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Newly Synthesized Human δ Opioid Receptors Retained in the Endoplasmic Reticulum Are Retrotranslocated to the Cytosol, Deglycosylated, Ubiquitinated, and Degraded by the Proteasome*

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We have previously shown that only a fraction of the newly synthesized human δ opioid receptors is able to leave the endoplasmic reticulum (ER) and reach the cell surface (Petäjä-Repo, U. E, Hogue, M., Laperrière, A., Walker, P., and Bouvier, M. (2000) *J. Biol. Chem.* 275, 13727–13736). In the present study, we investigated the fate of those receptors that are retained intracellularly. Pulse-chase experiments revealed that the disappearance of the receptor precursor form (M_r 45,000) and of two smaller species (M_r 42,000 and 39,000) is inhibited by the proteasome blocker, lactacystin. The treatment also promoted accumulation of the mature receptor form (M_r 55,000), indicating that the ER quality control actively routes a significant proportion of rescuable receptors for proteasome degradation. In addition, degradation intermediates that included full-length deglycosylated (M_r 39,000) and ubiquitinated forms of the receptor were found to accumulate in the cytosol upon inhibition of proteasome function. Finally, coimmunoprecipitation experiments with the β-subunit of the Sec61 translocon complex revealed that the receptor precursor and its deglycosylated degradation intermediates interact with the translocon. Taken together, these results support a model in which misfolded or incompletely folded receptors are transported to the cytoplasmic side of the ER membrane via the Sec61 translocon, deglycosylated and conjugated with ubiquitin prior to degradation by the cytoplasmic 26 S proteasomes.

The endoplasmic reticulum (ER)[1] quality control scrutinizes newly synthesized proteins entering the secretory pathway and ensures that only correctly folded and, in the case of multimeric proteins, fully assembled complexes are deployed to distal cellular compartments (1). Those that fail to fulfill these criteria are retained in the ER and subsequently degraded. Even minor changes in the protein primary structure can cause retention in the ER. This is exemplified in many diseases, including cystic fibrosis and cases of α_1-antitrypsin deficiency and familial hypercholesterolemia, which are characterized by an "ER storage phenotype" (2). Although less thoroughly characterized, numerous naturally occurring mutations leading to ER retention of G protein-coupled receptors (GPCRs) have also been evoked as the cause of inherited diseases. For example, ER-retained mutants of rhodopsin, the luteinizing hormone receptor, and the V2-vasopressin receptor have been implicated in some forms of retinitis pigmentosa, male pseudohermaphroditism, and nephrogenic diabetes insipidus, respectively (3–6).

Emerging evidence indicates that degradation of aberrant ER proteins is mediated by the cytosolic multiprotease complex, the 26 S proteasome. Indeed, an increasing number of misfolded or unassembled yeast and eukaryotic integral membrane and secreted proteins have been shown to be substrates for this disposal mechanism (7–26). However, proteasome-mediated degradation is not restricted to misfolded or unassembled proteins, as evidenced by the observation that some resident ER proteins are substrates for this degradation pathway in response to cellular signals (27–29). It can thus be hypothesized that this degradation pathway might have a role in regulating steady state levels of normal wild type proteins traversing the secretory pathway, as has been described for many cytosolic and nuclear regulatory molecules (for review, see Refs. 30 and 31). Reported examples pointing to this phenomenon are, however, scarce, exceptions being apolipoprotein B100 (32, 33), apolipoprotein(a) (34), and tyrosinase (35). In a recent report, Schubert and co-workers (36) showed that at least 30% of newly synthesized proteins might be degraded by the proteasomes. Whether these degraded proteins represent defective ribosomal products resulting from errors in translation, as suggested, or might also include correctly translated but incompletely folded proteins remains to be determined.

Proteasome-mediated ubiquitin-dependent degradation of cytosolic and nuclear proteins is a well described phenomenon (for review, see Refs. 30 and 31), but the detailed mechanisms involved in the ER-associated proteasomal degradation remain

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[1] The abbreviations used are: ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator; DDM, *n*-dodecyl-β-D-maltoside; DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor; hδOR, human δ opioid receptor; HEK-293S, human embryonic kidney 293S; Hsp70, heat shock protein 70; MG-132, Z-Leu-Leu-Leu-CHO; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PNGase F, peptide-*N*-glycosidase F; PSI, Z-Ile-Glu(OtBu)-Ala-Leu-CHO; STI, soybean trypsin inhibitor.

unclear. Recent findings have led to the emergence of models suggesting that misfolded or incompletely folded ER proteins are retrotranslocated to the cytosolic side of the ER membrane through the Sec61 translocon complex and conjugated with ubiquitin before hydrolysis by the 26 S proteasomes (for review, see Refs. 37–39).

We have previously shown that the human δ opioid receptor (hδOR) is an example of integral membrane proteins that are partially retained in the ER, possibly due to difficulties in the folding process (40). Another example is the cystic fibrosis transmembrane conductance regulator (CFTR) (for review, see Ref. 41). Only about 40% and 25% of the newly synthesized wild type hδOR and CFTR molecules, respectively, are able to leave the ER and reach the cell surface. We thus set out to determine whether the ER-retained hδORs are targeted for degradation by the 26 S proteasomes and, if so, to delineate the mechanisms involved in this process. The results presented here indicate that these receptors are transported back to the cytosol, in a process that involves the Sec61 translocon, and deglycosylated before being degraded by the proteasomes. Furthermore, inhibition of the proteasomal pathway led to the accumulation of ubiquitinated receptor forms, pointing to the fact that polyubiquitination may be a targeting signal for disposal of misfolded or incompletely folded hδOR molecules.

EXPERIMENTAL PROCEDURES

Materials—EXPRE^{35}S^{35}S protein labeling mix (1175 Ci/mmol) and [9-^{3}H]bremazocine (26.6 Ci/mmol) were purchased from PerkinElmer Life Sciences. Recombinant peptide-*N*-glycosidase F (PNGase F) of *Flavobacterium meningosepticum*, purified from *Escherichia coli* (EC 3.5.1.52) was from Roche Molecular Biochemicals. The proteasome inhibitors lactacystin, Z-Leu-Leu-Leu-CHO (MG-132) and Z-Ile-Glu-(OtBu)-Ala-Leu-CHO (PSI) were obtained from Calbiochem. Cell culture reagents were either from Life Technologies Inc. or Wisent. The anti-FLAG M2 monoclonal antibody, the anti-FLAG M2 affinity resin, and the FLAG peptide were products of Sigma. The anti-calnexin (SPA-860), the anti-heat shock protein 70 (Hsp70) (SPA-820), and the anti-ubiquitin (SPA-200) antibodies were from Stressgen Biotechnologies Corp. and Protein G-Sepharose from Amersham Pharmacia Biotech. The anti-Sec61β antibody was a generous gift from Dr. Tom A. Rapoport (Harvard Medical School, Boston, MA). All the other reagents were of analytical grade and obtained from various commercial suppliers.

Cell Culture—Cells were cultured in either 25-cm^2 or 75-cm^2 culture flasks and grown to 80–90% confluence at 37 °C in a humidified atmosphere of 5% CO_2. Human embryonic kidney 293S (HEK-293S) cells stably expressing the hδOR tagged at the C terminus with the FLAG epitope (DYKDDDDK) (40) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 1000 units/ml penicillin, 1 mg/ml streptomycin, 1.5 μg/ml fungizone (complete DMEM) and 400 μg/ml Geneticin. A clone expressing 10 pmol of receptor/mg of membrane protein was chosen for this study.

Metabolic Labeling with [^{35}S]Methionine/Cysteine—For [^{35}S]methionine/cysteine labeling, cells were first preincubated in methionine and cysteine-free DMEM for 60 min at 37 °C, and the labeling was performed in fresh medium containing 150 μCi/ml [^{35}S]methionine/cysteine. After an incubation of 60 min at 37 °C, the pulse was terminated by washing the cells twice with the chase medium (complete DMEM supplemented with 5 mM methionine) and chased for different periods of time as specified in the figures. When labeling was performed in the presence of the proteasome inhibitors, the reagents were added 3 h before the pulse labeling at a final concentration of 10 μM (lactacystin) or 25 μM (MG-132 and PSI). After the labeling, cells were washed with and harvested in phosphate-buffered saline and, unless otherwise indicated, quick-frozen in liquid nitrogen and stored at −80 °C.

Immunoprecipitations—Total cellular lysates were prepared in buffer A (0.5% *n*-dodecyl-β-D-maltoside (DDM) (w/v), 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor (STI), 10 μg/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM 1,10-phenanthroline). Insoluble material was removed by centrifugation at 5000 × *g* for 30 min, and bovine serum albumin was added to a final concentration of 0.1% (w/v). The receptor was immunoprecipitated using the anti-FLAG M2 affinity resin as described (40). For the Sec61β immunoprecipitation, DDM was replaced by digitonin while NaCl, EDTA, and 1,10-phenanthroline were omitted from the buffer. For the ubiquitin immunoprecipitation, *N*-ethylmaleimide (NEM) was included in all buffers to distinguish the high molecular weight ubiquitinated receptor forms from putative aggregated receptors. Before immunoprecipitation of calnexin, Hsp70, Sec61β, and ubiquitin, samples were precleared for 60 min with 15 μl of Protein G-Sepharose. The appropriate antibody (dilutions: 1:200, 10 μg/ml, 1:100, and 1:100, respectively) and 15 μl of Protein G-Sepharose were then added and incubated overnight at 4 °C with gentle agitation. Following washing of the resin as described (40), the bound antigens were eluted in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromphenol blue) at 95 °C for 5 min. If two or more consecutive immunoprecipitation steps were performed, the antigens were eluted with 1% (w/v) SDS, 25 mM Tris-HCl, pH 7.4, at 95 °C for 5 min and the eluates diluted 10-fold with buffer A prior to the next immunoprecipitation.

Cell Fractionation—Cells were harvested and homogenized in buffer B (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml STI, 10 μg/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM PMSF, 2 mM 1,10-phenanthroline) with a Dounce homogenizer (Fig. 3, *A–C*). Alternatively (Figs. 3 (*D and E*) and 4*B*), cells were homogenized by freezing and thawing the cell suspension and passing it 10 times through a 26-gauge needle. Fractionation was performed as described (42). Briefly, homogenates were first centrifuged at 1000 × *g* for 10 min to pellet unbroken cells and nuclei. The supernatant was then centrifuged at 10,000 × *g* for 30 min to pellet the crude membranes, and the supernatant was further centrifuged at 100,000 × *g* for 60 min to clarify the cytosolic fraction. DDM was added to the soluble fraction to a final concentration of 0.5% (w/v) to decrease nonspecific binding of proteins to either the anti-FLAG M2 affinity resin or Protein G-Sepharose. Pellets were washed with buffer B, solubilized in buffer A, and insoluble material removed by centrifugation at 100,000 × *g* for 60 min. Immunoprecipitations from the soluble fraction and the solubilized membranes were carried out as described above.

Deglycosylation of the Immunoprecipitated HδORs—The immunoprecipitated receptors were deglycosylated following elution from the anti-FLAG M2 affinity resin with 1% (w/v) SDS, 50 mM sodium phosphate, pH 7.5. Before the enzyme reaction, the eluates were diluted 10-fold with 0.5% (w/v) DDM, 50 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.5 mM PMSF, 2 mM 1,10-phenanthroline, 5 μg/ml leupeptin, 5 μg/ml STI, 10 μg/ml benzamidine, and 1% 2-mercaptoethanol. PNGase F was added at a final concentration of 0.01–10 units/ml, samples incubated at 37 °C for 16 h, and the reaction terminated by the addition of SDS-sample buffer.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was performed as described (43), using 4% stacking gels and 10% separating gels. Samples were heated at 95 °C for 2 min in the presence of 50 mM dithiothreitol and run on a Bio-Rad Mini-Protein II apparatus. Molecular weight markers (Bio-Rad) detected by staining with Coomassie Brilliant Blue (Bio-Rad) were used to calibrate the gels. For the detection of radioactivity, the gels were treated with EN^{3}HANCE® (PerkinElmer Life Sciences) according to the manufacturer's instructions, dried, and exposed at −80 °C for 1–15 days using Biomax MR film and intensifying screens (Eastman Kodak Co.). The relative intensities of the labeled bands on the fluorographs were analyzed by densitometric scanning with an Agfa Arcus II laser scanner, and the data were quantified using the NIH image program, version 1.61, subtracting the local background from each lane.

Miscellaneous Procedures—Total protein was measured using the Bio-Rad DC assay kit and bovine serum albumin as a standard. Binding assays were carried out using [^{3}H]bremazocine, essentially as described (40).

RESULTS

Degradation of ER-retained Newly Synthesized hδORs Is Mediated by the Proteasomal Pathway—We have previously shown that a large fraction of newly synthesized hδORs in HEK-293S cells remains in a pre-Golgi compartment as core-glycosylated M_r 45,000 precursors that are not transported to the cell surface (40). Now we set out to investigate the fate of these intracellularly retained receptors. To determine whether proteasomes are involved in the degradation of these receptor forms, cells were incubated with lactacystin, a specific inhibitor of the 26 S proteasome (44, 45). HEK-293S cells stably expressing the receptor carrying a C-terminal FLAG epitope (HEK-293S-hδOR-FLAG cells) were metabolically labeled with [^{35}S]methionine/cysteine and after a chase in a medium con-

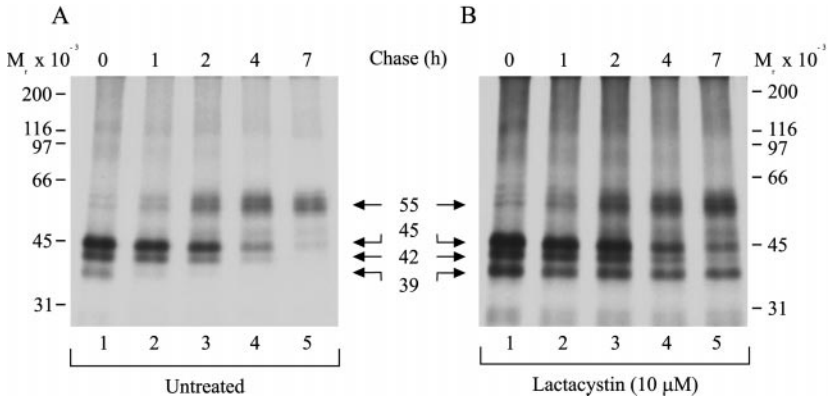

FIG. 1. **HδOR synthesis and maturation in untreated and lactacystin-treated HEK-293S cells.** HEK-293S-hδOR-FLAG cells were untreated (*A*) or treated with 10 μM lactacystin (*B*) for 3 h, pulse-labeled for 60 min with [^{35}S]methionine/cysteine, and then chased for the indicated times in the absence (*A*) or continued presence of the proteasome inhibitor (*B*). Cellular lysates were prepared and the solubilized receptors isolated by a two-step immunoprecipitation using the anti-FLAG M2 antibody as described under "Experimental Procedures." The samples were analyzed by SDS-PAGE and fluorography and the dried gels exposed to x-ray films. The fluorographs shown are representative of four independent experiments. *Arrows* indicate the molecular weights of the different receptor forms. Markers used to calibrate the gels are indicated (from the top: myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase).

taining an excess of unlabeled methionine, cell lysates were prepared and the receptors isolated by immunoprecipitation (Fig. 1). A major band of M_r 45,000 and two minor ones of M_r 42,000 and 39,000 were apparent in untreated cells at the end of the pulse (Fig. 1*A*). During the subsequent chase, label incorporated into these bands decreased and a slower, more diffusely migrating band of M_r 55,000 appeared, representing the fully mature receptor (40). When the cells were treated with lactacystin, an increase in labeling of all receptor species was detected (Fig. 1*B*). Furthermore, proteasome inhibition significantly retarded the disappearance of the M_r 45,000, 42,000, and 39,000 receptor species, the half-life of the last one being the most dramatically affected (Table I). Similar findings were obtained using two other proteasome inhibitors, the peptide aldehydes MG-132 and PSI (45) (data not shown). Taken together, these results suggest an active role of proteasomes in the elimination of newly synthesized hδORs. The fact that lactacystin promoted increase in receptor labeling, even within a shorter pulse period of 15 min, suggests that targeting to degradation begins very early, either cotranslationally or immediately after the receptor synthesis is complete. Inhibition of this rapid degradation is most likely responsible for the increase in the amount of mature receptors observed in pulse-chase labeling experiments (compare *lanes 4* and *5* in Fig. 1, *A* and *B*). This increase was accompanied by a 38% increase in the number of [^{3}H]bremazocine-binding sites detected. Our findings are not due to the presence of a FLAG-epitope tag at the C terminus of the receptor since the same results were also obtained using a receptor harboring the tag at its N terminus (data not shown). Unfortunately, the lack of a high affinity and selective antibody for the hδOR makes it impossible to carry out these experiments on the native untagged receptor.

The M_r 42,000 and 39,000 hδOR Species Represent Receptors Carrying One or No N-Linked Oligosaccharides, Respectively—It has been reported previously that *N*-linked oligosaccharides of newly synthesized glycoproteins are removed before proteasomal degradation (13–17, 26, 35, 42, 46). We therefore assessed whether the smaller molecular weight receptor species of M_r 42,000 and 39,000 represent deglycosylated receptors rather than proteolytic fragments. For this purpose, immunoprecipitated [^{35}S]methionine/cysteine pulse-labeled samples were subjected to enzymatic deglycosylation using PNGase F to remove the two *N*-linked glycans from the receptor (40). As can be seen in Fig. 2 (*lane 5*), complete deglycosylation of the M_r 45,000 receptor precursor led to the appearance of a single

TABLE I
Half-lives of the hδOR species in the absence and presence of lactacystin

Relative intensities of the labeled receptor species were quantified from fluorographs of pulse-chase experiments, an example of which is presented in Fig 1, by densitometric scanning. The half-lives of the different receptor species were determined using the GraphPad Prism program, version 2.01, and the data represent the mean ± S.E. of four independent experiments. Statistical significance of the differences was assessed using the two-tailed Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$.

Receptor species	Half-life	
	Untreated	Lactacystin
M_r	*min*	
45,000	125 ± 11	230 ± 49**
42,000	97 ± 7	184 ± 29***
39,000	37 ± 2	413 ± 88***

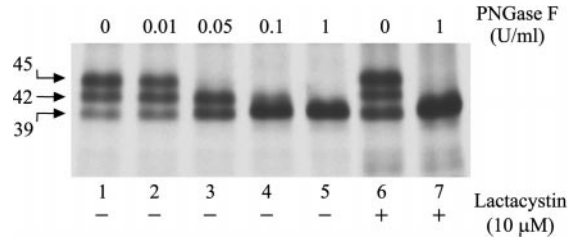

FIG. 2. **Deglycosylation of the metabolically labeled hδOR with PNGase F.** HEK-293S-hδOR-FLAG cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine in the absence or presence of lactacystin as indicated. Receptors were then isolated from cellular lysates by immunoprecipitation as described in Fig. 1. The immunoprecipitates were incubated for 16 h at 37 °C with increasing concentrations (0.01–1 unit/ml) of PNGase F as described under "Experimental Procedures." The enzyme-free controls (*lanes 1* and *6*) contained buffer only. Reactions were stopped by adding SDS-sample buffer followed by SDS-PAGE and fluorography.

species migrating at M_r 39,000. The fact that the M_r 42,000 species was found to be an intermediate in the stepwise deglycosylation of the receptor precursor suggests that this species represents a receptor carrying one *N*-linked glycan. The mobility of the M_r 39,000 species that was stabilized in lactacystin-treated cells was indistinguishable from that of the PNGase F-digested core-glycosylated M_r 45,000 receptor form (Fig. 2, compare lanes *5*–*7*), confirming the fact that this species represents an intact receptor polypeptide with no *N*-linked glycans. Protein translocation into the ER membrane and core

glycosylation are believed to occur cotranslationally in mammalian cells (47). Thus, it is very likely that the M_r 39,000 receptor species represents a previously core-glycosylated receptor that has been deglycosylated and is an intermediate in the degradation pathway.

The M_r 39,000 hδOR Species Dislocates from the ER Membrane and Accumulates in the Cytosol—To further verify the nature and the subcellular localization of the M_r 39,000 hδOR species, fractionation of cellular homogenates was performed after metabolic labeling (Fig. 3). Receptors were immunoprecipitated from four fractions: 1000 × *g* pellet containing cellular debris, nuclei and trapped soluble proteins, 10,000 × *g* pellet containing crude membranes, 100,000 × *g* pellet containing residual microsomal membranes, and 100,000 × *g* supernatant containing cytosolic proteins. As a control, the ER membrane protein, calnexin, and the cytosolic protein Hsp70 were immunoprecipitated from aliquots of the same four fractions. Calnexin was detected in the membrane fraction (Fig. 3*B*, *lanes 3* and *4*) but not in the cytosolic one (*lanes 7* and *8*), whereas the opposite was true for Hsp70 (Fig. 3*C*). As expected, both proteins were also detected in the 1000 × *g* pellet (Fig. 3, *B* and *C*, *lanes 1* and *2*). As can be seen in Fig. 3A, in addition to be found in the membrane fraction, the M_r 39,000 receptor species was recovered from the cytosolic fraction of lactacystin-treated (*lane 8*) but not of untreated cells (*lane 7*). This is in agreement with the notion that it represents an intermediate in the degradation pathway that would normally be broken down by the cytosolic proteasomes. Higher molecular weight bands forming a regularly spaced ladder, of which the smallest had an apparent molecular weight of 45,000, were also found in the cytosolic fraction of lactacystin-treated cells (Fig. 3*A*, *lane 8*). This M_r 45,000 species does not represent the core-glycosylated receptor precursor seen in Fig. 3*A* (*lanes 1–4*), because PNGase F was not able to decrease its molecular weight (Fig. 3*D*, *lanes 3* and *4*). It is more likely that the ladder and the accompanying high molecular weight smear comprise polyubiquitinated forms of the M_r 39,000 receptor (see below).

The fact that inhibition of proteasomal degradation significantly increased the amount of the M_r 39,000 hδOR species in the cytosolic fraction (Fig. 3*A*, *lanes 7* and *8*), suggests that this species may dislocate from the ER membrane and accumulate in the cytosol prior to proteasomal degradation. To test this hypothesis, receptors were immunoprecipitated from the soluble fraction after pulse-chase labeling of lactacystin-treated cells. As seen in Fig. 3*E*, a small amount of the M_r 39,000 receptor species was present in the cytosol at the end of the pulse. However, the amount detected clearly increased during the chase, indicating that the M_r 39,000 species originates from a pool of newly synthesized receptors that are retained in the ER and not from a failure or inefficiency of the translocation machinery. Thus, this species most likely results from deglycosylation of the M_r 45,000 receptor precursor that is destined for degradation and eventually dislocates from the ER membrane. Removal of the *N*-linked glycans most likely occurs prior to retrotranslocation to the cytosol since the deglycosylated hδOR species was found both in the cytosolic and the membrane fractions (Fig. 3*A*, *lanes 8* and *4*, respectively).

Inhibition of Proteasomal Degradation Leads to Accumulation of Polyubiquitinated hδORs—To directly test the hypothesis that the hδOR is modified by ubiquitination, cellular lysates from [^{35}S]methionine/cysteine pulse-labeled cells were subjected to sequential immunoprecipitation. First, the hδORs were immunoprecipitated with the anti-FLAG M2 antibody and the purified material was denatured by SDS. Proteins were then reimmunoprecipitated using an anti-ubiquitin antibody. All these immunoprecipitation steps (Fig. 4, *A* and *B*) were carried out in the presence of NEM to prevent inappropriate aggregation of proteins. In lactacystin-treated cells, the immunoprecipitated material migrated as a high molecular weight smear that extended upwards toward the top of the gel (Fig. 4*A*, *lane 4*), a feature that is characteristic of ubiquitinated

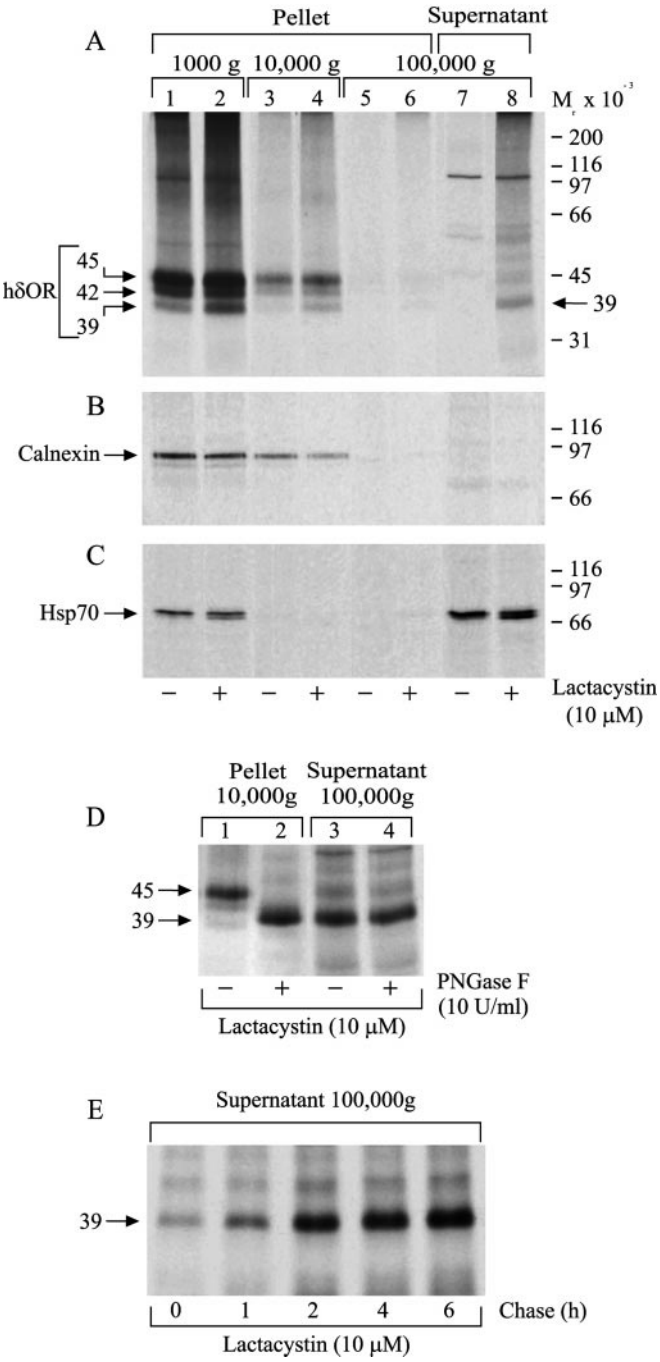

FIG. 3. **Subcellular fractionation of metabolically labeled HEK-293S-hδOR-FLAG cells.** HEK-293S-hδOR-FLAG cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine in the presence or absence of lactacystin as indicated and either harvested immediately (*A–D*) or chased for the indicated times in the continued presence of the proteasome inhibitor (*E*). The total cellular homogenates were fractionated by differential centrifugation as described under "Experimental Procedures." HδOR (*A*, *D*, and *E*), calnexin (*B*), and Hsp70 (*C*) were immunoprecipitated from the fractions obtained using the appropriate antibodies. The immunoprecipitates were then either analyzed directly by SDS-PAGE and fluorography (*A–C* and *E*) or deglycosylated with PNGase F prior to SDS-PAGE (*D*). *Arrows* indicate migration of the different receptor species, calnexin and Hsp70. Results shown are representative of four (*A–C*) or two (*D* and *E*) independent experiments.

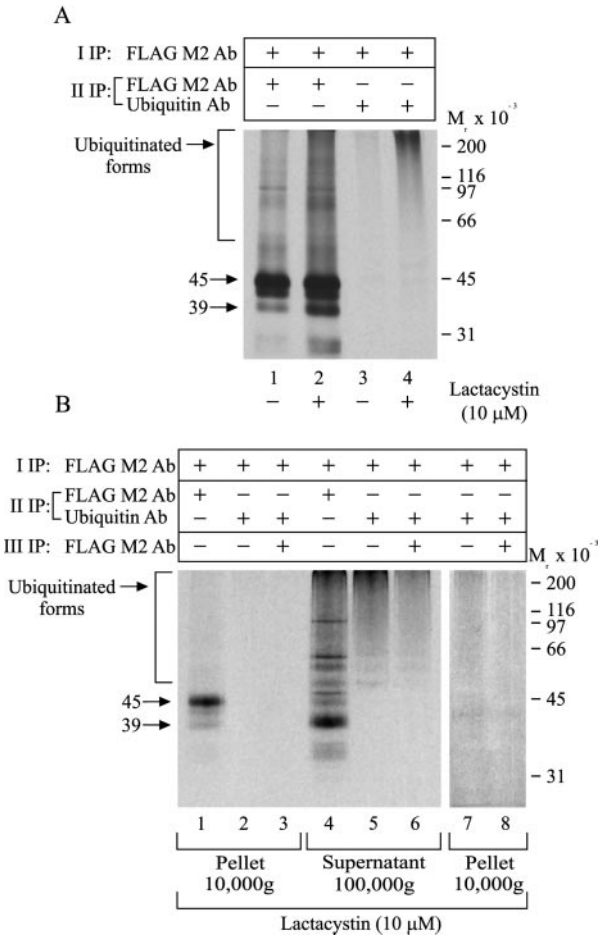

FIG. 4. **Immunoprecipitation of the ubiquitinated hδOR species from metabolically labeled HEK-293S-hδOR-FLAG cells.** HEK-293S-hδOR-FLAG cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine in the absence or presence of lactacystin as indicated, and cellular lysates were prepared (*A*) or membrane (*B*, *lanes 1–3*, *7*, and *8*) and soluble fractions (*B*, *lanes 4–6*) were isolated. The total pool of receptors (*A* (*lanes 1* and *2*) and *B* (*lanes 1* and *4*)) and the ubiquitinated receptor forms (*A* (*lanes 3* and *4*) and *B* (*lanes 2*, *3*, and *5–8*)) were isolated by sequential immunoprecipitation using the anti-FLAG M2 and the anti-ubiquitin antibodies as indicated. The immunoprecipitates were then subjected to SDS-PAGE and fluorography. *Lanes 7* and *8* in *B* represent longer exposures of *lanes 2* and *3*, respectively. Results shown are representative of three independent experiments.

proteins (8, 10, 17, 20, 22, 33, 35, 48, 49). This smear is probably due to the variable number of ubiquitin molecules that are added to individual receptor molecules during the process of polyubiquitination. Ubiquitinated proteins were barely detectable in the absence of lactacystin (Fig. 4*A*, *lane 3*), indicating that they normally are efficiently disposed by the proteasomes.

To assess whether the ubiquitinated hδOR species are associated with the ER membranes or represent dislocated cytosolic receptors, [^{35}S]methionine/cysteine-labeled receptors were subjected to sequential immunoprecipitation following fractionation of lactacystin-treated cells. As seen in Fig. 4*B*, the presence of ubiquitinated receptors in the cytosolic fraction could be easily detected following re-immunoprecipitation of the purified hδORs with the anti-ubiquitin antibody (*lane 5*). Although hardly any ubiquitinated material could be seen in the membrane fraction (*lane 2*), longer exposure revealed that some ubiquitinated receptors were also associated with this fraction (*lane 7*). These results suggest that ubiquitinated receptors accumulate in the cytosol after the receptor dislocates from the ER membrane but that addition of ubiquitin molecules to the receptor begins prior to dislocation. To further verify that the receptor itself was being ubiquitinated and did not coimmunoprecipitate with other ubiquitinated proteins, the anti-ubiquitin antibody immunoprecipitates were purified a second time with the anti-FLAG M2 antibody following SDS denaturation. As expected for an ubiquitinated receptor, the diffusely migrating material was recovered again (Fig. 4*B*, *lane 6*). The distinct higher molecular weight species detected in Fig. 4 (*panels A* (*lane 2*) and *B* (*lane 4*) but not in *panels A* (*lane 4*) and *B* (*lane 5*)) most likely represent labeled proteins that interact with the newly synthesized receptor in a SDS-resistant manner or receptor oligomers that are resistant to NEM. Polyubiquitination of the receptor did not result from the presence of a FLAG-epitope tag at its C terminus since a N-terminally tagged receptor form was also found to be ubiquitinated (data not shown).

The observation that a significant proportion of the soluble receptors migrates as a nonubiquitinated M_r 39,000 species (Fig. 4*B*, *lane 4*) is consistent with previous observations (8, 17, 35, 48). Such nonubiquitinated species probably results from constant deubiquitination by ubiquitin isopeptidases (50). It is very unlikely that this form could result from a lactacystin-mediated interference in cotranslational protein translocation into the ER membrane since this species accumulates in the cytosol during the chase period of the metabolic labeling experiment shown in Fig. 3*E*.

The ER-retained Newly Synthesized hδORs Are Retrotranslocated to the Cytosol via the Sec61 Complex—The cellular fractionation experiments clearly showed that the ER-retained newly synthesized hδORs dislocate from the ER membrane into the cytosol (Figs. 3 (*A* and *E*) and 4*B*). To assess whether this dislocation involves retrotranslocation through the Sec61 translocon, as has been reported for other membrane proteins that are degraded by the proteasomes (13, 17, 21), coimmunoprecipitation experiments with the β-subunit of the Sec61 complex were performed. Cells treated or not with lactacystin were pulse-labeled with [^{35}S]methionine/cysteine and chased for 60 min. As shown in Fig. 5*A* (*lanes 5* and *6*), re-immunoprecipitation of the Sec61β immunoprecipitates with the anti-FLAG M2 antibody recovered the M_r 45,000, 42,000, and 39,000 receptor species, suggesting that they interact with the translocon. This interaction was shown to be specific because these species were absent when the anti-Sec61β antibody was replaced with a preimmune serum in the first immunoprecipitation step (Fig. 5*A*, *lanes 3* and *4*) or when the anti-FLAG M2 affinity resin was replaced with the anti-mouse IgG agarose in the second step (data not shown). Although the three receptor species were found to coimmunoprecipitate with the β-subunit of the Sec61 complex in both untreated and lactacystin-treated cells, treatment with the proteasome inhibitor increased their amount and the M_r 39,000 species became prevalent (Fig. 5*A*, compare *lanes 5* and *6*). The sensitivity of the M_r 45,000 and the M_r 42,000 receptors to PNGase F (Fig. 5*B*, *lanes 3* and *4*) confirmed their identity as the precursor and the deglycosylation intermediate, respectively. Because the cells were chased for 60 min before the lysates were prepared, the receptor species that interact with the translocon are more likely to be in transit to exit the ER rather than being translocated into the membrane. The fact that the fully deglycosylated M_r 39,000 receptor as well as the glycosylated M_r 42,000 and 45,000 species all associate with the Sec61 complex confirms that deglycosylation occurs before complete retrotranslocation of the receptor to the cytosol.

DISCUSSION

The results of the present study indicate that core-glycosylated hδOR precursors that are not competent to enter the maturation pathway are transported to the cytosol in a process

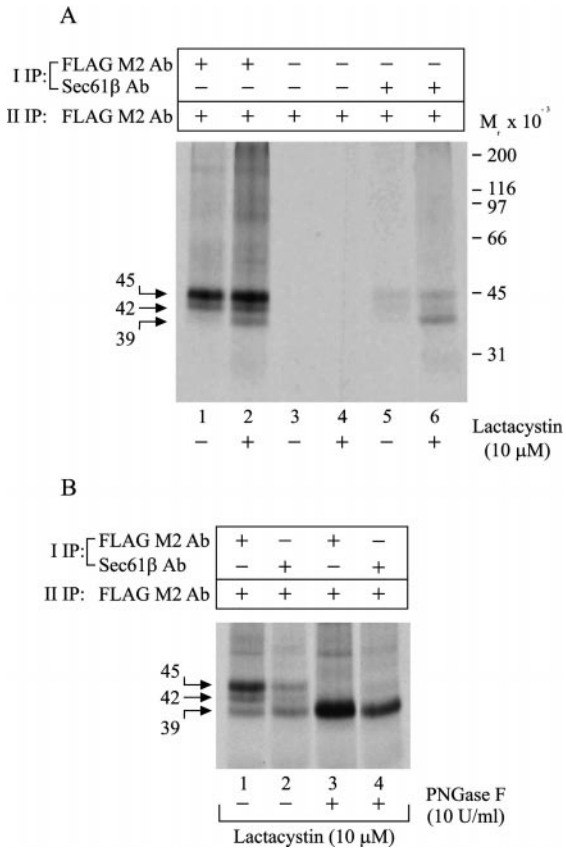

FIG. 5. **Immunoprecipitation of the Sec61 complex-associated hδOR species from metabolically labeled HEK-293S-hδOR-FLAG cells.** HEK-293S-hδOR-FLAG cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine and chased for 60 min in the presence or absence of lactacystin as indicated. The total pool of receptors (*A* (*lanes 1* and *2*) and *B* (*lanes 1* and *3*)) and those interacting with the Sec61 complex (*A* (*lanes 5* and *6*) and *B* (*lanes 2* and *4*)) were isolated from total lysates by sequential coimmunoprecipitation using the anti-FLAG M2 and the anti-Sec61β antibodies as indicated, and the immunoprecipitates were subjected to SDS-PAGE and fluorography either directly (*A*) or after PNGase F digestion (*B*). The results shown are representative of three independent experiments.

that involves the Sec61 translocon, removal of *N*-linked oligosaccharides, and addition of ubiquitin molecules. This process leads to targeting of a significant proportion of newly synthesized receptors for degradation by the 26 S proteasomes. The hδOR thus appears to be a protein that, due to inherent difficulties in acquiring its native conformation, is very efficiently disposed of by the ER quality control. This elimination could result either from a high level of irreversible misfolding or from limitations in the folding kinetics. Because proteasomal blockade was found to enhance ER export and maturation of the receptor, kinetic limitations leading to the premature degradation of folding intermediates most likely contribute to the observed phenomenon. Irrespective of the nature of the receptor forms that are targeted for degradation (irreversibly misfolded or folding intermediates), the present results show that the ER quality control can eliminate a high proportion of wild type proteins. This does not seem to be unique to the hδOR since Schubert and colleagues (36) recently suggested that at least 30% of newly synthesized proteins are degraded by the proteasomes (36). It was hypothesized that degradation of what was referred to as defective ribosomal products could serve as an important adaptation for early antigen presentation during viral infection (36, 51). Whether the high rate of degradation of the newly synthesized hδORs also serves a biological purpose remains to be determined. Alternatively, this process could merely reflect the exceedingly stringent control system monitoring the rapid but error-prone protein synthesis machinery. Because expression to a relatively high level (~10 pmol/mg of protein for the clone used) is needed to perform the type of studies presented here, one cannot entirely exclude that overexpression is in part responsible for the proteasomal-mediated degradation observed. However, we feel that this is unlikely since identical data were obtained using a clone expressing a smaller number of receptors (4 pmol/mg of protein; data not shown). Additionally, the fact that several endogenously expressed ER proteins have been shown to be targeted for proteasomal degradation in a regulated manner (27–29) reinforces the idea that this proteolytic pathway is a general mechanism used to regulate protein biosynthesis.

Several lines of evidence demonstrate the role of cytoplasmic proteasomes in the high degradation rate of newly synthesized hδORs. First, the turnover rate of the core-glycosylated M_r 45,000 precursor as well as that of the two smaller receptor species of M_r 42,000 and 39,000 was reduced considerably in cells treated with proteasomal inhibitors, lactacystin, MG-132, and PSI. Second, the amount of the fully mature receptor was increased upon proteasomal inhibition. Finally, the M_r 39,000 receptor and its polyubiquitinated forms accumulated in the cytoplasm following proteasomal blockade. Given that the M_r 39,000 receptor species contains the full-length receptor polypeptide, these data support the hypothesis that integral membrane proteins are released completely from the ER membrane prior to proteasomal degradation rather than cleaved partially within the membrane (13–15, 17, 42).

Association of the hδOR precursor and that of the two full-length deglycosylated intermediates with the Sec61 translocon supports the recently proposed model, suggesting that retrotranslocation of misfolded or incompletely folded ER proteins to the cytoplasm is mediated by the Sec61 complex (13, 17, 21, 46, 52, 53), the multimeric protein complex that is also responsible for the cotranslational translocation of proteins into the ER (54). Like the hδOR, the major histocompatibility class I molecule and the ΔF508 mutant of the CFTR were found to interact with the Sec61 translocon during their transit out of the ER, which leads to their degradation by the proteasomes (13, 17). It can be hypothesized that dislocation of these integral membrane proteins may involve a reverse process of membrane integration involving lateral opening of the translocon toward the lipid bilayer (39, 54). However, how these misfolded or incompletely folded proteins are recognized and delivered to the dislocation machinery is unknown. Similarly, the precise mechanism by which these proteasomal substrates are extracted from the lipid bilayer as well as the origin of the driving force for their retrotranslocation are still unresolved issues.

The finding that the M_r 39,000 hδOR degradation intermediate accumulates in the cytosol following lactacystin treatment suggests that proteasomal function is not obligatory for dislocation of membrane proteins from the ER membrane. However, this does not rule out the possibility that, in normal conditions (*i.e.* when proteasomes are not impaired), dislocation and degradation of misfolded or incompletely folded proteins may be tightly coupled events. In fact, three observations in the present study suggest coupling between proteasome function and dislocation of proteins from the ER membrane. Inhibition of the proteasome was found to: 1) increase the amount and reduce the turnover-rate of the membrane bound M_r 42,000 and 45,000 forms of the receptor, 2) increase the total amount of receptor coimmunoprecipitated with the β-subunit of the Sec61 complex, and 3) increase the relative proportion of the translocon-associated M_r 39,000 degradation intermediate that ultimately dislocates from the ER membrane into

the cytosol. Thus, although proteasomal function is not obligatory for dislocation, its impairment appears to lead to attenuation of the process and prolongation of association with the translocon. These results are consistent with findings showing that mutant (21) and chimeric (55) transmembrane proteins were stabilized within the ER membrane of yeast mutants expressing functionally impaired proteasomes. Coupling of dislocation and degradation could be achieved by direct transfer of protein substrates to the proteasomes. Indeed, although proteasomes are mainly detected in the cytosol and the nucleus, some have been found to be associated with ER membranes (56). Coupling of membrane dissociation and proteolysis would clearly increase the efficiency of proteolysis, because direct transfer of the unfolded retrotranslocated proteins to the proteasome would eliminate the need for additional unfolding of the substrate that is a prerequisite for proteasomal degradation (31). At the same time, the proteasome itself, via its regulatory subcomplex, PA700, could provide the driving force for the retrotranslocation (31). Such coupling would also be a very effective way to prevent cytoplasmic aggregation of dislocated membrane proteins. In this context, it is noteworthy that proteasomal blockade has been shown to promote the formation of high molecular weight insoluble aggregates (8, 16). For example, both wild type and ΔF508 mutant forms of the CFTR were shown to accumulate, together with cytosolic chaperones, in a specific pericentriolar structure, called the aggresome (57, 58).

Inhibition of proteasomal degradation resulted in accumulation of polyubiquitinated forms of the hδOR in the cytosol, suggesting that this modification may target the misfolded or incompletely folded receptors to the proteasome. Based on the finding that a small amount of ubiquitinated receptor forms was detected in the membrane fraction, it can also be envisioned that polyubiquitination could provide a direct signal for proteasome recruitment to the ER membrane. However, it is unclear whether ubiquitination is a prerequisite for the rapid breakdown of the receptor, as has been shown for many other integral membrane and secretory proteins that are degraded by the proteasomes. For example, many misfolded or unassembled ER proteins were shown to be stabilized by mutant ubiquitin-activating (8, 46, 48) or ubiquitin-conjugating enzymes (9, 10, 18, 20, 21, 59) or by dominant-negative ubiquitin mutants that prevent polyubiquitination of substrate proteins (8–10, 18, 21, 48, 60). When considering the enzymes that could be responsible for the ubiquitination of the hδOR while it is still membrane-bound, it is noteworthy that two yeast ubiquitin-conjugating enzymes, the Ubc6p and the Ubc7p, have been implicated in the ubiquitination and degradation of ER proteins (9, 10, 18, 20, 21, 28, 59). Both of these enzymes are localized to the ER, thus possibly enabling ubiquitination of misfolded or incompletely folded proteins prior to their dislocation from the membrane. The Ubc6p is an integral membrane protein, which is anchored to the membrane via a C-terminal hydrophobic sequence (61), whereas the Ubc7p is docked onto the membrane protein Cue1p (59). In a recent report, several mammalian homologs of the Ubc6p were identified (62). Whether these enzymes are actually involved in ubiquitination and degradation of ER proteins remains to be determined.

Proteasomal degradation of the hδOR is preceded by removal of the two *N*-linked oligosaccharides from the M_r 45,000 receptor precursor. This is in agreement with previous reports on glycoproteins for which proteasomal degradation has been described (13–17, 26, 35, 42, 46). Although PNGase-type activity has been detected in both cytosolic and microsomal membrane fractions of mammalian tissues (63, 64), the enzymes mediating the deglycosylation of misfolded or incompletely folded proteins as well as their precise location have remained elusive. However, recent cloning of a cytosolic yeast PNGase (65) may be pointing to the first candidate involved in deglycosylation of proteasomal substrates. In any case, our data suggest that deglycosylation of the hδOR occurs prior to dislocation from the ER membrane and the translocon. Indeed, neither the M_r 45,000 receptor precursor nor the partially deglycosylated M_r 42,000 form were found in the cytosolic fraction. Furthermore, the fully deglycosylated M_r 39,000 degradation intermediate was found to coimmunoprecipitate with the β-subunit of the Sec61 complex.

Whether the degradation pathway uncovered for the hδOR in the present study is a unique characteristic of this receptor or represents a general mechanism regulating the maturation and cell surface expression of most GPCRs remains an open question. Indeed, very little is known about the processes involved in the folding/maturation/degradation of other GPCRs. Inhibition of proteasome function was found to lead to an increase in the β_2-adrenergic receptor expression in HEK-293 cells (66) and only three GPCRs, rhodopsin (67) and the yeast α- and a-mating factor receptors (68, 69), have previously been shown to be ubiquitinated. For rhodopsin, no specific role has yet been attributed to the modification whereas ubiquitination has been proposed to be an internalization signal for the activated α-mating factor receptor, leading to its lysosomal/vacuolar degradation (68, 70–72). Interestingly, monoubiquitination rather than polyubiquitination normally associated with the proteasome degradation pathway appears to be sufficient to promote such internalization (70, 72). It will be interesting to determine whether ubiquitination of the hδOR and other mammalian GPCRs can, in addition to its role in the proteasome-mediated degradation of ER-retained receptors, also be implicated in their internalization and lysosomal degradation. Such a dual role for ubiquitination has been proposed for a number of yeast and mammalian membrane proteins (38) but has not been documented yet for mammalian GPCRs.

Results of the present study support the notion that the cytosolic proteasomal disposal mechanism is involved in the elimination of newly synthesized hδORs, resulting ultimately in degradation of even salvageable folding intermediates. This raises several important questions. For example, it becomes important to determine the mechanisms by which the stringent ER monitoring system discriminates conformational variants of the receptor and sorts them either for degradation or allows their transport downstream within the secretory pathway. Future studies are also needed to identify the pathways and components that facilitate folding of the receptor and to determine whether these are under the control of dynamic regulatory processes. Although inhibiting proteasomal function favored ER export and maturation of the receptor, a large proportion of the newly synthesized receptors still remained entrapped within the ER. Whether approaches acting at earlier off-pathway steps could be more effective in enhancing receptor folding still remains to be investigated.

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Newly Synthesized Human δ Opioid Receptors Retained in the Endoplasmic Reticulum Are Retrotranslocated to the Cytosol, Deglycosylated, Ubiquitinated, and Degraded by the Proteasome

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