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P2X$_5$ Subunit Assembly Requires Scaffolding by the Second Transmembrane Domain and a Conserved Aspartate*  

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Functional homomeric and heteromeric ATP-gated P2X receptor channels have been shown to display a characteristic trimeric architecture. Of the seven different isoforms (designated P2X$_1$-P2X$_7$), P2X$_5$ occurs in humans primarily as a non-functional variant lacking the C-terminal end of the ectodomain and the outer half of the second transmembrane domain. We show that this truncated variant, which results from the splice-skipping of exon 10, is prone to subunit aggregation because the residual transmembrane domain 2 is too short to insert into the membrane. Alleviation of the negative hydrophobic mismatch by the addition of a stretch of moderately hydrophobic residues enabled formation of a second membrane-spanning domain and strictly parallel homotrimerization. Systematic mutagenesis identified only one transmembrane domain 2 residue, Asp$^{355}$, which supported homotrimerization.  

Asp$^{355}$ seems to assist by simultaneously driving intramembrane helix interactions. Overall, these results indicate a complex interplay between topology, helix-helix interactions, and oligomerization to achieve a correctly folded structure.  

P2X receptors comprise a family of ligand-gated ion channels that are activated by extracellular ATP and mediate rapid signaling in a large variety of cells including neurons, smooth and cardiac muscles, epithelia, and lymphocytes (1). Seven subunit isoforms, designated P2X$_1$-P2X$_7$, have been identified and share a common topology with cytosolic N- and C-terminal domains and two membrane-spanning regions (transmembrane (TM) 1 and TM2)$^3$ connected by a large N-glycosylated ectodomain comprising 10 conserved cysteine residues. Like all other known ligand-gated ion channels, P2X receptors are oligomers of identical (homomeric) or homologous (heteromeric) subunits. Biochemical (2–4), biophysical (5), and functional studies (6) revealed that a trimeric architecture driven by non-covalent intermolecular interactions is a structural hallmark of functional homomeric and heteromeric P2X receptors. The three subunits are thought to arrange circularly, contributing one or both transmembrane domains to form a central cation-conducting pore.  

The assembly pathway leading to this trimeric structure is not well characterized. The question of which molecular determinants lead to the assembly of subunits into P2X receptors has so far been addressed by examining the ability of deletion mutants and chimeric constructs to associate with full-length P2X subunits in a co-immunoprecipitation assay (7). Neither the N- nor C-terminal cytoplasmic domain was found to be important for assembly. However, a deletion mutant terminating 25 amino acids before the start of the TM2 domain was unable to interact with either of the wild type subunits or with itself. This suggested that either TM2 or a region immediately upstream of TM2 carried a critical determinant of specific subunit-subunit interactions, although the possibility that the lack of co-assembly was the result of an inadequate secondary or tertiary structure was not ruled out (7).  

To further examine the role of both TM2 and the pre-TM2 region in P2X receptor assembly, we utilized the P2X$_3$ isoform, which occurs in humans predominantly as a natural deletion mutant (designated hP2X$_{3\Delta 328-349}$) and lacks much of the TM2 and pre-TM2 regions as a result of the splicing-out of exon 10 (8). The hP2X$_{3\Delta 328-349}$ cDNA does not encode functional channels, but a receptor chimera consisting of the N-terminal and C-terminal halves of the human and the rat P2X$_5$ subunits, respectively, has been expressed in Xenopus laevis oocytes and is functional (8). A full-length hP2X$_5$ subunit generated by incorporating a sequence corresponding to exon 10 (as identified by a TBLASTN search of human genomic DNA) was expressed well in human embryonic kidney 293 cells and provided robust ATP-dependent currents (9). In this report we sequentially generated a full-length hP2X$_5$ subunit cDNA by progressively and repeatedly inserting codons for 4–8 amino acids of the spliced-out exon 10 and then determined the assembly state of the resultant constructs by blue native PAGE. We also challenged homotrimerization of the full-length P2X$_5$ subunit by block replacements of amino acids throughout the pre-TM2 and TM2 regions to test for the presence of residues important for P2X$_5$ subunit-subunit recognition. Our results

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3 The abbreviations used are: TM, transmembrane; NTA, nitrilotriacetic acid; h- human; r-, rat; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild type.
Role of TM2 and Asp^{355} in P2X_5 Homotrimerization

are consistent with the view that TM2 supports homotrimerization in a relatively sequence-unspecific manner by tethering the C-terminal end of the large ectodomain to the membrane; thus, by defining the overall shape of the ectodomain, TM2 spatially constrains folding events in a productive way. We detected a specific role in assembly recognition for only one TM2 residue, Asp^{355}, which is fully conserved among P2X family members and may stabilize helix-helix interactions by satisfying hydrogen bonding groups in the membrane.

MATERIALS AND METHODS

P2X cDNA Constructs—To indicate the species origin, P2X subunit names are preceded by “h” or “r” for human or rat, respectively. Constructs from previous work include His-rP2X_1 (2) and His-rP2X_5 (4), which encode the rat P2X_1 and P2X_5 subunit with N-terminal His tags. The original rP2X_5 clone was kindly provided by Dr. Florentina Soto (10). A cDNA encoding the hP2X_5 subunit (GenBank™ accession number AAC51931 (8)) was isolated by PCR from a human brain cDNA library (Invitrogen) using sequence-specific primer pairs (forward, aaagaattcCATGGGCGAGCGGAGTCACA; reverse, aaagaattcGAGGCAAGTCCACGTGTCTGTTGCGCT; EcoRI cloning sites are underlined) subcloned into the oocyte expression vector pNKS2 (11) and entirely sequenced. Codon insertions and replacement mutations were introduced by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA).

P2X Receptor Expression in X. laevis Oocytes—Defolliculated X. laevis oocytes injected with capped cRNAs as described previously (12) were kept at 19°C in sterile frog Ringer’s solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, and 10 mM Hepes, pH 7.4) supplemented with 50 μg/ml gentamycin. Two days after cRNA injection, ATP responses were measured by two-electrode voltage-clamp recording at holding potential of −60 mV as described previously (13). Capping the N-terminal end of the rP2X_5 subunit with a His tag for one-step affinity purification had virtually no effect on the electrophysiological phenotype of the corresponding receptor in X. laevis oocytes (results not shown).

Radiolabeling and Affinity Purification of P2X_5 Receptor Constructs—For metabolic radiolabeling, cRNA-injected oocytes and non-injected controls were incubated overnight with L-[35S]methionine (>40 TBq/mm mol, Amersham Biosciences) at about 100 MBq/ml (0.4 MBq per oocyte) in ORi at 19°C and then chased for 24 h. His-tagged receptors were purified by Ni^{2+}-nitrolotriacetic acid (NTA)-agarose (Qiagen, Hilden, Germany) chromatography from digitonin (1.0%) extracts of oocytes as detailed previously (2, 14). Proteins were eluted from Ni^{2+}-NTA-agarose with non-denaturing buffer consisting of 250 mM imidazole/HCl, pH 7.4, and 1% digitonin (buffer 1) and then kept at 0°C until analysis, which was carried out on the same day as purification.

P2X_5 receptor constructs at the plasma membrane were selectively labeled by incubating oocytes 2 days after cRNA injection with 125I-labeled sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate, a membrane-impermeant derivative of the Bolton-Hunter reagent (15) exactly as described previously (2, 4). Proteins were purified from digitonin extracts of the oocytes by Ni^{2+}-NTA-agarose chromatography as detailed above.

Trypsin Resistance Assay—P2X_5 receptor constructs, purified by non-denaturing Ni^{2+}-affinity chromatography, were treated with 10–1000 μg/ml of bovine trypsin (Sigma-Aldrich) for 15 min on ice in buffer 1. Reactions were terminated by the addition of a 5-fold excess of soybean trypsin inhibitor. Digested samples were then analyzed by Tricine-SDS-PAGE with PhosphorImager scanning (see below).

Blue Native PAGE and SDS-PAGE—Blue native PAGE (16, 17) was carried out as described (2) using gradient gels (4–20% acrylamide). For partial dissociation of natively purified P2X receptors into lower-order complexes down to monomers, samples were treated for 1 h at 37°C with 0.1% SDS or 4–8 M urea as indicated. For SDS-PAGE or Tricine-SDS-PAGE (18), proteins were supplemented with the appropriate SDS-PAGE sample buffer containing 20 mM dithiothreitol followed by heating to 56°C for 15 min and electrophoresed in parallel with 1^4C-labeled molecular mass markers (Rainbow™, Amersham Biosciences). In some experiments samples were treated before SDS-PAGE for 2 h at 37°C with either endoglycosidase H or peptide:N-glycosidase F (PNGase F) (New England Biolabs, Frankfurt, Germany) in the presence of 1% Nonidet P-40 to decrease inactivation of PNGase F. Both SDS-PAGE gels and blue native PAGE gels were fixed, dried, exposed to a PhosphorImager screen, and scanned using a Storm 820 PhosphorImager (Amersham Biosciences). Individual bands were quantified with the ImageQuant software.

RESULTS

Functional Rat P2X_5 Receptors Possess a Homotrimeric Architecture—All P2X_5 constructs in this study were N-terminally-tagged with a hexahistidyl sequence to allow for protein purification by metal affinity chromatography after expression in X. laevis oocytes. Oocytes expressing WT rP2X_5 subunits or His-rP2X_5 subunits responded to ATP with a non-desensitizing inward current of similar shape and magnitude. A typical current trace in response to ATP from His-rP2X_5 subunit-expressing oocytes is shown in Fig. 1A. The shape of the current trace was notably changed when rP2X_5 receptors were repeatedly challenged with ATP. We observed the development of a slowly activating inward current, which was most likely mediated by secondary activation of oocyte endogenous channels since it was greatly reduced by flufenamic acid, an inhibitor of Ca^{2+}-activated Cl^- channels (19). Flufenamic acid did not affect the shape of the current component attributable to the ATP-activated rP2X_5 channel (results not shown).

We have previously demonstrated that the blue native PAGE technique has the capacity to correctly display the quaternary state of receptor channels and transporters. These include the known pentameric structure of Cys-loop receptors (2, 20–22) and the trimeric structure of glutamate transporters (23), which has also been visualized by x-ray crystallography (24). Like other oocyte-expressed P2X receptors except for hP2X_2 (4), the plasma membrane-bound rP2X_5 receptor migrated on the blue native PAGE gel as a distinct protein band (Fig. 1B, lane 1). Treatment with urea (lanes 2 and 3) resulted in a ladder-like pattern of three bands, each separated by the approximate mass of an rP2X_5 monomer. Using this ladder as a mass marker, a trimeric state can be assigned to the non-denatured rP2X_5 receptor.
Role of TM2 and Asp$^{355}$ in P2X$_5$ Homotrimerization

Consistent with previous studies (8), expression of hP2X$_5$$^{\Delta328-349}$ in Xenopus oocytes did not lead to the formation of an ATP-gated cation channel. Because trimerization is essential for P2X receptor function, we performed a blue native PAGE analysis to investigate the assembly status of this splice variant. Fig. 2B, lane 1, shows that the [${}^{35}$S]methionine-labeled His-hP2X$_5$$^{\Delta328-349}$ protein was poorly expressed and migrated on the blue native PAGE gel as an amorphous mass of protein (indicative of aggregates) rather than as a distinct band. In contrast, full-length His-rP2X$_5$ subunits migrated largely as homotrimers (Fig. 2B, lanes 10 and 11). We conclude that the lack of exon 10 results in a severe assembly defect that prevents the formation of functional homotrimeric hP2X$_5$ receptor channels at the level of subunit assembly in the endoplasmic reticulum.

Not All Exon 10-Encoded Residues Are Necessary for the Trimerization and Plasma Membrane Localization of hP2X$_5$ Subunits—To examine which of the 22 amino acids encoded by exon 10 were necessary for proper trimer formation and ion channel function, we progressively rebuilt exon 10 by inserting codons for 4–8 consecutive amino acids at a time, thus generating by stages a full-length hP2X$_5$ subunit cDNA (Fig. 2B). The inserted codons correspond to those previously identified by a TBLAST search of human genomic DNA corresponding to exon 10 (9). Expression of the various hP2X$_5$ constructs in X. laevis oocytes revealed that insertion of a minimum of 11 amino acids (residues 328–338; construct 3) from the total of 22 amino acids encoded by exon 10 was required to mediate significant homotrimer formation.

Trimerization was associated with increased protein stability and with plasma membrane localization of properly assembled trimers (Fig. 2D) but not with the formation of a functional receptor channel (Fig. 2C). ATP-gated inward currents could be elicited only from oocytes expressing the full-length His-hP2X$_5$ subunit (Fig. 2C, lane 8). The trimerization-defective constructs His-hP2X$_5$$^{\Delta328-349}$ and His-hP2X$_5$$^{\Delta335-349}$ were neither functional (Fig. 2C, lanes 1 and 2) nor exported to the cell surface (Fig. 2D, lane 2). Accordingly, once the span of inserted residues specified by exon 10 is of adequate length to allow for trimer formation, the resultant protein complex must have a sufficiently native-like conformation to be no longer recognized and retained by the endoplasmic reticulum quality control system, which retains malfolded proteins.

Insertion of Alanines, but Not Serines, Supports Trimerization as Efficiently as the Insertion of Genuine Exon 10 Residues—Insertion of seven alanine or serine residues instead of the genuine 335PTIINVG 341 sequence and examined whether inserted residues specified by exon 10 is of adequate length to allow for trimer formation, the resultant protein complex must have a sufficiently native-like conformation to be no longer recognized and retained by the endoplasmic reticulum quality control system, which retains malfolded proteins.

Plasma membrane-bound rP2X$_5$ subunits bear three N-glycans, which add $\approx$10 kDa of carbohydrate to the protein core (Fig. 1C). The mass calculated from the protein sequence is 52 kDa including the His tag, which is in good agreement with the experimental mass of $\approx$54 kDa of the fully deglycosylated protein. The different glycosylated states obtained by combined deglycosylation with endoglycosidase H and submaximally effective concentrations of peptide:N-glycosidase F indicate that all three predicted N-glycosylation sites (Asn$^{277}$NTT, Asn$^{107}$NST, 202NFS) were occupied, one with a high mannos-type glycan and two with complex-type glycans (Fig. 1D).

Human P2X$_5$ Subunits Lacking Exon 10 Are Trimerization-defective—Using gene-specific primer pairs, we PCR-amplified an hP2X$_5$ cDNA from a human brain cDNA library. The deduced amino acid sequence was identical to a previously reported hP2X$_5$ splice variant that lacked exon 10 (8). We refer to this splice variant as hP2X$_5$$^{\Delta328-349}$ to indicate that codons 328–349 of the full-length P2X$_5$ subunit are missing. The loca-
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Role of TM2 and Asp\textsuperscript{355} in p2X\textsubscript{5} Homotrimerization

Trimerization Parallels the Formation of a Second Membrane-spanning Segment—A possible explanation of these results is that alanine but not serine residues are capable of complementing the missing outer portion of TM2, thus allowing for membrane anchoring of the C-terminal end of the ectodomain. To address this possibility, we tried to exploit the fact that the hP2X\textsubscript{5} subunit carries an N-glycosylation sequence, \textsuperscript{430}NGS (numbering refers to the full-length hP2X\textsubscript{5} subunit), which is normally topologically inaccessible because it resides on the C-terminal endodomain (cf. topology model in Fig. 2A). This site may be expected to be N-glycosylated when TM2 is excluded from the lipid bilayer, thus forcing the C-terminal tail to remain aberrantly located in the endoplasmic reticulum lumen. Deglycosylation analysis, however, provided no evidence for hyperglycosylation of the hP2X\textsubscript{5} polypeptide, which could be shown to carry two N-glycans (results not shown); this is fully consistent with the occurrence of two N-glycosylation sites in the ectodomain.

\textsuperscript{430}NGS may remain unused as an acceptor site because of its short distance of only 15 residues to the C-terminal end (25). Indeed, the corresponding Gly\textsubscript{330}-Ile\textsubscript{362} mutant was more likely to aggregate than to homotrimerize (Fig. 4A, lane 3). Overall, alanine block mutations were well tolerated. Only one of the mutants, \textsuperscript{330}AA\textsubscript{331}-hP2X\textsubscript{5}, was more likely to aggregate than to homotrimerize (Fig. 4A, lane 3). The ATP-gated inward current amplitude was similarly reduced to \textasciitilde one-third of that mediated by the WT hP2X\textsubscript{5} receptor (Fig. 4B, lane 3). Cell surface radioiodination verified that the \textsuperscript{330}AA\textsubscript{331}-hP2X\textsubscript{5} mutant appeared exclusively as properly assembled trimers at the surface, albeit at lower levels than the WT hP2X\textsubscript{5} receptor (Fig. 4C). Strikingly, none of the pentalanine block mutations covering the entire span of TM2 affected either subunit homotrimerization (Fig. 4A) or export to the plasma membrane (Fig. 4, B and C). Robust inward cur-

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**FIGURE 3.** A hydrophobic mismatch prevents formation of a second transmembrane domain. A, PhosphorImager scan of an SDS-PAGE gel. Where indicated, the wild type residue Glu\textsuperscript{380} was replaced by Asn to generate a glycosylation reporter site (\textsuperscript{380}NDS) 65 residues from the C-terminal end. Construct numbers are the same as those used in Fig. 2. Triply glycosylated protein bands are indicated by an orange dot. B, topology schematic illustrating the position of natural glycosylation acceptor sites (green circles) and the engineered reporter site (orange circle). If two membrane-spanning domains are formed, only the two natural sites \textsuperscript{77}NTS and \textsuperscript{250}NFS are accessible for N-glycosylation. The third natural glycosylation site, \textsuperscript{430}NGS, is too close to the C-terminal end to be used if topologically accessible.

**FIGURE 2.** Contribution of exon 10-encoded residues to hP2X\textsubscript{5} subunit assembly and function. A, topology model of the full-length His-hP2X\textsubscript{5} construct, comprising all exon 10-specified residues plus the subsequent 12 residues of the inner half of TM2 (Fig. 4). Overall, alanine block mutations were well tolerated. Only one of the mutants, \textsuperscript{330}AA\textsubscript{331}-hP2X\textsubscript{5}, was more likely to aggregate than to homotrimerize (Fig. 4A, lane 3). The ATP-gated inward current amplitude was similarly reduced to \textasciitilde one-third of that mediated by the WT hP2X\textsubscript{5} receptor (Fig. 4B, lane 3). Cell surface radioiodination verified that the \textsuperscript{330}AA\textsubscript{331}-hP2X\textsubscript{5} mutant appeared exclusively as properly assembled trimers at the surface, albeit at lower levels than the WT hP2X\textsubscript{5} receptor (Fig. 4C). Strikingly, none of the pentalanine block mutations covering the entire span of TM2 affected either subunit homotrimerization (Fig. 4A) or export to the plasma membrane (Fig. 4, B and C). Robust inward cur-

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FIGURE 4. Assembly and electrophysiological functioning of alanine-scanning mutants of pre-TM2 and TM2 regions of the hP2X5 subunit. A, bottom panel, TM2 residues are highlighted in yellow, and WT residues mutated as a whole to alanines are indicated by gray boxes. Superscript numbers refer to amino acid positions of the full-length WT-hP2X5 subunits. Top panel, PhosphorImager scan of the indicated [35S]methionine-labeled and natively purified hP2X5 proteins resolved on a blue native PAGE gel. WT residues replaced as a whole by alanines are indicated. Where indicated, proteins were partially denatured by 0.1% SDS (1 h at 37 °C). Note that all hP2X5 alanine replacement mutants migrated as homotrimers.

B, oligomeric state versus electrophysiological function. Top panels and bottom panels show quantitative scans of the indicated lanes of the blue native PAGE gel of A and representative current traces elicited by 1 mM ATP (denoted by gray areas), respectively, from oocytes expressing the same hP2X5 mutants. C, both electrophysiologically functional and non-functional alanine replacement mutants appear as homotrimers at the cell surface. The indicated proteins were purified from surface-radioiodinated oocytes, resolved on a blue native PAGE gel, and visualized by PhosphorImager scanning.
rents in response to ATP were mediated by most of the mutants. Only penta-alanine substitutions in the inner third of TM2 (residues 352–361) abolished channel function, but these mutants also appeared as homotrimers at the cell surface (Fig. 4, B and C). To examine if trimer formation is also possible when bulkier residues than alanine are substituted, we performed a similar mutagenesis of TM2 with penta-leucine and penta-glutamine substitutions. Results obtained by leucine replacements of residues 337–361 resembled those obtained by equivalent alanine replacements except in one respect; the 352FFCDL mutant trimerized only weakly (Fig. 5A, lane 5). All the other leucine replacement mutants assembled into homotrimers as efficiently (lanes 2–4 and 6) as the parent hP2X5 subunit (lane 1). Even when 15 consecutive residues constituting the N-terminal two-thirds of TM2 (residues 337–351) were simultaneously replaced by leucines, homotrimers formed and appeared at the cell surface (Fig. 5B). However, inclusion of the 352FFCDL sequence to replace 20 consecutive residues by leucines virtually abolished trimer formation (results not shown). Responses to ATP could be elicited from mutants carrying leucine replacements at the membrane entry (residues 337–341) or exit sites of TM2 (residues 357–361), but penta-leucine replacements in the center of TM2 resulted in non-functional trimers (Fig. 5A, lanes 1–6, lower panels).

Penta-glutamine block replacements of the same TM2 residues resulted in all cases in severe assembly defects, as judged by the inability of these mutants to migrate as defined oligomers on blue native PAGE gels (Fig. 5A, lanes 9–13). Moreover, all the penta-glutamine replacement mutants were non-functional in terms of mediating ATP-gated inward currents (lanes 9–13, lower panels). Introducing the same glycosylation reporter sequence380NDS as described above (cf. Fig. 3B) into two of the glutamine mutants resulted in a 2–3-kDa increase in molecular mass (Fig. 5C). This mass shift indicates usage of Asn380 and, accordingly, exclusion of the penta-glutamine block-containing TM2 sequence from the membrane.

To address the impaired trimerization of the 352FFCDL leucine replacement mutant in more detail, single, double, and triple mutants were generated. All of the mutants that included the D355L mutation trimerized very weakly even if singly mutated (Fig. 6A, lanes 2–4). In contrast, leucine replacement of 355FFC alone, thus excluding Asp355, did not lower the trimerization efficiency (Fig. 6A, lane 5). Quantification of radioactivity showed that about 10-fold fewer trimers were formed from the D355L-containing mutants than from the WT hP2X5 subunit or from the 352FFCDL leucine replacement mutant. In addition, a subset of the D355L-containing mutants

FIGURE 5. Oligomeric state and electrophysiological functioning of penta-leucine and penta-glutamine scanning mutants of the TM2 region of the hP2X5 subunit. The initial residue of the TM2 scan, Ile337, is indicated by a red arrow in the bottom panel of Fig. 3A, A. PhosphorImager scan of [35S]methionine-labeled and natively purified hP2X5 proteins resolved on a blue native PAGE gel. WT residues replaced as a whole by leucines or glutamines are indicated. Bottom panels show representative current traces elicited by 1 mM ATP (denoted by gray areas) from oocytes expressing the same hP2X5 mutants. B, as a whole substitution of 15 consecutive TM2 residues (337–351) by leucines did not impair homotrimer formation. Total and Cell surface refer to the [35S]methionine-labeled total protein (both surface and intracellular) and the [35S]methionine-labeled surface form of the protein, respectively. Where indicated, proteins were partially denatured by 0.1% SDS (1 h at 37 °C). C, glutamine block mutations prevent formation of a second transmembrane domain. Glutamine mutants bearing an engineered glycosylation reporter site (Asn380, cf. model in Fig. 3B) instead of the WT Glu380 residue migrate as triply glycosylated protein bands (orange dot) in the SDS-PAGE gel, indicating luminal location of the C-terminal tail.

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Role of TM2 and Asp\textsuperscript{355} in P2X\textsubscript{5} Homotrimerization

FIGURE 6. Leucine substitution of Asp\textsuperscript{355} located in TM2, greatly impairs homotrimerization. A, PhosphorImager scan of \([\textsuperscript{35}S]\)methionine-labeled and natively purified hP2X\textsubscript{5} proteins resolved on a blue native PAGE gel. Residues substituted in the native \(352\textsuperscript{FFCD}\) sequence are underlined. B, quantitative scans of the indicated lanes of the PhosphorImager scan in A display the relative amounts of dimers, trimers, and aggregates (indicated by hatched areas).

FIGURE 7. Effect of hP2X\textsubscript{5} subunit truncation on homopolymerization. A, PhosphorImager scan of a blue native PAGE gel of \([\textsuperscript{35}S]\)methionine-labeled and natively purified hP2X\textsubscript{5} proteins truncated at the indicated C-terminal positions. Trimer formation required the entire TM2 and three C-terminal basic residues, \(363\textsuperscript{KKR}\), the positions of which are marked by red arrows in the topology model in Fig. 2A. B, PhosphorImager scan of a reducing SDS-PAGE gel of the same samples as in A.

The Initial Proteolytic Events Are the Most Critical for Unraveling Structural Features, since Proteins Fragments are More Easily Degraded as a Result of Their Enhanced Flexibility than the Intact Protein (27). Therefore, we quantified trypsin resistance of TM2 mutants by relating the amount of the two major products formed at the lowest trypsin concentration to the amount of the non-trypsin-treated intact protein (27). We, therefore, deduced from these results that particularly the ectodomain of the WT hP2X\textsubscript{5} receptor must be tightly folded to resist full digestion by a trypsin concentration sufficient to completely digest aggregated hP2X\textsubscript{5} polypeptides. This result further suggests that hP2X\textsubscript{5} polypeptides contained in aggregates exist in a substantially unfolded state.

Next, we systematically tested the trypsin susceptibility of five leucine replacement mutants that cover the entire TM2 region and that all assembled as trimers, although with distinct differences in efficiency. Three of the mutants covering Ser\textsuperscript{342}–Leu\textsuperscript{366} were non-functional. Under identical digestion conditions, identical patterns of tryptic fragments were produced from the WT hP2X\textsubscript{5} receptor (Fig. 8B, lanes 1–4) and from both the functional (lanes 5–8 and 21–24) and non-functional mutants (lanes 9–20).

The initial proteolytic events are the most critical for unraveling structural features, since proteins fragments are more easily degraded as a result of their enhanced flexibility than the intact protein (27). Therefore, we quantified trypsin resistance of TM2 mutants by relating the amount of the two major products formed at the lowest trypsin concentration to the amount of the non-trypsin-treated intact polypeptide. Non-functional leucine replacement mutants were as resistant to trypsin digestion as the WT hP2X\textsubscript{5} receptor or the functional mutants (Fig. 8C). The TM2 mutants that contained a D355L exchange existed mostly as aggregates and less as trimers (cf. Fig. 6). Accordingly, the intense non-trypsin-treated polypep-
Because trypsin treatment rapidly degrades hP2X5 mutants predominantly from aggregates and much less from trimers. The strong increase in trimer formation revealed an extreme trypsin susceptibility of aggregates that is consistent with the view that aggregates consist of essentially unfolded polypeptides.

In contrast, trimerization-competent constructs, as judged by blue native PAGE, exhibited substantial trypsin resistance in the native state and were all exported to the cell surface. The virtually identical degree of resistance to trypsin digestion of the WT hP2X5 receptor and all the TM2 mutants studied must be very similar if not identical.

DISCUSSION

P2X subunits trimerize cotranslationally in the endoplasmic reticulum and reside in intracellular compartments and in the plasma membrane as permanently assembled trimeric receptor complexes (2) (“obligomers” (28)). Here we investigated the contribution of residues encoded by exon 10 and of the subsequent residues in TM2 to hP2X5 subunit trimerization. We demonstrated that the hP2X5^{328–349} splice variant forms aggregates instead of trimers, indicating that a severe defect in assembly is one reason for the known inability of this polypeptide to form functional ATP-gated receptors (8, 9). Consistent with the view that the absence of a defined trimeric state on the blue native PAGE gel in general reflects a non-native conformation of a P2X receptor, aggregated hP2X5 proteins, including the hP2X5^{328–349} splice variant, were entirely retained in the endoplasmic reticulum by the quality control system. Moreover, structural probing by limited proteolysis revealed an extreme trypsin susceptibility of aggregates that is consistent with the view that aggregates consist of essentially unfolded polypeptides.

The important contribution of Asp^{355} to efficient trimerization and, accordingly, to overall trypsin resistance is also apparent from a leucine block mutant that explicitly retained Asp^{355} (Fig. 8B, lanes 25–28). The strong increase in trimer formation (cf. Fig. 6) was accompanied by an equivalent increase in trypsin resistance (Fig. 8C).

To further strengthen our view that trimers represent the trypsin-resistant entity, we plotted the amount of trimers (normalized to monomers) against trypsin resistance (Fig. 8D). Data points originating from functional and non-functional mutants are both located on one straight line. Linear regression analysis yielded a significant correlation ($r = 0.76$, $p < 0.0001$), indicating that trypsin resistance refers indeed to the fraction of hP2X5 polypeptides that are assembled as trimers. The close relationship suggests further that the overall folding of the WT hP2X5 receptor and all the TM2 mutants studied must be very similar if not identical.

FIGURE 8. Probing the structure of P2X5 receptor mutants by limited proteolysis. Proteolysis was carried out on the purified non-denatured hP2X5 receptor and its mutants at the indicated trypsin concentrations (in mg/ml) for 15 min at 4°C. A and B show PhosphorImager scans of reducing Tricine SDS-PAGE step gels (10%/H9004, 16% acrylamide). The positions of molecular mass markers (in kDa) are shown on the left of each panel. a, b, and c indicate major proteolytic fragments of 57, 51, and 17 kDa, respectively, of the 61-kDa intact hP2X5 subunit. a, trimerization-inefficient P2X5 receptor mutants are highly trypsin-sensitive. b, trimerization-competent P2X5 receptor mutants exhibit trypsin resistance comparable with the WT hP2X5 receptor. c, quantification of trypsin resistance. The trypsin resistance of the indicated P2X5 receptor constructs was expressed as the percentage of radioactivity incorporated into trimers as resolved on blue native PAGE gels to the radioactivity of the corresponding intact P2X5 polypeptide of the non-trypsin-treated sample. Data are from B except for the additional D355L-hP2X5 single mutant, which was analyzed on a separate gel not shown. Filled columns, D355L-containing mutants. D, relationship between trimerization efficiency and trypsin resistance. Trypsin resistance data are from C. The relative amount of trimers was calculated for equivalent samples by normalizing the radioactivity incorporated in the two bands that detectable in the corresponding intact P2X5 polypeptide of the non-trypsin-treated sample. Data are from D except for the additional D355L-hP2X5 single mutant, which was analyzed on a separate gel not shown. Data of the D355L-hP2X5 mutant not included in B are also shown. In C and D, error bars indicate S.D. values of duplicate determinations; where absent, single determinations are shown.

Tide band in the SDS-PAGE gel (Fig. 8B, lane 17) originates predominantly from aggregates and much less from trimers. Because trypsin treatment rapidly degrades hP2X5 mutants contained in aggregates, the weak bands that are left (lane 18) must originate from the small fraction of trimers, which apparently resisted degradation.

The important contribution of Asp^{355} to efficient trimerization and, accordingly, to overall trypsin resistance is also apparent from a leucine block mutant that explicitly retained Asp^{355} (Fig. 8B, lanes 25–28). The strong increase in trimer formation (cf. Fig. 6) was accompanied by an equivalent increase in trypsin resistance (Fig. 8C).
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from the total of 22 exon 10-encoded residues. These polypeptides appeared at the cell surface exclusively as fully assembled trimers but were unable to mediate an ATP-gated current.

TM2 Plays an Indispensable Scaffolding Role in P2X$_5$ Subunit Homotrimerization—An involvement of TM2 in the assembly of P2X receptors has previously been inferred from co-immunoprecipitation assays (7). A P2X$_5$ deletion mutant terminating 25 amino acids N-terminal to TM2 did not co-precipitate with other subunits or with itself, suggesting that the missing region of the protein participates in assembly. In contrast, a truncation located C-terminal to TM2 at position Tyr$^{362}$ of the P2X$_2$ subunit (corresponding to Tyr$^{468}$ of P2X$_5$) enabled co-isolation. These and additional data obtained by studies of chimeras led to the conclusion that TM2 is a critical determinant for productive P2X subunit assembly (7).

Our present data offer a substantially extended perspective on this issue by showing that TM2 contributes to assembly first of all merely as a hydrophobic membrane anchor rather than by providing specific subunit recognition information. This conclusion is supported by the following data. First, blocks of five consecutive amino acid residues could be replaced by alanines throughout the entire pre-TM2 and TM2 regions without impairing homotrimer formation. Second, the absence of the outer half of TM2 residues did not prevent efficient homomeric assembly if a sufficiently long alanine stretch was present in the pre-TM2 region in order to alleviate the hydrophobic mismatch, thus enabling formation of a second membrane-spanning segment. Third, all sequence manipulations that prevented the formation of a second membrane-spanning segment also led to subunit aggregation and accelerated protein degradation. This was true for the exon 10-lacking hP2X$_5$$^{$\Delta$128$^{349}$} variant, for a series of pre-TM2 and TM2 mutants with original residues replaced by blocks of polar residues (serines or glutamates), and for C-terminal truncation mutants, which included all pre-TM2 and TM2 residues but lacked a positively charged cap, $^{$363$}$KKR$^{$365$}$, at their free C-terminal end. In summary, we note a close relationship between correct lipid bilayer insertion of a hydrophobic domain in N$_{exo}$-C$_{cys}$ Orientation and proper P2X$_5$ subunit trimerization.

P2X$_5$ Subunit Assembly Is Not Driven by Specific Packing Interactions of TM2 Residues—Major driving forces for association of helices in membrane proteins are (i) van der Waals interactions of TM2 residues are (i) van der Waals interactions of TM2 residues or the abundant GXXXG motif of bitopic proteins (30) and (ii) hydrogen bonding between polar residues. TM domains of P2X receptors do not contain a leucine zipper-like motif, but small residues (Gly, Ala, Ser) are over-represented in the outer half of TM2 of all P2X subunits and may allow for tight van der Waals packing between neighboring helices. The outer TM2 half of the hP2X$_5$ subunit contains as many as 8 small residues (4 Gly, 3 Ala, 1 Ser) over a span of 11 residues. Of these, the sequence $^{340}$VGSGVA$^{345}$ (GXXXA) is reminiscent of the common pattern defined by the GXXXG-containing assembly motif of glycoporin A; small residues are next to the larger side chains of the $\beta$-branched amino acids valine and isoleucine (31), enabling strong (SDS-resistant) self-association by a ridge-into-groove mode of packing (32). However, in contrast to the exquisite specificity of TM-mediated dimerization of glycoporin A, which is destabilized significantly by small interfacial changes (33–35), homotrimerization of hP2X$_5$ subunits was entirely insensitive to such mutations. Even when 15 consecutive TM2 residues, Ile$^{377}$–Ala$^{391}$, including all eight small residues, were mutated to leucines, homotrimerization was unimpaired. Pure polyleucine-based coiled-coil helices mediate only relatively weak associations between TM domains in the absence of a polar residue (36). Thus, despite the fact that small residues occur abundantly in helix interfaces of membrane proteins and mediate tight helix-helix interactions (37), small residues in TM2 are not necessary for efficient P2X$_5$ subunit trimerization.

Intramembrane Hydrogen Bonding May Play a Role in Initiation of Homotrimerization—hP2X$_5$ homotrimer formation was remarkably tolerant to TM2 substitutions by small (alanine) and large (leucine) residues except in one position, Asp$^{355}$, which represents the only charged TM2 residue. Recent evidence indicates that single strongly polar residues (Asp, Asn, Glu, Gln) in a transmembrane helix can be sufficient to drive self-association of transmembrane helices through the formation of side-chain-side-chain interhelical hydrogen bonds in the absence of detailed packing (36, 38–40). The importance of Asp$^{355}$ and, accordingly, of hydrogen bonding as a hotspot in P2X$_5$ subunit assembly is limited insofar as that (i) the D355L mutation reduced, but did not abolish hP2X$_5$ subunit homotrimerization, and (ii) substitution of Asp$^{355}$ by alanine did not impair homotrimerization. To account for these observations, we propose that Asp$^{355}$, by satisfying hydrogen bonding groups in the membrane, functions at an early stage of the assembly process (41) to drive intersubunit interactions to form higher order oligomers. A locally increased effective subunit concentration may provide an environment that promotes interactions between specific assembly interfaces, which are evidently located in the ectodomain. This model can explain the observation that Asp$^{355}$ increases the yield of homotrimers during biosynthesis but is not essential for the continued structural maintenance of the fully assembled homotrimer. Admittedly, the undisturbed assembly of the D355A mutant cannot be directly integrated in this model, and additional experiments are needed to assess the extent to which intramembrane hydrogen bonding contributes to hP2X$_5$ subunit homotrimerization.

Negative Hydrophobic Mismatch Results in hP2X$_5$ Subunit Aggregation—The inability of the splice variant hP2X$_5$$^{$\Delta$128$^{349}$} to form a second membrane-spanning domain can be directly attributed to the absence of the exon 10-encoded pre-TM2 region and to the absence of the outer half of TM2. Using a helix translation of 1.5 Å per residue, the calculated hydrophobic length of the existing inner half of TM2, $^{350}$GAFFCDLV-LIYLI$^{362}$, is around 20 Å, which is clearly too short to span the 30-Å hydrophobic core of the membrane. Studies with synthetic hydrophobic peptides consisting of a polyleucine or polyleucine/alanine hydrophobic stretch have demonstrated that helices too short to span the phospholipid membrane (referred to as negative hydrophobic mismatch) are excluded from the lipid bilayer. Because of the general insolvability of hydrophobic segments in the aqueous phase, peptides with a negative hydrophobic mismatch have a strong tendency to aggregate (42, 43). Proteins such as bacteriorhodopsin (44,
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P2X$_5$ Homotrimerization Is Further Supported by Residues That Anchor TM2 in the Cytoplasmic Membrane-Water Interface—Truncation of the full-length P2X$_5$ subunit C-terminal to the entire TM2 region also resulted in aggregation as long as the C-terminal tail of the hydrophobic segment was not flanked by the naturally occurring residues $^{363}$KKR$^{365}$. Positive charges in the region flanking a transmembrane segment are known to represent topological determinants that tend to have cytoplasmic localizations (50). Moreover, flanking lysine residues are thought to electrostatically anchor the position of the edge of a TM helix in the acidic phospholipid head-group region of the membrane-water interface, thus securing a stable transmembrane orientation of the helix more or less perpendicular to the bilayer surface (51). The importance of flanking lysine residues as anchors for a stable transmembrane orientation is particularly obvious in experiments with synthetically designed transmembrane peptides, which also show that basic flanking residues disfavor extensive peptide aggregation (52).

Interestingly, sufficiently long hydrophobic peptides that are flanked by lysine residues only at the C-terminal end have the peculiar feature of spontaneously adopting a transmembrane orientation (53). Overall, several mechanisms may account for the crucial role of the $^{363}$KKR$^{365}$ sequence in the assembly of C-terminal-truncated P2X$_5$ subunits. However, within the context of the full-length P2X$_5$ subunit, the $^{363}$KKR$^{365}$ sequence appears of limited importance for assembly.4

TM2 Side-chain Chemistry and Channel Function—A detailed characterization of the electrophysiological properties of the various P2X$_5$ mutants was beyond the scope of the present study, and thus, only maximum current amplitudes elicited by a supersaturating concentration of ATP (1 mM) were recorded. hP2X$_5$ channel function was not abolished by pentalanine substitutions in the outer half of TM2 (as summarized in the model in Fig. 9) even when the sole non-hydrophobic residue of this region, Ser$^{342}$, was also replaced by alanine. Most of this tolerant region bears small residues including glycine, so their mutation to alanine (Gly$^{344}$–Ala$^{351}$) caused only moderate changes in side-chain volume. Block substitutions by pentaleucines were less well accommodated and ablated function except at the outer and inner end of TM2 ($^{337}$INVG$^{341}$ and $^{357}$VLIYL$^{361}$), where bulky hydrophobic residues (Ile, Leu, Val) already prevailed in the WT sequence. Apparently, small side chains are important in the center of TM2. Of these, Gly$^{342}$ or Gly$^{344}$ has been suggested to constitute a part of the channel gate (54), similar to the glycine hinge of K$^+$ channels (55). Smooth helical faces that result from having small interfacial residues may provide the flexibility needed for the conformational change in the center of TM2 (56) or, alternatively, may ensure a wide pathway through the channel for ions to reach the selectivity filter.

The sole stretch of TM2 residues that could not be block-substituted to alamines or to leucines without abolishing function was $^{352}$FFCDL$^{356}$. Function was also abolished when Asp$^{355}$ was singly mutated to alanine or leucine,4 indicating that Asp$^{355}$ has a dual role, being both structurally and functionally important. The aspartate corresponding to Asp$^{355}$ of the P2X$_5$ subunit is conserved among all seven P2X isoforms and has been shown by cysteine-accessibility scanning to be located on the intracellular side of the channel gate of the P2X$_2$ receptor (57). Our data fit well with those of a tryptophan-scanning study on the P2X$_4$ receptor that showed that non-functional mutations clustered in the inner TM2 region (58). To account for the dual structural and functional roles of Asp$^{355}$ that emerge from our study, we propose that Asp$^{355}$ serves to control and stabilize the positioning of inner TM2

4 W. Duckwitz, R. Hausmann, and G. Schmalzing, unpublished results.
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residues during the conformational transitions that accompany channel gating.

**TM2 and Asp\textsuperscript{355} Function as Scaffolds in P2X\textsubscript{5} Subunit Assembly**—Our data are most compatible with the view that TM2 makes two essential contributions to hP2X\textsubscript{5} subunit oligomerization. First, the entire TM2 domain exerts a role that seems to have its basis in constraining the spatial mobility of the polypeptide chain, when the C-terminal end of the ectodomain is tethered to the membrane to form a loop-like structure. As long as certain threshold values for the hydrophobicity and length of this domain meet the requirements for membrane insertion, homotrimerization occurs. Second, TM2 formation results in an intramembrane localization of Asp\textsuperscript{355}, which may initiate homooligomerization by hydrogen bond-driven transmembrane helix-helix associations. We suggest that TM2 formation and the intramembrane presentation of Asp\textsuperscript{355} constitute scaffolds, which drive productive assembly by assisting in the creation and correct positioning of specific recognition surfaces that are evidently located in the ectodomain.

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P2X₅ Subunit Assembly Requires Scaffolding by the Second Transmembrane Domain and a Conserved Aspartate

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