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Export from the Endoplasmic Reticulum Represents the Limiting Step in the Maturation and Cell Surface Expression of the Human δ Opioid Receptor*

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Synthesis and maturation of G protein-coupled receptors are complex events that require an intricate combination of processes that include protein folding, post-translational modifications, and transport through distinct cellular compartments. Relatively little is known about the nature and kinetics of specific steps involved in these processes. Here, the human δ opioid receptor expressed in human embryonic kidney 293S cells is used as a model to delineate these steps and to establish the kinetics of receptor synthesis, glycosylation, and transport. We found that the receptor is synthesized as a core-glycosylated M_r 45,000 precursor that is converted to the fully mature M_r 55,000 receptor with a half-time of about 120 min. In addition to trimming and processing of two *N*-linked oligosaccharides, maturation involves addition of O-glycans containing *N*-acetylgalactosamine, galactose, and sialic acid. In contrast to *N*-glycosylation, which is initiated co-translationally and is completed when the protein reaches the *trans*-Golgi network, *O*-glycosylation was found to occur only after the receptor exits from the endoplasmic reticulum (ER) and was terminated as early as the *trans*-Golgi cisternae. Once the carbohydrates are fully processed and the receptor reaches the *trans*-Golgi network, it is transported to the cell surface in about 10 min. The exit from the ER was found to be the limiting step in overall processing of the receptor. This indicates that early events in the folding of the receptor are probably rate-limiting and that receptor folding intermediates are retained in the ER until they can adopt the correct conformation. The overall low efficiency of receptor maturation, less than 50% of the precursor being processed to the fully glycosylated protein, further suggests that only a fraction of the synthesized receptors attain properly folded conformation that allows exit from the ER. This indicates that folding and ER export are key events in control of receptor cell surface expression. Whether or not the low efficiency of the ER export is a general feature among G protein-coupled receptors remains to be investigated.

G protein-coupled receptors (GPCRs)[1] constitute a true superfamily of membrane proteins with more than 1000 individual members. A variety of chemical messengers, such as neurotransmitters and hormones, act on these receptors, and they also serve as sensory molecules in the olfactory and visual systems. Although overall they do not share extensive sequence identity, they are believed to have a similar three-dimensional structure that is characterized by seven hydrophobic transmembrane domains connected by alternating intracellular and extracellular loops (1–5). The N terminus is extracellular, whereas the C terminus lies within the cytosol. Several post-translational modifications, most notably glycosylation, phosphorylation, and palmitoylation, also have an impact on the structure and function of these important membrane proteins.

The GPCRs are expressed at very low levels in natural tissues and cells, and thus progress in biochemical characterization of these proteins has been greatly facilitated by the use of heterologous expression systems. Over the last few years, this approach has been used to address a number of fundamental questions related to both structural and functional features of these proteins. In some cases, however, expression of wild type (6, 7) or mutant (8–11) receptors in transfected cells has resulted in low levels of functional receptors at the cell surface. Similarly, a number of inherited disorders have been traced to mutations within the GPCRs that result in proteins with reduced cell surface expression (12–16). In many cases this was proposed to be due to misfolding and/or deficient targeting of the protein. Recently, a few GPCRs, such as olfactory receptors (17), visual opsins (18–20), the calcitonin gene-related peptide receptor, and the adrenomedullin receptor (21), were shown to need special chaperone/escort proteins to be expressed at the cell surface. In the case of the γ-aminobutyric acid type B-1 receptor, heterodimerization with another GPCR, the γ-aminobutyric acid type B-2 receptor, was shown to be required (22). In general, however, very little is known about how the GPCRs assume their correct conformation and acquire their covalent modifications and are trafficked from the endoplasmic reticulum (ER) to the plasma membrane.

In an attempt to delineate the basic steps involved in the biosynthesis, maturation, post-translational modifications and

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; BFA, brefeldin A; BSA, bovine serum albumin; DDM; n-dodecyl-β-D-maltoside: DMEM, Dulbecco's modified Earl's medium; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; hδOR, human δ opioid receptor; HEK-293S; human embryonic kidney 293S; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PNGase F, peptide-*N*-glycosidase F; PAGE, polyacrylamide gel electrophoresis; SNC-80, ((+)-4-[(αR)-α-((2S, 5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]N, N,-diethylbenzamidin); STI, soybean trypsin inhibitor; WGA, wheat germ agglutinin.

targeting of these proteins, the human δ opioid receptor (hδOR) was used as an experimental model. This receptor, along with the other opioid receptors, μ and κ, mediates the actions of endogenous opioid peptides as well as opiate alkaloids in the nervous system (23, 24). The deduced primary structure of the hδOR predicts a protein of 372 amino acids with two consensus *N*-glycosylation sites in its N-terminal extracellular domain and a calculated weight of approximately 40,000 (25, 26). To date no study has directly investigated the steps leading from the newly synthesized polypeptide to the mature receptor at the plasma membrane. Here, using a heterologous expression system, we studied the rates of biosynthesis, maturation, and metabolic turnover of the hδOR in discrete cellular compartments in relation with the glycosylation events that occur during the transit of the protein from the ER to the cell surface. We found that protein export from the ER represents the rate-limiting step in the processing of the newly synthesized receptor.

EXPERIMENTAL PROCEDURES

Materials—EXPRE^{35}S^{35}S protein labeling mix (1175 Ci/mmol), [9-^{3}H(N)]bremazocine (26.6 Ci/mmol), [2,8-^{3}H]adenine (31.7 Ci/mmol), and D-[6-^{3}H(N)]glucosamine (22.7 Ci/mmol) were purchased from NEN Life Science Products. Endo-β-*N*-acetylglucosaminidase H (Endo H) of *Streptomyces plicatus*, recombinant from. *Escherichia coli* (EC 3.2.1.96), peptide-*N*-glycosidase F (PNGase F) of *Flavobacterium meningosepticum*, recombinant from *E. coli* (EC 3.5.1.52), and *O*-glycosidase from *Diplococcus pneumoniae* (EC 3.2.1.97) were obtained from Roche Molecular Biochemicals. Neuraminidase from *Vibrio cholerae* (EC 3.2.1.18), monensin, and brefeldin A (BFA) were from Calbiochem. Cell culture reagents were either from Life Technologies, Inc. or Wisent. Tunicamycin, wheat germ agglutinin (WGA)-agarose, anti-FLAG M2 monoclonal antibody, anti-FLAG M2 affinity resin, and FLAG peptide were products of Sigma. EZ-linked™ sulfo-*N*-Hydroxysuccinimide (NHS)-biotin and Immunopure® immobilized streptavidin were from Pierce and the opioid agonists and antagonists from RBI, except SNC-80, which was purchased from Tocris. All the other reagents were of analytical grade and obtained from various commercial suppliers.

DNA Constructs and Site-directed Mutagenesis—The hδOR cDNA was subcloned into the pcDNA3 expression vector (Invitrogen) as described (27). The receptor was tagged at the C-terminal end with the FLAG epitope (DYKDDDDK) using the CLONTECH site-directed mutagenesis kit that is based on the method of Deng and Nickoloff (28). The construction was verified and confirmed by restriction enzyme mapping and DNA sequencing.

Cell Culture and Transfection—Human embryonic kidney 293S (HEK-293S) cells were grown and maintained in Dulbecco's modified Earl's medium (DMEM) containing 10% (v/v) fetal calf serum, 1000 units of penicillin/ml, 1 mg streptomycin/ml, and 1.5 μg of fungizone/ml (complete DMEM) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were transfected by a modified calcium-phosphate precipitation method (29), and clones of stably transfected cells were selected using 400 μg/ml of G-418 (Calbiochem). Saturation binding of [^{3}H]bremazocine (see below) was used to estimate the level of receptor expression.

Radioligand Binding Assays—Cells were harvested in phosphate-buffered saline (PBS) at 80–90% confluency, quick frozen in liquid nitrogen, and stored at −80 °C. After thawing, the cell pellets were suspended in buffer A (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor (STI), and 10 μg/ml benzamidine) and homogenized with a Polytron homogenizer (Ultra-Turrax T-25, Janke and Kunkel) using three bursts of 5 s at maximum setting. Homogenates were centrifuged at 700 × *g* for 5 min, and the pellets were rehomogenized and recentrifuged. The combined supernatants were further centrifuged at 27,000 × *g* for 20 min, and the pellets were washed twice with buffer A. The pellets containing the crude membrane fractions were resuspended in buffer A at a concentration of 1–5 mg/ml and either used immediately or quick frozen in liquid nitrogen and stored at −80 °C. Protein was assayed by using Bio-Rad's DC assay kit with bovine serum albumin (BSA) as a standard.

Saturation and competition binding assays for the wild type and the FLAG-tagged hδOR were carried out using 5 μg of membrane protein in a final volume of 300 μl of buffer A supplemented with 0.1% BSA (w/v). For saturation experiments, triplicates contained 0.2–20 nM [^{3}H]bremazocine. For competition experiments, triplicates contained 1 nM [^{3}H]bremazocine and either SNC-80, Leu-enkephalin, or naloxone at final concentrations ranging from 0.1 pM to 100 μM. Nonspecific binding was determined using 10 μM naltrexone. After incubation at 25 °C for 60 min, the membranes were harvested by rapid filtration over Whatman GF/C filters presoaked for 60 min in 0.1% polyethyleneimine (v/v)/0.1% BSA (w/v) and washed three times with cold 25 mM Tris-HCl, pH 7.4. The radioactivity on the filters was determined after an overnight incubation in UniverSol™ scintillation fluid (ICN). Curve fitting and analysis of the binding data were performed using the GraphPad Prism program version 2.01.

cAMP Accumulation Assay—Intracellular cAMP levels were determined by measuring the conversion of [^{3}H]ATP to [^{3}H]cAMP following metabolic labeling with the [^{3}H]adenine, as described (30). Stably transfected HEK-293S cells were subcultured into 6-well culture plates at a density of 100,000 cells/well and allowed to recover for 48 h before the experiments. The cells were incubated at 37 °C for 16 h in complete DMEM containing 2 μCi/ml of [^{3}H]adenine, washed twice with PBS, and incubated further for 15 min in medium supplemented with 1 mM 3-isobutyl-1-methylxanthine, 100 μM forskolin, and SNC-80 at a final concentration of 1 fM to 1 μM. The reaction was stopped by aspirating the medium and adding 1 ml of ice-cold 5% trichloroacetic acid (w/v). After adding 100 μl of 5 mM ATP/5 mM cAMP, cells were lifted by a rubber policeman, and cellular proteins were removed by centrifugation (700 × *g*, 10 min). [^{3}H]ATP and [^{3}H]cAMP were measured in the supernatant following separation by sequential chromatography on Dowex and alumina columns, as described (31). Analysis of the data was performed using the GraphPad Prism program, version 2.01.

Purification of the Solubilized HδOR by Lectin Affinity Chromatography—Total cellular membranes prepared as described above were extracted (1 mg of membrane protein/ml) with buffer B (0.5% *n*-dodecyl-β-D-maltoside (DDM) (w/v), 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 140 mM NaCl, 5 μg/ml leupeptin, 5 μg/ml STI, 10 g/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM 1,10-phenantroline) by mixing the suspension on a magnetic stirrer for 60 min at 4 °C. After centrifugation at 100,000 × *g* for 60 min, the solubilized receptor was purified batchwise from the supernatant fraction by affinity chromatography on immobilized WGA. The resin (100 μl/mg of membrane protein) was incubated with the supernatant overnight at 4 °C with gentle agitation, pelleted, and washed five times with 1 ml of buffer C (buffer B containing 0.1% instead of 0.5% DDM (w/v)). The bound proteins were eluted by incubating the resin for 30 min at 4 °C under gentle agitation with 300 μl of buffer C containing 0.3 M *N*-acetylglucosamine. This was repeated three times, and the eluates were combined. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), aliquots of the solubilized material, unbound fraction, and the eluate were diluted 2-fold with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.001% (w/v) bromphenol blue).

Metabolic Labeling with [^{35}S]Methionine/Cysteine and [^{3}H]Glucosamine—Cells were subcultured in either 25 cm^2 or 75 cm^2 culture flasks and grown to 80–90% confluency. For [^{35}S]methionine/cysteine labeling, the 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 of [^{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 and the text. When labeling was performed in the presence of monensin, BFA, or tunicamycin, the reagents were added either 1 h (BFA and monensin) or 3 h (tunicamycin) before the pulse labeling at a final concentration of 10 μM (monensin) or 5 μg/ml (BFA and tunicamycin). For [^{3}H]glucosamine labeling, the cells were preincubated for 2 h in complete DMEM containing 1 g of glucose/liter and labeled for 6 h in the same medium containing 70 μCi/ml of [^{3}H]glucosamine.

Immunoprecipitation of the Metabolically Labeled HδOR—After the metabolic labeling, the cells were washed with and harvested in PBS, quick frozen in liquid nitrogen, and stored at −80 °C. After thawing, the cells were sonicated with a tip sonicator twice for 15 s in buffer D (25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml STI, 10 μg/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM PMSF, and 2 mM 1,10-phenantroline). Total cellular membranes were recovered by centrifugation at 27,000 × *g* for 20 min, washed twice with buffer D, and extracted with buffer E (0.5% DDM (w/v), 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 140 mM NaCl, 5 μg/ml leupeptin, 5 μg/ml STI, 10 μg/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM PMSF, and 2 mM 1,10-phenantroline). After centrifugation at 100,000 × *g* for 60 min, the receptor was immunoprecipitated from the supernatant fraction by using anti-FLAG M2 antibody resin. The polyclonal antibody used for Western blotting

TABLE I

Pharmacological comparison of the wild-type hδOR and the FLAG epitope-tagged hδOR expressed in HEK-293S cells

Ligand binding assays were carried out using membranes prepared from stably transfected HEK-293S cells expressing the wild type hδOR or the FLAG epitope-tagged hδOR, whereas cAMP accumulation assays were performed using intact cells. Saturation binding assays were performed using 0.2–20 nM [^{3}H]bremazocine to obtain the K_d and B_{max} values. K_i values were determined by competition of 1 nM [^{3}H]bremazocine with the indicated unlabeled opioid ligands (0.1 pM–100 μM). The inhibition of forskolin-stimulated (100 μM) cAMP accumulation by SNC-80 (1 -M–1 μM) was carried out to obtain the IC_{50} values. Analysis of the data was performed using the GraphPad Prism program, version 2.01. Data represent the means ± S.E. of three to five independent experiments performed in triplicate. There were no statistical differences between the values for the wild type and the epitope-tagged hδOR for any parameter.

Receptor	Ligand binding					cAMP accumulation SNC-80[d]
	K_i[a]			[^{3}H]Bremazocine		
	Naloxone	Leu-enkephalin	SNC-80	K_d[b]	B_{max}[c]	
	nM			*nM*	*pmol/mg protein*	*nM*
hδOR	88.1 ± 9.7	2.27 ± 0.43	0.76 ± 0.12	0.94 ± 0.13	24.2 ± 3.0	1.25 ± 0.87
hδOR-FLAG	99.4 ± 5.4	2.59 ± 0.27	0.73 ± 0.09	1.18 ± 0.26	23.9 ± 2.3	1.36 ± 0.74

[a] Equilibrium inhibition constant.
[b] Equilibrium dissociation constant.
[c] Maximal specific binding.
[d] IC_{50}, half-maximal effective inhibitory concentration.

(see below) that recognizes the native C-terminal end of the receptor was not suitable for immunoprecipitation. 20 μl of antibody-coupled resin equilibrated in buffer E supplemented with 0.1% BSA (w/v) was used to purify the receptor from one 25-cm^2 flask. The resin was incubated with the supernatant overnight at 4 °C with gentle agitation, pelleted, and washed twice with 500 μl of buffer E and four times with 500 μl of buffer F (buffer E containing 0.1% instead of 0.5% DDM (w/v)). The receptor was eluted by incubating the resin for 10 min at 4 °C with 100 μl of buffer F containing 175 μg of FLAG peptide/ml. This was repeated three times, and the eluates were combined. For SDS-PAGE, 100 μl of the combined eluates was concentrated down to 25 μl by membrane filtration over Microcon-30 concentrators (Millipore), and 25 μl of SDS sample buffer was added.

Biotinylation and Isolation of Cell Surface Proteins—For cell surface biotinylation, cells were washed three times with warm Dulbecco's PBS, cooled on ice, and incubated with gentle agitation at 4 °C for 30 min in Dulbecco's PBS containing 0.5 mg/ml of sulfo-NHS-biotin. Excess biotin was quenched by adding Tris-HCl, pH 7.4, to a final concentration of 50 mM and incubating the cells further for 10 min at 4 °C. The cells were then washed twice with cold Dulbecco's PBS and harvested.

Biotinylated cell surface proteins were isolated using immobilized streptavidin after solubilizing the total cellular membranes as described above. 25 μl of resin was used for 1 mg of solubilized membrane proteins. The resin was incubated with the 100,000 × *g* supernatant containing 0.1% BSA (w/v) for 2 h at 4 °C with gentle agitation, pelleted and washed five times with 500 μl of buffer E. The biotinylated proteins were eluted with 100 μl of SDS sample buffer containing 50 mM dithiothreitol by incubating the resin first for 15 min at 25 °C and then for 5 min at 95 °C. When cell surface proteins were isolated from metabolically labeled cells, the elution was carried out using 100 μl of 1% SDS (w/v), 25 mM Tris-HCl, pH 7.4, which was diluted with 900 μl of buffer E prior to purifying the receptor by immunoprecipitation.

Deglycosylation of the Solubilized and the Purified HδOR—Thawed total cellular membranes were washed with either buffer G (50 mM sodium phosphate, pH 5.5, 50 mM EDTA, 0.5 mM PMSF, 2 mM 1,10-phenantroline, 5 μg/ml leupeptin, 5 μg/ml STI, 10 μg/ml benzamidine; Endo H) or buffer H (50 mM Na-phosphate, pH 7.5, 50 mM EDTA, 0.5 mM PMSF, 2 mM 1,10-phenantroline, 5 μg/ml leupeptin, 5 μg/ml STI, and 10 μg/ml benzamidine; PNGase F). After pelleting the membranes at 27,000 × *g* for 20 min, they were extracted in the appropriate buffers containing 0.5% DDM (w/v) at 1 mg of membrane protein/ml on a magnetic stirrer for 60 min at 4 °C. Solubilized receptors were recovered in the supernatant of a 100,000 × *g* centrifugation for 60 min at 4 °C, and the enzymes were added at final concentrations of 100 milliunits/ml (Endo H) and 0.001–20 milliunits/ml (PNGase F). Samples were incubated at 37 °C for 16 h, and the reaction was terminated by adding SDS sample buffer.

The metabolically labeled purified receptor was deglycosylated in a similar manner following elution from the anti-FLAG M2 antibody resin with 1% (w/v) SDS, 50 mM sodium phosphate, pH 7.5. The enzymes were added at final concentrations of 25 milliunits/ml (Endo H), 20 units/ml (PNGase F) and 50 milliunits/ml (neuraminidase and *O*-glycosidase). Before the enzyme reaction, the eluates were diluted 10-fold with 0.5% (w/v) DDM in buffer G containing 1% 2-mercaptoethanol (Endo H), buffer H containing 1% 2-mercaptoethanol (PNGase F), or 50 mM sodium phosphate, pH 6.0, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml STI, and 10 μg/ml benzamidine (neuraminidase and *O*-glycosidase).

SDS-PAGE and Western Blotting—SDS-PAGE was performed as described (32), 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® (NEN Life Science Products) according to the manufacturer's instructions, dried, and exposed at −80 °C for 1–15 days, using Biomax MR film and intensifying screens (Kodak). The relative intensities of the labeled bands on the autoradiograms 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 a local background from each lane.

For Western blotting, the proteins resolved in SDS-PAGE were transferred electrophoretically from the gels onto nitrocellulose (Amersham Pharmacia Biotech) (50 mA, 16 h, Bio-Rad Mini-Trans Blot apparatus). The nitrocellulose-bound proteins were probed with either a polyclonal receptor antiserum directed against a peptide (SDGPGGGRAA) corresponding to the 10 amino acids at the native C-terminal end of the receptor (a generous gift from Dr. Robert Elde, University of Minnesota) or a monoclonal anti-FLAG M2 antibody (Sigma) recognizing the FLAG epitope tag at the C terminus of the receptor, according to the protocol described by the manufacturer of the latter antibody. Horseradish peroxidase-conjugated secondary antibodies from Amersham Pharmacia Biotech, and enhanced chemiluminescence Western blotting detection reagents from NEN Life Science Products were used to reveal the blotted proteins. The electroblotted molecular weight markers were detected by staining with Ponceau S (Sigma).

RESULTS

Functional Characterization of the HδOR Expressed in HEK-293S Cells—The FLAG epitope was introduced to the C-terminal end of the hδOR coding sequence to facilitate immunological detection and purification of the receptor protein. An antiserum recognizing the last 10 amino acids of the native receptor was used for Western blotting (see below), but it did not allow efficient immunopurification of the receptor. Plasmids encoding either the wild type or the tagged hδOR were stably transfected into HEK-293S cells. Characterization of the pharmacological properties of the two constructs revealed that the addition of the epitope did not significantly affect ligand binding or G protein coupling (Table I). The K_d for the agonist [^{3}H]bremazocine, the K_i for all the ligands tested in binding competition assays as well as the potency of SNC-80 to inhibit forskolin-stimulated cAMP accumulation were found to be identical for the FLAG epitope-modified hδOR and the wild type receptor and in close agreement with the values reported previously (23, 24).

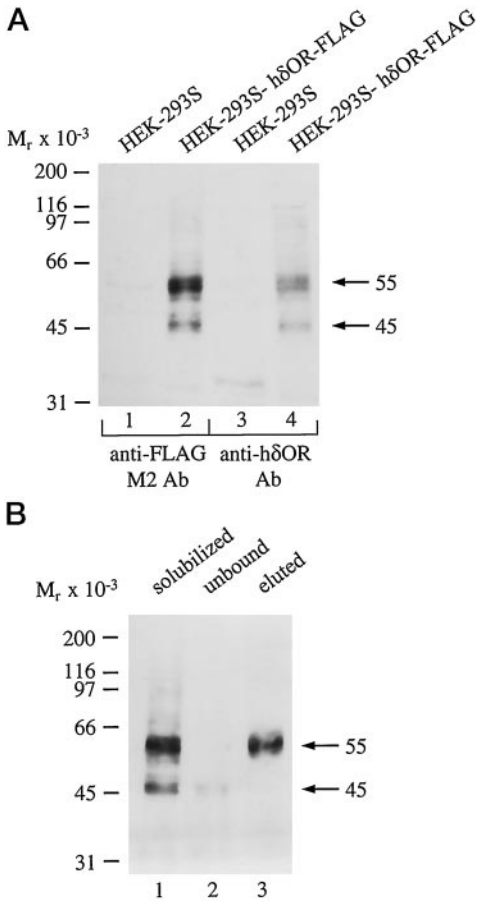

FIG. 1. **Identification of the hδOR expressed in HEK-293S cells.** *A*, total cellular membranes from nontransfected HEK-293S cells (*lanes 1* and *3*) and HEK-293S cells stably transfected with hδOR-FLAG cDNA (HEK-293S-hδOR-FLAG, *lanes 2* and *4*) were isolated and extracted in DDM. The solubilized proteins were subjected to SDS-PAGE and electroblotted on nitrocellulose sheets, which were incubated with either anti-FLAG M2 antibody (*lanes 1* and *2*) or anti-hδOR-antiserum (*lanes 3* and *4*). *B*, solubilized receptors from HEK-293S-hδOR-FLAG cells were subjected to batchwise purification on WGA-agarose as described under "Experimental Procedures." Aliquots of the solubilized material, the unbound fraction, and the eluate were loaded on *lanes 1*, *2*, and *3*, respectively. Anti-FLAG M2 antibody was used to probe the electroblotted receptor. Molecular weight markers are indicated on the *left* (from the *top*, myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase).

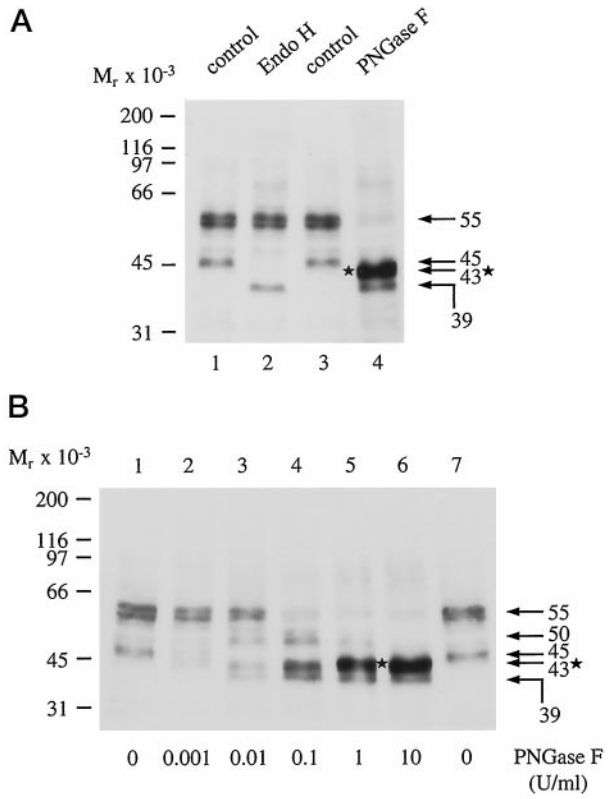

FIG. 2. **Deglycosylation of the hδOR with Endo H and PNGase F.** Total cellular membranes from HEK-293S-hδOR-FLAG cells were solubilized in DDM, and the solubilized receptors were incubated for 16 h at 37 °C (*A*) with (*lane 2*) or without (*lane 1*) Endo H (100 milliunits/ml), with (*lane 4*) or without (*lane 3*) PNGase F (20 units/ml) or (*B*) with an increasing concentration of PNGase F as described under "Experimental Procedures." The enzyme-free controls contained buffer only. Reactions were stopped by adding SDS sample buffer, followed by SDS-PAGE and immunoblotting, using the anti-FLAG M2 antibody.

Identification of the HδOR Species Expressed in HEK-293S Cells—To identify the FLAG epitope-tagged hδOR expressed in HEK-293S cells, the cells were homogenized, and DDM-solubilized membranes were subjected to SDS-PAGE and immunoblotting (Fig. 1*A*). Anti-FLAG M2 antibody recognized a broad, heterogeneous band with an apparent molecular weight of 55,000 and a sharper one of 45,000 (Fig. 1*A*, *lane 2*). The same polypeptides were also detected by a polyclonal antiserum that was raised against the native C-terminal hδOR decapeptide (Fig. 1*A*, *lane 4*). These two bands were not present in nontransfected HEK-293S cells (Fig. 1*A*, *lanes 1* and *3*), indicating that they specifically represent the expressed hδOR. Occasionally, higher molecular weight species were detected that could represent dimeric forms of the receptor (33–35).

The identities of the two receptor species were further studied by subjecting the solubilized receptor to purification on WGA-agarose (Fig. 1*B*). Only the higher molecular weight receptor species was specifically bound to and eluted from the resin with 0.3 M *N*-acetylglucosamine (Fig. 1*B*, *lane 3*). This indicates that the M_r 55,000 receptor species is a glycoprotein and demonstrates that it represents the full-length receptor polypeptide. This is based on the facts that the only putative *N*-glycosylation sites are located in the N-terminal portion of the receptor (Asn-18 and Asn-33) and that the epitope recognized by the antibody is localized in the C-terminal end of the protein. Two possibilities can be envisioned to explain why the M_r 45,000 species does not bind to the lectin. First, this species could be a degradation product of the M_r 55,000 receptor form lacking carbohydrate moieties required for high affinity lectin binding. Second, it could be a full-length receptor containing oligosaccharides that have low affinity for WGA, such as unprocessed high mannose type glycans (36).

To investigate further the latter possibility that the two molecular forms of the hδOR expressed in HEK-293S cells contain the same polypeptide backbone but differ in their carbohydrate structures, the solubilized receptors were treated with glycosidases (Fig. 2). Endo H selectively removes unprocessed high mannose type oligosaccharides from glycoproteins but does not cleave complex type fully processed glycans (37). Treatment of the solubilized proteins with this enzyme did not change the molecular weight of the M_r 55,000 receptor species but reduced that of the M_r 45,000 one to about M_r 39,000 (Fig. 2*A*, *lane 2*). This demonstrates that the M_r 45,000 receptor species does not represent a degradation product lacking *N*-glycosylation sites but rather a full-length receptor bearing high mannose-type oligosaccharides.

The resistance of the M_r 55,000 receptor species to Endo H treatment (Fig. 2*A*, *lane 2*) coupled to the observation that it binds avidly to WGA (Fig. 1*B*, *lane 3*) suggests that this species contains complex-type *N*-linked oligosaccharides. This was fur-

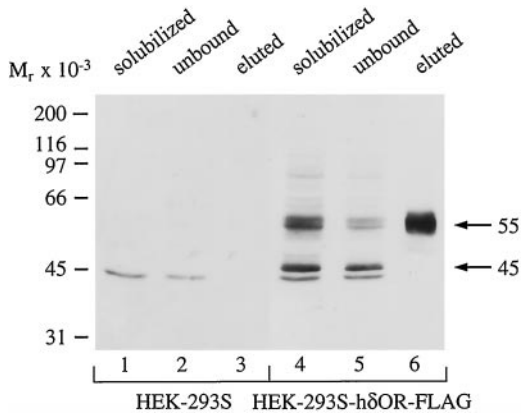

FIG. 3. **Isolation of the hδOR expressed at the cell surface.** Cell surface proteins were labeled by incubating either HEK-293S cells (*lanes 1–3*) or HEK-293S-hδOR-FLAG cells (*lanes 4–6*) with sulfo-NHS-biotin (0.5 mg/ml) at 4 °C for 30 min. Total cellular membranes were isolated and extracted in DDM, and the biotinylated proteins in the detergent extracts were adsorbed to and eluted from immobilized streptavidin as described under "Experimental Procedures." Aliquots of the detergent extracts (*lanes 1* and *4*), the unbound fractions (*lanes 2* and *5*), and the eluates (*lanes 3* and *6*) were analyzed by SDS-PAGE and immunoblotting, using the anti-FLAG M2 antibody.

ther supported by the observation that PNGase F, an enzyme that can remove all types of *N*-linked oligosaccharides from glycoproteins (37), was able to digest the M_r 55,000 receptor (Fig. 2*A*, *lane 4*). The broadness of the band and the occasional detection of discrete species around M_r 55,000 most likely represent *N*-glycosylation heterogeneity because this heterogeneity was abolished by the PNGase treatment. As anticipated, PNGase F was also able to increase the electrophoretic mobility of the M_r 45,000 receptor form (Fig. 2*A*, *lane 4*). Interestingly, however, deglycosylation of the two receptor species by this enzyme led to the formation of two distinct molecular weight species of 43,000 and 39,000 respectively. Provided that complete enzymatic cleavage of *N*-linked carbohydrates was achieved, as indicated by the PNGase F concentration series presented in Fig. 2*B*, these data suggest that the higher molecular weight receptor species has an additional modification that changes its electrophoretic mobility on SDS-PAGE. The nature of this modification will be addressed in a later section.

Concerning the glycosylation, the difference between the apparent molecular weight of the major hδOR species estimated from SDS-polyacrylamide gels (55,000) and the predicted polypeptide size (about 40,000) indicates that both putative *N*-glycosylation sites of the receptor are occupied by carbohydrate. This was confirmed by stepwise, concentration-dependent digestion of the solubilized receptor by PNGase F from the M_r 55,000 species to the M_r 43,000 product via a M_r 50,000 intermediate form that most likely represents a receptor with a single *N*-glycan (Fig. 2*B*). Increasing the enzyme concentration up to 20 units/ml did not decrease the molecular weight of the M_r 43,000 band any further, indicating that the reaction was complete.

To further investigate the nature of the M_r 55,000 and the M_r 45,000 receptor species observed on Western blots, we assessed their cellular location (*i.e.* intracellular *versus* cell surface). Intact cells were labeled with a membrane impermeable biotinylation reagent, sulfo-NHS-biotin, and cellular membranes were isolated. After solubilization, the biotinylated proteins were purified by immobilized streptavidin, and the receptor was revealed on a Western blot using the anti-FLAG M2 antibody (Fig. 3). The selectivity of the cell surface biotinylation assay was confirmed by the absence of calnexin, an ER resident transmembrane protein, from the streptavidin-purified fraction (data not shown). This indicates that the sulfo-NHS-biotin does not have access to the intracellular compartments in the conditions used. As shown in Fig. 3 (*lane 6*), the M_r 55,000 receptor species was purified by streptavidin-agarose, suggesting that this receptor form is expressed at the cell surface. In contrast, the M_r 45,000 receptor did not bind to streptavidin and thus is confined to an intracellular compartment that is inaccessible to the extracellular sulfo-NHS-biotin. Taken together, these observations indicate that the M_r 55,000 hδOR species may represent the fully mature receptor form that is transported to the cell surface, whereas the M_r 45,000 is a precursor form that is found in an intracellular compartment, most likely in the ER.

Biosynthesis, Processing, and Trafficking of the Newly Synthesized HδOR—To further confirm the potential precursor-product relationship between the two hδOR species expressed in HEK-293S cells, pulse-chase experiments were performed (Fig. 4). Cells were labeled for 60 min in a methionine/cysteine-free medium containing 150 μCi/ml of [^{35}S]methionine/cysteine and subsequently chased for periods of up to 22 h in a medium containing an excess of unlabeled methionine. At each time point, cells were harvested and membranes were prepared. The solubilized receptors were immunoprecipitated and subjected to SDS-PAGE followed by fluorography. As shown in Fig. 4*A*, the different receptor forms do not appear simultaneously but rather in a specific sequence and with quite different kinetics. The M_r 45,000 band was the first component to be labeled along with two minor ones with apparent molecular weights of about 42,000 and 39,000. None of these three bands was apparent in nontransfected HEK-293S cells (data not shown). During the subsequent chase, label incorporated into these bands decreased in parallel with the appearance of a slower, more diffusely migrating one of M_r 55,000, confirming the precursor-product relationship between the M_r 45,000 and the M_r 55,000 hδOR species and the fact that the latter receptor species corresponds to the fully mature form of the receptor.

The kinetic relationships between the different hδOR species were assessed by densitometric scanning of autoradiograms to construct progress curves (Fig. 4*C*). As can be seen, addition of the terminal sugars reached completion with a half-time of about 120 min (121 ± 4 min). The half-life for the M_r 55,000 mature receptor species was approximately 15 h (15.3 ± 2.7 h), whereas the turnover rate for the smaller receptor forms was substantially faster (M_r 45,000, 134 ± 8 min; M_r 42,000, 99 ± 5 min; and M_r 39,000, 35 ± 4 min). The M_r 42,000 receptor form most likely represents a partially glycosylated form of the receptor, whereas the M_r 39,000 one is the nonglycosylated polypeptide. Indeed, like the M_r 45,000 form, the M_r 42,000 species was found to be sensitive to both Endo H and PNGase F treatments leading to a form that comigrates with the M_r 39,000 species (see Fig. 7, *lane 1*, in *A*, *B*, and *C*). No precursor-product relationships could be established between the M_r 39,000, the M_r 42,000, and the M_r 45,000 forms, even when the pulse was shortened to 15 min (data not shown), leaving the possibility open that they may represent either incompletely translocated and glycosylated species or deglycosylated forms of the receptor precursor that are destined to a degradation pathway (38–41). The relatively long half-time for the conversion of the precursor to the mature form (120 min) suggests that export from the ER is a limiting step in the overall maturation process of the receptor.

One apparent fact of hδOR biosynthesis seen in Fig. 4 (*A* and *C*) is that the efficiency of maturation of the receptor is rather low. The fractional conversion of precursor to the mature form was less than 50% (40.5 ± 3.5%, a mean of eight experiments) of maximum label incorporated into the receptor precursor.

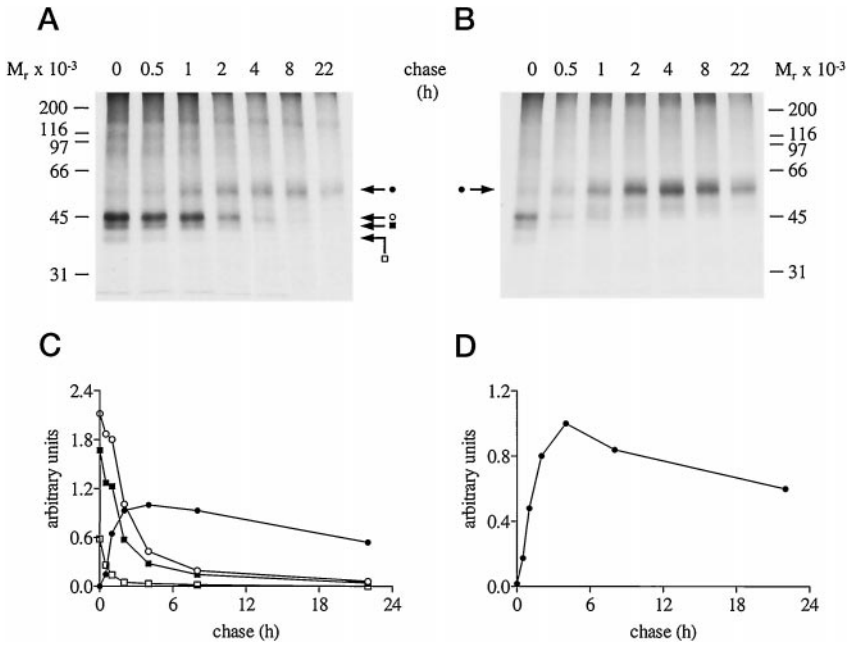

FIG. 4. **Pulse-chase analysis of the synthesis, processing, and trafficking of the hδOR.** HEK-293S-hδOR-FLAG cells were labeled for 60 min with 150 μCi/ml of [^{35}S]methionine/cysteine and then chased with complete medium supplemented with 5 mM methionine for the indicated times. Cell surface proteins were biotinylated as described in Fig. 3, and the total cellular membranes were isolated and extracted in DDM. Receptors from one-fourth of the extracts were immunoprecipitated using immobilized anti-FLAG M2 antibody as described under "Experimental Procedures" to determine total labeled receptors (*A*). The rest of the extracts were used to isolate the cell surface receptors by sequential precipitations using immobilized streptavidin and immobilized anti-FLAG M2 antibody (*B*). The samples were analyzed by SDS-PAGE and fluorography, and the dried gels were exposed to x-ray film to obtain the fluorographs shown. The results shown are representative of four independent experiments. *Arrows* indicate the different receptor forms. ●, M_r 55,000; ○, M_r 45,000; ■, M_r 42,000; □, M_r 39,000. *C* and *D* describe the time course of appearance and disappearance of the different labeled receptor species in the total and cell surface pool of receptors, respectively. The *symbols* refer to those used to identify the different receptor forms. Intensities of the immature and the mature hδOR species were obtained by densitometric scanning and the values were normalized to the maximum labeling of the mature receptor at 4 h of chase.

Deglycosylation did not increase the intensity of the band corresponding to the mature receptor (see Fig. 7, compare *lane 4* in *A* and *C*), ruling out the possibility that the amount of the M_r 55,000 receptor species was underestimated as a consequence of glycosylation heterogeneity. It could be argued that the inefficient conversion of the receptor to the mature form is an artifact of the high level of expression achieved in HEK-293S cells (~24 pmol/mg protein). However, this is unlikely because a similar low efficiency (39.5%) of maturation was observed when the kinetics of receptor turnover was studied in cells expressing a lower level of receptors (3 pmol/mg protein) Although this expression level is still supraphysiological, the data allow us to argue that the inefficient conversion to a form bearing complex type *N*-linked oligosaccharides is an intrinsic property of the hδOR polypeptide and does not reflect congestion of a saturable process. Such a saturation would have lead to a lower efficiency in cells expressing higher number of receptors.

To define the time course over which newly synthesized receptors are transported to the cell surface, cells were subjected to biotinylation following different times of chase in the pulse-chase labeling experiments (Fig. 4*B*). [^{35}S]methionine/cysteine-labeled receptors that had reached the plasma membrane were distinguished from the total pool of labeled receptors by their ability to undergo biotinylation when intact cells were incubated with sulfo-NHS-biotin. The biotinylated cell surface proteins were then isolated by streptavidin-agarose, and the receptors were purified by immunoprecipitation. As can be seen from Fig. 4 (compare panels A and B), the mature M_r 55,000 receptor form was the predominant one at the cell surface and was very stable ($t_{1/2}$ = 19.6 ± 2 h). It accumulated at the cell surface with a half-time of 134 ± 3 min. Because the half-time for the maturation of the receptor was about 120 min (Fig. 4*C*), there does not seem to be any lag time between final maturation and transport from the Golgi to the cell surface.

O-Glycosylation of the HδOR—As mentioned previously, removal of *N*-linked oligosaccharides from the precursor and the mature forms of the hδOR led to species with distinct electrophoretic mobilities (M_r 39,000 and M_r 43,000 for the precursor and the mature forms, respectively; Fig. 2*A*). This indicates that the hδOR undergoes an additional post-translational processing step during its transit along the secretory pathway prior to its transport to the cell surface. In addition to *N*-glycosylation, two other modifications, namely *O*-glycosylation and phosphorylation, have been shown to decrease the apparent mobility of membrane-bound proteins when analyzed by SDS-PAGE (42–44). The possible contribution of phosphorylation as an explanation for our findings was ruled out by the inability of alkaline phosphatase treatment to increase the mobility of the deglycosylated M_r 43,000 hδOR (data not shown). Thus, addition of oligosaccharides to serine or threonine acceptor sites on the receptor is the most probable cause for the additional decreased electrophoretic mobility of the hδOR.

To test the hypothesis that the hδOR is *O*-glycosylated, we first performed metabolic labeling with [^{3}H]glucosamine (Fig. 5) that labels both *N*-linked and *O*-linked oligosaccharides. This is a technique that has been successfully used previously to demonstrate that some cell surface receptors contain *O*-linked glycans (42, 45). After labeling, the immunoprecipitated receptors were subjected to glycosidase treatment to remove the *N*-linked oligosaccharides. As can be seen in Fig. 5*A* (*lane 1*), label was detected in bands of M_r 45,000 and M_r 55,000, corresponding to the precursor and the mature forms of the hδOR. PNGase F treatment did not completely abolish the label from the [^{3}H]glucosamine-labeled mature receptor because the M_r 43,000 receptor species that resulted from *N*-

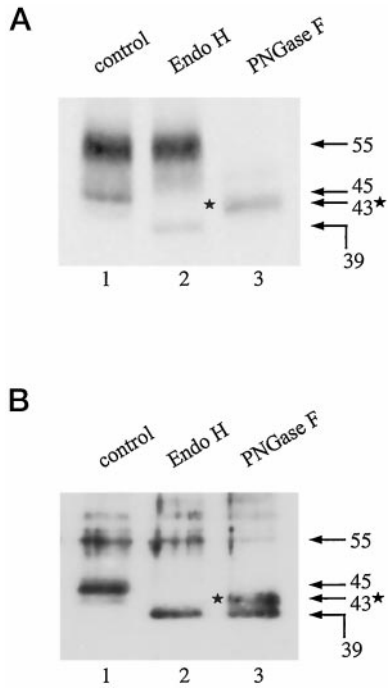

FIG. 5. **Metabolic labeling of the hδOR with [^{3}H]glucosamine.** HEK-293S-hDOR-FLAG cells were labeled with 70 μCi/ml of [^{3}H]glucosamine for 6 h, isolated membranes were solubilized in DDM, and the immunoprecipitated receptors were either incubated in the absence (*lane 1*) or presence of 25 milliunits/ml of Endo H (*lane 2*) or 20 units/ml of PNGase F (*lane 3*) as described under "Experimental Procedures." Four-fifths of the samples were subjected to SDS-PAGE and fluorography (*A*), and the rest of the samples were subjected to immunoblotting (*B*), using the anti-FLAG M2 antibody.

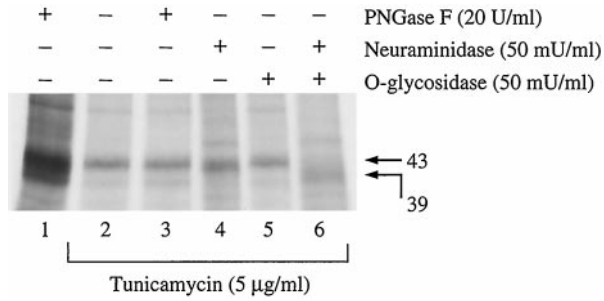

FIG. 6. **Deglycosylation of the hδOR by sequential neuraminidase and *O*-glycosidase treatment.** HEK-293S-hδOR-FLAG cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine and chased for 5 h. The cells were treated with (*lanes 2–6*) or without (*lane 1*) tunicamycin (5 μg/ml) by adding the drug to the medium 3 h prior to the labeling. The immunoprecipitated receptors were treated with glycosidases as described under "Experimental Procedures" and subjected to SDS-PAGE and fluorography.

deglycosylation of the M_r 55,000 form still contained 10–15% of the label (Fig. 5*A*, *lane 3*). This strongly suggests that the mature hδOR indeed contains *O*-linked oligosaccharides. Furthermore, because PNGase F was able to completely remove the label from the precursor (compare *lane 3* in Fig. 5, *A* and *B*), it can be concluded that the addition of the *O*-linked oligosaccharides occurs after the receptor has been transported to the Golgi. Consistent with this notion, deglycosylation of the immunoprecipitated receptors after [^{35}S]methionine/cysteine pulse-chase labeling revealed that the kinetics of acquisition of Endo H-resistance and the addition of *O*-linked oligosaccharides (conversion of the M_r 39,000 *N*-deglycosylated receptor to the M_r 43,000 one) were indistinguishable (data not shown). The remaining ^{3}H label in the Endo H-treated precursor (Fig. 5*A*, *lane 2*) can be explained by the fact that in contrast to PNGase F that releases intact *N*-linked oligosaccharides from glycoproteins, Endo H leaves one *N*-acetyl-glucosamine residue attached to the protein (37).

As a second approach to determine whether the hδOR contains *O*-linked oligosaccharides, we subjected the [^{35}S]methionine/cysteine-labeled receptor to enzymatic deglycosylation using O-glycosidase (Fig. 6). This is an enzyme that is able to remove unsubstituted *O*-linked galactose-*N*-acetyl galactosamine disaccharides from glycoproteins (46) after the terminal sialic acids have been removed by neuraminidase. To follow the subtle changes in the migration of the receptor in SDS-PAGE after the enzyme treatment, cells were treated with tunicamycin to prevent addition of *N*-linked glycans to the receptor. The major receptor form that was immunoprecipitated from tunicamycin-treated cells after a 5-h chase (Fig. 6, *lane 2*) comigrated with the *N*-deglycosylated mature receptor of control cells (M_r 43,000) (Fig. 6, *lane 1*). Neuraminidase was able to decrease the molecular weight of the M_r 43,000 receptor species only slightly (Fig. 6, *lane 4*), but a sequential treatment with neuraminidase and *O*-glycosidase resulted in a clear decrease in its apparent molecular weight down to M_r 39,000 (Fig. 6, *lane 6*). *O*-Glycosidase by itself was without any effect (Fig. 6, *lane 5*). These results confirm that the hδOR contains *O*-linked oligosaccharides and further indicate that these glycans are sialylated species with a core disaccharide of galactose and *N*-acetylgalactosamine.

Having demonstrated that the hδOR is modified by the addition of *O*-linked oligosaccharides, apparently after exit from the ER, we next proceeded in characterizing further in which subcompartment of the secretory pathway this modification occurs. To this end, HEK-293S cells expressing the receptor were labeled with [^{35}S]methionine/cysteine in the presence of either monensin or BFA (Fig. 7). These drugs have an effect on protein transport through the secretory pathway, and thus they interfere with the processing of the *N*-linked as well as of the *O*-linked oligosaccharides. They prevent terminal processing of oligosaccharides that occurs in the *trans*-Golgi cisternae and the *trans*-Golgi network, respectively. Monensin induces neutralization of the *trans*-Golgi, which affects the activity of the glycosyltransferases located there (47), whereas BFA induces a rapid disassembly of the Golgi and redistribution of its resident proteins into the ER. These proteins include enzymes that normally reside in the *cis*, *medial* and *trans*-Golgi cisternae but none of those that reside in the *trans*-Golgi network (48–50). As seen in Fig. 7*A* (*lanes 4–6*), the apparent molecular weight of the receptor after the 5-h chase period was clearly smaller in monensin- and BFA-treated cells compared with control cells. This suggests that the processing of the oligosaccharides was not complete, although the M_r 46,000 receptor species had undergone trimming of its *N*-linked oligosaccharides, as evidenced by its resistance to Endo H treatment (Fig. 7*B*, *lanes 4–6*). To find out, if processing of both the *N*-linked and the *O*-linked glycans was affected, the *N*-linked oligosaccharides were removed by treating the immunoprecipitated receptors with PNGase F (Fig. 7*C*, *lanes 4–6*). The apparent molecular weight of the *N*-deglycosylated receptor in BFA-treated cells was identical (M_r 43,000) to that in control cells (compare *lanes 4* and *6*, Fig. 7*C*), indicating that the final processing of the *O*-linked oligosaccharides of the hδOR occurs before the protein enters the *trans*-Golgi network. In contrast, the final processing of the *N*-linked glycans of the receptor requires transport to the *trans*-Golgi network (compare *lanes 4* and *6*, Fig. 7*A*). The *N*-deglycosylated receptor of monensin-treated cells (M_r 40,000) migrated more quickly than the *N*-deglycosylated receptor of BFA-treated cells (M_r 43,000) (compare *lanes 5* and *6*, Fig. 7*C*) but slightly more slowly than the

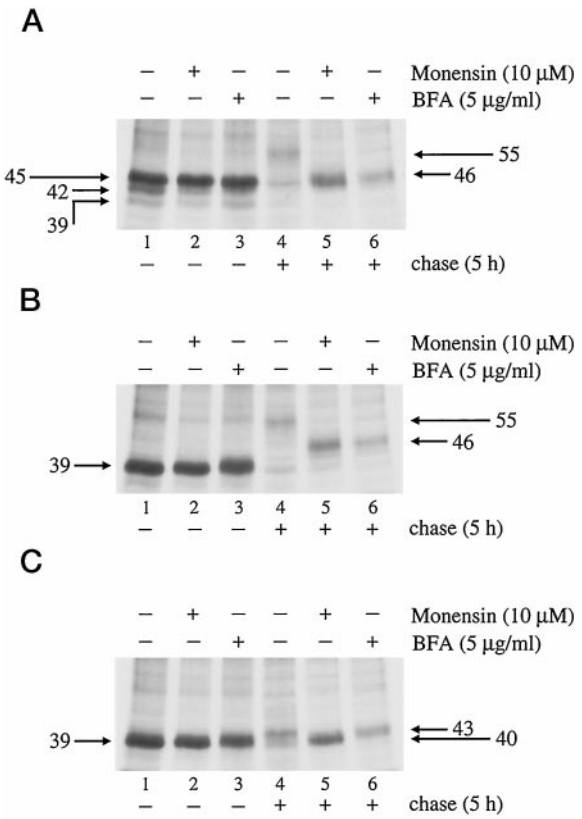

FIG. 7. **Analysis of the hδOR species synthesized in the presence of monensin and BFA.** HEK-293S-hδOR-FLAG cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine and harvested directly or chased first for 5 h. Monensin (10 μM) or BFA (5 μg/ml) was added to the medium 1 h prior to the labeling. The immunoprecipitated receptors were analyzed by SDS-PAGE and fluorography after incubation in the absence (*A*) or presence of either 25 milliunits/ml of Endo H (*B*) or 20 units/ml of PNGase F (*C*) as described under "Experimental Procedures."

N-deglycosylated precursor of control cells (M_r 39,000) (compare *lanes 1* and *5*, Fig. 7*C*). These data are consistent with the idea that 1) the addition of monosaccharides to the serine or threonine acceptor sites of the receptor begins prior to the monensin block, either in the *cis* or *medial* compartments of the Golgi (explaining the M_r 39,000 to M_r 40,000 shift) and 2) that the final processing of the *O*-linked sugars requires the integrity of the *trans*-Golgi (explaining the M_r 40,000 to M_r 43,000 difference). Thus, the M_r 40,000 receptor species is apparently an intermediate in the processing of the *O*-linked glycans and probably represents a species in which at least the first *N*-acetylgalactosamine has been added to the protein, whereas the M_r 43,000 one would represent a receptor form with fully processed *O*-linked glycans.

Taken together, our results strongly suggest that the hδOR is modified not only by *N*-linked glycosylation but also by addition of *O*-linked oligosaccharides. Initiation of the *O*-linked glycans by addition of *N*-acetylgalactosamine occurs after the protein has exited the ER and further addition of other monosaccharides, including galactose and sialic acid, is completed before it enters the *trans*-Golgi network. In contrast, the final processing of the *N*-linked oligosaccharides occurs in the *trans*-Golgi network.

DISCUSSION

The results obtained in the present study allow us to propose the following model for the synthesis, maturation, and plasma membrane targeting of the hδOR. The protein is initially synthesized as a M_r 45,000 precursor that is converted in the Golgi to the mature M_r 55,000 receptor with a half-time of about 120 min. This conversion involves processing of two high mannose-type *N*-linked oligosaccharides as well as addition of *O*-linked glycans containing *N*-acetylgalactosamine, galactose, and sialic acid. The fully mature receptor is then transported to the cell surface without delay in about 10 min. Once at the cell surface, the mature receptor is very stable with a half-life of about 20 h. Intriguingly, the overall process of maturation of the receptor was found to be inefficient as only 40% of the precursor was converted to the mature form and eventually reached the cell surface. This low efficiency most likely results from slow folding and ER export steps.

N-Glycosylation is a well characterized modification of GPCRs, but *O*-glycosylation has gone unnoticed until very recently, when Sadeghi and Birnbaumer (51) showed that the V_2 vasopressin receptor contains *O*-linked glycans. In the present study, *O*-glycosylation of the hδOR was demonstrated by two different approaches: metabolic labeling using [^{3}H]glucosamine and sequential enzymatic deglycosylation using neuraminidase and *O*-glycosidase. There are several reasons that could explain why the presence of *O*-linked oligosaccharides in the GPCRs has, until now, eluded firm biochemical identification. First, parameters that determine sites of *O*-glycan attachment to glycoproteins are poorly understood, and no obvious consensus sequence motif has emerged (52). This is in clear contrast to *N*-glycosylation for which a well defined consensus motif has been determined (53). Another reason that may have hindered the study of *O*-glycans is that they have no common core structure. This is related to the fact that *O*-glycosylation starts post-translationally with the addition of a single monosaccharide catalyzed by several different glycosyltransferases, whereas *N*-glycosylation is initiated in the ER by the co-translational addition of a large oligosaccharide unit to the polypeptide by a unique oligosaccharyltransferase. The initial reaction in *O*-linked oligosaccharide biosynthesis is most commonly the transfer of *N*-acetyl-galactosamine to a serine or threonine residue on the protein acceptor. However, this is not always the case, and, for example, fucose-type *O*-glycosylation has also been described (54). The analysis of *O*-linked oligosaccharides has also been hampered by the poor availability of *O*-glycosylation inhibitors and of enzymes that can selectively remove these types of oligosaccharides from glycoproteins. Finally, GPCRs are likely to belong to those glycoproteins that contain only a limited number of *O*-linked oligosaccharides. Their primary sequences do not show extensive clusters of putative acceptor serine and threonine residues on their extracellular domains (2), a feature that is typical for many heavily *O*-glycosylated membrane proteins, such as the low density lipoprotein receptor (45) and the glycophorins (55).

In contrast to the *N*-linked oligosaccharides, the biosynthesis and topology of which are well documented (53), the site of initiation of *O*-glycosylation is still somewhat controversial. Whereas the elongation steps of *O*-glycosylation are commonly believed to occur in the Golgi, the addition of the first monosaccharide has been placed in the ER, the ER-Golgi intermediate compartment, or the Golgi (56–59), possibly reflecting variations in the *N*-acetyl-galactosamine transferase localization among different cell types. Our data indicate that the *O*-glycosylation of the hδOR in the HEK-293S cells is initiated after the protein exits the ER, because the receptor precursor was not *O*-glycosylated. It was shown to contain *O*-linked glycans only when Golgi enzymes were allowed to redistribute into the ER by treating the cells with BFA. The data obtained using monensin-mediated interference in protein transport were consistent with the idea that the site of addition of the first *O*-linked monosaccharides occurs in the *cis* or the *medial* com-

partments of the Golgi. Furthermore, our results also showed that the final processing of the *O*-linked and the *N*-linked glycans of the hδOR occurs in different compartments of the cell. For the *N*-linked glycans, this step occurs in the *trans*-Golgi network, whereas for the *O*-linked ones it takes place in an earlier compartment, the *trans*-Golgi cisternae. These findings agree with those reported by Kirjnse Locker *et al.* (43) for the coronavirus M protein and vesicular stomatitis virus G protein, showing that the terminal processing of the *O*-linked glycans by the addition of sialic acids occurs in the *trans*-Golgi, whereas the *N*-linked glycans are sialylated in the *trans*-Golgi network. The terminal processing of the oligosaccharides of the hδOR probably also involves addition of sialic acids as was evidenced by the observation that the mature receptor displays high affinity for WGA and was sensitive to neuraminidase treatment.

The kinetic data obtained from the pulse-chase labeling experiments suggest that the hδOR spends most of its intracellular residence time in the ER and is converted to a form containing *O*-linked glycans and Endo H-resistant *N*-linked glycans quite rapidly after exit from this organelle. The kinetics of oligosaccharide maturation of the receptor parallels that of the appearance of the protein at the cell surface. This implies that the time period between the removal of the mannose residues from the core oligosaccharides, the addition of the first *N*-acetylgalactosamine, and the subsequent transport of the protein to the plasma membrane is relatively short. Because there is no apparent lag time before the fully mature hδOR is transported to the cell surface, it is unlikely that there is a significant pool of intracellular newly synthesized mature receptors. This contrasts with findings for some other GPCRs, like the thrombin receptor (60), the *N*-formyl peptide receptor (61), and one of the α_2-adrenergic receptor subtypes (62), for which a significant reserve of intracellular mature receptors was proposed. This may either indicate that there is an intrinsic difference between the maturation process of the hδOR and that of these receptors or else that the reserve observed in the previous studies included recycling receptors that were first targeted to the cell surface. Once at the cell surface, the mature M_r 55,000 form of the hδOR is very stable with a half-life of about 20 h. This turnover rate is somewhat slower than that reported for other GPCRs in stably transfected cells: 8–10 h and 6 h for the three α_2-adrenergic receptor subtypes and the V_2 vasopressin receptor, respectively (63, 64).

From the above discussion, we can conclude that export from the ER represents the rate-limiting step in the overall processing of the hδOR. In addition, this step appears to be quite inefficient because only 40% of the M_r 45,000 precursor is converted to the mature receptor form. The rest are probably retained in the ER by its quality control mechanisms and may eventually be targeted for degradation. It has been shown for several other proteins that nascent molecules that fail to fold correctly are not deployed to distal compartments of the secretory pathway and only correctly and completely folded molecules are transported out of the ER (65). One possibility that could explain the inefficient processing of the hδOR is that overexpression in the HEK-293S cells causes overloading of the capacity of the cells to properly handle the receptor protein. Several enzymes and proteins are involved in the processing and transport of nascent proteins along the secretory pathway (65, 66). It could be envisioned that in a heterologous expression system any one of these factors could become limiting, accounting for the deficiency in processing of the newly synthesized hδOR to the fully mature protein. Although this may be a reasonable explanation that cannot completely be ruled out, we consider this an unlikely possibility, because we found no correlation between the efficiency of maturation of the hδOR and the level of receptor expression in the stably transfected cells. If saturation of a limiting factor is responsible for the inefficient processing, one would expect to observe a lower percentage of mature receptors at the higher expression level, and this was not the case. An identical efficiency of maturation was observed in cells expressing 3 and 24 pmol of receptor/mg of protein. The situation may be different, however, in transient expression systems. Indeed, Birnbaumer and co-workers observed a lower processing efficiency of the V_2 vasopressin receptor when it was expressed at high levels in transient *versus* stable expression systems (64). In contrast, we consider it more likely that in HEK-293S cells the disposal mechanisms that are responsible for removing the misfolded receptors from the ER are overloaded, because at steady state a considerable proportion of the hδOR was in a precursor form. Similar findings have been reported for another inefficiently processed membrane protein, the cystic fibrosis transmembrane conductance regulator (67).

Given the results obtained in the present study, it is quite reasonable to hypothesize that the low efficiency of processing of the hδOR is an intrinsic property of the protein molecule itself and is related to folding difficulties. Whether this occurs in other heterologous expression systems and is a common feature among GPCRs remains to be determined. Nevertheless, it can be reasoned that the low efficiency of folding and ER export may be related to the complex structure of the GPCRs. The transmembrane domains of these proteins are believed to be arranged in a couterclockwise bundle with interhelical hydrogen bonds and a constraining disulfide bond between the first and the second extracellular loops (2–4). Complex structure of the GPCRs coupled to the need for precise intramolecular interactions may result in slow folding rate and high susceptibility to misfolding. This is exemplified by the numerous naturally occurring mutations among the GPCRs that result in intracellular trapping of the misfolded protein (12–16). In some cases, these misfolded proteins can be rescued by incubating cells at a reduced temperature (68), indicating that folding kinetics may be important. Moreover, folding difficulties do not always follow from deviations from the wild type structure by mutations but may be typical for the wild type protein itself. For example, Fishburn *et al.* (69) showed that the two isoforms of the D_2 dopamine receptor (D_{2L} and D_{2S}) are processed differently when expressed at comparable level in Chinese hamster ovary cells. The short isoform was processed fully after 3 h of chase, whereas about 20% of the long isoform were still in an immature form. Accumulation of receptor precursor forms has been shown to occur not only in heterologous expression systems but also in tissues expressing native receptors. For example, a large pool of precursor forms of the luteinizing hormone receptor and the follicle-stimulating hormone receptor was detected in testes and ovaries (70–72). Whether this also applies to the endogenous hδOR remains an open question. Unfortunately, the lack of an antibody that would allow an efficient immunopurification of the native receptor precludes realization of these experiments at the moment.

Interestingly, in some special cases normal folding and transport of GPCRs to the plasma membrane appear to involve chaperone- or escort-like proteins. This is the case for the rhodopsin in *Drosophila* (18, 19) and cows (20). Indeed, mutants of a specific cyclophilin-like chaperone in flies lead to accumulation of rhodopsin in the ER. It was speculated that the existence of a specific chaperone for this GPCR was necessary for optimization of the synthesis and maturation of the receptor, expressed at exceedingly high level in photoreceptor cells. Recently, distinct escort systems have also been described

for the olfactory receptors (17), the calcitonin gene-related peptide receptor, the adrenomedullin receptor (21), and the γ-aminobutyric acid type B-1 receptor (22), indicating that the complex folding of these GPCRs may require specialized proteins even when expressed at lower level. Whether similar systems are necessary for other GPCRs in general remains speculative.

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Export from the Endoplasmic Reticulum Represents the Limiting Step in the Maturation and Cell Surface Expression of the Human δ Opioid Receptor

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