Is hexamerin receptor a GPI-anchored protein in *Achaea janata* (Lepidoptera: Noctuidae)?

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The process of uptake of hexamerins during metamorphosis from insect haemolymph by fat body cells is reminiscent of receptor-mediated endocytosis. Previously, we had identified a hexamerin-binding protein (HBP) and reported for the first time that uptake of hexamerins is dependent on the phosphorylation of HBP partly by a tyrosine kinase, which is, in turn, activated by 20-hydroxyecdysone (20E). However, the exact nature of HBP and the mechanism of interaction are still unknown. Here we report the possibility of HBP being a GPI-anchored protein in the fat body of *Achaea janata* and its role in the tyrosine-kinase-mediated phosphorylation signalling. Digestion of fat body membrane preparation with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), and the subsequent recognition by antibodies specific for the cross-reacting determinant (CRD), revealed that HBP is glycosylphosphatidylinositol (GPI)-anchored protein and, further, that the hexamerin binding to HBP was inhibited after digestion. Hexamerin overlay assay (HOA) of co-immunoprecipitated *in vitro* phosphorylated HBP showed exclusive binding to ~120 kDa protein. Lectin-binding analysis of hexamerins revealed the presence of *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GluNAc), whereas HBP showed the presence of GalNac alone. Mild chemical deglycosylation studies and binding interaction in the presence of sugars revealed that glycan moieties are possibly not involved in the interaction between HBP and hexamerins. Taken together, these results suggest that HBP may be a GPI-anchored protein, and interaction and activation of HBP is through lipid-linked non-receptor *src* tyrosine kinases. However, additional studies are needed to prove that HBP is a GPI-anchored protein.

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1. Introduction

During final instar development, holometabolan lepidopteran insect larvae undergo dramatic physiological changes to prepare themselves for pupation and metamorphosis. As pupae do not feed, they depend on hexamerins, a central source of a storage pool of amino acid resources for complete metamorphic development of the adult moth (Haunerland 1996). Hexamerins are storage proteins synthesized from the fat body of a wide range of lepidopteran and other insect orders, and these are the major serum proteins in the haemolymph. Hexamerins are taken up by the fat body shortly before pupation and stored in protein granules. The rise in the level of the steroid hormone 20-hydroxyecdysone (20E) at the end of larval life triggers the incorporation of these hexamerins from the

Keywords. Ecdysone; fat body; GPI anchor; haemolymph; hexamerin-binding protein; lepidopteran; CRD epitope

Abbreviations used: ALP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3'-indolyl phosphate; CRD, cross-reacting determinant; 20E, 20-hydroxyecdysone; ELI, early-last instar; GalNAc, *N*-acetylgalactosamine; GluNAc, *N*-acetylglucosamine; GPI, glycosylphosphatidylinositol; HBP, hexamerin-binding protein; HOA, hexamerin overlay assay; LLI, late-last instar; MLI, mid-last instar; NBT, Nitro Blue Tetrazolium; PI-PLC, phosphatidylinositol-specific phospholipase C; PP, prepupae; SBA, soybean agglutinin; WGA, wheat germ agglutinin

haemolymph into the fat body (Burmester and Scheller 1997; Kirankumar *et al*. 1997). Even though the endocytosis of vitellogenins by oocytes is well established, the regulation of hexamerin uptake and their delivery to protein storage granules of the fat body remains elusive, in spite of extensive studies that proposed to investigate whether the uptake process is a 'classical' endocytosis or anything different (Burmester and Scheller 1999). The uptake of hexamerins by the insect fat body cells exhibits several interesting and unusual aspects, and it is still uncertain whether this process fits into the standard scheme of eukaryotic endocytosis at all (Burmester and Scheller 1999). Besides this, cloning and molecular characterization of the putative hexamerin receptor have been reported in few insect species, but it is still not clear whether the receptor responsible for uptake is a transmembrane or membrane-anchored protein (Wang and Haunerland 1993; Burmester and Scheller 1995; Burmester and Scheller 1999; Persaud and Haunerland 2004; Manohar *et al*. 2010). Interestingly, none of the receptor sequences showed the features of classical endocytic sorting signals to confirm it as a transmembrane protein (Burmester and Scheller 1995; Hansen *et al*. 2003; Persaud and Haunerland 2004).

Glycosylphosphatidylinositol (GPI)-anchored proteins are membrane-anchored proteins, possess a covalently linked glycosylated phosphatidylinositol moiety that attaches the protein portion of the molecule to the cell surface lipid bilayer (Hooper 2001; Kasahara *et al.* 2002). Proteins linked to the cell surface via a GPI-anchor are involved in a wide variety of cellular functions, including cell activation, transduction of extracellular stimuli, hydrolysis of extracellular matrix proteins and cell–cell adhesion (Brown 1994; Cherr *et al*. 2001; Tam *et al.* 2001; Kasahara *et al*. 2002). In addition, GPI-anchored proteins have been reported to be responsible for mediating sequestration or uptake of ligands by phosphorylation (Freedman *et al.* 1998; Solomon *et al.* 1998). Earlier our group reported that tyrosine-kinase-mediated phosphorylation of the ~120 kDa putative hexamerin-binding protein (HBP) in the membrane fraction of the fat body (Kirankumar *et al.* 1997; Arif *et al.* 2003) is responsible for the uptake of hexamerins in lepidopteran moth *Corcyra cephalonica* and this process is regulated by a steroid hormone, 20-hydroxyecdysone (20E).

On the basis of these observations and the fact that tyrosine phosphorylation of cellular substrates has been used as an indication of GPI-anchored-protein-mediated signalling, we attempted to investigate whether the HBP is also a GPI-anchored protein and binding of hexamerins to ~120 kDa fat body membrane protein initiates a signalling event, which is responsible for sequestration. We also wished to directly demonstrate the role of glycan moieties in the interaction and uptake of hexamerins by its putative receptor.

2. Materials and methods

2.1 *Experimental animals*

Achaea janata larvae were obtained from the Directorate of Oil Seeds Research, Hyderabad, India, and maintained in the rearing facility. The larvae were reared on fresh castor leaves at 25±1°C and 70% RH (relative humidity) with a photoperiod of 12 h light and 12 h dark. Based on the age and body weight, the last instar larvae were categorized as early-last instar (ELI), mid-last instar (MLI), and late-last instar (LLI) (Budatha *et al*. 2007). In the present study ELI, MLI, LLI and PP were used. The insects were chilled on ice for 15 min, and the haemolymph was collected by puncturing the prolegs. Further, insects were dissected to isolate the fat body tissue.

2.2 *Preparation of hexamerins and generation of polyclonal antibodies*

Hexamerins were purified from the haemolymph of 5th instar larvae as described previously (Kirankumar *et al*. 1997). To facilitate studies on developmental profile of hexamerins and their uptake, polyclonal antibodies against hexamerins were generated in male New Zealand variety rabbits.

2.3 *Preparation of fat body membranes*

The fat body tissues from larvae were dissected out and stored in buffered insect saline (5 mM HEPES, pH 8.5, 130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$) kept on ice. The membrane preparation was carried out according to the method of Kirankumar *et al*. (1997).

2.4 *Hexamerin overlay assay*

The fat body membrane proteins were separated by 7.5% SDS-PAGE and electrotransferred to nitrocellulose membranes. After blocking the membrane for 1 h in blocking buffer [3% (w/v) BSA in TBS (Tris-buffered saline)], the blot was incubated in the blocking buffer containing biotin-labelled hexamerins [100 µg of purified hexamerins were biotinylated using the protein biotinylation module kit (Banglore Genei, India) and then purified with Sephadex G25 columns]. After incubation, the blots were washed with TBS containing Tween 20 and then incubated for 2 h in blocking buffer containing streptavidin–ALP conjugate. Bound hexamerins were detected using the ALP substrate BCIP-NBT.

2.5 *Immunobloting*

Electrophoretically separated polypeptides were transferred to nitrocellulose membranes. After blocking the membrane for 1 h in blocking buffer, the blot was incubated in the blocking buffer containing primary antibodies raised against hexamerins. After incubation, the blot was washed with TBS containing Tween 20 and then incubated for 2 h in blocking buffer containing ALP-conjugated goat anti-rabbit IgG. Finally, detection was carried out using the ALP substrate BCIP-NBT. To detect tyrosine phosphorylation, immunoblot analysis was carried out using anti-phosphotyrosine antibody (Santa Cruz Biotechnology, USA).

2.6 *Lectin blot analysis of the fat body membrane proteins*

Electrophoretically separated fat body membrane proteins, transferred to nitrocellulose membrane, were incubated with either biotin conjugated soybean agglutinin (SBA) or wheat germ agglutinin (WGA). The lectin-bound proteins were subsequently detected with streptavidin–ALP conjugate.

2.7 *The GPI anchor detection with anti-CRD antibody*

The larval fat body membrane preparation was digested with 5 units/10 ml phosphatidylinositol phospholipase C (PI-PLC, Molecular probes) at 30°C for 90 min and subjected to 7.5% SDS-PAGE. Proteins were electro-transferred to nitrocellulose membrane and the presence of cleaved GPI epitope was detected using 1:200 dilution anti-cross-reacting determinant antibody (anti-CRD) (Broomfield and Hooper 1992). Further, the binding of hexamerin to PI-PLC-cleaved fat body membrane preparation was analysed by hexamerin overlay assay (HOA).

2.8 *In vitro phosphorylation of proteins*

In vitro phosphorylation of fat body membrane proteins was carried out according to the method described previously (Shanavas *et al.* 1998) with slight modifications. The reaction mixture of 40 µl contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 µM ATP, 10 µM sodium orthovanadate and 20 µg of fat body homogenate/membrane protein. When required, 20E (80 nM) was added. The reaction mixture was preincubated for 5 min at 30°C. Phosphorylation was initiated by addition of 4 µCi of [γ32P] ATP. The reaction was terminated after 1 min by the addition of 20 µl of SDS sample buffer (0.188 M Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol and 0.003% bromophenol blue) followed by immersion in boiling water for 3 min. The phosphorylated proteins (10 µg) were subjected to 7.5% SDS-PAGE. The gels were vacuum-dried and exposed to Kodak X-Omat AR film at −70°C for autoradiography.

2.9 *Immunoprecipitation of phosphorylated hexamerin-binding proteins*

In vitro phosphorylated fat body membrane proteins were incubated with purified hexamerins (5 µg) in binding buffer (50 mM sodium phosphate, pH 7.5, 50 mM NaCl and 3 mM $MgCl_2$) for 3 h at 4°C, followed by incubating with 5 µl of hexamerin antisera for another 3 h. Subsequently, 100 µl of protein A-Sepharose 4B was added and incubated for 2 h at 4°C on a rotatory shaker. The Sepharose beads were pelleted, washed six times with PBS buffer, resuspended in 20 µl of SDS sample buffer containing β-mercaptoethanol and heated at 100°C for 5 min. Eluted proteins were subjected to 7.5% SDS-PAGE and electro-transferred to nitrocellulose membrane. The membrane was then processed for HOA and subsequently subjected to autoradiography.

2.10 *Mild periodate oxidation*

Western blots of fat body membrane preparations were subjected to mild chemical deglycosylation as described elsewhere (Knight *et al.* 2004). Briefly, the blots were equilibrated in 50 mM sodium acetate buffer, pH 4.5, then exposed to 10 mM periodate and dissolved in the same buffer for 1 h in the dark. After extensive washing with water, the blots were reduced with 50 mM sodium borohydride in phosphate-buffered saline (PBS), pH 7.4, for 30 min. After extensive washing with PBS, the blots were blocked and incubated with biotin-labelled hexamerins or lectins. Control blots were treated identically except that no periodates was added to the pH 4.5 buffer at the oxidation stage.

2.11 *Effect of sugars on hexamerin binding to its receptors*

Fat body membrane proteins were incubated with purified hexamerins, with the inclusion of sugars *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GluNAc) where appropriate, at 100 mM. After 60 min incubation at room temperature, bound hexamerin was separated from free hexamerin by centrifugation at 12500*g* for 10 min. Samples of the pellets and supernatants were subjected to SDS-PAGE, transferred to nitrocellulose membrane and hexamerin was detected by immunoblotting.

3. Results

3.1 *Purification of hexamerins and immunoblotting*

Results in figure 1A show hexamerins purified from haemolymph of 5th instar larvae of *A. janata*. The polyclonal antibodies generated against purified hexamerins showed selective cross reactivity with hexamerins (86 kDa and 84 kDa) alone present in the haemolymph (figure 1B).

3.2 *Developmental profile and stage-specific uptake of hexamerins by the fat body*

We traced the stage-specific presence of hexamerins in the fat body by immunoblotting. Hexamerins found to be fairly high in prepupal fat body when compared with ELI, MLI and LLI (figure 1C). It is well known that the prepupal fat body cells do not synthesize hexamerins and proteins present at this stage represents that the hexamerins have been incorporated from the haemolymph.

3.3 *Identification of hexamerin-binding protein in the fat body membrane*

As the exact nature and molecular weight of hexamerin receptor is ambiguous, in order to make sure that the HBP identified in all experiments is the same, fat body membrane preparation (including samples digested with PI-PLC) were subjected to SDS-PAGE and transferred to nitrocellulose membrane at the same time. Required lanes were cut into individual strips and used for separate experiments, and the experiments were carried out in triplicate. The presence of HBP/receptor of ~120 kDa has been already documented in a few lepidopteran insects (Chung *et al.* 1995; Kirankumar *et al.* 1997; Arif *et al.* 2003). Hence, it was tempting to check whether or not the larval fat body of *A. janata* also has a similar binding protein. Membrane fractions of the fat body cells from LLI larvae of *A. janata* were subjected to SDS-PAGE (figure 2A, lane 1) subsequent to HOA. The biotinylated hexamerin bound to a protein of ~120 kDa (lane 2). These results show that, indeed, a ~120 kDa protein in the fat body of *A. janata* is able to bind to hexamerin *in vitro*. Therefore, this is considered as the HBP of *A. janata*.

3.4 *20E-induced phosphorylation of the ~120-kDa protein is tyrosine kinase dependent*

The tyrosine-kinase-mediated phosphorylation of the ~120 kDa protein (Kirankumar *et al.* 1997; Arif *et al.* 2003) is responsible for the uptake of hexamerins in *Corcyra cepholonica*, and this process is enhanced in presence of 20E. The *in vitro* phosphorylation of *A. janata* larval fat body membrane proteins in the presence of 20E led to the increased phosphorylation of the ~120 kDa protein (figure 2B, lane 2) in comparison with the control where 20E was not included (lane 1). It has been demonstrated that GPI-anchored proteins can mediate cell signalling through phosphorylation by *src* tyrosine kinases (Harder and Simons 1999). Hence, tyrosine phosphorylation of ~120 kDa proteins was analysed by immunoblotting of SDS-solubilized fat body membrane extracts with an anti-phosphotyrosine antibody, and the difference in banding patterns was measured between the 20E treatments. As compared with 20E untreated (figure 2C, lane 1), the 20E treated phosphorylated ~120 kDa protein (figure 2C, lane 2)

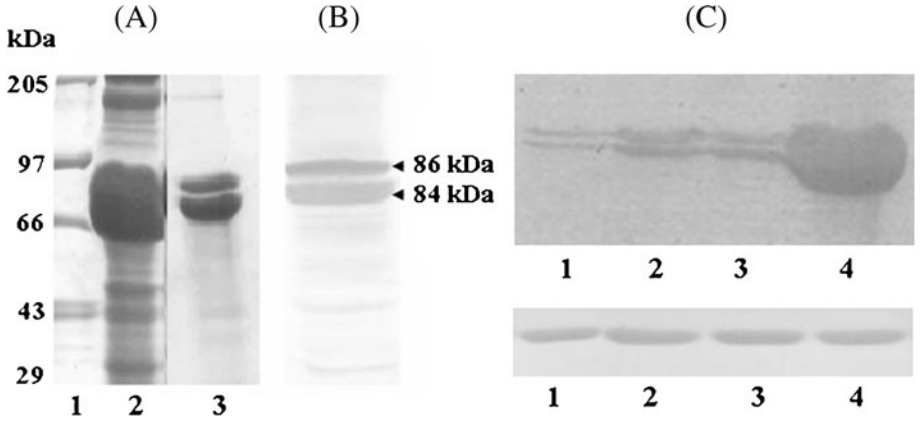

Figure 1. (**A**) SDS-PAGE analysis of hexamerins: molecular weight marker (lane 1) crude haemolymph (lane 2) and purified hexamerins (86 and 84 kDa) (lane 3). (**B**) Immunoblot analysis of hexamerins. (**C**) Immunoblot analysis showing the developmental profile of hexamerins in the fat body homogenates: ELI (lane 1), MLI (lane 2), LLI (lane 3) larvae and prepupae (lane 4). The blot was probed with hexamerin antibodies. The lower panel shows β actin control.

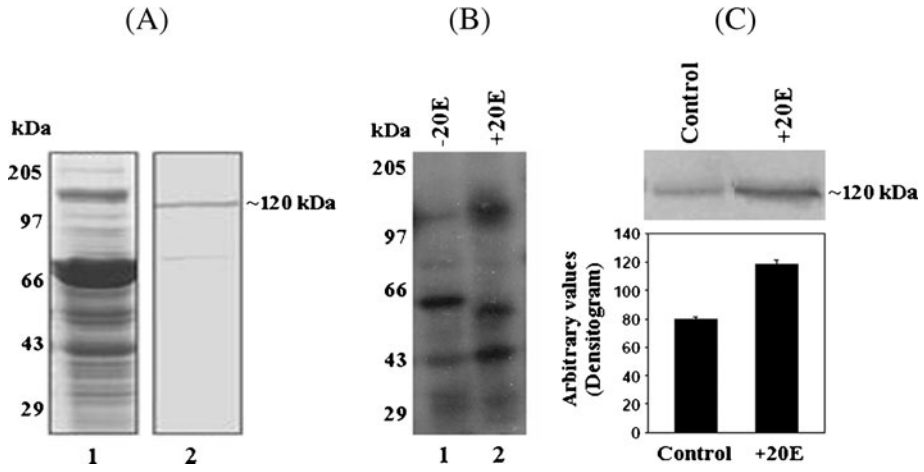

Figure 2. (**A**) Detection of hexamerin-binding proteins by hexamerin overlay assay (HOA). *A. janata* fat body membrane proteins separated by SDS-PAGE and transferred to nitrocellulose membrane and overlaid with biotin labeled hexamerin. SDS-PAGE of fat body membrane proteins (lane 1); hexamerin overlay assay (lane 2). (**B**) *In vitro* phosphorylation of fat body proteins of *A. janata* under different phosphorylating conditions. Autoradiograph of the *A. janata* fat body protein phosphorylation in the absence of 20E (lane 1) in the presence of 80 nM 20E (lane 2). (**C**) Immunoblot indicating the cross-reaction of the ~120 kDa protein with anti-phosphotyrosine antibody. Phosphorylation in the absence of 20E (lane 1) in the presence of 80 nM 20E (lane 2). The lower panel shows the densitogram of the blot.

showed a higher cross-reactivity, which confirms that tyrosine kinase mediates the ~120 kDa protein phosphorylation induced by 20E. The specificity of anti-phosphotyrosine immunoblotting was verified by the elimination of the band in the presence of L-phosphotyrosine (data not shown). Genistein, an inhibitor of tyrosine kinase, partly inhibited the 20E-induced phosphorylation of the ~120 kDa protein (figure 3A, lane 4) when compared with the 20E alone treated sample (figure 3A, lane 3), whereas the treatment with genistein did not affect the basic phosphorylation (figure 3A, lanes 1 and 2).

3.5 *The phosphorylated ~120 kDa is a hexamerin-binding protein*

In order to confirm that the phosphorylated ~120 kDa protein is a HBP, we co-immunoprecipitated the phosphorylated ~120 kDa protein from the fat body membrane

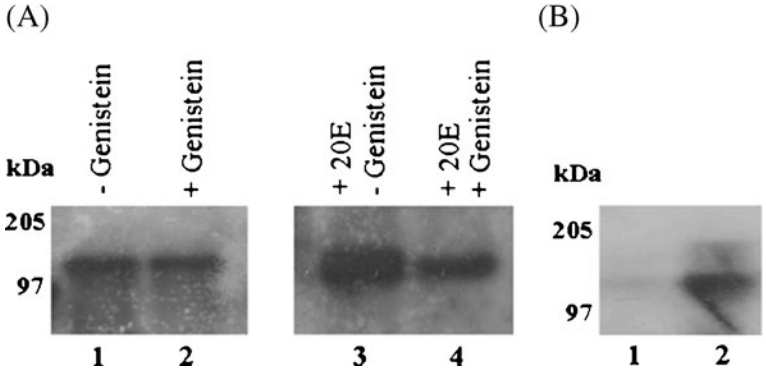

Figure 3. (**A**) Autoradiograph showing the effect of genistein on the phosphorylation of the ~120 kDa protein. (**A**) Phosphorylation in the absence of genistein (lane 1), in the presence of genistein (lane 2), in the presence of 20E (lane 3) and in the presence of 20E+ genistein (lane 4). (**B**) Hexamerin overlay analysis of co-immunoprecipitated *in vitro* phosphorylated fat body membrane proteins. The membrane was subsequently processed for autoradiography. Co-immunoprecipitation in the absence of hexamerins (lane1); co-immunoprecipitation in the presence hexamerins and hexamerin antisera (lane 2).

preparation and assessed its binding with hexamerins using HOA and subsequently subjected to autoradiography. In this experiment, only the ~120 kDa protein was observed on the autoradiogram (figure 3B, lane 2), whereas in the control, there is no signal (lane 1).

3.6 *Identification of GPI moiety on hexamerin-binding protein*

PI-PLC digestion was used to determine whether the ~120 kDa protein was anchored to fat body membrane through the GPI moiety in *A. janata*. As shown in figure 4A, after PI-PLC digestion, anti-CRD sera recognized the ~120 kDa protein in the fat body membrane (figure 4A, lane 2), suggesting that this protein is anchored to the fat body membrane through the GPI moiety. The cross-reactivity in the absence of PI-PLC (lane 1) may be due to cleavage by endogenous phospholipases.

3.7 *PI-PLC treatment releases hexamerin-binding protein from fat body membrane*

We further found that treatment of fat body membrane preparation with PI-PLC releases HBP and the binding of hexamerins to HBP was not observed (figure 4B, lane 2), whereas binding was observed in control (lane1). This result suggests that hexamerins bind to the identified GPI-anchored protein.

3.8 *Role of glycan moieties on hexamerin-binding protein*

To investigate the oligosaccharides present on HBP, lectin blotting analysis was performed using selected lectins (figure 4C). After lectin blotting, HBP was recognized by SBA (lane 1) but not by WGA (lane 2). Following lectin blotting, these membranes were subjected to HOA, in order to confirm that the lectin-binding proteins were actually the HBP being investigated (data not shown)

Further, we examined if the sugar moieties present on the binding protein had any role in the interaction with hexamerins. To test this, mild periodate deglycosylation (Knight *et al*. 2004) studies were carried out after transferring the fat body membrane proteins from SDS-PAGE to nitrocellulose membrane. After this, HOA was carried out. The binding of hexamerin to the ~120 kDa protein was not eliminated (figure 4C, lane 3) after mild chemical deglycosylation. This result suggests that the carbohydrate residues on HBP are not involved in binding of hexamerins.

3.9 *Identification of glycan moieties on hexamerins and their role in the HBP interaction*

Hexamerins are reported to be highly glycosylated haemolymph proteins in insects. Hence, lectins were used in conjunction with Western blots to identify carbohydrate structures covalently attached to the *A. janata* hexamerins.

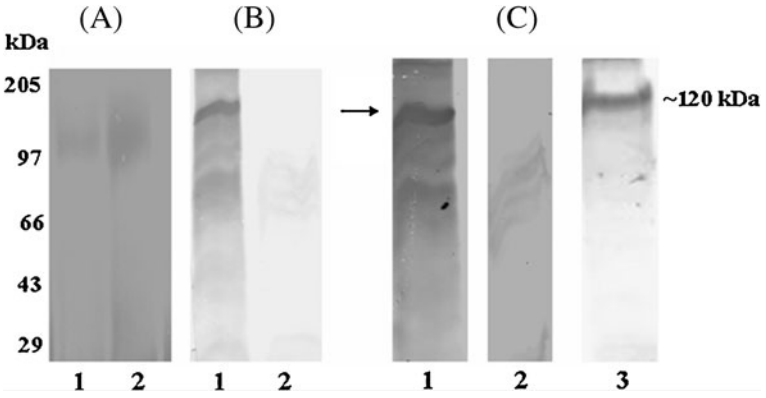

Figure 4. (**A**) Detection of the cleaved GPI anchors. Fat body membrane proteins digested with 5 units of PLC/10 ml, subjected to SDS/PAGE and the proteins transferred on to a nitrocellulose membrane. The blot was incubated with anti-CRD antibodies and cleaved GPI anchors were detected with ALP-conjugated secondary antibody. Fat body membrane protein without PLC digestion (lane 1); fat body membrane protein digested with PLC (lane 2). (**B**) Hexamerin overlay analysis of intact and PLC-digested fat body membrane protein. PLC untreated (lane 1); PLC digested (lane 2). (**C**) Lectin-binding assays. Fat body membrane protein preparations was separated by SDS-PAGE (7.5% gels) and transferred on to nitrocellulose membranes. The membranes were incubated either with biotin conjugated SBA or WGA and lectin-bound proteins were subsequently detected with streptavidin–ALP conjugate. SBA (lane 1); WGA (lane 2). Effect of chemical deglycosylation on the interaction between HBP and hexamerin: membrane protein preparations were separated by SDS/PAGE (7.5% gels) and transferred to nitrocellulose membrane. After the chemical deglycosylation, the blot was subjected to hexamerin overlay assay (lane 3).

Samples of purified *A. janata* hexamerins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane and probed with biotin-labelled SBA and WGA lectins. Both lectins bound to the hexamerins, and binding pattern of two lectins suggests that *A. janata* hexamerins contains GluNAc (figure 5A, lane 1) and GalNAc moieties (figure 5A, lane 2). Since hexamerins bind to specific lectins on Western blots, we examined whether these glycans have any role in binding to HBP under native conditions. Purified hexamerins were incubated with fat body membrane preparation in the presence of either GalNAc or GluNAc and separated into precipitating aggregates and soluble supernatant. When pellets and supernatant from respective experiments were subjected to immunoblotting after SDS-PAGE, hexamerin was detected mostly in pellets (figure 5B, lanes 4 and 6), suggesting that possibly there is no role for hexamerin glycans in the interaction between hexamerins and HBP.

4. Discussion

A. janata is a lepidopteran insect and an economically important pest of castor crop found in most parts of south Asia. Until now, there is no information on the characterization of hexamerins and their uptake by the fat body cells in this insect. This information is very important because uptake of hexamerins is a vital process that allows holometabolous insects to survive the non-feeding pupal period. Therefore, specific compounds that interfere with hexamerin endocytosis could probably be useful to serve as highly selective insecticides that do not hazard vertebrates. To develop such insecticides, it is imperative to identify the nature of binding proteins and the mechanism of interaction with hexamerins (Burmester and Scheller 1999). In the present study, we have demonstrated the tyrosine-kinase-mediated phosphorylation of a ~120-kDa HBP and showed that phosphorylation was enhanced in the presence of 20E. This result is in corroboration with our earlier report, where we projected that the hexamerin receptor is activated through phosphorylation by a tyrosine kinase and showed that enhanced phosphorylation in the presence of 20E is responsible for the uptake of hexamerins in *Corcyra cephalonica* (Arif *et al.* 2003). The hexamerin receptor cDNAs have been cloned from *Calliphora vicina* as well from *Sarcophaga peregrine*. The presence of receptor cleavage product has been demonstrated, but typical transmembrane domain or endocytosis sorting signals were not reported in these hexamerin receptor sequences (Burmester and Scheller 1997); therefore, it is still uncertain how the receptors are linked to the fat body cell membrane. The authors reported that arylphorin-binding protein will be in the cleaved form during larval stage and, by PP stage, fragments will be united and responsible for the uptake. Later they suggested that it is difficult to conceive a specific mechanism for joining the two smaller proteins to form the 120-kDa protein in the pupal fat body (Chung *et al.* 1995).

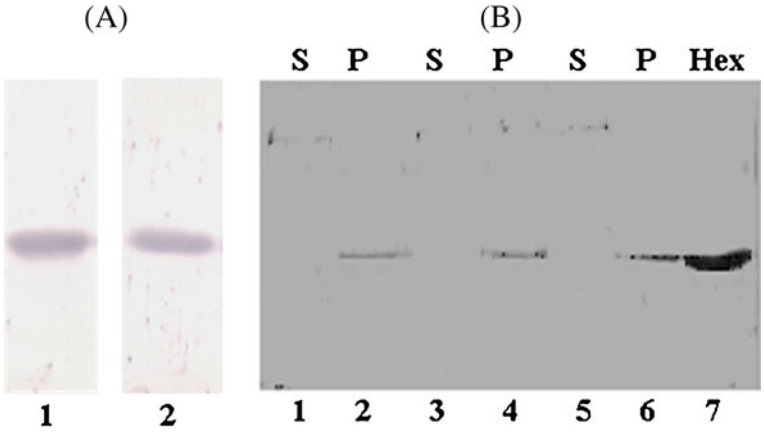

Figure 5. (**A**) Lectin binding assays. Purified hexamerin were separated by SDS/PAGE (7.5% gels) and transferred to nitrocellulose membranes. The membranes were incubated either with biotin conjugated SBA or WGA and lectin bound proteins were subsequently detected with streptavidin–ALP conjugate. SBA (lane 1); WGA (lanes 2). (**B**) Effect of sugars on hexamerin binding to HBP. Hexamerins were incubated with fat body membrane preparation with the inclusion of sugars, where appropriate, at 100 mM. After 60 min incubation at room temperature, bound hexamerins were separated from free hexamerins by centrifugation at 12500*g* for 10 min. Samples of the supernatants (lanes 1, 3 and 5) and pellets (lanes 2, 4 and 6) were subjected to SDS-PAGE, transferred to nitrocellulose paper and the hexamerin detected by immunoblotting. Fat body membrane proteins and hexamerin with no sugars (lanes 1 and 2); fat body membrane proteins and hexamerin with 100 mM GalNAc (lanes 3 and 4); fat body membrane proteins and hexamerin with 100 mM GluNAc (lanes 5 and 6) and pure hexamerin (Hex) (lane 7).

The other reports suggested that hexamerin receptor is similar to its own ligand (Burmester and Scheller 1995), and Haunerland's group reported that receptor will be similar to juvenile hormone suppressible proteins and VHDL receptors (Persaud and Haunerland 2004). Hence, upto now the exact nature of HBP/receptor is unclear. Based on our earlier report that the tyrosine-kinase-mediated phosphorylation is responsible for uptake, we assumed that it could be a GPI-anchored protein.

The cross-linking or binding of ligands to GPI-anchored proteins triggers the sequestration of proteins into specialized glycolipid-based microdomains, which assemble signalling molecules and causes activation of certain cell types (Mayor *et al*. 1998). GPI-anchored proteins are physically associated with lipid-linked signal transduction molecules and co-immunoprecipitated with heteromeric G-proteins and *src* tyrosine kinases. GPI-anchored proteins lack a cytoplasmic domain and cannot interact directly with the clathrin endocytic machinery on the cytoplasmic face of the plasma membrane. Hence, lipid-linked signal transduction molecules such as *src* family non-receptor tyrosine kinases and heterotrimeric G proteins are reported to be responsible for the signalling by GPI-anchored proteins (Freedman *et al*. 2001; Togo and Morisawa 2004). Based on these, we speculated that this protein may be GPI-anchored and the signalling is regulated by *src* tyrosine kinases. Hence, the tyrosine-kinase-mediated phosphorylation of HBP lead us to check its status on the membrane.

Bacterial PLC is known to cleave the GPI anchor, causing the exposure of the CRD epitope. The CRD epitope is exposed when the protein is converted into a hydrophilic form by PI-PLC, and its recognition by an anti-CRD antibody is the strongest evidence for the presence of a GPI anchor. Upon treatment of fat body membrane proteins with PLC, we detected the exposed CRD epitope using anti-CRD antibody (Broomfield and Hooper 1992; Hooper *et al*. 1997). In addition, several properties offered by the GPI anchor are likely to favour the process of hexamerin uptake. The GPI anchor might provide an increased spatial flexibility required for proper interaction between the hexamerin and the receptor, and local increase in hexamerin concentration leads polymerization by increasing the probability of collision between the hexamerin molecules (Nohe *et al*. 2006). In addition, the lipid anchor (*versus* a transmembrane segment) increases the lateral mobility of the receptor in the plane of the membrane (Hooper 2005). It was proposed that the preferential association of the ligand (hexamerin) with lipid rafts, through binding to GPI-anchored proteins, is likely to increase the local ligand (hexamerin) concentration and thereby promote polymerization, a step that is a prerequisite for uptake (Abrami *et al*. 2003).

Furthermore, most of the reported GPI-anchored proteins are known to be glycoproteins. Hence, we checked the role of glycan moieties that are present on the ~120 kDa HBP. Our study clearly ruled out involvement of glycan in the interaction between hexamerin and HBP. The structures of the oligosaccharides attached to hexamerins display significant variations based on the season during which larvae were reared (Kim *et al*. 2003). It is possible that glycosylation of hexamerins has an important role in folding and subunit assembly to achieve the mature protein in the haemolymph (Marinotti *et al*. 1988; Kim *et al*. 2003). Further, *N*-glycans could have a significant role in keeping this large, hydrophopic protein in the haemolymph to improve its transport to the ovary (Sappington and Raikhel 1998). The present study clearly ruled out the role of hexamerin glycan moieties in interaction with HBP. This result is supported by the evidence that the carbohydrate moiety of housefly, *Musca domestica*, arylphorin, was not involved in its uptake by the fat bodies and integument (Marinotti *et al*. 1988). In contrast, Vg uptake into the oocytes is known to be mediated by receptors and the glycan moiety reported to play a role in recognition and receptor-mediated endocytosis. In this way, hexamerin binding and its uptake process are reported to be different from other endocytic processes. Hence, the interaction between hexamerins and HBP is most likely through GPI moieties or lipid rafts.

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