Membrane Topology of an ATP-gated Ion Channel (P2X Receptor)*

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Alison Newbolt‡, Ron Stoop§, Caterina Virginioò, Annmarie Surprenant†, R. Alan North*, Gary Buell, and François Rassendren**

From the Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, Plan-les-Ouates, 1228 Geneva, Switzerland

Western blots of *Xenopus* oocyte membrane preparations showed that the apparent molecular mass of the wild type P2X2 receptor (about 65 kDa) was reduced by pretreatment with endoglycosidase H. Mutagenesis of one or more of three potential asparagines (N182S, N239S, and N298S) followed by Western blots showed that each of the sites was glycosylated in the wild type receptor. Functional channels were formed by receptors lacking any single asparagine, but not by channels mutated in two or three positions. Artificial consensus sequences (N-X-S/T) introduced into the N-terminal region (asparagine at position 9, 16, or 26) were not glycosylated. Asparagines were glycosylated when introduced at the C-terminal end of the first hydrophobic domain (positions 62 and 66) and at the N-terminal end of the second hydrophobic domain (position 324). A protein in which the C terminus of one P2X2 subunit was joined to the N terminus of a second P2X2 subunit (from a concatenated cDNA) had twice the molecular mass of the P2X2 receptor subunit, and formed fully functional channels. The experiments provide direct evidence for the topology originally proposed for the P2X receptor, with intracellular N and C termini, two membrane-spanning domains, and a large extracellular loop.

The extracellular signaling properties of nucleotides are mediated through two distinct families of membrane proteins. These are the P2Y receptors, coupled to G proteins and second messenger pathways, and the P2X receptors, which are ligand-gated ion channels (1). Seven subunits of the P2X receptor family (P2X1–7) have been characterized at the molecular level (reviewed in Refs. 2 and 3). These show a broad expression of the P2X receptor subunits are 36–48% identical to one another at the amino acid level. All seven proteins have similar hydrophobicity profiles, with only two hydrophobic regions sufficiently long to span the plasma membrane. These regions display the features often seen in transmembrane segments such as aromatic residues at interfacial regions, and they have an excess of positively charged amino acids at their presumed cytoplasmic ends (7). Together with the absence of signal peptide sequence after the initiating methionine, this suggests that P2X receptors may have intracellular N and C termini, and two transmembrane domains separated by a large extracellular loop (8, 9). This proposed topology differs from that of the nicotinic and glutamate superfamilies of ligand-gated ion channels (reviewed in Ref. 10), but resembles that shown for the pore-forming subunits of epithelial sodium channels (11, 12). There is, however, no detectable similarity of amino acid sequence between P2X receptors and epithelial sodium channels (3).

There are other experimental results that are consistent with this topological model of P2X receptor subunit. First, P2X1 receptors are activated by the ATP analog βmeATP, 1 whereas P2X2 receptors are not; transferring the putative extracellular loop (approximately residues 50–320) from the P2X1 receptor into the P2X2 receptor confers βmeATP sensitivity (13). Second, changing one amino acid within the loop of the P2X4 receptor (E249K) causes a large increase in the blocking action of the slowly reversible antagonist pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid (14). Third, the difference in sensitivity to pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid between human and rat P2X4 receptors can be transferred by exchange of a segment within the first half of this loop (15). Fourth, evidence for N-glycosylation of Asn194 of the P2X1 receptor has been obtained, indicating that this must be located extracellularly (16).

The recent identification of residues contributing to the pore of the P2X2 receptor has also provided evidence for an intracellular location for the C-terminal part of the protein (17). Amino acids in and around the second hydrophobic domain of the P2X2 receptor (residues 316–354) were mutated individually to cysteine, the proteins were expressed, and ATP-activated currents were measured. Inhibition of the current by polar methanethiosulfonate derivatives was then used to determine whether the residue was likely exposed to the aqueous solution. One substitution was identified (D349C) at which extracellularly applied methanethiosulfonate ethylamine inhibited the ATP-evoked current. However, the inhibition required channel opening. Because methanethiosulfonate ethylamine can permeate the open channel, these results indicate that Asp349 normally lies internal to the channel “gate.” The residue is close to the C-terminal end of the second hydrophobic...

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‡ Present address: School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom.
§ Present address: Institute Biologique Cellulaire et Morphologie, University of Lausanne, CH1005 Lausanne, Switzerland.
¶ Present address: Dept. of Pharmacology, Glaxo Wellcome Research and Development, 37135 Verona, Italy.
† Present address: Inst. of Molecular Physiology, University of Sheffield, Sheffield 510 2TN, United Kingdom.
** To whom correspondence should be addressed. Tel.: 41-22-706-9739; Fax: 41-22-794-6965; E-mail: rassendren@serono.com.

1 The abbreviations used are: βmeATP, adenosine 5′-(β,γ-methylene)triphosphate; Endo H, endoglycosidase H; MTSET, methanethiolosulfonate trimethylammonium; EGFP, enhanced green fluorescent protein.
region; this therefore implies that the C terminus of the protein is within the cytoplasm, because there is no further hydrophobic domain long enough to span the plasma membrane.

These results are consistent with the topology for P2X receptors initially proposed (8, 9), but alternative models are also possible. For example, the data generated so far on cloned P2X receptors might as well be explained with a model in which only the second hydrophobic domain spans the plasma membrane, placing the entire N-terminal part of the protein extracellularly. Therefore, we have used the N-glycosylation site tagging approach to determine the precise topology of P2X receptors. During protein biosynthesis, extracellular domains of the protein are facing the lumen of the endoplasmic reticulum where they are able to be glycosylated. Due to the strict compartmentalization of N-glycosylating enzymes at the luminal face of the endoplasmic reticulum, it is possible to assess the extracellular location of different region of a given protein by N-glycosylation site tagging. This approach has been used to determine the topology of other membrane proteins including channels and transporters (18, 19).

We first identified the endogenous N-glycosylation of P2X receptor, and then by mutagenesis obtained a form in which no natural N-glycosylation of the protein remained. The cDNA for this modified form of the receptor was then used as a background plasmid to engineer artificial N-glycosylation sites at different locations in the protein, with respect to the two hydrophobic domains. The extent of N-glycosylation was assayed by gel shift assay after functional expression of epitope-tagged forms of the P2X receptor in Xenopus oocytes. Finally, we concatenated cDNAs and expressed the tandem constructs to show that the N and C termini reside on the same side of the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—In all experiments, a P2X receptor cDNA was used that carried a C-terminal epitope (DPGLNEYMPME), cloned into the expression vector pcDNA3 (Invitrogen) (17, 20). Point mutations in P2X receptor cDNA were introduced by full-length polymerase chain reaction amplification of plasmid DNA with sense and antisense mutated primers. The polymerase chain reaction (PCR) protocol (23) was used to reduce the rate of contaminating mutations. Parental (wild type) DNA was digested with 10 units of *DpnI* (New England Biolabs) for 1 h at 37 °C; 2.5 µl of the reaction was then directly used for transformation of *Escherichia coli* DH5α strain. DNA fragments carrying the mutation were digested with appropriate restriction enzymes and subcloned in a background vector that had not been amplified. All mutants were sequenced on both strands.

**Construction of Dimeric cDNAs**—A concatenated tandem P2X receptor cDNA was constructed in pBluescript vector. Briefly, two P2X cDNAs were modified by in-frame addition of an EcoRI site either at the 5′ or 3′ end of the coding sequence, respectively. These two cDNAs were ligated between the EcoRI site and a unique site from the backbone of the vector. The resulting construct contains two P2X subunits linked from the C terminus of one (-KGLAQ) to the N terminus of another (MVRLA-); the deduced amino acid sequence at the junction site is from the C terminus of one (-KGLAQ) to the N terminus of another ligated through the *EcoRI* site. The resulting construct contains two P2X2 subunits linked through the *EcoRI* site.

**Identification of Natural N-Glycosylation**—We determined if it was possible to detect tagged P2X receptor by Western blot from a crude oocyte membrane preparation. As shown in Fig. 1, when oocytes were injected with the wild type P2X2 cDNA, the anti-EE antibody recognized a protein of apparent molecular mass around 65 kDa. The calculated size of the P2X receptor protein is 52.6 kDa; the observed difference is due to N-glycosylation because a mutated subunit lacking all three asparagines found at consensus glycosylation sites (N-X-S/T) migrated at approximately 50 kDa. The broad smear of the wild type Western blot may result from the fact that crude membrane preparations were used; these would include not only plasma membrane but also the membrane of intracellular organelles (endoplasmic reticulum and Golgi) in which the identity and length of sugars added to proteins might be variable. Un.injected or EGFP-injected oocytes showed no staining or very weak bands (Fig. 1B).

The P2X receptor primary sequence contains three consensus sites for N-glycosylation (N-X-S/T); these are Asn182, Asn239, and Asn298. To determine if any or all of these were glycosylated, we constructed a series of mutants in which one, two, or three sites were removed. As shown in Fig. 1 (C and D), all three sites were glycosylated in *Xenopus* oocytes. This was evidenced by the progressive reduction of the apparent molecular mass of P2X protein as the number of N-glycosylation sites decreases (Fig. 1C). No differences were observed among the three single mutants (N182S, N239S, and N298S) or among the three double mutants (N182S/N239S, N182S/N298S, and N239S/N298S), suggesting that all three asparagines are modified (Fig. 1C). This was further demonstrated by the effect of endoglycosidase H (Endo H). Membrane preparations of oocytes expressing wild type P2X2 receptors and the three double mutants were treated with Endo H; in each case, this led to the appearance of a band that co-migrated with the trypsin mutant receptor (*ΔN-P2X2*) (Fig. 1D). A fraction of the protein in each case was resistant to Endo H; this may be because Endo H only hydrolyzes high mannose oligosaccharides specific to the endoplasmic reticulum and does not affect asparagines added in other compartments such as the Golgi. Higher concentrations of enzyme or longer incubation times did not change the pattern of action of Endo H. In the case of the *ΔN-P2X2* receptor, Endo H had no effect on the apparent molecular mass of the protein (data not shown).

Mutants lacking one, two, or three N-glycosylation sites were...
transfected in HEK293 cell and tested electrophysiologically for the presence of ATP-induced currents. In HEK293 cells expressing each of the single mutants, ATP (30 μM) evoked inward currents that were not obviously different from those observed in cells expressing wild type receptors (Fig. 2). These results indicate both that full glycosylation is not required for normal function, and that the point mutations introduced did not critically disrupt the overall structure of the protein. However, in cells expressing the double mutations ATP evoked only very small currents, and on the triple mutant receptor (Δ3N-P2X2) ATP had no detectable effect.

Introduction of Artificial N-Linked Glycosylation Sites—We introduced three point mutations resulting in consensus sites for N-glycosylation into the Δ3N-P2X2 receptor between the initiating methionine and the beginning of the first hydrophobic regions (C9N, Y16N, and R28T) (Fig. 3A). The latter possibility is favored by the observations that I66S, I66L, and I66C all provided functional channels with ATP (30 or 300 μM) in HEK293 cells transfected with the indicated P2X2 receptor. WT, wild type.

Fig. 1. Native N-glycosylation of the P2X2 receptor. A, model of P2X2 receptor indicating three Asn residues within N-X-S/T consensus sites. B, control immunoblot of membranes from oocytes expressing wild type (WT) P2X2 receptor and Δ3N-P2X2 receptor and GFP, nothing (uninjected), or GFP alone. Arrowsheads indicate nonspecific binding of the antibody. C, characterization of native N-glycosylation sites by gel shift assay. Sites were removed by mutagenesis singly (N182S, N239S, N298S), doubly (N182S/N239S, N182S/N298S, N239S/N298S) or triply (N182S/N239S/N298S: Δ3N). Note the progressive reduction in the apparent molecular mass of the protein as the number of N-glycosylation sites decreases. D, in wild type and doubly mutant proteins, Endo H causes a reduction in the apparent molecular mass to that of the Δ3N-P2X2 receptor. Note that, under these conditions, Endo H does not completely deglycosylate the wild type P2X2 receptor.

Fig. 2. Functional P2X receptors may lack one N-linked glycan. A, examples of currents evoked by ATP (30 or 300 μM) in HEK293 cells transfected with the indicated P2X2 receptor. B, histogram of amplitude of currents evoked by maximum ATP concentrations in cells transfected with the indicated cDNAs. Currents evoked by ATP were normal in cells expressing receptors with any single asparagine mutated; however, currents were greatly reduced or absent in cells transfected with constructs in which more than one asparagine was mutated. WT, wild type.
normal responses to ATP (currents were $3.6 \pm 0.7 \, nA$, $3.9 \pm 0.4 \, nA$, and $4.1 \pm 0.6 \, nA$, respectively; $n = 6$ in each case).

Close to the second hydrophobic domain, the mutations K324N, I328N, and A335T were made so as to introduce consensus sequences N-F-S, N-P-T, and N-L-T (Fig. 5). Only K324N displayed a shift in mobility; as for P62N, two bands were seen, with one migrating at the same size as the Δ3N-P2X$_2$ receptor (Fig. 5B). The higher molecular weight band resulted from a glycosylated receptor because it was abolished by preincubation of the membrane preparation with Endo H (Fig. 5C). The lack of glycosylation of I328N might reflect the introduction of the sequence N-P-T; it has been previously shown that a proline at position X in the consensus sequence N-X-T/S strongly reduces the likelihood of N-glycosylation (24).

We made the double mutant I328N/P329F, which creates the sequence N-F-T. This was also not glycosylated, but a shorter form of the protein was consistently observed (Fig. 5D). When the double mutation I328N/P329F was introduced in the P2X$_2$ receptor, which carried the three native glycosylation sites, no functional expression was obtained (data not shown). However, the mutation A335T in this “wild type” background resulted in apparently normal responses to ATP.

**Expression of Dimeric cDNAs**—The failure to observe glycosylation when consensus sequences were introduced into the region N terminus to the first hydrophobic domain is unhelpful with respect to assignment of this domain to one or other side of the membrane, because not all consensus sites are used (24). We therefore sought to determine whether the N and C termini were located on the same side of the membrane by expressing a concatenated cDNA (25). Fig. 6G shows a Western blot from oocytes injected with the dimeric construct. A single band was observed at a molecular mass of about 120 kDa, indicating that the intact dimeric protein is expressed. Recordings made from the same oocytes, prior to membrane preparation for Western blotting, showed inward currents evoked by ATP (30 μM) which were not obviously different from those seen in oocytes expressing the monomeric wild type protein (data not shown). We have previously shown that substitution of Cys for Thr at position 336 in the P2X$_2$ receptor produces a channel in which methanethiosulfonate ethyltrimethylammonium (MTSET) almost completely blocks the current evoked by ATP (17). MTSET inhibited the currents by about 50% when the Cys to Thr substitution was made in either the first or second (Fig. 6C) domain of the dimeric construct, and by close to 100% when the mutation was in both domains (25).

**DISCUSSION**

These experiments show that the P2X$_2$ receptor is a glycoprotein, in which all three consensus sequences for N-linked glycosylation (N-X-S/T) are used. Among the seven P2X receptor subunits, the number of such consensus sequences ranges from three (P2X$_2$, P2X$_3$, P2X$_9$) to seven (P2X$_5$), and in no case is the consensus sequence found at a corresponding position in all seven sequences. However, the sequence around Asn$^{256}$ of the P2X$_2$ receptor (N-F-T) is present in all except the P2X$_5$ receptor, and it is also found within a region of the protein showing relatively high homology among all seven subunits. This suggests that it may be glycosylated in them all (except P2X$_2$); indeed, the corresponding residue in the P2X$_2$ receptor provides the only previously known glycosylation site in the P2X receptor family (16). Our finding that fully functional ATP-gated channels are found with the point mutation N182S is consistent with the inference from the P2X$_5$ receptor sequence that glycosylation at this position is not essential for channel assembly and function.

The second of the two sites identified in the present study (Asn$^{239}$ of N-F-T) also occurs in a relatively highly conserved region of the protein, but in this case the consensus sequence is found only in the P2X$_2$ and P2X$_7$ receptors. The third of the three sites found to be glycosylated is the Asn$^{296}$ of N-G-T; the consensus sequence is found at this position also in the P2X$_1$ and P2X$_3$ receptors but not in the other receptors. A further conserved consensus site that is missing in the P2X$_2$ receptor is found in receptors P2X$_{4–7}$ (corresponding to Asn$^{355}$ in the P2X$_4$ receptor), and it will be interesting to determine whether this is also glycosylated.

We found that the currents induced by ATP in HEK293 cells expressing P2X$_2$ receptors lacking any one of the three asparagines were essentially normal (Fig. 2), suggesting that none of the attached sugars play a significant role in agonist recognition and binding. On the other hand, receptors in which two or three asparagines were substituted by serine responded to ATP either very poorly or not at all (Fig. 2). We have visualized the cells immunohistochemically using standard fluorescent or confocal laser microscopy: all showed a pattern of plasma membrane localization similar to that seen in the wild type, but the fluorescence intensity was significantly reduced in the recep-
tors lacking two or three (but not one) glycosylation sites. This finding implies that full glycosylation is not necessary for correct protein folding and transport to the plasma membrane. However, further immunohistochemical studies of these receptors tagged in the extracellular domain would be useful to examine whether the unglycosylated protein is correctly inserted in the plasma membrane.

The main aim of the present work was to delineate the boundaries of the putative transmembrane domains. We interpret the finding of glycosylation at residues P62N, I66N, and K324N to indicate that these positions are exposed at the extracellular aspect of the membrane; Pro62 is the 11th amino acid residue following the final hydrophobic residue of the first hydrophobic domain (Val51). The failure to observe glycosylation at sites closer to the hydrophobic domain (Q52N, Q56N, and S58N) might be because they are situated within the interfacial region and inaccessible to glycosylating enzymes (23), or because these mutations prevent normal folding and membrane expression. The latter interpretation seems less

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2 A. Newbolt, R. Stoop, C. Virginio, A. Surprenant, R. A. North, G. Buell, and F. Rassendren, unpublished observations.

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![Diagram](image-url)
likely in view of the finding of normal functional responses in the wild type P2X2 receptor carrying mutations Q52N, S58N, and P62N. The residues corresponding in position to Gln52, Ser58, and Pro62 are highly variable among the seven P2X family members, has intracellular N and C termini, two hydrophobic domains (approximately residues 31–51 and 331–353), and a large extracellular loop (52–330). We cannot exclude the possibility that regions of this long loop fold back into the plasma membrane, although we note that any such regions that are predominately hydrophobic are only six or seven amino acids in length. The identification of the natural glycosylation sites indicates that residues 184, 239, and 298 are extracellular. This overall topology is thus similar to that described for the α subunit of the epithelial sodium channels (11). The sequence homology between the epithelial sodium channels and the FMRFamide-activated (27) and proton-activated (28) channels makes it likely that these other ligand-gated channels share the same overall pattern of membrane insertion, with two transmembrane domains and a large, cysteine-rich extracellular loop. This contrasts clearly with the other large families of ligand-gated ion channels which have either three (glutamate family) or four (nicotinic acetylcholine family) membrane-spanning segments per subunit (see Ref. 10).

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