Roles of Individual N-Glycans for ATP Potency and Expression of the Rat P2X<sub>1</sub> Receptor*

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P2X<sub>1</sub> receptor subunits assemble in the ER of Xenopus oocytes to homotrimers that appear as ATP-gated cation channels at the cell surface. Here we address the extent to which N-glycosylation contributes to assembly, surface appearance, and ligand recognition of P2X<sub>1</sub> receptors. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of glycan minus mutants carrying Gln instead of Asn at five individual NTX/S sequons reveals that Asn<sup>286</sup> remains unused because of a proline in the +4 position. The four other sites (Asn<sup>123</sup>, Asn<sup>184</sup>, Asn<sup>210</sup>, and Asn<sup>300</sup>) carry N-glycans, but solely Asn<sup>190</sup> located only eight residues upstream of the predicted reentry loop of P2X<sub>1</sub> acquires complex-type carbohydrates. Like parent P2X<sub>1</sub>, glycan minus mutants migrate as homotrimers when resolved by blue native PAGE. Recording of ATP-gated currents reveals that elimination of Asn<sup>123</sup> or Asn<sup>210</sup> diminishes or increases functional expression levels, respectively. In addition, elimination of Asn<sup>210</sup> causes a 3-fold reduction of the potency for ATP. If three or all four N-glycosylation sites are simultaneously eliminated, formation of P2X<sub>1</sub> receptors is severely impaired or abolished, respectively. We conclude that at least one N-glycan per subunit of either position is absolutely required for the formation of P2X<sub>1</sub> receptors and that individual N-glycans possess marked positional effects on expression levels (Asn<sup>123</sup>, Asn<sup>210</sup>) and ATP potency (Asn<sup>300</sup>).

Like virtually all membrane proteins, P2X receptors are glycoproteins. A multitude of functional aspects of oligosaccharides in glycoproteins have been described (for reviews, see Refs. 20 and 21), including influence on topogenesis (22), aid of proper protein folding (chaperone function) (23), quality control in the secretory pathway (24), prevention of marginally stable proteins from unfolding, as well as protein-protein interactions, ligand binding (25), and intracellular trafficking (26). The role of a particular N-glycan in a given protein, however, has been found to be rather unpredictable and may comprise several of the above mentioned functions, or it may have no known function at all and be completely dispensable.

The protein sequence of the rat P2X<sub>1</sub> subunit shows five putative N-glycosylation sites consisting of the NTX/S sequon (where X denotes any amino acid except proline), which are all located in the predicted P2X<sub>1</sub> ectodomain (1). We have recently shown that four of these five putative N-glycosylation sites of the rat P2X<sub>1</sub> subunit are used in Xenopus oocytes (9). Notably, only one of the four N-glycans acquires Endo H<sup>+</sup> resistance in route to the plasma membrane. Since P2X<sub>1</sub> subunits assemble rapidly and quantitatively to noncovalently linked homotrimers soon after synthesis while still in the ER, it seems likely that the three-dimensional structure of the fully assembled and properly folded P2X<sub>1</sub> receptor sterically hindered the access of particular Golgi enzymes to three of the four N-glycans. Hence, we considered that one or several of these inaccessible N-glycans play a role in stabilizing these interactions or even directly participate in subunit interactions in a lectin-like manner.

To examine whether N-glycans are important for P2X subunit assembly, surface expression, or ligand recognition, we eliminated N-glycosylation sites by site-directed mutagenesis and analyzed the oligomerization and ATP-gated currents of the corresponding receptors in Xenopus oocytes by SDS-PAGE, blue native PAGE, and two-electrode voltage clamp measurements, respectively. An additional aim of the study was to examine the effect of N-glycans on the migration of P2X receptor complexes in the blue native PAGE system. Our results show that any two out of the four naturally occurring N-glycans are sufficient for robust functional expression of P2X<sub>1</sub> receptors at the cell surface and that the N-glycan in position 210 contributes to ATP potency.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The construction of the His-P2X<sub>1</sub> plasmid encoding the rat P2X<sub>1</sub> subunit with an N-terminal hexahistidyl tag has been...
described previously (9). His-P2X mutants lacking one or several consensus sequences for N-glycosylation were generated by changing the Asn of existing NXST/T codons to Gln using a commercially available kit (QuikChange mutagenesis kit from Stratagene). The resulting His-P2X mutants were named ΔN1–ΔN5 (in sequence order) for single mutants (Fig. 1), ΔN12, ΔN13, ΔN15, ΔN23, ΔN25, and ΔN35 for double mutants, ΔN123 and ΔN235 for triple mutants, and ΔN1235 for the quadruple mutant lacking all N-glycosylation sites. All mutations were verified by diodeoxynucleotide sequencing (27).

**cRNA Synthesis**—Capped cRNAs were synthesized from EcoRI-linearized templates with SP6 RNA polymerase (Amersham Pharmacia Biotech), purified by Sepharose chromatography and phenol-chloroform extraction, and dissolved in 25 mM Tris/HCl, pH 7.2, at 0.5 µg/µl, using the optical density reading at 260 nm for quantitation (OD 1.0 = 40 µg/µl).

**Injection and Maintenance of Xenopus oocytes**—Xenopus laevis females received from South Africa were anesthetized with MS222 (tricaine) for surgical removal of ovaries. After collagenase treatment (28) defolliculated stage V or VI oocytes were manually selected and injected with 50-nl aliquots of cRNAs. Injected oocytes were kept in parallel with noninjected controls at 19 °C in sterile oocyte Ringer’s solution (ORi: 90 mM NaCl, 1 mM KCl, 2 mM MgCl2, and 5 mM Hepes, pH 7.4) supplemented with 50 mM NaCl, 1 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM Hepes, pH 7.4 supplemented with radioactive label of oocytes and Protein Purification—cRNA-injected oocytes and noninjected controls were metabolically labeled by ATP at 1-min intervals, low pass-filtered at 100 Hz, and sampled at 200 Hz using the Turbo TEC-05 amplifier (NPI Electronics, Tamm, Germany). All measurements were performed at room temperature (20–22 °C).

**RESULTS**

The P2X1 Polypeptide Carries Glycans at Asn153, Asn184, Asn210, Asn300, but Not at Asn284—The P2X1 cDNA has been originally cloned from rat vas deferens, where the P2X1 receptor plays a role in muscle contraction (1), which propels sperm into the ejaculate (34). The P2X1 polypeptide chain is 399 amino acids long. For affinity purification after synthesis in Xenopus oocytes, we tagged the rat P2X1 polypeptide at its N-terminal end with six histidine residues, which had no effect on ATP-induced currents (9). The His-P2X subunit can be isolated from cRNA-injected and [35S]methionine-labeled oocytes by using the two-electrode voltage clamp technique in oocytes injected with His-P2X, cRNA 3 days earlier. The electrodes contained 3 mM KCl and had resistances of 0.5–2 MΩ. The superfusion solution consisted of 90 mM NaCl, 1 mM KCl, 2 mM MgCl2, and 5 mM Hepes/NaOH, pH 7.4. Calcium salts were omitted to avoid activation of endogenous Ca2+-dependent Cl− channels. A fast and reproducible solution exchange was achieved as described previously (9).

Current signals were elicited by ATP at 1-min intervals, low pass-filtered at 100 Hz, and sampled at 200 Hz using the Turbo TEC-05 amplifier (NPI Electronics, Tamm, Germany). All measurements were performed at room temperature (20–22 °C).

**Blue Native PAGE and SDS-PAGE**—Blue native PAGE (30, 31) was carried out as described previously (9, 32, 33). Just before gel loading, purified proteins were supplemented with blue native sample buffer to final concentrations of 10% glycerol, 0.2% Serva blue G, and 20 mM sodium 6-amino-6-caproate and applied onto polyacrylamide gradient slab gels. Molecular mass markers (Combithek II, Roche Molecular Biochemicals) were run on both borders of the gel and were visualized by Coomassie blue staining. For SDS-PAGE, proteins were supplemented with SDS sample buffer containing DTT and electrophoresed in parallel with [14C]-labeled molecular mass markers (Rainbow, Amersham Pharmacia Biotech) on SDS-polyacrylamide gradient gels. When indicated, samples were treated prior to SDS-PAGE with either Endo H or PNGase F (New England Biolabs) in the presence of 1% octyl guoside to diminish inactivation of PNGase F. Gels were fixed, dried, and exposed at –80 °C to BioMax MR or MS film (Kodak) as appropriate. In some experiments, radioactive bands were quantified with an image analyzer (PhosphorImager 445 SI, Molecular Dynamics).

Fig. 1. Linear model of rat P2X1 showing approximate positions of N-glycosylation sequences and corresponding glycan minus mutants. N, cytoplasmic N terminus; M1 and M2, membrane spanning segments; N1–N5, asparagine residues of NXT/S tripeptides at position 153 (N1), 184 (N2), 210 (N3), 284 (N4), and 300 (N5). The 10 cysteine residues (S), which are conserved among all seven P2X isoforms and which are assumed to form five intramolecular disulfide linkages, are also indicated; an additional conserved cysteine residue (not shown) is located in M2.

Fig. 2. N-Glycosylation of His-P2X, mutants each lacking one out of five N-glycosylation sites. Oocytes injected with 23 ng of the indicated cRNAs were metabolically labeled by overnight incubation with [35S]methionine. After an additional 24-h chase interval, digitonin extracts were prepared. Parent His-P2X and the various glycan minus mutants were isolated by Ni2+-NTA chromatography, eluted with non-denaturing elution buffer, and then supplemented with SDS sample buffer, 20 mM DTT (final concentration). Samples were incubated for 15 min at 37 °C in the absence (A) or for 2 h at 37 °C in the presence of Endo H (B) or PNGase F, 1% octyl guoside (C), and then analyzed by SDS-PAGE (10% acrylamide) followed by autoradiography.
Currents were activated at a holding potential of −40 mV by 30 μM ATP, which elicits maximum responses at parent His-P2X1 receptor. Data are given as means ± S.E. from 20–48 oocytes per column of two to six experiments with 6–10 oocytes per experimental condition. Currents significantly different from that of parent His-P2X1 are denoted by asterisks (*, p < 0.05; **, p < 0.001).

Enzymes of three of the N-glycans were sterically hindered, most likely as a result of folding and oligomerization of the P2X1 chains. Fig. 2B shows that like the parent His-P2X1 receptor (lane 1) all the P2X1 receptor mutants (lanes 2–6) except ΔN5 (lane 7) acquired Endo H-resistant carbohydrates. Since the P2X1 receptor mutant ΔN5 appears at the plasma membrane (cf. Figs. 3–5), the absence of Endo H-resistant carbohydrates can be directly conferred to the lack of the sole N-glycan that can acquire complex-type carbohydrates during transit of the Golgi apparatus. This indicates that the glycans at N1 and N3 play opposing roles for the expression level of functional P2X1 receptors.

At Least One N-Glycan per Subunit at Either Position is Needed for P2X1 Receptor Formation—To examine whether the elimination of a particular N-glycan affects the expression level of functional P2X1 receptors, we recorded current responses to 30 μM ATP at −40 mV. This ATP concentration elicited different yet maximum responses at the parent P2X1 receptor and all receptor mutants, since activation with 100 μM ATP did not result in a further increase of currents. Like the parent P2X1 receptor, also all receptor mutants lacking one N-glycan per subunit exhibited large ATP-gated currents, indicating that no particular N-glycan is essential for receptor function (Fig. 3). There are, however, marked positional effects of the N-glycans. While the glycans at N2 or N5 were without significant effect on the expression level of functional receptors, elimination of the glycan at N1 or N3 resulted in a 30% decrease or 70% increase, respectively, of the current response (Fig. 3). Likewise, reduced currents were recorded from double mutants that lacked N1 together with N2 or N5, whereas elimination of N3 consistently resulted in increased currents even when N1 was simultaneously eliminated (Fig. 3). These findings suggest that the glycans at N1 and N3 play opposing roles for the expression level of functional P2X1 receptors.

Elimination of any three N-glycans did also not prevent P2X1 receptor formation, but the functional expression level decreased markedly to <10% of that of the parent P2X1 receptor. The currents were particularly low when the three acceptor sites that were eliminated included N1 (Fig. 3). No ATP-induced currents could be recorded from oocytes after removal of all canonical N-glycosylation sites. Taken together, these results implicate that despite their marked positional effects, only one N-glycan at any locus is required for the formation of functional P2X1 receptors, and two such sites are sufficient for robust functional expression.

Elimination of the Glycan at Asn210 Decreases ATP Potency—Since N-glycans are bulky and can shield a large section of the protein surface, we examined whether elimination of N-glycans affects ATP potency. To this end, we recorded receptor currents elicited by 0.3 and 30 μM ATP and determined the ratio of the current responses at these two ATP concentrations. As apparent from Fig. 4C, all the receptor mutants lacking N3 (Asn210) showed a current ratio of 5–7% independent of simultaneous elimination of additional N-glycosylation sites. In contrast, all glycan minus receptor mutants that contained N-glycans N2 and N5 showed a ratio of 26–37% similar to the 32% for the parent His-P2X1 receptor. Since N3 turned out to be crucial for the reduced potency, concentration response curves for ATP of ΔN3 and parent His-P2X1 were recorded. Fitting the Hill equation to the data for the parent His-P2X1 receptor yielded an EC_{50} value of 0.7 μM (Fig. 4D). In contrast, the glycan minus

FIG. 3. Comparison of maximum inward current responses generated by parent P2X1, and glycan minus mutants. Oocytes were injected with 23 ng of the indicated cRNAs. After 3 days at 19 °C, currents were activated at a holding potential of −40 mV by 30 μM ATP, which elicits maximum responses at parent His-P2X1. Data are given as means ± S.E. from 20–48 oocytes per column of two to six experiments with 6–10 oocytes per experimental condition. Currents significantly different from that of parent His-P2X1 are denoted by asterisks (*, p < 0.05; **, p < 0.001).

FIG. 4. Comparison of ATP potency between parent His-P2X1 and glycan minus mutants. Inward current traces were recorded at the indicated ATP concentrations and a holding potential −40 mV from oocytes injected 3 days earlier with 23 ng of cRNA for parent His-P2X1 (A) or the glycan minus mutant ΔN3 (B). C, the ratio between currents activated at −40 mV by 0.3 and 30 μM ATP was calculated as a measure of the ATP potency at the various glycan mutants (n = 5–6, mean ± S.E.). Note that all mutants lacking N-glycosylation site N3 exhibit a markedly decreased current ratio indicative of a reduced ATP potency. D, ATP dose-response curve for parent His-P2X1 (A) and mutant ΔN3 (B). Currents were elicited by the respective ATP concentration in 1-min intervals. Continuous lines represent least squares fits of the Hill equation to the data points, yielding EC_{50} values of 0.7 ± 0.1 μM (Hill coefficient of 1.1 ± 0.1) and 2.1 ± 0.2 μM (Hill coefficient of 1.3 ± 0.1) for parent His-P2X1, and mutant ΔN3, respectively (n = 6–7, mean ± S.E.).
To display the oligomeric state of these receptor complexes, we exposed the natively eluted His-P2X1 mutant receptors to DTT in the presence of Coomassie Blue and sodium 6-aminon-caproate. This treatment causes a partial dissociation of the parent His-P2X1 receptor to the monomer and dimer of apparent masses similar to 80 and 170 kDa, respectively, indicating that the nondenatured 250-kDa protein band must be a His-P2X1 homotrimer (9). Likewise, the His-P2X1 glycan minus receptor mutants lacking one or two N-glycans per subunit dissociated into dimers and monomers when exposed to DTT (Fig. 5B). Quantification of the bands by phosphor image analysis shows that the extent of dissociation induced by DTT is virtually identical for the three N-glycan minus mutant receptors. It should be noted that the dissociating effect of DTT does not result from a cleavage of intersubunit disulfide linkages, but from a perturbation of noncovalent subunit interactions. Dissociation of P2X1 complexes into monomers could also be produced by other denaturing additions such as urea or SDS, which by themselves will not affect disulfide bonds (9). In summary, receptor mutants lacking up to three N-glycans per subunit exist in a homotrimeric state like the wild type P2X1 receptor. A mutant that lacked all four N-glycans could neither be detected by two-electrode voltage clamp measurements nor by Ni2+ chelate chromatography followed by SDS-PAGE and autoradiography.

**Discussion**

**Factors That Control N-Glycosylation of P2X1 Receptors—**

The P2X1 receptor influences its own N-glycosylation at two levels: (i) at the level of its primary sequence and (ii) the level of its three-dimensional structure. First, the N284LS tripeptide of its three-dimensional structure. First, the N284LS tripeptide

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**N-Glycans and Membrane Folding of P2X1—**

The two-dimensional orientation of P2X subunits in the plasma membrane has been predicted from hydrophathy plots, usage of natural N-glycosylation sites (9), and N-glycan scanning mutagenesis studies (38, 39). The five putative N-glycosylation sites of the P2X1 polypeptide are all located in the predicted ectodomain.

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**N-Glycans and P2X1 Receptor Function**

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**Fig. 5.** Glycan minus mutants migrate as trimers like parent P2X1, when analyzed by blue native PAGE. Oocytes injected with the indicated cRNA 3 days earlier were surface-labeled with membrane impermeant 125I-sulfo-SHPP and then isolated the His-tagged proteins were then purified by Ni2+ NTA chromatography under nondenaturing conditions and resolved by PAGE followed by autoradiography. A. proteins were resolved immediately after native elution by blue native PAGE (4-10% acrylamide front). B. proteins were analyzed after native elution either without further treatment or after incubation with 100 mM DTT for the indicated time period. Numbered arrows indicate positions of monomers, dimers, and trimers.
upstream of the conserved lipophilic domain (Fig. 6), which has been postulated to constitute a reentry loop immediately before M2 like the H5 domain of K⁺ channels and hence to contribute to the pore (2). Using a cell-free translation system, it has been demonstrated that oligosaccharide transfer occurs only when 12–14 amino acids downstream to a sequon have been translated (40). The efficient N-glycosylation of N5 and its ready accessibility for Golgi enzymes, i.e., within the fully assembled P2X₁ receptor argue against a significant membrane embedding of this lipophilic domain. The glycan may serve to shield a hydrophobic surface at this locus.

Positional Effects of N-Glycans on Surface Expression of P2X₁ Receptor Protein—The most proximal glycan (N1) appears to be especially important for high surface expression levels of functional P2X₁ receptors, whereas N2 and N5 are dispensable in this respect. The surprising observation that the expression level of functional receptors increased upon elimination of N3 may be correlated with an increased number of P2X₁ receptors at the plasma membrane, as evident from most though not all surface radioiodination experiments (results not shown), rather than by altered receptor function. The mechanism of this increase is not clear yet. The peculiar role of the glycan at N3 for ATP potency is discussed below.

If the modulatory roles of individual N-glycans are neglected, it becomes obvious that the elimination of two of the four canonical N-glycosylation sites per subunit is quite well tolerated. This suggests that the number of N-glycans per P2X₁ subunit is redundant, at least for robust expression. A large decline of the expression level occurs only after elimination of three sites per subunit both in terms of maximum current amplitudes and receptor protein at the cell surface. Taking the trimeric architecture of P2X receptors into account, this means that a total of six N-glycans are required per receptor complex for robust functional expression. A reduction of surface appearance has been observed for the nonglycosylated forms of a large variety of glycoproteins, but the mechanisms that account for this phenomenon appear to be different. Many nonglycosylated forms of glycoproteins accumulate in the ER, aggregate, and do not exit (41). Others exit the ER, but are retained in the Golgi apparatus, apparently because N-linked oligosaccharides are required for efficient transport from the Golgi apparatus to the cell surface (42). Since nonglycosylated His-P2X₁ receptor subunits or complexes accumulate neither in the ER nor in the Golgi apparatus, rapid degradation soon after synthesis is likely to account for the low functional expression levels and low amounts of receptor protein at the cell surface of mutants carrying solely one N-glycan.

Elimination of all four glycosyl acceptor sites abolished the appearance of His-P2X₁ at the cell surface. An essential role of N-glycans has been also described for the formation of functional P2X₉ receptors in HEK293 cells (43). This raises the possibility that interaction with lectin-like chaperones such as calnexin and/or calreticulin (23) is crucial at certain stages of the folding process and for oligomerization of P2X subunits, for instance by providing additional time to allow for productive collisions between P2X monomers. Incompletely folded monomers that lack N-glycans as functional tags may no longer be retained in the ER and eventually get degraded (44).

Conservation of N-Glycosylation Sites among P2X Isoforms—the four N-glycans of P2X₁ are distributed over two-thirds of the ectodomain like the ten cysteine residues that are assumed to be involved in disulfide formation (Fig. 1). In contrast to the cysteines, however, which are totally conserved among all P2X isoforms, only P2X₂ exhibits a N-glycan distribution that overlaps exactly with that of P2X₁. Sequons corresponding to N1 and N2 of P2X₁ are present on almost all isoforms, whereas the sequon corresponding to N5 (Asn₁⁰⁰) is found on P2X₂, P₂X₃ only. The number of N-glycosylation sites likely to be used amount to three (P₂X₄, P₂X₅, P₂X₆) or four (P₂X₁, P₂X₃), six (P₂X₇) and seven (P₂X₉), i.e., all isoforms carry at least three N-glycans. Since only two such sites are actually required for robust receptor formation, and no particular N-glycan is absolutely important, variation in acceptable positions of glycans can occur. Hence, the redundancy in the number of N-glycans provides at least a partial explanation for the imperfect conservation of the number and positions of N-glycans among the P2X isoforms.

N-Glycans and Ligand Recognition—Elimination of N-glycans has only occasionally been observed to be associated with altered ligand recognition. Lack of N-glycosylation of the glucose transporter GLUT1 decreased its apparent affinity for glucose (45). In contrast, the nonglycosylated transporter for serotonin (46) or norepinephrine (47), like the Na⁺/glucose symporter SGLT1 (48), exhibited no alteration of substrate recognition. Elimination of the four N-glycosylation sites of the insulin receptor β subunit also did not affect the affinity for insulin, but blocked signal transduction (49). The single N-glycan of the human T-cell surface glycoprotein CD2 was first suggested by cell adhesion assays to be required for binding of the counter receptor CD58. However, structure resolution by NMR revealed that this N-glycan is not directly involved in ligand binding, but crucial for the stabilization of the folded protein structure (50). Presence of a glycan in the vicinity of the α-bungarotoxin binding site has been demonstrated to confer α-bungarotoxin resistance to the nicotinic acetylcholine receptor (51). Most likely, the bulky glycan moiety imposes steric hindrance for toxin binding. Referred to the present work, one would expect a gain of function, i.e., an increase in ATP potency, once a bulky N-glycan is removed in the vicinity of the ligand binding site. The observation of a decrease in the potency for ATP, i.e., a loss of function, makes it more likely that the glycan at Asn²¹⁰ imposes structural alterations, which may act to stabilize a folded domain essential for ATP binding. A contribution of this region to the ATP binding site can be inferred from the recent observation that neutralization of the conserved residues Lys¹⁹⁰ and Lys²¹⁵ by alanine substitution produced also a slight (2–5-fold) decrease of ATP potency (52).
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