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Expression of the Chicken Hepatic Glycoprotein Receptor in *Xenopus* Oocytes: Conservation of Ligand and Receptor Targeting Signals

Doris A. Wall and Raisa Holguin

Department of Drug Delivery (UW2111), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-0939

Abstract We have obtained expression of the β-N-acetylglucosamine-binding receptor from chicken hepatocytes in *Xenopus* oocytes by injecting mRNA synthesized in vitro from a full length cDNA cloned into an expression vector (Mellow et al: J. Biol Chem 263:5468–5473, 1988). Immunoprecipitation of the receptor after labeling of oocytes with [^{35}S]-methionine for times ranging from 6 to 72 h revealed 4–5 closely spaced bands of 25–30 kDa after SDS-PAGE. Although these bands were largely resistant to endoglycosidase H cleavage, endoglycosidase F reduced the size of all bands to a single species at 23–24 kDa, indicating that they resulted from heterogeneity in glycosylation of a single polypeptide. Incubation of oocytes expressing this receptor with [^{125}I]-GlcNAc-BSA resulted in 1.8 to 10 × higher levels of cell-associated ligand in mRNA-injected vs. water-injected control oocytes, 2–35% of cell-associated counts was removed by EGTA rinse at 20°C, suggesting that most ligand was inaccessible (presumably intracellular). Immunoprecipitation of sucrose gradient fractions detected receptor molecules predominantly in a light organelle at 1.09–1.12 g/cc (the density of early endosomes and plasma membrane vesicles), with no evidence of the receptor in much heavier yolk platelet fractions even in the presence of ligand. In contrast, internalized [^{125}I]-GlcNAc-BSA was found either at the top of the gradients or in organelles at 1.09–1.17 g/cc and in yolk platelets. TCA precipitation indicated that much intracellular ligand was degraded to acid-soluble fragments. Addition of vitellogenin (the yolk protein precursor) to the medium together with the [^{125}I]-GlcNAc-BSA shifted much of the ligand into yolk platelets. These data indicate that the chicken glycoprotein receptor expressed in oocytes mediates binding and internalization of this ligand into an organelle in which ligand-receptor dissociation occurs, allowing for separation of these two molecules into different compartments. The behavior of ligand in *Xenopus* oocytes expressing the chicken receptor closely resembles its behavior in hepatocytes.

Key words: receptor-mediated endocytosis, chicken hepatic lectin, glycoprotein, cell fractionation, lysosomes

Chicken hepatocyte plasma membranes contain a 26 kDa receptor (the chicken hepatic lectin, or CHL) which mediates specific binding and clearance of serum glycoproteins with terminal N-acetylglucosamine (GlcNAc) residues (Kawasaki and Ashwell, 1977). Cross-linking studies indicate that the CHL is hexameric in hepatocyte membranes, with each receptor polypeptide having GlcNAc binding ability (Loeb and Drickamer, 1987). The relatively small size of this receptor and the fact that it is comprised of a single polypeptide species make it a particularly favorable candidate for structure/function analysis via site-directed mutagenesis.

Determination of the complete amino acid sequence of the CHL (Drickamer, 1981) has allowed cloning of the gene encoding this molecule using synthetic oligonucleotide probes to screen a λgt11 cDNA library (Mellow et al., 1988). Expression of cloned receptors in rat fibroblasts allows these cells to bind, internalize, and degrade glycoproteins with N-acetylglucosamine-terminating carbohydrate residues, indicating that intracellular signals allowing completion of this endocytic pathway were highly

Abbreviations used: CHL, chicken hepatic lectin; VG, vitellogenin; MVB, multivesicular body; GlcNAc-BSA, N-acetylglucosaminated bovine serum albumin; Endo H, endoglycosidase H; Endo F, endoglycosidase F.

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Address reprint requests to Doris Wall, Department of Drug Delivery, (UW2111), SmithKline Beecham Pharmaceuticals, P.O. Box 1539, 709 Swedeland Road, King of Prussia, PA 19406-0939.

Raisa Holguin's present address is BRL Life Technologies, Frederick, MD, 21701.

conserved during evolution (Mellow et al., 1988; Verrey et al., 1990).

Xenopus oocytes are a popular system for the expression of injected mRNA species obtained from a wide variety of sources, ranging from viruses to humans (Lane, 1983). The ability of this amphibian cell to translate foreign messengers from distantly related species, and to target many of these proteins to their correct intracellular destinations, make the oocyte a very useful expression system to study effects of mutagenesis on protein function. Oocytes have the advantage that expression can be analyzed relatively rapidly (compared with transfection of cell lines) and that the level of translation obtained is sufficient to allow detection of synthesized proteins by immunoprecipitation or by antibody or ligand binding to a single cell. In addition, their large size (1.2 mm diameter) allows analysis of protein distribution by subcellular fractionation of as few as 10 microinjected cells.

A number of plasma membrane receptors have been shown to be targeted to the plasma membrane of oocytes, where they function in ligand binding (e.g., Sakmann et al., 1985; Peacock et al., 1988; Sehgal et al., 1988; Coats and Navarro, 1990; Wafford et al., 1990). However, the intracellular fate of internalized ligands and receptors has received little attention in these studies.

We have used microinjection of CHL mRNA followed by cell fractionation to investigate the behavior of this receptor in *Xenopus* oocytes. In this report, we present data indicating that functional CHLs are targeted normally to the oocyte plasma membrane, where they mediate ligand internalization and partial degradation. The amount of GlcNAc-BSA transported to yolk platelets is increased by addition of vitellogenin to the medium, indicating that these two ligands share parts of the endocytic pathway. Receptors appear to dissociate from their ligand and remain in an early endocytic compartment. These molecules therefore appear to behave in the same manner within frog oocytes as they do in their normal cellular environment, demonstrating that the mechanisms by which intracellular sorting of ligand and receptor occur after endocytosis are compatible across large evolutionary distances. The oocyte system therefore has considerable potential for analysis of the behavior of modified receptors prepared by in vitro mutagenesis.

METHODS

Reagents

Fsp1 restriction endonuclease was purchased from New England Biolabs (Beverly, MA). Collagenase (type IV), hyaluronidase (type III), p-nitrophenyl-N-acetyl-β-D-glucosaminide, and protein A-Sepharose were from Sigma Chemical Co. (St. Louis, MO). The glycoconjugate ligand acetylglucosamine (GlcNAc)-β-O-BSA was obtained from Pierce Chemical Co. (Rockford, IL), SP6 RNA polymerase from Promega (Madison, WI), and Enzymobeads for radioiodination of proteins from BioRad (Rockville Centre, NY). Vitellogenin was purified as described previously (Wall and Patel, 1987). Endoglycosidase F was purchased from NEN Research Products (Boston, MA), and tunicamycin and endoglycosidase H from Boehringer-Mannheim (Indianapolis, IN). All other chemicals used were reagent grade.

Animals and Oocytes

Adult female *Xenopus laevis* were purchased from Nasco Biologicals (Fort Atkinson, WI), and were maintained on a diet of frog brittle from the same supplier. Ovarian tissue was obtained by partial ovariectomy and maintained in solution O-R2 (Wallace et al., 1973) for up to 1 day before use. Individual stage V–VI oocytes (Dumont, 1972) were separated for microinjection by incubation for 1–2 h at room temperature in a collagenase/hyaluronidase solution (4 mg/ml of each enzyme in Ca^{2+}-free O-R2), followed by several rinses in O-R2 without enzymes. Cells obtained in this manner were usually left overnight after dissociation, and healthy oocytes were selected the next day for injection. Oocytes were manually dissected from their follicles for experiments involving VG endocytosis since levels of VG internalized by collagenase treated cells are often low.

Preparation of Plasmid DNA and Transcription of RNA In Vitro

A plasmid containing the full coding sequence for the chicken hepatic lectin inserted behind the SP6 promoter in the expression vector pSP64 was provided by Dr. Kurt Drickamer (Columbia University College of Physicians and Surgeons). Transformation of *E. coli* strain HB101, and preparation of plasmid DNA by the alkaline lysis procedure were performed by standard protocols (Maniatis et al., 1982). The DNA was linear-

ized by Fsp1 cleavage, and RNA transcripts prepared in vitro as described by Melton et al. (1984), using [^{32}P]-UTP to monitor incorporation. mRNA was capped co-transcriptionally by addition of 2.5 mM G(5′)ppp(3′)G (Pharmacia LKB Biotechnology, Piscataway, NJ) to the reaction mixture. The mRNA was analyzed by electrophoresis on 1.4% agarose-formaldehyde gels, which revealed two different transcripts differing by approximately 200 kB present in approximately equal amounts. We do not know whether or not both of these are translated in oocytes, which synthesizes only a single polypeptide species (see Results). RNA synthesized in vitro was treated with RNase-free DNase (Promega), extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 10 μl of sterile water with 1 μl of RNasin added; 2 μl aliquots of this RNA were frozen at −70°C and thawed as needed for microinjection.

Oocyte Microinjection, Metabolic Labeling, and Immunoprecipitation

Oocytes were injected with approximately 50 nl of mRNA solution at 100–200 ng/μl, or with water as a control. They were incubated overnight at 19–20°C in O-R2 containing 2,000 units/ml penicillin and 0.3 mg/ml streptomycin, and damaged cells were removed the following day. For metabolic labeling, groups of 10–15 oocytes were incubated in O-R2 with antibiotics and [^{35}S]-methionine (translation grade, > 800 Ci/mmol, New England Nuclear/Dupont, Wilmington, DE) at 1 mCi/ml, using 10 μl of medium per cell; 0.5 ml additional O-R2 was added to batches of oocytes which were incubated for times longer than 24 h. At appropriate times, cells were rinsed with O-R2, 100 μl per oocyte of cold lysis buffer (0.1M NaCl, 5 mM $MgCl_2$, 1% Triton-X-100, 0.5% SDS, 1% Na deoxycholate, 0.1M Tris, pH 8.2, 1 mM PMSF, 5 μg/ml Pepstatin A, and 1 μg/ml leupeptin) was added, and the cells were homogenized by drawing them up and down 4–8 times in a Pasteur pipet whose tip had been pulled out in a flame.

Homogenates were spun 5 min in a microfuge to pellet pigment and other insoluble material, and the supernates used for immunoprecipitation. Immune precipitations were performed as described (Faust et al., 1987), except that 100 μl of homogenate was diluted to 1 ml with lysis buffer before addition of 4 μl of polyclonal anti-CHL antiserum (Loeb and Drickamer, 1987; antiserum was provided by Dr. Kurt Drickamer). Samples were analyzed by SDS-PAGE under reducing conditions (Maizel, 1971), and radioactive bands were visualized in dried gels by fluorography with PPO-DMSO.

Iodination of GlcNAc-BSA and Binding Assays

GlcNAc-BSA was iodinated by the chloramine T (Greenwood et al., 1963) or lactoperoxidase/glucose oxidase (Hubbard and Cohn, 1972) procedures, yielding a ligand of 5–9 × 10^6 cpm/μg. [^{125}I]-GlcNAc-BSA was stored in 50–100 μl aliquots at −70°C until use. Binding assays were performed with groups of 10–15 oocytes in O-R2 + 0.5% BSA and 3–68 ng/μl of [^{125}I]-GlcNAc-BSA (0.02–1.0 μM ligand). The cells were incubated in this medium for 2–24 h, cooled 10 min on ice, then rinsed with 6 changes of cold O-R2 + 0.5% BSA and transferred to clean tubes before determination of cell-associated radioactivity in a gamma counter. Oocytes were then rinsed with 6 changes of cold O-R2 + 0.5% BSA containing 10 mM EGTA and were transferred to fresh tubes before counting again to determine the % EGTA-inaccessible radioactivity.

Cell Fractionation

Sucrose gradient fractionation of oocytes was performed as described previously (Wall and Patel, 1987; Wall and Meleka, 1985), except that any visible fragments of plasma membrane complexes remaining after the initial homogenization were removed with a Pasteur pipet, disrupted by pipeting vigorously up and down in 0.5 ml of homogenization buffer, and then combined with the rest of the homogenate before application to sucrose gradients. This procedure improved recovery of internalized ligand. Homogenates were not filtered through Nitex screen before application to gradients, since filtration often resulted in loss of many cell-associated counts.

Protein and Enzyme Assays

Protein levels were determined by the Bio-Rad procedure (BioRad, Rockville Centre, NY), using the protocol supplied with the dye reagent concentrate and bovine γ-globulin as protein standard. β-N-acetylglucosaminidase activity was measured by a slight modification of the procedure of Hubbard and Cohn (1975), as described (Wall and Patel, 1987).

Endoglycosidase Treatment

Immunoprecipitation of 50 μl of oocyte lysates from [^{35}S]-methionine-labeled cells was performed as described above. Proteins bound to protein-A-Sepharose beads were treated with endoglycosidase F by incubation for 16 h to 2 days at 37°C in a mixture of 0.1 M sodium phosphate, pH 6.1, 50 mM EDTA, 1% NP-40, 0.02% SDS, 1% β-mercaptoethanol, with 4.4 units of enzyme added initially, and 1 unit additional endoglycosidase F added after 24 h of incubation. After incubation, beads were washed twice with 0.1 M Tris-HCl, pH 8.0, and the samples solubilized and analyzed by SDS-PAGE on 8–15% linear gradient gels. Endoglycosidase H digestion was performed either under the same incubation conditions as endoglycosidase F digestion, or in 0.5 M sodium acetate, pH 5, 1% Triton-X-100, 0.02% SDS, 1% β-mercaptoethanol, and 0.5 mM PMSF, with 10 munits of enzyme added at the start of the incubation, and 1 additional munit after 24 h at 37°C.

Tunicamycin Treatment of Oocytes

Inhibition of glycosylation with tunicamycin was performed according to the procedure of Colman et al. (1981). Briefly, inhibitor was added both to the mRNA which was injected (40 μg/ml tunicamycin) and to the O-R2 in which cells were incubated after injection (2 μg/ml tunicamycin). At 24 h after injection, groups of 15 cells were labeled with [^{35}S]-methionine for an additional 24 h before lysis and immunoprecipitation. Control cells were incubated in the same manner without tunicamycin addition.

Trichloroacetic Acid Precipitation

Samples were brought to a volume of 1 ml on ice, and an equal volume of cold 20% TCA was added. 50 μl of 10% BSA was added before precipitation to samples of the oocyte incubation medium, which contained very low concentrations of protein. Samples were left on ice for 30 min to overnight, and precipitated material was removed by centrifugation at 1,730*g* for 25 min. Supernates and pellets were then separated and counted to determine the percentage of acid soluble radioactivity.

RESULTS

Chicken Hepatic Glycoprotein Receptors Are Expressed in Oocytes

Xenopus oocytes were injected with either CHL mRNA or an equivalent amount of water, incubated overnight, and then transferred to medium containing [^{35}S]-methionine for times ranging from 6 to 72 h. The cells were lysed in detergent buffer, and aliquots equivalent to 1–2 oocytes were immunoprecipitated with anti-receptor antiserum and analyzed by SDS-PAGE. mRNA-injected cells expressed 4–5 closely-spaced bands at 25–30 kDa which were absent in water-injected control oocytes (Figs. 1, 2). This size range is consistent with the expected molecular weight of this protein (apparent molecular weight 25–26 kDa determined by gel electrophoresis: Kawasaki and Ashwell, 1977). However, the multiple bands found in oocytes are not seen either in chicken hepatocytes (Drickamer and Mamon, 1982; Loeb and Drickamer, 1988), or when this receptor is expressed by transfection in rat fibroblasts (Mellow et al., 1988) or MDCK cells (Graeve et al., 1989). Although the higher molecular weight forms of the receptor became relatively more prominent between 6 h and the later time points, all bands persisted over the time course of this experiment.

Considerable variation in level of receptor expression was seen between batches of oocytes obtained from different animals, even when the same mRNA preparation was used for injection of the cells.

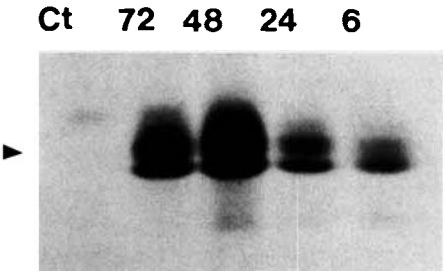

Fig. 1. Expression of CHL in *Xenopus* oocytes. The CHL was immunoprecipitated from lysates of [^{35}S]-methionine labeled oocytes which had been injected with either water (lane Ct) or CHL mRNA 24 h prior to radioactive labeling. mRNA-injected oocytes were incubated with the radioactive amino acid for 6, 24, 48, or 72 h before homogenization to examine the time course of receptor synthesis. Water-injected controls were incubated for 72 h before homogenization. Multiple receptor bands were seen at all time points, with some increase in higher molecular weight forms between 6 and 24 h. Although the relative amount of [^{35}S]-methionine labeled receptor which could be precipitated increased consistently with time, the apparent loss of receptor protein between 48 and 72 h seen here was not found consistently in all experiments. The arrowhead indicates the position of the molecular weight marker chymotrypsinogen at 26 kDa.

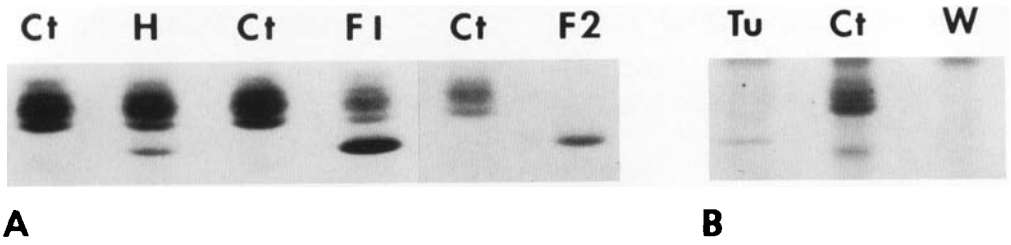

Fig. 2. Glycosylation heterogeneity is responsible for the multiple forms of CHL seen in oocytes. **(A)** Immunoprecipitated receptors from [^{35}S]-methionine labeled cells were treated with either Endo F or H, or were incubated without addition of any enzymes (lanes marked Ct). Complete digestion after incubation with Endo F for 48 h reduced the pattern to a single sharp receptor band with an apparent molecular weight of approximately 23 kDa (lane F2). Partial digestion after 16 h incubation with the same enzyme (lane F1) suggested that the enzyme was cleaving all forms of the receptor at approximately the same rate, since all bands disappeared with similar kinetics. Although Endo H cleaved a small percentage of total radiolabeled receptor to a single band whose size was similar to that of the Endo F digestion product, the majority of the CHL was resistant to treatment with this enzyme. **(B)** Oocytes were treated with tunicamycin to examine the effect of inhibition of N-linked glycosylation on CHL synthesis. In the presence of inhibitor, [^{35}S]-methionine labeled oocytes synthesized a single immunoprecipitable receptor species, whose size was similar to that of the Endo F treated receptor (lane Tu). This band was not seen in immunoprecipitates of water-injected control cells (lane W). Although considerably lower amounts of precipitable CHL were detected in [^{35}]S-methionine labeled cells treated with tunicamycin, the single receptor species synthesized was similar in size to that of the Endo F treated receptor seen in Figure 2A. Endoglycosidase treatment therefore appeared to cleave all N-linked carbohydrate residues from CHL molecules made by the *Xenopus* oocyte.

Multiple Forms of the CHL Result From Variations in Glycosylation

To determine whether or not the multiple bands seen after immunoprecipitation were due to addition of heterogeneous carbohydrate residues to this receptor or to synthesis of several different protein species, we digested the molecules with endoglycosidases F and H. While treatment with endoglycosidase H only cleaved a small amount of the labeled receptor, endoglycosidase F reduced all of the 25–30 kDa bands to a single polypeptide species at approximately 23 kDa (Fig. 2A). This result suggests that oocytes attach carbohydrate moieties which are predominantly of the complex type and that differences in structure of these carbohydrate chains produces the pattern of several closely-spaced bands seen in gels. Since the CHL has only one N-linked glycosylation site (Drickamer, 1981; Mellow et al., 1988), variation due to attachment of more than one carbohydrate residue seems unlikely. Absence of a 23 kDa unglycosylated precursor band after metabolic labeling for times as short as 6 h (Fig. 1) suggests that carbohydrate is added to the CHL very rapidly after synthesis, in contrast to other receptors expressed in the oocyte system (see Discussion).

Tunicamycin Treatment Results in Synthesis of a Single CHL Species

Since little is known about the structure of carbohydrate side chains synthesized by amphibian cells, we were concerned that endoglycosidase cleavage might not remove all sugar residues. Therefore, we used tunicamycin treatment to block addition of N-linked carbohydrate residues to the receptor as it was being synthesized (Tkacz and Lampen, 1975; Takatsuki and Tamura, 1971). Tunicamycin-treated or control oocytes were incubated for 24 h after injection, labeled with [^{35}S]-methionine for an additional 24 h, and receptors were immunoprecipitated from cell lysates (Fig. 2B). Although the amount of TCA-insoluble material labeled with [^{35}S]-methionine was equivalent in both tunicamycin treated and control cells, oocytes exposed to inhibitor appeared to synthesize less immunoprecipitable CHL (Fig. 2B). Nevertheless, the results confirmed the endoglycosidase cleavage data, since cells treated with inhibitor synthesized only a single receptor band at 23 kDa, similar in size to the species seen after enzymatic treatment.

The CHL Expressed in Oocytes Is Transported to the Cell Surface Where It Binds Ligand

mRNA and water-injected control cells were incubated with [^{125}I]-GlcNAc-BSA to determine whether or not receptors were present at the cell surface, and whether or not they could mediate ligand binding and internalization. Binding activity was generally higher as cells were incubated for longer times after injection: we rou-

tinely waited 72–96 h in the experiments reported here.

Levels of ligand associated with mRNA-injected cells increased rapidly at GlcNAc-BSA levels up to 0.1 μM, then began to plateau at higher ligand concentrations (Fig. 3). In contrast, ligand associated with control oocytes increased linearly over the entire concentration range up to 0.5 μM. Consequently, the ratio of specific:nonspecific ligand incorporation became progressively lower as the GlcNAc-BSA concentration was increased. Since degraded ligand is not released from *Xenopus* oocytes (see below and Opresko et al., 1980b), cell-associated ligand does not reach a steady state, but rises continually with time. This increase was usually linear over at least 5.5 h of incubation. Based on these data, at ligand levels > 0.1 μM mRNA-injected oocytes had 1.6–4.7 × 10^9 molecules of specifically associated [^{125}I]-GlcNAc-BSA/cell/h at 20°C. (specific internalization was determined by subtraction of non-specific levels of uptake seen in water-injected control cells).

At ligand concentrations between 25 and 370 nM, 1.8 to 10 times more [^{125}I]-GlcNAc-BSA was associated with CHL mRNA-injected oocytes than with H_2O-injected control cells after 2.5–6 h incubations at 20°C ($\bar{x}$ = 3.6 ± 1.9, n = 17). Absolute levels of ligand incorporation by mRNA-injected cells ranged from 0.084–0.76 ng/oocyte/h and in H_2O-injected cells from 0.015–0.20 ng/oocyte/h at the different ligand concentrations used. Some of this variation could be attributed to differences in levels of cell-associated ligand between individual cells in any one experiment, which varied by as much as 12-fold. In addition, groups of cells from different animals gave different absolute levels of both specific and non-specific ligand association.

Addition of a 75- to 150-fold excess of unlabeled GlcNAc-BSA to the binding assay medium of mRNA-injected cells resulted in a reduction of specific cell-associated radiolabeled ligand by 76–79% compared with control levels obtained without competitor. In contrast, the competitor reduced [^{125}I]-GlcNAc-BSA binding to water-injected oocytes by 27–29% of control levels.

The Majority of Cell-Associated Ligand Is Internalized at 20°C

Since binding of GlcNAc-BSA to the CHL is strictly dependent on calcium (Kawasaki and Ashwell, 1977; Loeb and Drickamer, 1988), we used EGTA treatment after cooling cells to 4°C

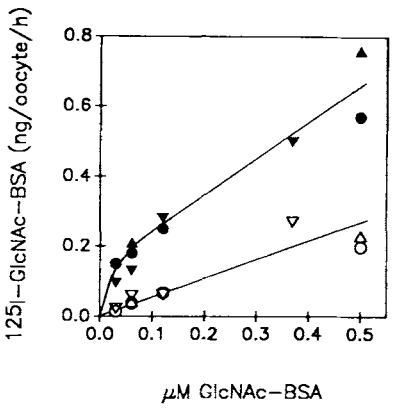

Fig. 3. Concentration-dependence of [^{125}I]-GlcNAc-BSA binding by oocytes. Oocytes were incubated for 72 h after injection with either water or CHL mRNA, and were then switched into fresh medium with ligand for 3–6 h at [^{125}I]-GlcNAc-BSA concentrations ranging from 0.05 to 0.5 μM. Results of three representative experiments are presented here using different symbols for each set of data. Non-specific endocytosis of the tracer increased linearly with increasing ligand input in control (water-injected) cells (open symbols). However, in those oocytes expressing the CHL (filled symbols), the amount of cell-associated ligand was consistently higher than in controls, rising sharply at concentrations between 0.05 and 0.1 μM, and then increasing much more gradually at higher ligand input. Each symbol represents the value obtained from 6–20 oocytes which were counted together as a group, with the total counts divided by the number of cells to obtain an average value.

to determine whether or not cell-associated ligand was at the cell surface or in internal compartments. EGTA rinsing removed 2–35% of cell-associated counts in cells which had been incubated with ligand at 20°C for 2.5–6 h ($\bar{x}$ = 17 ± 10% for water-injected and 13 ± 8% for mRNA-injected cells: no correlation with time of incubation with ligand was seen). As expected, a much greater percentage of cell-associated ligand bound at 4°C was removed by the EGTA rinse, with 69–76% of [^{125}I]-GlcNAc-BSA lost from mRNA-injected and 43–73% removed from water-injected control cells. Amphibian oocytes continue to endocytose vitellogenin slowly at 4°C, a temperature which does not completely block endocytosis by these cells. Therefore, the relatively high amount of undissociated ligand after 4°C binding may be either internal ligand or cell surface ligand which is non-specifically bound and therefore not released by EGTA treatment. Using the values for EGTA-dissociable ligand associated with mRNA-injected oocytes at 4°C from these experiments, each cell had 0.97 × 10^9 molecules of [^{125}I]-GlcNAc-BSA bound at the surface, approximately two times greater than the number of

ligand molecules which associated with oocytes each h at 20°C.

Since these data suggested that the CHL was mediating ligand internalization, we next examined the subcellular distribution of both receptor and GlcNAc-BSA by sucrose gradient fractionation.

Intracellular Distribution of the CHL

To determine the density of organelles containing the CHL, we labeled oocytes with [^{35}]S-methionine for 24 h, then homogenized the cells and fractionated them on sucrose gradients. The receptor was immunoprecipitated from each fraction, and its distribution determined by cutting out radioactive CHL bands and counting them. All of the multiple receptor bands had a similar density profile, with a single peak of receptor centered at 1.11 g/cc, and no evidence of CHL molecules in much denser yolk platelet fractions at 1.20–1.24 g/cc (Fig. 4A,B). Addition of 1 μM GlcNAc-BSA to the medium of cells for 5 h prior to their homogenization had no effect on receptor distribution (Fig. 4C). These results suggested that the receptor remained predominantly in an early organelle of the endocytic pathway, analogous to its recycling behavior in chicken hepatocytes.

To determine the effect of glycosylation on intracellular distribution of the CHL, we also examined the distribution of the single CHL species synthesized by oocytes treated with tunicamycin. Blockage of N-linked glycosylation had little effect on CHL distribution in sucrose gradients: The receptor peak was seen in a low-density organelle at 1.11–1.12 g/cc, despite the lack of carbohydrate addition (data not shown).

Ligand Distribution After Endocytosis

The density distribution of intracellular [^{125}I]-GlcNAc-BSA was examined after 5.5 h of incubation with ligand and removal of cell surface ligand with EGTA treatment. In this experiment, a portion of the GlcNAc-BSA appeared in low-density organelles in a similar region of the gradient as the CHL (Fig. 5A). However, a large proportion of radioactivity also appeared with *non-sedimentable* material remaining at the top of the gradients, and in both light (centered at 1.21 g/cc) and heavy (centered at 1.23–1.24 g/cc) yolk platelet fractions. Since 44% of total cell-associated radioactivity was due to non-specific incorporation in this experiment (596 cpm/oocyte for H_2O-injected vs. 1,345 cpm/oocyte for mRNA-injected cells), we also examined ligand distribution in H_2O-injected controls (Fig. 5A). In controls, ligand was found predominantly at the top and in platelet regions of the gradient, with very little in low-density organelles. Comparison of control and mRNA-injected gradient profiles clearly demonstrates that ligand is present in low-density organelles only after specific (i.e., receptor-mediated) endocytosis (Fig. 5A).

Although 85–90% of the material in low-density organelles of mRNA-injected cells was intact (trichloroacetic acid insoluble), only 17–62% of the radioactivity at the top of gradients and in platelet fractions appeared to be acid insoluble (Fig. 5B). This was also true for water-injected control cells. These data indicated that a good deal of intracellular radioactivity was degraded within oocytes, consistent with the normal behavior of this ligand in hepatocytes. Similar results were obtained with [^{125}I]-GlcNAc-BSA prepared by either chloramine T or lactoperoxidase/glucose oxidase procedures. Relatively inefficient hydrolysis and retention of much of the degradation products are characteristic features of the *Xenopus* oocyte system (Opresko et al., 1980b).

Time Course of Ligand Distribution

Changes in distribution of intact and degraded ligand were examined at times of incubation with [^{125}I]-GlcNAc-BSA ranging from 2 to 21 h (Fig. 6). After 2 h, the majority of ligand was found in low-density organelles between 1.07 and 1.15 g/cc sucrose. This was predominantly intact (TCA insoluble) protein. In addition, radioactivity was present in light and heavy yolk platelets at densities greater than 1.20 g/cc in both degraded and undegraded form. Oocytes incubated with the ligand continuously for 5 h had considerably more radioactive tracer in yolk platelets and at the top of gradients, although some ligand still remained in low-density organelles which predominated at 2 h of labeling. The relative amount of ligand in low-density organelles at 5 h varied somewhat from experiment to experiment: The two examples shown here represent the two extremes (contrast Fig. 6B with Fig. 5B). When oocytes were kept for an overnight incubation after 5 h with [^{125}I]-GlcNAc-BSA, the relative amount of radioactivity detected at densities between 1.10 and 1.15 was still further reduced, and the majority of ligand was degraded (TCA soluble). Since non-specific

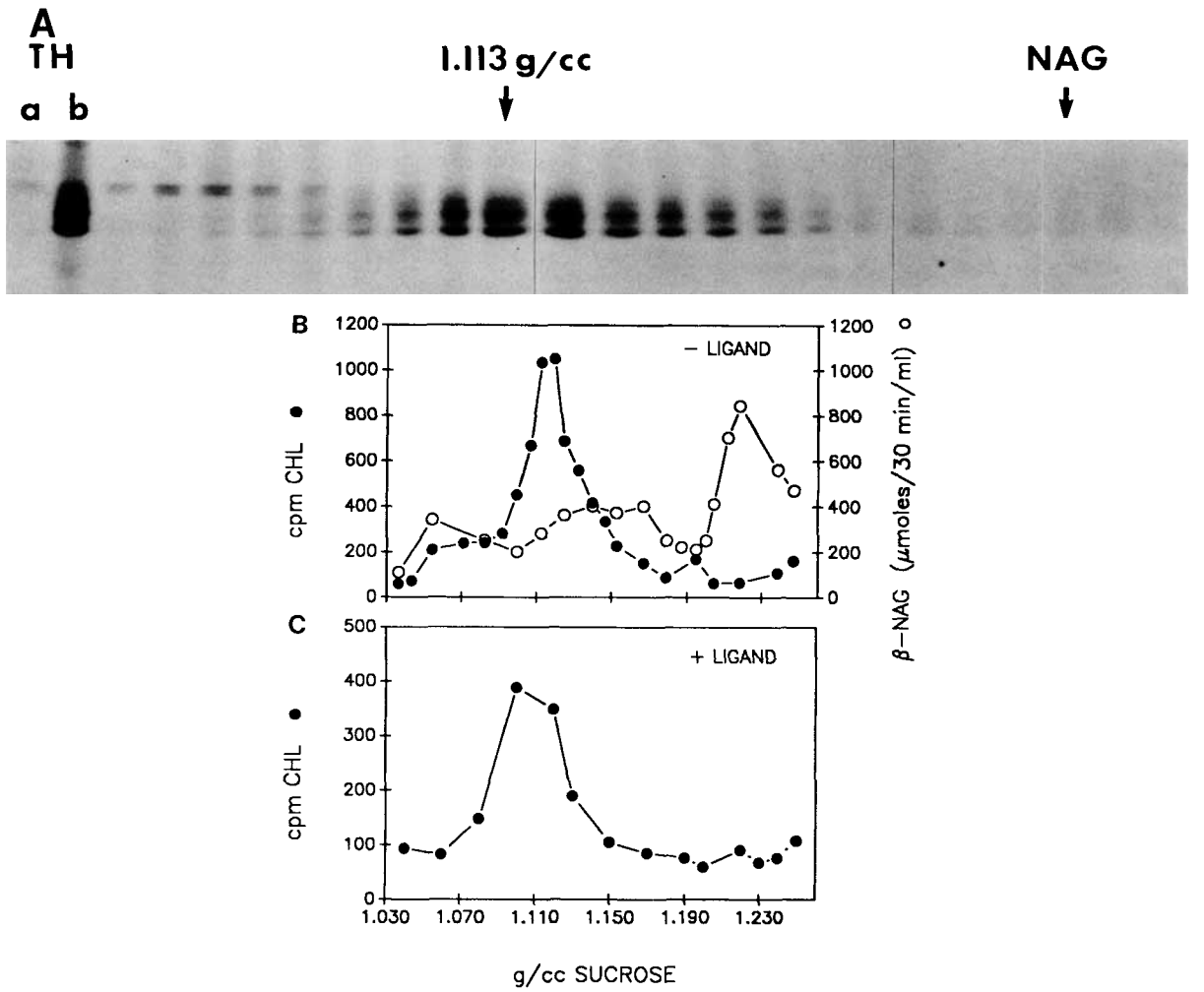

Fig. 4. Intracellular distribution of the CHL. Oocytes which had been injected with CHL mRNA were labeled for 24 h with [^{35}S]-methionine, then homogenized and analyzed by sucrose gradient fractionation. (**A**) 100 microliters of each gradient fraction were used for receptor immunoprecipitation and analysis by SDS-PAGE and fluorography. TH, total homogenates of (a) water-injected control oocytes and (b) the sample applied to the sucrose gradient, both immunoprecipitated with anti-receptor antiserum. All forms of the receptor had a similar distribution and were found predominantly in organelles of very low density, in a peak centered at 1.11–1.12 g/cc. The position of the lysosomal enzyme activity peak (NAG) is indicated. (**B**) Radioactive bands corresponding to the CHL were cut out of the dried gel used for the fluorogram shown in A, and counted to quantitate the distribution of receptor in each gradient fraction (●). The distribution of the enzyme β-N-acetylglucosaminidase (β-NAG) was also determined as a marker for the oocyte lysosomal compartment (○). (**C**) Receptor distribution is unaffected by addition of 1 μM GlcNAc-BSA to the medium for 5 h before homogenization of [^{35}]S-methionine labeled oocytes.

incorporation by water-injected control oocytes ranged from 16% to 24% in these experiments, the amount of ligand degraded exceeded the amount which could be attributed to fluid-phase endocytic internalization.

Addition of Vitellogenin Shifts Previously Internalized [^{125}I]-GlcNAc-BSA Into Yolk Platelet Compartments

Since internalized vitellogenin is rapidly transported to light yolk platelets at 1.21 g/cc, we asked whether or not [^{125}I]-GlcNAc-BSA in low-density organelles could be shifted into the vitellogenin pathway by addition of 1 mg/ml of the yolk protein precursor to the medium of cells for the final 1.5 h of a 5.5-h incubation with [^{125}I]-GlcNAc-BSA. The results show that a substantial portion of intact ligand in low density organelles between 1.10 and 1.15 g/cc is shifted into denser organelles when VG was present, with a peak in the light platelet region (Fig. 7). This was accompanied by an increase in the

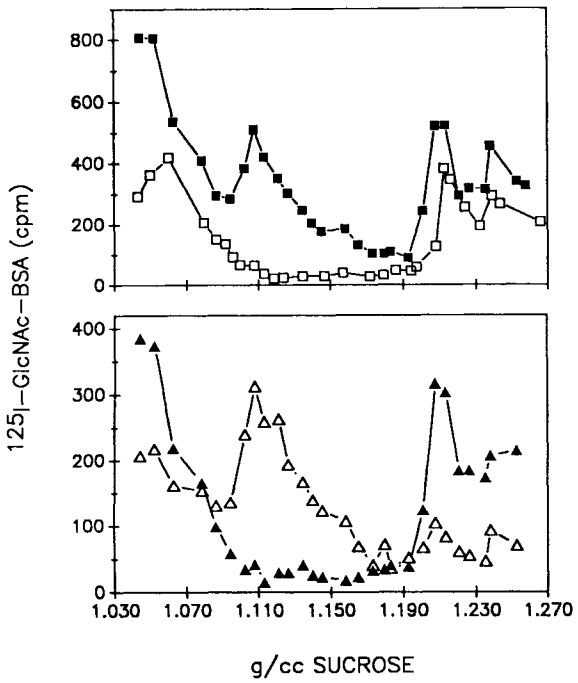

Fig. 5. [^{125}I]-GlcNAc-BSA distribution after endocytosis by CHL-mRNA and water-injected oocytes. **(Top)** Oocytes were incubated for 72 h after injection, then placed in O-R2 containing 0.05 μM ligand for 5.5 h at 20°C. After an EGTA rinse to remove surface-bound ligand, the cells were homogenized, fractionated on 1.04–1.26 g/cc linear sucrose gradients, and the distribution of radioactivity and refractive index determined for each fraction. [^{125}I]-GlcNAc-BSA incorporation by water-injected control oocytes was 44% of the level incorporated by mRNA-injected cells in this experiment. Oocytes expressing the CHL (■) exhibited peaks of ligand at the densities of light and heavy yolk platelets (1.21 and 1.23 g/cc, respectively), in low-density organelles centered at 1.11 g/cc, and at the top of the gradient (non-sedimentable material). In contrast, water-injected control cells (□) showed ligand only at the top of the gradient and in yolk platelet fractions, with little material in low-density regions. **(Bottom)** Trichloroacetic acid precipitation of the CHL-mRNA-injected oocyte gradient fractions revealed that the peak centered at 1.11 g/cc contained intact (acid-insoluble: △) ligand, but that a large proportion of the GlcNAc-BSA was apparently degraded (acid-soluble: ▲) in platelet fractions and at the top of the gradient.

amount of acid-soluble radioactivity in yolk platelet fractions.

DISCUSSION

The results presented in this report demonstrate that *Xenopus* oocytes are capable of expressing functional CHL molecules at the cell surface after microinjection of mRNA coding for this receptor. Ligand internalized via this receptor separates from the receptor and is delivered to a degradative compartment, while receptors remain in organelles of lower density, where plasma membrane and endosomes are found.

Receptor Glycosylation

Both tunicamycin and endoglycosidase experiments indicate that the oocyte attaches carbohydrate residues of heterogeneous composition to a single 23 kDa receptor polypeptide. The extent of heterogeneity seen in oocytes apparently exceeds the variation (due to different levels of sialylation and phosphorylation) that the CHL exhibits in its native environment, where at most two bands are resolved by SDS-PAGE on 15% gels (Drickamer and Mamon, 1982). An unusually high degree of glycosylation heterogeneity is also found for rat hepatocyte glycoprotein receptors when expressed in oocytes (D. Wall, unpublished data), although other receptor proteins appear no different in oocytes than in their tissue of origin (e.g., Sehgal et al., 1988; Peacock et al., 1988; Wall et al., 1988). Although little is known about the detailed structure of carbohydrate groups attached to proteins by *Xenopus* oocytes, the resistance of CHL molecules to Endo H digestion and susceptibility to Endo F suggests that they resemble the usual complex type carbohydrate structures sufficiently to be discriminated by these enzymes. In this regard, glycosylation of the CHL differs from that of the α subunit of human chorionic gonadotropin, which appears to have only Endo H sensitive carbohydrate groups attached when synthesized within *Xenopus* oocyte cytoplasm (Mous et al., 1980).

Absence of a demonstrable unglycosylated precursor band in oocytes at any time of the pulse-chase experiments suggests that glycosylation of this molecule occurs very rapidly after its synthesis. The CHL is quite unusual in this regard, since labeling periods of as little as 6 h readily reveal precursor forms of a number of other exogenous glycoproteins destined for the cell surface in *Xenopus* oocytes. These include nerve growth factor receptors (Sehgal et al., 1988), LDL receptors (Peacock et al., 1988), rat hepatic glycoprotein receptors (D. Wall, unpublished observations), and mouse macrophage/lymphocyte Fc receptors (Wall et al., 1988). Since all of these proteins possess multiple glycosylation sites, while the CHL has only one, the difference in rate of glycosylation may result from the need to add more than one carbohydrate residue to these other proteins. Precursor molecules of the rat hepatic glycoprotein recep-

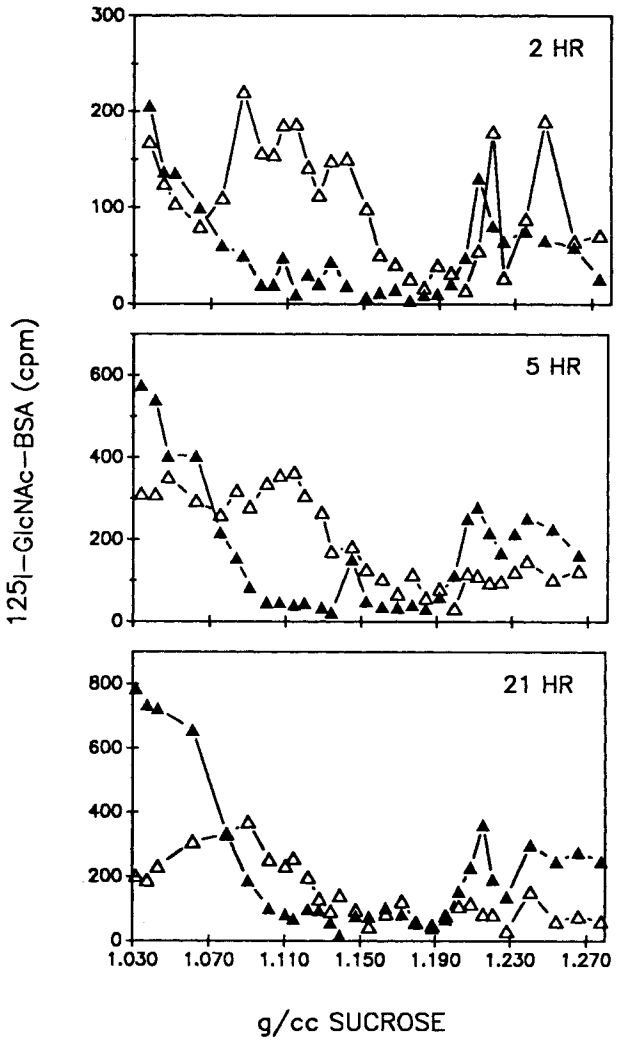

Fig. 6. Time course of ligand distribution. Oocytes were incubated for 72 h after injection with CHL mRNA or H_2O, and groups of 10 cells were then incubated with 0.1 μM [^{125}I]-GlcNAc-BSA in OR-2 at 20°C for 2 h (**Top**), 5 h (**Middle**), or for 5 h followed by a 16 h chase without ligand (**Bottom**). At the end of each incubation period, oocytes were rinsed with EGTA, homogenized, and fractionated on sucrose gradients to examine ligand distribution. Non-specific incorporation of ligand by H_2O-injected control cells was 16–24% of mRNA-injected levels after the EGTA rinse in this experiment. Trichloroacetic acid precipitation was performed to determine the amount of intact (△) and degraded (▲) ligand in each gradient fraction. Relatively more ligand was found in low-density organelles between 1.10 and 1.15 g/cc at the 2-h time point. At later times of incubation, the increased amounts of cell-associated counts were found predominantly at the top of the gradient and in yolk platelets, and most was degraded to an acid-soluble form.

tor are seen at a higher density (1.13–1.15 g/cc) in sucrose gradients than fully glycosylated forms (D. Wall, unpublished observations). Similarly, human cathepsin D precursors expressed in oocytes appear to undergo mannose-6-PO_4 addition while they reside in an organelle of about 1.154 g/cc density (Faust et al., 1987). Failure to detect the 23 kDa form of the CHL at this density suggests that glycosylation is completed so rapidly that these molecules do not accumulate to any significant extent in an organelle (presumably rer/Golgi) of the biosynthetic pathway. In contrast, molecules with multiple glycosylation sites appear to remain for some time in the processing compartment during completion of their carbohydrate side chains before transport to their final intracellular destination. Inhibition of N-linked glycosylation by tunicamycin had no effect on the density distribution of the CHL, suggesting that lack of glycosylation did not block intracellular transport of this molecule.

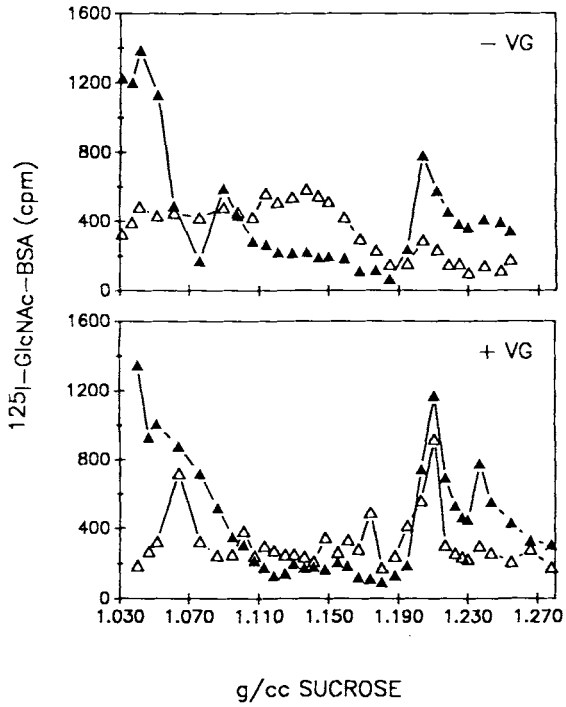

Fig. 7. Addition of VG shifts [^{125}I]-GlcNAc-BSA into yolk platelet fractions. Two groups of 10 manually-dissected oocytes were incubated for 5.5 h with 0.1 μM [^{125}I]-GlcNAc-BSA, with an additional 1 mg/ml VG added to one set of cells 1.5 h before the end of the incubation. Oocytes were rinsed with EGTA, homogenized, and fractionated on sucrose gradients to determine the effect of VG addition on [^{125}I]-GlcNAc-BSA distribution. TCA precipitation was performed to distinguish intact (△) and degraded (▲) BSA. The peak of intact ligand between 1.10 and 1.15 g/cc sucrose was significantly reduced in oocytes exposed to VG, with increased amounts of intact ligand seen in the light yolk platelet region at 1.21 g/cc. Addition of VG had less effect on the distribution of degraded ligand, although the amount of TCA-soluble material in platelets also appeared to increase.

Intracellular Receptor Behavior

CHL molecules expressed in oocytes are found predominantly in low-density organelles, and not in yolk platelets whether or not ligand is present in the medium. Since our sucrose gradient fractionation procedure does not resolve plasma membrane from early endocytic vesicles, the CHL peak centered at 1.11 g/cc could be derived from either location. Behavior of the CHL in oocytes is therefore consistent with its recycling pathway in chicken hepatocytes, where this receptor avoids degradation through dissociation from a ligand which is delivered to lysosomes (Kawasaki and Ashwell, 1977; Loeb and Drickamer, 1988). Since the trigger for ligand-receptor dissociation is likely to be conformational changes in the CHL molecule resulting from acidification of an early endosomal compartment (Loeb and Drickamer, 1988), separation of these two molecules after internalization by oocytes provides indirect evidence for acidification of oocyte endosomes.

Intracellular Ligand Behavior

Behavior of cell-associated ligand is in striking contrast to that of the CHL, since the majority of intracellular GlcNAc-BSA accumulates with time in yolk platelet fractions and at the top of gradients, and much of the material in both of these locations is degraded. Newly internalized intact ligand is found in organelles of low density (1.07–1.15 g/cc), and chases out of this compartment during the next few hours. This differs markedly from the transport of VG given to oocytes at physiological concentrations, which rapidly appears in platelets at 1.21 g/cc after endocytosis by oocytes, with little accumulation in lighter organelles (Wall and Meleka, 1985).

The apparently inefficient internalization of GlcNAc-BSA seen here (approximately 2 times the number of cell surface receptors/h) is similar to the rate of endocytosis of ligands bound to LDL receptors expressed in *Xenopus* oocytes, where 7–10-fold more LDL was internalized during an 8-h period than was bound at the cell surface at 0°C (Peacock et al., 1988). GlcNAc-BSA endocytosis was 3 times slower than the rate of anti-Fc receptor Fab internalization in oocytes expressing mouse Fc receptors (Wall et al., 1988). Cell-associated GlcNAc-BSA rose linearly for at least 5.5 h in our experiments, suggesting that internalization rates were not slowing with time. Further work will be required to determine what factors limit the rate of ligand endocytosis in this system.

The presence of vitellogenin in the medium shifted GlcNAc-BSA from low-density organelles to yolk platelet compartments (particularly light yolk platelets). In this regard, the intracellular behavior of GlcNAc-BSA resembles that of vitellogenin which enters oocytes at low extracellular concentrations (Wall and Patel, 1987), suggesting that these two molecules share at least part of the VG endocytic pathway. Appearance of much of the internalized GlcNAc-BSA in yolk platelets further supports this idea. However,

some differences exist between the behavior of these two ligands: 1) VG is undegraded (although it does undergo limited proteolytic processing to mature yolk proteins: Wallace et al., 1983), while a substantial portion of cell-associated GlcNAc-BSA is acid-soluble, and 2) much of the GlcNAc-BSA enters the platelet compartment without requiring high concentrations of VG, while VG entry into these storage organelles is concentration-dependent (Wall and Patel, 1987).

Lack of VG degradation likely results from the unusual structure of this protein, which contains both highly phosphorylated domains and hydrophobic domains associated with a considerable amount of lipid (Ohlendorf et al., 1977; Wiley and Wallace, 1981). Oocyte lysosomes are also unusual in their lack of acid phosphatase and other hydrolytic enzymes associated with this organelle in most cell types (Wall and Meleka, 1985). Unglycosylated BSA labeled by the chloramine T procedure (see below) is degraded in *Xenopus* oocytes in a biphasic manner, with approximately 30% hydrolyzed rapidly (t = 5.5 h), and the remainder slowly (t = 90 h: Opresko et al., 1980a). Thus, VG and GlcNAc-BSA could traverse the same intracellular compartments with one ligand undergoing degradation and the other not. The rapid phase of the BSA degradation pathway may occur in MVBs, an endocytic compartment of low density in which proteolytic processing of VG appears to occur (Wall and Patel, 1987). The site of the slow phase may be light yolk platelets, since both undegraded and degraded GlcNAc-BSA are found at this site. However, the persistence of undegraded BSA in fractions of very low density, and our inability to be certain we have eliminated all cell-surface or non-specifically bound ligand with the EGTA rinse before fractionation (see Results) complicates this picture. EM localization of GlcNAc-BSA-colloidal gold complexes did not clarify this issue, since these complexes gave a much higher level of non-specific binding to control oocytes than GlcNAc-BSA (data not shown).

Failure of VG to appear in yolk platelets after it enters oocytes at low extracellular concentration may result from a need for condensation and/or crystallization of this protein before fusion of MVBs containing VG with light yolk platelets or for their conversion to light platelets (Wall and Patel, 1987). These intracellular fusion events might be triggered by ligand-receptor dissociation (Opresko and Karpf, 1987), or by other factors associated with the condensation process (e.g., condensation of the ligand may be necessary for segregation of lysosomal enzymes and VG into different compartments during yolk platelet formation). Our data on the intracellular behavior of GlcNAc-BSA are consistent with either model: In this case ligand-receptor dissociation could be accomplished by low endosomal pH (which does *not* cause VG dissociation from its receptor; Opresko and Wiley, 1987), and the GlcNAc-BSA pathway involves no condensation process.

Comparison With FcR Behavior

The mouse macrophage Fc receptor is a molecule which normally recycles to the surface together with its ligand when a monovalent molecule is bound (Mellman et al., 1984). Expression of this molecule in oocytes results in the synthesis of a molecule which is found at the same low-density as the CHL, as would be expected for a molecule which recycles from an early endosomal compartment (Wall et al., 1988). However, the monovalent ligand anti-receptor Fab remains in organelles of the same low density after endocytosis by oocytes expressing Fc receptors, in contrast to the behavior of GlcNAc-BSA shown here. In addition, Fab associated with oocytes shows no evidence of degradation as determined by TCA-solubility. These results suggest that the intracellular fate of each ligand in oocytes is consistent with its normal behavior: that is, Fab is recycled and GlcNAc-BSA is at least partly transported to lysosomes.

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