A   A   R   D   I   G   S   N   I   K   A̲   D̲   K̲   D̲   K̲   L̲   Q̲   I̲   N̲   L̲
        GACGTGCAGCATTTCTCGCCTGAGGAGATCTCGGTGAAGACGGCGGACGGGTTCGTGGTG    300
     81 D̲   V̲   Q̲   H̲   F̲   S̲   P̲   E̲   E̲   I̲   S̲   V̲   K̲   T̲   A̲   D̲   G̲   F̲   V̲   V̲
        GTGGAAGGCAAGCATGAGGAGAAGAAGGACGAGCACGGCTACATCTCTCGGCAGTTCGTG    360
    101 V   E   G   K   H   E   E   K   K   D   E   H   G   Y   I   S   R   Q   F   V
        CGGCGGTACGCGCTGCCGGAGGGCGCGGCGTCGGAGACTGTGGAGTCGCGGCTGTCGTCC    420
    121 R   R   Y   A   L   P   E   G   A   A   P   E   T   V   E   S   R   L   S   S
        GGCGGCGTGCTGACCATCACCGCGCCGCTGAAGGTGCCCGACGCGGTCAAGGGTGAGCGC    480
    141 G   G   V   L   T   I   T   A   P   L   K   V   P   D   A   V   K   G   E   R
        AAAGTGCCCATCGCTCAAACGGGACCCGTGCGCAAGGAGATCAAGGATCAGAGCGGAAAG    540
    161 K   V   P   I   A   Q   T   G   P   V   R   K   E   I   K   D   Q   S   G   K
        GAGAAAGGG**AATAAA**TAATTTTCACTAGGTATTTTAAAAGTTTATTTTCAGACTGATACT    600
    181 E   K   G   N   K
        GTGATCTCCTAGGTTTAATTTACTTGCTTGTAATTTTTTTTTTTTATTTGA**AATAAA**GTAC    660

        AATAATTACCGTGTAAAAAA                                            680
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Fig. 1.   The nucleotide sequence and predicted amino acid sequence for clone Pi-*hs25*-8 cDNA. Linker sequences at the 5′ and 3′ ends of the clone are omitted. The adenine of the first methionine codon is designated as +1 in the nucleotide sequence. The predicted amino acid sequence of the 185-codon open reading frame is shown under the nucleotide sequence in the single-letter amino acid code. The single underlined sequence is identical to the amino acid sequence from an endopeptidase Lys-C fragment of αCP25 polypeptide reported previously (Shirk and Zimowska, 1997). The bold sequences show the poly(A)⁺ addition signals at 552 and 650.
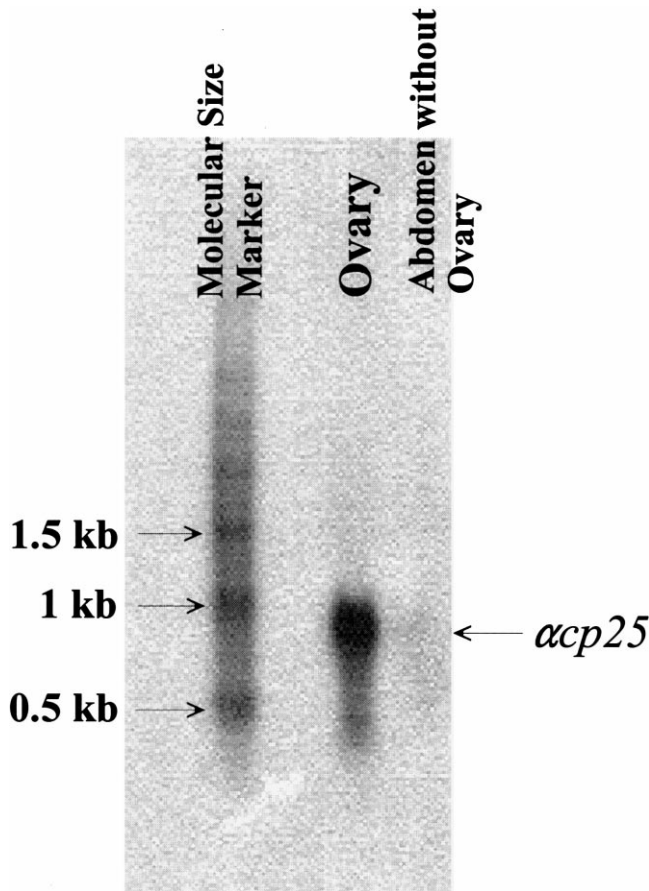
Fig. 2. Northern blot analysis of *αcp25* transcripts from vitellogenic pharate adult female *P. interpunctellla*. Total RNA from ovaries and non-ovarian tissues of the abdomen were resolved and blotted to membrane. The position of the RNA molecular size markers bracketing the transcripts is shown on the left.

αcp25 (beginning at amino acid 70 of the predicted sequence), the *αcp25* protein and *l(2)efl* protein had a highly conserved sequence of 56 amino acids that showed 82% similarity. The predicted amino acid sequence of *αcp25* also shared considerable similarity with the α-crystallin A chain proteins from the lenses of vertebrate eyes. The region following amino acid 70 extending to amino acid 125 was similar to the conserved region shared between the α-crystallin proteins and the small heat shock proteins that was observed previously (Ingolia and Craig, 1982). In addition to the conserved C-terminus, the N-terminal hydrophobic aggregation site present in α-crystallin proteins (James et al., 1994) was also present in both the *αcp25* protein in *P. interpunctella* and the *l(2)efl* protein in *D. melanogaster* (Fig. 3, double underlined sequence).

To establish an index of relatedness between these six proteins, a relative genetic distance dendogram was constructed for the *αcp25* protein and the six related proteins examined above using the Neighbor-Joining method as described (Fig. 4). The dendogram broke out as three grouped pairs of related sequences. The *αcp25* and

l(2)efl proteins were placed together, and they were more closely related to the α-crystallin protein than to the small heat shock proteins from *Drosophila*. The sequence similarity between *αcp25* and *l(2)efl* and other α-crystallin proteins was consistently greater than the similarity with the small *hsps* from *Drosophila* (data not shown).

A dendogram was also constructed for the cDNA sequences of these six genes. The dendogram again broke out into three pairs of related sequences (Fig. 5). As in the dendogram for the proteins, the cDNA sequences for *αcp25* and *l(2)efl* were found to be the most closely related. However, the dendogram shows that the *αcp25* cDNA sequence was more closely or just as related to the sequences for the small *hsps* from *Drosophila* than to the α-crystallin protein DNA sequence.

3.3. Functional analysis of αCP25 binding activities

To determine the nature of the association between αCP25 and FEYP in materials originating in the egg, native semi-purified protein fractions from DEAE-Sepharose chromatography were resolved in non-denaturing gel electrophoresis and electroblotted. The αCP25 antiserum reacted with multiple high molecular weight proteins up to approximately 400 000 in the αCP25/FEYP fraction, although no bands in the 18 000 to 29 000 mass range representing monomers of the αCPs were observed (Fig. 6, Panel B lanes a and b). The αCP25 antiserum did not react with any proteins in the vitellin fraction (Fig. 6, Panel B lanes c and d). Previously, only two forms of native FEYP had been described that had a maximum molecular weight of 237 000 (Bean et al., 1988). However, in this experiment the FEYP antiserum reacted with multiple high molecular weight bands up to 400 000 forming a banding pattern similar to, but not identical with, the band pattern observed for the multiple high molecular weight proteins identified with the αCP25 antiserum. The vitellin antiserum reacted primarily with one major protein band at approximately 460 000 in the vitellin fraction (Fig. 7, Panel C lanes c and d). Unlike the αCP25 antiserum, the FEYP antisera reacted with minor amounts of FEYP protein present in the vitellin fraction (Fig. 6, Panel A, lanes c and d), and similarly the vitellin antiserum reacted with minor amounts of vitellin protein present in the αCP25/FEYP fraction (Fig. 6, Panel C, lanes a and b).

The sequence similarities between the *αcp25* protein and the α-crystallin proteins/small *hsps* and the fact that αCP25 co-purifies with FEYP (Shirk and Zimowska, 1997) and forms high molecular weight complexes with the FEYP of *P. interpunctella* led us to assess the binding activities of αCP25. An electroblot/protein binding assay was developed to determine what proteins the αCPs from *P. interpunctella* were capable of binding

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Piac25ᵃ      1   MSLLPFVFGY  ESPRYYHHSW  PSRLIDQNFG  LALTPDEMLT  AV--ACP-LL  STDYYR----  ------PWRQ
Dml2eflᵇ     1   ..vv.lm.RD  WWDELDFPMr  T...l..H..  QG.KR.dlmS  S.WNSR.Tv.  RSG.L.----  ------..hT
Hsacᶜ        1   .DVTIQHPWF  KR-TL-GPFY  ...F..F..  EG.FEYdl.P  FL--SST--i  .P-...----  ------QSLF
Clhs27ᵈ     33   AFGv.RLPDE  W.QwfSAAG.  .G-Yv-RPlP  A.TAEGP--A  ..--.LAAP.  AAPAfh----  ------RALN
Dmhs23ᵉ      1   .ANI.lLLSL  ADDLGRMSMV  .FYEPYYCQR  QRNPYLAlVG  PMEQQLRQ.E  ----------  --------k.
Dmhs26ᶠ      1   ...STlLSLV  DELQEPRSPI  YELGlGLHPH  SRYVLPLGTQ  QRRSINGCPC  ASPICPSSPA  GQVLALRREM


Piac25      58   LAAAARDIGS  NIKADKDKLQ  INLDVQHFSP  EEISVKTADG  FVVVEGKHEE  KKDEHGYISR  QFVRRYALPE
Dml2efl     60   NsLQKQes..  TlnI.Se.fe  vI....q...  S..t..V..K  ..i.......  .Q....v..  ..S...Q..S
Hsac        51   RTVLDSG.-.  EVRS.R..fV  .F...K....  .Dlt..VQ.D  ..EiH...N.  rQ.d......  E.H...R..S
Clhs27      88   R-QLsSGV-.  E.rQTA.rWR  vS...N..A.  ..lt...Ke.  V.EiT....  rQ.......  C.T.k.T..P
Dmhs23      53   Vg.ssgss.A  VS.IG..Gf.  vCm..S..K.  S.lV..VQ.N  S.l...N...  rE.d..f.t.  h........P
Dmhs26      72   ANRNDIHWPA  TAHVG..Gf.  vCm..Aq.K.  S.lN..VV.D  Sil......  rQ.d..H.M.  h.....Kv.d


Piac25     128   GAASETVESR  LSSGGVLTIT  APLKVPDAVK  G---ERKVPIA  QTG-PVRKEI  KDQSGKEKGN  K
Dml2efl    130   DVNPd..T.S  ...D.L...K  ..m.ALPPPQ  T----..L.Q.T  ...-.SS..D  NAKKVETSTA  -
Hsac       120   NVDQSALSCS  ..AD.M..FC  G.-.IQTGLD  AHT-..Ai.vS  REEK.TSAPS  S--------  --
Clhs27     156   .VDPTL.S.S  ..PE.T..ve  ...PKTATQS  A---..ITi.vT  FEARAQIGGQ  EAGKSEQS.A  .
Dmhs23     123   .YEAdK.A.T  ...D.....K  V.KPPAIED.  .N---.I.Q.T  .V.-.AhLNV  keNP-..AVE  Q
Dmhs26     142   .YKA.Q.V.Q  ...D....vS  I.KPQAVED.  SK--..IiQ.Q  .V.-.AhLNV  kANESEV..K  E
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Fig. 3. Alignment of the deduced amino acid sequence of *αcp25* from *P. interpunctella* with α-crystallins and small heat shock proteins showing homologous domains. ᵃPiac25, deduced amino acid sequence of *αcp25*. The sequences for the α-crystallins and small heat shock proteins from vertebrates were chosen as the highest scored representatives of the protein class that had both amino acid and nucleic acid sequences available. The small heat shock proteins from *D. melanogaster* were chosen as the highest ranking invertebrate sequences. The single underlined sequence represents the identified 30 amino acid internal fragment of αCP25 reported previously (Shirk and Zimowska, 1997) and the C-terminal conserved region that is important for the protective function of protein (James et al., 1994). The double underlined sequence shows the N-terminal hydrophobic aggregation site of α-crystallins (James et al., 1994). Abbreviations: "." = identity; "upper case letters" = non-identity; "lower case letters" = conservative substitution; and "-" = insertion; ᵃPiac25, predicted α-crystallin cognate 25 sequence of *P. interpunctella*; ᵇDml(2)efl, embryonic lethal *l(2)efl*, of *D. melanogaster* (Kurzik-Dumke and Lohman, 1995; smallest sum probability = $5.1 \times e^{-52}$); ᶜHsac, αA-crystallin a chain protein of *Homo sapiens* (Jaworsky, 1995; smallest sum probability = $6.8 \times e^{-38}$); ᵈClhs27, small heat shock protein *hsp27* of *Cricetulus longicaudatus* (Lavoie et al., 1990; smallest sum probability = $3.5 \times e^{-37}$); ᵉDmhs23, small heat shock protein *hsp23* of *D. melanogaster* (Ingolia and Craig, 1982; smallest sum probability = $8.3 \times e^{-35}$); ᶠDmhs26, small heat shock protein *hsp26* of *D. melanogaster* (Ingolia and Craig, 1982; smallest sum probability = $5.5 \times e^{-24}$).

(Fig. 7). Three different protein fractions containing semi-purified αCPs plus FEYP, FEYP and vitellin were resolved by SDS-PAGE and electroblotted to PVDF membrane. The electroblots were then exposed to varying solutions of salts and/or proteins before they were reacted with antisera for selected proteins and then color developed. Alkaline phosphatase was used as the marker for the second antibody and the reactions were intentionally over developed. This was done to provide the greatest level of sensitivity even though it results in an increase in the non-specific background. When reacted with antiserum to αCP25, the presence and their migrational positions for the four αCPs in the αCPs-FEYP fraction was established (Fig. 7A). The αCPs were also present in the FEYP fraction which indicates that the αCPs were a minor contaminant in the FEYP fraction. The αCPs were not present in the vitellin fraction. When reacted with antiserum to YP4 (Fig. 7E), YP4 was detected in all three fractions although only a small amount was present in the vitellin fraction (Fig. 7E, lane c). YP4 antiserum did not react (even with the inclusion of non-specific material) with any proteins smaller than 33 000 molecular weight. However, when the electroblot was reacted with a solution of native

FEYP before the reaction with the YP4 antiserum, band corresponding with the four αCPs also reacted with the YP4 antiserum (Fig. 7B) indicating that YP4 was bound to the αCPs bands. YP4 antiserum binding with these bands was not detected when the electroblots were further incubated with either an ATP solution or a pH 2.5 TBS buffer before the reaction with the YP4 antiserum (Fig. 7C, D). This indicates that either the YP4 bound to the αCPs bands lost its antigenic conformation or YP4 was released from the αCPs bands under these two reaction conditions. When vitellin was used instead of FEYP, the YP3 (a vitellin subunit) antiserum did not react with the αCPs bands in the αCPs/FEYP or FEYP lanes (Fig. 7F).

4. Discussion

A full length cDNA clone, *αcp25*, for the αCP25 protein in *P. interpunctella* was isolated from a cDNA expression library constructed of mRNA from pharate adult female ovary. The cDNA sequence contained a single open reading frame that encoded for a 185 amino acid protein. One section of the predicted amino acid
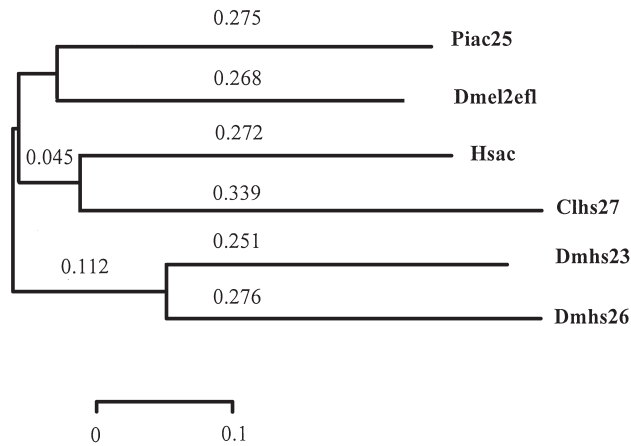
Fig. 4. Homology dendogram for amino acid sequence relationships amongst *αcp25* from *P. interpunctella*, *α*-crystallins, and small heat shock proteins from various vertebrate species and *D. melanogaster*. The sequences were chosen for comparison on the basis of the ranking from the smallest sum probability from the BLAST search. The unit scale for the branch lengths of the dendogram represents the relative genetic distance. Designations for amino acid sequences: Piac25, *αcp25* from *P. interpunctella*; Dml(2)efl; embryonic lethal, *l(2)efl*, from *D. melanogaster* (Kurzik-Dumke and Lohman, 1995; smallest sum probability = $5.1 \times e^{-52}$); Hsac, *α*-crystallin A chain protein from *Homo sapiens* (Jaworsky, 1995; smallest sum probability = $6.8 \times e^{-38}$); Clhs27, small heat shock protein *hsp27* of *Cricetulus longicaudatus* (Lavoie et al., 1990; smallest sum probability = $3.5 \times e^{-37}$); Dmhs23, *hsp23* from *D. melanogaster* (Ingolia and Craig, 1982; smallest sum probability = $8.3 \times e^{-35}$); Dmhs26, *hsp26* from *D. melanogaster* (Ingolia and Craig, 1982; smallest sum probability = $5.5 \times e^{-24}$).
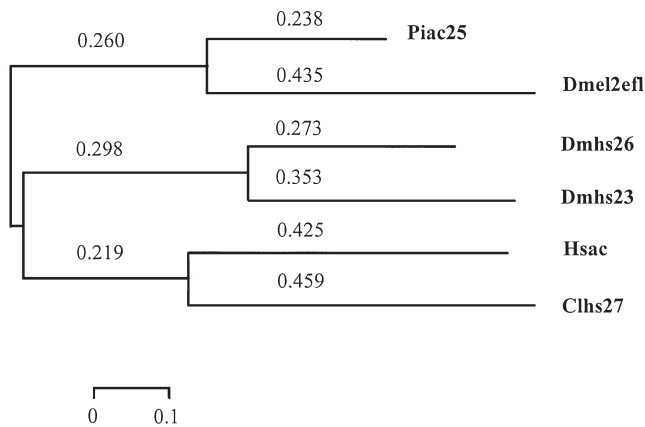

Fig. 5. Homology dendogram for DNA sequence relationships amongst *αcp25* from *P. interpunctella*, *α*-crystallins, and small heat shock proteins from various vertebrate species and *D. melanogaster*. The DNA sequences were the same as those selected for the comparison of amino acid sequences. Designations for DNA sequences: Piac25, *αcp25* cDNA sequence from *P. interpunctella*; Dml(2)efl; embryonic lethal, *l(2)efl*, gene from *D. melanogaster* (Kurzik-Dumke and Lohman, 1995); Dmhs23, *hsp23* gene from *D. melanogaster* (Ingolia and Craig, 1982); Dmhs26, *hsp26* gene from *D. melanogaster* (Ingolia and Craig, 1982); Hsac, *α*-crystallin A chain protein gene from *Homo sapiens* (Jaworsky, 1995); Clhs27, small heat shock protein *hsp27* gene of *Cricetulus longicaudatus* (Lavoie et al., 1990).

sequence had total identity with a 30 amino acid sequence derived from an internal fragment of purified αCP25 that was described previously (Shirk and Zimowska, 1997).

The predicted amino acid sequence for *αcp25* shared considerable sequence similarity with an embryonic lethal in *D. melanogaster* and the *α*-crystallin proteins from vertebrate lenses. Sequence similarity was shared to a lesser degree with the small *hsps* from vertebrates and insects. The Neighbor-Joining dendogram for the predicted proteins placed *αcp25* most closely related to the embryonic lethal *l(2)efl* protein in *D. melanogaster* (Kurzik-Dumke and Lohman, 1995). On the basis of its predicted amino acid sequence, *l(2)efl* was characterized as a member of the small heat shock family. However, the evolutionary dendograms presented here show that the amino acid sequences for both *αcp25* in *P. interpunctella* and *l(2)efl* in *D. melanogaster* are more closely related to the *α*-crystallin proteins from vertebrates and even some of the small *hsps* in vertebrates than to the small *hsps* in *D. melanogaster*.

The *αcp25* protein, as well as the *l(2)efl* protein, contains two regions that are highly conserved with the *α*-crystallin proteins. First *α*-crystallin proteins and small *hsps* have a highly conserved region that corresponds to the sequence of *αcp25* from amino acid 70 through to amino acid 125. This sequence represents a C-terminal region that is involved in the protective functions of the *α*-crystallin proteins and small *hsps* (Ingolia and Craig, 1982; James et al., 1994). *αcp25* also has an N-terminal sequence, amino acid residues 25 through 29, that is highly conserved with a RLFDQFF hydrophobic site in the N-terminus of *α*-crystallin proteins. This hydrophobic site has been shown to be important for aggregation and formation of the large *α*-crystallin complexes found in the vertebrate lens (James et al., 1994). The RLFDQFF hydrophobic site is also present in *l(2)efl* but is not found in the small *hsps* of *D. melanogaster*. The presence of these two conserved regions and the overall amino acid sequence similarity with the *α*-crystallin proteins supports our characterization of *αcp25* as an *α*-crystallin protein.

Unlike the predicted amino acid sequences, the cDNA sequences for *αcp25* and *l(2)efl* are more closely related to the small *hsps* genes in *D. melanogaster* than they are to the genes for the *α*-crystallin proteins. This suggests that, similar to the *α*-crystallin genes, *αcp25* in *P. interpunctella* and *l(2)efl* in *D. melanogaster* have common ancestral genes with the small *hsps* (Wistow and Piatigorsky, 1988; de Jong et al., 1993).

The presence of the conserved N-terminal hydrophobic aggregation site and the C-terminal protective binding region suggests that the amino acid sequences for *αcp25* and *l(2)efl* have converged on a protein structure common to the *α*-crystallins. This convergence may also suggest that the common structural features confer
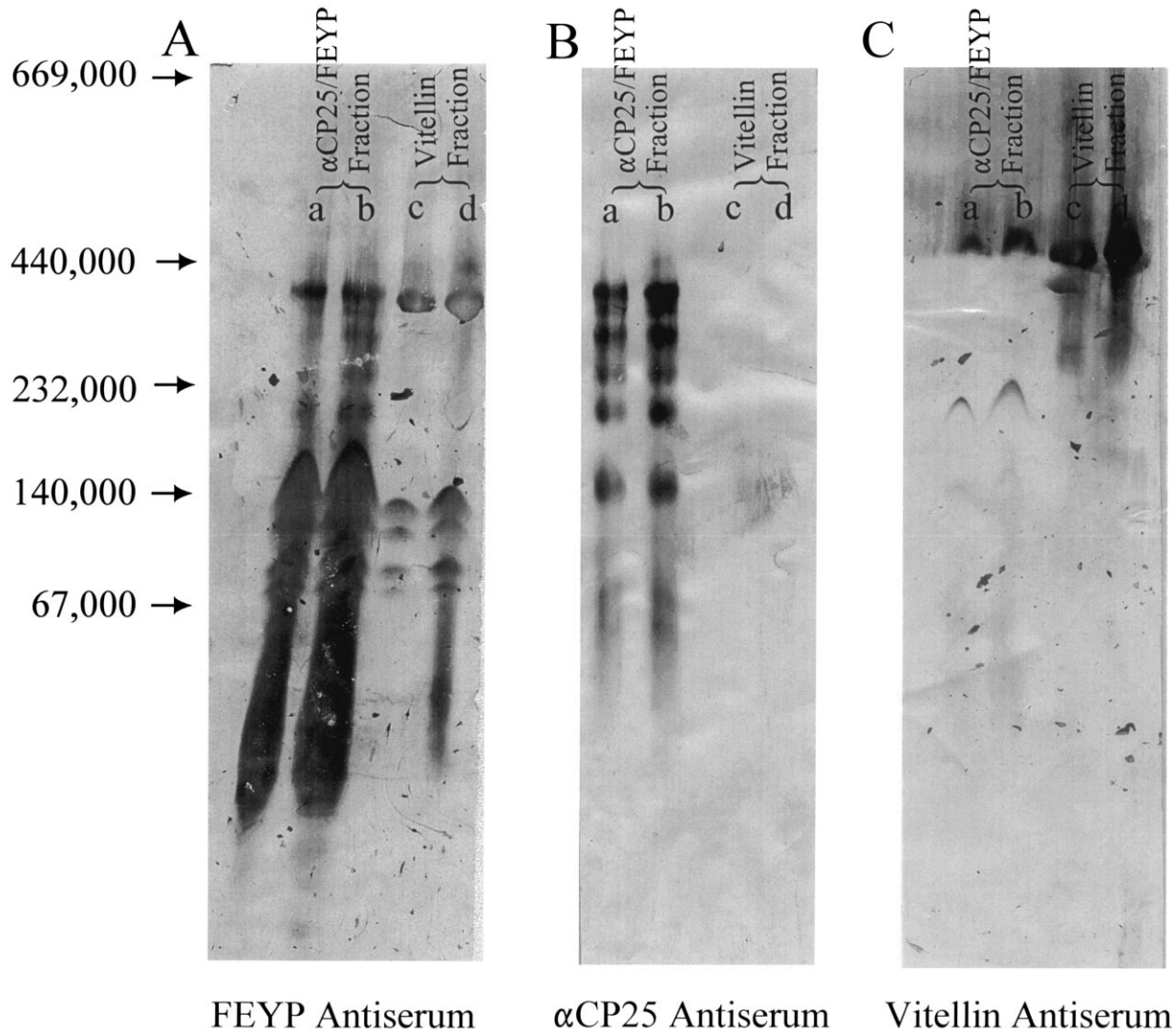
Fig. 6. α-Crystallins from eggs of *P. interpunctella* associate with FEYP in native conditions. An electroblot of a native gel to resolve the α-CP25/FEYP fraction (lanes a and b) and vitellin fraction (lanes c and d) was reacted with antiserum to FEYP (Panel A), α-CP25 (Panel B) or vitellin (Panel C). The position of the molecular weight standards is marked on the left of the gel.

αcp25 and *l(2)efl* with functional activities similar to those of the α-crystallin proteins as well. The major function of α-crystallins in lenticular tissues is to form heteropolymer aggregates with high molecular weights, up to 800 000, providing a protein complex with properties of transparency and proper light refraction necessary for vision (Wistow and Piatigorsky, 1988; de Jong et al., 1993). The formation of the heteropolymer aggregates is dependent on the presence of the N-terminal sequences (Merck et al., 1993) and appears to involve the RLFDQFF hydrophobic site mentioned above (James et al., 1994). Variations in the heteropolymeric mixture of the α-crystallins with other crystallins provides the appropriate changes in the refractive index of the lens material to provide the physical characteristics necessary for correct focusing by the lens.

The α-crystallins in vertebrates also function as mol-ecular chaperones for various proteins in both lenticular and non-lenticular tissues. Binding with α-crystallins prevents thermal aggregation of enzymes (Horowitz, 1992) and other crystallins (Rao et al., 1995) into insoluble precipitates. They also prevent steroid-induced inactivation of catalase by binding directly with the catalase enzyme to prevent attack by reactive carbonyl groups of the steroid on the α- or ϵ-amino groups of lysine residues (Hook and Harding, 1996). In addition, the α-crystallins have been shown to inhibit the assembly of intermediate filaments in both lenticular and non-lenticular cells and may play a role in the control of cytoskeleton assembly and remodeling during development (Nicholl and Quinlan, 1994).

In a similar fashion with the α-crystallins, αCP25 also appears to function as a chaperone during embryogenesis in *P. interpunctella*. The initial isolation of αCP25 was
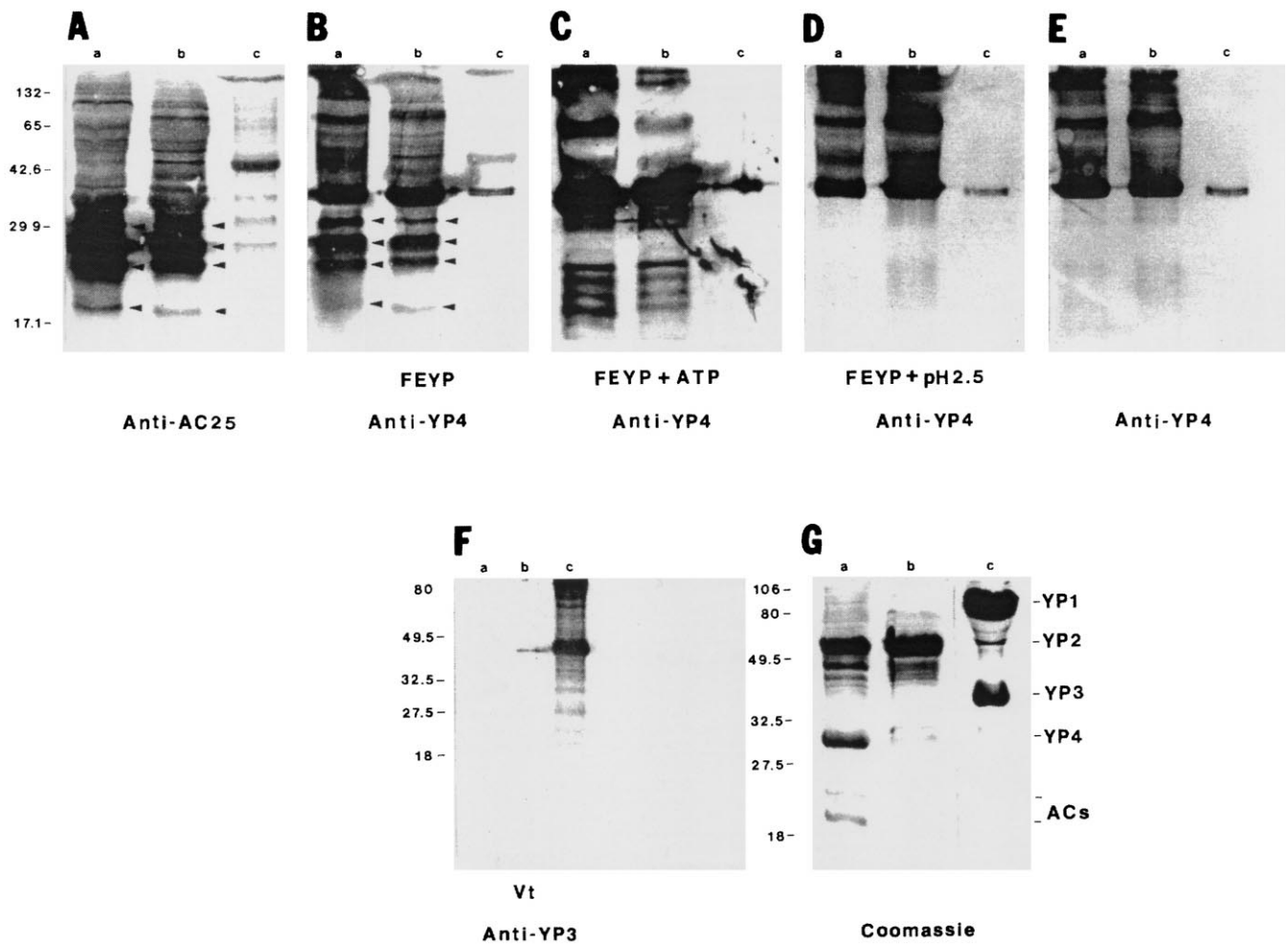
Fig. 7. α-Crystallins from eggs of *P. interpunctella* bind reversibly with FEYP. Immobilized α-Crystallins were exposed to various native proteins and assessed for binding interactions. The protein fractions (lane a, FEYP + acs; b, FEYP; c, vitellin) were resolved by SDS-PAGE and electroblotted to Trans-Blot membrane as described. Panel A; Electroblotted protein fractions reacted with αCP25 antiserum (Arrow heads show the position of the four αCPs proteins). Panel B; Electroblotted protein fractions incubated with native FEYP and then reacted with YP4 antiserum (Arrow heads show the position of the four *acs* proteins). Panel C; Electroblotted protein fractions incubated with native FEYP, then incubated with 100 mM ATP, and then reacted with YP4 antiserum. Panel D; Electroblotted protein fractions incubated with native FEYP, then washed with pH 2.5 buffer, and then reacted with YP4 antiserum. Panel E; Electroblotted protein fractions reacted with YP4 antiserum. Panel F; Electroblotted protein fractions incubated with native vitellin and then reacted with YP3 antiserum. Panel G; Coomassie Brilliant Blue 254 stained SDS-PAGE gel of the three protein fractions. The migration positions of the molecular weight markers are shown on the left of Panels A, F, and G. The positions of the four yolk polypeptides are shown on the right of Panel G.

in conjunction with the purification of the yolk proteins from the eggs of *P. interpunctella* (Shirk and Zimowska, 1997). A reaction that contained both FEYP and the four α-crystallin cognates was eluted from DEAE-Sepharose chromatography and after immunoblot analysis showed that the α-crystallins and FEYP formed high molecular weight complexes. On the other hand, the α-crystallins and the vitellin did not form any high molecular weight complexes, suggesting a specificity to the binding between the α-crystallins and the FEYP. Using an electroblot binding assay, we have demonstrated that αCP25 can bind selectively with FEYP, but not with other proteins, and that the binding is reversible under denaturing conditions and in the presence of ATP. The release of binding complexes under the influence of MgATP is

typical of chaperones such as *E. coli* groEL and *hsp70* (for review see Ellis and van der Vies, 1991). Because we find αCP25 and FEYP as a complex in developing embryos and can simulate the binding *in vitro*, this leads us to conclude that αCP25 can function as a chaperone for FEYP in the embryo.

The binding between αCP25 and FEYP would take place only after fertilization when the yolk spheres begin to break down, because αCP25 is present only in the cytoplasm and FEYP is contained in the yolk spheres (Shirk and Zimowska, 1997). During embryogenesis, active yolk degradation appears to be regulated by acidification of the yolk spheres in both invertebrates and vertebrates (Fagatto, 1991; Nordin et al., 1991; Mallya et al., 1992; Fagatto and Maxfield, 1994). That αCP25

binds with FEYP under an acidic environment is corroborated in the conditions of the electroblot binding assay; the maximum binding activity of αCP25 for FEYP was found to be pH 6.5 (data not shown). Additionally, the predicted amino acid sequence for $\alpha cp25$ has a pI of 8.2 which suggests that the protein would be charged under the acidic conditions of the degenerating yolk spheres. In fact, the pI is the one major difference between αCP25 and other related proteins: the pI for the predicted *l(2)efl* protein in *D. melanogaster* is 5.8, for *hsp23* in *D. melanogaster* it is 5.5, and for the vertebrate α-crystallins it is 5.7. This implies that the sequence for $\alpha cp25$ has shifted from the archetypal form shared by the related proteins to accommodate its function as a chaperone for FEYP in the acidified environment of the yolk spheres during embryogenesis.

The function of αCP25 as a chaperone in the germ cells may be critical to cellular activities as suggested by the finding that the closely related protein *l(2)efl* is an embryonic lethal in *D. melanogaster* (Kurzik-Dumke and Lohman, 1995). This is not the case for the small *hsps* that are expressed in the germ cells of *D. melanogaster* (Zimmerman et al., 1983; Mason et al., 1984; Glaser et al., 1986). Neither deletion of the 67B locus (Sirotkin et al., 1986) nor insertional mutation at the *hsp28* locus (Eissenberg and Elgin, 1987) causes the same phenotype as *l(2)efl*. Analysis of the possible functions of $\alpha cp25$ and *l(2)efl* in germ cells may provide a means to examine the unique physiological needs of germ cells.

Acknowledgements

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