Glycosylation, Palmitoylation, and Localization of the Human D_{2S} Receptor in Baculovirus-Infected Insect Cells†

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ABSTRACT: In order to evaluate the baculovirus expression system as a means for high-yield production of homogeneous D_{2S} receptor, we have expressed various D_{2S} receptor constructs in two *Spodoptera frugiperda* cell lines, a *Trichoplusia ni* and a *Mammestra brassicae* cell line. To improve expression yield, the environment of the polyhedrin gene translational initiation site was retained by fusing the first 12 codons of the polyhedrin gene to the 5′-end of the D_{2S} receptor coding sequence. The pharmacological profile of the expressed D_{2S} receptor was similar to that reported for neuronal D_2 receptors. Sf9 and Tn cells were best suited for overexpression, yielding about 2×10^6 and 4×10^6 receptors/cell, respectively, corresponding to 6 pmol/mg of cell protein in Sf9 cells and 10 pmol/mg of cell protein in Tn cells. We have developed a D_2 receptor-specific anti-peptide antibody to study glycosylation, palmitoylation, and localization of the heterologously produced receptor. Immunoprecipitation of digitonin/cholate-solubilized receptor from control and tunicamycin-treated Sf9, Tn, and Mb cells revealed an apparent molecular mass of 47−48 kDa for the glycosylated receptor and of 39−40 kDa for the unglycosylated receptor. Although pulse−chase studies showed that glycosylation occurred rapidly and efficiently, the glycosylated receptor only constituted a small fraction of the overall produced receptor protein, which was mainly located intracellularly. The glycosylation of the receptor was of the high-mannose-type in contrast to the complex-type glycosylation found in native tissue. The glycosylated D_{2S} receptor was palmitoylated. Glycosylation, however, was not a prerequisite for palmitoylation which was insensitive to tunicamycin, brefeldin A, and monensin. NH_2-terminal addition of the signal sequence of prepromelittin to the D_{2S} receptor increased expression levels 2−3-fold and significantly enhanced membrane insertion and processing, resulting in increased targeting of the synthesized receptor to the plasma membrane.

Dopamine receptors belong to the superfamily of seven-transmembrane domain G protein-coupled receptors (GPCRs) which play a key role in central nervous system (CNS) neurotransmission. Dopamine has been implicated in several disorders of the CNS, including Parkinson's disease and schizophrenia (Lee et al., 1978; Seeman, 1987). Originally, two dopamine receptor subtypes, D_1 and D_2, were recognized based on their opposing effects on adenylyl cyclase activity and their disparate pharmacological profiles (Kebabian & Calne, 1979). Molecular cloning has to date identified five receptor subtypes designated D_{1A}, D_2, D_3, D_4, and D_5 (D_{1B}), thus pointing to the complexity of the dopaminergic system [summarized in Civelli et al. (1993) and Gingrich & Caron (1993)]. The D_2 subtype is of particular interest as it is thought to be the target for many antipsychotic drugs, known to be dopamine antagonists (Reynolds, 1992; Seeman, 1992), but also exists in two alternatively spliced isoforms (Dal Toso et al., 1989; Giros et al., 1989; Monsma et al., 1989). The two splice variants, D_{2S} and D_{2L}, differ by an insert of 29 amino acids in the third intracellular loop, which is a major site of interaction with G proteins (Dohlman et al., 1991). At the molecular level, activation of D_2 dopamine receptors results in inhibition of adenylyl cyclase and in a variety of cell-type specific responses [reviewed in Vallar & Meldolesi (1989) and Civelli et al. (1993)].

Knowledge of the three-dimensional structure of the D_2 receptor as well as other receptors would significantly add to our understanding of their molecular mechanisms and be useful in the search for functionally more discriminating drugs. Three-dimensional structural analysis requires milligram quantities of pure and homogeneous protein. Since the D_2 receptor like other neuroreceptors is not very abundant in its native tissue and usually occurs together with several subtypes, overexpression of these receptors is a necessary first step. We chose to overexpress the human dopamine D_{2S} receptor in baculovirus-infected insect cells. The baculovirus expression system has proved to be a convenient and powerful method for the overexpression and characterization of GPCRs, which appear to be biochemically, pharmacologically, and functionally similar to those in native membranes. Moreover, a variety of GPCRs heterologously expressed in Sf9 insect cells are able to couple to endogenous G proteins and are subject to posttranslational modifications like phosphorylation and palmitoylation (Mouillac et al., 1992; Richardson & Hosey, 1992; Ng et al., 1994a,b).

The D_2 receptor sequence reveals three potential N-glycosylation sites near its N-terminus, consensus sequences for phosphorylation in the third intracellular loop, and a conserved C-terminal cysteine which most likely is the site of palmitoylation (Ng et al., 1994b). Co- and posttranslational modifications including incomplete processing are among the main reasons for the structural heterogeneity of overexpressed membrane proteins. This heterogeneity often does not affect functional studies. Overexpression for structural studies, however, should result in structurally

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homogeneous protein. In this study, we compare the expression of several human D_{2S} receptor constructs in four different insect cell lines and examine glycosylation, palmitoylation, and localization of the overexpressed D_{2S} receptor using a specific anti-D_{2S} receptor−peptide antiserum. Since the human D_{2S} receptor like many other GPCRs lacks a cleavable signal sequence (Grandy et al., 1989), we added the cleavable signal sequence of *A. melifica* (honeybee) prepromelittin to the N-terminus of the receptor. This signal sequence impressively improved expression yield and processing, so that all solubilizable receptor is fully glycosylated and palmitoylated.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Spiperone ([^{3}H]SPIRO; 700−900 GBq/mmol), [^{3}H]methylspiperone ([^{3}H]MS; 3108 GBq/mmol), [^{35}S]methionine (43 TBq/mmol), and [^{3}H]palmitic acid (1700−1900 GBq/mmol) were purchased from DuPont NEN. *R*(−)-apomorphine hydrochloride (APO), *R*(−)-propylnorapomorphine hydrochloride (NPA), (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN), haloperidol (HAL), spiperone hydrochloride (SPIRO), and *S*(+)-butaclamol hydrochloride (BUT) were obtained from Research Biochemicals Inc. (Natick, MA). Dopamine (DA), domperidone (DOM), metoclopramide (MET), brefeldin A (BFA), tunicamycin, monensin, glutaraldehyde, and goat anti-mouse IgG (alkaline phosphatase-labeled) were from Sigma. Leupeptin, aprotinin, pepstatin A, and chymostatin were from Serva. Keyhole limpet hemocyanin (KLH) was purchased from Pierce (Rockford, IL). The anti-c-*myc* antibody 9E10 was obtained from Cambridge Research Biochemicals. The secondary goat anti-rabbit IgG antibody (alkaline phosphatase-labeled) was obtained from Biozol (Eching, FRG). Benzonase was purchased from Merck (Darmstadt, FRG), endoglycosidase H (Endo H) from Boehringer (Mannheim, FRG), and *N*-glycosidase F (PNGase F) from New England Biolabs. Activated CH Sepharose 4B and protein A−Sepharose were obtained from Pharmacia. Ethylene glycol monomethyl ether and the scintillation cocktail Rotiscint eco plus were from Roth (Karlsruhe, FRG). All other chemicals were of reagent grade, obtained primarily from Sigma. Sf9 and Sf21 cells were purchased from ATCC (Rockville, MD). Tn 368 cells were kindly provided by Dr. J. Klier (Dortmund, Germany), and Mb cells were a gift from Dr. G. Fertig (Darmstadt, Germany). Cell culture medium ingredients were obtained from Sigma and Gibco BRL. Modified baculovirus DNA (BaculoGold) was obtained from Pharmingen (San Diego, CA). The transfer vectors pAc360 and pVL1393 and wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were kindly provided by Dr. M. Summers (Texas A&M, College Station, TX). Plasmid pD2 containing the sequence of the human dopamine D_{2S} receptor was a generous gift from Dr. M. Caron (Durham, NC).

Antibody Production. The peptide sequence AARRAQELEMEML (amino acids 243−255), selected for immunization, is located within the third intracellular loop of the D_2 receptor and has been shown to be suitable for anti-D_2 receptor antibody production previously (Chazot et al., 1993). The peptide was synthesized using standard Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] chemistry on solid phase. The crude peptide was purified by high-performance liquid chromatography and coupled to the carrier protein KLH by glutaraldehyde (Reichlin, 1980). Two New Zealand White rabbits were injected subcutaneously at multiple sites with about 1.4 mg of peptide−KLH conjugate in complete Freund's adjuvant (at least 0.2 μmol of peptide/immunization). The rabbits were boosted with the same amount of conjugate in incomplete Freund's adjuvant at 4-week intervals. Blood was collected 10 days after boosting and serum stored at −70 °C. For affinity purification of the site-directed antibodies, the peptide was coupled to activated CH Sepharose 4B by a standard protocol suggested by the supplier (Pharmacia, Uppsala, Sweden). The column was equilibrated at 4 °C with 10 mM Tris-HCl, pH 7.6, 0.5 M NaCl, and 0.02% Tween 20 (TNT-buffer), and antiserum D2/1 (diluted 10-fold with the same buffer) was applied and allowed to circulate through the column overnight. After the column was washed with TNT-buffer, antibodies were eluted with 0.1 M glycine hydrochloride, pH 2.8, into tubes containing 1 M Tris-HCl, pH 8, for neutralization. After dialysis against PBS, the antibodies were concentrated by ultrafiltration, stabilized with 1% BSA, and stored at −70 °C.

Construction of Recombinant Baculoviruses. The recombinant transfer vector pAcPolD2S was constructed by digesting plasmid pD2 (kindly provided by Dr. M. Caron) with *Bam*HI and *Kpn*I and ligation of the 1.45 kbp DNA fragment into the appropriately digested transfer vector pAc360. This insert contained the entire coding region of the human D_{2S} receptor with the exception of the start codon (ATGGATCC; restriction site *Bam*HI underlined, ATG is the start codon of the D_{2S} receptor). The recombinant transfer vector pAcPolD2SH6 was constructed analogously by excising the His-tagged coding sequence of the D_{2S} receptor from plasmid pKSD2SH6 (constructed from pD2 by T. Lenhard). The recombinant transfer vector pVLD2S was constructed by ligation of the pD2-derived *Sma*I/*Kpn*I-DNA fragment (≈1490 bp) into the appropriately digested transfer vector pVL1393. For construction of the recombinant transfer vector pVLMelD2SH6, the *Bam*HI/*Kpn*I-DNA fragment encoding the His-tagged D_{2S} receptor was inserted into vector pAcC4s (Chai et al., 1993). pVLMelMycD2S was constructed by inserting the *Bam*HI/*Kpn*I fragment obtained from pVLD2S into pVL93MelMyc (Lenhard et al., 1996). All constructs (see Figure 1) were verified by restriction analysis and DNA sequencing. Recombinant baculoviruses were produced by cotransfecting 1 μg of genomic AcMNPV-DNA and 10 μg of recombinant transfer vector, or 0.1 μg of linearized BaculoGold-DNA (Pharmingen) and 0.4 μg of pVLMelMycD2S, using cationic liposomes (Lipofectin, GIBCO, BRL), according to the manufacturer's specifications. Screening for recombinant baculoviruses was performed as described in Reiländer et al. (1991). Viruses were amplified, and virus titers were determined by end-point dilution as described in O'Reilly et al. (1992).

Insect Cell Culture, Infection with Recombinant Baculoviruses, and Membrane Preparation. The insect cell lines Sf9, Sf21, Tn 368, and Mb were routinely propagated in TNM-FH medium supplemented with 5% fetal calf serum and 50 μg/mL gentamycin as described by Summers and Smith (1987). For time-course and labeling experiments, 5×10^6 (Sf9 or Sf21), 4×10^6 (Tn), or 3×10^6 (Mb) cells were seeded in 6 cm petri dishes and infected with wild-type or recombinant baculovirus at a moi (multiplicity of infection) of 10−20. At the indicated times after infection,

cells were either metabolically labeled or harvested and resuspended in cold 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 10 mM EDTA (TEN-buffer) supplemented with 5 μg/mL leupeptin and aprotinin, 1 μg/mL pepstatin A, 1 μg/mL chymostatin, 5 μg/mL soybean trypsin inhibitor, and 1 mM pefabloc (TEN/PI) at a density of 1×10^7 cells/mL for receptor binding and immunological analysis. For some experiments, tunicamycin was added to a final concentration of 10 μg/mL 30 h before harvesting. For membrane preparation, Sf9 cells were cultured in 250 mL spinner flasks (Techne, U.K.) at 70 rpm. At a cell density of $(1.5-2) \times 10^6$ cells/mL, the cells were infected with recombinant baculovirus at a moi of about 10; 60 h after infection, cells were harvested, washed twice in cold 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TN-buffer), and lysed by nitrogen cavitation (Parr Instruments) in TEN/PI at 500 psi (3.4 MPa) for 30 min. Lysates were centrifuged at 500*g* for 10 min at 4 °C to remove nuclei and cell debris. The supernatant was pelleted at 100000*g* for 1 h at 4 °C. The crude membrane pellet was resuspended in TEN/PI at a protein concentration of 10−13 mg/mL, snap-frozen in liquid nitrogen, and stored at −70 °C. The protein concentration was determined using the BCA reagent (Pierce, Rockford, IL).

Immunoblot Analysis. Uninfected or infected insect cells (10^7 cells/mL) were lysed in Laemmli sample buffer, DNA was digested with Benzonase (Merck, Darmstadt, FRG), and the sample was centrifuged at 12000*g* for 5 min. Protein in the supernatant was resolved by 10% SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (10 μL ∼ 100 000 cells/lane) (Laemmli, 1970) and electrophoretically transferred to Immobilon-P poly(vinylidene difluoride) membranes (Millipore Corp., Bedford, MA) in Towbin transfer buffer [25 mM Tris, 192 mM glycine, and 20% (v/v) methanol; Towbin et al. (1979)] containing 0.02% SDS. Immobilon-P membranes were blocked for 1 h at room temperature with 5% non-fat dry milk powder in PBST (phosphate-buffered saline containing 0.2% Tween 20). Then, the membranes were probed either with the affinity-purified anti-peptide D_2 antibody D2/1, diluted 1:500 in the blocking solution, or with the primary monoclonal anti c-*myc* antibody 9E10, diluted 1:2000 (Evan et al., 1985). Protein bands were visualized by incubating with the appropriate alkaline phosphatase-labeled IgG. Antibody incubations were for 1 h at room temperature each followed by three 5 min washes in PBST. The blots were developed in AP-buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) using 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidinium salt (BCIP) and nitroblue tetrazolium chloride (NBT).

Metabolic Labeling. Cells were seeded in 6 cm culture dishes and infected as described above. One hour before labeling, the regular TNM-FH medium was replaced by Grace's medium which, for [^{35}S]methionine labeling, was free of methionine. Then, cells were labeled either with [^{35}S]-methionine (500 kBq/mL) or with [^{3}H]palmitic acid (4−7 MBq/mL) for 4 h at 27 °C. For pulse−chase studies, the cells were pulsed with [^{35}S]methionine for 15 min and chased with TNM-FH medium supplemented with 1 mg/mL methionine for various periods. Metabolic labeling was terminated by centrifugation. The cells were washed twice in TEN-buffer and solubilized as described below. For some experiments, 5 μg/mL tunicamycin was added 12 h before labeling, or 5 μg/mL brefeldin A (BFA) or 15 μM monensin was added at the time of the addition of the deficient medium and was maintained until the cells were harvested.

Solubilization and Immunoprecipitation of Metabolically Labeled Products. Metabolically labeled cells were solubilized with a mixture of 1.25% digitonin and 0.25% cholate for 30−60 min at 4 °C. After ultracentrifugation at 100000*g* for 20 min at 4 °C, the supernatant was diluted 2-fold in TEN/PI-buffer and incubated with 0.01 volume of the anti-peptide antiserum D2/1 or preimmune serum for 1−2 h at 4 °C. Immunocomplexes were precipitated for 1−2 h at 4 °C with 40 μL of protein A−Sepharose (prewashed with 0.5% BSA in TEN). Following three washes in TEN-buffer containing 0.6% digitonin and 0.12% cholate, samples were eluted into Laemmli sample buffer at 10 °C and analyzed by 10% SDS−PAGE and subsequent fluorography. Gels were Coomassie-stained, incubated for 1 h in 1 M sodium salicylate, pH 6, dried, and exposed to Kodak X-Omat AR film at −70 °C.

Analysis of Incorporated [^{3}H]Palmitic Acid. The ^{3}H-receptor region of the SDS−polyacrylamide gel was excised. The gel slice was homogenized, and [^{3}H]palmitic acid was released by saponification and analyzed by thin-layer chromatography as described in O'Dowd et al. (1989), except that the chromatogram was developed with toluene/methanol/acetic acid (80:20:1).

Endoglycosidase H and N-Glycosidase F Digestions. A total of 3×10^7 cells were metabolically labeled with [^{35}S]-methionine and harvested as outlined above. The cells were sheared using an Ultra-Turrax (5×10 s) followed by sonication for 15 s, and crude membranes were prepared as described above. For glycosylation analysis, solubilized and immunoprecipitated receptor was eluted from 10 μL of protein A−Sepharose with 5 μL of elution buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 0.1 M β-mercaptoethanol) and digested either with 10 IUB milliunits of PNGase F in 100 mM sodium phosphate buffer, pH 8.6, 1% *n*-octyl glucoside, or with 5 milliunits of Endo H in 50 mM sodium citrate, pH 5.5, 1% *n*-octyl glucoside, in a total volume of 25 μL for 11 h at 25 °C. The digestion was stopped as described above and analyzed by 10% SDS−PAGE and subsequent fluorography.

Receptor Binding Assays. [^{3}H]Methylspiperone saturation binding was performed on membranes prepared from AcPolD2S-infected Sf9 cells in 500 μL of TEN-buffer containing 50−100 pM binding sites for 2 h at 25 °C. For ligand competition binding, membranes were diluted into TEN-buffer to 40−70 pM [^{3}H]spiperone binding sites in a total volume of 1 mL and incubated for 2 h at 25 °C with increasing concentrations of agonists or antagonists in the presence of about 600 pM [^{3}H]methylspiperone. The incubations were terminated by the addition of 3.5 mL of cold 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TN-buffer), and filtering with a Brandel cell harvester through Whatman GF/F filters presoaked in 0.3% poly(ethylenimine) (Sigma). The filters were washed twice with 3.5 mL of cold TN-buffer and the filter disks counted for tritium using a Canberra-Packard Tri-Carb scintillation counter. For estimating total [^{3}H]spiperone binding sites, approximately 100 000 cells per tube were incubated in TEN-buffer in a final volume of 500 μL with 4 nM [^{3}H]spiperone at 30 °C for 30 min. The incubation was terminated as described above. Nonspecific binding was determined in the presence of 2 μM (+)-butaclamol. Binding data were analyzed by nonlinear least-

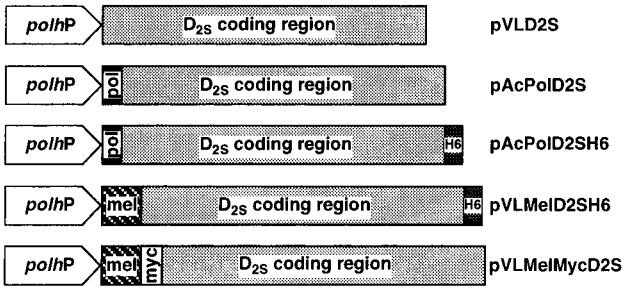

FIGURE 1: Schematic representation of various recombinant baculovirus transfer vectors for heterologous expression of the human D_{2S} receptor in insect cells. *polh*P, polyhedrin promotor; pol, the first 12 codons of the polyhedrin coding sequence; mel, coding sequence for the prepromelittin signal sequence; myc, coding region for the c-*myc* epitope; H6, His-tag consisting of six consecutive histidine codons.

squares fitting using the program KaleidaGraph. Competition curves were fit to the four-parameter logistic equation.

Electron Microscopy (Fixation, Embedding, and Immunogold Staining). Infected Sf9 or Tn cells were fixed *in situ* with 4% parafomaldehyde and 1% glutardialdehyde in 0.1 M PBS, pH 7, for 2 h at room temperature. After being washed in PBS, the cells were treated with 2% glycine in PBS. For pre-embedding immunostaining, the cells were incubated first with 1% BSA in PBS, then with 0.1% BSA in PBS, and then with either the monoclonal anti-c-*myc* antibody 9E10 (1:2000) or the polyclonal anti-peptide D_2 antiserum D2/1 (1:2000) at 4 °C overnight. After being washed, the cells were incubated with the secondary goat anti-mouse or anti-rabbit antibody (diluted 1:20−1:40 in PBS) coupled to 10 nm gold particles (Amersham-Buchler, Braunschweig, FRG) for 90 min. After immunogold labeling, the cells were refixed in 1% glutardialdehyde in 0.1 M PBS, pH 7, for 30 min, washed with 0.15 M sodium cacodylate, enclosed in 4% agar−agar, postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer, and stained en bloc with 1% uranyl acetate. The samples were dehydrated with ethanol, embedded in Spurr's resin (Spurr, 1969), and polymerized at 70 °C. For post-embedding immunostaining, glycine-treated cells were directly enclosed in 4% agar−agar, dehydrated, and infiltrated with LR White resin, and samples were polymerized at 58 °C. Ultra-thin sections were sequentially incubated in saturated sodium metaperiodate (1 h), water (10 min), 2% glycine in PBS (10 min), 1% BSA in PBS plus 0.2% Tween 20 (20 min), and 0.1% BSA in PBS plus 0.2% Tween 20 (10 min). After immunogold labeling which was performed as described above, the sections were refixed in 1% glutardialdehyde in 0.1 M PBS, pH 7 for 30 min, washed, and dried. In some experiments, gold staining was enhanced using the silver enhancement kit IntenSEM (Amersham-Buchler, Braunschweig). The sections were double-stained with 2% uranyl acetate and lead citrate and viewed in an electron microscope (Philips EM 300, Eindhoven, The Netherlands).

RESULTS

Time-Dependent Expression of the Human D_{2S} Receptor in Different Insect Cell Lines Infected with the Recombinant Baculovirus AcPolD2S. For expression of the human D_{2S} receptor in insect cells, various recombinant baculovirus transfer vectors, schematically depicted in Figure 1, were constructed. The resulting recombinant baculoviruses were

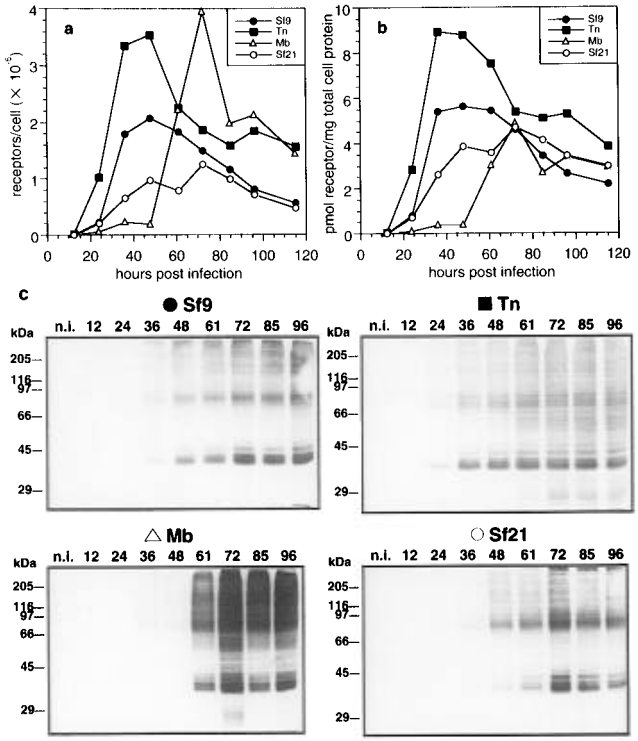

FIGURE 2: Time-dependent expression of the human D_{2S} receptor in AcPolD2S-infected insect cell lines. Sf9, Sf21, Tn, and Mb cells were infected with recombinant baculovirus AcPolD2S, and at the indicated times after infection (12, 24, 36, 48, 61, 72, 85, 96, and 115 h; n.i. = noninfected), the cells were harvested and subjected to ligand binding assays (a), protein determination (b), and immunoblot analysis (c) as described under Experimental Procedures. The results of one out of two similar experiments are shown.

termed VLD2S, AcPolD2S, AcPolD2SH6, VLMelD2SH6, and VLMelMycD2S. The transfer vector pVLD2S contains the D_{2S} receptor gene without any modifications, whereas in the transfer vectors pAcPolD2S and pAcPolD2SH6, the 5′-end of the D_{2S} coding sequence was fused to the first 12 codons of the viral polyhedrin gene (Pol-sequence).

Figure 2 shows the time course of D_{2S} receptor synthesis in AcPolD2S-infected cells of four different insect cell lines: the two *Spodoptera frugiperda* cell lines Sf9 and Sf21, the *Trichoplusia ni* cell line Tn 368, and a *Mammestra brassicae* cell line (Mb). Expression of the receptor was monitored by ligand binding using the radioactive antagonist [^{3}H]spiperone and by immunoblot analysis with affinity-purified polyclonal antibody D2/1 raised against a peptide sequence of the putative third intracellular loop. Receptor expression peaked between 36 and 48 h in Tn cells, at about 48 h in Sf9 cells, and at 72 h in Sf21 and Mb cells. Receptor expression was lowest in Sf21 cells. Tn and Mb cells expressed twice as much receptor as Sf9 cells on a per cell basis with up to 4×10^6 receptors per cell. However, when looking at the specific activity of the expressed receptor in picomoles of receptor per milligram of total cell protein, Tn and Sf9 cells are superior to Mb cells. Mb cells are substantially larger which may account in part for this difference. Noninfected insect cells or cells infected with wild-type baculovirus did not show any specific [^{3}H]-spiperone binding (data not shown).

Immunoblottable receptor protein was first detectable in Tn cells 24 h after infection. In Sf9 cells, immunostained protein bands appeared 36 h after infection, in Sf21 cells at 48 h, and in Mb cells only at about 60 h post-infection in

accordance with the binding data presented in Figure 2a. The D_2 receptor antibody D2/1 reacted specifically with the cloned D_{2S} receptor, since baculovirus wild-type-infected Sf9 cells at different times post-infection (data not shown) as well as noninfected cells (first lane in all four blots of Figure 2c) did not reveal any significant signal. In all four cell lines tested, the antibody labeled a dominant broad set of bands between 38 and 41 kDa, a thinner band at about 44 kDa, and a band at about 87 kDa. The molecular mass of these bands falls within the range of apparent molecular masses found for the native D_2 receptor (Grigoriadis et al., 1988; Jarvie et al., 1988; Clagett-Dame & McKelvy, 1989) as well as for recombinant D_{2S} or D_{2L} receptors (Javitch et al., 1994; Ng et al., 1994b; Fishburn et al., 1995; Boundy et al., 1996). In addition, a lot of aggregated and degraded protein can be seen. Clearly, aggregation and degradation were most serious in Tn and Mb cells. As opposed to cell lines derived from *Spodoptera frugiperda*, baculovirus infection does not result in a shutdown of host cell protein production in Tn and Mb cells, so that in these cells protein degradation pathways most likely are still fully functional.

Sf9 cells infected with the baculovirus VLD2S maximally produced up to 1.5 million receptors per cell. Therefore, this recombinant virus was not selected for further biochemical and pharmacological characterization.

Characterization of AcPolD2S-Produced D_{2S} Receptor by Immunoprecipitation. In light of the variety of bands specifically recognized by the D2/1 antibody in Western blots of AcPolD2S-infected insect cells (Figure 2c), it was difficult to assign any specifically processed or unmodified receptor forms to specific bands. Therefore, infected insect cells were metabolically labeled with [^{35}S]methionine and lysed using a digitonin/cholate detergent mixture. Under these mild lysis conditions, functional receptor could be solubilized, separated from D_{2S} receptor aggregates by centrifugation, and analyzed by immunoprecipitation (Figure 3). In all three cell lines, Sf9, Tn, and Mb, which were infected with the recombinant baculovirus AcPolD2S, the D2/1 antiserum identified the same two prominent broad protein bands with apparent molecular masses centered around 47 and 39 kDa (Figure 3a, lane 3; 3b, lane 2; autoradiographs of Tn and Mb cells not shown). In addition to these monomeric receptor forms, the D2/1 antiserum identified a 70 kDa, a 140 kDa, and a 170 kDa band, which most likely correspond to receptor aggregates. The 70 kDa band was compressed by the large amount of partly reduced IgG and corresponds to the 87 kDa band seen on immunoblots. A similar band of 93 kDa was found for the long isoform D_{2L} expressed in Sf9 cells and interpreted as dimer or receptor−G protein complex (Ng et al., 1994b). In insect cells infected with wild-type baculovirus, these bands were not present, and virtually no protein could be precipitated by the polyclonal antiserum (Figure 3a,b, first lanes). Preimmune serum did not precipitate any protein in D_{2S} receptor-expressing cells (Figure 3a, lane 2). This gives further evidence that the antibody reaction is absolutely specific and that the precipitated protein represents the D_{2S} receptor.

Glycosylation of the D_{2S} Receptor: Effect of Tunicamycin and Brefeldin A on AcPolD2S-Infected Cells. Tunicamycin which inhibits N-linked glycosylation (Tkacz & Lampen, 1975), and brefeldin A (BFA), which inhibits transport of proteins out of the ER and redistributes *cis*/medial Golgi components to the ER (Misumi et al., 1986; Lippincott-

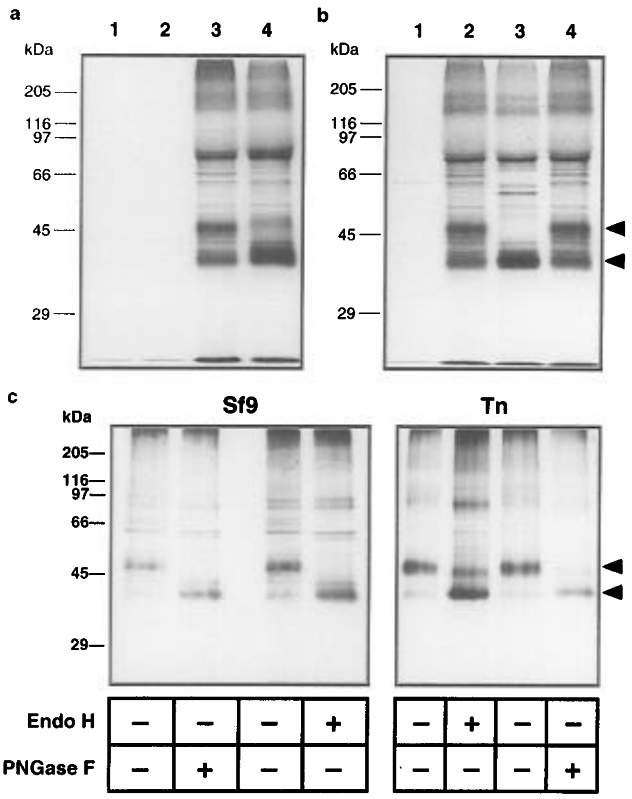

FIGURE 3: Glycosylation of the immunoprecipitated D_{2S} receptor of [^{35}S]methionine-labeled, baculovirus-infected insect cells. (a) Sf9 cells were infected with either wild-type baculovirus (lane 1), recombinant virus AcPolD2S (lanes 2, 3), or AcPolD2SH6 (lane 4). 68 h after infection, the cells were labeled for 4 h with [^{35}S]-methionine, and after solubilization, protein was immunoprecipitated with either preimmune serum (lane 2) or anti-peptide antiserum D2/1 (lanes 1, 3, 4). The samples were separated on a 10% SDS−polyacrylamide gel and autoradiographed. (b) Sf9 cells were infected with either wild-type baculovirus (lane 1) or recombinant virus AcPolD2S (lanes 2, 3, 4) and labeled with [^{35}S]methionine, and expressed receptors were immunoprecipitated as described under Experimental Procedures. 10 μg/mL tunicamycin (lane 3) or 5 μg/mL brefeldin A (lane 4) was added 12 h or 1 h before labeling with [^{35}S]methionine, respectively. Arrows indicate the glycosylated and nonglycosylated D_{2S} receptor forms. (c) AcPolD2S-infected Sf9 and Tn cells were labeled with [^{35}S]methionine. Crude membranes were prepared, and immunoprecipitated samples were split into two and incubated in the presence or absence of either Endo H or PNGase F as described.

Schwartz et al., 1989), were used to determine the effect of glycosylation on the size of the D_{2S} receptor in insect cells. Treatment of AcPolD2S-infected Sf9 cells with tunicamycin reduced the amount of functional receptor as measured by ligand binding on whole cells to about 40% of that detected in nontreated cells. But tunicamycin did not change the pattern of bands on the immunoblots, and the similar intensity of immunostained bands of tunicamycin-treated and non-treated cells suggests that tunicamycin did not reduce receptor synthesis (Figure 6, lanes 1, 2). Addition of tunicamycin, however, led to the disappearance of the immunoprecipitated 47 kDa band in all three cell lines, and at the same time, the 39 kDa band became more intense (Figure 3b, lane 3, data for Tn and Mb cells not shown). Thus, the 47 and 39 kDa bands represent the glycosylated and unglycosylated form of the D_{2S} receptor. The 47 kDa band could not be detected on Western blots of AcPolD2S-infected cells, suggesting that the glycosylated form of the D_{2S} receptor only represents a small fraction of the overall produced receptor protein. On

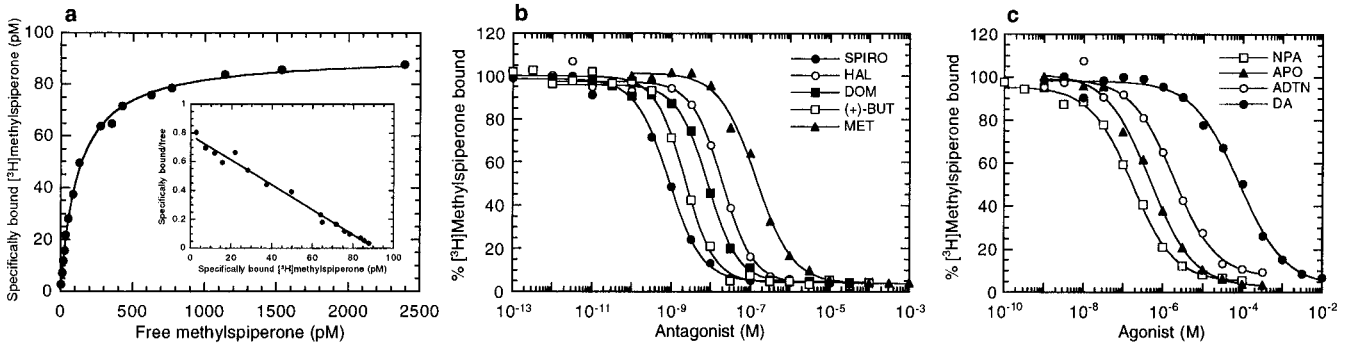

FIGURE 4: Radioligand binding studies on membranes of AcPolD2S-infected Sf9 cells. Infection, membrane preparation, and binding assays were performed as outlined under Experimental Procedures. The results shown are from one of at least two independent experiments. (a) Saturation isotherm of [^{3}H]methylspiperone. (b) Antagonist displacement curves: spiperone hydrochloride (SPIRO), haloperidol (HAL), domperidone (DOM), *S*(+)-butaclamol hydrochloride [(+)-BUT], metoclopramide (MET). (c) Agonist displacement curves: *R*(−)-propylnorapomorphine hydrochloride (NPA), *R*(−)-apomorphine hydrochloride (APO), (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN), dopamine (DA). K_i values for displacing drugs are given in Table 1.

the other hand, the 44 kDa band seen on Western blots only appeared as a faint band on autoradiographs of solubilized and immunoprecipitated protein. It most likely represents misfolded receptor protein which is barely solubilized under the mild digitonin/cholate conditions used and may not be fully denatured on a SDS gel. BFA did not have any influence on the apparent molecular mass of the human receptor in any of the three insect cell lines tested, suggesting that, in these cells, no major oligosaccharide processing occurs beyond the *cis*/medial Golgi. This is further supported by studies of endoglycosidase H digestion of the [^{35}S]-methionine-labeled, immunoprecipitated receptor. This enzyme hydrolyzes the core di-*N*-acetylchitobiose linkage in all high-mannose and some hybrid oligosaccharides (before being processed by mannosidase II in the medial Golgi). Results shown in Figure 3c illustrate that the human D_{2S} receptor heterologously expressed in Sf9 insect cells was completely hydrolyzed by this enzyme, reducing the apparent molecular mass similar to tunicamycin from 47 kDa to 39−40 kDa. This change of about 7−8 kDa is in agreement with the postulated presence of three oligosaccharide chains according to the three potential N-terminal N-glycosylation sites. A control digestion with PNGase F, which cleaves the glycosamine linkage between the oligosaccharide and the asparagine regardless of oligosaccharide class, showed the same molecular mass shift (Figure 3c). Therefore, glycosylation of the D_{2S} receptor expressed in Sf9 insect cells is of the high-mannose type. A similar result was obtained in Tn cells except for a residual band at 46 kDa in both Endo H and PNGase F digestions (Figure 3c). This band most probably represents residual glycosylated receptor.

Pharmacological Characterization of the Expressed D_{2S} Receptor. Specific binding of [^{3}H]methylspiperone to membranes of AcPolD2S-infected Sf9 cells expressing D_{2S} receptors at 60 h after infection was saturable and of high affinity, with Scatchard analysis indicating the presence of a homogeneous binding site (Figure 4a). The equilibrium dissociation constant of [^{3}H]methylspiperone was 98 ± 14 pM (n = 4) and equalled that of [^{3}H]spiperone (86 ± 19 pM, n = 5). These values are in good agreement with results obtained for D_2 receptors in human retinas (87 ± 26 pM) (McGonigle et al., 1988) as well as for recombinant human D_{2S} receptors expressed in COS-7 (70 ± 13 pM) (Dearry et al., 1991) and human embryonic kidney 293 cells (≈50 pM) (Dal Toso et al., 1989). The ability of a number of subtype-selective dopaminergic agonists and antagonists to compete for the binding of [^{3}H]methylspiperone to membranes of infected Sf9 cells is shown in Figure 4b,c. The inhibition constants, K_i, for the dopaminergic ligands (summarized in Table 1) agree well with previously published values [summarized in Falardeau (1994)]. The rank order of potency for agonists (NPA > APO > ADTN ≫ DA) and antagonists [SPIRO > (+)BUT > DOM > HAL > MET] was as expected for a D_2 receptor (Bunzow et al., 1988; Grandy et al., 1989; Dearry et al., 1991). While the antagonist competition curves all displayed a pseudo-Hill coefficient of 1, the slope factor of the agonist competition curves was less than unity. However, agonist inhibition of [^{3}H]methylspiperone binding was not guanine nucleotide-sensitive (data not shown).

Table 1: Pharmacological Profile of the D_{2S} Receptor Expressed in Sf9 Cells by Infection with AcPolD2S[a]

ligands	K_i (nM)	n
antagonists		
spiperone	0.11 ± 0.02	1.0 ± 0.06
haloperidol	3.2 ± 0.7	1.1 ± 0.10
domperidone	1.4 ± 0.3	1.0 ± 0.1
(+)-butaclamol	0.31 ± 0.01	1.15 ± 0.08
metoclopramide	18 ± 1	0.93 ± 0.05
agonists		
R(−)-NPA	22 ± 1	0.85 ± 0.04
R(−)-apomorphine	58 ± 2	0.78 ± 0.03
(±)-6,7-ADTN	217 ± 24	0.9 ± 0.1
dopamine	9800 ± 700	0.72 ± 0.07

[a] Membranes prepared from Sf9 cells expressing the D_{2S} receptor at 40−60 h after infection were incubated with varying concentrations of competing drugs in the presence of about 0.6 nM [^{3}H]methylspiperone as described under Experimental Procedures. Values were determined from two experiments performed in duplicate and analyzed using the four-parameter logistic function by KaleidaGraph. K_i values were calculated from IC_{50} by the Cheng−Prusoff relation $K_i = IC_{50}/(1 + L/K_D)$, where L is the concentration and K_D is the dissociation constant of [^{3}H]methylspiperone (Cheng & Prusoff, 1973).

Influence of the Prepromelittin Signal Sequence and the His-Tag on Expression Levels and Processing of the Human D_{2S} Receptor in Insect Cells. In order to improve folding, processing, and yield of the heterologously produced D_{2S} receptor, we replaced the N-terminal 12 amino acids of polyhedrin by the cleavable prepromelittin signal sequence, resulting in the baculovirus transfer vectors pVLMelMycD2S and pVLMelD2SH6. The first construct contains the c-*myc* epitope to allow immunological detection of the N-terminal

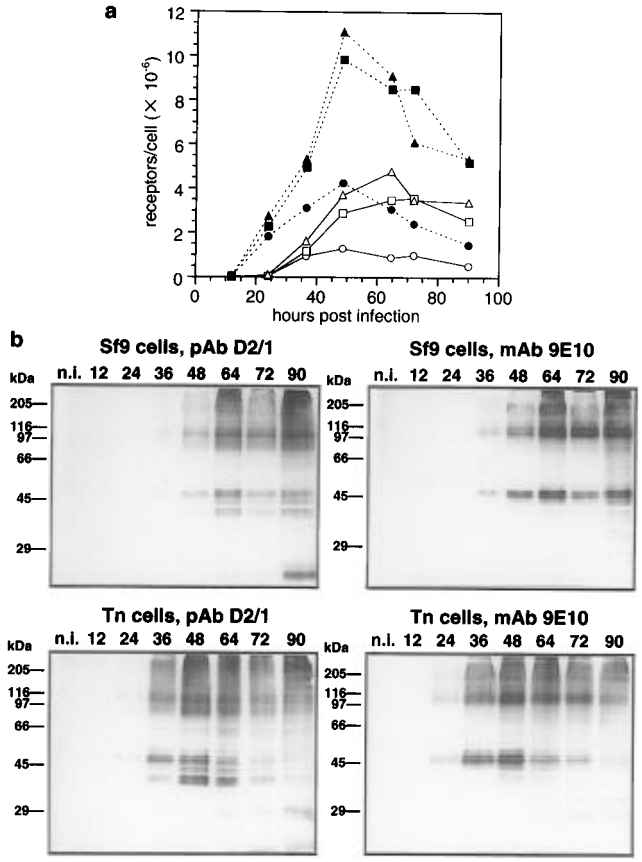

FIGURE 5: Influence of the prepromelittin signal sequence and the His-tag on time-dependent expression of the human D_{2S} receptor in recombinant baculovirus-infected insect cells. (a) Sf9 (solid lines, open symbols) and Tn (dotted lines, closed symbols) cells were infected with the following recombinant baculoviruses: AcPolD2SH6 (○, ●); VLMelMycD2S (△, ▲); VLMelD2SH6 (□, ■). At the indicated times after infection (12, 24, 36, 48, 64, 72, and 90 h; n.i. = noninfected), the cells were harvested and receptor levels measured using [^{3}H]spiperone. (b) Immunoblot analysis of the VLMelMycD2S-infected cells harvested as in (a) using either the D_2-specific anti-peptide antibody D2/1 or the monoclonal anti-c-*myc* antibody 9E10.

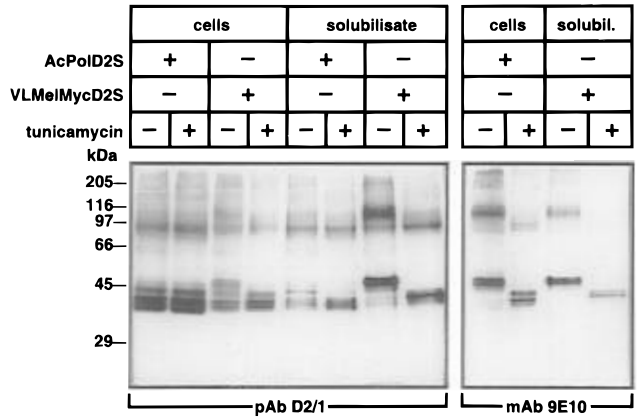

FIGURE 6: Immunoblot analysis of tunicamycin-treated Sf9 cells expressing the D_{2S} receptor containing either the Pol sequence or the prepromelittin signal sequence and the c-*myc* epitope at the N-terminus. Sf9 cells were infected with recombinant baculovirus AcPolD2S or VLMelMycD2S. 28 h after infection, tunicamycin was added to a final concentration of 10 μg/mL. 30 h later, cells were harvested and either solubilized in digitonin/cholate or directly lysed in Laemmli sample buffer. Samples were separated on a 10% polyacrylamide gel and analyzed by immunoblotting with either the polyclonal antibody D2/1 or the monoclonal anti-c-*myc* antibody 9E10. The samples loaded per lane equalled 70 000 cells extracted in sample buffer or 110 000 cells extracted with digitonin/cholate.

end. In view of a possible purification, six histidine residues (His-tag) were added at the C-terminus of the D_{2S} receptor in the second construct. Figure 5 compares the time-dependent expression of the D_{2S} receptor containing the prepromelittin signal sequence followed by the c-*myc* epitope at the N-terminus and of the His-tagged receptor with either the N-terminal Pol sequence or the prepromelittin signal sequence in Sf9 and Tn cells. In accordance with the AcPolD2S infections shown in Figure 2, receptor expression was 2−3 times higher in Tn cells than in Sf9 cells with all three recombinant baculoviruses. AcPolD2SH6 infections reached the same receptor expression levels as AcPolD2S infections in both cell lines, and immunoblot analysis revealed the same pattern of bands (data not shown). However, immunoprecipitation of functionally solubilized receptor of AcPolD2SH6-infected Sf9 cells shows that the His-tag has a negative influence on processing. While the receptor from AcPolD2S-infected cells gives rise to two bands of about the same intensity for the glycosylated and unglycosylated form, the His-tagged receptor is mostly unglycosylated as shown by the intense lower band of 39 kDa and the weak upper band of 48 kDa (Figure 3a, lanes 3, 4). Replacement of the Pol sequence by the prepromelittin signal sequence visibly improved expression levels and processing. Receptor levels in VLMelMycD2S- or in VLMelD2SH6-infected cells reached (10−11) × 10^6 receptors per cell in Tn cells and (4−5) × 10^6 receptors per cell in Sf9 cells. Here, immunoblot analysis of VLMelMycD2S-infected Sf9 or Tn cells revealed a (triplet) band at 48 kDa in addition to a weak 44 kDa band and the 38−41 kDa bands detected in AcPolD2S- or AcPolD2SH6-infected cells (Figure 5b). This upper 48 kDa band was PNGase F- and Endo H-sensitive (data not shown) and was lacking on Western blots of tunicamycin-treated VLMelMycD2S-infected cells (Figure 6), resulting in basically the same pattern of bands as on blots of AcPolD2S-infected cells. Therefore, this 48 kDa band corresponds to the glycosylated form of the receptor which, contrary to AcPolD2S-infected cells, was produced in amounts high enough to be detected on immunoblots of whole cell extracts from VLMelMycD2S-infected cells. As found for AcPolD2S-infected cells, tunicamycin reduced the [^{3}H]spiperone binding sites in VLMelMycD2S-infected cells by 50−60%.

Interestingly, the anti-c-*myc* antibody 9E10 mainly labeled the glycosylated receptor at 48 kDa and a possible dimer at about 110 kDa. The 44 kDa band and the consecutive bands between 38 and 40 kDa, stained by the polyclonal antibody D2/1, were not detected. These bands, therefore, most probably are due to proteolysis. Only in immunoblots of tunicamycin-treated VLMelMycD2S-infected cells, a 44 kDa band and a 40 kDa band were labeled by the monoclonal antibody 9E10. It is mainly this 40 kDa band which was solubilized by digitonin/cholate (Figure 6) in these cells in accordance with the results obtained by immunoprecipitation of the D_{2S} receptor expressed in AcPolD2S-infected cells. A comparison of the intensities of the Western blot bands in lanes 1 and 3 of Figure 6 shows a much stronger signal for the AcPolD2S construct than for the VLMelMycD2S construct although the latter expresses about twice as much receptors per cell (cf. Figures 2 and 5). Therefore, the

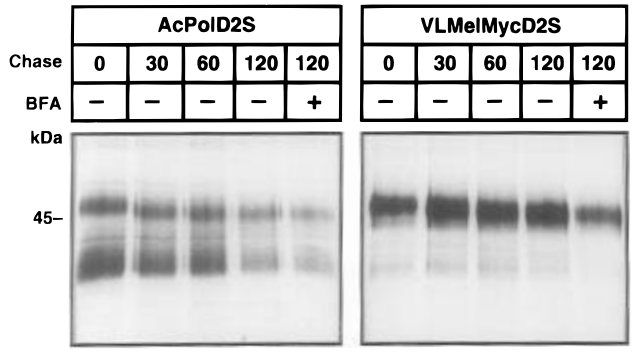

FIGURE 7: Pulse−chase analysis of *de novo* synthesized D_{2S} receptor in AcPolD2S- and VLMelMycD2S-infected Sf9 cells. Infected cells were pulsed with [^{35}S]methionine for 15 min. After the indicated chase time, cultures were harvested, and immunoprecipitates were analyzed. Brefeldin A (5 μg/mL) was added to one of the cultures 1 h before starting the pulse and maintained until the cells were harvested.

AcPolD2S-baculovirus must produce much more inactive receptor than the VLMelMycD2S-baculovirus.

The prepromelittin signal sequence also seems to overcome the negative influence of the His-tag. Immunoblot analysis of VLMelD2SH6-infected cells revealed the same pattern of bands (not shown) as the corresponding blots of VLMelMycD2S-infected cells shown in Figure 5b. Furthermore, virtually all of the functionally solubilized and immunoprecipitated receptor bearing the prepromelittin signal sequence was found to be glycosylated despite the presence of the His-tag, whereas at least 50% of the solubilizable and immunoprecipitable receptor containing the Pol sequence was unglycosylated and migrated with an apparent molecular mass of about 38−40 kDa. In order to investigate whether this unglycosylated receptor form was subject to further processing, infected cells were pulsed for 15 min with [^{35}S]-methionine and then chased with cold methionine for various time points (Figure 7). With the MelMycD2S construct, it seemed that basically all of the newly translated receptor protein may undergo N-linked glycosylation during the 15-min [^{35}S]methionine labeling period, giving rise to a 50 kDa band. After 30 min of chase, this band slightly dropped by 1−2 kDa. This small decrease in molecular mass could be interpreted as trimming of the primary oligosaccharide. BFA did not prevent this drop; however, less protein was precipitated. The reason could be either lower protein synthesis in the presence of BFA or less solubilizable protein. As expected, only about half of the solubilized receptor was glycosylated using the AcPolD2S construct. After 30 min of chase, the glycosylated form also decreased in size by about 1 kDa, but, surprisingly, the nonglycosylated form of the receptor did not dissappear with prolonged chase. So, in the constructs where the receptor contains an N-terminal Pol sequence, it seems that about half of the solubilizable protein cannot be processed correctly.

Palmitoylation. Figure 8 compares palmitoylation of the D_{2S} receptor in AcPolD2S-, AcPolD2SH6-, and VLMel-D2SH6-infected Sf9 cells. Cells expressing the D_{2S} receptor were cultured in serum-free media in the presence of either [^{3}H]palmitic acid as substrate for palmitoylation or [^{35}S]-methionine as a control to metabolically label the receptors. Figure 8a shows a fluorograph of whole cell extracts. Only a few proteins were palmitoylated. The faint band at 47 kDa might represent the D_{2S} receptor. Figure 8b shows a fluorograph of the corresponding functionally solubilized and immunoprecipitated protein. A distinct single band was precipitated with all three recombinant receptor constructs migrating at the same apparent molecular mass as the glycosylated form of the [^{35}S]methionine-labeled receptor. Occasionally, higher bands could be seen, which most likely represent receptor dimers and higher multimers. Immunoprecipitation of solubilized protein from baculovirus wild-type-infected cells did not reveal any bands (data not shown). The identity of the incorporated [^{3}H]palmitic acid was verified by saponification of the protein band followed by thin-layer chromatography (data not shown). Sequence analysis suggests that the cytoplasmic C-terminus of the D_{2S} receptor only consists of about 14 amino acids and the only cysteine that could be palmitoylated is the very last amino acid of the protein. So, it is interesting to note that the His-tag did not hinder palmitoylation (Figure 8b, lanes 1, 2). Since only the glycosylated form of the receptor was palmitoylated, [^{3}H]palmitic acid labeling, solubilization, and immunoprecipitation were done with AcPolD2S-infected Sf9 cells which had been treated either with tunicamycin, with BFA, or with monensin (Figure 8c). Neither of these compounds showed any influence on receptor palmitoylation. As already seen before in [^{35}S]methionine-labeled cells, tunicamycin caused a decrease in the apparent molecular mass of about 8 kDa.

Localization of the D_{2S} Receptor Heterologously Produced in Sf9 and Tn Cells. Although heterologous expression of the D_{2S} receptor with the different baculoviruses described above resulted in several million binding sites per cell, immunoblot analysis revealed the presence of aggregated protein, and immunoprecipitation of functionally solubilized receptor showed differences in the extent of glycosylation. To examine whether the recombinant D_{2S} receptor synthesized by insect cells after infection was transported to the cell surface, electron microscopic immunogold staining was performed. Figure 9a−c shows post-embedding immunogold staining of Sf9 cells infected with AcPolD2S, AcPolD2SH6, or VLMelD2SH6 using the polyclonal antiserum D2/1. Gold particles were predominantly found associated with dense patches in the perinuclear cytoplasm. In the cell periphery, the cytoplasm often appeared less dense and more flocculated. The plasma membrane was devoid of specific labeling using this technique. Pre-embedding immunogold staining with the D2/1 antiserum directed against an intracellular epitope was not successful under conditions where the cells had to be permeabilized before performing the immunoreactions. In order to directly compare both techniques, the baculovirus VLMelMycD2S was used for receptor expression. Here, the receptor contains the c-*myc* epitope fused to its N-terminal end; therefore, pre-embedding immunogold staining does not require cell permeabilization in order to detect receptors in the plasma membrane. Post-embedding immunogold staining with the mAb 9E10 gave the same result as that obtained with antiserum D2/1. Occasionally, single gold particles were located at the plasma membrane (Figure 9e). In contrast, pre-embedding labeling resulted in convincing labeling on the extracellular site of the cell membrane (Figure 9g). Obviously, embedding the cells before performing the immunoreactions destroyed or reduced the antigenicity of both epitopes such that the fraction of receptors eventually present in the cell membrane cannot be detected any longer.

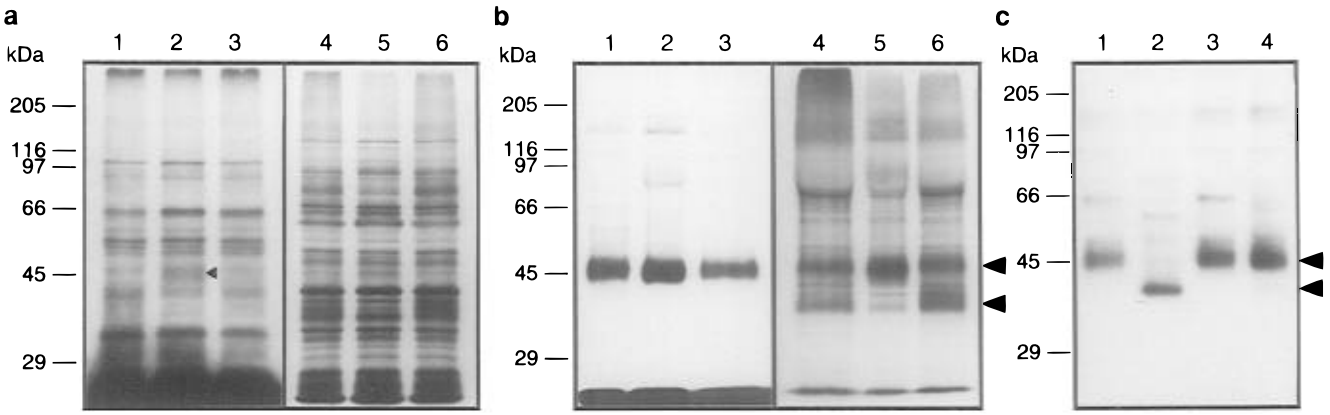

FIGURE 8: Palmitoylation of the D_{2S} receptor in Sf9 insect cells. (a) Sf9 cells were infected with either AcPolD2S (lanes 1 and 6), AcPolD2SH6 (lanes 3 and 4), or VLMelD2SH6 (lanes 2 and 5) and labeled either with [^{35}S]methionine (lanes 4−6) or [^{3}H]palmitic acid (lanes 1−3). A cell aliquot was lysed in Laemmli sample buffer and analyzed by 10% SDS−PAGE. (b) The cells obtained in (a) were solubilized in digitonin/cholate, and immunoprecipitated D_{2S} receptor was analyzed by 10% SDS−PAGE. The order of the gel lanes is as in (a). (c) Sf9 cells were infected with AcPolD2S and metabolically labeled with [^{3}H]palmitic acid. 12 h before labeling, tunicamycin was added to a concentration of 10 μg/mL (lane 2). 1 h before labeling, either 5 μg/mL brefeldin A (lane 3) or 10 μM monensin (lane 4) was added. Expressed D_{2S} receptor was immunoprecipitated and analyzed by 10% SDS−PAGE.

In Tn cells, basically the same results were obtained, except that post-embedding immunogold staining with the mAb 9E10 was more frequently found at the plasma membrane than in Sf9 cells (not shown).

DISCUSSION

The purpose of this study was to evaluate the baculovirus system as a means for high-yield production of homogeneous D_{2S} receptor by examining expression and processing of various D_{2S} receptor constructs in infected insect cells. The polyhedrin promotor is a very strong and efficient promotor, which, together with an efficient translation, leads to the production of milligrams of soluble protein per liter of culture (Bishop, 1990). The results presented here show that the D_{2S} receptor also is efficiently produced.

Two strategies were applied to improve receptor yield. First, recombinant baculoviruses were constructed which contained the first 12 codons of the polyhedrin gene fused to the 5′-end of the D_{2S} receptor coding sequence. Retention of the environment of the polyhedrin initiation site has been reported to increase translation and, consequently, protein yield (Matsuura et al., 1987). Second, the signal sequence of *A. melifica* (honeybee) prepromelittin was fused to the N-terminus of the D_{2S} receptor. This signal sequence has been shown to enhance the expression and secretion of recombinant propapain (Tessier et al., 1991) and TNF-β (Chai et al., 1993) in recombinant baculovirus-infected Sf9 cells. The oxytocin (Gimpl et al., 1995) and the $5HT_{5A}$ receptor (Lenhard et al., 1996) have been successfully expressed in Sf9 cells using this signal sequence. Yet, its influence on receptor expression has not been examined.

Addition of the Pol sequence increased receptor expression from about 1.5×10^6 to 2×10^6 receptors/cell. This effect of the Pol sequence was by far not as striking as has been reported for the D_4 dopamine receptor (Mills et al., 1993). D_{2S} receptor levels were comparable to the 5−8 pmol/mg of membrane protein reported for both D_2 receptor isoforms in Sf9 cells (Boundy et al., 1996) but 4 times higher than the levels of $(3-5) \times 10^5$ receptors per cell (Javitch et al., 1994) or 5−10 times higher than the level of 1−2 pmol/mg of membrane protein (Ng et al., 1994b) reported for the D_{2L} isoform in Sf9 insect cells.

The pharmacological characteristics of the human D_{2S} receptor overexpressed in insect cells were as expected for a D_2 receptor and agreed with results obtained for the rat D_2 receptor isoforms (Boundy et al., 1996) and for the human D_{2L} isoform expressed in insect cells (Javitch et al., 1994; Ng et al., 1994b). The human D_{2S} receptor did not display any high-affinity agonist binding sites when heterologously expressed in insect cells. This is in agreement with results reported by Boundy et al. (1996), who showed that the rat D_2 receptor isoforms expressed in Sf9 cells only displayed GTP-sensitive high-affinity binding when expression levels were below 1 pmol/mg of membrane protein. These findings suggest that the amount of endogenous insect cell G protein capable of coupling to the overexpressed receptor is limiting and/or that coupling is not efficient. Thus, only a small fraction of the overall produced receptor can be converted to the high-affinity state, and this fraction can obviously only be detected at very low expression levels. Despite the lack of guanine nucleotide sensitivity, the slope factor of the agonist competition curves was less than unity. The same phenomenon has been reported earlier for the binding of the agonist quinpirole to the D_{2L} receptor expressed in Sf9 cells (Javitch et al., 1994). We think that the very high overexpression of the receptor in these cells may lead to receptor aggregation and artifactual binding especially of agonists. The question of G protein coupling has been addressed in detail in the accompanying report (Grünewald et al., 1996), where we show that the heterologously produced D_{2S} receptor is able to couple to G proteins and, therefore, is fully functional.

Immunoprecipitations of digitonin/cholate extracts of AcPolD2S-infected cells revealed an apparent molecular mass of 47−48 kDa for the glycosylated form and of about 39−40 kDa for the unglycosylated monomer of the D_{2S} receptor. The apparent molecular mass of the unglycosylated form is lower than the molecular mass of 47 kDa predicted from the protein sequence. Nevertheless, the results agree well with those obtained by Fishburn et al. (1995), who reported an apparent molecular mass of 37 kDa for the full-length translation product and of 43 kDa for a partially processed form of the D_{2S} receptor expressed in CHO cells. Moreover, there are several indications suggesting that the

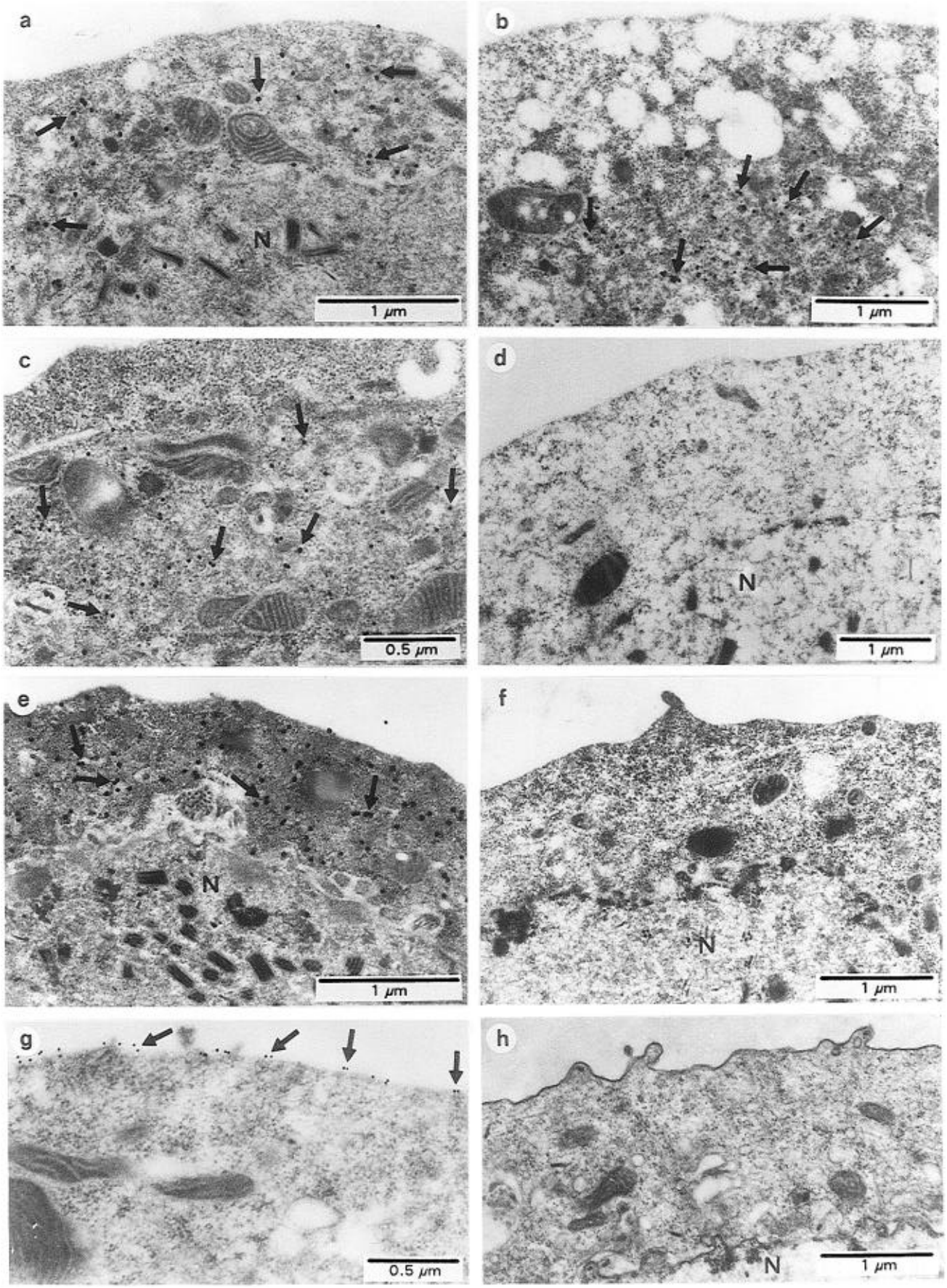

FIGURE 9: Localization of the recombinant D_{2S} receptor in infected Sf9 cells by electron microscopy. Post-embedding immunogold staining was performed with the D_2-anti-peptide antiserum D2/1 on Sf9 cells infected with the following baculoviruses: (a) AcPolD2S; (b) AcPolD2SH6; (c) VLMelD2SH6; (d) wild-type AcNMPV. The anti c-*myc* antibody 9E10 was used for post- (e and f) and pre-emmbedding (g and h) immunogold staining of Sf9 cells infected either with recombinant virus VLMelMycD2S (e and g) or, as control, with the BaculoGold virus. N, nucleus.

39−40 kDa band represents the full-length translation product. A band of this size is also obtained after deglycosylation of immunoprecipitated receptor by PNGase F or Endo H, indicating the presence of the glycosylated N-terminus. Furthermore, a band with the same molecular mass appeared when palmitoylation was studied in tunicamycin-treated cells, indicating an intact C-terminus. It is not unusual for membrane proteins to migrate with a lower apparent molecular mass on SDS−PAGE than predicted from their protein sequence. This behavior, for instance, has also been found for the sugar transport protein lactose permease (Wright et al., 1983). Moreover, anomalous migration of the immunoprecipitated D_{2S} receptor turned out to be a function of the concentration of polyacrylamide in the separating gel, such that the glycosylated and unglycosylated receptor forms with an apparent molecular mass of 47−48 and 39−40 kDa on a 10% gel migrated at 45 and 53 kDa on a 13% gel (S. Grünewald, unpublished observation). A similar observation was reported earlier for the rat striatal D_2 receptor (Clagett-Dame & McKelvy, 1989).

In Sf9 and Tn insect cells, glycosylation of the heterologously produced D_{2S} receptor is of the high-mannose type. In contrast, the native D_2 receptor in brain tissue contains complex-type oligosaccharides (Grigoriadis et al., 1988; Jarvie et al., 1988; Clagett-Dame & McKelvy, 1989). Similar results were obtained with other GPCRs in insect cells such as the β_2-adrenergic receptor (Parker et al., 1991; Reiländer et al., 1991) and the rat m3 muscarinic receptor (Vasudevan et al., 1995). In most cases, N-glycosylation of recombinant proteins produced in insect cells is of the high-mannose type, although insect cells seem to possess the potential for synthesis of complex-type *N*-glycans (März et al., 1995). Indeed, glycosylation of the gastrin-releasing peptide receptor expressed in Sf9 cells was reported to be entirely biantennary complex. But full glycosylation was not achieved either, since the native receptor contains tri- and tetraantennary complex N-linked oligosaccharides (Kusui et al., 1995). In contrast to a number of peptide hormone receptors, glycosylation is not essential for receptor function in the case of neurotransmitter receptors, including the D_2 receptor (Jarvie et al., 1988; Clagett-Dame & McKelvy, 1989). Expression of the D_{2S} receptor in tunicamycin-treated cells, however, suggests that glycosylation might stabilize the inserted receptor and help in folding and targeting the receptor to the plasma membrane.

The D_{2S} receptor protein sequence ends with a cysteine residue which is a potential palmitoylation site. Palmitoylation in Sf9 insect cells has been shown for several GPCRs such as the β_2-adrenergic receptor (Mouillac et al., 1992), the $5HT_{1B}$ (Ng et al., 1993), the D_1 receptor (Ng et al., 1994a), and the D_{2L} isoform (Ng et al., 1994b). Immunoprecipitation experiments demonstrated that the glycosylated, but not the unglycosylated, form of the D_{2S} receptor was palmitoylated in Sf9 insect cells. This is in contrast to Ng et al. (1994b), who reported palmitoylation of the unglycosylated form of the D_{2L} isoform in Sf9 cells. However, their assignment of the detected band to the unglycosylated receptor form was only based on the apparent molecular mass without any further studies on glycosylation. Considering the phenomenon of anomalous migration of membrane proteins on SDS−PAGE discussed above, this assignment is not reliable. Glycosylation is not a prerequisite for palmitoylation since in tunicamycin-treated cells the unglycosylated receptor still is palmitoylated. Tunicamycin does not seem to decrease receptor biosynthesis in AcPolD2S-infected cells as judged from the immunoblots. It does decrease, however, receptor binding by about 60% and also lowers the fraction of palmitoylated receptor as judged qualitatively from the autoradiograph. These results, combined with the fact that the unglycosylated receptor form does not get further processed, suggest that this form either does not reach the cellular site at which fatty acid addition occurs or is misfolded and, therefore, is no longer a substrate for palmityl transferase. Similar observations were reported for the unglycosylated transferrin receptor in tunicamycin-treated CCRF-CEM cells (Omary & Trowbridge, 1981). BFA and monensin had no influence on fatty acylation, indicating that in Sf9 insect cells palmitoylation takes place either in the ER or in intermediate compartments between the ER and *cis*-Golgi in agreement with previously published evidence for a "post-ER/pre-Golgi location" of palmitoylation in mammalian cells (Bonatti et al., 1989).

The pattern of bands detected on immunoblots of AcPolD2S-infected cells extracted with SDS−Laemmli sample buffer differed from that obtained by immunoprecipitation and fluorography of metabolically labeled and functionally solubilized receptor. The protein bands specifically recognized on immunoblots of whole cell extracts mostly represent misfolded or proteolytic receptor forms. This is supported by the observation that most of the produced receptor is localized intracellularly, most probably associated with ER or Golgi membranes. Only a minor fraction of the overall produced receptor protein is transported to the plasma membrane. This fraction, which most likely represents the glycosylated receptor form, is too small to be detected on immunoblots within SDS-extracted protein.

The addition of the signal sequence of prepromelittin enhanced the expression of functional receptors (as assessed by ligand binding) 2−3-fold. Although not explicitly shown for the D_{2S} receptor constructs, the prepromelittin signal peptide can be readily recognized and correctly cleaved in Sf9 cells as has been demonstrated for recombinant propapain (Tessier et al., 1991), TNF-β (Chai et al., 1993), and N-terminal flag-epitope-tagged serotonin $5HT_{5A}$ receptor (H. Reiländer, unpublished results) expression. A similar increase in expression of functional β_2-adrenergic receptors in Sf9 cells as well as in a cell-free system was observed by adding a cleavable signal sequence from influenza hemaglutinin to the N-terminus of the receptor (Guan et al., 1992). This increase in functional D_{2S} receptor correlated with an increase of glycosylated receptor, which was then produced in amounts high enough to be seen on Western blots. Part of the expressed receptor was still located intracellularly, but pre-embedding immunogold labeling showed that a significant fraction was transported to the plasma membrane. In contrast to the AcPolD2S construct, virtually all of the digitonin/cholate-solubilized receptor bearing the prepromelittin signal sequence was glycosylated. Pulse−chase studies showed that glycosylation occurred efficiently and rapidly within 15 min, which also has been observed in CHO cells (Fishburn et al., 1995). Glycosylation occurs in the lumen of the ER, therefore indicating that the N-terminus of the receptor has been properly translocated. Since glycosylation seems to be very rapid and efficient, the fact that about half of the digitonin/cholate solubilizable receptor in AcPolD2S-infected cells lacks glycosylation may indicate that the

N-terminus of this receptor fraction is either not translocated or not properly folded.

The first step in the synthesis of secretory or integral membrane proteins is the recognition of a signal sequence and targeting of the ribosome to the ER, while continued elongation of the polypeptide chain is delayed or even arrested. Obviously, the receptor expressed from the AcPolD2S construct is the product of a very efficient translation. But here, membrane insertion seems to be slow, and chain elongation may lead to incorrect folding and aggregation. Addition of the His-tag to this construct increased the fraction of nonglycosylated receptor. The six consecutive histidine residues are partly uncharged under physiological conditions and might increase the potential for aggregation and incorrect folding.

The D_2 dopamine receptor (Grandy et al., 1989) like the β_2-adrenergic receptor and most GPCRs does not contain a cleavable signal sequence. Addition of the prepromelittin signal sequence leads to a more efficient insertion of the receptor into the ER. Whether in general membrane insertion of proteins with cleavable signal sequences is more efficient than membrane insertion of proteins with noncleavable signal sequences or whether the internal sequence of the D_{2S} receptor, which serves as noncleavable signal sequence, is not recognized as efficiently as the prepromelittin signal sequence, therefore resulting in less efficient translocation, is not clear at the moment.

In summary, the limiting factor for the synthesis of membrane proteins in the baculovirus system does not seem to be transcription or translation, but membrane insertion, folding, and processing of the protein. Addition of a well-recognized cleavable signal sequence to the N-terminus of the D_{2S} receptor permits production of fully glycosylated and palmitoylated protein in the plasma membrane in amounts high enough to initiate purification and biophysical studies.

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