Vitellin and Formation of Yolk Spheres in Vitellogenic Follicles of the Moth, *Plodia interpunctella*

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Vitellin (Vt) was found not to be critical to the formation or structure of yolk spheres in oocytes of the moth, *Plodia interpunctella* (Hübner). Vitellogenic activities of the follicular tissues were determined by visualizing the immuno-cytolocalization of Vt subunits (YP1 and YP3) and of a follicular epithelium yolk protein (FEYP) subunit (YP2) in ultrathin sections or in whole-mounted tissues. Vitellogenin was detectable in the inter-follicular epithelial cell (FC) spaces of patent, vitellogenic follicles of normal females. When the follicles entered terminal growth phase, the inter-FC spaces closed equatorially around the follicle which excluded vitellogenin from that region. The closure of the spaces spread towards the poles in more mature follicles. Vt was immunolocalized to yolk spheres of vitellogenic and terminal growth phase oocytes. To examine the role of Vt in formation of yolk spheres, ovaries were transplanted into males. Vt was not detected in the inter-FC spaces, vitelline membrane, or yolk spheres of follicles from transplanted ovaries developing in males. However, the FEYP subunit YP2 was detected in the Golgi apparatus and secretory vesicles of columnar FC and in the yolk spheres of the oocytes from transplanted ovaries. During the late vitellogenic period, late yolk spheres appeared in the cortical region of the oocytes. In addition, YP2 was detected in the electron-translucent vitelline membrane of terminal growth phase follicles. We conclude that Vt is not required for the formation of yolk spheres or the electron-translucent layer of vitelline membrane.

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Key words: oogenesis, yolk proteins, vitelline membrane, immunofluorescent staining, immunogold labeling

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INTRODUCTION

The major yolk proteins of Lepidoptera are produced in two tissues and are then taken up by vitellogenic oocytes (cf. Englemann, 1979; Hagedorn and Kunkel, 1979; Telfer et al., 1981; Kunkel and Nordin, 1985). Proteins from the fat body (i.e., vitellogenin, microvitellogenin, and lipophorin) and from the follicular epithelial cells (FC) are packaged in yolk spheres to form proteinaceous yolk within the oocytes during vitellogenesis. Although yolk proteins are most likely the major source of amino acids during embryogenesis, the importance of each of these proteins to the formation and function of the yolk spheres and to embryogenesis is not clear.

In the silkworm, Bombyx mori, oocytes completed development normally even without the presence of vitellin (Yamashita and Irie, 1980). After transplantation of immature ovaries into male pupae before initiation of adult development, chorionated eggs developed in the ovaries during metamorphosis even though males do not produce vitellogenin. Those eggs that developed in males were later shown to contain egg-specific protein, which is produced by the FC, and a 30 kDa non-sex-specific hemolymph protein in normal levels but no vitellin (Zhu et al., 1986). Although the eggs that developed in males lacked vitellin, normal larvae, pupae, and adults developed parthenogenically from these eggs (Yamashita and Irie, 1980). Formation of chorionated eggs that lack vitellin has also been observed in ovaries that developed in males of Hyalophora cecropia (Telfer, 1954), Antheraea polyphemus (Telfer and Rutberg, 1960), and Lymantria dispar (Ballarino et al., 1991a,b).

Vitellogenic processes in the Indianmeal moth, Plodia interpunctella (Hübner), are typical of those observed for other Lepidoptera but with unique features that may provide a means to further assess the role of vitellin in the formation of yolk spheres (Shirk et al., 1990; Zimowska et al., 1991, 1994; Shaaya et al., 1993). Following the major ecdysteroid peak (Shaaya et al., 1993), the terminal follicles within the ovarioles become vitellogenic 105 h after pupation (Zimowska et al., 1994). The two major yolk protein precursors, vitellogenin and follicular epithelium yolk protein (FEYP), are taken up and accumulate in the yolk spheres of oocytes during vitellogenesis (Shirk et al., 1984; Bean et al., 1988; Zimowska et al., 1994). Based on in vitro amino acid labeling of proteins, we found that the fat body secretes vitellogenin, which consists of two subunits, YP1 (Mr = 153,000) and YP3 (Mr = 43,000) (Shirk et al., 1984; Bean et al., 1988). Vitellogenin is accumulated, without major change, in yolk spheres within oocytes as vitellin (Mr = 475,000). The hemolymph titers of vitellogenin began to increase just before the initiation of vitellogenesis in terminal follicles and continued to increase into the adult stage (Shirk et al., 1992). FC secrete FEYP (Mr = 235,000) that is comprised of subunits (YP2 (Mr = 69,000) and YP4 (Mr = 33,000) (Shirk et al., 1984; Bean et al., 1988; Zimowska et al., 1994). FEYP is accumulated in the yolk spheres in a homogeneous mixture with vitellin (Zimowska et al., 1994). Terminal follicles complete vitellogenesis within 2–6 h after adult eclosion, which occurs 136 h after pupation, and initiate choriogenesis (Zimowska et al., 1991).

During termination of vitellogenesis, a specialized form of yolk spheres, the late yolk spheres, appears in the cortical region of the oocyte (Zimowska
et al., 1995). The late yolk spheres were morphologically characterized by irregular shapes, a variable density of contents, associations with membrane-bound vesicles, and the presence of internal vesiculations and/or electron-dense inclusions. Some of the late yolk spheres apparently fuse with the oolemma, and yolk proteins, that may have originated in the late yolk spheres, were immunologically detectable in the electron-translucent layer of the vitelline membrane. Examination of the formation of late yolk spheres in *P. interpunctella* provides a system to examine whether vitellin influences the formation of these organelles and their potential function.

We examined changes in the follicles during the termination of vitellogenesis and initiation of vitelline membrane synthesis using specific antisera for vitellin and FEYP subunits coupled with immunofluorescent or immunogold labeling of tissues to visualize the subcellular distribution of vitellin. By comparing organelle structure and protein distribution in normal ovaries with ovaries that developed in males, it was shown that yolk sphere formation was normal in oocytes that lacked vitellin.

**MATERIALS AND METHODS**

**Insect Preparations**

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.

**Ovariectomies and Ovarian Transplantations**

Wandering last instar larvae were narcotized by immersion in ice-cold saline (Weevers, 1966). The ovariectomies and transplantations were completed while the larvae were immersed in saline. The ovaries were removed through a small incision made on the dorsal-lateral surface of the fifth abdominal segment. The larvae were then removed from the saline and dried, and the wound was sealed with Krazy glue® (Borden, Inc., Columbus, OH).

For ovarian transplantations, the ovaries were removed from the females and transplanted into males through an incision in the mid-dorsal cuticle of the fourth abdominal segment. Once the ovaries were placed within the hemocoel, the male was removed from the saline and dried, and the wound was sealed with Krazy glue®. The male larvae were placed under normal culture conditions and allowed to complete development.

**Immunohistochemistry**

Immunofluorescent detection of yolk polypeptides YPI and YP3 in whole-mounts of ovaries was performed as described previously (Zimowska et al., 1991). Immunogold localization of YP1, YP2, and YP3 in ultrathin sections was performed as described previously (Zimowska et al., 1994). The polyclonal rabbit antisera for YP1, YP2, and YP3 used in the immunoreactions were described in Bean et al. (1988). Preabsorbed antisera for YP1, YP2, and YP3 were prepared by serially exposing each antiserum to the corresponding antigen electroblotted to nitrocellulose (Shirk et al., unreported data).
Polyacrylamide Gel Electrophoresis and Western Blotting

The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to Trans-Blot membrane (BioRad, Richmond, CA) as described previously (Bean et al., 1988). The immunoblots were blocked and reacted as described previously (Shirk et al., 1992). The individual blots were reacted with polyclonal antisera for YP1, YP2, and YP3 (Bean et al., 1988) and polyclonal antiserum for YP4. The immunoreactive bands were visualized with an Immun-Blot color assay (BioRad) using horseradish peroxidase-linked goat anti-rabbit IgG as the second antibody.

Vitelline Membrane/Chorion Purification

One-hour-old embryos were washed twice in 0°C distilled water and once in 0°C homogenization buffer (40 mM Tricine, pH 6.8, 20% sucrose, 150 mM NaCl, 1 mM MgCl2, 0.1 mM PMSF, 1% Triton X-100). The embryos were disrupted in homogenization buffer by 1 min of sonication with a cup-horn sonicator (Heat Systems Ultrasonics, Inc., Farmingdale, NY). The homogenate was centrifuged at 50g for 5 min at 4°C. The pellet was resuspended in homogenization buffer, sonicated, and pelleted by centrifugation six times. The pellet was then resuspended in homogenization buffer, the large pieces of chorion were allowed to settle by gravity for 30 min, and the supernatant was removed. The washing and settling of the chorion pieces was repeated five times until no yolk spheres were present by visual inspection with a microscope (data not shown). The preparation was placed in SDS-sample buffer (Shirk et al., 1984) and boiled for 5 min. The proteins were resolved by SDS-PAGE and electroblotted to Trans-Blot membrane (BioRad) as described previously (Bean et al., 1988). The immunoblots were blocked and reacted as described previously (Shirk et al., 1992). The individual blots were reacted with polyclonal antisera for YP1 or YP3 (Bean et al., 1988). The immunoreactive bands were visualized with an Immun-Blot color assay (BioRad) using horseradish peroxidase-linked goat anti-rabbit IgG as the second antibody.

RESULTS

Termination of Patency in the Follicular Epithelium

The inter-FC spaces of vitellogenic follicles were large, and the follicles were considered patent. Immunofluorescent staining of whole-mounted ovarioles for YP1 showed large amounts of the stained material around the perimeter of the FCs, indicating that vitellogenin was present in the inter-FC spaces of the vitellogenic follicles (Fig. 1A, follicles 4–8; Fig. 2A). The YP1 staining material could also be seen pooled between the basal surface of the FCs and the overlying tunica propria (Fig. 2A). When immunofluorescently stained whole-mounted ovarioles were embedded in Lowicryl and sectioned, the semithick sections showed that the YP1 reactive material was present in the inter-FC spaces (Fig. 1B).

As the follicles entered the terminal growth phase, YP1 staining material was progressively excluded from the inter-FC spaces as observed in whole-mounted ovaries (Fig. 1A, follicles 1–3). The narrowing of the inter-FC spaces
Fig. 1. Inter-FC spaces in vitellogenic and terminal growth phase follicles from newly eclosed adult female *P. interpunctella*. A: A linear series of follicles in a whole-mounted ovariole immunofluorescently stained for YP1. Follicle 2 is the first follicle in terminal growth phase. B: A glancing section through two adjoining follicles that had been immunofluorescently stained in whole-mounted ovarioles as in A. The preparation was postfixed in 0.1% glutaraldehyde and embedded in lowyl before sectioning. The yolk spheres within the oocyte were not immunofluorescently stained in these sections because the antiserum did not penetrate well through the vitelline membrane in the original whole-mounted preparation. fc, follicular epithelial cell; nc, nurse cell cap; oo, oocyte; sp, inter-FC space. Scale bar for A = 50 μm and for B = 25 μm.
was not uniform around the oocytes and appeared to begin equatorially in the most mature vitellogenic follicles (Fig. 1A, oocyte 4) and spread progressively towards the poles of the oocytes during the successive stages of the terminal growth phase (Fig. 1A; compare follicles 3, 2, and 1). By the end of the terminal growth phase, the only remaining YP1 staining material was collected in large pools at the polar regions of the follicles (Fig. 1A). In the first follicles in terminal growth phase, the FC had formed an occlusion zone around the oocyte, and the inter-FC spaces had narrowed (Zimowska et al., in press). The amount of YP1 staining material still present in the inter-FC spaces of the first terminal growth phase follicles was reduced compared with vitellogenic follicles (compare Fig. 2A,B). In follicles that had completed the terminal growth phase, only traces of YP1 staining material were observed in the spaces (Fig. 2C).

**Vitellin Deposition in Yolk Spheres**

The yolk spheres of vitellogenic follicles contain vitellin. Immunogold labeling of ultrathin sections from vitellogenic follicles for YP1 showed that the vitellin subunit was present in the nascent and mature yolk spheres in the cortical cytoplasm of the oocyte (Fig. 3A); immunolabeling for YP3 was identical to that for YP1 (data not shown). YP1-labeled material was also observed within the brush border and the broad electron-translucent vitelline membrane present in follicles of this stage. In late vitellogenic follicles, electron-translucent vesicles (Fig. 3B,C) as well as cytoplasmic invaginations (Fig. 3D) were observed in association with yolk spheres in the cortical region. These yolk spheres were also intensely labeled for YP1.

As described previously, in follicles that were completing vitellogenesis in
Vitellin and Yolk Spheres in *P. interpunctella*

Fig. 1. Inter-FC spaces in vitellogenic and terminal growth phase follicles from newly eclosed adult female *P. interpunctella*. A: A linear series of follicles in a whole-mounted ovariole immunofluorescently stained for YP1. Follicle 2 is the first follicle in terminal growth phase. B: A glancing section through two adjoining follicles that had been immunofluorescently stained in whole-mounted ovarioles as in A. The preparation was postfixed in 0.8% glutaraldehyde and embedded in Lowicryl before sectioning. The yolk spheres within the oocyte were not immunofluorescently stained in these sections because the antiserum did not penetrate well through the vitelline membrane in the original whole-mounted preparation. fc, follicular epithelial cell; nc, nurse cell cap; oo, oocyte; sp, inter-FC space. Scale bar for A = 50 µm and for B = 25 µm.
Fig. 3. Vitellin was present in yolk spheres of oocytes. Ultrathin sections were immunogold-labeled for YP1. A: The vitelline membrane, brush border, and the yolk spheres of a vitellogenic follicle. B,C: Vesicles associated with late yolk spheres in the cortical region of late vitellogenic follicles. D: Cytoplasmic invaginations in late yolk spheres from late vitellogenic follicles. Arrows in D indicate inclusions within the late yolk spheres. Gold particles are 20 nm electron-dense spheres. bb, brush border; lys, late yolk spheres; v, vesicle; vm, vitelline membrane; ys, yolk sphere. Scale bars = 1 μm.
P. interpunctella, late yolk spheres appeared in the cortical region of the oocyte (Zimowska et al., 1995). These organelles were characterized by irregular shapes, variable amounts of electron-dense material in the matrix, and the presence of vesiculations and/or inclusions within the limiting membranes. These morphological features of late yolk spheres were also observed in preparations where the presence of vitellin was demonstrated by immunogold labeling (Fig. 3B–D). The relative amount of immunogold labeling for YP1 and YP3 in the late yolk spheres was proportionate with the relative amount of electron-dense material in the matrix (Fig. 4). Some of the late yolk spheres were juxtaposed to the oolemma of the most mature vitellogenic follicles or had apparently fused with the oolemma, with their contents exposed to the electron-translucent layer of the vitelline membrane (Fig. 4B).

To assess the importance of vitellin to the formation and structure of yolk spheres, ovaries were transplanted into males to permit the development of oocytes that lack vitellin. The lack of vitellin within oocytes developed in males was initially confirmed by subjecting oocyte homogenates to immunodetection on Western blots. The oocytes that developed in males contained immunodetectable YP2 and YP4, subunits of FEYP, whereas YP1 and YP3, the subunits of vitellin, were not detectable (Fig. 5A).

To establish the presence of the FEYP subunits in vitellogenic follicles developing in males, ultrathin sections of these follicles were immunogold-labeled for YP2. Labeling for YP2 was observed in the Golgi apparatus and secretory vesicles of the FC, in the electron-translucent vitelline membrane, and in the yolk spheres in the oocytes (Fig. 6A); immunogold labeling for YP4 was identical (data not shown). However, immunogold labeling for YP3 showed no detectable labeling for the vitellin subunit in the vitelline membrane or yolk spheres of the vitellogenic follicles that developed in males (Fig. 6B); similarly, YP1 labeling was not detectable (data not shown).

Yolk spheres of oocytes that developed in males had structural features similar to those from normal oocytes. Most yolk spheres were spherical and contained evenly distributed amounts of electron-dense material in the matrix, as did yolk spheres from normal oocytes. Immunogold labeling for YP2 in ultrathin sections from oocytes that developed in males demonstrated the presence of FEYP in all of the yolk spheres in these oocytes (Figs. 6A, 7A–D). In addition to vitellogenic yolk spheres, late yolk spheres appeared in the cortical region of oocytes during termination of vitellogenesis. Late yolk spheres in oocytes from ovaries developed in males had characteristics of these organelles from normal oocytes: irregular shapes, electron-dense inclusions (Fig. 7A,D), variable electron density in the matrix (Fig. 7B,D), and vesiculations (Fig. 7C). Some of the late yolk spheres were also observed apparently fused with the oolemma (Fig. 7D), as described previously (Zimowska et al., 1995). To determine if vitellin became a permanent component of the vitelline membrane, a purified vitelline membrane/chorion from early eggs preparation was examined using Western blots. YP1 and YP3 were immunodetectable in the vitelline membrane preparation (Fig. 5B).
Fig. 4. The cortical region of late vitellogenic follicles contained late yolk spheres. Ultrathin sections were immunogold-labeled with YP1 (A) or YP3 (B). A: Late yolk spheres contained variable amounts of electron-dense material and the vitellin subunit YP1. B: Electron-translucent late yolk spheres were juxtaposed to the oolemma. Gold particles are 20 nm electron-dense spheres. The stars in each panel are presented as a relative scale of electron-dense material and immunogold labeling in yolk spheres and late yolk spheres: ★ = weak density; ★★ = moderate density; ★★★ = high density. bb, brush border; lys, late yolk spheres; v, vesicle; vm, vitelline membrane; ys, yolk sphere. Scale bars = 1 μm.
Fig. 5. Vitellin subunits YP1 and YP3 were not immunodetectable in ovaries that developed in males. Homogenates of ovaries were prepared from normal females and from ovaries that had completed development in males. After resolution by SDS-PAGE, a Western blot was made of the gel. The size of the molecular weight standards (× 10^3) are marked on the left of each panel, and the position for the YPs is marked on the right. A: Identical blots that were exposed to YP1 antiserum (lanes b,c), YP2 antiserum (d,e), YP3 antiserum (f,g), and YP4 antiserum (h,i). Lane a: Molecular weight standards. Lanes b,d,f,h: Homogenate of ovary from normal female. Lanes c,e,g,i: Homogenate from ovary that completed development in a male. MTO, homogenate of ovary transplanted into male; NO, homogenate of normal ovary. Yolk proteins were immunodetectable in a purified vitelline membrane/chorion preparation. Vitelline membrane and chorion were purified from 1-h-old eggs, resolved by SDS-PAGE, and electroblotted. B: Identical blots that were exposed to YP1 antiserum (b,c) and YP3 antiserum (d,e). Equivalent amounts of protein were applied to each lane. Lane a: Molecular weight standards. EH, homogenate of 1 h eggs; VM, purified vitelline membrane.
DISCUSSION

The vitellogenin subunits were immunofluorescently detected within the inter-FC spaces of patent vitellogenic follicles of *P. interpunctella*, as reported previously (Zimowska et al., 1994). However, once a follicle switched from vitellogenesis to the terminal growth phase and the FC began to form an occlusion zone around the oocyte, vitellogenin became excluded from the inter-FC spaces. The exclusion process began equatorially and spread towards the poles of the oocyte and was followed by an apparent pooling of vitellogenin in the interfollicular areas anterior and posterior to the follicle. The polar pooling of vitellogenin is similar to that observed at the poles of follicles initiating vitellogenesis (Zimowska et al., 1994).

The vitellin subunits were also detected in the yolk spheres of oocytes from *P. interpunctella* by immunogold labeling. The presence of vitellin in yolk spheres was previously demonstrated for the moths *B. mori* (Takesue et al., 1983) and *Manduca sexta* (van Antwerpen et al., 1993) and the flies *A. aegypti* (Raikhel, 1984, 1987) and *D. melanogaster* (Butterworth et al., 1992). To determine the importance of the presence of vitellin to the formation and functioning of yolk spheres, ovaries were transplanted into males, which do not produce vitellogenin (Shirk et al., 1984), to undergo metamorphosis and vitellogenesis. Previous reports have shown that ovaries transplanted into males of *A. polyphemus* (Telfer and Rutberg, 1960), *B. mori* (Yamashita and Irie, 1980; Zhu et al., 1986), *H. cecropia* (Telfer, 1954), and *L. dispar* (Ballarino et al., 1991a,b) all lack vitellin. The yolk spheres of ovaries transplanted into males of *P. interpunctella* likewise did not contain vitellin.

In normal ovaries, small yolk spheres that contain only YP2 are formed during the provitellogenic stage, and, when the FC becomes patent, the yolk spheres increase in size and contain both vitellin and FEYP (Zimowska et al., 1994). Although morphometric measurements were not made to determine the precise size range of the mature yolk spheres in the oocytes of ovaries transplanted into males, it was apparent that the size of the yolk spheres increased when the follicles became vitellogenic. This indicates that the transition from the provitellogenic stage to the vitellogenic stage was not inhibited. In addition, the lack of vitellin did not interfere with the normal fusion of endosomes during formation of mature yolk spheres. The corollary of this observation is that the proteins produced by the follicular epithelial cells (e.g., FEYP) are adequate to provide sufficient materials to support yolk sphere formation.

Late yolk spheres that lacked vitellin were also observed in the late vitellogenic oocytes of ovaries transplanted into males. The late yolk spheres had morphologies similar to normal late yolk spheres and were localized in the cortical region. In addition, some of the late yolk spheres were observed to fuse with the oolemma in terminal growth phase follicles. The electron-translucent layer of the vitelline membrane of normal terminal growth phase follicles was immunogold-labeled for vitellogenin subunits. This was not so for vitelline membrane of terminal growth phase follicles from ovaries transplanted in males. As expected, the FEYP subunits were detected in the electron-translucent layer of the vitelline membrane, but no labeling was observed.
Fig. 6. Follicles that developed in males contained FEYP but not vitellin. A: Ultrathin sections of follicles that developed in males that were immunogold-labeled for the FEYP subunit YP2. B:
Fig. 7. Late yolk spheres produced in oocytes that developed in males without vitellin had morphologies typical of late yolk spheres from normal ovaries. Ultrathin sections were immunogold-labeled for FEYP subunit YP2. Oocytes from follicles during termination of vitellogensis formed late yolk spheres with electron-dense inclusions (A, arrow). Irregular shapes (A,B,D), uneven distribution of electron density in the matrix (B,D), and internal vesiculations (B,C) were also observed morphological characters of late yolk spheres. The late yolk spheres were restricted to the cortical region of the oocyte, and an occasional fusion with the oolemma was observed (D). Gold particles are 20 nm electron-dense spheres. bb, brush border; li, lipid droplet; lys, late yolk sphere; oo, oocyte; v, vesicle; vm, vitelline membrane. Scale bars = 1 μm.

Immunogold labeling for YP3 in ultrathin sections from these follicles was not above background in vitelline membrane or yolk spheres. Gold particles are 20 nm electron-dense spheres, and in B the ▽ points to immunogold particles. bb, brush border; fc, follicular epithelial cell; lys, late yolk sphere; oo, oocyte; sp, inter-FC space; sv, secretory vesicle; v, vesicle; vm, vitelline membrane. Scale bars = 1 μm.
for the vitellogenin subunits. Thus, the formation of the late yolk spheres and their fusion with the oolemma was not disrupted by the lack of vitellin. This evidence is consistent with our hypothesis (Zimowska et al., 1995) that the oocyte, as well as the FC, contributes to the production of the impermeable egg membranes by releasing yolk proteins into the vitelline membrane.

The importance of the role of vitellogenin/vitellin in oocyte maturation and embryonic development of Lepidoptera has obviously been in question since the astounding observation of Yamashita and Irie (1980) that oocytes matured in males of B. mori could parthenogenically develop into normal adults. This study examined several aspects of vitellin accumulation during the maturation of oocytes to assess whether the presence of vitellin influenced the formation or function of yolk spheres. Vitellin was not present in oocytes of ovaries transplanted into males, and the lack of vitellin did not interfere with the transitions in yolk sphere morphology that occur during the changes from the provitellogenic to vitellogenic and vitellogenic to postvitellogenic stages of follicle development. These findings suggest that the products of the follicular epithelial cells may play critical roles in the transport, uptake, and formation of yolk in the oocytes.

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