

# N-linked Glycosylation Is Required for Plasma Membrane Localization of D5, but Not D1, Dopamine Receptors in Transfected Mammalian Cells

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## ABSTRACT

We have analyzed the role of *N*-linked glycosylation in functional cell surface expression of the D1 and D5 dopamine receptor subtypes. Treatment of transfected HEK 293 cells with tunicamycin, an inhibitor of *N*-linked oligosaccharide addition, was found to prevent localization of D5 receptors in the plasma membrane. In contrast, tunicamycin treatment had no effect on the plasma membrane localization of the D1 receptor. Polymerase chain reaction mutagenesis was used to generate a panel of D5 receptors containing mutations in the three predicted sites of *N*-linked glycosylation. Expression of mutant receptors indicated that glycosylation of residue N7 was the major determinant of D5 receptor plasma membrane localization. Mutation

of a comparable site in the D1 receptor at position N5 had no effect on the delivery of the D1 receptor to the cell surface. Tunicamycin treatment during receptor biosynthesis, but not *N*-glycosidase F digestion of mature receptors, abrogated binding of the D5 receptor antagonist [<sup>3</sup>H]SCH23390, suggesting that while oligosaccharide moieties play a key role in the cell surface expression of D5 receptors, they do not appear to contribute to the receptor's ligand binding properties. Together, our data indicate a differential requirement for *N*-linked glycosylation in functional cell surface expression of D1 and D5 dopamine receptors.

The D1 and D5 subtypes belong to the D1-like class of dopamine receptors. These seven transmembrane-spanning domain receptors are known to couple to the G<sub>s</sub> GTP binding protein and thereby stimulate adenylyl cyclase activity (Dohlmann et al., 1991; Gingrich and Caron, 1993). In transfected mammalian cells, D5 receptors appear to be constitutively active and exhibit a 10-fold higher affinity for dopamine than the D1 receptor (Sunahara et al., 1991; Tiberi and Caron, 1994). Transfected cells expressing the D5 receptor also show a lower agonist-stimulated adenylyl cyclase activity than do cells expressing the D1 receptor (Tiberi and Caron, 1994). Whether the D1 and D5 receptors play distinct roles in mediating neurotransmission in brain is an issue that has not yet been clearly elucidated.

Studies with D1 and D5 subtype-specific antibodies suggest that the two receptors are coexpressed within the same pyramidal neurons of both cerebral cortex and hippocampus (Bergson et al., 1995b). Electron microscopic analysis further revealed D1 receptor antibody staining of pyramidal cell

dendritic spines, whereas D5 receptor antibodies localized mainly to dendritic shafts (Bergson et al., 1995b). On the basis of these results, it has been proposed that D1 receptors are targeted to dendritic spines to regulate glutamatergic inputs, whereas D5 receptors on dendritic shafts function to modulate inhibitory input from GABAergic interneurons (Bergson et al., 1995b). Detection of D1 and D5 receptor subtypes concentrated within different regions of pyramidal cell dendrites raised a number of questions regarding the mechanisms governing the transport of D1 and D5 receptors to the plasma membrane.

Transport to the cell surface of several receptors and membrane proteins, including the Ca<sup>2+</sup> receptor (Fan et al., 1997), the lutropin receptor (Liu et al., 1993), and the norepinephrine transporter (Nguyen and Amara, 1996), has been shown to be dependent on *N*-linked glycosylation. These proteins require the post-translational addition of oligosaccharides to extracellular asparagines within the sequence motif Asn-X-Ser/Thr to achieve plasma membrane localization. Because both the D1 and D5 receptors contain predicted sites for *N*-linked glycosylation, we have examined the role that *N*-linked sugars play in trafficking D1 and D5 receptors to the plasma membrane in transfected mammalian fibro-

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blasts. By using tunicamycin to prevent the addition of *N*-linked carbohydrates and by analyzing glycosylation-deficient dopamine receptor mutants, we have found the D5 receptor, but not the D1 receptor, to be dependent on *N*-linked glycosylation for plasma membrane localization in transfected HEK 293 cells. Furthermore, we show that glycosylation is not required for D5 receptor ligand interaction once the receptor is associated with the plasma membrane, but prevention of oligosaccharide addition to D5 during biosynthesis eliminates receptor-ligand binding.

## Materials and Methods

**DNA Constructs and Site-Directed Mutagenesis.** Full-length human D1 (Zhou et al., 1990) and D5 receptor (Grandy et al., 1991) cDNAs were utilized for site-directed mutagenesis studies. There are two predicted consensus sites for *N*-linked glycosylation (N-X-S/T) in the human D1 receptor (amino acid positions 5 and 175) and three predicted consensus sites within the human D5 receptor (positions 7, 198, and 222). Glycosylation-deficient mutants were generated by polymerase chain reaction mutagenesis as described by Nelson and Long (1989). Single-base changes were introduced at Asn residues within consensus sites for *N*-linked glycosylation to create the following panel of mutant D5 receptors: N7Q, N7T, N198T, and N222Q. A mutant D1 receptor carrying an N5Q substitution was also generated. D5 double mutants were generated by exchanging segments between two singly mutagenized constructs. The D5 triple mutant, N7Q-N198T-N222Q was generated by domain swapping between the N7Q mutant and the N198T-N222Q mutant. Full-length mutant receptor cDNAs were subcloned into the tetracycline-inducible pTetSplice vector (Gossen and Bujard, 1992) or the eukaryotic expression vector pCB6 (Brewer and Roth, 1991). Each mutant D1 and D5 receptor was verified by DNA sequencing with an ABI Automated DNA Sequencer (Perkin-Elmer, Foster City, CA).

**Cell Culture and DNA Transfection.** HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum. Transfections were carried out by the calcium phosphate coprecipitation method as previously described (Canfield et al., 1996). Cells transfected with pTetSplice vectors were maintained in Dulbecco's modified Eagle's medium supplemented with 2  $\mu$ g/ml tetracycline (Sigma, St. Louis, MO). Expression of pTetSplice constructs was induced by removing tetracycline from the culture medium. Cells were grown for 24 h in the absence of tetracycline before harvesting for Western blot or immunofluorescence analysis. To inhibit addition of *N*-linked sugars, transiently transfected HEK 293 cells were grown in the presence of 2  $\mu$ g/ml tunicamycin (Boehringer Mannheim, Indianapolis, IN) for 16 h, then harvested for Western blot or immunofluorescence analysis.

**Generation of D5 Receptor Monoclonal Antibodies.** Female BalbC mice were immunized with the maltose binding protein-D5 purified fusion protein. This fusion protein contains amino acid residues 375 to 477 of the human D5 dopamine receptor fused to the carboxyl terminus of *Escherichia coli* maltose binding protein (Bergson et al., 1995b). Monoclonal antibodies (MAbs) were generated by standard techniques as described previously (Kimball et al., 1994). Hybridomas were screened by enzyme-linked immunosorbent assay for antibody reactivity to glutathione *S*-transferase-D5 fusion protein. Positive clones were subcloned by limiting dilution and subsequently expanded. Medium harvested from clones was tested for antibody reactivity on immunoblots. The specificity of antibodies produced from one positive clone, 1G1, for the D5 receptor was established using the following criteria: 1) MAbs purified from clone 1G1 recognized epitope-tagged D5 receptors expressed in transfected CV-1 cells. 2) MAbs reacted with D5 receptors expressed in baculovirus-infected Sf9 cells. 3) MAbs did not react with immunoblots

containing membranes prepared from Sf9 cells expressing D1 dopamine receptors.

**Tissue Preparation and Immunoblotting.** Crude membrane fractions from transfected HEK 293 cells were prepared as previously described (Jorgensen, 1974; Shyjan and Levenson, 1989; Bergson et al., 1995b; Canfield et al., 1996). Briefly, cells were collected in homogenization buffer consisting of 250 mM sucrose, 30 mM histidine, and 1 mM phenylmethylsulfonyl fluoride, homogenized in a glass homogenizer with a tight-fitting pestle, and centrifuged at 5000g for 10 min at 4°C. The pellet was resuspended by homogenization and centrifuged again at 5000g for 10 min at 4°C. The combined supernatants from the two centrifugations were centrifuged at 125000 X g for 1 h at 4°C. The pellet was resuspended in homogenization buffer. Protein concentrations were determined by the method of Bradford (1976). Solubilized membrane fractions were separated on SDS-containing 12% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (ICN Biomedicals, Aurora, OH). Filters were blocked for 2 h in phosphate-buffered saline (PBS) containing 10% dry milk and 5% goat serum, and then incubated with either polyclonal D5 antibodies (1:500) (Bergson et al., 1995b), D5-specific mouse MAb 1G1 (1:100), or a D1-specific rat MAb (1:300) obtained from Research Biochemicals Inc. (Natick, MA). Blots were rinsed with PBS and then incubated with horseradish peroxidase-conjugated goat anti-rabbit, mouse, or rat secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL) using an ECL Plus kit (Amersham, Piscataway, NJ).

To deglycosylate D5 receptors, microsomes prepared from transfected HEK 293 cells were digested with *N*-glycosidase F (Boehringer Mannheim, Indianapolis, IN) as previously described (Bergson et al., 1995b). Protein samples were denatured in 125 mM NaPO<sub>4</sub> (pH 7.4), 10 mM EDTA, 1% SDS for 1 h at 37°C before the addition of Triton X-100 to 1% (Smith et al., 1987). Samples were then digested with 1.0 unit of *N*-glycosidase F for 1 h at 37°C.

**Immunofluorescence and Confocal Microscopy.** Transfected HEK 293 cells grown on glass coverslips were examined 72 h after transfection. Cells were fixed in 1:1 methanol/acetone (v/v) solution and blocked with PBS containing 2% bovine serum albumin and either 10% goat serum (for D5 staining) or 10% donkey serum (for D1 staining) at room temperature for 1 h and subsequently incubated in blocking solution containing anti-rabbit D1 (Bergson et al., 1995b) or anti-mouse D5 MAbs. Cy-3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) secondary antibody was diluted 1:800, whereas fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) was diluted 1:200 and applied in the same buffer. Confocal laser scanning microscopy was performed using a Zeiss LSM 210 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

**Ligand Binding.** To analyze the effect of deglycosylation on D5 receptor binding sites, membrane fractions were prepared from stably transfected CV-1 cells in which expression of the D5 dopamine receptor can be induced by removing tetracycline from the culture medium (Gossen and Bujard, 1992; Bergson et al., 1995a). Addition of *N*-linked oligosaccharides was prevented by inducing D5 receptor expression in the presence of 1  $\mu$ g/ml tunicamycin for 16 h. Alternatively, membrane fractions prepared from cells expressing glycosylated D5 receptors (i.e., in which receptor expression was induced in the absence of tunicamycin) were incubated with *N*-glycosidase F (0.2 units/ $\mu$ g protein for 16 h) in buffer containing 125 mM NaPO<sub>4</sub> (pH 7.4), 10 mM EDTA. Ligand binding to D5 receptors was carried out essentially as described (Lidow et al., 1989). Membrane fractions were incubated at room temperature for 1 h with 0.1 to 24 nM [<sup>3</sup>H]SCH23390 (New England Nuclear, Boston, MA) in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>. To block serotonin receptor binding sites, 1  $\mu$ M mianserin (ICN, Costa Mesa, CA) was added to the incubation buffer. Nonspecific binding was determined by incubating samples in the presence of 1  $\mu$ M *cis*-flupentixol (ICN, Costa Mesa, CA). Specific

and nonspecific binding assays were carried out in triplicate at each concentration of agonist. Incubations were terminated by rapid filtration through Whatman GF/B filters (Fisher, Pittsburgh, PA) using an M-242 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), and radioactivity was measured with a Packard 3320 liquid scintillation counter (Packard, Instrument Co., Meriden, CT). Data were analyzed using the nonlinear curve-fitting computer program EDBA/LIGAND (Elsevier Biosoft, Cambridge, UK).

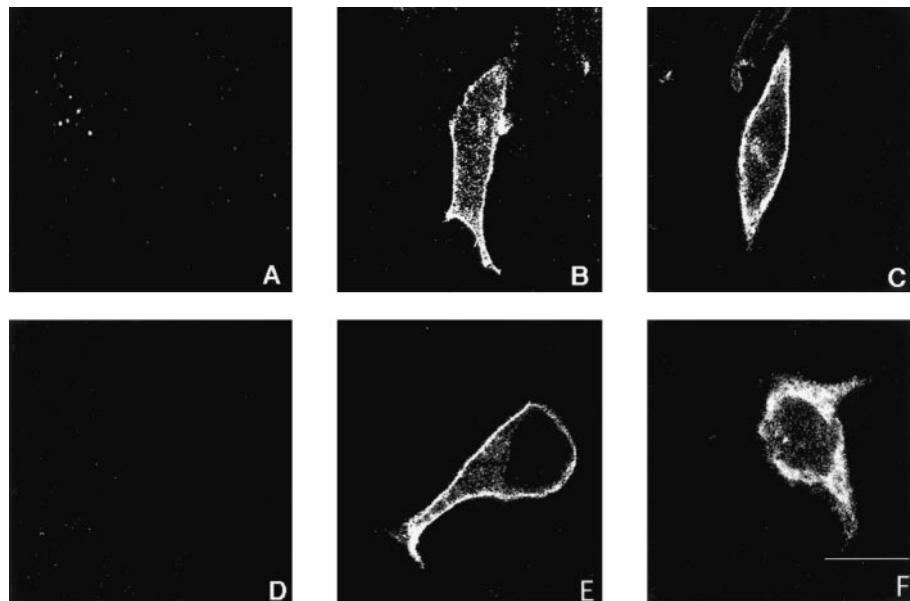
## Results

**Localization of D1 and D5 Receptors in Tunicamycin-Treated Cells.** We used confocal laser microscopy to analyze the subcellular distribution of D1 and D5 receptors in tunicamycin-treated, transiently transfected HEK 293 cells. Tunicamycin is a specific inhibitor of *N*-linked glycosylation (VanBerkel et al., 1996). As shown in Fig. 1B, cells transfected with D1 receptors were reactive with anti-rabbit D1-specific antibodies. Strong D1 receptor staining was observed at cell margins, indicating targeting of D1 receptors to the plasma membrane. A similar staining pattern was observed in D1 receptor-expressing cells grown in the presence of tunicamycin (Fig. 1C), suggesting that inhibition of *N*-linked glycosylation did not affect the plasma membrane localization of D1 receptors. Untransfected cells were devoid of staining with either D1 (Fig. 1A) or D5-specific antibodies (Fig. 1D). Cells transfected with the D5 receptor and probed with the 1G1 MAb were stained predominantly at the cell margins (Fig. 1E), indicating targeting of D5 receptors to the plasma membrane. However, D5 receptors showed a predominantly intracellular distribution in cells grown in the presence of tunicamycin (Fig. 1F). Tunicamycin treatment produced similar effects on localization of D1 and D5 receptors when expressed in NIH 3T3 cells and CV-1 cells (data not shown). These results suggest that *N*-linked glycosylation is required for the plasma membrane-targeting of the D5, but not the D1, dopamine receptor.

We analyzed immunoblots containing microsomes prepared from tunicamycin-treated, D1- or D5-expressing HEK 293 cells in order to evaluate the effect of the drug on receptor

glycosylation. Figure 2A shows that D1 antibodies react predominantly with a broad, diffuse band ~60–90 kDa in size in transient transfectants grown in the absence of tunicamycin. This band corresponds well with the size of the glycosylated D1 receptor expressed in monkey brain (Bergson et al., 1995b). In cells treated with tunicamycin, only the 48 kDa D1 receptor core protein reacted with D1-specific antibodies (Fig. 2A). D5-specific antibodies reacted with two distinct bands of ~60 and ~68 kDa in size (Fig. 2B). These bands appear to represent differentially glycosylated forms of the D5 receptor (Bergson et al., 1995a). Tunicamycin treatment produced a single immunoreactive band 50 kDa in size. This band appears to represent the unglycosylated D5 receptor and corresponds in size to the D5 receptor core protein generated by *N*-glycosidase F treatment (Bergson et al., 1995a). These results demonstrate that tunicamycin treatment prevents the addition of *N*-linked sugars to D1 and D5 receptor polypeptides.

**Expression of Mutated D1 and D5 Receptors.** To determine whether the failure of D5 receptors to localize to the plasma membrane was due directly to the effect of tunicamycin on D5 receptors, rather than D5-receptor-associated proteins, we analyzed the effect of mutating consensus glycosylation sites on receptor localization. Mutant receptors were transiently expressed in HEK 293 cells, and then analyzed by confocal microscopy. Representative examples of cells are shown in Fig. 3. Wild-type D5 receptors were predominantly localized in the plasma membrane as evidenced by strong D5 immunoreactivity at cell margins (Fig. 3A). In contrast, the N7Q mutant gave strong cytosolic and virtually no plasma membrane staining (Fig. 3B). The N198T mutant localized within the cytosol and at cell margins (Fig. 3D), whereas the N222Q mutant gave strong plasma membrane and light cytosolic staining (Fig. 3E). These results suggest that the N7Q mutation gives the strongest mislocalization of the D5 receptor. To rule out the possibility that mislocalization was due to the presence of Q, at position 7, we generated an N7T mutant D5 receptor. Expression of this receptor in HEK 293 cells produced a staining pattern very similar to that of the N7Q mutant (data not shown). We also generated a D5 receptor



**Fig. 1.** Effect of tunicamycin treatment on cell surface expression of D1 and D5 dopamine receptors. HEK 293 cells transiently expressing D1 and D5 receptors were labeled with either anti-D1 polyclonal antibodies (A–C) or the anti-D5 MAb 1G1 (D–F). Fluorescein isothiocyanate-conjugated anti-rabbit and Cy3-conjugated anti-mouse secondary antibodies were used to detect D1 and D5 receptor immunoreactivity, respectively. A and D, untransfected HEK 293 cells; B, D1-expressing cell; C, D1-expressing cell grown in the presence of 2  $\mu$ g/ml tunicamycin; E, D5-expressing cell; F, D5-expressing cell grown in the presence of 2  $\mu$ g/ml tunicamycin. Antibody staining was visualized by confocal laser microscopy. Magnification, 4000 $\times$ . Bar, 10  $\mu$ m.

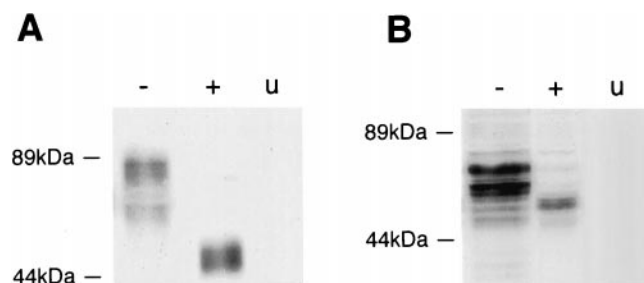


mutant T9V, which eliminated T within the *N*-linked glycosylation consensus sequence motif N-X-T. Expression of T9V (Fig. 3C) produced a strong cytosolic staining pattern similar to that of the N7Q mutant. These results indicate that mutations within the consensus site for *N*-linked glycosylation at position 7 prevent plasma membrane localization of the D5 dopamine receptor.

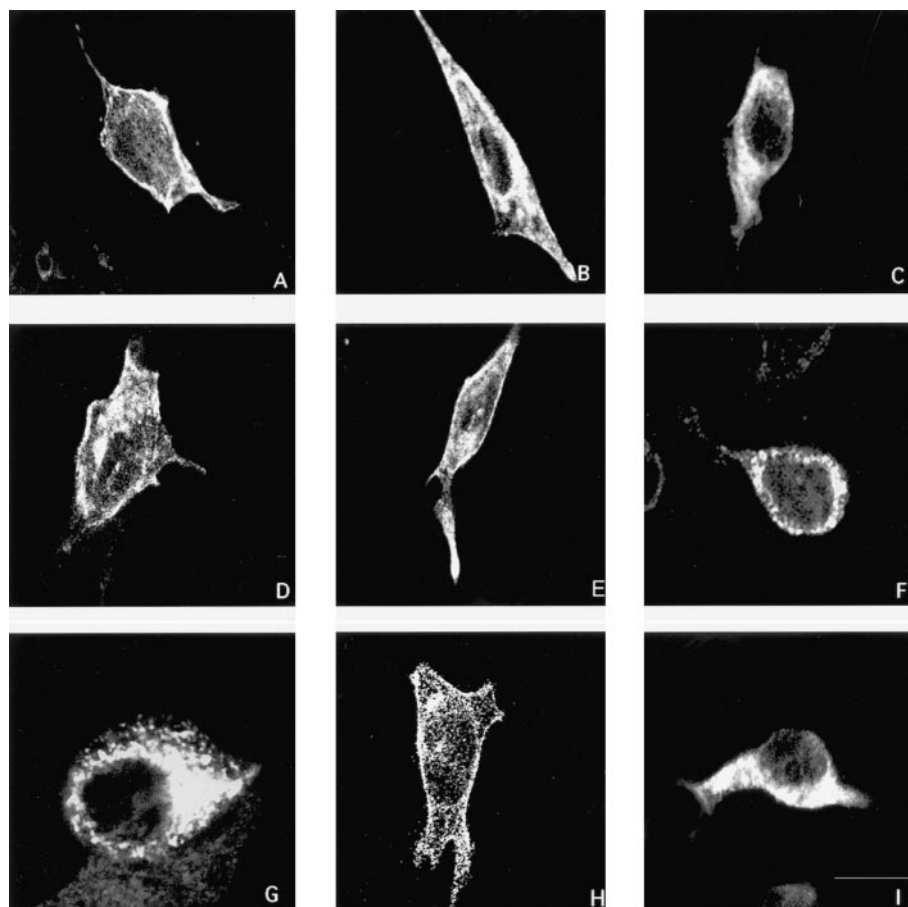
We next examined the effect of double and triple mutations on D5 receptor localization. Expression of the N7Q-N198T (Fig. 3F) and N7Q-N222Q (Fig. 3G) double mutants produced intense perinuclear staining with D5 MAbs. Virtually no

plasma membrane D5 receptor staining was observed in N7Q-N198T or N7Q-N222Q expressing cells. A series of horizontal cross-sections (Z sections) taken through a single cell expressing the N7Q-N198T mutant (Fig. 4) confirms the lack of any detectable plasma membrane-associated D5 receptor immunoreactivity. In contrast, the double mutant, N198T-N222Q, exhibited D5 immunoreactivity predominantly at the cell margins (Fig. 3H). Expression of the triple mutant, N7Q-N198T-N222Q exhibited strong perinuclear, and virtually no plasma membrane staining (Fig. 3I). Together, these data are consistent with the view that glycosylation at N7 is a major determinant of D5 receptor plasma membrane localization.

Expression of D1 receptors in tunicamycin-treated HEK 293 cells indicated that unglycosylated D1 receptors can traffic to the plasma membrane. Like the D5 receptor, the D1 receptor contains a consensus site for *N*-linked glycosylation (N5) at the amino terminus of the polypeptide. To determine whether glycosylation at this site is required for plasma membrane localization, we expressed and localized an N5Q mutant D1 receptor. As shown in Fig. 5, D1 antibodies strongly stained the margins of cells transfected with either wild-type (Fig. 5A) or N5Q mutant (Fig. 5B) D1 receptors. To confirm that residue N5 is a site of *N*-linked oligosaccharide addition, we probed immunoblots of microsomes prepared from cells expressing N5Q mutant receptors with D1 receptor antibodies (Fig. 6C). The N5Q mutation yielded two prominent bands, a band of approximately 55 kDa that migrated much faster than wild-type receptor, and a band of approximately 48 kDa band that comigrated with the core D1 recep-



**Fig. 2.** Effect of tunicamycin on glycosylation of D1 and D5 dopamine receptors. Solubilized microsomal proteins were prepared from HEK 293 cells transiently transfected with D1 or D5 dopamine receptor cDNAs, fractionated by SDS-polyacrylamide gel electrophoresis, then transferred to PVDF membranes. A, proteins were resolved on a 12% gel, and the filter was probed with anti-D1 Mabs; B, proteins were fractionated on a 10% gel, and the filter was probed with anti-D5 polyclonal antibodies. Cells were grown either in the absence (–) or presence (+) of 2  $\mu$ g/ml tunicamycin. Membranes from untransfected cells (U) were run as a control. Molecular weight markers are shown on the left.



**Fig. 3.** Localization of D5 dopamine receptor glycosylation mutants. D5 receptors carrying mutations in consensus sites for *N*-linked glycosylation were transiently expressed in HEK 293 cells. D5 receptor reactivity was detected using the anti-D5 MAb 1G1 and visualized by confocal laser scanning microscopy. A, wild-type D5 dopamine receptor; B, single mutant N7Q; C, single mutant T9V; D, single mutant N198T; E, single mutant N222Q; F, double mutant N7Q-N198T; G, double mutant N7Q-N222Q; H, double mutant N198T-N222Q; I, triple mutant N7Q-N198T-N222Q. Magnification, 3780 $\times$ . Bar, 10  $\mu$ m.

tor protein, suggesting that N5 and the predicted glycosylation site at N175 are glycosylated. Because confocal immunofluorescence studies showed that both the N5Q mutant D1 receptor (Fig. 5B) and the fully unglycosylated (tunicamycin-treated) D1 receptor (Fig. 1C) localize to the plasma membrane, these results support the notion that *N*-linked glycosylation is not required for plasma membrane targeting of the D1 dopamine receptor.

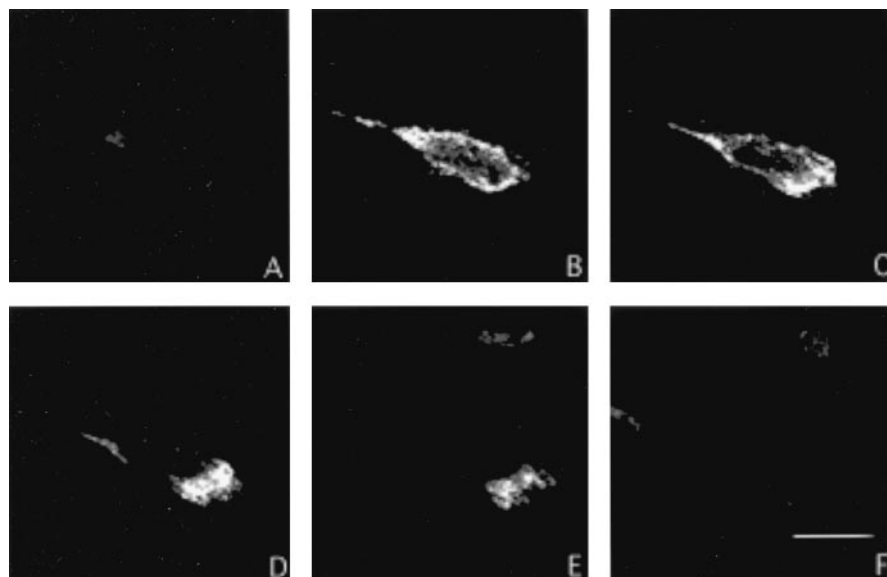
#### Differential Glycosylation of Mutant D5 Receptors.

To determine the glycosylation state of D5 receptor mutants, immunoblots containing microsomes prepared from transfected cells were probed with the D5-specific MAb, 1G1. As shown in Fig. 6 (A and B), the D5 MAb reacted predominantly with bands ~60 and ~68 kDa in size in cells expressing wild-type D5 receptors. A faint band of ~50 kDa in molecular mass, the size of the predicted D5 core protein, was also detected. Treatment of wild-type D5 receptors with *N*-glycosidase F reduced the bulk of the immunoreactive material to the size of the 50-kDa D5 core polypeptide. Transient expression of the N7Q mutant produced two prominent bands, ~50 kDa and ~60 kDa in size. The 50-kDa band comigrates with the D5 core protein and probably represents unglycosylated D5 receptors generated by overexpression of transfected D5 cDNA. The 60-kDa band most likely represents D5 receptors glycosylated at positions N198, N222, or both. Expression of N198T produced a prominent band of ~50 kDa that comigrates with the D5 core protein, as well as an abundant band that appears to comigrate with the 68-kDa glycosylated D5 receptor species. In contrast, the N222Q mutant produced bands of ~50, ~60, and ~68 kDa that appeared to comigrate with wild-type D5 receptor polypeptides. These results suggest that the D5 receptor is glycosylated at positions N7 and N198, but not at N222.

We next analyzed expression of double and triple D5 receptor glycosylation mutants (Fig. 6B) to confirm our single mutant analyses. The N198T-N222Q and N7Q-N222Q double mutants each produced a polypeptide migrating with an apparent molecular mass of ~55–60 kDa. These higher molecular weight bands were not detectable with expression of the N7Q-N198T mutant D5 receptor. Likewise, the most abundant form of the N7Q-N198T-N222Q triple mutant was

a polypeptide that migrated with a mass of ~50 kDa. The similarity in size of the receptors produced by the N7Q-N198T and N7Q-N198T-N222Q mutations supports the view that N222 is not normally a site of *N*-linked glycosylation, and that the wild-type D5 receptor is glycosylated at positions N7 and N198.

**Ligand Binding.** To determine whether *N*-linked glycosylation plays a role in D5 receptor ligand binding, we performed filter binding assays on crude membrane fractions prepared from stable transfectants in which D5 receptor expression was induced by activation of the tetracycline-regulatable promoter. Ligand binding of D5 receptors induced in the presence of tunicamycin, and therefore not glycosylated, was compared with that of receptors enzymatically deglycosylated following induction. Immunoblot analyses revealed comparable levels of D5 receptor protein present in membrane fractions used for ligand binding assays (Fig. 7A). Control membrane fractions from D5 CV-1 cells induced in the *absence* of tunicamycin exhibited significant levels of saturable binding of the D1-like receptor antagonist [<sup>3</sup>H]SCH23390 (Fig. 7B). Membranes from cells in which D5 receptor expression was induced in the *absence* of tunicamycin, but later treated with *N*-glycosidase F, exhibited a similar level of ligand binding to that of control (Fig. 7C). As shown in Table 1, treatment of membranes with *N*-glycosidase F did not significantly alter D5 receptor binding of [<sup>3</sup>H]SCH23390. In contrast, membranes from cells in which the induction of D5 receptors was carried out in the *presence* of tunicamycin, exhibited a greater than 90% decrease in saturable ligand binding compared to control CV-1 cells (Fig. 7D). Taken together, these results indicate that glycosylation is not required for D5 receptor ligand binding once the receptor is associated with the plasma membrane. However, preventing oligosaccharide addition to the D5 polypeptide during biosynthesis appears to abrogate ligand binding. The inability of unglycosylated D5 polypeptide to bind [<sup>3</sup>H]SCH23390 could reflect the fact that the receptor is not properly folded (preventing the formation of a ligand binding pocket), or that the receptor requires the presence of factors located at the plasma membrane in order for its activation to occur.



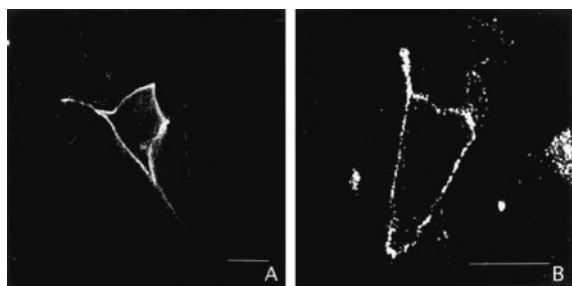
**Fig. 4.** Cytosolic localization of the D5 receptor double mutant N7Q-N198T. HEK 293 cells expressing the N7Q-N198T mutant were stained using the D5 1G1 MAb and visualized with Cy3-conjugated anti-mouse secondary antibodies. Each panel represents a serial section scanned at 1- $\mu$ m intervals through the Z-axis of a single expressing cell. Magnification: 4000 $\times$ . Bar, 10  $\mu$ m.

## Discussion

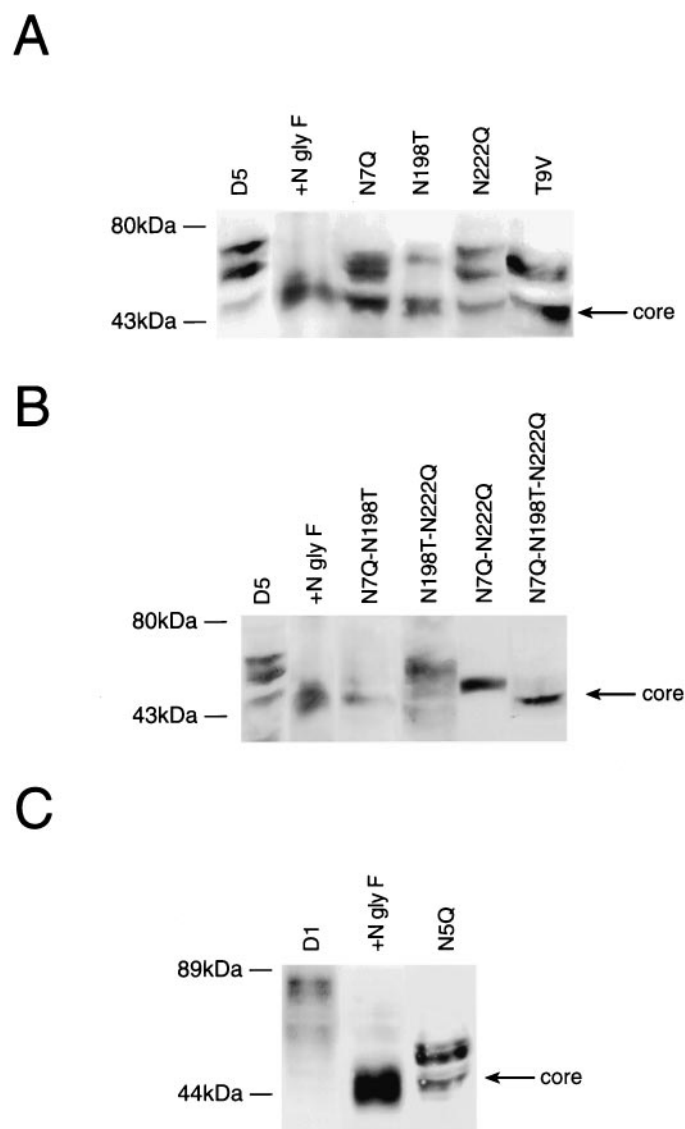
Our results suggest that *N*-linked glycosylation is required for the functional expression of D5, but not D1, dopamine receptors at the plasma membrane of transfected cells. Both tunicamycin-mediated inhibition of oligosaccharide addition and mutation of consensus sites for *N*-linked glycosylation prevent cell surface expression of the D5 receptor. D5 receptors lacking *N*-linked sugars at position N7 showed a predominantly cytoplasmic localization, whereas single and double D5 glycosylation mutants with N7 intact appeared to localize to the plasma membrane. Furthermore, unglycosylated D5 receptors were unable to bind the D1-like dopamine receptor antagonist SCH23390. Lack of ligand binding to unglycosylated D5 receptors may reflect the fact that glycosylation is required for proper folding of the receptor, and that misfolded receptors are unable to interact with ligand. Alternatively, glycosylation may play an important role in the formation of protein interactions involving the D5 receptor, and these interactions may be critical for ligand binding.

There are three potential sites for *N*-linked oligosaccharide modification of asparagine residues within the D5 receptor polypeptide. Immunoblot analyses of D5 receptors containing substitution of candidate asparagines at residue N7 and N198 with either glutamine or threonine exhibited a banding pattern distinct from wild-type D5 receptor. In contrast, mutation of N222 does not alter the banding profile of D5 receptors on SDS-polyacrylamide gel electrophoresis. These results suggest that residues N7 and N198, two of the three potential sites, but not the third site at residue N222, are modified by oligosaccharides when D5 receptors are expressed in transfected cells. In addition, we found that the N7Q-N198T double mutant comigrates with the deglycosylated D5 receptor core protein, as well as with the N7Q-N198T-N222Q triple mutant. The similarity in size of the receptors produced by the N7Q-N198T and N7Q-N198T-N222Q mutations indicates that residue N222 is not normally glycosylated in the wild-type D5 receptor. However, when N7 or N198 are mutated, introduction of a second mutation at N222 appeared able to influence the processing of the mutated D5 receptor polypeptide (Fig. 6, A and B). The N222Q also markedly reduced the plasma membrane delivery of D5 receptors containing the N7Q mutation (Fig. 3, A and G). There are two potential explanations that could account for the enhanced phenotype of the N222Q-containing double mutants. One possibility is that in the double mutants, N7Q-N222Q and N198T-N222Q, alteration of residue

N222 serves to indirectly reduce the extent of *N*-linked glycosylation remaining at positions at N7 or N198. Alternatively, it is possible that prevention of glycosylation at either N7 or N198 somehow leads to aberrant glycosylation at position N222. Both possibilities would suggest variability in the process of *N*-linked oligosaccharide addition, either in the extent of modification of, or in the choice of candidate asparagine residues. Heterogeneous use of *N*-linked glycosylation sites has been observed for the hemagglutinin-neuraminidase glycoprotein of the Newcastle disease virus and human lactoferrin (VanBerkel et al., 1996; McGinnes and Morrison, 1997). In the case of lactoferrin, site-specific mutagenesis of *N*-linked glycosylation consensus sequences showed that the extent of glycosylation at one asparagine in the wild-type



**Fig. 5.** Plasma membrane localization of a D1 dopamine receptor glycosylation mutant. The wild-type D1 (A) and the single D1 (B) glycosylation mutant N5Q were transiently expressed in HEK 293 cells. Reactivity was detected using anti-D1 polyclonal antibodies and visualized by confocal laser microscopy. Magnifications: 2000 $\times$  (A); 4000 $\times$  (B). Bar, 10  $\mu$ m.



**Fig. 6.** Expression of D1 and D5 receptor glycosylation mutants. Single D5 receptor mutants (A), double and triple D5 receptor mutants (B), and the N5Q D1 receptor mutants (C) were transiently expressed in HEK 293 cells. Solubilized microsomal proteins prepared from transfected cells were fractionated by electrophoresis through a 12% polyacrylamide gel containing SDS, transferred to a PVDF membrane, and probed with the anti-D5 MAb 1G1 (A, B) or anti-D1 MAb (C). The D1 or D5 receptor mutant is indicated above each lane. +NglyF indicates wild-type D1 (C) or D5 (A, B) receptors treated with *N*-glycosidase F. Molecular weight markers are shown at the left.



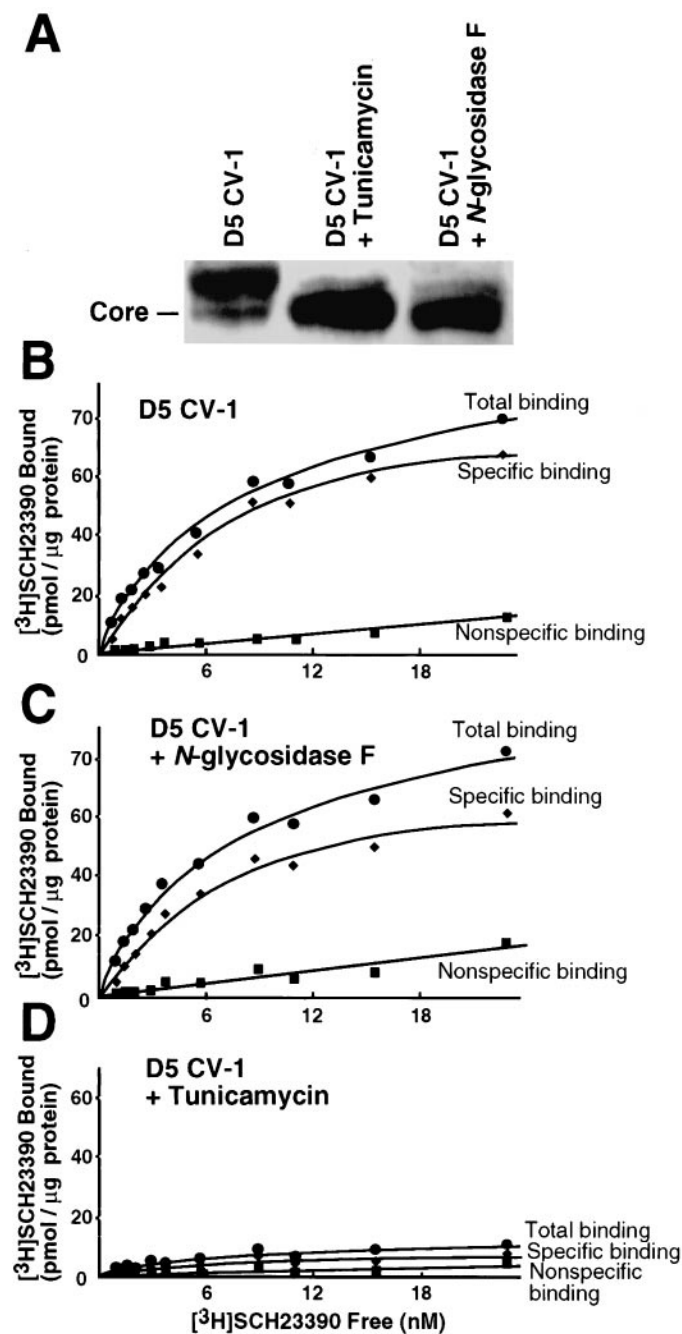
protein was altered when either one of two other glycosylated asparagines was mutated to glutamine (VanBerkel et al., 1996). While the mechanism underlying heterogeneous use of glycosylation sites is still unclear, studies with the hemagglutinin-neuraminidase glycoprotein of the Newcastle disease virus would indicate that events affecting local folding of nascent polypeptide chains (e.g., oligosaccharide addition or

disulfide bond formation) may limit accessibility of candidate asparagine residues to oligosaccharyl transferase (McGinness and Morrison, 1997).

The mechanisms underlying carbohydrate-mediated cell surface transport and function are still not well understood. Post-translational oligosaccharide addition has been shown to be important for the plasma membrane localization of a variety of polypeptides, including the norepinephrine transporter (Nguyen and Amara, 1996), lutropin receptor (Liu et al., 1993),  $\alpha$  subunit of the insulin receptor (Collier et al., 1993), and  $\text{Ca}^{2+}$  receptor (Fan et al., 1997). As is the case with the D5 dopamine receptor, mutation of consensus *N*-linked glycosylation sites has been found to cause mislocalization of each of these polypeptides. On the other hand, many proteins such as the rhodopsin receptor (Kaushal et al., 1994) and the luteinizing hormone receptor (Zhang et al., 1995) are capable of reaching the plasma membrane even when glycosylation of these proteins is blocked by inhibitor treatment or mutation. Likewise, tunicamycin treatment did not prevent the D1 dopamine receptor from trafficking to the plasma membrane. Sequence motifs other than carbohydrate moieties are thus likely to play a role in the targeting of these polypeptides to the plasma membrane.

The sorting of several membrane proteins has recently been shown to depend on discrete cytoplasmic targeting determinants. Di-leucine motifs appear to mediate interaction with Golgi adaptor proteins and have been shown to act as plasma membrane sorting signals in a variety of proteins, including the FcR2-B2 receptor (Hunziker and Fumey, 1994) and human CD4 (Marks et al., 1996). Tyrosine-based (Y-X-X-F) sequence motifs appear to specify basolateral delivery of influenza hemagglutinin and vesicular stomatitis virus glycoproteins (Keller and Simons, 1997). How these signals function, and the distinguishing features of individual signals that are responsible for directing proteins to the plasma membrane, however, are not well defined. In this context, it is of interest to note that the D1 and D5 dopamine receptors contain multiple di-leucine- and tyrosine-based sequence motifs. The D1 receptor contains six cytoplasmically disposed tyrosine-based and three di-leucine motifs, whereas the D5 receptor contains six tyrosine-based and two di-leucine motifs. Analysis of D1 and D5 receptors carrying mutations in these sorting sequences may provide additional insight into how these receptors are differentially trafficked to the plasma membrane.

Ligand binding assays were performed to examine the role of *N*-linked glycosylation on D5 receptor function. Prevention of nascent D5 receptor glycosylation by tunicamycin prohibited D5 receptor-ligand interaction. However, removal of *N*-linked sugars from D5 receptors already resident in the plasma membrane (using *N*-glycosidase F) had no apparent effect on ligand binding. The inability of unglycosylated D5 receptors (tunicamycin-treatment) to bind ligand is most



**Fig. 7.** Effect of glycosylation on D5 receptor binding of D1-like receptor antagonist [ $^3\text{H}$ ]SCH23390. **A**, immunoblot of solubilized microsomal proteins isolated from stable transfected D5 CV-1 cells grown for 16 h in tetracycline-free media (lanes D5 CV-1 and D5 CV-1 + *N*-glycosidase F), or the same media containing 1.0  $\mu\text{g}/\text{ml}$  tunicamycin (lane D5 CV-1 + tunicamycin). Microsomal proteins were digested with *N*-glycosidase F (D5 CV-1 + *N*-glycosidase F) following purification. **B–D**, saturation binding studies of [ $^3\text{H}$ ]SCH23390 to microsomal fractions prepared from D5 CV-1 cells grown and treated as described above. **B**, D5 CV-1; **C**, D5 CV-1 + *N*-glycosidase F; and **D**, D5 CV-1 + tunicamycin.

**TABLE 1**

The effect of *N*-glycosidase F and tunicamycin on D5 ligand binding

The effect of *N*-glycosidase F and tunicamycin on  $B_{\text{max}}$  and  $K_d$  values of [ $^3\text{H}$ ]SCH23390 binding to crude membrane fractions prepared from D5 CV-1 cells

Cells	$B_{\text{max}}$ (pmol/ $\mu\text{g}$ protein)	$K_d$ (nM)
Untreated control	64	6.5
Treated with <i>N</i> -Glycosidase F	57	4.1
Treated with tunicamycin	4	0.53

likely due to the expression of a misfolded polypeptide. The addition of *N*-linked sugars occurs immediately following the emergence of the nascent polypeptide in the lumen of the endoplasmic reticulum and, therefore, occurs before protein folding and assembly. It is likely that large, hydrophilic carbohydrate chains added to the D5 receptor facilitate the process of receptor folding. Without the addition of *N*-linked sugars, the D5 receptor may fail to achieve the conformational structure necessary to bind ligand (Fig. 7D).

It is apparent that functional expression of D5 dopamine receptors at the cell surface of HEK 293 cells requires the addition of *N*-linked carbohydrates, whereas D1 receptor trafficking is governed by other, as yet, unidentified factors. Specific sorting sequences located within the D1 and D5 receptors may also be involved with the functional expression of these receptors at the cell surface. In fact, recently, a new family of proteins that serve to regulate plasma membrane delivery and function of membrane-spanning proteins has been identified. These proteins, termed RAMPs (receptor-activity modifying proteins) (McLatchie et al., 1998) are sensitive to the glycosylation state of the seven transmembrane domain receptors with which they interact and aid in directing the receptors to the plasma membrane. Analysis of protein interactions involving D1 and D5 dopamine receptors may identify RAMPs or other accessory/regulatory molecules involved with the trafficking of these dopamine receptors in vivo.

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