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Characterization of the solubilized oocyte membrane receptor for insecticyanin, a biliprotein of the hawkmoth, *Manduca sexta*

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Abstract

We report the solubilization and characterization of the oocyte membrane receptor for insecticyanin, a blue biliprotein of the hawkmoth *Manduca sexta*. The insecticyanin receptor was solubilized using 40 mM CHAPS. Strong binding affinity of [^{125}I]insecticyanin to its solubilized receptor was demonstrated to be heat-labile, pH-dependent, Ca^{2+}-dependent, and saturable. The binding was inhibited by excess unlabeled insecticyanin, but not by two other major hemolymph and oocyte proteins, vitellogenin and lipophorin. The receptor for insecticyanin showed tissue specificity: it was present only in oocyte membranes, not in membranes of fat body, midgut or ovariole sheath. The equilibrium data for the solubilized receptor, K_d and B_{max}, were estimated to be 17 nM and 11.4 pmol/mg solubilized proteins, respectively. The results from co-immunoprecipitation showed that the apparent molecular mass for the insecticyanin receptor is approximately 185 kDa while chemical crosslinking of the insecticyanin–receptor complex revealed a product with a molecular mass near 10^3 kDa. This suggests that the insecticyanin receptor has a multimeric structure, or that four receptor molecules can bind to one insecticyanin tetramer.

Keywords: Insecticyanin; Endocytosis; Receptor; Immunoprecipitation; Crosslinking; (*Manduca*)

1. Introduction

The construction of insect eggs involves the internalization of proteins and lipids from the circulating hemolymph and this internalization process is thought to be mediated in large part by membrane-associated receptors. The principal protein component thus imported is vitellogenin, a major storage protein used by the developing embryo. It has been demonstrated that numerous proteins other than vitellogenin are also taken up from hemolymph by developing oocytes. In the case of *Lepidoptera*, these include insecticyanin (Ins) [1], other lipoproteins, microvitellogenin and pigmented proteins [2,3]; in the case of locusts, a small protein [4]; and in the case of mosquitoes, proenzymes and other small proteins [5]. Current efforts are under way to isolate and characterize

Abbreviations: BS^3, bis(sulfosuccinimidyl)suberate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammoniol]-1-propane sulfonate; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; DTSP, dithiobis(succinimidyl propionate); HDLp, high density lipophorin; Ins, insecticyanin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Vg, vitellogenin

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endocytotic receptors of insect oocytes that are responsible for the sequestration of these hemolymph proteins. The ultimate goal of these studies is to design specific methods that interfere with the ligand–receptor interactions, which may provide new ways to control insects that are harmful to humans. Most attention has been directed toward isolation of receptors for vitellogenin and other yolk proteins, which has so far been accomplished for the locust *Locusta migratoria* [6], the mosquito *Aedes aegypti* [7] and the fruitfly *Drosophila melanogaster* [8].

Very little is known about the detailed mechanism for interactions between vitellogenins and their receptors. One of the difficulties in working with a vitellogenin receptor, or lipophorin receptor [9] is that the ligand is a large, complex structure that contains lipids, carbohydrates and polypeptides with extensive post-translational modifications. This complicates detailed studies of receptor–ligand interactions. We therefore reasoned that if an endocytotic receptor for a relatively small and well-characterized protein can be found, future studies of the receptor–ligand interactions will be facilitated. Ins, a blue hemolymph protein of the hawkmoth *Manduca sexta* seems to be an ideal candidate for this purpose.

Ins, providing camouflage for the larvae and the eggs [10], is synthesized in the epidermis [11] of larvae. Then the major isoform is secreted into hemolymph [12], where it persists into the adult stage and is incorporated into eggs [13,14]. Biochemical studies, including X-ray crystallography [15] and chemical crosslinking reported in the current study, indicated that Ins is a tetrameric protein composed of identical subunits with a mass of 21 378 Da [16]. The amino acid sequence [16] and cDNA sequence [11] of Ins have been determined. Using in vitro uptake, binding assays and histological autoradiography, we have previously demonstrated that the internalization of Ins into the developing oocyte follows a receptor-mediated endocytotic pathway [1].

In this study we report the solubilization and further characterization of the *M. sexta* oocyte receptor for Ins. Our results show that the receptor is a 185 kDa protein that specifically binds Ins with high affinity. Co-immunoprecipitation and chemical crosslinking demonstrate that Ins and its receptor form a multimeric complex with a molecular mass of about 1000 kDa.

2. Materials and methods

2.1. Animals

M. sexta was reared as previously described [17]. Two-day-old adult female animals were used to prepare solubilized oocyte membrane proteins.

2.2. Buffer designations and components

Affinity-column elution buffer: 0.1 M acetate, 0.5 M NaCl (pH 4.0);
Binding buffer: 20 mM MES (2-(N-morpholino) ethanesulfonic acid), 150 mM NaCl, 5 mM $CaCl_2$ and 1% BSA (bovine serum albumin) (pH 7.0);
Lepidopteran saline: 110 mM KCl, 4 mM NaCl, 15 mM $MgCl_2$, 5 mM phosphate (pH 6.5).

2.3. Solubilization of membrane proteins by detergent

Preliminary experiments were performed to determine the solubilizing capacities of different detergents, Triton X-100, octyl-β-D-glucoside and CHAPS, and the optimal concentration of the chosen detergent, based on the protein recoveries from solubilized membranes. While the differences between the detergents in their effects on solubilization of membranes were not significant, 40 mM of CHAPS gave slightly higher membrane protein yield than 15 mM and 25 mM (data not shown). Therefore 40 mM CHAPS was used for subsequent solubilization of membrane proteins in this study.

All operations were carried out at 0°C unless otherwise indicated. Membranes were prepared as previously described [1] except that the buffer used to wash membranes was the lepidopteran saline given above instead of the binding buffer. After homogenization of oocytes and removal of soluble proteins by ultracentrifugation, the membrane preparation was adjusted to a protein concentration of 5 mg/ml with lepidopteran saline. CHAPS (1 M) in the same buffer was added to a final concentration of 40 mM to solubilize membrane proteins. The suspension was kept on ice for 1 h with occasional vortexing, then diluted to 15 mM CHAPS using lepidopteran saline, followed by centrifugation at $100\,000 \times g$ for 1 h, 4°C. The supernatant containing solubilized proteins was further diluted to 5 mM CHAPS, and was even-

tually brought to a minimum volume using a Centricon-30 concentrator unit (Amicon). The proteins were aliquoted and stored at −80°C. The solubilization of membrane proteins from fat body, midgut and ovariole sheath of *M. sexta* were performed as described above.

2.4. Protein purification

Hemolymph was collected from female adults by the 'flushing out' method [18] and centrifuged (8000 × *g*, 4°C) to remove hemocytes. Established procedures were then used to purify Ins [16], vitellogenin [19] and high density lipophorin [20], respectively. Protein concentrations were determined spectrophotometrically using the BCA (bicinchoninic acid) protein assay kit (Pierce) [21].

2.5. Iodination of proteins

Ins and solubilized oocyte membrane proteins were radioiodinated with $Na^{125}I$ (17.4 Ci/mg, DuPont-New England Nuclear, Wilmington, DE), using iodobeads (Pierce), according to the manufacturer's instructions. 1 mCi $Na^{125}I$ was used for iodinating 20–100 μg protein and the incorporation efficiency was 5–20%.

2.6. Ins–receptor binding assays

The binding assay was designed following published work [6,7,9]. All binding assays were performed in polyethylene Eppendorf tubes (1.5 ml). A typical incubation mixture consisted of 100 μl binding buffer containing 5 mM CHAPS, indicated amounts of solubilized membrane proteins, [^{125}I]Ins, or other proteins as indicated in the figure legends. Nonspecific binding was determined by carrying out the binding assay in the presence of a 75-fold excess of unlabeled Ins in a parallel incubation. Each experiment consisted of three determinations. After incubation at 4°C for 90 min, the volume of each incubation was brought to 1 ml by adding ice-cold binding buffer. The diluted incubation mixture was then filtered through a polyvinylidene fluoride membrane with a pore size of 0.22 μm (Millipore, GVWP 02500), which had been pre-incubated overnight at 4°C with the binding buffer supplemented with 0.01% Ins to minimize nonspecific binding of [^{125}I]Ins to filters. Each filter was washed six times using iced binding buffer, 1 ml per wash. The washed filters were then placed into a plastic counting vial and the radioactivity remaining on the filters was measured in a gamma counter (Wallac 1282 High Energy Compu Gamma CS). The specific binding was calculated as the difference between the total and nonspecific binding.

2.7. Immunoprecipitation of [^{125}I]Ins–receptor complex

In order to remove endogenous Ins remaining in the membrane proteins, and to remove labeled membrane components that might nonspecifically bind to immune complexes or protein-A agarose gels, aliquots of iodinated solubilized oocyte membrane proteins were first kept in binding buffer lacking Ca^{2+} for several hours to dissociate the bound Ins from its receptor. This solution was then incubated with antibodies against Ins (a gift from Dr. Ralph R. Martel, Biochemistry Department, University of Arizona) at 4°C for 2 h, followed by incubation with protein-A agarose gel for 2 h. The mixture was centrifuged and the supernatant was saved for the next round of treatment. This process was repeated a total of three times. The final supernatant was supplemented with Ca^{2+} (to a final concentration of 5 mM) and used in the subsequent binding and immunoprecipitation experiments. Typically the binding reaction consisted of ^{125}I-membrane proteins, Ins and 5 mM CHAPS (to prevent aggregation of membrane proteins). The incubation mixture (100 μl) was kept at 4°C for 90 min with gentle shaking. 20 μl Ins antibodies was added and incubated for another 2 h, at 4°C. Then 50 μl of 50% (v/v) protein-A agarose suspension (Sigma) was added and further incubated at 4°C for 2 h. The protein-A gel was pelleted in a microcentrifuge for 15 s and the supernatant was discarded. The pellet was resuspended by briefly vortexing in 0.5 ml binding buffer containing 0.01% Ins and subjected to centrifugation again. This washing step was repeated 4–5 times until the radioactivity in the supernatant was barely detectable. The last washed protein-A pellet was violently vortexed in 50 μl SDS-PAGE sample buffer for 30 s and boiled for 15 min. The resulting sample was briefly centrifuged and then subjected to SDS-PAGE and autoradiography. One

control for this immunoprecipitation assay lacked the ligand Ins, one was supplemented with 100-fold excess unlabeled membrane proteins, and another one was treated with an antibody against cI (a repressor protein of phage λ) (Pharmacia), rather than against Ins.

2.8. Affinity chromatography of receptor for Ins

An Ins affinity column was prepared by coupling 1 mg Ins to a CNBr-activated Sepharose 4B Column (1 ml capacity, Pharmacia) according to the manufacturer's instructions. After the column was washed with affinity-column elution buffer and subsequently equilibrated with binding buffer, solubilized ^{125}I-membrane proteins were injected into the affinity column and incubated at 4°C for 2 h. Then the column was washed with binding buffer until the radioactivity in the effluent approached the background. The bound ^{125}I-receptor was eluted from the column using affinity-column elution buffer containing 0.01% Ins. The eluate was concentrated using a Centricon-30 concentrator unit and analyzed by SDS-PAGE.

2.9. Affinity crosslinking conditions

Conditions for crosslinking varied among experiments and can be found in the legends of figures. In general, the concentration of crosslinking reagents used was 5 mM and crosslinking time was 1 h at room temperature.

For crosslinking of [^{125}I]Ins to crude oocyte membrane preparations, the membrane preparations were first incubated with [^{125}I]Ins, in 100 μl binding buffer at 4°C for 2 h, in the presence or absence of 150-fold excess unlabeled Ins. The incubations were washed with binding buffer twice to remove unbound [^{125}I]Ins. The pellets were resuspended in 100 μl of 0.1 M Hepes buffer (pH 7.5). The appropriate crosslinking reagent dissolved in DMSO was added so that the final concentration of DMSO was 5%. Crosslinking reactions were stopped by Tris buffer (final concentration of 20 mM, pH 8.5). CHAPS detergent was then added to a final concentration of 40 mM and incubated on ice for 1 h to solubilize membrane proteins.

The crosslinking of ^{125}I-membrane proteins to Ins was carried out in the same way as described above, except that both components were incubated in 50 μl binding buffer, in the presence or absence of 100-fold excess unlabeled membrane proteins. After incubation of ^{125}I-membrane with Ins, to each reaction was added 50 μl of 0.2 M Hepes buffer (pH 7.5) and crosslinking reagent. Crosslinking reactions were stopped as described above and the crosslinking reagent was removed by ultrafiltration to minimize the possible crosslinking of ^{125}I-membrane proteins to antibody or protein-A components. The resulting sample was then used for immunoprecipitation experiment as described previously.

2.10. Electrophoresis and autoradiography

Reducing SDS-PAGE was carried out basically according to Laemmli [22]. When 1–30% gradient gels were used, a modification was made in that 0.5% of agarose (low melting point grade) was included in the lower concentration gel preparation and stacking gel (1%). After electrophoresis, the gels were exposed to Kodak XAR-5 film at −80°C to obtain an autoradiogram. Kaleidoscope protein molecular weight markers (Bio-Rad) and IgM (970 kDa, Pierce) were used to estimate the protein sizes of interest.

2.11. Computations

The dissociation constant, K_d (ligand concentration at which receptor is half-saturated) and B_{max} (maximum specific binding per unit membrane proteins) in this study were estimated by fitting saturation binding curves using the built-in equations from the software GraphPad Prism™ (1994, GraphPad Software, San Diego). Molarities were calculated assuming the molecular masses of the proteins as follows: Ins, 88 000 [16]; vitellogenin, 500 000 [19]; and lipophorin, 420 000 [23].

3. Results

3.1. Optimization of ligand–receptor interaction

In order to obtain the optimal conditions for the interactions between Ins and its solubilized receptor, we first carried out experiments to determine the time

required for the binding to reach equilibrium, the optimal pH values and the requirement of Ca^{2+} for the binding. As shown in Fig. 1a, the binding increased rapidly over the first 30 min and reached equilibrium in about 90 min while the nonspecific binding, determined by including a 75-fold excess of unlabeled Ins in parallel incubations, did not increase over the same period. The binding of Ins to the receptor varied with pH: the optimal pH was approximately 7.0 (Fig. 1b). Ca^{2+} was required for the formation of receptor–Ins complex in that the specific binding increased with Ca^{2+} concentrations of up to 5 mM, while this increase in Ca^{2+} concentration did not affect the nonspecific binding (Fig. 1c). When the solubilized membrane proteins were heated at 80°C for 15 min prior to incubation with Ins, the specific binding capacity was greatly diminished (Fig. 1d). The subsequent binding assays were therefore performed for 90 min, at 4°C, pH 7.0, and with 5 mM Ca^{2+} in the binding buffer.

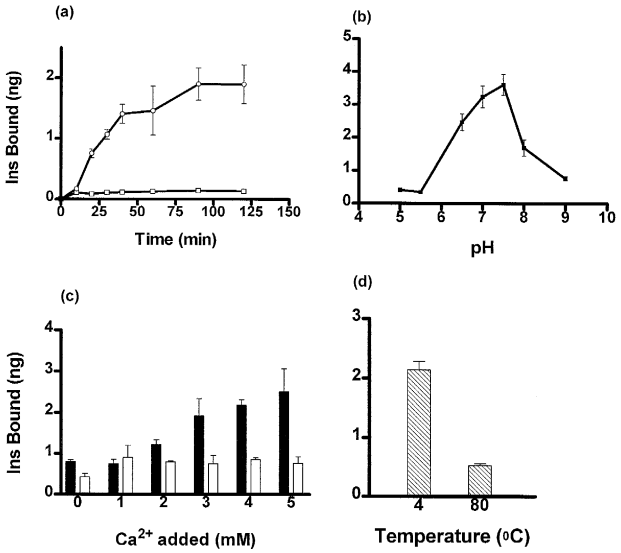

Fig. 1. Parameters affecting binding of Ins to its receptor. (a) Time dependence: in each tube, 2 μg of solubilized membrane proteins and 2 μg [^{125}I]Ins (900 000 cpm/μg) were incubated in binding buffer (pH 7) at 4°C for indicated time intervals. Nonspecific binding was determined by including 75-fold excess unlabeled Ins in a parallel incubation. The specific binding was calculated as the difference between the total and nonspecific binding. The circles represent specific binding and squares represent nonspecific binding. The data points are the means ± S.D. of three determinations. The incubation and washing conditions for b, c and d were the same as for a, except for incubation time being 90 min and other changes as indicated. (b) pH dependence: pH values were varied using a 20 mM MES-Tris buffer system. (c) Effect of Ca^{2+}: For 0 mM concentration, Ca^{2+} was chelated with 5 mM EGTA. The solid columns stand for specific binding and the open columns for nonspecific binding. (d) Test of heat lability: the membrane proteins of one mixture were heated at 80°C for 15 min prior to incubation.

3.2. Binding properties of the solubilized Ins receptor

When a fixed amount of solubilized oocyte membrane proteins was incubated with increasing amounts of [^{125}I]Ins under equilibrium conditions, the specific binding exhibited a saturable mode while the nonspecific binding increased very slowly in a linear manner (Fig. 2a). The apparent K_d and the B_{max}, given by the saturation curve fitting, were 17 nM and 11.4 pmol/mg solubilized membrane proteins, respectively. The results were very close to those estimated from Scatchard plot [24], as shown in the inset of Fig. 2a. On the other hand, when a fixed amount of [^{125}I]Ins was incubated with increasing amounts of membrane proteins, the specific binding increased linearly (Fig. 2b).

Competition between Ins and two other hemolymph proteins, vitellogenin and lipophorin, both of which are also taken up by the developing oocytes via membrane-associated receptors [20,25], was examined by incubating membrane proteins with radioactively labeled Ins and excess molar concentration of unlabeled vitellogenin or high density lipophorin. The results shown in Fig. 3a indicated that these two major hemolymph proteins had no apparent binding affinity for the Ins receptor. In order to test the tissue specificity of Ins for its receptor, solubilized membrane proteins from other tissues of *M. sexta*, fat body, midgut and ovariole sheath, were prepared and tested for binding of Ins. As shown in Fig. 3b, the total binding of these preparations were less than 15% as effective as oocyte membrane proteins for binding Ins, which is equivalent to nonspecific background binding.

3.3. Visualization of Ins receptor using co-immunoprecipitation and SDS-PAGE

Because repeated attempts to visualize the receptor for Ins by ligand blotting have failed, we adapted a co-immunoprecipitation strategy reported by Kessler

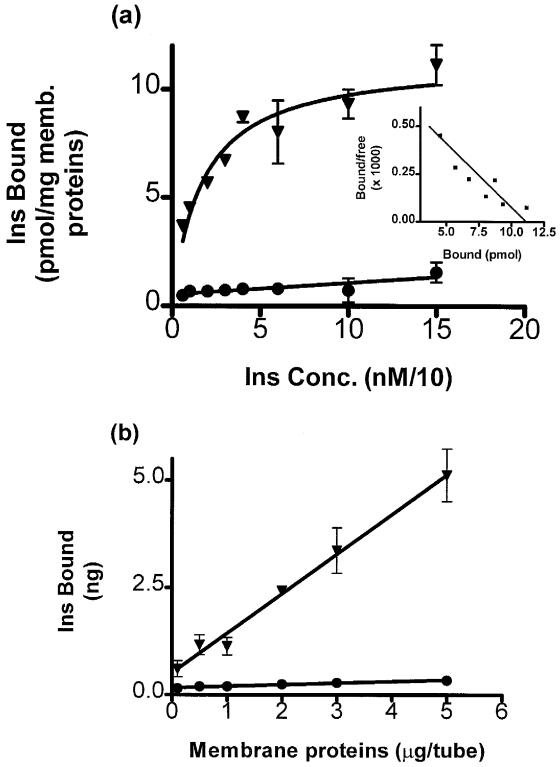

Fig. 2. Concentration-dependent binding of [^{125}I]Ins to its receptor. (a) Constant membrane protein vs. varied [^{125}I]Ins concentrations. 2 μg solubilized membrane proteins and increasing amounts of [^{125}I]Ins were incubated in binding buffer at 4°C for 90 min. Triangles represent specific binding and circles represent nonspecific binding. Data points are the means ± S.D. of three determinations. Through the fitted curve for specific binding, K_d (equilibrium dissociation constant) and B_m (maximum specific binding) were estimated as 17 nM and 11.4 pmol/mg solubilized membrane proteins respectively. A Scatchard plot of specific binding data is given in the inset. (b) Constant [^{125}I]Ins vs. varied membrane protein concentrations. 0.2 μg of [^{125}I]Ins and the indicated amounts of solubilized membrane proteins were incubated in the binding buffer at 4°C for 90 min. Triangles stand for specific binding, solid circles stand for nonspecific binding. Nonspecific binding was determined by including 75-fold excess unlabeled Ins in a parallel incubations. The specific binding was calculated as the difference between the total and nonspecific binding. Data points are the means ± S.D. of three determinations.

[26], which exploits the binding affinity between two proteins and the affinity of one of the proteins for its antibody. In our procedure, Ins was first incubated with solubilized ^{125}I-membrane proteins. Then antibodies against Ins were added to form a non-covalent receptor–ligand–antibody complex. This immune complex was then precipitated using protein-A agarose gel which has high affinity for the Fc portion of IgG. The unbound radioactive membrane proteins were removed by repeated washing of the protein-A gel using binding buffer, while the bound protein complex was readily dissociated from protein-A by SDS-PAGE sample buffer. On the autoradiogram derived from a 4–20% gradient SDS-PAGE gel, the selectively enriched membrane proteins gave a prominent band at the position of 185 kDa (Fig. 4A, lane 1). The presence of 150-fold excess of unlabeled oocyte membrane proteins effectively diminished this band (Fig. 4A, lane 2). In our preliminary experi-

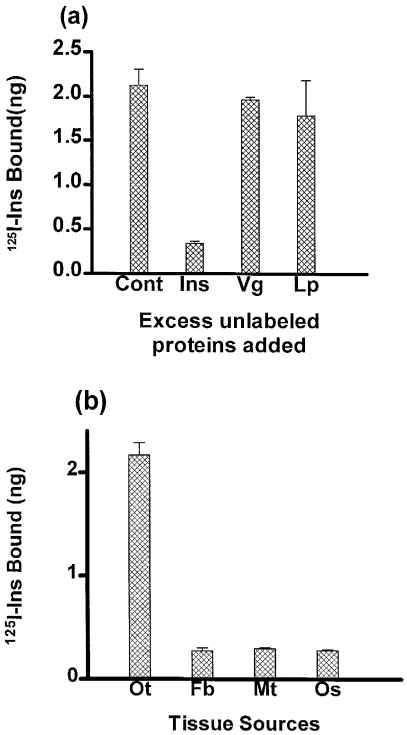

Fig. 3. Ligand selectivity and tissue specificity of Ins receptor. (a) Competition of [^{125}I]Ins by unlabeled hemolymph proteins for binding to Ins receptor. 2 μg solubilized membrane proteins and 0.2 μg [^{125}I]Ins (900 000 cpm/μg) were incubated in the presence of 20 μg of one of the unlabeled proteins as indicated, at 4°C for 90 min. Cont, control, without addition of unlabeled protein; Vg, vitellogenin; Lp, lipophorin. (b) Tissue specificity in binding of Ins to its receptor. 0.2 μg [^{125}I]Ins (900 000 cpm/μg) was incubated with 2 μg of the solubilized membrane proteins from different tissues of *M. sexta* as indicated at 4°C for 90 min. Ot, oocyte; Fb, fat body; Mt, midgut; Os, ovariole sheath. The data points are the means ± S.D. of three determinations.

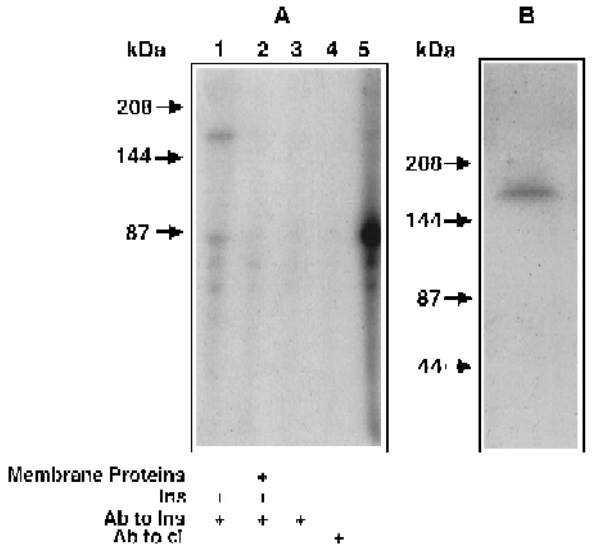

Fig. 4. SDS-PAGE of [^{125}I]Ins–receptor complex. (A) 10 μg of solubilized ^{125}I-membrane proteins ($14 \cdot 10^5$ cpm/μg) were pretreated with antibody against Ins and Protein A gel as described in Section 2 to remove any Ins bound to membrane proteins. The resulting final solution was divided into four equal parts, 100 μl each, and used for the following experiments. Lane 1: ^{125}I-solubilized membrane proteins were incubated with 0.2 μg Ins in 200 μl binding buffer supplemented with 5 mM CHAPS, at 4°C for 90 min. Then 20 μl of antibody against Ins was added to the incubation mixture and incubated for 2 h at 4°C followed by addition of 50 μl of Protein-A slurry (50% in binding buffer). The incubation was continued at 4°C for 2 h and Protein-A gel was pelleted by brief centrifugation. The resultant pellet was washed repeatedly with binding buffer (4–5 times) until the radioactivity of the effluent was barely detectable. 50 μl SDS-PAGE sample buffer was added to the Protein-A pellet and mixed by vortexing. The mixture was centrifuged briefly and the supernatant was loaded onto a 4–20% gradient gel to resolve the receptor–Ins–antibody immune complex. The electrophoresis was carried out at a constant voltage of 200 V for 3 h. The gel was autoradiographed at −80°C for 24 h. The band which appears to be at 185 kDa is believed to be the Ins receptor. The treatments for the samples presented in the other lanes were the same as for lane 1, but with a change for each incubation as described in the following. Lane 2: 150 μg unlabeled solubilized oocyte membrane proteins were included in the incubation. Lane 3: Ins was omitted in the incubation. Lane 4: antibody against Ins was replaced by that against cI (a prokaryotic protein). Lane 5: was loaded with 25 ng of ^{125}I-solubilized membrane proteins($14 \cdot 10^5$ cpm/μg). (B) 20 μg of ^{125}I-solubilized membrane proteins (25 000 cpm/μg) were injected into the Ins-affinity column and washed. The bound receptor was then eluted as described in Section 2. The eluted radioactivity accounted for some 0.1% of the total amount injected. The eluate was concentrated using a Centricon-30 (Amicon) and loaded onto 4–20% gradient SDS-PAGE gel, and electrophoresed at a constant voltage of 200 V for 3 h. The gel was autoradiographed at −80°C for 96 h.

ments, a control in which no exogenous Ins was added to the incubation, a weaker band was inconsistently observed at the position where the Ins receptor was identified (data not shown). We reasoned this band might be caused by endogenous Ins which remained bound to membrane proteins during the process of solubilizing membrane proteins. When the ^{125}I-membrane proteins were thoroughly pretreated with antibodies against Ins, this background was eliminated (Fig. 4A, lane 3). In order to see if the band denoting putative Ins receptor was an artifact caused by radioactively labeled high molecular mass proteins which might be nonspecifically bound to antibodies, a control in which the antibodies against Ins were replaced by an antibody against a protein of phage λ was performed. The result revealed that the signal was not caused by nonspecific binding of ^{125}I-membrane component to antibodies (Fig. 4A, lane 4). Moreover, the ^{125}I-membrane protein eluted from the Ins-affinity column gave a single band on SDS-PAGE gel, which had the same size as detected before (Fig. 4B). From these data we conclude that this 185 kDa protein is, or is part of, the oocyte receptor for Ins.

3.4. Crosslinking of Ins to its receptor

In order to obtain further information about the Ins–receptor complex, the receptor was covalently crosslinked to [^{125}I]Ins and analyzed by SDS-PAGE. Initially we tested the effects of three commonly used crosslinkers: DSS, DTSP and BS^3 (Pierce), on crosslinking of ^{125}Ins to its oocyte membrane receptor. The results showed that both DSS and DTSP were effective in crosslinking [^{125}I]Ins to critical membrane component(s) while BS^3 was quite inefficient (data not shown). The extent of the crosslinking increased with time of exposure and the concentration of the reagent. Exposure to 5 mM reagent for 1 h appeared to be optimal (data not shown). Considering the high efficiency and low cost, we chose to use DSS as crosslinker for the rest of crosslinking experiments. [^{125}I]Ins was first incubated with crude oocyte membrane preparations as described in the legend of Fig. 5. After unbound [^{125}I]Ins was removed by centrifugation, the [^{125}I]Ins–receptor complex was chemically crosslinked by incubation with DSS. The mixture was then solubilized, separated by SDS-

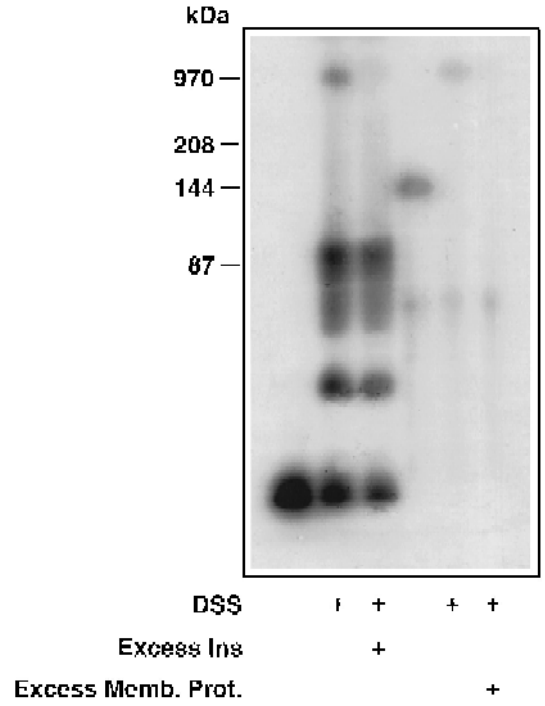

Fig. 5. Reciprocal crosslinking of [^{125}I]Ins to membrane proteins and ^{125}I-solubilized membrane proteins to Ins. Binding reactions were performed at 4°C for 2 h. Crosslinking reactions were carried out at room temperature for 1 h, followed by addition of Tris (pH 8.5) to a final concentration of 20 mM to stop crosslinking. 100 μl SDS-PAGE sample buffer (non-reducing) was added to each sample, boiled for 15 min and subjected to 1–30% gradient SDS-PAGE. Kaleidoscope protein marker and IgM were run in a parallel lane as molecular weight standards. A constant voltage of 200 V was applied for 8 h. Finished gel was subjected to autoradiography at −80°C for 20 h. Lanes 1 to 3 show crosslinking of [^{125}I]Ins to membrane proteins where each reaction contained 5 μl of [^{125}I]Ins (0.43 μg/μl, 900 000 cpm/μg), 2 μl membrane proteins (1.5 μg/μl) or other component as indicated in the figure. For lanes 4 to 6, the reaction mixture was precipitated with antibodies to Ins, and the precipitate was loaded on the gel. These lanes exhibit the crosslinking of ^{125}I-membrane proteins to Ins where each reaction contained 0.2 μg ^{125}I-membrane proteins, 0.5 μg Ins or other component as indicated in the figure. DSS, the addition of DSS with a final concentration of 5 mM after binding reaction; Excess Ins, the addition of 150-fold excess unlabeled Ins; Excess Membrane proteins, addition of 100-fold excess unlabeled membrane proteins. In the reactions corresponding to lane 1 and lane 4, DSS was substituted by equal volume (5 μl) of the solvent DMSO.

PAGE electrophoresis and autoradiographed. Several bands were detected on the autoradiogram (Fig. 5, lane 2). The band at the top, with similar molecular mass to IgM (970 kDa) corresponded to the [^{125}I]Ins–receptor complex since it was eliminated by excess unlabeled Ins (Fig. 5, lane 3). The other 4 bands (Fig. 5, lanes 2 and 3), ranging from 22 kDa to 88 kDa, represent the [^{125}I]Ins monomer, dimer, trimer and tetramer respectively. In order to confirm the receptor identity of the species with high molecular mass, we reciprocally crosslinked ^{125}I-membrane proteins to Ins, followed by immunoprecipitation of the Ins–receptor complex and electrophoretic analysis, as described above. This identified an Ins–receptor complex (Fig. 5, lane 5), with the same mobility on gel as identified by the alternative crosslinking (Fig. 5, lane 2) and this band could be specifically competed out by excess unlabeled membrane proteins (Fig. 5, lane 6). When only the solvent DMSO was included in the reaction mixture, neither intramolecular nor intermolecular crosslinking occurred (Fig. 5, lanes 1 and 4). However, the band of 185 kDa was present in Fig. 5, lane 4 where no crosslinking took place, since the ^{125}I-receptor for Ins was still co-immunoprecipitated.

4. Discussion

The *M. sexta* larva is provided with a perfect coloration to camouflage it among the green plants of its habitat [10]. The tint is produced by combining dietary carotenoids (especially lutein) with an endogenous blue biliprotein, Ins. Ins is produced in larval epidermis [11] and is abundant in the hemolymph. During the adult life of the female, Ins, along with yellow pigments, is sequestered into the developing oocyte to provide the mature eggs with a protective green coloration by allowing them to blend in with the color of the leaf on which they are deposited.

Hemolymph proteins are internalized into developing insect oocytes largely via receptor-mediated endocytosis [2,3,5]. The initial event in this endocytosis is thought to be binding of the proteins to a specific oocyte surface protein or receptor. There is, therefore, great interest to identify these receptor species and to study their interactions with ligand proteins and other possible membrane components. For the Ins receptor, much information has been gathered by classical binding studies and other assays (Ref. [1]; this study).

The solubilized Ins receptor exhibited the common features shared by the insect receptors so far identified [5]. Binding of Ins to this solubilized receptor was saturable over time, which basically differed from the mode of nonspecific binding. This binding depended on the pH, with optimal pH in proximity of 7.0. Ca^{2+} increased binding of Ins to its receptor, suggesting that it is important to the ligand–receptor complex formation. Heating of the solubilized receptor abolished binding of Ins, indicating that the receptor is a protein.

It was reported that in another lepidopteran insect, *Hyalophora cecropia*, the same receptor that is responsible for vitellogenin uptake may also bind the major hemolymph lipoprotein, lipophorin [27]. The electron microscopic observation of endocytotic pathway of several proteins including vitellogenin and lipophorin has been described in *M. sexta* [28]. Therefore it is of interest to investigate if the endocytosis of Ins shares a common mechanism with the uptake of vitellogenin or lipophorin. Our results indicated that the endocytotic receptor for Ins is distinct from that for vitellogenin or lipophorin, as the binding of Ins to its receptor is not affected by the presence of excess vitellogenin or lipophorin of *M. sexta*. It was also observed that in *M. sexta* vitellogenin and lipophorin did not compete for binding to oocyte membranes [20,25]. The Ins–receptor interactions also exhibit explicit tissue specificity: a significant amount of [^{125}I]Ins is only bound to membrane proteins from the developing oocyte, not to solubilized membrane proteins from fat body, midgut or ovariole sheath of *M. sexta*. These findings are consistent with data we previously reported on the binding of Ins to crude oocyte membranes [1].

The dissociation constant K_d of 17 nM determined with solubilized membrane proteins was lower than that (40 nM) with crude membrane extracts [1]. We postulate that the removal of nonspecific Ins binding sites by the solubilization process may account, at least in part, for this lower K_d value. Actually the phenomenon that an increase in relative purity of receptor proteins is associated with a decrease in K_d value has been reported for the vitellogenin receptor of the locust [29,30], mosquito [7,31,32] and chicken [33].

We estimated the apparent molecular size of Ins receptor as 185 kDa by co-immunoprecipitation and SDS-PAGE analysis. This size is comparable to that for a mosquito vitellogenin receptor of 205 kDa [7] and a locust vitellogenin receptor of 180 kDa [6], but different from that for a fat body lipophorin receptor of 120 kDa in *M. sexta* [9]. Ligand blotting has been employed in some insects to estimate the apparent sizes of receptors in eggs for *L. migratoria* [6], *Schistocerca gregaria* [34], *Nauphoeta cinerea* [35] and *Aedes aegypti* [7,32], and receptor in fat body for *M. sexta* [9]. A common feature of the above reported receptors is that the intactness of certain disulfide bonds is necessary for the ligand–receptor interaction, as only in the absence of reducing agents such as 2-mercaptoethanol or dithiothreitol, were the ligands able to bind to their receptors on blots. However, we did not succeed in visualizing the Ins receptor by following or modifying the published procedures for ligand blotting. One of the possible causes for this failure might be that when the Ins receptor is denatured in the SDS-PAGE, it does not refold into a native structure as exactly as other receptors even after SDS is removed, and thus no longer binds to its ligand. Therefore we adapted the strategy of co-immunoprecipitation which has been widely used in receptor studies in vertebrates. This method exploits several advantages: Ins–receptor binding under optimum conditions, the high specificity of Ins for antibody, the high affinity of protein-A for IgG and the expediency of SDS-PAGE analysis for resolving the immune complex. This circumvents the technical difficulty in binding Ins to the denatured or incompletely renatured receptor on blotting membranes. This technique may be especially useful when a receptor possesses a structure of noncovalently associated homo- or hetero-oligomers and the cooperativity of the subunits is a requirement for its binding to ligand, where the ligand blotting is obviously not applicable.

Further information on the Ins–receptor interaction was obtained by chemical crosslinking experiments. To our knowledge, this was the first time that this method was used in the study of an insect receptor. Chemical crosslinking is a valuable tool to study the protein–protein interaction and has been extensively applied to gather information about the sizes and spatial organizations of receptors in mammalian species [36]. Many parameters of the crosslinking reagent, such as the nature of reactive groups, the

length of arm between reactive groups, solubility or chemical stability, could play a part in determining if a crosslinking reaction is successful. In this study we have tested three commonly used homobifunctional NHS (*N*-hydroxysuccinimidyl)-ester crosslinkers: DSS, DTSP and BS^3 for their effects on crosslinking Ins to its receptor. DSS and DTSP are water-immiscible while BS^3 is water-soluble. Our results revealed that DSS and DTSP are effective in crosslinking Ins to its membrane receptor, while the efficiency of BS^3 was poor.

When [^{125}I]Ins was crosslinked to its binding site on *M. sexta* oocyte membranes and analyzed on SDS-PAGE gel, the apparent molecular mass of the crosslinked species was approximately 1000 kDa. This high molecular mass band was eliminated by including excess unlabeled Ins, which indicated the interaction between the ligand and receptor was specific. More convincingly, when ^{125}I-membrane proteins from *M. sexta* oocytes were reciprocally crosslinked to Ins, followed by immunoprecipitation with antibodies against Ins and SDS-PAGE analysis, a species of the same size was detected, which was also competed out by excess unlabeled membrane proteins. A possible interpretation of these results is that the Ins receptor is composed of several (probably four) subunits. It is common that endocytotic receptors in their native state possess multimeric organization which may be held together through disulfide linkages or through non covalent interaction [37]. The human transferrin receptor, for example, is a disulfide-linked homodimer [38] while the mammalian low-density lipoprotein receptor (LDLR) and the chicken hepatic lipoprotein receptor are non-covalent oligomers [37,39]. An oligomeric structure was also observed for the mosquito oocyte vitellogenin receptor [7]. A second possibility is that the receptor is normally monomeric, but when it binds one of the four identical Ins subunits, it clusters with other receptors which bind the additional subunits of a single Ins molecule, resulting in four receptors binding to one Ins molecule. This would be similar to the electron microscopic observation made by Delain et al. in that two and three α_2-macroglobulin receptors simultaneously binding to its tetrameric ligand in solution [40]. Further characterization of the Ins receptor will be necessary before we can decide between these possibilities.

In summary, we have solubilized *M. sexta* oocyte receptor for Ins, characterized its binding properties and visualized apparent molecular size of this receptor and its possible higher-order structure. The information obtained through this study will help to design methods for purification or molecular cloning of the receptor.

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References

[1] Kang Y., Kulakosky, P.C., Van Antwerpen, R. and Law, J.H. (1995) Insect Biochem. Mol. Biol. 25, 807–817.
[2] Telfer, W.H. and Pan, M.L. (1988) Arch. Insect Biochem. Physiol. 9, 339–355.
[3] Law, J.H. (1990) in Advances in Invertebrate Reproduction 5 (Hoshi, M. and Yamashita, O., eds.), pp. 97–102, Elsevier Science Publishers, Amsterdam.
[4] Zhang, J., McCracken, A. and Wyatt, G.R. (1993) J. Biol. Chem. 268, 3282–3288.
[5] Raikhel, A.S. and Dhadialla, T.S. (1992) Annu. Rev. Entomol. 37, 217–251.
[6] Hafer, J. and Ferenz, H.-J. (1991) Comp. Biochem. Physiol. 100 B, 579–586.
[7] Sappington, T.W., Hays, A.R. and Raikhel, A.S. (1995) Insect Biochem. Mol. Biol. 25, 807–817.
[8] Schonbaum, C.P., Lee, S. and Mahowald, A.P. (1995) Proc. Natl. Acad. Sci. USA 92, 1485–1489.
[9] Tsuchida, K. and Wells, M.A. (1990) J. Biol. Chem. 265, 5761–5767.
[10] Kawooya, J.K., Keim, P.S., Law, J.H., Riley, C.T., Ryan, R.O. and Shapiro, J.P. (1985) in Bioregulators for Pest Control (Hedin, P.A., ed.), pp. 511–521, ACS Symp. Series 276, Washington, DC.
[11] Kiely, M.L. and Riddiford, L.M. (1985) Roux's Arch. Dev. Biol. 194, 325–335.
[12] Riddiford, L.M., Palli, S.R., Hiruma, K., Li, W.-C., Green, J., Hice, R.H., Wolfgang, W. and Webb, B.A. (1990) Arch. Insect Biochem. Physiol. 14, 171–190.
[13] Cherbas, P.T. (1973). Ph.D. Thesis, Harvard University.
[14] Trost, J.T. and Goodman, W.G. (1986) Insect Biochem. 16, 353–358.
[15] Holden, H.M., Rypniewski, W.R., Law, J.H. and Rayment, I. (1987) EMBO J. 6, 1565–1570.
[16] Riley, C.T., Barbeau, B.K., Keim, P.S., Kezdy, F.J., Hein-

rikson, R.L. and Law, J.H. (1984) J. Biol. Chem. 259, 13159–13165.
[17] Prasad, S.V., Ryan, R.O., Law, J.H. and Wells, M.A. (1986) J. Biol. Chem. 261, 558–562.
[18] Chino, H., Hirayama, Y., Kiyomoto, Y., Downer, R.G.H. and Takahashi, K. (1987) Insect Biochem. 17, 88–97.
[19] Osir, E.O., Wells, M.A. and Law, J.H. (1986) Arch. Insect Biochem. Physiol. 3, 217–233.
[20] Osir, E.O. and Law, J.H. (1986) Arch. Insect Biochem. Physiol. 3, 513–528.
[21] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provezano, M.D., Fujimoto, E.K., Koeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Shapiro, J.P., Law, J.H. and Wells, M.A. (1988) Annu. Rev. Entomol. 33, 297–318.
[24] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[25] Kawooya, J.K., Osir, E.O. and Law, J.H. (1988) J. Biol. Chem. 263, 8740–8747.
[26] Kessler, S.W. (1975) J. Immunol. 115, 1617–1624.
[27] Kulakosky, P.C. and Telfer, W.H. (1990) Arch. Insect Biochem. Physiol. 14, 269–285.
[28] Van Antwerpen, R., Conway, R. and Law, J.H. (1993). Tiss. Cell 25, 205–218.
[29] Röhrkasten, A. and Ferenz, H.-J. (1986) Int. J. Invert. Reprod. Dev. 10, 133–142.
[30] Röhrkasten, A. and Ferenz, H.-J. (1986) Biochim. Biophys. Acta 10, 141–149.
[31] Dhadialla, T.S. and Raikhel, A.S. (1991) Arch. Insect Biochem. Physiol. 18, 55–70.
[32] Dhadialla, T.S., Hays, A.R. and Raikhel, A.S. (1992) Insect Biochem. Mol. Biol. 22, 803–816.
[33] Stifani, S., George, R. and Schneider, W.J. (1988) Biochem. J. 250, 467–475.
[34] Hafer, J. and Ferenz, H.-J. (1994) Arch. Insect Biochem. Physiol. 25, 107–120.
[35] Indrasith, L.S., Kindle, H. and Lanzrein, B. (1990) Arch. Insect Biochem. Physiol. 15, 213–228.
[36] Wong, S.S. (1991) Chemistry of Protein Conjugate and Crosslinking, CRC Press, Boca Raton.
[37] Van Driel, I.R., Davis, C.G., Goldstein, J.L. and Brown, M.S. (1987) J. Biol. Chem. 262, 16127–16134.
[38] Newman, R., Schneider, C., Sutherland, R., Vodinelich, L. and Greaves, M. (1982) Trends Biochem. Sci. 7397–400.
[39] Loeb, J.A. and Drickamer, K. (1987) J. Biol. Chem. 262, 3022–3029.
[40] Delain, E., Darray, M., Tochon, F., Gliemann, J. and Moestrup, S.K. (1994) Ann. NY Acad. Sci. 737, 202–211.