N-Linked Glycosylation Is Essential for the Functional Expression of the Recombinant $P2X_2$ Receptor†

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ABSTRACT: P2X receptors are integral membrane proteins that belong to the growing family of transmitter-gated ion channels. The extracellular domain of these receptors contains several consensus sequences for N-linked glycosylation that may contribute to the functional expression of the channel. We have previously reported the extracellular orientation of asparagine residues 182, 239, and 298 of the $P2X_2$ receptor subunit by showing that the protein is glycosylated at each site [Torres, G. E., et al. (1998) *FEBS Lett. 425*, 19−23 (*1*)]. In this study, we focused on the consequences of removing N-linked glycosylation from the $P2X_2$ receptor by using two different approaches, tunicamycin treatment or site-directed mutagenesis. HEK-293 cells stably transfected with the $P2X_2$ receptor subunit showed little or no response to ATP after tunicamycin treatment. In addition, loss of function was observed with the elimination of all three N-linked glycosylation sites from $P2X_2$. Cell surface labeling with biotin or indirect immunofluorescence revealed that the expression of the nonglycosylated receptors produced by either tunicamycin or site-directed mutagenesis is greatly reduced at the cell surface, indicating that the nonglycosylated $P2X_2$ receptors are retained inside the cell. These data provide the first direct evidence for a critical role of N-linked glycosylation in the cell surface expression of a P2X receptor subunit.

In addition to its classical role as a bioenergetic molecule, ATP is now recognized as an important transmitter in both the central and peripheral nervous systems. Extracellular ATP elicits its responses by activating two distinct classes of purinergic receptors of the P2 subclass (*2*). P2Y, P2U, and P2T receptors are members of a large family of G-protein-coupled receptors, whereas P2X receptors are ligand-gated ion channels. Recently, seven subtypes of P2X receptors ($P2X_1$−$P2X_7$) have been cloned from different tissues (*3*−*8*). The gene products of these clones share an overall amino acid identity of approximately 40% and contain two putative transmembrane domains connected by a large extracellular loop. This topological arrangement differs considerably from those of classical members of the transmitter-gated ion channel family such as the nicotinic acetylcholine, glutamate, and GABA receptors (*9*), but it resembles the structure of a distinct class of channels including the sodium-selective channel found in epithelia and brain (*10*), the Phe-Met-Arg-Phe-amide-gated sodium channel (*11*), the acid-sensing ionic channel (*12*), and the mechanosensitive channel of *Caenorhabditis elegans* (*13*).

The heterologous expression of the cloned P2X subunits results in the formation of functional nonselective cation channels gated by ATP. The phenotypes of the ATP responses differ among the P2X subunits with respect to rates of desensitization and sensitivities to the agonist $\alpha\beta$-methylene-ATP and the antagonists PPADS and suramin (*14*). Structure−function studies based on a combination of mutagenesis and electrophysiology have identified important structural elements that underlie many of the physiological and pharmacological properties of P2X receptors. Examples of such investigations include the identification of residues from the second transmembrane domain of $P2X_2$ that are implicated in ion channel conduction (*15*, *16*), the involvement of the intracellular amino and carboxyl termini in desensitization (*17*), and the identification of extracellular residues that contribute to determining the PPADS affinity of P2X receptors (*6*, *18*).

Contrary to the growing body of structure−function studies, nothing is known about the role of posttranslational modifications in determining P2X channel function. This is relevant to P2X function since the deduced amino acid sequence of all cloned subunits contains consensus sequences for phosphorylation and N-linked glycosylation. N-Linked glycosylation has been implicated in many diverse functions of membrane-bound receptors, including intracellular trafficking, stability, and/or cell surface expression (*19*, *20*). In the ligand-gated channel family, the contribution of N-linked glycosylation to channel expression and function is variable. Inhibition of N-linked glycosylation of the nicotinic AchR α1 subunit by either tunicamycin or site-directed mutagenesis reduced the expression of the receptors at the cell surface (*21*, *22*). Similarly, N-linked glycosylation is required for the expression of functional homomeric AchR α7 subunits in *Xenopus* oocytes (*23*). In contrast to the clear role of N-linked glycosylation in AchR expression and function, the importance of carbohydrate moieties in the function of

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ionotropic glutamate receptors is controversial. It has recently been reported that N-linked glycosylation is not required for the expression and function of recombinant glutamate receptors of the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate subclasses (*24*), which is in disagreement with previous studies supporting a critical role of N-glycosylation for the functional expression of recombinant AMPA receptors, and native ionotropic glutamate receptors (*25*−*27*).

The involvement of N-linked glycosylation in channel expression or function for the P2X receptor class has not been investigated. Several potential consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr) are found in the large extracellular loop of all cloned subunits. Interestingly, some of these sites are well conserved in mouse, rat, and human clones of the P2X family (*18*, *28*, *29*), suggesting that the binding of carbohydrate moieties to the protein may be important for the expression or the function of the channel. We addressed this hypothesis by examining whether N-linked glycosylation is necessary for the functional expression of the $P2X_2$ receptor subunit. Thus, using transfected HEK-293[1] cells, our approach involved tunicamycin treatment to inhibit N-glycosylation and deletion of N-glycosylation consensus sequences by site-directed mutagenesis. Our results demonstrate that N-linked glycosylation is essential for the normal expression of the $P2X_2$ receptor subunit at the cell surface.

EXPERIMENTAL PROCEDURES

Materials. Enzymes for cloning and sequencing were obtained from Promega. Vent DNA polymerase used for PCR-based mutagenesis was purchased from New England Biolabs; MEM medium, glutamine, fetal bovine serum (FBS), lipofectamine, and oligonucleotides were obtained from GIBCO BRL. Gel extraction kit and plasmid DNA isolation kit were from Qiagen. Tunicamycin was from Boehringer Mannheim, sulfosuccinimidyl 6-(biotinamido)-hexanoate (Sulfo-NHS-LC-biotin) and Protein G Gamma-Bind agarose were from Pierce Chemical Co.; [^{35}S]dATP for sequencing, ECL detection reagents, streptavidin coupled to horseradish peroxidase (HRP), and anti-mouse IgG/HRP conjugate were from Amersham. M2 anti-FLAG monoclonal antibody was from Kodak, and pCDNA-3 was obtained from Invitrogen. All other chemicals used in this study were purchased from Sigma.

DNA Constructs. Creation of the epitope-tagged $P2X_2$ wild-type and N-glycosylation mutants by site-directed mutagenesis has been described previously (*1*). All mutations were verified by DNA sequencing with the dideoxynucleotide chain termination method, utilizing the T7 sequenase kit from Amersham.

Transfection of HEK-293 Cells. The full-length rat $P2X_2$ receptor subunit cDNA, containing the FLAG sequence (DYKDDDDK) at the carboxyl terminus, was subcloned into the pCDNA-3 vector. HEK-293 cells were stably transfected with this construct by incubating the cells with 1 μg of cDNA mixed with 6 μL of lipofectamine in 1 mL of serum-free medium. After 5 h at 37 °C, the medium was replaced with MEM, and 24 h later, transfected cells were incubated in MEM containing 400 μg/mL G418. Resistant colonies were characterized electrophysiologically or by immunoblot analysis. A cell line termed clone 5 was selected for the present studies. Average whole cell capacitance of clone 5 cells was 43 ± 4 pF (mean ± SEM). For transient transfections of N-glycosylation mutants, 3×10^5 cells were plated in 35-mm dishes 24 h before transfection. Cells were transfected with lipofectamine as described above and incubated in MEM for 24−48 h.

Electrophysiological Recordings. Whole cell recordings were obtained from single cells kept at a holding voltage of −40 mV. ATP (30 μM) was applied by manually moving the electrode and attached cell into the line of flow of solutions exiting an array of inlet tubes lying side by side.

Tunicamycin Treatment. Stably transfected HEK-293 cells were cultured in MEM with or without 10 μg/mL tunicamycin for 72 h. Cells were then harvested for electrophysiological and immunoblot analysis.

Biotinylation and Immunoprecipitation Experiments. Confluent monolayers of HEK-293 cells in 35-mm dishes were washed 3 times with PBS and then incubated with gentle agitation for 30 min at RT with 0.5 mL of 0.5 mg/mL Sulfo-NHS-LC-biotin in PBS supplemented with 0.1 mM Hepes, pH 8.0. The reaction was quenched by incubating the cells for an additional 10 min with 50 mM ammonium chloride in PBS. The cells were then washed 3 times in PBS, and incubated in solubilization buffer (PBS, 1% NP-40, 1 mM PMSF, 1 mM AEBSF, 10 μg/mL leupeptin) at 4 °C for 1 h. Immunoprecipitation was carried out using the M2 anti-FLAG antibody (5 μg/mL) in the presence of 50 μL of Protein G Gamma-Bind agarose. Immunoprecipitates were washed 5 times with solubilization buffer and resuspended in protein sample buffer. Samples were boiled for 5 min, and proteins were analyzed by SDS−polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose filters. The filters were blocked overnight in TBS-T (20 mM Tris, pH 7.6, 145 mM NaCl, 0.05% Tween 20) containing 2% bovine serum albumin, and incubated for 1 h with streptavidin coupled to horseradish peroxidase. Filters were washed extensively in TBS-T, and immunoreactivity was visualized by enhanced chemiluminescence using an ECL kit. In some experiments, transfected HEK-293 cells were solubilized without immunoprecipitation, analyzed by SDS−PAGE, and transferred to nitrocellulose filters. Filters were incubated with 10 μg/mL anti-FLAG antibody. After several washes with TBS-T, filters were incubated with peroxidase -conjugated sheep anti-mouse antibody for 1 h. Filters were washed extensively in TBS-T, and immunoreactivity was detected with the ECL detection kit.

Immunofluorescence and Confocal Microscopy. Transiently transfected HEK-293 cells grown on glass coverslips were fixed with fresh 2% paraformaldehyde in PBS for 10 min at room temperature. After three washes with PBS, cells were permeabilized in PBS containing 0.05% NP-40 for 10 min at room temperature, and incubated in blocking solution (PBS, 1% bovine serum albumin, 5% goat serum) containing 10 μg/mL anti-FLAG antibody. Cells were washed in blocking solution prior to incubation with FITC-conjugated sheep anti-mouse antibody at a dilution of 1:200 for 1 h at

[1] Abbreviations: HEK, human embryonic kidney; MEM, minimal essential medium; Sulfo-NHS-LC-biotin, sulfosuccinimidyl 6-(biotinamido)hexanoate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; NP-40, nonidet P-40; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

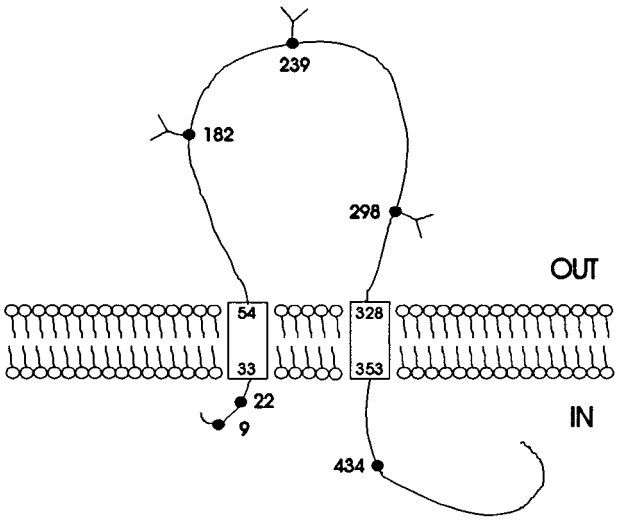

FIGURE 1: Transmembrane topology of the rat $P2X_2$ receptor subunit. Boxes correspond to presumptive transmembrane domains. Approximate locations of N-linked glycosylation consensus sequences are indicated by "trees".

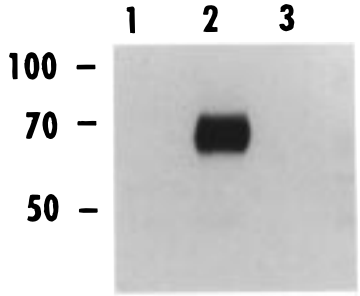

FIGURE 2: Immunoblot of HEK-293 cells stably expressing the $P2X_2$ receptor subunit. Fifty micrograms of protein from HEK-293 cells was separated on a 10% acrylamide gel, transferred to nitrocellulose, and probed with the anti-FLAG antibody. Lane 1, lysates from nontransfected cells; lane 2, lysates from HEK-293 cells stably transfected with the epitope-tagged $P2X_2$ receptor subunit; lane 3, same as lane 2 except that the immunoblot was performed in the presence of 10 ng/mL FLAG peptide.

room temperature. The cells were then mounted in Vectashield, and representative fields were selected using a MRC 1024 confocal laser scanning microscope. Control experiments included nontransfected cells and cells transfected with the wild-type untagged $P2X_2$ receptor subunit.

RESULTS

The amino acid sequence of the rat $P2X_2$ receptor subunit contains three consensus sequences for N-linked glycosylation within the extracellular loop of the receptor, located at amino acid positions 182, 239, and 298 (Figure 1). We have previously shown that the $P2X_2$ receptor is glycosylated at each site, demonstrating the extracellular orientation of those residues (*1*). Here, we extend those results by examining the role that N-linked glycosylation plays in $P2X_2$ receptor expression and function. As a first step, we have created a cell line that stably expresses the $P2X_2$ receptor subunit tagged with the FLAG epitope to allow for immunodetection. Figure 2 shows immunoblot analysis of lysates from nontransfected cells and from a clonal HEK-293 cell line stably expressing the FLAG-$P2X_2$ receptor subunit (clone 5). Lysates from nontransfected cells show no immunoreactivity (Figure 2, lane 1), whereas in lysates from clone 5 cells, the

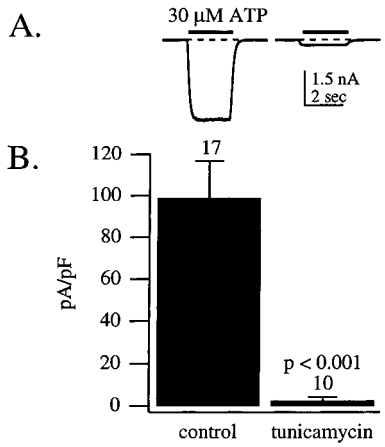

FIGURE 3: (A) Tunicamycin prevents the effect of 30 μM ATP on $P2X_2$ receptors. Representative recordings corresponding to ATP-gated currents in control cells (left) and tunicamycin-treated cells (right). (B) HEK-293 cells stably transfected with the $P2X_2$ receptor subunit (clone 5) were treated without (left bar, $n = 17$ cells tested) or with 10 μg/mL tunicamycin (right bar, $n = 10$ cells tested) for 72 h and assayed for ATP-mediated inward currents. (Statistical analysis was performed using the Student's *t*-test.)

anti-FLAG antibody detects a predominant band with an approximate molecular mass of 70 kDa (Figure 2, lane 2). Since activation of the $P2X_2$ receptor generates nondesensitizing inward currents (*4*), we examined the effect of ATP on cells stably expressing the $P2X_2$ receptor subunit. In each of 17 cells tested, ATP produced robust nondesensitizing inward currents ranging in size from 27 to 297 pA/pF (99 $\pm$ 18, mean $\pm$ SEM). ATP had no effect in nontransfected cells ($n = 45$), as has been reported previously (*1*, *3*, *16*). These results indicate that functional $P2X_2$ receptors are expressed in all cells of the stable line, which provides a suitable model to examine the involvement of postranslational modifications in ion channel expression and function.

A well-established approach to study the role of N-linked glycosylation of membrane-bound receptors is the use of specific inhibitors of the enzymes responsible for the formation of carbohydrate moieties of proteins. Tunicamycin specifically inhibits the addition of *N*-acetylglucosamine to the carrier dolicholphosphate, which is the first step in the formation of the core oligosaccharide in N-linked glycosylation. Thus, we examined the effect of tunicamycin on the function of the $P2X_2$ receptor. Clone 5 cells treated with 10 μg/mL tunicamycin for 72 h show little or no ATP-gated currents compared with the robust response seen in control cells. Of the 10 tunicamycin-treated cells tested, only 5 responded to ATP, and these currents were much smaller (0.3−15 pA/pF) than control (Figure 3). Next, we examined the molecular mass of receptor proteins expressed in cells treated with or without tunicamycin. When analyzed on SDS−polyacrylamide gels, the apparent molecular mass of $P2X_2$ decreases to approximately 53 kDa in tunicamycin-treated cells (Figure 4), a size consistent with the molecular mass predicted from the primary amino acid sequence of $P2X_2$. Moreover, the size of the protein expressed in tunicamycin-treated cells was identical to that of a mutant $P2X_2$ receptor lacking the three glycosylation sites (Figure 6, lanes 2 and 3), indicating that the tunicamycin treatment brought about complete inhibition of N-glycosylation. Hence, these results establish a correlation between the decreased response of ATP in treated cells and the absence of N-linked

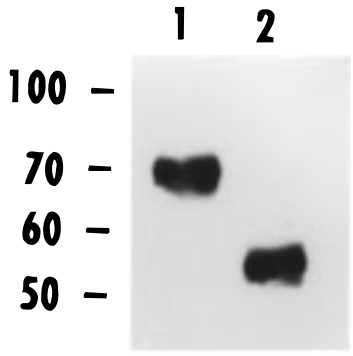

FIGURE 4: Tunicamycin treatment prevents N-linked glycosylation of the $P2X_2$ receptor subunit. Clone 5 cells stably expressing the $P2X_2$ receptor were incubated for 72 h in the absence (lane 1) or presence of 10 μg/mL tunicamycin (lane 2). Samples containing equal amounts of protein were resolved on a 10% SDS−PAGE gel, transferred to nitrocellulose, and probed with an anti-FLAG antibody.

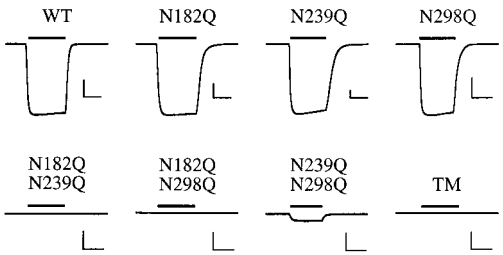

FIGURE 5: Whole-cell currents recorded from HEK-293 cells expressing single, double, or triple N-linked glycosylation $P2X_2$ mutants. Recordings were obtained 36−48 h after cells were transfected with the respective cDNAs as described under Experimental Procedures. The currents were elicited by 30 μM ATP from a holding potential of −40 mV. Note: only 2 out of 34 cells tested that were transfected with the N238Q/N298Q double mutant gave ATP-induced currents, and these were smaller than those elicited at wild-type or single mutant receptors. Scale bars are equal to 1 nA, 1 s.

glycosylation of the $P2X_2$ receptor subunit. There are two possible interpretations that would account for the lack of response observed with the nonglycosylated receptor. One possibility is that the carbohydrate moieties bound to the protein may be necessary for the functional expression of the channel. Alternatively, these results could be explained by the deglycosylation of a regulatory protein essential for the functional expression of P2X receptors.

To distinguish between these two possibilities, we analyzed the functional expression of WT and mutant $P2X_2$ receptors lacking one (single mutants), two (double mutants), or all three (triple mutant) glycosylation sites present in $P2X_2$. All of these N-glycosylation mutants, carrying the FLAG epitope to allow for immunodetection, were transiently transfected into HEK-293 cells. As shown in Figure 5, simply removing individual N-glycosylation sequences had no effect on functional expression of $P2X_2$ receptors in HEK-293 cells ($n \geq 20$ cells tested). Cells expressing these mutants gave robust and reproducible nondesensitizing currents in response to 30 μM ATP in at least 85% of the cells tested. In contrast, no ATP responses were observed in cells expressing two double mutants (N182Q, N239Q; and N182Q, N298Q) or the triple N-linked glycosylation mutants ($n \geq 20$ for each clone, Figure 5). In 34 cells transfected with the N239Q/N298Q mutant, only 2 exhibited small ATP-gated currents (Figure 5). Thus, it appears that at least two N-linked

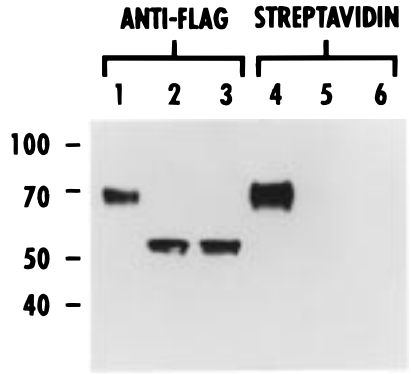

FIGURE 6: Biotinylation of cell surface P2 × 2 receptors. HEK-293 cells were transfected with the epitope-tagged $P2X_2$ receptor in the absence (lanes 1 and 4) or the presence of 10 μg/mL tunicamycin (lanes 2 and 5), or cells were transfected with the triple glycosylation mutant (lanes 3 and 6). Cells were labeled with biotin, and solubilized receptors were immunoprecipitated with the anti-FLAG antibody and subjected to immunoblot analysis using HRP-conjugated streptavidin (lanes 4−6). Proteins from similar dishes were solubilized and subjected to immunoblot analysis using the anti-FLAG antibody followed by HRP-conjugated anti-mouse antibody (lanes 1−3).

glycosylation sites are required for the full functional expression of the receptor; however, there does not appear to be any specificity as to which sites must be glycosylated. Another possible explanation for the lack of ATP-induced current in the cells transfected with the double or triple mutants might relate to transfection and/or translational difficulties with these particular cDNAs. We think this unlikely, as we observed no difference in either the relative levels of protein by Western blotting (e.g., Figures 6 and 7) or the relative number of immunoreactive cells per field when confocal microscopy was used to assess cell sorting of the proteins (presented later in this section). Thus, we would have expected that the percentage of cells tested that would have been transfected was similar, regardless of which construct was used. These results with the N-linked glycosylation mutants were consistent with those of the tunicamycin experiments, and suggest that the lack of response observed with the double and triple $P2X_2$ N-linked glycosylation mutants could be due to the inability of these receptor mutants to either respond to ATP or be targeted to the cell membrane.

To assess whether nonglycosylated $P2X_2$ receptors are found on the cell surface, intact cells transfected with the $P2X_2$ receptor in the presence of tunicamycin, or cells transfected with the N-glycosylation mutants, were incubated with Sulfo-NHS-LC-biotin. This compound binds to free amino groups of proteins, and since it is membrane-impermeant to intact cells, it can be used to distinguish between cell surface and intracellular proteins. Receptors labeled with Sulfo-NHS-LC-biotin were subjected to immunoprecipitation with the anti-FLAG antibody, separated on SDS−PAGE, transferred to nitrocellulose, and detected with streptavidin. Cells expressing the wild-type $P2X_2$ receptor were labeled with Sulfo-NHS-LC-biotin (Figure 6, lane 4), whereas no immunoreactivity was detected in cells expressing the $P2X_2$ triple mutant or cells expressing the $P2X_2$ receptor in the presence of tunicamycin (Figure 6, lanes 5 and 6). As a control for the expression of protein receptors, samples from dishes with equivalent numbers of cells were

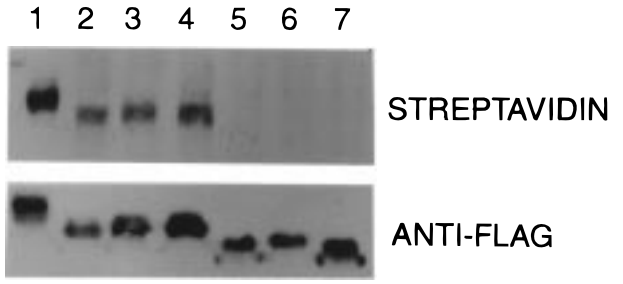

FIGURE 7: Biotinylation of single and double N-linked glycosylation mutants. HEK-293 cells were transfected with wild-type, single, and double N-linked glycosylation $P2X_2$ receptor mutants. Top panel: samples were labeled with biotin, immunoprecipitated with the anti-FLAG antibody, and immunoblotted with HRP-conjugated streptavidin. Bottom panel: proteins from similar dishes were solubilized and subjected to immunoblot analysis using the anti-FLAG antibody followed by HRP-conjugated anti-mouse antibody. Lane 1, FLAG-$P2X_2$ receptor; lanes 2, 3, and 4, the single mutants FLAG-$P2X_2$N182Q, FLAG-$P2X_2$N239Q, and FLAG-$P2X_2$N298Q, respectively; lanes 5, 6, and 7, the double mutants FLAG-$P2X_2$-N182Q/N239Q, FLAG-$P2X_2$N182Q/N298Q, and FLAG-$P2X_2$-N239Q/N298Q, respectively.

solubilized and subjected to immunoblot analysis carried out with the anti-FLAG antibody. Figure 6 shows that the nonglycosylated proteins were indeed expressed at similar levels compared to the fully glycosylated $P2X_2$. In addition, consistent with the functional studies, single mutants but not double N-linked glycosylation mutant receptors were biotinylated (Figure 7). Together, these results indicated that nonglycosylated $P2X_2$ receptors were retained inside the cell.

To analyze the subcellular localization of the wild-type $P2X_2$ receptor and the N-linked glycosylation mutants, immunofluorescence staining was performed using the anti-FLAG antibody. As shown in Figure 8A, confocal microscopy of permeabilized HEK-293 cells expressing the wild-type $P2X_2$ receptor exhibits bright fluorescent staining at the perimeter of the cell, indicating that the protein is distributed predominantly on the cell surface. No immunofluorescence was observed when nontransfected HEK-293 cells or cells transfected with the untagged $P2X_2$ receptor were subjected to immunofluorescence staining (data not shown). Cells expressing the single glycosylation mutants showed similar patterns of immunofluorescence staining with respect to the wild-type receptor (Figure 8B−D), indicating that the single mutants were also located on the plasma membrane. In contrast, cells expressing the double mutants and the triple glycosylation mutant exhibited a different distribution pattern from that observed in cells expressing the wild-type $P2X_2$ receptor. Confocal microscopy clearly showed a diffuse intracellular staining, consistent with greatly reduced expression of the receptor on the plasma membrane and a concomitant increase of tagged protein in the intracellular compartment.

DISCUSSION

The present studies are the first to show the role of a posttranslational modification in the normal activity of purinergic receptors of the P2X subclass. Our results demonstrate that N-linked glycosylation of the $P2X_2$ receptor subunit is essential for its expression at the cell membrane. That conclusion is supported by the evidence obtained from two different approaches. First, in HEK-293 cells stably transfected with the $P2X_2$ receptor subunit, tunicamycin, an inhibitor of N-linked glycosylation, caused a significant decrease in ATP-mediated inward currents. The average ATP responses were approximately 10% of control. Failure to completely prevent ATP-activated inward currents in a few cells probably reflects the existence of mature $P2X_2$ protein synthesized before tunicamycin treatment. The effect of tunicamycin treatment on ATP-gated currents correlated with an inhibition of N-linked glycosylation as evidenced by a shift in the molecular weight of the protein in tunicamycin-treated cells. We did not observe any apparent reduction in the amount of immunoreactivity of tunicamycin-treated cells with respect to control cells, which indicates that tunicamycin did not produce a significant effect on receptor synthesis. In addition, tunicamycin treatment produced nonglycosylated receptors that were not labeled with the membrane-impermeant Sulfo-NHS-LC-biotin. These findings suggest that inhibition of N-linked glycosylation of the $P2X_2$ receptor subunit affects the ability of the protein to assemble into a functional receptor or impairs sorting to the plasma membrane. However, as always with the use of general glycosylation inhibitors, we could not exclude the possibility that the effect on $P2X_2$ receptor function results from an indirect effect of tunicamycin, since this agent has a general action on glycoprotein biosynthesis.

To provide direct evidence on the functional role of N-linked glycosylation, and because the use of tunicamycin does not allow analysis of the contribution of individual N-linked glycosylation sites, our second approach involved the use of site-directed mutagenesis to remove systematically the consensus N-linked glycosylation sequences from the $P2X_2$ receptor subunit. Receptor subunits containing two oligosaccharide moieties at either of the three normal sites appeared to be expressed nearly as efficiently as the wild-type receptor. In general, current amplitude, kinetics, and desensitization properties were similar to the wild-type receptor. In addition, the immunofluorescent pattern of cells transfected with P2X receptors lacking individual glycosylation sites resembled that of cells transfected with the wild-type receptor. Thus, preventing glycosylation at any one site does not produce a dramatic effect on the functional expression of $P2X_2$. In contrast, removal of a second N-linked glycosylation site greatly impairs the functional expression of the receptor. Two of the double mutants, N182Q/N239Q and N182Q/N298, failed to form functional channel receptors, whereas in cells transfected with the N239Q/N298Q mutant, we observed small currents in less than 10% of cells tested. In addition, removal of the third N-linked glycosylation site produced a nonfunctional channel. Two independent sets of experiments, cell surface labeling with biotin and indirect immunofluorescence, were carried out to determine whether these mutants were expressed at the cell membrane. Our results show that when two or all three glycosylation sites are mutated, the protein cannot be detected at the cell surface.

Since the loss of N-linked glycosylation impairs the expression of the $P2X_2$ receptor at the cell membrane, we could not assess whether the carbohydrate moieties play a direct role in channel function. In the context of the results obtained with the single glycosylation mutants, it seems that individual N-linked glycosylation sites do not play a critical role in any of the general properties of the receptor, i.e., ATP

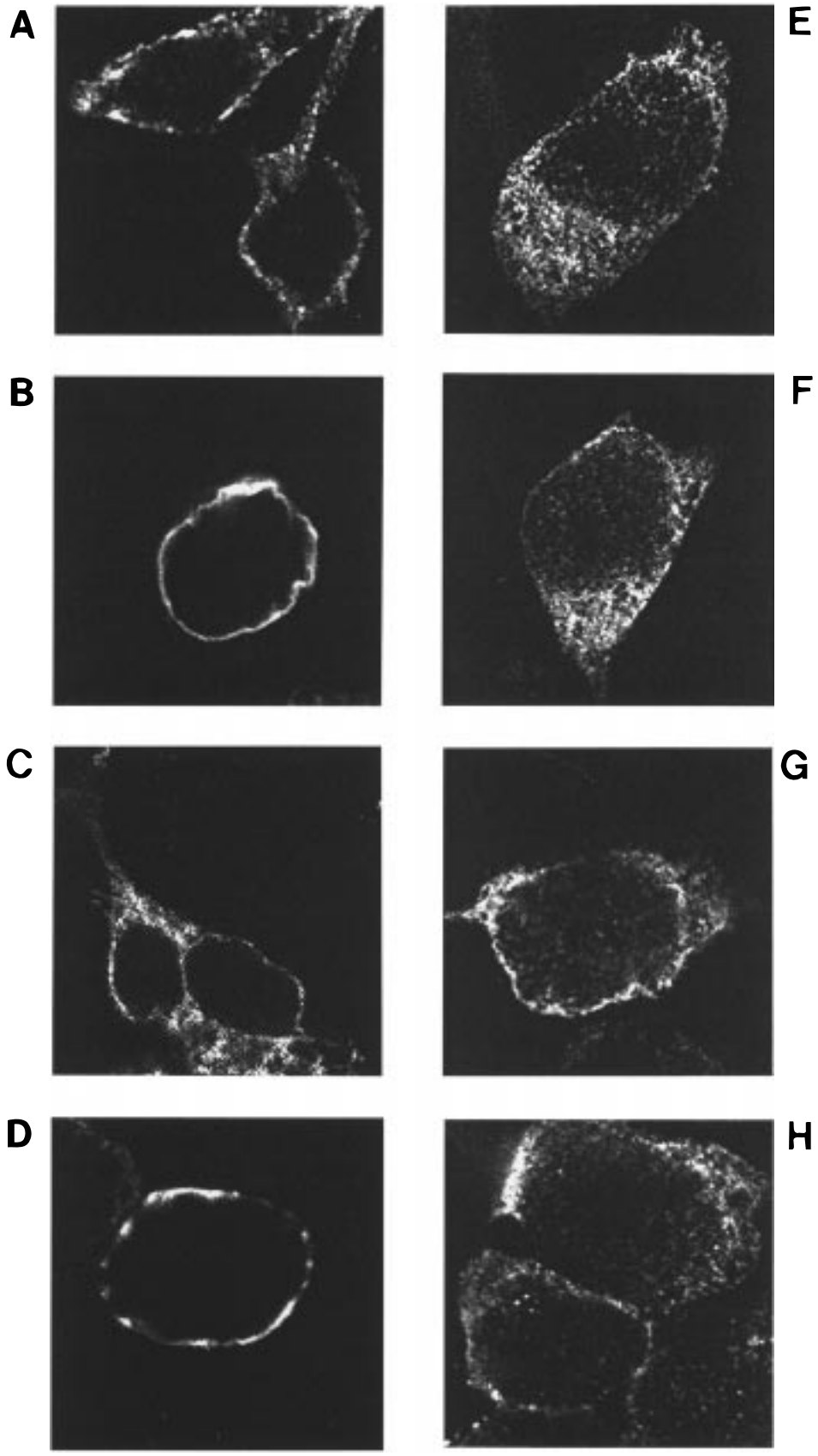

FIGURE 8: Immunofluorescence labeling of HEK-293 cells expressing wild-type and N-linked glycosylation $P2X_2$ mutants. HEK-293 cells were transfected with the wild-type FLAG-$P2X_2$ receptor (A); the single mutants FLAG-$P2X_2$N182Q (B), FLAG-$P2X_2$N239Q (C), and FLAG-$P2X_2$N298Q (D); the double mutants FLAG-$P2X_2$N182Q/N239Q (E), FLAG-$P2X_2$N182Q/N298Q (F), and FLAG-$P2X_2$N239Q/N298Q (G); and the triple mutant FLAG-$P2X_2$N182Q/N239Q/N298Q (H). Cells were then fixed, permeabilized, and stained with M2 anti-FLAG antibody followed by FITC-conjugated anti-mouse antibody. Representative fields were selected using a Bio-Rad confocal microscope.

binding, kinetics of activation, and/or desensitization, since those properties were not modified by the single mutations. However, we cannot exclude an additional, subtle role for N-linked glycosylation in either homomeric or heteromeric channel function.

The carbohydrate moieties of glycoproteins in general are believed to be important for a variety functions including normal protein folding, protection from proteolytic degradation, intracellular trafficking, and/or cell surface targeting (*19*, *20*). As integral membrane proteins, many ion channels have N-linked glycosylation consensus sequences. However, the role that N-linked glycosylation plays in the normal expression and function of ion channel proteins is variable, with nonuniform effects on ligand binding, cell surface expression, and/or direct channel function. For instance, the loss of N-linked glycosylation sites was found to produce changes in the open probability of the inward rectifier potassium channel ROMK1 (*30*). In the case of sodium channels, N-linked glycosylation was required for correct subunit assembly and transport to the cell membrane (*31*). Similarly, the nicotinic acetylcholine receptor α1 subunit failed to assemble into toxin binding receptors on the membrane of cells treated with tunicamycin (*21*). On the other hand, in contrast to the direct role of N-linked glycosylation in ion channel expression and function, other ion channel proteins, such as the Shaker potassium channel, are fully active in the absence of glycosylation (*32*). Thus, the loss of N-linked glycosylation appears to have a spectrum of effects on specific channel receptors, and no general predictions can be made based exclusively on the occurrence of glycosylation for any given protein.

One important aspect of studying the inhibition of glycosylation of ion channels is that it may help to understand the process by which these membrane-bound glycoproteins are synthesized, assembled, and targeted to the cell membrane as functional channel complexes. In the case of $P2X_2$, the molecular mechanisms by which N-linked glycosylation impairs the cell surface expression of the protein are not clear. One possible explanation is that the oligosaccharides are important in the initial protein folding of the receptor for further intracellular transport to the cell membrane. An alternative hypothesis that accounts for the failure of the nonglycosylated receptor to reach the cell surface is that removing the carbohydrate moieties affects the assembly of channel subunits. Further experiments are needed to answer those questions.

In summary, the experiments described in the present study demonstrate the critical role that the carbohydrate moieties attached to the $P2X_2$ receptor subunit play in cell surface expression of the protein. By analogy with other transmitter-gated channels, it is likely that the functional consequences of removing N-linked glycosylation from $P2X_2$ will be shared by other members of the P2X family.

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