The human low density lipoprotein (LDL) receptor is shown to carry out efficient receptor-mediated endocytosis in Xenopus laevis oocytes. Microinjection of mRNAs encoding the human receptor led to synthesis of a 120-kDa precursor possessing high mannose N-linked sugars and core O-linked sugars. During its transport to the cell surface, the protein increased in apparent size to 160 kDa, which is similar to the change that occurs in human cells. This increase was not seen when the receptor lacked the serine/threonine-rich region that undergoes O-linked glycosylation. The surface receptors bound 125I-LDL at 0 °C and internalized it with a half-time of 2 min when the cells were warmed to 19 °C. The rate of internalization was slowed by 7-fold when a single residue in the cytoplasmic domain (Tyr<sup>683</sup>) was changed to a cysteine, an alteration that slows incorporation into coated pits in mammalian cells. Deletion of the cytoplasmic domain abolished rapid internalization. We conclude that the signals for O-linked glycosylation and receptor-mediated endocytosis of the LDL receptor have been conserved throughout vertebrate evolution.

The signals that direct proteins to their sites of function within cells are generally conserved in evolution. Thus, a similar class of signals is responsible for targeting nascent membrane proteins to the endoplasmic reticulum in animal cells and to plasma membranes in bacteria (Sabatini et al., 1982), for guiding soluble proteins to mitochondria of yeast and animal cells (Schatz, 1987), and for translocating soluble proteins to the cell nucleus of yeast and animal cells (Dingwall and Laskey, 1986). Moreover, the mammalian mannose 6-phosphate-dependent pathway for targeting lysosomal enzymes to lysosomes is functional in Xenopus oocytes (Faust et al., 1987). For one class of signals, however, the degree of evolutionary conservation has not been established. These are the signals that direct cell surface receptors to coated pits, which leads to the rapid endocytosis of receptor-bound ligands. In the case of the receptors for low density lipoprotein (LDL), transferrin, and epidermal growth factor, this movement depends on sequences that project into the cytoplasm (Goldstein et al., 1985). These sequences function in a variety of mammalian cells, but it is not known whether they would function in lower vertebrates, such as birds and amphibians.

The oocytes of lower vertebrates are known to engage in receptor-mediated endocytosis. Chicken oocytes ingest up to 1 g of vitellogenin and very low density lipoproteins/day via this route (Wallace, 1985). In amphibians such as Xenopus laevis, the receptor-mediated endocytosis of yolk proteins is also quite rapid. The role of coated pits and coated vesicles in this uptake has been recognized since the early studies of Roth and Porter (1964) on mosquito oocytes. Coated pits and coated vesicles are also abundant in oocytes from frogs (Wallace, 1985) and chickens (Perry and Gilbert, 1979). The uptake of yolk lipoproteins in coated pits has been demonstrated by electron microscopy in chicken oocytes (Perry and Gilbert, 1979), and the rapid internalization of receptor-bound vitellogenin in Xenopus oocytes (Opresko and Wiley, 1987a, 1987b) is consistent with a coated pit-mediated process in this species as well.

In cells of higher vertebrates, much is beginning to be learned about receptor-mediated endocytosis, largely through the study of naturally occurring and induced mutations in the receptor genes (Goldstein et al., 1985). These studies have revealed that certain receptors, such as those for LDL, transferrin, and asialoglycoproteins, migrate spontaneously into clathrin-coated pits and undergo repeated internalization and recycling even in the absence of ligand. Other receptors, typified by the receptor for epidermal growth factor, are believed to remain quiescent on the cell surface, migrating into coated pits only after the ligand is bound (Schlessinger, 1980).

Rapid internalization of the LDL receptor is dependent on a short COOH-terminal cytoplasmic segment (Goldstein et al., 1985). Of the 839 amino acids in the human LDL receptor, 76 face the extracellular environment. This portion contains the cysteine-rich binding site for LDL, a region of 400 amino acids homologous to a segment of the precursor of epidermal growth factor, and a serine- and threonine-rich region that undergoes O-linked glycosylation. The COOH-terminal 50 amino acids extend into the cytoplasm. We have identified three different naturally occurring human mutations in the LDL receptor that prevent internalization (Lehrman et al., 1985; Davis et al., 1986b), and all three of these mutations alter the cytoplasmic domain. One, designated FH 683, is a

---

* This work was supported in part by research Grant HL-20948 from the National Institutes of Health and the Lucille P. Markey Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of Student Research Fellowship Award 86503 from the American Heart Association.

‡ Recipient of National Institutes of Health Research Career Development Award HL-02187.

The abbreviations used are: LDL, low density lipoprotein; CHO, Chinese hamster ovary; endo H, endo-β-N-acetylglucosaminidase H; MBSS, modified Barth's saline solution; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
nonsense mutation that truncates the protein so that only two amino acids project into the cytoplasm (Lehrman et al., 1985). Another, designated J.D. or FH 380, has a cysteine substitution for tyrosine at position 807, which is 18 amino acids into the cytoplasmic domain (Davis et al., 1986b). Studies with in vitro mutagenesis techniques revealed a relative, but not absolute, requirement for an aromatic amino acid (tyrosine, phenylalanine, or tryptophan) at position 807 (Davis et al., 1987b). When other amino acids were substituted at position 807, the rate of internalization was diminished by 80%. The third naturally occurring mutation, designated FH 763, is a frameshift that alters the sequence of most of the cytoplasmic domain (Lehrman et al., 1985).

If the cytoplasmic signals required for internalization of the LDL receptor were recognized in Xenopus oocytes, it might be possible to use the powerful techniques of oocyte microinjection to define general mechanisms for receptor-mediated endocytosis. Xenopus oocytes have been used extensively as recipients of mRNAs encoding receptors that form ion channels (Sumikawa et al., 1984; Takai et al., 1985; Schofield et al., 1987) or interact with adenylate cyclase (Koblika et al., 1987). When the messenger RNAs encoding various subunits of the acetylcholine receptor are injected into Xenopus oocytes, the receptor assembles correctly, moves to the cell surface, and exhibits ligand-dependent conductance similar to that of the native acetylcholine channels (Sakmann et al., 1985). Whether endocytosis-mediated receptors would also perform their functions in oocytes is unknown.

In this study, we have microinjected the mRNA for the human LDL receptor into Xenopus oocytes. We found that the receptor is synthesized, subjected to O-linked glycosylation, and transported to the cell surface as in mammalian cells. The receptor also undergoes rapid internalization. Mutant receptors with alterations in the cytoplasmic domain that are not internalized efficiently in mammalian cells also were not internalized efficiently in Xenopus oocytes. These findings suggest that the signals that make the receptor a target for O-linked glycosylation and rapid internalization are similar in Xenopus and mammalian cells, and they raise the possibility that this system may be useful for the detailed molecular dissection of receptor-mediated endocytosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained [3H]methylionine (110 Ci/mmol) from Du Pont-New England Nuclear; protease inhibitors, trypsin, type II collagenase (C5661), and Protein A-Sepharose from Sigma; Pronase (165921) and endo-β-N-acetylglucosaminidase H (endo H) from Boehringer Mannheim; type III collagenase (243134) from Behring Diagnostics; and the GEM-3 vector, SP6 RNA polymerase, and RNAsin (ribonuclease inhibitor) from Promega Biotec. Human LDL (d 1.019–1.063 g/ml) (Goldstein et al., 1983) and IgG 15C8, a monoclonal antibody directed against the LDL receptor (Russell et al., 1984; Beisiegel et al., 1981), were prepared and radiolabeled with 111In as described in the indicated references. Human and newborn calf lipoprotein-deficient sera (d > 1.215 g/ml) were prepared as described (Goldstein et al., 1983). X. laevis females were obtained from Nasco (Fort Atkinson, WI).

**Construction of Plasmids and in Vitro Transcription**—The SP6 LDL receptor transcription plasmid was constructed by inserting fragments from pLDLR-2, a human LDL receptor CDNA clone (Yamamoto et al., 1984), into GEM-3. Specifically, an XbaI/Smal fragment encompassing the full LDL receptor coding sequence flanked by a small amount of 5'- and 3'-noncoding sequence, and a 197-base pair AaIII/BamHI fragment from the extreme 3'-untranslated region of the cDNA that contains a poly(A) tract were ligated into XbaI/BamHI double-digested GEM-3. The resulting template is designated "normal LDL receptor." Three mutant receptor plasmid templates, designated "A-O-linked LDL receptor," cytoplasmic domain receptor," and "J.D. receptor," were prepared by oligonucleotide-directed mutagenesis of the normal human LDL receptor cDNA and have been described previously (Davis et al., 1986a, 1987b). The O-linked sugar receptor lacks a 48-amino acid region (residues 700–747 of the human receptor) that is rich in serine and threonine residues and is the site of O-glycosylation in the normal human LDL receptor (Davis et al., 1986a). The Δ cytoplasmic domain receptor lacks the 245 residues (700–944) encompassing the cytoplasmic domain of the normal human LDL receptor (Davis et al., 1987b); this mutation corresponds to a natural mutation in an individual with the internalization-defective form of familial hypercholesterolemia (FH 683) (Lehrman et al., 1985). The J.D. receptor contains a substitution of a cysteine for a tyrosine at residue 807 in the cytoplasmic domain; this mutation corresponds to a natural mutation in an individual with the internalization-defective form of familial hypercholesterolemia (FH 380 or J.D.) (Davis et al., 1986b).

The plasmid templates described above were linearized at the Smal site located in the polylinker region of pGEM-3. mRNAs containing a 5'-GppG cap were prepared from the linearized plasmid templates using SP6 RNA polymerase as previously described (Melton et al., 1984; Konarska et al., 1984). The standard transcription reaction (20 μl) contained 40 mM Tris chloride (pH 7.5); 6 mM MgCl2; 10 mM diithiothreitol; 0.5 mM spermidine; 0.5 mM each ATP, UTP, and CTP; 0.2 mM GTP; 0.5 mM GpppG (Pharmacia LKB Biotechnology Inc.); 0.5 mM SP6 RNA polymerase; and 20 μg/mL of RNase-free DNA. Transcription was initiated by the addition of 0.2 μg of linearized plasmid DNA. Incubations were at 40 °C for 1 h. Aliquots of the transcription reaction were stored at −20 °C and injected directly into oocytes without further purification of the RNA.

**Injection of Oocytes**—Xenopus oocytes were prepared, and RNA was injected essentially as described by Colman (1984). Individual stage 5 and 6 oocytes were manually dissected from surgically removed ovaries and were maintained at 19 °C for up to 48 h prior to injection in modified Barth’s saline solution (MBSS). Oocytes were injected with 20–50 nl of the in vitro transcription reaction (described above) and then allowed to recover in MBSS at 19 °C for 4–5 h. The healthiest oocytes were selected for experiments (described below).

**Immunoprecipitation of 35S-Labeled LDL Receptors**—Injected oocytes (50 nl of transcription reaction) were radiolabeled at 19 °C for 4 h in five oocyte cultures of MBSS containing 0.1 mg/ml endo H digestion, 10 μg/ml calf serum and 1 μCi/ml [3H]methionine. After radiolabeling, the oocytes were washed, and the oocytes were homogenized at 4 °C in 0.2 ml of buffer containing 0.1 mM Tris chloride (pH 8.0), 0.1 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 50 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride, and 0.22 unit/ml aprotinin. Oocyte extracts were centrifuged in an Eppendorf centrifuge at 4 °C for 1 min, and then in a Beckman Airfuge at 20 psi for 5 min at 4 °C. Aliquots of the oocyte extracts (equivalent to 0.1 oocyte) were immunoprecipitated with 0.2 ml of the buffer described by Colman (1984) with the addition of protease inhibitors at the concentrations above. The extracts were incubated for 1 h at 4 °C with Protein A-Sepharose and 50 μg/ml nonimmune rabbit IgG and centrifuged 1 min, after which the cleared extract was incubated overnight at 4 °C with a 50 μg/ml concentration of a polyclonal antibody directed against the human LDL receptor (Russell et al., 1984). Immunocomplexes were harvested with Protein A-Sepharose and analyzed by electrophoresis on 7% SDS-polyacrylamide gels, followed by autoradiography. The dried gels were exposed to Kodak XAR-5 film for 6–24 h at −70 °C. Immunoprecipitation of [35S]-labeled LDL receptor synthesized by TR 715–19 cells, a stable line of hamster cells transfected with the human LDL receptor cDNA, was performed as previously described (Davis et al., 1986a). Apparent molecular sizes of the radiolabeled proteins were calculated from the positions of migration of standard proteins (Davis et al., 1986a).

**Ligand Binding Experiments**—Oocytes used for ligand binding experiments were incubated prior to injection in MBSS (300 oocytes/0.5 ml) containing 1 mg/ml type II collagenase for 1 h at room temperature. The collagenase-treated oocytes were washed in MBSS containing 1 mg/ml bovine serum albumin and maintained in this same medium. Oocytes were injected with 20–30 nl of the in vitro transcription reaction incubated in MBSS containing 10 μg/ml leupeptin and 1% (v/v) calf liver lipoprotein-deficient serum for 36–48 h at 19 °C. Binding assays were performed on groups of three or four oocytes in 50 μl of MBSS containing 5% (v/v) human lipoprotein-deficient serum and either 125I-LDL (250–550 cpm/ng) or 125I-IgG 15C8 (500–700 cpm/ng) for 1 h at room temperature, temperature, and ligand concentrations as indicated in the figure legends. Total binding was measured by layering the oocytes onto 0.2 ml of a 1:1.5 mixture of dibutyl
FIG. 1. Expression of human LDL receptor in Xenopus oocytes. Left, oocytes were injected with water (lane 1) or LDL receptor mRNA (lane 2), radiolabeled with 1 mCi/ml [35S]methionine for 7 h, immunoprecipitated with an antibody against the human LDL receptor, and subjected to SDS gel electrophoresis as described under "Experimental Procedures." Lane 3 shows the immunoprecipitated LDL receptor synthesized by transfected hamster TR 715-19 cells pulse-labeled for 90 min with [35S]methionine. An autoradiogram of the SDS gel is shown. Center, oocytes were injected with LDL receptor mRNA, after which they were preincubated in MBSS containing 10% fetal calf serum for 2 h at the indicated pulse temperature and then pulse-labeled with 1 mCi/ml [35S]methionine for 10 h at the indicated temperature. Oocytes in lanes 4–6 were harvested immediately. Oocytes in lanes 7–9 were incubated for an additional 12 h (chase) at 19°C in MBSS containing 10% fetal calf serum and 10 mM unlabeled methionine. Oocytes were processed for immunoprecipitation, SDS gel electrophoresis, and autoradiography. Lane 10 shows the immunoprecipitated LDL receptor synthesized by transfected hamster TR 715-19 cells pulse-labeled for 90 min with [35S]methionine at 37°C. Right, oocytes injected with LDL receptor mRNA were labeled for 8 h at 19°C with 1 mCi/ml [35S]methionine. The oocytes in lanes 11 and 12 were processed for immunoprecipitation as described above. The immunoprecipitate in lane 12 was digested with 0.1 unit of endo H for 6 h at 37°C according to the supplier’s specifications (Boehringer Mannheim). Oocytes in lanes 13–15 were washed and incubated for an additional 40 min at 37°C in 0.1 mg/ml type II collagenase. The collagenase was removed, and the oocytes were incubated for 30 min at 37°C in MBSS alone (lane 13) or in MBSS containing either 50 μg/ml trypsin (lane 14) or 50 μg/ml Pronase (lane 15). The oocytes were washed once in MBSS containing 10 μg/ml soybean trypsin inhibitor, 50 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride and then processed for immunoprecipitation. All immunoprecipitates were subjected to SDS gel electrophoresis and autoradiography.

RESULTS

In the experiment of Fig. 1, Xenopus oocytes were injected with mRNA for the human LDL receptor, which was prepared by in vitro transcription. After a 7-h labeling period with [35S]methionine, the injected oocytes were homogenized and solubilized; and the LDL receptor was identified by immunoprecipitation, SDS gel electrophoresis, and autoradiography. Two immunoprecipitable bands were visualized (lane 2). For comparative purposes, lane 3 shows the electrophoretic behavior of immunoprecipitated receptors that were synthesized in Chinese hamster ovary (CHO) cells (TR 715 cells) that were transfected with the human LDL receptor cDNA (Davis et al., 1986a). The lower band in lane 2 (approximately 120 kDa) is similar in migration to the receptor precursor, which contains high mannose asparagine-linked carbohydrate chains as well as serine- and threonine-linked N-acetylgalactosamine (Cummings et al., 1983). The upper band (160 kDa) corresponds in molecular size to the mature LDL receptor in which the asparagine-linked carbohydrates have been processed to siaiylated forms and the O-linked sugars have been lengthened by addition of galactose and sialic acids (Cummings et al., 1983).

Fig. 1 (center) shows that the processing of the receptor from the precursor to the mature form was temperature-dependent. When injected oocytes were incubated with [35S]methionine at 4 or 10°C, the precursor was the only form visualized (lanes 4 and 5). When the incubation was performed at 19°C, the precursor and the mature forms were both seen (lane 6). When cells were pulse-labeled at 4°C and shifted to 19°C after removal of the [35S]methionine and dilution with unlabeled methionine, the 120-kDa precursor (lane 4) was processed to the 160-kDa mature form (lane 7). The precursor was also converted into the mature form when the cells were warmed to 19°C after labeling at 10°C (lane 8 versus lane 5). When the pulse and chase were both performed at 19°C, all of the label was found in the mature form (lane 9). Again, for comparative purposes, lane 10 shows the precursor and the mature forms of the receptor synthesized in transfected CHO cells.

The precursor form of the LDL receptor synthesized in oocytes was reduced in apparent molecular weight when treated with endo H (Fig. 1, lane 12). The mature form of the receptor also appeared to be partially endo H-sensitive, indicating that some of the asparagine-linked sugars remained in the high mannose form. To determine whether the mature receptor was accessible to external proteases, we found it necessary to pretreat the cells with collagenase to allow the proteases to penetrate to the surface of the oocyte. This pretreatment with protease-free collagenase had no effect on the radiolabeled LDL receptors (lane 13). When collagenase was followed by treatment with trypsin (lane 14) or Pronase (lane 15), the 160-kDa form of the receptor was destroyed, but the 120-kDa form of the receptor was protected. A similar
The result was obtained in cultured human fibroblasts (Tolleshaug et al., 1983). These results indicate that the 160-kDa form of the receptor, but not the 120-kDa form, is on the cell surface.

Most of the increase in apparent molecular size of the LDL receptor from 120 to 160 kDa is attributable to the elongation of the O-linked sugar chains (Cummings et al., 1983). When CHO cells are transfected with an LDL receptor cDNA from which the region encoding the O-linked sugar domain is deleted, the precursor form of the receptor is reduced in apparent molecular size to 100 kDa, and there is only a 20-kDa increase after processing of the carbohydrate, largely owing to the processing of the asparagine-linked sugars (Davis et al., 1986a). When a mutant mRNA lacking the coding region for the O-linked sugar domain was injected into Xenopus oocytes, the precursor also appeared smaller than normal (Fig. 2, lane 3 versus lane 1). When the incubation was prolonged for 22 h, the mature form of the mutant receptor was seen (lane 5). It was reduced in molecular weight by approximately 40,000 when compared with the normal (lane 1). Whereas the precursor form was sensitive to endo H (lane 4), the mature form appeared to be partially endo H-resistant (lane 6). Thus, in the absence of the O-linked sugar region, the LDL receptor failed to show its normal increase in apparent molecular weight. It did reach the cell surface, however, as evidenced by the ability of the injected oocytes to bind 125I-LDL (see below).

To determine whether the normal human LDL receptor synthesized by Xenopus oocytes was functional, we first treated oocytes with collagenase and then injected the receptor mRNA. After 44 h, we incubated the cells with 125I-LDL (Fig. 3). When the 125I-LDL was added at 0 °C, the amount of cell-associated radioactivity reached a plateau by 4 h. When the incubation with 125I-LDL was performed at 4 or 19 °C, the accumulation of 125I-LDL progressively increased. At 8 h, the total uptake was six times greater at 19 °C than it was at 0 °C. The oocytes were injected with a similar volume of water or transcription buffer, there was no measurable uptake of 125I-LDL at any temperature (Fig. 3, open symbols).

To determine whether the increased uptake at 19 °C was attributable to internalization of the 125I-LDL, we injected oocytes with LDL receptor mRNA, incubated them for 36 h, and then allowed them to take up 125I-LDL for 8 h at either 0 or 19 °C (Table I). The cells were then washed and treated with suramin, a polyanionic compound that releases LDL from its receptor (Schneider et al., 1982). When the cells were incubated at 0 °C, a total of 7 ng of 125I-LDL was bound per oocyte, of which 84% was released by suramin. When the cells had been incubated at 19 °C, the total uptake was increased to 44 ng/oocyte, but the amount releasable by suramin was the same as at 0 °C. The enhanced uptake at 19 °C was due entirely to the accumulation of material that was resistant to release by suramin, suggesting that it had entered the cell.

Internalization was demonstrated in a different way by treating the injected oocytes with formaldehyde and then determining their binding activity (Table II). In human fibroblasts, these concentrations of formaldehyde prevent internalization of receptor-bound LDL but do not interfere with receptor binding (Brown et al., 1976). In control cells, there was a 5-fold higher uptake of 125I-LDL at 19 °C than at 0 °C. When the cells had been fixed with formaldehyde, binding at 0 °C was not significantly affected, but there was no longer any significant increase in binding at 19 °C.

FIG. 3. Uptake of 125I-LDL by Xenopus oocytes injected with normal human LDL receptor mRNA as a function of temperature and time. Collagenase-treated oocytes were injected with water (O, A, □) or LDL receptor mRNA (□, △, ▪). After incubation at 19 °C for 44 h, the injected oocytes were incubated with 20 μg of protein/ml of 125I-LDL (500 cpm/ng of protein) for varying times at the indicated temperature. Total binding of 125I-LDL was determined for individual groups of four oocytes by the phthalate centrifugation procedure as described under "Experimental Procedures." Each point represents the average of duplicate incubations (total of eight oocytes).

TABLE I
Release of receptor-bound 125I-LDL from surface of Xenopus oocytes by suramin

| Incubation temperature | Total 125I-LDL bound | Suramin-releasable fraction | Suramin-resistant fraction |
|------------------------|----------------------|-----------------------------|---------------------------|
| °C         | ng/oocyte | % | ng/oocyte | % |
| 0         | 7.0       | 5.9 (84) | 1.1 (16) |
| 19        | 44        | 5.7 (13) | 38 (87)  |

* Values in parentheses denote percent of total 125I-LDL bound.

Human LDL Receptors in Xenopus Oocytes

![Image](image_url)

**FIG. 2.** Expression in Xenopus oocytes of a mutant human LDL receptor lacking the clustered O-linked sugar domain. Oocytes were injected with normal LDL receptor mRNA (lanes 1 and 2) or the Δ O-linked receptor mRNA (lanes 3–6) and labeled with 1 μCi/ml [35S]methionine for 7 h (lanes 1 and 2), 6 h (lanes 3 and 4), or 22 h (lanes 5 and 6). Immunoprecipitates were incubated at 37 °C overnight in the absence or presence of 0.1 unit of endo H as indicated and then subjected to SDS gel electrophoresis and autoradiography. Lane 7 shows the immunoprecipitated LDL receptor synthesized by transfected hamster TR 715-19 cells pulse-labeled for 2 h with [35S]methionine.
TABLE II

Binding of 125I-LDL to formaldehyde-fixed Xenopus oocytes

Collagenase-treated oocytes were injected with normal LDL receptor mRNA and incubated for 44 h at 19 °C. The oocytes were then incubated for 1 h at 0 °C in 100 μl of phosphate-buffered saline (control) or phosphate-buffered saline containing 2% (v/v) paraformaldehyde (fixed). The oocytes were then washed five times in 1 ml of MBSS containing 1 mg/ml bovine serum albumin and incubated with 20 μg of protein/ml of 125I-LDL for 8 h at 0 or 19 °C as indicated. Total binding for groups of three oocytes was measured by the phthalate centrifugation procedure as described under “Experimental Procedures” except that 1 mg/ml bovine serum albumin was included in the binding medium. Each point represents the average of duplicate incubations (total of six oocytes).

| Oocyte treatment | 125I-LDL bound 0 °C (a) | 19 °C (b) | Ratio (b/a) |
|------------------|-------------------------|----------|------------|
| Control          | 11 ng/oocyte            | 56       | 5.1        |
| Fixed            | 7 ng/oocyte             | 8.9      | 1.3        |

![Graph](image)

**Fig. 4.** Saturation curves for binding of 125I-LDL (A) and 125I-labeled anti-receptor monoclonal antibody (B) to human LDL receptors expressed in Xenopus oocytes. Collagenase-treated oocytes were injected with water (C, D) or LDL receptor mRNA (C, A). After incubation at 19 °C for 48 h, the injected oocytes were incubated with the indicated concentration of 125I-LDL (500 cpm/ng of protein) or 125I-IgG-15Cb (600 cpm/ng) for 8 h at 0 °C (C, A) or at 19 °C (D, C). Total binding of 125I-ligand was determined for groups of four oocytes. Each point represents the average of duplicate incubations (total of eight oocytes).

about 10 μg of LDL protein/ml. The injected oocytes also bound a monoclonal antibody directed against the LDL receptor (Fig. 4B). Again, the affinity was similar at 0 and 19 °C. The maximal amount of binding was higher at 19 °C than at 0 °C, suggesting that some of the monoclonal antibody had been internalized. This monoclonal antibody is known to be internalized in human fibroblasts (Beisiegel et al., 1981) and in CHO cells that have been transfected with the LDL receptor cDNA.

Oocytes expressing the receptor that lacks the O-linked sugar region bound 125I-LDL (Fig. 5B), confirming that this mutant receptor reaches the cell surface. The amount of binding was similar to that seen in cells injected with mRNA encoding the normal receptor in the same experiment (Fig. 5A). The absence of the clustered O-linked sugar region did not prevent the binding and uptake of 125I-LDL at 19 °C (Fig. 5B), although the amount of this uptake was somewhat reduced when compared with the cells expressing the normal receptor (Fig. 5A). This receptor lacking the O-linked sugar region is internalized normally in transfected CHO cells (Davis et al., 1986a).

![Graph](image)

**Fig. 5.** Binding of 125I-LDL to Xenopus oocytes expressing the human LDL receptor lacking the clustered O-linked sugar domain. Collagenase-treated oocytes were injected with in vitro synthesized normal LDL receptor mRNA (A) or Δ O-linked receptor mRNA (B) and incubated for 36 h at 19 °C. Groups of three oocytes were then incubated with 20 μg of protein/ml of 125I-LDL (400 cpm/ng at 0 °C (●) or at 19 °C (△)) for the times indicated, and total binding was determined. Each point represents the average of duplicate incubations (total of six oocytes).

![Graph](image)

**Fig. 6.** Expression in Xenopus oocytes of a mutant human LDL receptor lacking the cytoplasmic domain. Oocytes were injected with normal LDL receptor mRNA or Δ cytoplasmic domain receptor mRNA. The oocytes were pulse-labeled with [35S]methionine for 8 h, followed by a chase in the presence of 10 mM unlabeled methionine for the indicated time. Oocytes were processed for immunoprecipitation, SDS gel electrophoresis, and autoradiography as described under “Experimental Procedures.”

To determine whether the internalization of human LDL receptors in Xenopus oocytes is dependent upon the cytoplasmic domain, we injected oocytes with a mutant mRNA that contained a premature termination codon at position 792, which leaves only two amino acids in the cytoplasm. The receptor encoded by this mRNA is known to be internalized slowly in human fibroblasts because it migrates slowly into coated pits (Lehrman et al., 1985). In oocytes, the mutant mRNA evoked synthesis of a precursor that was approximately 5 kDa smaller than normal (Fig. 6, lane E), consistent with the absence of the 5-kDa cytoplasmic domain. With time, this precursor was processed to a mature form whose apparent molecular weight remained slightly lower than normal (lane H). The rate of processing was slower than was observed with the normal receptor. In human fibroblasts, this mutant receptor is also transported to the surface at a relatively slow rate (Lehrman et al., 1985).

At 0 °C, oocytes injected with the LDL receptor mRNA lacking the cytoplasmic domain were releasable by suramin. At 19 °C, the mutant receptor showed...
Experimental Procedures." Each value represents the average of triplicate incubations (total of nine oocytes). After incubation for 30 min, they had internalized an amount of $^{125}$I-LDL equal to that bound at the cell surface. These cells required 6 h to internalize an amount of $^{125}$I-LDL equal to that bound at the cell surface.

To study internalization of surface-bound $^{125}$I-LDL directly, we incubated the oocytes with $^{125}$I-LDL at 0°C, washed the cells, and then warmed them to 25°C. Cells expressing normal or mutant receptors showed similar binding at 0°C (zero time in Fig. 9, A and B). When the cells were warmed, those expressing the normal receptor showed a prompt decrease in suramin-releasable radioactivity and a corresponding increase in suramin-resistant radioactivity (Fig. 9A). By 15 min, near all of the surface-associated material had been internalized. On the other hand, the cells expressing the mutant receptor lacking the cytoplasmic domain showed only a slight increase in the suramin-resistant fraction upon warming, and the suramin-releasable fraction remained high throughout the experiment (Fig. 9B). These data indicate that the mutant LDL receptor lacking the cytoplasmic domain was internalized much more slowly than the wild-type receptor in Xenopus oocytes.

The tyrosine at position 807 in the cytoplasmic domain of the LDL receptor plays a crucial role in migration into coated pits in mammalian cells (Davis et al., 1987b). When it is switched to a cysteine, the receptor in transfected CHO cells is internalized at only one-fifth the normal rate (Davis et al., 1987b). Fig. 10 shows that this mutant receptor was also internalized more slowly when the mutant mRNA was injected into Xenopus oocytes. In three experiments, the mean time for internalization of half of the surface-bound $^{125}$I-LDL was 1.8 min (range of 1.5–2.5 min) when the normal mRNA was injected and 12.8 min (range of 10.5–14.5 min) when the Cys807 mRNA was injected.

**FIG. 7.** Binding at 0°C of $^{125}$I-LDL to Xenopus oocytes expressing a mutant human LDL receptor lacking the cytoplasmic domain. Collagenase-treated oocytes were injected with mRNA encoding the normal LDL receptor (A) or the mutant receptor lacking the cytoplasmic domain (B) and then incubated at 19°C for 48 h. Groups of three oocytes were then incubated with 20 μg of protein/ml of $^{125}$I-LDL (450 cpm/ng) at 0°C for the indicated time. The oocytes were washed at 0°C and assayed for suramin-resistant (internalized) or suramin-releasable (surface-bound) $^{125}$I-LDL as described under "Experimental Procedures." Each point represents the average of duplicate incubations (total of six oocytes).

**FIG. 8.** Binding at 19°C of $^{125}$I-LDL to Xenopus oocytes expressing a mutant human LDL receptor lacking the cytoplasmic domain. The experimental procedures were identical to those of Fig. 7 except that the injected oocytes were incubated with $^{125}$I-LDL at 19°C rather than at 0°C.

**FIG. 9.** Temperature-dependent internalization of receptor-bound $^{125}$I-LDL by Xenopus oocytes expressing normal human LDL receptor (A) or mutant J. D. receptor (B). Collagenase-treated oocytes were injected with mRNA encoding the indicated receptor and incubated for 48 h at 19°C. Groups of three oocytes were then incubated in the presence of 20 μg of protein/ml of $^{125}$I-LDL (575 cpm/ng) for 4 h at 0°C. The oocytes were washed at 0°C in MBSS containing 2 mg/ml bovine serum albumin and then incubated in this same medium for the indicated time at 25°C. The amounts of suramin-releasable (surface-bound) and suramin-resistant (internalized) $^{125}$I-LDL were determined as described under "Experimental Procedures." Each value represents the average of triplicate incubations (total of nine oocytes).

A higher level of steady-state suramin-releasable binding than did the normal receptor (Fig. 8). In the cells expressing the normal receptor, the amount of suramin-resistant binding rose progressively with time at 19°C (Fig. 8A). Within 30 min, they had internalized an amount of $^{125}$I-LDL that was equal to the amount bound at the surface. Internalization was much slower in the cells expressing the mutant receptor (Fig. 8B). These cells required 6 h to internalize an amount of $^{125}$I-LDL equal to that bound at the cell surface.

This study demonstrates that Xenopus oocytes recognize the signals on the human LDL receptor that mediate rapid

**DISCUSSION**

This study demonstrates that Xenopus oocytes recognize the signals on the human LDL receptor that mediate rapid
endocytosis. The rate of internalization of receptor-bound LDL, as determined by the warm-up experiment of Figs. 9A and 10A, was as fast as it is in human fibroblasts (Goldstein et al., 1977). Rapid internalization was dependent upon the presence of the cytoplasmic domain. When this domain was removed, rapid internalization did not occur. When Tyr867 in the cytoplasmic domain was changed to cysteine, the rate of internalization was reduced 7-fold, a decrease that is similar to the decrease measured in CHO cells (Davis et al., 1986b, 1987b).

From the quantitative data on 125I-LDL binding, we calculate that the injected oocytes express approximately 10^10 receptors/cell. This is somewhat lower than the reported number of receptors for vitelligenin on Xenopus oocytes (2-28 x 10^9/cell) (Opreseko and Wiley, 1987a).

Since rapid internalization of the LDL receptor in oocytes depends on the cytoplasmic signal that is known to be required for incorporation into coated pits, we infer that rapid internalization is mediated by coated pits. However, we have not yet demonstrated this event directly. In our initial attempts to study internalization by electron microscopy through the use of gold-coupled LDL, we encountered technical problems, apparently owing to a non-uniform distribution of LDL around the periphery of the oocyte. Further attempts are being made to study this process morphologically.

Whereas in other cell types the receptor-mediated endocytosis of LDL leads to prompt and complete proteolytic degradation, the same was not observed in Xenopus oocytes. Indeed, we were not able to demonstrate the secretion of any trichloroacetic acid-soluble radioactivity after incubation with 125I-LDL at 19°C. More than 80% of the intracellular radioactivity remained precipitable by trichloroacetic acid after incubation for up to 8 h (data not shown). This lack of complete proteolysis indicates that the LDL is being routed to a site other than classic lysosomes. When vitelligenin enters oocytes, it is carried to yolk platelets, where specific proteolytic cleavages occur; but the protein is not degraded completely as it would be in lysosomes (Wall and Meleka, 1985).

A second difference in the behavior of the LDL receptor in Xenopus oocytes versus human fibroblasts relates to the relative affinity of the receptor for ligands at different temperatures. In human fibroblasts, the affinity for LDL and for monoclonal antibodies IgG-C7 and IgG-15C8 increases by more than 10-fold when the cells are cooled from 37 to 4°C (Beisiegel et al., 1981; van Driel et al., 1987). However, when the LDL receptor was expressed in Xenopus oocytes, the affinity for LDL and the affinity for IgG-15C8 were the same at 19 and 0°C (Fig. 4). The affinities for both ligands were similar to the affinities seen in human fibroblasts at 37°C. Thus, the oocytes did not show the dramatic increase in affinity that develops in human fibroblasts at low temperatures.

From detailed study of binding kinetics, Opreseko and Wiley (1987b) concluded that in Xenopus oocytes the vitelligenin receptor enters the cell and recycles in the absence of ligand. We do not know whether the LDL receptor behaves similarly in oocytes. Over an 8-h period, the cells internalized 7-10-fold more LDL than was initially bound to the surface (Figs. 4A and 8A). Over this time period, there was a slow decline of about 50% in surface LDL receptors as defined by suramin release (Fig. 8A). The failure of surface receptors to decline rapidly upon internalization of LDL could mean that the receptors recycled, or it could mean that all of the receptors were internalized and replaced by newly synthesized receptors. The traditional way to study recycling is to block the synthesis of new receptors with cycloheximide and to determine whether uptake continues. Unfortunately, this type of experiment was inconclusive in the Xenopus oocytes because the cells contain a large pool of unprocessed receptor precursor. Uptake of 125I-LDL continued in the presence of cycloheximide, but we do not know whether this was due to the recycling of the receptor or to the resupply of receptors from the pool of receptor precursor.

In the biosynthesis experiments, the observed rate of processing of the receptor from the precursor to the mature form was much slower in Xenopus oocytes than it is in human fibroblasts and transfected hamster cells. In mammalian cells, the receptor is completely processed within 1 h of synthesis (Goldstein et al., 1985). In the Xenopus oocytes after an 8-h incubation with [35S]methionine, approximately half of the receptors were in the mature form, and half remained in the precursor form (Fig. 6, lane A). Even after a further 4-h chase, some of the receptor appeared to remain in the precursor form. After processing to the higher molecular weight form, some of the carbohydrate remained endo H-sensitive (Fig. 1, lane 12). In mammalian cells, the high molecular weight form of the receptor is completely resistant to endo H.

The increase in apparent molecular weight from 120,000 to 180,000 in oocytes was dependent upon the region of the LDL receptor that is known to undergo O-linked glycosylation. When this region was deleted, no such processing occurred (Fig. 2). These findings strongly suggest that O-linked glycosylation occurs in Xenopus oocytes. However, we have been unable to provide direct evidence for this modification. Multiple attempts to remove the O-linked sugars by treatment with several types of neuraminidase followed by O-glycanase failed. The failure of these enzymes may be due to the occurrence of unusual sialic acid linkages that are used in Xenopus oocytes. There has been no comprehensive characterization of O-linked glycosylation in these cells. However, these cells are known to use different carbohydrates than are used by mammalian cells (Mous et al., 1982).

The human LDL receptor lacking the cytoplasmic domain was processed in Xenopus oocytes to the mature form, but this processing occurred at about one-fourth the normal rate (Fig. 6). In human fibroblasts, this mutant receptor was also processed somewhat slowly, i.e. at about half the normal rate (Lehrman et al., 1985). We do not believe that this slow processing relates to the inability of the receptor to bind to coated pits. The receptors in which a cysteine was substituted for a tyrosine at position 807 moved to the surface of oocytes for a tyrosine at position 807 moved to the surface of oocytes at a nearly normal rate even though they do not bind efficiently to coated pits.

This study establishes Xenopus as a model system in which to study receptor-mediated endocytosis. The great advantage of the system is the ability to test rapidly many different forms of mutant receptors that are prepared by oligonucleotide-directed mutagenesis of cDNAs. It should also now be possible to inject mRNAs for different receptors and to determine whether they compete with each other for access to coated pits, ar. experiment that has not yet been possible in transfected mammalian cells.

Acknowledgments—We thank Phyllis Faust and Stuart Kornfeld (Washington University Medical School, St. Louis, MO) for teaching us the procedure of oocyte microinjection and Jolanda Schreurs (DNAX, Inc., Palo Alto, CA) for communicating techniques used for the ligand binding assay. Jacky Dailey and Shirley Hall provided excellent technical assistance.

REFERENCES

Beisiegel, U., Schneider, W. J., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1981) J. Biol. Chem. 256, 11923-11931
