

Role of the domain encompassing Arg³⁰⁴–Ile³²⁸ in rat P2X₂ receptor conformation revealed by alterations in complex glycosylation at Asn²⁹⁸

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The final 25 amino acids of the ectodomain of the P2X receptors, immediately prior to the second TM (transmembrane domain) (pre-TM2: Arg³⁰⁴–Ile³²⁸ in rat P2X₂), are highly conserved. Whole-cell patch clamp recordings showed that single cysteine substitutions in the N-terminal half of pre-TM2 (Arg³⁰⁴–Ile³¹⁴) led to loss of function at Arg³⁰⁴, Leu³⁰⁶, Lys³⁰⁸ and Ile³¹². Cysteine substitutions within this region also resulted in a significant reduction in the apparent molecular mass of receptors, due to loss of complex glycosylation at the nearby acceptor site Asn²⁹⁸, which was not seen for the C-terminal portion of pre-TM2 (Asp³¹⁵–Ile³²⁸). The reduction in complex glycosylation was not due to reduced cell-surface presentation, demonstrating that glycosylation at Asn²⁹⁸ was acting as a sensor of subtle changes

in receptor conformation within the pre-TM2 region. When this N-glycan site was repositioned closer to the plasma membrane by mutagenesis (N298S together with G299N, T300N, T301N or T303N), glycosylation was restored at G299N and T300N, but was impaired for T301N and completely absent for T303N. These results suggest that the region in the vicinity of Asp³¹⁵ is at the plasma membrane interface and that the N-terminal portion of pre-TM2 (Arg³⁰⁴–Ile³¹⁴) is important for the correct conformation of the receptor at the extracellular face of the membrane.

Key words: ion channel, pre-transmembrane segment 2 (TM2), topology, scanning cysteine mutagenesis.

INTRODUCTION

P2X receptors are a family of ligand-gated ion channels that play key roles in diverse physiological processes such as nerve transmission, control of smooth muscle tone and the response to inflammation [1]. Functional P2X receptors are trimers. Each of the three subunits is composed of intracellular N- and C-termini, two TMs (transmembrane domains) and a large ectodomain [2–4]. One of the most highly conserved parts of the P2X receptor protein sequence is the final 25 amino acids of the ectodomain which immediately precedes the second TM (pre-TM2; Arg³⁰⁴–Ile³²⁸ in the rat P2X₂ receptor sequence). This region has been proposed to be a signal transduction module, linking the conformational change associated with ATP binding to the opening of the channel pore [5,6]. Residues within this region, in particular Lys³⁰⁸, have been implicated in both ATP binding and channel gating [7,8]. Previous studies using cysteine mutants within the pre-TM2 region of human P2X₁ [5] and alanine mutants within the pre-TM2 region of rat P2X₄ [6] have highlighted the importance of Arg³⁰⁵, Lys³⁰⁹ and Phe³¹¹ in P2X₁ (Arg³⁰⁴, Lys³⁰⁸ and Tyr³¹⁰ in P2X₂) and Tyr³¹⁵, Gly³¹⁶ and Arg³¹⁸ in P2X₄ (Tyr³¹⁰, Gly³¹¹ and Arg³¹³ in P2X₂) for optimal channel function. It has also been proposed that this region of the receptor might contain a membrane-permeant loop similar to that seen in voltage-gated potassium channels [9].

N-glycosylation of P2X receptors has been shown significantly to affect both protein folding and function [2,10–13]. Mature rat P2X₂ receptors are glycosylated at Asn¹⁸², Asn²³⁹ and Asn²⁹⁸; removal of any single glycan by mutagenesis does not significantly impair receptor function [2], but removal of two or more glycans leads to incorrect folding and loss of surface presentation of the receptor [2,13]. In the present study on the function of rat P2X₂ receptors with cysteine substitutions in the pre-

TM2 region, we observed marked differences in the apparent molecular mass of the mutated receptors when expressed in HEK (human embryonic kidney)-293 cells. In the present study, we show that the reasons for these differences have to do with altered complex glycosylation at Asn²⁹⁸, reflecting a subtle change in the conformation of the receptor in the pre-TM2 region.

MATERIALS AND METHODS

Molecular and cell biology

The wild-type rat P2X₂ cDNA used in the present study has been described previously [14]; it contains a C-terminal EYMPME epitope. Single point mutations were introduced into wild-type or mutant cDNAs using the QuikChange[®] site-directed mutagenesis protocol (Stratagene) and the coding regions of each mutant were fully sequenced. cDNAs corresponding to wild-type or mutant receptors were transiently transfected into 35 mm dishes of near-confluent HEK-293 cells using Lipofectamine[™] 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were incubated for 48 h (0.1 μg of cDNA/dish) or 24 h (1 μg of cDNA/dish) to allow protein expression.

Electrophysiology

Whole-cell patch clamp experiments were performed as described previously [7]. Cells were held at –60 mV, and agonists were applied for a 2 s duration. EC₅₀ values for agonists were determined by least-squares curve fitting to the Hill equation: $I/I_{\max} = 1/[1+(EC_{50}/A)^n]$, where I is the current as a fraction of the maximum (I_{max}) and [A] is the agonist concentration, and n is the Hill coefficient.

Abbreviations used: EndoH, endoglycosidase H; HEK, human embryonic kidney; TM, transmembrane domain.

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Solubilization of HEK-293 cell protein and Western blotting

Transfected cells were washed twice in PBS (pH 7.4). Following pelleting, cells were solubilized in 50 μ l of RIPA buffer [20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂ and protease inhibitors (Complete-EDTA; Roche)] containing 2% (v/v) Triton X-100 (w/v) for 1 h at 4°C. Insoluble material was pelleted by centrifugation at 16 000 *g* for 2 min, the protein content in the supernatant was assayed using the Bio-Rad protein assay kit, and 10 μ g or 1 μ g of total protein samples were taken. Samples were boiled for 2 min at 100°C in SDS/PAGE sample buffer [4% SDS and 10% (v/v) 2-mercaptoethanol] and loaded on to Nu-PAGE gels (4–12% gradient gels; Invitrogen) according to the manufacturer's protocol. After running, protein was transferred on to PVDF membranes and Western blotting was performed according to standard protocols. Both the primary antibody against the EE-tag (rabbit anti-Glu-Glu; Universal Biologicals) and the secondary antibody [HRP (horseradish peroxidase)-conjugated goat anti-rabbit; DAKOCytomation] were used at a dilution of 1:5000. Bands were visualised using the ECL[®] plus kit (GE Healthcare) and Kodak Biomax MS film (Sigma).

Cell-surface biotinylation

Biotinylation was performed at 0°C to prevent internalization of the label. Transfected cells were washed twice in PBS (pH 7.4), and once in PBS (pH 8.0). Cells were incubated in 1 mg/ml sulfo-NHS-biotin (sulfo-*N*-hydroxysuccinimidobiotin; Pierce) in PBS (pH 8.0) for 30 min. Unreacted biotin was quenched with two washes of PBS containing 192 mM glycine and protein was solubilized and assayed as above. Total protein samples (10 μ g) were taken, and 300 μ g of protein was incubated with streptavidin beads (Pierce) overnight at 4°C to bind cell-surface protein. Beads were washed three times and samples were boiled for 5 min at 100°C in SDS/PAGE sample buffer to liberate cell-surface protein. Samples of both total and cell-surface protein were analysed by Western blotting as above.

EndoH (endoglycosidase H) treatment

Total protein samples were prepared as above, and 100 μ g of samples were incubated with EndoH (Roche; 2 units) for 1 h at 37°C. Following this treatment, 20 μ g of equivalent protein was loaded on to SDS/PAGE gels for Western blotting.

Data analysis

All of the data, where appropriate, are presented as means \pm S.E.M. Statistical analyses were performed using two-way ANOVA [post-hoc Tukey's HSD (Honestly Significant Differences)].

RESULTS

Cysteine mutants in the pre-TM2 region display reduced molecular mass

The effects of cysteine substitution in the pre-TM2 domain on functional receptor expression were characterized using whole-cell patch clamp recording. Figure 1(A) shows the EC₅₀ values calculated from ATP concentration–current response curves. Either no currents were observed or the currents were too small to determine the EC₅₀ values at the mutants R304C, L306C, K308C and I312C (indicated with #). ATP potency was significantly reduced for the cysteine mutant at Arg³¹³ ($P < 0.01$ compared with wild-type); no significant differences from wild-type were

observed in any other mutants. Therefore most disruption to channel function was observed for mutants between positions Arg³⁰⁴ and Ile³¹⁴ (black bars), whereas mutations in the region Asp³¹⁵–Ile³²⁸ had little effect on functional receptor (grey bars). Three mutants (V318C, H319C and G320C) appeared to display increased ATP sensitivity; however, these values were not significantly different from wild-type ($P > 0.05$).

Western blotting with an antibody directed against the C-terminal EYMPME epitope tag was used to assess the total protein expression of each cysteine mutant. Several mutants displayed a reduced apparent molecular mass compared with wild-type, indicated by a reduction in, or loss of, the upper band representing protein of higher molecular mass (Figure 1B; broken lines); for example, the upper band was almost completely absent in the mutants R304C, L306C, Y310C, I312C and R313C. Scanning densitometry was used to calculate the percentage of upper band density relative to total band density (Figure 1C). The percentage of upper band observed for wild-type P2X₂ was $46 \pm 1\%$ ($n = 36$). Cysteine substitutions in the region Arg³⁰⁴–Ile³¹⁴ almost all showed a reduced amount of upper band (black bars), whereas those in the region Asp³¹⁵–Ile³²⁸ did not (grey bars), with the exceptions of K324C and F325C.

We tested the effects of amino acid substitutions other than cysteine on the amount of higher molecular mass form observed in Western blots. P2X₂ receptors with K308A, K308C or K308R have been shown to be significantly impaired in terms of ion-channel function [7]. However, the proportion of P2X₂ receptor protein running at the higher molecular mass was quite different for each of these mutants (Figures 2A and 2B). For K308A the upper band was virtually absent ($3 \pm 1\%$, $n = 9$; $P < 0.01$ compared with wild-type), for K308C the amount of upper band was also markedly reduced ($11 \pm 6\%$, $n = 4$; $P < 0.01$), but for K308R it was not different from wild-type protein ($45 \pm 1\%$, $n = 4$; $P > 0.05$). To ensure that the observed differences in the amount of upper band were not due to altered cell-surface expression, we performed cell-surface biotinylation assays to determine the proportion of mutant receptor expressed at the cell surface (Figure 2C). At the cell surface, the band profile of wild-type P2X₂ was somewhat altered; it appeared that a greater proportion of uppermost band was present in this sample. The mutant K308A was efficiently expressed at the cell surface, but displayed a complete absence of upper band, demonstrating that the presence of the upper band was not a requirement for cell-surface expression. Interestingly, the proportion of upper band present in the mutant K308C was much greater at the cell surface than in the total protein sample, suggesting that the high-molecular-mass form of this mutant was more efficiently expressed at the cell surface than the lower molecular mass forms. The cell-surface expression of K308R was identical with wild-type (results not shown). We quantified these data over several experiments using scanning densitometry (Figure 2D). For each mutant, the proportion of receptor at the cell surface was not significantly different from wild-type, even though the upper band was partially or fully absent. This data demonstrated that the reduction in the amount of higher-molecular-mass protein was not due to reduced cell-surface presentation, but that it reflected a disruption in protein conformation, the severity of which was dependent on the identity of the amino acid substituted.

Loss of complex glycosylation at Asn²⁹⁸ underlies the altered molecular mass

We hypothesized that the loss of the highest molecular mass of the P2X₂ mutants was likely to be due to a reduction in N-linked glycosylation as a result of altered protein conformation. To test

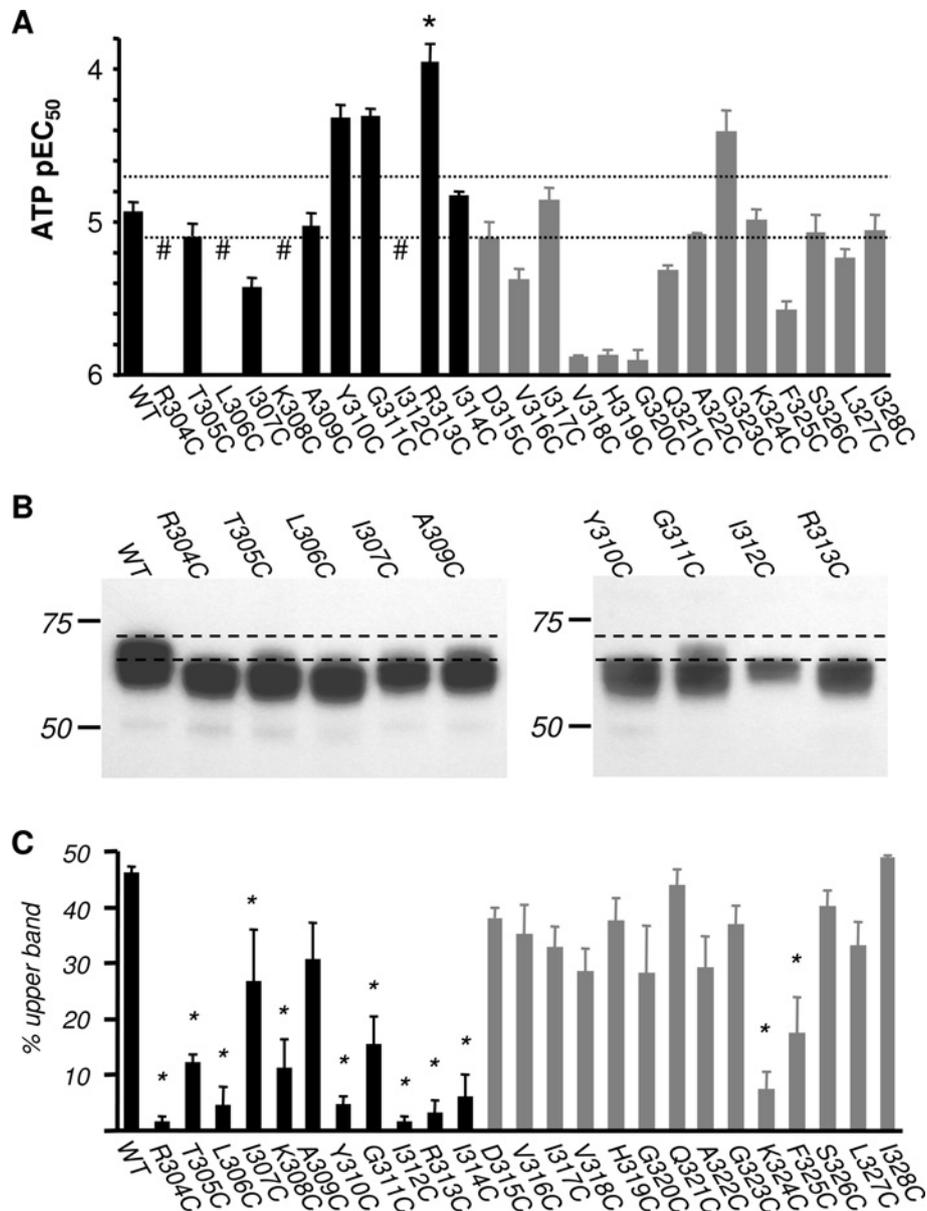


Figure 1 Effect of cysteine substitutions in the pre-TM2 region (Arg³⁰⁴-Ile³²⁸) on receptor function and glycosylation

(A) Summary of ATP pEC₅₀ values. * $P < 0.01$ compared with wild-type (WT) for R313C; all other mutants were not significantly different from wild-type ($P > 0.05$). $n = 3-5$ for all mutants. #, either no currents were observed or the currents were too small to allow determination of EC₅₀ values. Dotted lines represent S.D.s above and below the wild-type mean. (B) Sample Western blots of total protein from HEK-293 cells expressing wild-type (WT) or mutant rat P2X₂. Significant reductions in the amounts of upper band may be observed in several mutants. The molecular mass in kDa is indicated on the left-hand side. (C) Summary of scanning densitometry data ($n = 3-36$ for each mutant). Each value is expressed as a percentage of the upper band compared with the total band density. * $P < 0.01$ compared with wild-type (WT). Wild-type P2X₂ was included in every blot to control for variations between experiments and data was recorded only where the band densities were within the linear range of the sensitivity of the film.

this, we compared the expression of K308A, the N-glycan mutant N298S, and the double mutant N298S/K308A (Figure 3A). We observed a significant but differential loss of mass in both K308A and N298S; the mutant N298S induced a greater loss of mass than K308A (Figure 3A; lane 2 compared with lane 3). Interestingly, the reduction in mass observed in the double mutant N298S/K308A was the same as that of N298S (Figure 3A; lane 4 compared with lane 2). This result demonstrated that the mutation of Lys³⁰⁸ in the mutant N298S induced no further loss of mass, implying that the loss of mass seen in the mutant K308A was most probably due to an alteration in N-glycosy-

lation at Asn²⁹⁸, leading to a reduction in the mass of the glycan chain.

We reasoned that this might result from loss of complex N-glycosylation from Asn²⁹⁸, and therefore tested the sensitivity of wild type P2X₂, K308A and N298S to EndoH (Figure 3B). EndoH only cleaves core, high-mannose N-glycan chains; complex glycosylation acquired in the Golgi body is resistant to cleavage. Wild-type P2X₂ displayed partial resistance to EndoH treatment (Figure 3B; lane 2 compared with lane 1). In this blot the protein loading is lower than that in Figure 3(A), and it can be observed that wild-type P2X₂ expressed in HEK-293 cells

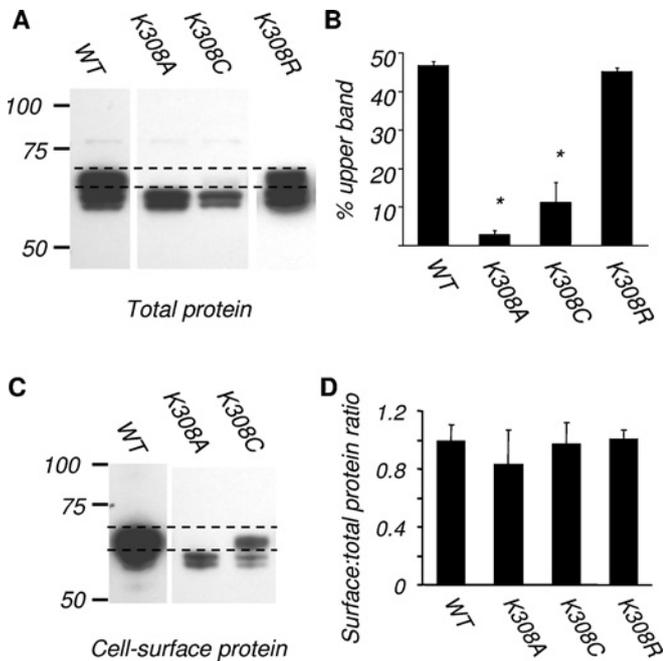


Figure 2 Effect of different substitutions at Lys³⁰⁸ on receptor glycosylation

(A) Western blot of total protein from HEK-293 cells expressing wild-type (WT), K308A, K308C or K308R mutant P2X₂ receptor. The upper band was absent in K308A, markedly reduced in K308C, but not different to wild-type in the case of K308R. (B) Summary of the data from three independent experiments. **P* < 0.01 compared with wild-type (WT). (C) Western blot of biotinylated cell-surface protein from HEK-293 cells expressing wild-type (WT), K308A or K308C mutant P2X₂ receptor. The upper band is absent in K308A, but enriched compared with total protein in K308C. (D) Summary of the cell-surface biotinylation data from 3–14 independent experiments. Values are presented as a ratio of cell-surface protein to total protein, normalized to wild-type (WT). No significant differences in cell-surface expression were observed for any of the mutants studied.

presents as three bands, reflecting different glycosylation states. Upon EndoH treatment, the upper band was lost, and a lower band at approx. 53 kDa (representing fully deglycosylated P2X₂) appeared. Additionally, a band of mass approx. 5 kDa lower than the uppermost band in the wild-type lane was present, representing EndoH-resistant protein. This result demonstrates that a proportion of rat P2X₂ expressed in HEK-293 cells acquires complex glycosylation. In contrast, the mutant N298S was fully cleaved by EndoH (lanes 5 and 6), demonstrating that, in this mutant, no complex glycosylation is present, and implying that Asn²⁹⁸ is likely to be the only site of complex glycosylation in the rat P2X₂ receptor. The loss of mass induced by EndoH treatment of wild-type P2X₂ (Figure 3B; lane 2) was slightly less than would be expected for the full cleavage of two core N-glycans (6 kDa). This might reflect anomalous running of this particular glycoform of P2X₂; it might be possible that full EndoH cleavage was not achieved in this sample or, alternatively, complex glycosylation might be added to Asn¹⁸² or Asn²³⁹, but only when Asn²⁹⁸ contains complex glycosylation. However, the important finding of this experiment was that K308A was fully cleaved by EndoH treatment, demonstrating a loss of complex glycosylation in this mutant, and implying that the loss of the upper band in this mutation was due to loss of complex glycosylation at Asn²⁹⁸.

Rat P2X₂ contains three N-glycan acceptor sequences, which may or may not be fully used when the protein is expressed in HEK-293 cells, and characterizing the glycosylation sites might help us to understand the Western blot profile. We expressed

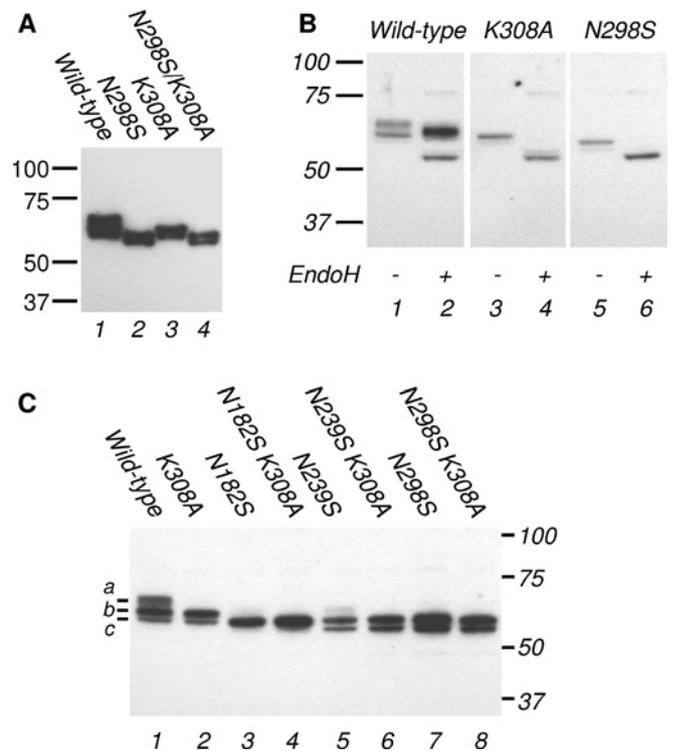


Figure 3 Difference in molecular mass is due to loss of complex glycosylation at Asn²⁹⁸

Western blot analysis of total protein from HEK-293 cells expressing wild-type or mutant rat P2X₂ receptor. (A) Removal of the N-glycan chain in the mutant N298S (lane 2) induced a reduction in molecular mass compared with wild-type (lane 1). Mutation of Lys³⁰⁸ to alanine (K308A; lane 3) also induced a reduction in mass; however, this was less than that induced by the mutant N298S. The double mutation N298S/K308A (lane 4) led to the same reduction in molecular mass as N298S, demonstrating that glycosylation at Asn²⁹⁸ was altered, but not completely abolished, in the single point mutant K308A. (B) K308A mutation results in loss of complex glycosylation from Asn²⁹⁸. Following EndoH treatment, partial cleavage of N-glycans was observed for wild-type P2X₂ (lane 2 compared with lane 1), indicating the presence of complex N-linked glycosylation. However, both point mutants N298S (lane 6 compared with lane 5) and K308A (lane 4 compared with lane 3) were fully cleaved, indicating that both mutants had lost complex glycosylation. Similar results were observed in three independent experiments. (C) Western blot analysis of total protein from HEK-293 cells transiently transfected with 1 μg of cDNA corresponding to wild-type or mutant P2X₂. Protein loading was purposefully low so as to enable visualization of each glycoform of P2X₂. Each of the three N-glycan acceptor sequences in rat P2X₂ (Asn¹⁸², Asn²³⁹ and Asn²⁹⁸) were mutated with or without K308A and the changes in molecular mass were analysed. The nature of the three distinct wild-type P2X₂ glycoforms (bands a, b and c) was deduced from the numbers of bands present in lanes corresponding to N182S (lane 3), N239S (lane 5) and N298S (lane 7) and the K308A double mutants (lanes 4, 6 and 8 respectively). The molecular mass in kDa is indicated on the left-hand side in (A) and (B) and on the right-hand side in (C).

serine mutants at each site (N182S, N239S and N298S) with or without the K308A mutation to determine how each site was utilized (Figure 3C). We intentionally loaded low amounts of protein (1 μg of protein per lane) to enable visualization of each glycoform. Mutants N182S (lane 3), N239S (lane 5) and N298S (lane 7) each displayed a reduction in overall mass. A faint upper band was also lost from both N182S and N239S when the mutation K308A was introduced (lanes 4 and 6), whereas N298S and N298S/K308A were unchanged (lanes 7 and 8). We argue that this faint upper band represents complex glycosylation at Asn²⁹⁸ which is lost in the mutant K308A. It was interesting to note that N182S presented as one major band with a faint upper band, N239S presented as two major bands with a faint upper band, and N298S presented as two major bands only.

This result tells us that the N-glycan site at Asn¹⁸² is only used in a proportion of P2X₂ receptors expressed in HEK-293 cells, whereas Asn²³⁹ and Asn²⁹⁸ are always glycosylated (since mutation of Asn¹⁸² leads to a reduction in the number of bands observed, what has been lost is a site that is only used in a proportion of receptors). The results of our EndoH studies (Figure 3B) showed that the complex glycosylation at Asn²⁹⁸ is only present in a proportion of receptors, and therefore we can assign each of the three bands observed for wild-type rat P2X₂ (Figure 3C, lane 1). Band (a) corresponds to fully glycosylated protein; band (b) is a mixture of core-glycosylated protein and protein which lacks glycosylation at Asn¹⁸², but contains complex glycosylation at Asn²⁹⁸; and band (c) corresponds to protein which is core-glycosylated at Asn²³⁹ and Asn²⁹⁸, but which lacks both glycosylation at Asn¹⁸² and complex glycosylation at Asn²⁹⁸.

The distance of glycosylation site Asn²⁹⁸ from the plasma membrane

We next examined the effect of moving the glycosylation acceptor sequence at Asn²⁹⁸ nearer to the plasma membrane. Second mutations in N298S were made so as to re-introduce the N-glycan acceptor sequence at positions 299, 300, 301 and 303 (Figure 4A). All sites were indicated to be good candidates for glycosylation using NetNGlyc (Expasy), and all constructs displayed channel function similar to wild-type P2X₂ receptors (results not shown). Analysis of total protein expression by Western blot (Figure 4B; summary histogram in Figure 4C) showed that moving the position of the N-glycan acceptor site to position 299 or 300 had no effect on full glycosylation. In contrast, moving the site to position 301 led to a reduction in full glycosylation of approx. 60%; this could either represent loss of complex glycosylation at position 301, or a reduction in the efficiency of core glycosylation at this residue. Moving the site to position 303 (N298S/T303N) gave rise to a band profile similar to that of the point mutant N298S (lane 6 compared with lane 2), implying that there was a complete absence of glycosylation at this residue. Previous work has demonstrated that the N-glycosylation acceptor sequence must be a minimum of 12–14 residues [or 30 Å (1 Å=0.1 nm)] away from the membrane to be efficiently core glycosylated [15]. Because core glycosylation was effectively abolished at position 303, we interpret that this position is 12–14 residues from the membrane, placing the region around Asp³¹⁵ at the membrane interface.

DISCUSSION

In the present study we have investigated the effects of introducing cysteine residues into the pre-TM2 region (Arg³⁰⁴-Ile³²⁸) of the rat P2X₂ receptor. This region is highly conserved in all mammalian P2X receptors [3], and we found that several residues were critical for efficient receptor function. In particular, cysteine substitution at Arg³⁰⁴, Leu³⁰⁶, Lys³⁰⁸ and Ile³¹² gave rise to non-functional channels, and substitution at Arg³¹³ led to a significant reduction in ATP potency. Our results correlate partially with those of Roberts and Evans [5], who showed that cysteine substitutions at Arg³⁰⁵, Lys³⁰⁹ and Phe³¹¹ in the human P2X₁ receptor (corresponding to Arg³⁰⁴, Lys³⁰⁸ and Tyr³¹⁰ in the rat P2X₂ sequence) significantly impaired receptor function. A study of the pre-TM2 region of rat P2X₄ [6] also showed that alanine substitutions at Tyr³¹⁵, Gly³¹⁶ and Arg³¹⁸ (Tyr³¹⁰, Gly³¹¹ and Arg³¹³ in the rat P2X₂ receptor) also inhibited receptor function. Taken together this work highlights the importance of the conserved pre-TM2 region centred on Arg³⁰⁴-Ile³¹⁴ for correct P2X receptor function.

A striking finding of the present study was that all but one (A309C) of the cysteine substitutions made in the region Arg³⁰⁴-

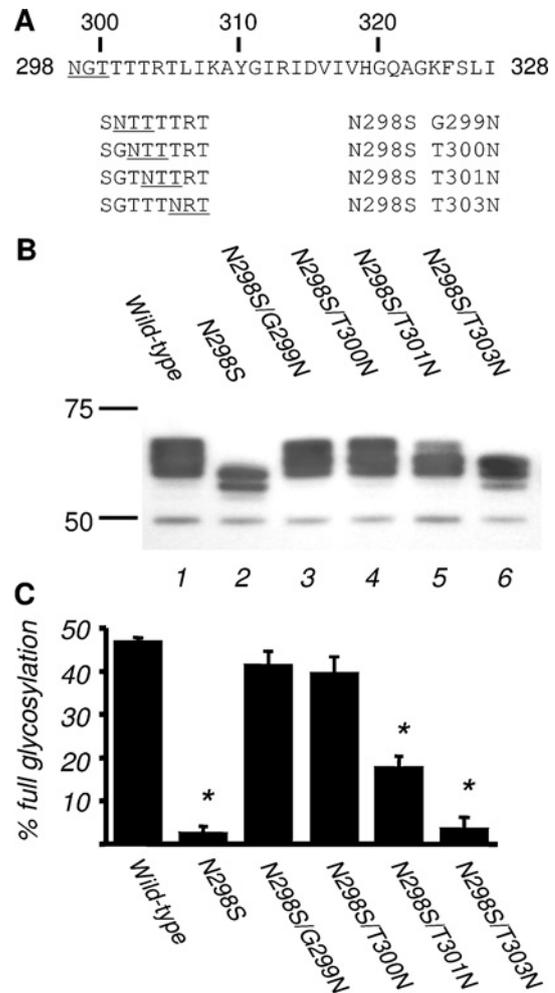


Figure 4 N-glycosylation is reduced by decreasing the number of residues between the glycosylation site (Asn²⁹⁸ or equivalent) and the membrane

(A) Point mutations in P2X₂ N298S used to move the position of the N-glycan acceptor sequence towards the C-terminus by one, two, three or five amino acids. (B) Western blot analysis of total protein from HEK-293 cells expressing wild-type or mutant P2X₂ receptors. Glycosylation was unaffected by moving the N-glycan acceptor position to 299 or 300, but was reduced at position 301 and was completely absent at position 303. The molecular mass is indicated on the left-hand side. (C) Histogram showing values from three independent experiments. **P* < 0.01 compared with wild-type.

Ile³¹⁴ led to substantial loss of complex glycosylation from Asn²⁹⁸. Asn²⁹⁸ itself is not required for either channel function or cell-surface presentation of the rat P2X₂ receptor [2], and we have demonstrated that the K308A mutation (which gives rise to loss of complex glycosylation from Asn²⁹⁸) is expressed at the cell surface in amounts similar to wild-type P2X₂ receptor; this implies that the loss of complex glycosylation does not indicate loss of cell-surface expression. The present study therefore suggests that complex glycosylation at Asn²⁹⁸ is acting as a reporter of a subtle change in the conformation of the receptor, and does not necessarily correlate with loss of ion-channel function (EC₅₀ values of the mutants T305C, I307C, Y310C, G311C and I314C were not significantly different to wild-type, whereas each mutant construct displayed significant reductions in levels of complex glycosylation).

It is clear from the present results that most of the mutations within the pre-TM2 region which affected channel function and/or

complex glycosylation were N-terminal to Asp³¹⁵; mutations within the C-terminal half of pre-TM2 had a much lesser effect. This suggests that the N-terminal half of pre-TM2 may be in an environment where tight structural contacts are more important for correct protein conformation, distinct from the environment occupied by the C-terminal half. We probed the environment of the pre-TM2 region by moving the position of the N-glycosylation acceptor sequence at Asn²⁹⁸ closer to the membrane. Our finding that Asn²⁹⁸ is approx. 15–17 residues distant from the membrane would indicate that the region encompassing Asp³¹⁵ is at the membrane interface. We do not believe that this region marks the start of TM2 in the P2X₂ receptor, as previous work demonstrates that the mutant V48C is able to form an intermolecular disulfide bond with the mutant I328C [17]. Val⁴⁸ is most probably positioned near the C-terminal end of TM1, and this would therefore position the N-terminal end of TM2 at or around Ile³²⁸. An intermolecular disulfide bond has also been demonstrated between the P2X₁ mutants K68C and F291C [18]; these residues correspond to Lys⁶⁹ and Arg²⁹¹ in the rat P2X₂ receptor. Val⁴⁸ and Lys⁶⁹ are 21 residues apart, whereas Arg²⁹¹ and Ile³²⁸ are 37 residues apart; in crude linear terms an additional 16 residues must be fitted in between Arg²⁹¹ and Ile³²⁸, and this is consistent with our observation that Asn²⁹⁸ is 15–17 residues from the membrane, with approx. 30 residues between Asn²⁹⁸ and Ile³²⁸. It is important to note that Newbolt et al [2] demonstrated an increase in N-glycosylation in the mutation K324N, using this result as evidence that Lys³²⁴ was on the cytoplasmic side of the membrane. However, in our studies the double mutant N298S/K324N appeared similar to N298S alone (results not shown), implying that glycosylation is not added to the asparagine residue at position 324 in this mutant.

Such a 'pairing-off' of the lengths of the polypeptide chain external to TM1 and external to TM2 would require approx. 14 'additional' amino acids between Asn²⁹⁸ and Ile³²⁸. For example, the region between Asp³¹⁵ and Ile³²⁸ may form a membrane-parallel structure (for example, an α -helix) which leads into TM2, thus holding the region Arg³⁰⁴–Ile³¹⁴ near the membrane. In an homology model of a fragment of the P2X₄ ectodomain based upon class II aminoacyl-tRNA synthetases, this portion of pre-TM2 is modelled as a helix extending from TM2 [6]. The residue Arg³¹⁸ (corresponding to Arg³¹³ in rat P2X₂) is placed towards the top of this helix, which is inconsistent with our observation that Arg³¹³ is very close to the membrane interface. One possible explanation, assuming that the model is reasonably accurate, is that, as this model did not include the TM2 region of P2X₄, there might be a bend between the helix and TM2, allowing placement of this helix parallel to the membrane. However, our finding that cysteine substitutions within the region between Asp³¹⁵ and Ile³²⁸ were well tolerated, both in terms of channel function and protein conformation as measured by the degree of complex N-glycosylation, suggests that this region of the molecule may not play a significant role in maintaining channel integrity, and is therefore unlikely to be in such a rigid conformation.

An intriguing possibility is that the region Asp³¹⁵–Ile³²⁸ forms a membrane-permeant loop, proposed by Brake et al. [9] when describing the cloning of rat P2X₂. This arrangement might explain why the mutants K324C and F325C displayed impaired complex glycosylation, because these residues would be placed at the membrane interface in a similar environment to the Arg³⁰⁴–Ile³¹⁴ region, which we have demonstrated to be important for correct protein conformation and function. To test this hypothesis, we aimed to probe the environment of the individual cysteine mutants in pre-TM2 using biotin-maleimide labelling. Unfortunately, and in contrast with P2X₁ [5], wild-type P2X₂ was efficiently labelled by maleimide (results not shown), meaning

that there was no suitable negative control for this experiment. Roberts and Evans [5] demonstrated that ATP-induced currents in the human P2X₁ receptor mutant G321C (corresponding to Gly³²⁰ in rat P2X₂) were markedly impaired upon labelling with thiol-reactive agents, and suggested that this residue might be involved in ion permeation. In agreement with this result, we also saw a similar effect for rat P2X₂ G320C labelled with methyl methanethiosulfonate (results not shown). Furthermore, Cao et al [7] have recently shown that Lys³⁰⁸ has a critical role in channel gating: this might be more readily interpreted if the first half of the pre-TM2 region was very near to the membrane, enabling it to have a direct influence on the permeation pathway [7]. Whether or not this 'loop region' is truly membrane-permeant might be tested by looking for intramolecular disulfide bond formation in double cysteine mutants around Gly³²⁰ and residues in TM1 or TM2.

In summary, we have exploited alterations in complex glycosylation at Asn²⁹⁸, induced by subtle changes in receptor conformation, to demonstrate that the region encompassing Arg³⁰⁴–Ile³¹⁴ in the rat P2X₂ receptor is important both for maintenance of correct receptor conformation and ion-channel function, and that it is likely to be positioned near to the plasma membrane. This finding should contribute to the evolving understanding of the structure–function relationship of the P2X receptors.

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REFERENCES

- 1 Khakh, B. S. and North, R. A. (2006) P2X receptors as cell-surface ATP sensors in health and disease. *Nature* **442**, 527–532
- 2 Newbolt, A., Stoop, R., Virginio, C., Surprenant, A., North, R. A., Buell, G. and Rassendren, F. (1998) Membrane topology of an ATP-gated ion channel (P2X receptor). *J. Biol. Chem.* **273**, 15177–15182
- 3 North, R. A. (2002) Molecular physiology of P2X receptors. *Physiol. Rev.* **82**, 1013–1067
- 4 Torres, G. E., Egan, T. M. and Voigt, M. M. (1998) Topological analysis of the ATP-gated ionotropic P2X₂ receptor subunit. *FEBS Lett.* **425**, 19–23
- 5 Roberts, J. A. and Evans, R. J. (2007) Cysteine substitution mutants give structural insight and identify ATP binding and activation sites at P2X receptors. *J. Neurosci.* **27**, 4072–4082
- 6 Yan, Z., Liang, Z., Obsil, T. and Stojilkovic, S. S. (2006) Participation of the Lys313–Ile333 sequence of the purinergic P2X₄ receptor in agonist binding and transduction of signals to the channel gate. *J. Biol. Chem.* **281**, 32649–32659
- 7 Cao, L., Young, M. T., Broomhead, H. E., Fountain, S. J. and North, R. A. (2007) Thr339-to-serine substitution in rat P2X₂ receptor second transmembrane domain causes constitutive opening and indicates a gating role for Lys308. *J. Neurosci.* **27**, 12916–12923
- 8 Wilkinson, W. J., Jiang, L. H., Surprenant, A. and North, R. A. (2006) Role of ectodomain lysines in the subunits of the heteromeric P2X_{2/3} receptor. *Mol. Pharmacol.* **70**, 1159–1163
- 9 Brake, A. J., Wagenbach, M. J. and Julius, D. (1994) New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**, 519–523
- 10 Hu, B., Senkler, C., Yang, A., Soto, F. and Liang, B. T. (2002) P2X₄ receptor is a glycosylated cardiac receptor mediating a positive inotropic response to ATP. *J. Biol. Chem.* **277**, 15752–15757
- 11 Jones, C. A., Vial, C., Sellers, L. A., Humphrey, P. P., Evans, R. J. and Chessell, I. P. (2004) Functional regulation of P2X₆ receptors by N-linked glycosylation: identification of a novel α β -methylene ATP-sensitive phenotype. *Mol. Pharmacol.* **65**, 979–985
- 12 Rettinger, J., Aschrafi, A. and Schmalzing, G. (2000) Roles of individual N-glycans for ATP potency and expression of the rat P2X₁ receptor. *J. Biol. Chem.* **275**, 33542–33547
- 13 Torres, G. E., Egan, T. M. and Voigt, M. M. (1998) N-Linked glycosylation is essential for the functional expression of the recombinant P2X₂ receptor. *Biochemistry* **37**, 14845–14851

- 14 Jiang, L. H., Rassendren, F., Surprenant, A. and North, R. A. (2000) Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor. *J. Biol. Chem.* **275**, 34190–34196
- 15 Nilsson, I. M. and von Heijne, G. (1993) Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.* **268**, 5798–5801
- 16 Reference deleted
- 17 Spelta, V., Jiang, L. H., Bailey, R. J., Surprenant, A. and North, R. A. (2003) Interaction between cysteines introduced into each transmembrane domain of the rat P2X₂ receptor. *Br. J. Pharmacol.* **138**, 131–136
- 18 Marquez-Klaka, B., Rettinger, J., Bhargava, Y., Eisele, T. and Nicke, A. (2007) Identification of an intersubunit cross-link between substituted cysteine residues located in the putative ATP binding site of the P2X₁ receptor. *J. Neurosci.* **27**, 1456–1466

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