**Xenopus Autosomal Recessive Hypercholesterolemia Protein Couples Lipoprotein Receptors with the AP-2 Complex in Oocytes and Embryos and Is Required for Vitellogenesis**

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ARH is required for normal endocytosis of the low density lipoprotein (LDL) receptor in liver and mutations within this gene cause autosomal recessive hypercholesterolemia in humans. xARH is a localized maternal RNA in Xenopus with an unknown function in oogenesis and embryogenesis. Like ARH, xARH contains a highly conserved phosphotyrosine binding domain and a clathrin box. To address the function of xARH, we examined its expression pattern in development and used pull-down experiments to assess interactions between xARH, lipoprotein receptors and proteins in embryo extracts. xARH was detected concentrated at the cell periphery as well as in the perinuclear region of oocytes and embryos. In pull-down experiments, the xARH phosphotyrosine binding domain interacted with the LDL and vitellogenin receptors found in Xenopus oocytes and embryos. Mutations within the receptor internalisation signal specifically abolished this interaction. The xARH C-terminal region pulled-down several proteins from embryo extracts including α- and β-adaptins, subunits of the AP-2 endocytic complex. Mutations in either of the two DxF/F motifs found in xARH abolished binding to α- and β-adaptins. Expression of a dominant negative mutant of xARH missing the clathrin box and one functional DxF/F motif severely inhibited endocytosis of vitellogenin in cultured oocytes. The data indicate that xARH acts as an adaptor protein linking LDL and vitellogenin receptors directly with the AP-2 complex. In oocytes, we propose that xARH mediates the uptake of lipoproteins from the blood for storage in endosomes and later use in the embryo. Our findings point to an evolutionarily conserved function for ARH in lipoprotein uptake.

In Xenopus, at least 8 months are required for stage I oocytes to develop into fully grown stage VI oocytes. One purpose of this lengthy process is to stockpile components required for early embryo development (1). Large amounts of macromolecules and nutrients in the form of serum-borne lipoproteins accumulate and the oocyte volume increases 10,000 times as a consequence. These lipoproteins are essential for the assembly of structures such as the plasma membrane in embryos. The major imported lipoproteins are very low density lipoproteins (VLDL) and vitellogenin (VTG). Vitellogenin is a lipophosphoglycoprotein produced under hormonal control in the liver and transported to the female gonads in the blood (2). Once internalized by receptor-mediated endocytosis, vitellogenin (~250 kDa) is cleaved into multiple polypeptides and then stored as microcrystals within membrane-bound structures called yolk platelets (3, 4). Yolk platelets are thought to be the oocyte equivalent of late endosomes or lysosomes but with low proteolytic activity (5). During embryogenesis, yolk platelets become progressively more acidic (pH < 5), allowing yolk proteins to be degraded and utilized as nutrients for early development (6). VTG is transported into oocytes through a specific receptor (VTGR) that is internalized in clathrin-coated pits (7). A mutant chicken strain carrying a single mutation in the VTGR gene cannot lay eggs (8). The oocytes of this mutant strain lose the ability to accumulate VTG and VLDL. In addition to VTG and VLDL, the VTG receptor also transports other molecules including riboflavin binding protein (9), α2-macroglobulin (10), and activin (11), suggesting that this receptor is important not only in nutrient uptake during oogenesis but also in the development of early embryos. For example, the mammalian VLDL receptor, closely related to the VTG receptor, is involved in brain development in mouse embryos (12).

To date, all molecularly characterized VTG receptors belong to the LDL receptor family (13–15). In mammals, the core members of this family include LDL receptor, LRP, VLDL receptor, ApoE receptor-2, LRPIb, megalin, and MEGF7. The cytoplasmic tails of these mammalian receptors contain one or more NPXY motifs, required for the endocytosis of the LDL receptor (16). However, this motif is not used universally within the LDLR family, as LRP requires an XXX motif for endocytosis (17).

Recently, genetic and biochemical studies have linked a gene to a disease in humans, autosomal recessive hypercholesterolemia (ARH) that clinically resembles familial hypercholesterolemia. The LDL receptors in ARH patients fail to efficiently clear LDL particles from the plasma, resulting in high blood cholesterol levels (18) despite increased levels of LDL receptors on the cell surface (19). These findings strongly suggest that hARH is required for LDL receptor endocytosis. ARH contains a phosphotyrosine binding (PTB) domain in the N-terminal

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region that is present in many adaptor proteins such as SHC, IRS-1, Dab-1, and Numb (20). PTB domains can interact with NPXY motifs as well as non-NPXY motifs (20, 21). hARH has been shown to be an adaptor protein coupling the LDLR and endocytic machinery (22, 23). However, the functions of ARH in other species or in other pathways have not been reported.

In a screen for localized RNAs in frog oocytes, we unexpectedly isolated the Xenopus homologue of ARH (24). We showed that maternal xARH, unlike hARH, is present as two transcripts, almost certainly the products of two genes, which differ in their 3′-untranslated regions. The longer transcript is found primarily in the vegetal cortex, whereas the shorter transcript is present almost exclusively in the oocyte animal half. Similar to hARH, xARH is found in the adult liver and spleen, but at low levels compared with oocytes. Other adaptor proteins mediating receptor endocytosis play important roles in development. A related PTB-containing protein, Drosophila Numb, links the Notch receptor with the endocytic machinery (25), which down-regulates the Notch signal (26) and thus specifies neuron cell fate (27). Additional examples of the endocytic regulation of signaling pathways in development are likely to be found (28).

Here we show that xARH can interact with the LDL and VTG receptors through the PTB domain. Affinity purification of proteins binding to the C-terminal region of xARH identified α-adaptin and β-adaptin, subunits of the adaptor protein complex 2 (AP-2) endocytic complex. DxF/FW motifs were required for the interaction between xARH and the AP-2 complex. Finally, we show that a dominant negative mutant of xARH containing a nonfunctional clathrin box and DxF/FW motif inhibits endocytosis of VTG in cultured oocytes. Our results strongly suggest that maternal xARH is required for vitelligenesis. Our data are consistent with xARH acting as an adaptor protein linking LDL receptor family members and the endocytic machinery in oocytes and embryos. Our findings point to an evolutionarily conserved function for ARH in nutrient uptake.
beads were washed three times with buffer H. The bound proteins were retrieved by boiling in SDS loading buffer and resolved by SDS-PAGE. Oocyte extracts routinely resulted in significantly higher background and more variable results and therefore were not used for these studies.

For mass spectrometry, the gels were stained with Coomassie Blue. The band of interest at 108 kDa was excised from the gels. A total of 200 ng of LC108 protein was obtained from two preparations and sent for mass spectrometry protein identification to the HHMI Biopolymer Laboratory and W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Site-directed Mutagenesis—Mutagenesis was carried out using the QuickChange method (Stratagene) according to the manufacturer’s protocol. Oligonucleotide primers were designed to mutate the D6(P/F) motif to an arginine/aspartate fusion protein to AAA (primers are shown 5’ to 3’: DLFA5, AATCATATGAGCATGTTGTGAGCCACCAAGGCCAAAAGGTTTGACAC; DLFC3, GTGTCAGAACATAGCTGCGGCATCTGCTTCTGC). DLFC5, GCAGAAGCAGATGCCGCAGCTATGTTCTGGAGCAAGGCTTGCC; DLFB3, GGCAAGCCTTGCTGCGGCAGCATCTGC). For the xARH-DM mutant, the clathrin box (LDDL) was mutated to LLAL (primers: LLALAF, GCAAAACCTTGTGTTGCGACGATCTACACAA; LLALAR, TTTTGTCAGCTGCAGCCAGGCGAAAAACTGGTTC- GC), and the D6(P/F) motif to258 was mutated to AAA (primers: DLFB5 and DLFB6). All mutant constructs were sequenced to confirm the presence of the designed mutations.

In Vitro Translation and GST Pull-down Assays—Oligonucleotides were synthesized to encode Xenopus LDLR-1 (nucleotides 2640–2804) and VTGR (nucleotides 2650–2811) cytoplasmic tails according to published sequence data (14, 33). A T7 promoter sequence (TAATACGACTCACTATAGGG) and Kozak sequence (GCCACCATGG) were added to the 5’-ends. The synthesized DNA fragments were translated using the Tnt Quick Transcription and Translation Coupled System from Promega.

5 μg of GST fusion proteins were dissolved in 500 μl of PBS + 0.1% Tween 20. 20 μl of glutathione beads (Amersham Biosciences) and 5 μl of an in vitro translation reaction (Tnt Quick System) containing LDLR or VTGR 35S-labeled cytoplasmic tails was added. After incubation at room temperature for 3 h, the beads were washed three times with 500 μl of PBS + 0.1% Tween 20. The bound proteins were released by boiling in SDS loading buffer and resolved on a 10% SDS-PAG. GST fusion proteins were visualized by Coomassie Blue staining. The candidate partners were visualized by exposing the dried gel to a PhosphorImager screen.

Purification and Labeling of Xenopus Vitellogenin—Vitellogenin purification was performed according to published procedures (4). Briefly, female frogs were injected with 17β-estradiol dissolved in propylene glycol (10 mg/ml) at a dose of 1.5 mg/100 g of frog on day 1 followed by a second injection on day 3. Plasma was collected on day 10 into 15-ml tubes containing 1 ml of 50% PBS and 70 mM sodium citrate. After centrifugation at 2500 × g for 15 min to remove the blood cells, the supernatant fraction was transferred to another tube. 10 ml of 20 mM EDTA, pH 7.7, for every 2.5 ml of plasma was added to the tube, and the contents were mixed gently. 0.5 ml of 0.5 M MgCl2 was added and mixed again. Vitellogenin was collected by centrifugation at 2500 × g for 15 min. The green pellet was dissolved in 1.5 ml of 1 M NaCl, 50 mM Tris, pH 7.5, and stored in aliquots at –20 °C (4).

To biotinylate vitellogenin, the protein was transferred into PBS by ultrafiltration, which was repeated three times using Microcon YM-30 filter devices (Millipore). 10 ng of vitellogenin was dissolved in 1 ml of PBS. 0.5 mg of Sulfo-NHS-LC-Biotin (Pierce) powder was added directly to the solution, mixed well, and incubated on ice for 8 h. The unconjugated biotin was removed using a desalting column (Amersham Biosciences) (molecular weight cutoff, 5,000). The biotinylated vitellogenin was concentrated with Microcon YM-30 and stored at –90% in 50% glycerol.

Vitellogenin Uptake Assay—Stage III/IV oocytes were obtained after collagenase treatment and incubated overnight in OCM (60% Liebovitz L-15 medium, 0.4 mg/ml bovine serum albumin, 1 mM glutamine, pH 7.6). Dominant negative mutant or control transcripts were injected into oocytes and the oocytes cultured overnight. Oocytes were then transferred into OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM Na2HPO4, 5 mM HEPEs, pH 7.5) containing 5 mg/ml bovine serum albumin and 0.25 mM biotinylated vitellogenin. After incubation at room temperature for varying periods of time, the oocytes were washed with OB2. Any labeled vitellogenin was removed from the surface by washing with 25 mM EDTA, 0.5 mM NaCl, 0.1 mM glycine-NaOH, pH 9.5, for 40 min (4). Oocyte samples were boiled in SDS loading buffer and analyzed by Western blot using Streptavidin-HRP (Pierce) at 1:50,000 dilution.

RESULTS

We had previously cloned two forms of xARH, xARHα and xARHβ, which appear to represent two genes that arose when the entire Xenopus genome duplicated an estimated 30 million years ago. Although xARHα RNA is the same length as hARH RNA, xARHβ has a much shorter 3’-untranslated regions (24). The two isoforms are distributed differentially along the animal/vegetal axis in fully grown oocytes, with xARHα concentrated predominantly within the vegetal cortex and xARHβ restricted to the animal hemisphere. The ARH proteins are 97% identical by sequence alignment, with amino acid changes found dispersed throughout the ORF (24). The xARHα clone was used in this study, and the results presented here are likely applicable to xARHβ as well, based on the virtual identity of their functional domains.

To begin to address the function of xARH in oocytes and embryos, we first examined its developmental expression pattern using a polyclonal antibody raised against the C-terminal region of xARH (Fig. 1, residues 217–309). This region includes sequences immediately after the conserved clathrin box and thus avoids any cross-reactivity with the conserved PTB domain found in other proteins. Within this 92-amino acid region, we detected two clones that represented the two isoforms (24). xARH was detected at all stages of oogenesis, and in most oocytes, it appeared more concentrated at the cell periphery (Fig. 2A). The signal could be eliminated almost completely if GST-C fusion protein was added to block the primary antibody (Fig. 2, control), confirming the specificity of the signal. Immunostaining for xARH in bisected late stage V oocytes shows the accumulation of xARH protein around the nucleus. In the egg and after nuclear (germinal vesicle) breakdown, xARH was detected distributed throughout the animal hemisphere (Fig. 2B). In embryos, xARH was present in all cells and at all stages examined, from early cleavage stage 5 to tail bud (Fig. 2C). As nuclei were formed in the early embryo, histological sections revealed the protein concentrated within the perinuclear and cortical region (Fig. 2D, brown signal distinguished from the black granular melanosomes). In human liver, ARH has also been localized near the plasma membrane in hepatocytes (34).

ARH homologues have been found in mosquito, ascidian, zebrafish, frog, mouse, and human (Fig. 1). In each, the PTB domain is highly conserved. xARH shows an overall 72% identity with hARH at the amino acid level, whereas the PTB domain is 91% identical (Fig. 1, shown underlined). PTB domains can recognize an NPXY motif present in the cytoplasmic region of certain receptors, which includes LDLR (35). The N-terminal 28 amino acids are also highly conserved (~90% identical) suggesting potential important functions for this region. A putative clathrin box (Fig. 1, boxed with broken line) is also conserved between Xenopus and human ARH. The C-terminal region of ARH is less well conserved (~50% identical) with the exception of short conserved stretches.

One function of xARH in oocytes and embryos may be in the uptake of nutrients through the LDLR. Indeed, reverse transcriptase-PCR analysis revealed that the LDL receptor is present both maternally as well as zygotically in later stage Xenopus embryos (Fig. 3A). To determine whether xARH interacts directly with LDLR, in vitro GST pull-down experiments were performed with purified components. The cytoplasmic tail of the LDL receptor (residues 859–909) was fused to GST, the fusion protein expressed in E. coli, and the protein was subsequently purified with glutathione-Sepharose beads. The N terminus of xARH, excluding the clathrin box and all downstream sequences (xARH-N, residues 1–212), was translated and la-
beled in vitro with \[^{35}\text{S}\]methionine. GST-LDLR pulled down xARH-N in this assay, whereas GST alone did not (Fig. 3B), indicating that the LDL receptor can interact with the xARH-N-terminal region containing the PTB domain.

The NPVY motif in the LDL receptor cytoplasmic tail is required for endocytosis of the receptor through clathrin-coated vesicles (16). To test whether the NPVY motif is required for xARH binding, a DNA fragment encoding the cytoplasmic tail of the \textit{Xenopus} LDL receptor was synthesized according to the published sequence (33). GST-xARH was expressed in \textit{E. coli}, purified with glutathione-Sepharose beads, and incubated with the labeled cytoplasmic tail of the LDL receptor. Full-length xARH was found to interact with the LDLR cytoplasmic region (Fig. 3C). A point mutation changing the required aromatic amino acid to alanine (Tyr to Ala) in the NPVY motif abolished the interaction (22). Therefore, the NPVY motif is required for the interaction between the LDL receptor and xARH (Fig. 3C).

However, a minor fraction of the input receptor tail was bound, suggesting the interaction between xARH and the LDL receptor may be weak and require an additional protein(s) for optimal binding in vivo.

xARH is expressed at high levels in vitellogenic oocytes. The VTG receptor is a member of the LDL receptor family and also has an NPVY motif in its cytoplasmic tail (14). The VTG receptor is expressed in \textit{Xenopus} oocytes and mediates the internalization of VTG as the precursor of yolk (14). The similarity between the VTG receptor and LDL receptor raised the possibility that maternal xARH may also interact with this receptor. The cytoplasmic tail of the \textit{Xenopus} VTG receptor (residues

| human | Xenopus alpha | zebrafish | ascidian | mosquito |
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| human | Xenopus alpha | zebrafish | ascidian | mosquito |
| human | Xenopus alpha | zebrafish | ascidian | mosquito |
| human | Xenopus alpha | zebrafish | ascidian | mosquito |

**Fig. 1.** Sequence alignment of ARH homologues from different species. Conserved residues are shaded using the Boxshade program. The PTB domain is underlined, the D/H/F/W motifs are boxed, and the putative clathrin box is boxed with broken lines. The phenylalanine residue required for the interaction between hARH and LDLR is marked with an arrow. The complete ascidian sequence is not available. The GenBank\textsuperscript{TM} accession numbers for the ARH homologues are as follows: zebrafish, BC045926; ascidian, AV384534; mosquito, AJ281467.

**Fig. 2.** xARH expression in oocytes and embryos. Affinity-purified mouse polyclonal anti-xARH-C antibody was used for whole-mount immunostaining of oocytes and embryos. No signal was detected in oocytes or embryos stained with antibody blocked with the purified antigen GST-C. A, in oocytes, ARH appears to be concentrated peripherally. B, in bisected stage V oocytes, ARH is perinuclear (arrow), and in bisected eggs after germinal vesicle breakdown, the signal is mostly in the animal hemisphere (arrow). N, nucleus. C, xARH protein is found broadly distributed in early stage embryos. D, in sectioned blastula stage embryos, ARH is found in the perinuclear (arrow) as well as the cortical region (arrow). Black pigment granules (open arrow) are distinct from the brown ARH-positive staining. Bar = 1 mm.
were visualized by exposure to a PhosphorImager screen. The GST-xARH protein was detected by Coomassie Blue staining. The GST fusion proteins and bound proteins were pulled down with glutathione-Sepharose beads and analyzed by SDS-PAGE. The GST fusion proteins were visualized by Coomassie Blue staining. The in vitro translated proteins were detected by exposure to a PhosphorImager screen. C, the cytoplasmic tails of the LDLR and VTGR were translated in vitro and labeled by 35S-Met incorporation. In mutants (mt), the NPVY motifs were changed to NPVA. Purified GST-xARH fusion protein from E. coli was mixed with the cytoplasmic tails and glutathione-Sepharose beads. The beads were spun down and boiled in SDS loading buffer (pellet). Both the supernatant (S) and pellet (P) fractions were resolved by SDS-PAGE. The cytoplasmic tails were visualized by exposure to a PhosphorImager screen. The GST-xARH protein was detected by Coomassie Blue staining.

The 108-kDa protein was selected for large scale affinity purification because it was relatively abundant and well separated from the other proteins. The purified protein was excised from polyacrylamide gels and subsequently analyzed by quadrupole time-of-flight (Q-TOF) mass spectrometry. All spectra were searched against the NCBI Non-redundant Protein Database. One spectrum matched a peptide sequence in α-adaptin (Fig. 4B). No other protein was identified from the analysis. α-Adaptin is a subunit of AP-2 that is involved in clathrin-mediated endocytosis (37). To confirm the identification by mass spectrometry, Western blot analysis was performed using a commercially available monoclonal anti-α-adaptin antibody. The antibody recognized a single protein of the expected mass (~100 kDa) in Xenopus embryo extracts (Fig. 5) and was taken to be Xenopus α-adaptin. In additional experiments, α-adaptin was pulled down by GST-C, but not by GST, confirming the interaction between α-adaptin and the xARH C-terminal region. About 6.8% of the input α-adaptin in embryo extracts was pulled down in this assay.

The AP-2 complex has four subunits consisting of α- and β2-adaptin (~100 kDa), μ2 adaptin (50 kDa) and σ2 adaptin (17 kDa). Several proteins affinity-purified by GST-C have similar molecular masses (Fig. 4A), suggesting that GST-C might pull down not only the α-adaptin subunit but also the entire AP-2 complex. To test this possibility, Western blot analysis was performed to determine whether β-adaptin was also pulled down by GST-C. Human Jurkat cell lysate containing the AP-2 complex was used as the positive control in these experiments. As expected, the anti-β-adaptin antibody detected a 106-kDa band in the Jurkat cell lysate (Fig. 5). The antibody also recognized a protein with the same molecular mass in Xenopus embryo extracts. GST-C, but not GST alone, pulled down ~5.6% of the input β-adaptin from embryo extracts. Therefore, GST-C pulled down α- and β-adaptin at comparable levels, a result consistent with the xARH C-terminal region interacting with the entire AP-2 complex.

Sequence analysis revealed that the C-terminal region of xARH has three D/C(F/W) motifs (φ, hydrophobic residue) at positions 235, 258, and 305 (Fig. 6A). These motifs in other endocytic proteins such as amphiphysin and epsin have been
shown to directly bind to the hydrophobic patch in the appendage domains of both α- and β₂-adaptins (38, 39). Motifs 258 and 305 are conserved between *Xenopus* and human ARH, whereas motif 235 is not conserved. To test whether these motifs are required for the binding of xARH to the AP-2 complex, three mutants were constructed in which the motifs were individually mutated to AAA (Fig. 6A). The results showed that the conserved motifs 258 and 305 were both required for xARH-C binding to 2-adaptin, whereas nonconserved motif 235 was not (Fig. 6B). DxF/W motifs bind to the AP-2 complex directly (38, 39), and this result indicates that xARH interacts with the AP-2 complex directly through the DxF/W motifs.

To directly address the function of xARH in oocytes, we designed a dominant negative interfering mutant of xARH based on the requirement for the conserved DxF/W motifs. In this mutant, xARH-DM, the putative clathrin box and DxF/W motif 258 were mutated within the context of the full-length xARH (Fig. 7A). xARH-DM will not interact with the endocytic machinery efficiently but will interact with the receptors and thus interfere with the function of the endogenous xARH protein. The effect of the mutant on the uptake of labeled VTG into cultured oocytes was tested. The mutant mRNA transcript was injected into stage III oocytes followed by an overnight culture period. Robust expression of the dominant negative mutant was confirmed by Western blot analysis (Fig. 7C). Injected oocytes were incubated for various periods of time in medium containing biotin-labeled VTG and washed extensively with a high pH buffer to remove surface-bound VTG (4). The internalized biotin-VTG was detected by blot analysis using streptavidin-HRP. As expected, the biotin-VTG accumulated in oocytes over time (Fig. 7B). Compared with the expression of the control galactosidase, expression of xARH-DM reduced the amount of biotin-VTG internalized by 47% (Fig. 7D). From these results we conclude that xARH is required for VTG endocytosis in oocytes.
DISCUSSION

The results from our study show that xARH interacts with LDLR and VTGR and directly with the AP-2 endocytic machinery. We show that during oogenesis, xARH is required for normal uptake of the nutrient yolk protein vitellogenin. Our results are consistent with ARH being an adaptor protein linking lipoprotein receptors to the endocytic machinery during oogenesis and embryogenesis.

xARH was selected originally in a screen for RNAs that localize to the vegetal cortex of oocytes in *Xenopus* (24). The longer xARHa transcript is uniformly distributed in previtellogenic stage I/II oocytes and is gradually localized to the vegetal cortex by the end of stage IV, during a time of intense endocytosis of vitellogenin and other lipoproteins. However, at the end of oogenesis, 30% of xARHa transcripts can be found in the animal hemisphere; these transcripts were likely synthesized after the localization machinery ceased to operate. The shorter β-transcript resides in the animal hemisphere throughout oogenesis.

In this study, xARH protein was detected throughout oogenesis and in embryos indicating that one or both RNAs are translated at the earliest stages, before their localization. As expected for an endocytic protein, immunostaining for xARH was predominantly cortical. Late stage oocytes (stages V/VI) showed a significant new accumulation of xARH protein around the nucleus, a region rich in endoplasmic reticulum. This protein likely represents a large maternal stockpile of xARH that is slowly released into the embryo. After germinal vesicle breakdown, xARH becomes distributed throughout the animal hemisphere, and as nuclei are formed in the embryo, xARH is again found in the perinuclear region of every cell. Therefore, although the mRNA for the xARH isoforms are ultimately polarized in their distribution in oocytes along the animal/vegetal axis, the protein does not appear to be distributed in that manner. The simplest explanation of these results is that the localization of xARH RNAs facilitates the uniform distribution of the xARH protein and allows a high concentration to develop cortically. Interestingly, human ARH protein localizes near the plasma membrane but not in the perinuclear region (34), consistent with this location being a repository for maternal protein for later use by the embryo. These studies show that xARH is present at the correct time and place to act as an adapter protein in receptor-mediated endocytosis of lipoproteins.

xARH interacts with the cytoplasmic tail of the LDL receptor in an in vitro GST pull-down assay, and this interaction requires the NPXY motif (Fig. 3C). These results are in agreement with two recent studies on human ARH protein (22, 23). We found that xARH can also interact with the cytoplasmic tail of the VTG receptor. This binding did not require the NPXY motif (Fig. 3C), the only motif found in all LDL receptor family members. Exceptions to the NPXY motif as the internalization signal for this family of receptors (16) are known. These exceptions suggest that different motifs may underlie the differences in specificity observed among adaptor proteins. A study on LRP has shown that an YXXφ motif, instead of the NPXY motif, is the endocytic signal for this receptor (17). The VTG receptor may use an YXXφ motif that lies just downstream of the NPXY motif for an endocytic signal. The YXXφ motif is conserved in *Xenopus*, chicken, and mammals (14). Certainly, the PTB domain can bind to motifs other than NPXY. The Numb PTB domain is instructive in this regard, as it binds to Lnx (ligand of Numb protein X) through an NPXY motif (40), to NAK (Numb-associated kinase) through a peptide without tyrosine (41), and to GPy-containing peptides in an expression library (42). These interactions have comparable affinities, revealing that PTB domains can have multiple modes of peptide recognition (21). The xARH PTB domain may recognize multiple receptors in the oocyte and embryo through recognition of different internalization motifs.

α- and β-adaptins were efficiently pulled-down at comparable levels from embryo extracts by the xARH C-terminal domain. Two Dφ(F/W) motifs in the C-terminal region of xARH are required for the interaction between xARH and the AP-2 complex (Fig. 6). In proteins such as amphiphysin and epsin, Dφ(F/W) motifs have been shown to bind directly to the hydro-
Phobic patch in the appendage domains of both α- and β₂-adaptins (38, 39). The appendage domains of α- and β₂-adaptins are very similar at the three-dimensional structural level but not at the amino acid sequence level. Therefore, the DPF motif in the appendage domain of α- and β₂-adaptins is relatively weak, with a $K_D$ in the $\sim 100$ μM range for a peptide with a single DPF motif (38).

The strength of binding appears to be correlated with the number of DPF motifs. One suggestion is that both α- and β₂-adaptin appendage domains bind simultaneously to different DPF motifs on a single target molecule (39). This could explain why both motifs 258 and 305 must be present in xARH for efficient interaction with the AP-2 complex. The DPF motifs are not the only regions required for the interaction between ARH and the AP-2 complex. The Arg-266 residue in xARH has also been shown to be critical for the interaction (22). This Arg residue is conserved in xARH and suggests a multi-contact model for interaction with the AP-2 complex. The requirement for DPF motifs in the interaction with the AP-2 complex and the direct interaction between DPF motifs and the appendage domains of α- and β₂-adaptins (38, 39) strongly suggest that xARH binds to the AP-2 complex directly in vivo.

Oocytes actively take up and store lipoproteins to meet the demands of embryo development (43). A large amount of lipid especially is required to accommodate the dramatic increase in the plasma membrane area that occurs during the rapid cell divisions in the cleaving embryo. Another lipoprotein, vitellogenin, is an essential nutrient in animal embryos as diverse as worms and birds (44). Expression of an xARH dominant negative mutant defective in AP-2 binding, and therefore in endocytosis, dramatically reduced VTG uptake in cultured oocytes. These results show that xARH is involved in VTG receptor endocytosis in vivo (Fig. 7). The LDL receptor is also expressed in oocytes (33), and xARH likely mediates the endocytosis of that receptor as well. Besides VTG, the VTG receptor can also mediate the endocytosis of non-lipoproteins such as riboflavin-binding protein (9), α₂-macroglobulin (10), and activin (11), raising the possibility that xARH is also involved in the internalization of those proteins. Recently a mouse model for ARH has been developed. Interestingly, in this mutant model, LRP was not affected and embryos developed normally (34). These results suggest that there may be a number of adaptor proteins with very specific binding affinities for receptors in specific tissues and that these may only be revealed by analysis in vivo. What other members of the LDL receptor family xARH may interact with is unknown and will require further studies.

A model for xARH function in oocytes and embryos in receptor-mediated endocytosis is shown in Fig. 8. In this model, xARH functions as an adaptor between the LDL receptor and the endocytic machinery. xARH is recruited to the cytoplasmic tail of the LDL and VTG receptors. Binding of the receptor is through the N-terminal PTB domain, but whether this interaction is direct or through other mediating proteins is not known. The xARH C terminus interacts directly with the endocytic machinery, AP-2 complex, and clathrin. The model predicts that the entire complex is then internalized into the endosome and stored there in the oocyte as yolk platelets. The receptors are then recycled back to the plasma membrane.

Previous studies on LDL receptor endocytosis have provided support for different models. In some studies, the LDL receptor cytoplasmic tail was shown to bind to the AP-2 complex (45, 46), whereas in other studies, using NMR, the LDL receptor cytoplasmic tail interacted directly with clathrin (47). Interestingly, over-expression of the LDL receptor saturated its own endocytosis but did not affect transferrin receptor endocytosis (48). These results indicate that the LDL receptor does not compete with other receptors for a common adaptor during endocytosis but uses a distinct partner(s) (48). xARH may be the distinct adaptor for LDL and VTG receptor endocytosis. The ability of the LDL receptor to interact with ARH, clathrin, and the AP-2 complex suggests that multiple pathways may exist in LDL receptor endocytosis. The relative importance of these pathways in vivo may be tissue specific. For example, the significantly elevated serum cholesterol levels in ARH patients clearly indicates the importance of the ARH-dependent pathway in vivo (18). At the same time, LDLR endocytosis is unaffected in fibroblasts of the same patients (18).

The interactions between xARH and the LDLR and VTGR were weak. Perhaps other factors may be required in vivo. It is possible that multiple adaptor proteins, including xARH, form a chain to connect the receptor and the endocytic machinery, similar to the Cbl-CIN85-endophilin complex, which mediates ligand-induced down-regulation of epidermal growth factor (EGF) receptors (49). Cbl functions as a negative regulator of epidermal growth factor receptor tyrosine kinase signaling in the Drosophila R7 photoreceptor development (50). In this regard, it is worth noting that five putative xARH binding proteins were pulled down and only p108 has been identified (Fig. 4). p53 and/or p48 may be the μ2-adaptin, whereas p119 and p35 may be components of the endocytic machinery or proteins with other functions. Identification of xARH-binding proteins such as p119 and p35 may provide further insight into the mechanism of receptor-mediated endocytosis during development.

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xARH Is Required for Vitellogenesis