Selective Yolk Deposition and Mannose Phosphorylation of Lysosomal Glycosidases in Zebrafish*

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The regulation and function of lysosomal hydrolases during yolk consumption and embryogenesis in zebrafish are poorly understood. In an effort to better define the lysosomal biochemistry of this organism, we analyzed the developmental expression, biochemical properties, and function of several glycosidases in zebrafish eggs, embryos, and adult tissues. Our results demonstrated that the specific activity of most enzymes increases during embryogenesis, likely reflecting a greater need for turnover within the embryo as yolk-derived nutrients are depleted. Analysis of glycosidase activity in zebrafish and medaka eggs revealed selective deposition of enzymes required for the degradation of N-linked glycans, including an abundance of acidic mannosidases. Treatment of zebrafish embryos with the \( \alpha \)-mannosidase inhibitor swainsonine resulted in the accumulation of glycosylated vitellogenin fragments and demonstrated a function for maternally deposited acid \( \alpha \)-mannosidase in yolk consumption. Surprisingly, we also found that, unlike mammals, acid \( \alpha \)-glucosidase from zebrafish and medaka does not appear to be modified with mannose 6-phosphate residues. We further showed these residues were not acquired on human acid \( \alpha \)-glucosidase when expressed in zebrafish embryos, suggesting unique differences in the ability of the human and zebrafish \( N \)-acetylglucosamine-1-phosphotransferase to recognize and modify certain lysosomal glycosidases. Together, these results provide novel insight into the role of acidic glycosidases during yolk utilization and the evolution of the mannose 6-phosphate targeting system in vertebrates.

Lysosomal catabolism and the subsequent recycling and utilization of metabolites are critical for many biological processes, as highlighted by the serious consequences to human and animal health when these functions are disrupted (1, 2). Degradation of macromolecules by lysosomal hydrolases is also essential for the utilization of the yolk during embryogenesis in fish, insects, and amphibians. Freshwater fish embryos such as zebrafish and medaka rely on the contents of their yolk cell as the primary source of amino acid, carbohydrate, and lipid building blocks throughout their early development. The cytoplasm of this yolk cell contains glycogen and protein granules, the latter of which is composed mainly of the yolk glycoprophoprotein, vitellogenin (Vtg).

This large multidomain protein is synthesized by the maternal liver, secreted into the bloodstream, internalized by the growing oocyte via receptor-mediated endocytosis, and then stored in specialized organelles, the yolk granules (yolk platelets) (3–5). In addition to vitellogenin, several other complex sialoglycoproteins and glycolipids have been characterized in the eggs of aquatic species, although their biological roles are not yet fully understood (6, 7).

Yolk granules and lysosomes share many common features; their intraluminal pH is tightly regulated, and they both appear to house all the hydrolytic enzymes necessary for the complete breakdown of their contents (8). In contrast to the constitutive activity of lysosomes, temporal regulation of yolk consumption is necessary to supply nutrients in a measured fashion to the developing tissues of the embryo and to ensure survival until it is capable of independent feeding. Several mechanisms of controlling yolk utilization have been described, including the regulation of pH within the yolk granules, fusion of granules with lysosomes, and enzymatic latency of the hydrolytic enzymes (8–10).

Although the identity and function of certain yolk-deposited proteases have been described (11–15), a characterization of the glycans-degrading capacity in the eggs of many species, including zebrafish, is lacking. Two endoglycosidases capable of removing N-linked oligosaccharides at distinct developmental stages have been identified in medaka (16, 17), and the presence of acid glycosidases within the yolk has been reported in *Xenopus laevis* eggs (18, 19). In both cases, however, the contribution of these glycosidases in the utilization of yolk granules or in the regulation of specific processes during oogenesis and/or embryogenesis has not been determined (18, 19). The catabolism of the glycans present on vitellogenin and other yolk-deposited molecules may serve as a source of carbohydrate for the developing embryo. Thus, understanding the developmental expression and biochemical properties of lysosomal glycosidases in the zebrafish yolk as well as the embryo represents an essential consideration for the development and subsequent interpretation of metabolic disease models within this organism, including emerging models of glycosylation-related disorders (20, 21).

To elucidate the developmental expression and physiological significance of glycosidases, we investigated the deposition,
post-translational modification, and function of these enzymes in the eggs and embryos of two common fish species, Danio rerio (zebrafish) and Oryzias latipes (Japanese medaka). Our results revealed that certain glycosidases are selectively deposited within the zebrafish and medaka yolk. In addition, we described a role for one hydrolase, α-mannosidase, in the end degradation and glycan trimming of glycosylated vitelligenin fragments. Furthermore, we uncovered a surprising lack of mannose phosphorylation on acid α-glucosidase in zebrafish and medaka. Together, these data provide new insight into the biological role of zebrafish glycosidases during yolk consumption and the evolution of the mannose 6-phosphate targeting pathway in vertebrates.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Swainsonine was purchased from Tocris Bioscience (Bristol, UK); the anti-vitellogenin monoclonal antibody (clone JE-10D4) was from Abcam (Cambridge, MA), and the X-gal substrate was obtained from Sigma. The HPC4 affinity matrix was from Roche Applied Science. The 4-methylumphanoside, and 4-MU α-glucosidase were also from Sigma, with the exception of the 4-MU α-iduronylpyranoside, 4-MU α-mannopyranoside, and 4-MU β-galactopyranoside that were purchased from Toronto Research Chemicals (Toronto, Canada). Biotinylated ConA was from Vector Laboratories (Burlingame, CA).

**Fish Strains, Embryo, and Yolk Lysate Preparation**—Wild type zebrafish were from Fish 2U (Gibsonton, FL), and wild type medaka (CAB strain) were obtained from the University of Georgia Aquatic Biotechnology and Environmental Laboratory. Both were maintained using standard protocols. For analysis of embryonic glycosidases, zebrafish embryos were dechondriated, if necessary, anesthetized with Tricaine, and deyolked by multiple passages through a glass Pasteur pipette. Lysates were prepared in 50 mM sodium citrate buffer, pH 5.0, with 1% Triton X-100 by brief sonication on ice and subjected to centrifugation (3500 × g, 10 min). For analysis of egg and yolk glycosidases, individual yolks were directly aspirated into a pipette tip and solubilized in sodium citrate buffer, pH 5.0, with 0.5% Triton X-100. Fertilized and unfertilized eggs were collected, disrupted by brief sonication in citrate buffer, and centrifuged to remove broken chorions. Protein concentration was determined using a micro BCA protein assay kit (Pierce), and aliquots were normalized for equivalent protein concentration.

**Enzyme Activity Assays**—Activity of lysosomal glycosidases was measured using the following fluorescent substrates: 4-MU β-galactopyranoside (for β-galactosidase), 4-MU α-galactopyranoside (for α-galactosidase), 4-MU β-glucuronidase (for β-glucuronidase), 4-MU β-N-acetylgalcosaminidase (for β-hexosaminidase), 4-MU α-mannopyranoside (for α-mannosidase), 4-MU β-mannopyranoside (for β-mannosidase), 4-MU α-idurylpyranoside (for α-iduronidase), 4-MU α-glucopyranoside (for acid and neutral α-glucosidase), and 4-MU β-glucopyranoside (for acid β-glucosidase). Tartrate-resistant acid phosphatase activity was measured in the presence of 100 mM sodium tartrate using 4-MU phosphate as a substrate. All substrates were prepared in 50 mM sodium citrate or 100 mM sodium acetate reaction buffers, pH 4.5, with 0.5% Triton X-100 to a final concentration of 3 mM except for 4-MU α-iduronylpyranoside, which was prepared in formate buffer, pH 3.5, to a final concentration of 0.5 mM. Reactions were incubated for 3–16 h at 37 °C and quenched with 0.2 M sodium carbonate buffer, and the fluorescence of 4-methylumbelliferylone was measured using a Turner 380 fluorometer. Specific activity was expressed as nanomoles of substrate cleaved (proportional to the fluorescence units read) per h per mg of total protein.

**Mannose 6-Phosphate Receptor and ConA Lectin Affinity Chromatography**—Lysates were fractionated using either a Man-6-P receptor affinity column or as described previously (21, 22) or a ConA lectin affinity column. The ConA lectin column was eluted sequentially with 10 mM α-methylglucoside and 100 mM α-methylmannoside in column buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂). Activity of lysosomal enzymes was measured in each column fraction using appropriate fluorescent substrate activity. Reactions were performed in 50 mM citrate buffer, pH 4.5, 0.5% Triton X-100 containing 3 mM of the respective substrates. The percentage of bound activity relative to total activity recovered from three independent experiments is shown.

**Determination of in Situ β-Galactosidase Activity**—For activity analysis in situ, zebrafish embryos were fixed in 4% paraformaldehyde for 2 h at room temperature or at 4 °C overnight, washed (three times for 10 min with PBS, pH 7.4), and then washed again (three times for 15 min) with PBS, pH 6.0. Embryos were stained with 1 mg/ml X-gal in PBS, pH 6.0, containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂ at 37 °C overnight. The embryos were photographed on an Olympus SZ-16 dissecting microscope fitted with a CCD camera.

**Vitellogenin Analysis and Lectin Blotting**—Yolk lysates were resolved by 10 or 15% SDS-PAGE, transferred to nitrocellulose, and either probed with a mouse monoclonal antibody (JE-10D4) against zebrafish vitellogenin (1:1000 dilution) or stained directly with Coomassie Blue as vitellogenin isoforms typically represent ~90% of the total protein in the yolk (19). In some experiments, lysates were treated with 10 units of Endo H in 50 mM sodium citrate buffer overnight at 37 °C prior to electrophoresis. ConA lectin blotting was performed by incubation of transferred proteins with 10 μg/ml biotinylated ConA lectin in 3% BSA for 1 h followed by extensive washing with PBS-T. Blots were then incubated with streptavidin-linked HRP, and bands were detected with standard ECL reagents.

**Expression and Analysis of HPC4-tagged Human Acid α-Glucosidase**—The plasmid carrying full-length human acid α-glucosidase tagged with HPC4 epitope at the N terminus was linearized with NotI, and in vitro transcription was performed with T7 promoter by using mRNA Machine kit (GE Healthcare). 200 pg of RNA was injected into zebrafish embryos at the one-cell stage. Deyolked embryos were collected at 30 h after injection and subjected to analysis using the cation-independent mannose 6-phosphate (CI-MPR) affinity column. For HPC4 immunoprecipitation experiments, the manufacturer’s instructions (Roche Applied Science) were followed. A total of 80 RNA-injected embryos at 30 h post-fertilization were deyolked, lysed in the 500 μl of lysis buffer by brief sonication, and cleared by centrifugation. The supernatant was incubated with

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50 μl of anti-protein C affinity matrix at 4 °C with slow rotation for 3 h. The matrix was rinsed three times and eluted with 200 μl of elution buffer without calcium. Aliquots of supernatant and eluted protein were assayed directly for acid-glucosidase and -galactosidase activity or subjected to Western blot analysis to assess efficiency of immunoprecipitation. The anti-human α-glucosidase monoclonal antibody was used at a 1:2500 dilution and the anti-HPC4 antibody at a 1:500 dilution. The blocking process was performed in the presence of 1 mM CaCl₂.

Inhibition of α-Mannosidase by Swainsonine—For inhibitor studies in living zebrafish embryos, 5 mM swainsonine was injected into the chorion of fresh laid eggs to increase inhibitor uptake, and the embryos were subsequently incubated in fish medium containing 20 μM swainsonine for 30 h. Embryos were then extensively rinsed in fish medium to remove any residual inhibitor prior to yolk collection, lysate preparation, and α-mannosidase activity assays. We achieved roughly 70% inhibition of α-mannosidase in activity assays when swainsonine was injected into the chorion.

RESULTS

Specific Activity and Expression of Lysosomal Glycosidases Increase during Embryonic Development—Fluorescence-based assays were used to determine the activity of several lysosomal glycosidases during the first 7 days of development. We chose lysosomal glycosidases that represent various types of macromolecule degradation as follows: glycoprotein (α-mannosidase, β-galactosidase, and β-hexosaminidase), glycosaminoglycan (α-iduronidase and β-glucuronidase), and glycogen (acid α-glucosidase). β-Galactosidase and β-hexosaminidase also play a role in the degradation of specific types of glycosaminoglycans and glycolipids. Zebrafish embryos were collected and deyolked to remove contamination by maternally deposited hydrolases, and acidic activity was measured in protein-normalized embryo lysates. The results of this analysis are shown in Fig. 1. The activity of most of the glycosidases rose steadily throughout the first 5 days of development. The increase in α-mannosidase activity was somewhat slower than β-galactosidase and β-hexosaminidase; however, at 7 days post-fertilization (dpf), the relative percent increase of activity was comparable. Relative to the other enzymes, low activity of both α-iduronidase and β-glucuronidase was observed at early developmental time points, but this activity rose substantially from 1 to 4 dpf before reaching a plateau at 5 dpf. The activity for glycogen-degrading acid α-glucosidase remained fairly constant during the 1st week of development, possibly reflecting the necessity for glycogen utilization in the muscle of the embryo throughout its early development.

Maternally Deposited β-Galactosidase Exhibits Granule-specific Activation in the Zebrafish Yolk—The expression of one of these lysosomal glycosidases, β-galactosidase (zGLB1), was investigated by in situ hybridization, RT-PCR, and colorimetric activity analyses. RT-PCR analysis revealed a constant level of zGLB1 transcripts throughout the first 5 days of development, and our in situ hybridization experiments showed that the majority of zGLB1 expression was present in the brain (data not shown). Staining for β-galactosidase activity with X-gal was undetectable in the embryo at the 4-cell stage but was evident by 3 dpf, particularly in the head region (Fig. 2, top panels). Conversely, substantial X-gal staining was visible within the yolk in 4-cell stage embryos, indicating that β-galactosidase is

![FIGURE 1](Image1.png)

![FIGURE 2](Image2.png)
deposited in an active form in the developing yolk cell. We next analyzed dissociated yolk cells from 4-cell stage and 3 dpf embryos by light microscopy following X-gal staining. As noted in Fig. 2, activation of β-galactosidase activity at early stages appears to be a granule-specific phenomenon, with some granules exhibiting strong X-gal staining, although others remain clear and crystalline in appearance. By 3 dpf, however, most of the yolk granules are X-gal-positive, indicating progressive activation.

Lysosomal Mannosidases Are Abundantly Deposited in Zebrafish and Medaka Yolks —In an effort to determine the specificity of enzyme deposition, we measured the activity of several glycosidases in zebrafish- and medaka-fertilized eggs. To minimize any contribution of enzymes from the developing zygotes, activity was measured in yolk lysates prepared from eggs not more than 1 h post-fertilization. These results shown in Fig. 3 show selective deposition of glycosidases in the yolk of both organisms, with α-mannosidase and β-mannosidase activity representing the highest specific activity in zebrafish and medaka yolk, respectively. The specific activity of some egg-deposited enzymes differed substantially when compared with the specific activity within early embryos. For example, only minimal β-hexosaminidase activity could be detected within zebrafish eggs, but this activity was among the highest measured in the embryo. The higher overall specific activity of most glycosidases in zebrafish compared with medaka appears to be a function of greater enzyme deposition (as opposed to lower total protein content) because equivalent concentrations of protein per egg were measured. The activity of β-galactosidase was readily detected in the yolk of both fish species and was present at higher levels than the glycosaminoglycan-degrading enzymes, α-iduronidase and β-glucuronidase. Although very little acid β-glucosidase activity could be measured in either species, acid α-glucosidase activity was also abundantly present in zebrafish and medaka eggs, a finding consistent with the presence of glycogen granules in the yolk. Collectively, these data demonstrate that lysosomal mannosidases and other glycosidases necessary for N-glycan catabolism are abundantly deposited into zebrafish yolks and are generally present at much higher specific activities when compared with medaka yolks.

Acidic α-Mannosidase Activity Represents the Primary α-Mannosidase Activity in Zebrafish Yolk —In addition to lysosomal α-mannosidase, which functions optimally at low pH, most mammalian cells also contain neutral mannosidases such as Golgi α-mannosidase and cytosolic α-mannosidase that are involved in N-glycan processing and the disposal of free N-glycans, respectively. In light of the fact that we detected such high levels of overall α-mannosidase activity in zebrafish eggs, we assayed egg and brain lysates across a pH range to estimate the potential contribution of neutral α-mannosidases. As shown in Fig. 4, only acidic α-mannosidase activity could be detected in zebrafish eggs, suggesting that neutral α-mannosidases are not present in the yolk cell. The pH profile of α-mannosidase activity in zebrafish brain demonstrated peaks at both acidic and neutral pH that likely indicates the presence of lysosomal α-mannosidase as well as cytosolic/Golgi α-mannosidases. We also analyzed α-glucosidase activities and found that both zebrafish and medaka contain both acid and neutral α-glucosidase activity within the yolk and brain (data not shown). Together, these findings demonstrate that acidic α-mannosidase represents the majority of α-mannosidase activity in zebrafish eggs.

Variable Mannose Phosphorylation of Lysosomal Glycosidases Is Observed in Zebrafish Brain and Yolk —Although most mammalian lysosomal glycosidases are mannose-phosphorylated, the extent to which zebrafish glycosidases are Man-6-P-
modiﬁed has not been fully characterized. To address this question, we fractionated zebrafish egg and adult brain lysates using a CI-MPR afﬁnity chromatography column and analyzed the relative activity of several glycosidases in unbound and Man-6-P-eluted fractions. The results of this analysis are shown in Fig. 5A. Consistent with our earlier experiments on whole zebrafish embryos, 15–20% of the acidic β-galactosidase, α-galactosidase, and α-mannosidase activity in brain lysates was shown to bind to the CI-MPR afﬁnity column (21). The percent of Man-6-P-modiﬁed activity, however, was considerably higher in the yolk lysates compared with the brain, with the majority of α-mannosidase (57%), β-galactosidase (54%), and α-galactosidase (78%) activity bound to the column. Surprisingly, all of the acid α-glucosidase activity from both sources was found exclusively in the unbound fractions. When fractionated using a ConA afﬁnity column, nearly 55% of the acid α-glucosidase activity was found in either the ﬂow-through fractions or those eluted with 10 mM α-methylglucoside. This indicates that the majority of enzyme is modiﬁed with only highly branched complex-type N-glycans, an indication that Man-6-P residues were never added to these glycans. The lack of mannose phosphorylation on acid α-glucosidase was also noted in medaka brains and yolk, demonstrating that this phenomenon is observed in other ﬁsh species (Table 1). By comparison, 24% of the acid α-glucosidase activity from ﬁsh brain, 51% of mouse brain activity, and 31% of the recombinant enzyme (MyozymeTM) were found to be mannose-phosphorylated. In an effort to rule out the possibility that a low level of mannose phosphorylation on acid α-glucosidase was removed upon fertilization, we also analyzed unfertilized egg lysates on the CI-MPR afﬁnity column, but again we failed to detect any measurable level of bound activity (Table 1).

The mannose phosphorylation of lysosomal hydrolases depends on their ability to bind to GlcNAc-1-phosphotransferase, the enzyme that initiates Man-6-P biosynthesis. To determine whether the lack of Man-6-P residues on acid α-glucosidase reﬂected differences between the mammalian and zebrafish protein, we next asked whether human acid α-glucosidase, a protein that has been demonstrated to be mannose-phosphorylated, was capable of being modiﬁed by zebrafish phosphotransferase. Human acid α-glucosidase tagged with HPC4 was injected into one-cell stage embryos, and embryos were collected at 2 dpf and analyzed for expression by activity assays and Western blots. We achieved 30–50% increase over the endogenous level of acid α-glucosidase activity in these embryos. Expression of the human enzyme was conﬁrmed by immunoprecipitation and detection using a monoclonal antibody to the HPC4 epitope. Consistent with our earlier experiments on whole zebrafish embryos, 15–20% of the acidic enzyme was modiﬁed with Man-6-P residues in zebrafish, conﬁrming the speciﬁcity of this method. To gauge whether the human enzyme was modiﬁed with Man-6-P residues in zebrafish, control and injected embryo lysates were fractionated on an anti-HPC4 afﬁnity column followed by analysis of unbound and bound aliquots using the mouse monoclonal antibody or anti-HPC4 antisera.

We detected only the 110-kDa form of human acid α-glucosidase, because further processing of this enzyme results in loss of the N-terminal HPC4 tag. Although acid α-glucosidase activity was readily measured in HPC4-bound fractions, no β-galactosidase activity could be immunoprecipitated demonstrating the speciﬁcity of this method. To determine whether the human enzyme is unable to become Man-6-P-modiﬁed by zebrafish phosphotransferase (Table 1).

Inhibition of α-Mannosidase Activity with Swainsonine Resulted in the Accumulation of Glycosylated Vtg Fragments—

The major protein deposited in the zebrafish yolk is vitelloge- nin, a large multidomain glycoprotein that is synthesized in the maternal liver, taken up into the ovaries from the serum, and packaged into yolk granules during oogenesis. The organization of zebrafish Vtg domains and the position of potential N-glycosylation sites are depicted in Fig. 6A. The full-length Vtg is proteolytically processed during oogenesis and embryonic development into smaller fragments that can be detected using either Coomassie staining or Western blot analysis with an anti-Vtg monoclonal antibody (Fig. 6B). Although this antibody can detect 160-kDa full-length Vtg, it does not appear to recognize the 110-kDa lipovitellin heavy chain subdomain, leading us to believe that the epitope lies within the C-terminal portion of the protein. Based on the predicted molecular

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**TABLE 1**

Mannose phosphorylation of acid α-glucosidase in fish and mammalian tissues

| Enzyme source | % Man-6-P-modified activity |
|---------------|-----------------------------|
|               | α-Glu | β-Gal |
| Zebrafish egg (fertilized) | <0.1 | 54 ± 3 |
| Zebrafish egg (unfertilized) | <0.1 | 48 ± 7 |
| Zebrafish brain | <0.1 | 16 ± 5 |
| Medaka egg | <0.1 | 60 ± 6 |
| Medaka brain | <0.1 | 21 ± 4 |
| Feline brain | 24 ± 7 | 63 ± 7 |
| Mouse brain | 51 | 77 |
| MyozymeTM | 31 | NA |
| Human acid α-glucosidase expressed in zebrafish | <0.1 | NA |

**FIGURE 5.** Mannose phosphorylation of glycosidases in zebrafish brain and yolk. A, zebrafish egg and adult brain lysates were fractionated on a Cl-MPR afﬁnity column, and activity of several glycosidases was measured in unbound and Man-6-P-eluted column fractions. The average of three independent experiments is shown with standard deviations. The glycosidase abbreviations are as follows: α-Gal, α-galactosidase; β-Gal, β-galactosidase; α-Glu, acid α-glucosidase; α-Man, acid α-mannosidase. n.d. means not detectable. **B**, HPC4-tagged human acid α-glucosidase was expressed in zebrafish embryos. Whole embryo lysates were resolved by SDS-PAGE and probed using a polyclonal antibody raised against human acid α-glucosidase (left panel). HPC4 bound and unbound fractions from injected (inj) embryo lysates were subjected to Western blot analysis using either the human acid α-glucosidase antibody (center panel) or a monoclonal antibody to the HPC4 epitope (right panel). ctr, control.
masses of known zebrafish Vtg domains and its apparent specificity for C-terminal fragments, the subdomains detected by Western blot with the anti-Vtg antibody (see Fig. 5E) most likely represent the light chain vitellin or lipovitellin light chain (≈30 kDa) and von Willebrand factor-like subdomains, β’ and CT (17 and 13 kDa, respectively) (23). Sequence analysis of the various Vtg isoforms in zebrafish demonstrated the presence of several potential N-linked glycan sites within these C-terminal Vtg domains. Glycomic analyses of vitellogenin in other species have indicated that Vtg precursors typically bear high mannose N-glycans (24, 25). To confirm the presence of high mannose N-glycans on zebrafish Vtg, we performed Coomassie stains and ConA lectin blots on control and Endo H-treated yolk lysates. As shown in Fig. 6C, the 13-kDa band shifted lower upon Endo H treatment, and mobility shifts in additional higher molecular weight bands could also be detected. Consistent with this finding, some of these low molecular weight bands were also reactive to the ConA lectin in control lysates (Fig. 6D). Upon Endo H treatment, the 50- and 13-kDa bands disappeared, suggesting that these Vtg fragments were modified by high mannose glycans. Thus, the glycosylated fragments we detected most likely correspond to the C-terminal domains of zebrafish Vtg.

Since the breakdown of high mannose N-glycans in the lysosomal compartment requires the action of acidic α-mannosidase, we postulated that the abundant deposition of this glycosidase in the zebrafish yolk serves to facilitate the utilization of Vtg during embryogenesis. To investigate this possibility, we treated embryos with the α-mannosidase inhibitor swainsonine and monitored the effects on the processing of zebrafish Vtg by Western blot analysis of yolk lysates. We first showed that swainsonine was able to potently inhibit zebrafish α-mannosidase activity (≈98% inhibition at 2 μM). Because in situ inhibition of α-mannosidase activity could not be performed due to the lack of an appropriate colorimetric α-mannosidase substrate, swainsonine uptake and in vivo inhibition of α-mannosidase activity (up to 70%) was indirectly assessed in embryos incubated with this inhibitor (see under “Experimental Procedures”). Swainsonine treatment was not toxic to the embryos nor did it appear to alter early development, even after exposure for up to 7 days at high concentrations (100 μM). Accumulation of the 17-kDa Vtg fragment in the swainsonine-treated yolks and greater heterogeneity of the glycosylated 13-kDa band could be detected by Western blot in 2 dpf yolk samples (Fig. 6E, see asterisks). Furthermore, higher levels of the 13-kDa band could easily be detected at 3 dpf. The relative amount of larger Vtg fragments in the swainsonine-treated samples was not altered, suggesting that α-mannosidase contributes to glycan trimming and end degradation of Vtg fragments but is not required for the initial steps in the digestion of this protein.

DISCUSSION

In this study, we undertook a characterization of the activity and biochemical properties of multiple glycosidases in zebrafish embryos and eggs and explored the function of these enzymes during embryogenesis. Our results demonstrated selective yolk deposition and mannose phosphorylation of these enzymes and suggest a role for acid α-mannosidase in the utilization of yolk glycoproteins. With regard to the developing embryo, we generally observed increasing activity of all the glycosidases tested throughout the first 5 days, at which time most of these activities either reached a plateau or began to decrease. The increased expression and activity of glycosidases within the embryo may reflect the need for greater turnover of glycosylated molecules in the embryo itself as the availability of nutrients from the yolk becomes depleted. Alternatively, up-regulation of glycosidase activity in the embryo could also be due to the differentiation or proliferation of specific cell types (such as chondrocytes) that express high levels of these enzymes.

Selective deposition of glycosidases within the yolk appears to be a common feature of both medaka and zebrafish, although there are interesting differences between these species, with regard to the specific activity of these enzymes within the yolk. Although both species deposit high levels of mannosidases in the yolk, the ratio of β-mannosidase and α-mannosidase is variable, and the overall specific activity of these and other glycosidases was considerably higher in zebrafish when compared with medaka. Furthermore, the specific activity of the glycosaminoglycan-degrading enzymes, β-glucuronidase and α-iduronidase, as well as the glycolipid-degrading acid β-glucosidase, was significantly lower compared with β-galactosidase and α-mannosidase. These observations may reflect variations in the composition of yolk glycoproteins or the general structure of glycans in these systems. The low specific activity of the glycosaminoglycan-degrading enzymes, β-glucuronidase and α-iduronidase, is somewhat surprising because substantial sulfatase activity in the zebrafish yolk has been reported previously (20). The molecular basis for glycosidase deposition is not well understood, but it does not appear to be mediated exclusively by the mannose 6-phosphate targeting system because certain enzymes that lack these residues, such as acid α-gluco-

FIGURE 6. Inhibition of α-mannosidase activity with swainsonine results in accumulation of low molecular weight glycosylated Vtg fragments. A, domain structure of zebrafish Vtg. Potential N-glycan sites within the C-terminal portion of the isoform VTGAo1 are marked. The location of these sites varies slightly in different Vtg isoforms. B, yolk lysates from 6 h post-fertilization zebrafish eggs were separated by SDS-PAGE using a 10% gel and analyzed by Coomassie Blue (CB) staining or Western blot (WB) with a monoclonal antibody raised against zebrafish vitellogenin. Note the unique profile of protein bands that can be detected with these two methods. C and D, yolk lysates from 2 dpf embryos were incubated for 4 h in the presence or absence of Endo H, separated on a 15% SDS-polyacrylamide gel to resolve the lower molecular weight bands, and stained with Coomassie Blue (C) or subjected to ConA lectin blot analysis as described under “Experimental Procedures.” D, yolk lysates from 2- and 3-dpf control and swainsonine-treated zebrafish embryos were separated using a 15% SDS-PAGE and subjected to Western blot analysis with a monoclonal antibody against vitellogenin (E). The asterisks denote the accumulation of the 17-kDa Vtg band and the increased heterogeneity of the 13-kDa band.

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sidase, are abundantly present within the yolk. It is possible that these enzymes interact directly with the yolk components that they will eventually degrade once activation of the yolk granules occurs. Indeed, vitellogenin contains multiple domains that are known to facilitate the binding of lipids and other molecules, including proteases (23). The basis for greater specific activity of the glycosidases in zebrafish, in particular the strikingly high deposition of acid α-mannosidase, is also not clear, but it does not depend on lower overall protein concentration in the zebrafish eggs because both species contain roughly equal protein content per fertilized egg. Medaka embryos develop slower when compared with zebrafish, whose development and utilization of the yolk by comparison are rapid. This necessity for rapid uptake of nutrients during zebrafish embryogenesis may therefore require a higher hydrolytic capacity compared with medaka. A more detailed characterization of the glycoprotein composition within the medaka and zebrafish yolk may highlight the basis for this selective glycosidase deposition.

Our findings strongly indicate a lack of mannose phosphorylation on acid α-glucosidase in all zebrafish and medaka tissues studied, including the yolk cell where levels of Man-6-P-modified glycosidases were found to be the highest. Because there was a fraction of acid α-glucosidase activity still capable of binding the mannose-specific lectin ConA, we cannot rule out the possibility that some zebrafish enzyme is modified to a low level by Man-6-P residues during biosynthesis and that these residues are subject to rapid removal within activated yolk granules. Although the zebrafish genome appears to contain all of the components of the Man-6-P targeting pathway (including subunits of GlcNAc-1-phosphotransferase and both mannos-6-phosphate receptors), these data suggest that acid α-glucosidase and possibly other glycosidases did not evolve to be high affinity substrates for GlcNAc-1-phosphotransferase in zebrafish. Interestingly, we were unable to achieve mannose phosphorylation of human acid α-glucosidase expressed in zebrafish embryos, suggesting that efficient mannose phosphorylation of acid α-glucosidase in higher vertebrates may have involved evolution of specific amino acids within the glycosidase as well as phosphotransferase. Comparison of the amino acid sequences of human and zebrafish acid α-glucosidase shows less sequence identity than other lysosomal glycosidases such as β-galactosidase and α-mannosidase, both of which exhibit high mannose phosphorylation levels. Although acid α-glucosidase lacks Man-6-P residues, its apparent optimal activity at low pH indicates that it functions within an acidic compartment (i.e., lysosomes). Thus, this enzyme likely relies on an ancestral method of transport to reach the lysosomal compartment in zebrafish and for its deposition into the yolk. Studies to determine the evolutionary node for the mannose phosphorylation of acid α-glucosidase in vertebrates are underway and should provide new insight into the development of the Man-6-P targeting pathway within the vertebrate lineage.

Our findings point to a likely role for acid α-mannosidase in the degradation of glycosylated Vtg fragments bearing high mannose-type N-glycans. We observed a clear difference in the profile of Vtg fragments upon swainsonine treatment despite the fact complete in vivo inhibition of acid α-mannosidase was not achieved. On a molecular level, the accumulation of the glycosylated fragments suggests that efficient removal of the glycans may be necessary for the final catabolism of these peptides. It is uncertain whether the sugars liberated from the catabolism of Vtg N-glycans are subsequently used to synthesize N-glycan precursors in the embryo or yolk cell. The fact that swainsonine treatment did not result in any noticeable development defects in zebrafish embryos might reflect the fact that incomplete inhibition of α-mannosidase was achieved or that glucose, not mannose, is the primary source of key glycosylation precursors in the yolk cell. Understanding the balance of monosaccharide utilization in the zebrafish yolk and embryo as well as the metabolic regulation of other precursor molecules such as lipids will no doubt be necessary to aid in the interpretation of disease models that are being developed in this versatile organism.

Although clearly localized to the yolk granule, the biological function of β-galactosidase with the yolk and its potential catabolic targets is not known. A wide range of carbohydrate-binding proteins, including galactose-specific lectins, are deposited in high concentrations in animal yolk cells, and robust staining with these lectins have been detected in a spatial and temporal manner during oogenesis and post-fertilization development (26–28). Thus, a requirement for removal of terminal galactose residues by β-galactosidase may serve an important role in the release of yolk membranes or the down-regulation of specific epitopes during these processes. Assessing the function of these abundant glycosidases and how they impact oogenesis and embryonic development represent an important consideration for future investigation.

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