

Mutation of Putative N-Linked Glycosylation Sites on the Human Nucleotide Receptor P2X₇ Reveals a Key Residue Important for Receptor Function[†]

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ABSTRACT: The nucleotide receptor P2X₇ is an immunomodulatory cation channel and a potential therapeutic target. P2X₇ is expressed in immune cells such as monocytes and macrophages and is activated by extracellular ATP following tissue injury or infection. Ligand binding to P2X₇ can stimulate ERK1/2, the transcription factor CREB, enzymes linked to the production of reactive oxygen species and interleukin-1 isoforms, and the formation of a nonspecific pore. However, little is known about the biochemistry of P2X₇, including whether the receptor is N-linked glycosylated and if this modification affects receptor function. Here we provide evidence that P2X₇ is sensitive to the glycosidases EndoH and PNGase F and that the human receptor appears glycosylated at N187, N202, N213, N241, and N284. Mutation of N187 results in weakened P2X₇ agonist-induced phosphorylation of ERK1/2, CREB, and p90 ribosomal S6 kinase, as well as a decreased level of pore formation. In further support of a role for glycosylation in receptor function, treatment of RAW 264.7 macrophages with the N-linked glycosylation synthesis inhibitor tunicamycin attenuates P2X₇ agonist-induced, but not phorbol ester-induced, ERK1/2 phosphorylation. Interestingly, residue N187 belongs to an N-linked glycosylation consensus sequence found in six of the seven P2X family members, suggesting this site is fundamentally important to P2X receptor function. To address the mechanism whereby N187 mutation attenuates receptor activity, we developed a live cell proteinase K digestion assay that demonstrated altered cell surface expression of P2X₇ N187A. This is the first report to map human P2X₇ glycosylation sites and reveal residue N187 is critical for receptor trafficking and function.

The nucleotide ATP can act as an important extracellular signaling molecule that regulates multiple processes, including neurotransmission and immune response mediator production (1, 2). ATP is released in millimolar concentrations from cells following infection or tissue injury and can stimulate cells in the microenvironment by binding to the P2 nucleotide receptors (3). The P2 nucleotide receptors have been divided into two major subfamilies: the G protein-coupled P2Y receptors and the ionotropic P2X cation channels (4).

The cation channel P2X₇ is considered an important component of the inflammatory response (5). Activation of P2X₇ by extracellular nucleotides leads to the processing of interleukin-1 β and the production of reactive oxygen species through the NADPH oxidase complex (6, 7). The P2X₇ receptor stimulates a number of downstream targets, including mitogen-activated protein kinases (MAPK)¹ and several transcription factors, including cyclic-AMP response element-binding protein (CREB) and activating transcription factor 1 (ATF1) (7–11). When the ligand

binds, P2X₇ mediates Ca²⁺ and Na⁺ influx and K⁺ efflux. Prolonged stimulation of P2X₇ can also promote the formation of a nonspecific pore, allowing molecules of up to 900 Da to enter the cell (4).

It has been proposed that P2X₇ is an attractive therapeutic target for inflammatory diseases (5). Animal studies have shown that pharmacological targeting of P2X₇ may be used to treat certain types of arthritis (12, 13), and a recent clinical study has correlated P2X₇ activity with virus-induced loss of asthma control (14).

Although numerous P2X₇ studies have focused on ATP-induced cell signaling, relatively few reports have examined the biochemical properties of the receptor. One aspect of P2X₇ biology that is poorly understood involves the glycosylation status of the receptor and how glycosylation contributes to receptor function. Such an analysis is important given that glycosylation of plasma membrane-bound proteins is often critical for numerous processes, including protein folding, cell adhesion, and pathogen recognition of host cells (15, 16).

In this study, we report that P2X₇ is N-linked glycosylated on five residues and this post-translational modification is important for P2X₇ agonist-stimulated signaling and pore formation. We present the first experimental evidence that human P2X₇ is glycosylated at residues 187, 202, 213, 241, and 284 and that mutation of the conserved amino acid 187 results in decreased nucleotide-induced signaling. In addition, we propose P2X₇ N187A exhibits attenuated activity because its expression on the plasma membrane is altered. A recent report has shown that two P2X₇ naturally occurring single nucleotide polymorphisms (SNPs) located near residue N187, E186K and L191P, exhibit decreased

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¹Abbreviations: ERK1/2, extracellular signal-regulated kinases 1 and 2; CREB, cyclic-AMP response element-binding protein; p90RSK, p90 ribosomal S6 kinase; MAPK, mitogen-activated protein kinase; ATF, activating transcription factor; BzATP, 2'-(3')-O-(4-benzoylbenzoyl)-ATP; PVDF, polyvinylidene fluoride; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; TM, transmembrane.

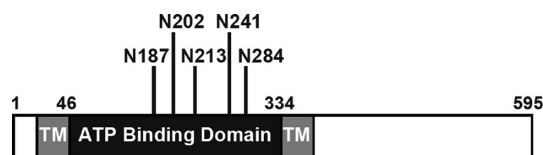


FIGURE 1: Graphical representation of the major P2X₇ domains and its putative N-linked glycosylation sites. The nucleotide receptor P2X₇ has two predicted transmembrane domains (TM) and an extracellular ATP binding domain. The extracellular portion of P2X₇ contains putative N-linked glycosylation sites at amino acids 187, 202, 213, 241, and 284. PredictProtein was used to determine where the putative transmembrane domains and N-linked glycosylation sites are located (31).

channel and pore activities (17). Those findings and this current report demonstrate that the extracellular region of P2X₇ surrounding the N187 glycosylation site is critical for receptor function.

MATERIALS AND METHODS

Reagents. The potent P2X₇ agonist, 2'-(3')-O-(4-benzoylbenzoyl)-ATP (BzATP), and tunicamycin were obtained from Sigma (St. Louis, MO). EndoH and PNGase F were purchased from New England Biolabs (Ipswich, MA), and proteinase K was purchased from Promega (Madison, WI). The anti-c-Myc antibody (catalog no. sc-40), the anti-P2X₇ antibody (catalog no. sc-25698) used for immunofluorescence and the immunoblot in Figure 7, and the anti-EGFR antibody (catalog no. sc-03) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PDI antibody (catalog no. SPA-891) was obtained from Stressgen (Ann Arbor, MI). The anti-ERK1/2 antibody (catalog no. 06-182) was purchased from Millipore (Billerica, MA); the anti-pERK1/2 antibody (catalog no. 44680G) was purchased from Invitrogen (Carlsbad, CA), and the anti-P2X₇ antibody (catalog no. 550694) used for the immunoblots in Figures 3, 6, and 10 was purchased from BD Biosciences (San Jose, CA). The anti-CREB (catalog no. 9197), anti-pCREB (catalog no. 9191), anti-p90RSK (catalog no. 9344), and anti- β -tubulin (catalog no. 2146) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture, Harvesting, and Protein Assays. Human HEK293 and monkey COS7 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum (Mediatech, Herndon, VA), 1% L-glutamine, and 100 units/mL penicillin/streptomycin, and the murine macrophage RAW 264.7 and RAW 264.7 SF cells were cultured in RPMI-1640 medium supplemented with 5% cosmic calf serum, 2 mM sodium pyruvate, and 1% L-glutamine. The cells were incubated at 37 °C under 5% CO₂. RAW 264.7 SF is a mutant cell line that contains a Ser to Phe mutation at amino acid 342 in the extracellular domain of P2X₇, rendering the receptor nonfunctional and the cell line a useful negative control (8, 18, 19). We generally prepared cell lysates by harvesting the cells in 2× sample buffer (20 mM Tris, 2 mM EDTA, 2 mM DTT, 1 mM Na₃VO₄, 2% SDS, and 20% glycerol) and boiling and sonicating the samples. Total protein concentrations of lysates were quantified using a BCA protein assay kit obtained from Thermo Scientific (Waltham, MA).

P2X₇ Constructs. Full-length human P2X₇ (GenBank accession number AAH11913) was subcloned into pcDNA3 and pCMV-Myc. Subcloning P2X₇ into pCMV-Myc yielded an N-terminally tagged receptor. The P2X₇ point mutants used in these studies were generated by site-directed mutagenesis using

the following primers and their reverse complements: P2X₇ N187A (5'-CAGTGCCGAAGCCTTCACTGTGCTC-3'), P2X₇ N187H (5'-CAGTGCCGAACACTTCACTGTGCTC-3'), P2X₇ N187Q (5'-CAGTGCCGAACAGTTCACTGTGCTC-3'), P2X₇ N202A (5'-CCGGCCACGCCTACACCACG-3'), P2X₇ N213A (5'-CCAGGTTTAGCCATCACTTGTACC-3'), P2X₇ N241A (5'-GAACAGGCGATGCTTTTTCAG-3'), and P2X₇ N284A (5'-CAAGACCACCGCCGTGTCCTTGTAC-3').

Glycosidase Assays. Cell lysates were treated with EndoH or PNGase F according to the manufacturer's instructions. Briefly, ~30 μ g of cell extract protein was denatured and then treated with 250 units of glycosidase at 37 °C for 1 h.

Immunofluorescent Staining. RAW 264.7 macrophages and COS7 cells were cultured on glass coverslips, fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with the indicated antibodies. The cells were then stained with the following secondary antibodies from Molecular Probes (Eugene, OR): Alexa Fluor 488 donkey anti-rabbit (used with anti-P2X₇ in Figures 4A and 9), Alexa Fluor 488 donkey anti-mouse (used with anti-PDI), and Alexa Fluor 594 donkey anti-rabbit (used with anti-P2X₇ in Figure 4C). The samples were imaged with either a Zeiss Axioplan 2 microscope (Figures 4A and 9) or a Bio-Rad Radiance 2100 MP Rainbow confocal microscope (Figure 4C). The ImageJ software colocalization plug-in was used to generate the colocalized image in Figure 4C.

YO-PRO-1 Dye Uptake Assay. COS7 cells were cultured on glass coverslips and were transfected with the indicated constructs using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche). The COS7 and RAW 264.7 cells were stimulated at room temperature in potassium glutamate buffer [130 mM potassium glutamate, 20 mM HEPES-KOH (pH 7.4), 5 mM KCl, 0.1% BSA, and 10 mM glucose] with 250 μ M BzATP or HEPES buffer control for 10 or 20 min in the presence of 1 μ M YO-PRO-1 (Molecular Probes). Potassium glutamate buffer is known to facilitate robust YO-PRO-1 uptake in response to BzATP, as previously reported (20, 21). The cells were then treated with 10 mM MgCl₂ to close the pore, fixed with 4% paraformaldehyde, and imaged with either a Zeiss Axioplan 2 microscope or an Olympus IX fluorescence inverted microscope (20, 21).

ERK1/2, CREB, and p90RSK Phosphorylation/Activation Assays. HEK293 cells were transfected with pcDNA3, P2X₇/pcDNA3, or the P2X₇ Asn to Ala mutants using FuGENE 6 transfection reagent, and G418 sulfate-resistant populations were selected. For the transient transfection experiments, HEK293 cells were transfected with pCMV-Myc, P2X₇/pCMV-Myc, P2X₇ N187H/pCMV-Myc, P2X₇ N187Q/pCMV-Myc, or the Myc-tagged P2X₇ Asn to Ala mutants. The cells were serum starved for 1–4 h, treated with 250 μ M BzATP for 5 or 10 min, and harvested, and cell extracts were immunoblotted as detailed below.

Immunoblotting. Proteins from cell lysates (~10–30 μ g) were separated by electrophoresis at 15–20 mA on 10% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked in 5% nonfat dry milk. The membranes were probed with the indicated primary antibodies, incubated with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and visualized by enhanced chemiluminescence.

Proteinase K Digestion Assays. HEK293 cells stably expressing wild-type or mutant P2X₇ were washed off the plate with media and pelleted. The cells were suspended in serum-free DMEM and treated with 333 μ g/mL proteinase K or buffer

P2X1	1	-----MARRFQEELAAFLHEYDTPRMVLVRNKKVGVIFRLIQLVVLVYVIGWVFLYEKGYS--GLISSVSV
P2X2	1	MAAAQPKYPAGATARRLARGCWSAFLWYETFKVIVVRNRRLGVLYRAVQLLILLYFVWVVFVQKSYQESGTPESSIT
P2X3	1	-----MNCISDEFTYETTKSVVVKSWTIGIINRVVQLLIIISYEVGWVFLHEKAYQVFDTAIESSVVT
P2X4	1	-----MAGCCAALAAFLHEYDTPRIVLIRSRKVGIMNRVAVQLLIIAYVIGWVFLWEKGYQETD-SVSSSVIT
P2X5	1	-----MGQAGCKGLCLSLFLYKTEKVIARNNKVGLLYRLIQASILAYLVVWVFLIKKGYQDVDTSLQSAVIT
P2X6	1	-----MGSPGAT-TGWGLLYKTEKVMTRNWFVGALQRLIQFGIVVYVVGWAILAKKGYQERDLEPQPSIIT
P2X7	1	-----MPACCS--CSDVFEYETNKVTRIQSMNYGTIKWFFHVIIIFYVC-FALVSDKLYQRKE-PVSSSVHT
P2X1	68	KIKGIATV-----QLPGLGPGVWDVADYVSPQGINSEFVMTNFIIVTPKQIQGYCPEHPG--GICKEDSGCTFGKAK
P2X2	81	KVKGITTS-----EHKVDVDEEYVKEPEGSSVFSLITRVEATHSQOGICPESIRVHNATCSIAICVAGELD
P2X3	63	KVKGSGLY-----ANRVVDVSDYVPEPGGTSVFVITTKMIVTENOMOGECPESE--EKYRCVSDSQCGP--ER
P2X4	67	KVKGIATV-----NTSKLGFRLWDVADYVPEQGENSIFVMTNFIIT-NQQLCPEHPDAT-TVCSIASCTAGSAG
P2X5	69	KVKGIATV-----NTSLGCRWDVADYVPEQGENVFVMTNFIIVTPNQKQVCEPENEIGIPDGACSKDSICHAGEAV
P2X6	68	KIKGVSVT-----QIKELGNRLWDVADYVPEQGENVFVMTNFIIVTPAQVQGCPEHPSPVLANCWDELCPBEGG
P2X7	64	KVKGIATVKEEIVENGVKKLHVSVEDIADYTFELQG-NSFFVMTNFIKTEGQEQRLCPEHPTRR-TLCSDRGCKKGMMD
P2X1	139	RKAGGIRTGKCVAFNDTVK-TCEIFGWCVPVEVDDIETPAILREAFNETLFIKNSISFPEKVNRRNLVEEVNAAHMKTC
P2X2	149	MLGNGLRTGRCVFEYQGPSKTCVEGWCVPVEGASVSQFLGT-MAFNETLFIKNSIHPKPFPSKGN-IADRTDGYIKSC
P2X3	127	LPGGGILTGRCVN-YSSLRTCEIFGWCVPEDVT-VETPIMM-EAFNETLFIKNSIRFPLFNEFKGNLENLTARDMKTC
P2X4	139	THNGVSTGRCVAFNGSVK-TCEVAAWCPVEDDTHVECPAHLKPAFNETLIMKNIMYPKFNESKRNLENITTYIKSC
P2X5	142	TAGNGVKTGRCRLRRENLAGRTCEIFAWCPLETSS-RPEEPHLKEADFTFIKNIHFPEKFNESKRNMDVKDRSFIKSC
P2X6	141	THSHGVKTGRCVWENGTH-TCEIWSWCPVESGV-VPSRPLAQA-NETLFIKNTVTFESKFNESKRNLETWDPYHKEC
P2X7	142	PQSKGIQTGRCVWHEGNQK-TCEVSAWCPLEAVEEAPRPAIINSAFNETVFIKNIIDFPNGYTRNLPGINIT----C
P2X1	218	LFHKTLLHPLCFVFCGLGVVQESGQNFSTIAERGGVVGITIDWECDLDNHVRHCSPIYEFHGLYE--EKNISPGENFREA
P2X2	227	TFHGEADLYCPIFKLGFIVEKAGESFTEIAHGGVIGVITINWDCDLDLPASECNEKYSERLDD--PKHVPASSGYNFRFA
P2X3	204	RFHPDKLPFCPIIRVGDVVKFAGQLEPAKIARTGGVLGKISWCDLDKAWDQCCEKYSERLDDSVSEKSSVSPGYNFRFA
P2X4	218	IYDAKTDFPCPIFRLGKIVENAGHSFQDMAVEGGIMGIQVWDCDLDRAASLCDFEYSERLDDTRDVEHNVSPGYNFRFA
P2X5	221	HFGPK-NHYCPIFRLGSVIRWAGSFQDIALBGGVIGITINWDCDLDKAASECCHYSERLDDNK-LSKSVSSGYNFRFA
P2X6	219	RYEPQFSFPCPIFRIGDILVAFAGSTFEDIALGGSGVIRVHWDCLDITGLSGCAPHYSFQLE-----KSYNFRFA
P2X7	217	TFHKTQNPFCPIFRLGDFRETGF-NESDIAIGGGIMGIEITWDCDLDLRFHHCCEKYSERLDDKTINVSLEPGYNFRFA
P2X1	295	RHEVENG-TNIRHLFKVHGIRFDILVGGAGKELIIPMTTIGSGIGIFGVATVLCDLLL-----LH-----
P2X2	305	KYYKIN-GTIRTLIKAYGIRIDVIVHGGAGKESLIPTIINLATALTSVGVSSFLCDWIL-----LT-----
P2X3	284	KYYKMENGSERTLLKAGIRFDILVVGAGKENIIPTISSVAFTSVGVSTVLCDIIL-----LN-----
P2X4	298	KYYRDLAGNERTLLIKAYGIRFDIVGGAGKELIIPMTINIGSGLALLGMATVLCDIIV-----LY-----
P2X5	299	KYYRDAAGMEERTLLKAYGIRFDIVVNGKG-----AFFCDLVL-----LY-----
P2X6	290	THWWEQFGMEERTLLKLYGIRFDILVGGAGKESLIPTAVTIGTGAAWIGVMTFFCDLL-----LY-----
P2X7	297	KYYKENN-VEKRTLLIKVHGIRFDILVGGTGGKELIILVYVIGSTLSYFGLAPVIFIDFLIDTYSSNCCRSHIYPWCKCCQ
P2X1	355	-ILFKRHYYKQK-----KFKYAEADMGP-AAERDLAATSSTLG---LQENMRTS-----
P2X2	365	-FMKNKVYSHK-----KFDKVTSPHPSGSGWPTILAR-----VLGQAPPEPGHRSEDQHPSPPSGQEGQGAEC
P2X3	345	-FLKGADQYKAK-----KFEEVN-----ETTLKI-----AALTNPVYPSDQTAEKQSTDSG-----
P2X4	359	-CMKKRLYYREK-----KYKYVEDYEQGLASELDQ-----
P2X5	338	-LIKKEFFYRK-----KYEBVGRLEDSSQEADEASGLGLSEQLTSGPGLGMPEQQLQEPPEAKRGSSSQKGN
P2X6	351	-VDREAHFYRT-----KYEBAKAPKATANSVWRELA-----FASQARLAECRLRSSAPAPTATAAGSQTP-
P2X7	376	PCVVNEYYYRKCESIVEPKPTLKYVSFVDESHRMVNOQLLGRSLQDVKGQEVPRPAMDFDLSRLPLALHDTPTPIPGQ
P2X1	399	-----
P2X2	430	GPAFPPLRPCPISAPSEQMVDTPASEPAQASTPTDPKGLAQL-----
P2X3	391	--AFS-----IGH-----
P2X4	388	-----
P2X5	409	GSVCP---QLLEPHRST-----
P2X6	414	GWPCPSSDTHLPTHSGSL-----
P2X7	456	PEBIQLLRKEATPRSRDSFVWCQCGSCLPSQLPESHRCLEELCCRKKPGACITTSSELFKRLVLSRHVLQFLLLYQEPLLA
P2X1	399	-----
P2X2	471	-----
P2X3	397	-----
P2X4	388	-----
P2X5	422	-----
P2X6	431	-----
P2X7	536	LDVDSTNSRLRHCAIRCYATWRFSGQDMADFAILPSCCRWRIRKEFPKSEGQYSGFKSPY

FIGURE 2: All human P2X family members contain putative N-linked glycosylation sites. The protein sequences of the seven human P2X family members were aligned using ClustalW (32), and NetNGlyc version 1.0 was used to identify potential N-linked glycosylation sites (highlighted in red). Identical residues are highlighted in black, and chemically similar residues are highlighted in gray. The following protein accession numbers were used in the alignment: P2X1, AAC24494; P2X2, Q9UBL9; P2X3, NP_002550; P2X4, NP_002551; P2X5, AAH39015; P2X6, AAF13303; and P2X7, AAH11913.

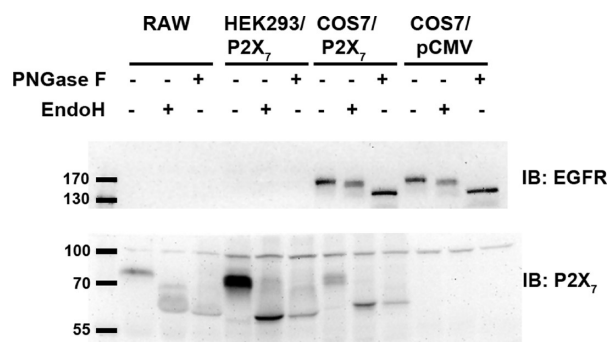


FIGURE 3: Endogenous and exogenous P2X₇ is sensitive to glycosidase treatment. Proteins from murine RAW 264.7 macrophages naturally expressing wild-type P2X₇ as well as human HEK293 and monkey COS7 cells transfected with human wild-type P2X₇ were denatured and treated with either EndoH or PNGase F for 1 h at 37 °C as described in Materials and Methods. The proteins were then resolved on polyacrylamide gels containing SDS and immunoblotted for P2X₇. Cleavage of EGFR was used as a positive control to show EndoH and PNGase F were active. These data are representative of three or more experiments.

control for 90 min at 37 °C. The samples were then boiled for 10 min, diluted in 2× sample buffer, sonicated, and immunoblotted.

RESULTS

P2X₇ Is Sensitive to Glycosidase Treatment. NetNGlyc (Technical University of Denmark, Copenhagen, Denmark) was used to identify putative N-linked glycosylation sites in the seven human P2X family members (Figures 1 and 2). N-Linked glycosylation sites contain the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline (22). Cell lysates from RAW 264.7 macrophages, which express endogenous P2X₇ (8), and HEK293 and COS7 cells expressing the exogenous human receptor were treated with the glycosidase EndoH or PNGase F and immunoblotted for P2X₇. We chose to include COS7 and HEK293 cells in our studies because they do not express detectable levels of endogenous P2X₇ (23). The enzyme EndoH cleaves high mannose and some hybrid N-linked glycosylation modifications, while PNGase F is less specific and can also cleave complex oligosaccharides with di-, tri-, and tetra-antennary arms (24). Both endogenous and exogenous P2X₇ are sensitive to EndoH and PNGase F as indicated by faster electrophoretic migration (Figure 3). The epidermal growth factor receptor (EGFR) was immunoblotted as a positive control to demonstrate the EndoH and PNGase F enzymes were functional (25).

Endogenous P2X₇ in RAW 264.7 and Transfected P2X₇ in HEK293 and COS7 Cells Are Functional. EndoH cleaves N-linked glycosylation modifications that are processed in the Golgi, and proteins that are insensitive to this enzyme are thought to have moved out of the Golgi and possibly to the plasma membrane (26). Because we did not observe a large EndoH-resistant population of P2X₇ in the HEK293, COS7, and RAW 264.7 cells, we tested the ideas that P2X₇ is primarily localized at intracellular sites and a small population of the receptor is expressed on the cell surface that is capable of eliciting P2X₇ agonist-induced events. As assessed by immunofluorescence, both exogenous P2X₇ in COS7 cells and endogenous P2X₇ in RAW 264.7 macrophages appear to be highly represented at intracellular sites (Figure 4A,C). In addition, P2X₇ in RAW 264.7 and COS7 cells colocalizes with the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI) (Figure 4C and data not shown). Although P2X₇ is not readily detected on the plasma membrane

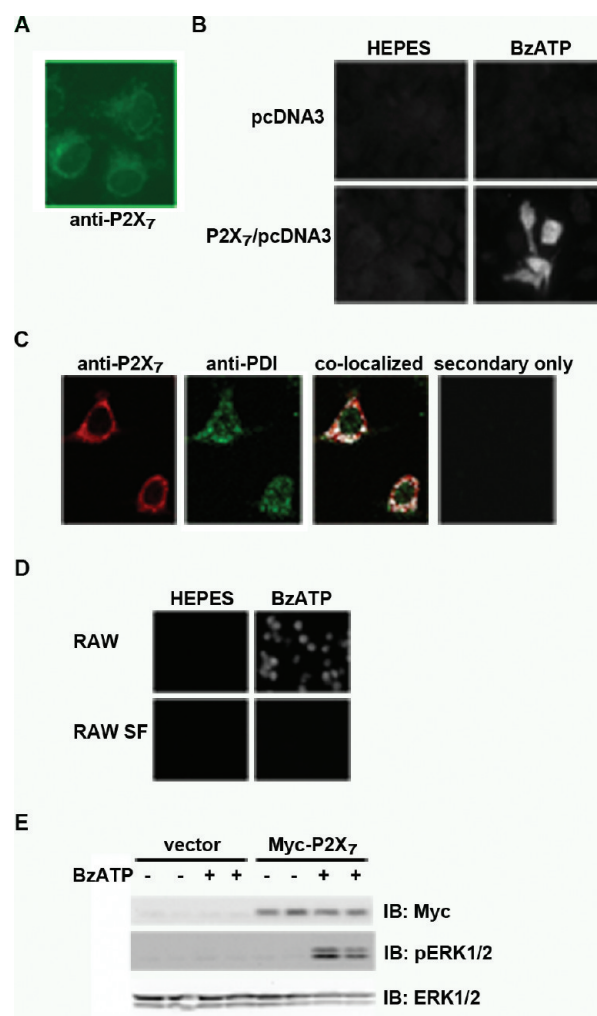


FIGURE 4: P2X₇ is highly localized in intracellular compartments but is activated in response to extracellular BzATP administration. (A) Human nucleotide receptor P2X₇ was expressed in COS7 cells, and the cells were stained with an anti-P2X₇ antibody and visualized with a Zeiss Axioplan 2 microscope. This image is representative of more than six experiments. (B) COS7 cells were transfected with either P2X₇/pCMV3 or pcDNA3 and were stimulated with either 250 μ M BzATP or HEPES buffer control in the presence of 1 μ M YO-PRO-1. The cells were fixed, and relative dye uptake was imaged with a Zeiss Axioplan 2 microscope. These data are representative of more than six experiments. (C) RAW 264.7 macrophages were fixed, stained with anti-P2X₇ and anti-PDI antibodies, and visualized with a Bio-Rad Radiance 2100 MP Rainbow confocal microscope. As a negative control, the cells were stained with only the secondary antibody. The represented images are of one z-stack. The anti-P2X₇ and anti-PDI images were merged using the ImageJ colocalization plugin. These data are representative of two experiments. (D) RAW 264.7 and RAW 264.7 SF macrophages were stimulated with either BzATP or HEPES buffer control in the presence of 1 μ M YO-PRO-1 and visualized as live cells using an Olympus IX fluorescence inverted microscope. These YO-PRO-1 dye uptake assays are representative of five experiments. (E) HEK293 cells were transfected with either P2X₇/pCMV-Myc or pCMV-Myc and were stimulated with either 250 μ M BzATP or HEPES buffer control for 5 min. Cell lysates were prepared and immunoblotted using anti-Myc, anti-pERK1/2, and anti-ERK antibodies. These data are representative of more than eight experiments.

via immunofluorescence even in naturally P2X₇-expressing RAW 264.7 macrophages, we observed that both the P2X₇-transfected COS7 and RAW 264.7 cells, but not the P2X₇-defective cell line RAW 264.7 SF, are capable of taking up the fluorescent dye YO-PRO-1 in the presence of BzATP (Figure 4B,D). BzATP is a

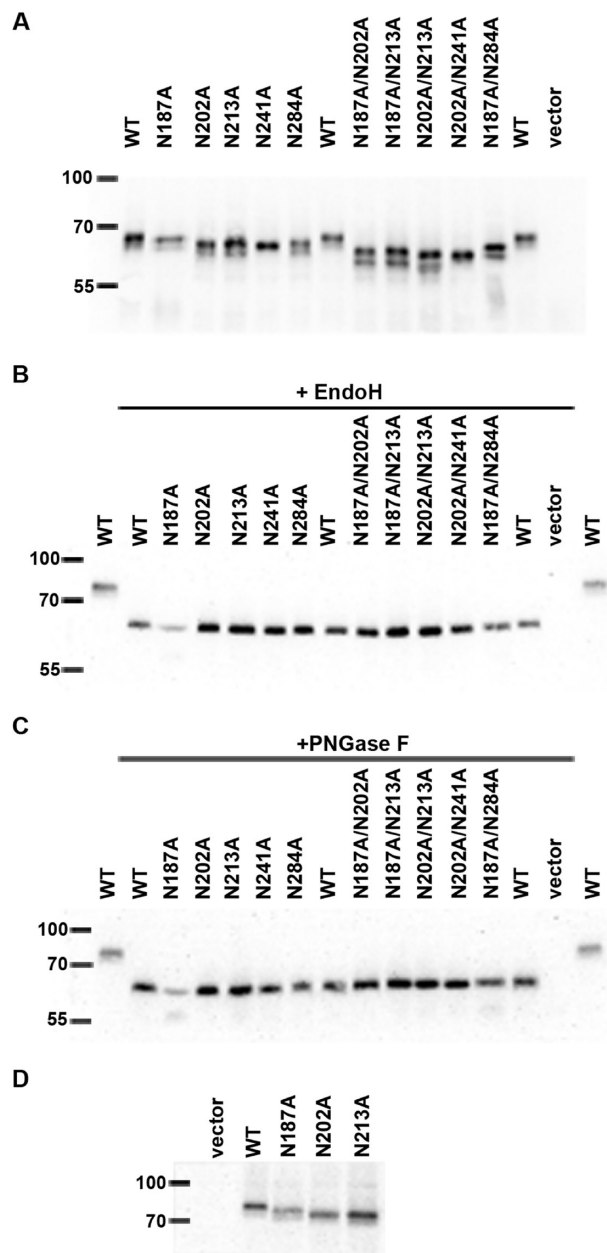


FIGURE 5: P2X₇ is glycosylated at N187, N202, N213, N241, and N284. (A) Wild-type P2X₇ (WT) and the Asn to Ala mutants were expressed in HEK293 cells, and cell lysates were immunoblotted with the anti-Myc antibody. The five single Asn to Ala mutants exhibited faster electrophoretic mobility than the WT receptor, and the double mutants migrated further than the single mutants. The proteins were denatured and treated with (B) EndoH or (C) PNGase F for ~1 h at 37 °C and immunoblotted with the anti-Myc antibody. The data shown in panels A–C are representative of two or more experiments. (D) P2X₇ WT and Asn to Ala mutants were expressed in HEK293 cells, and lysates were immunoblotted with the anti-Myc antibody. The P2X₇ N187A mutant does not migrate as fast as the other Asn to Ala mutants but does migrate faster than WT. These data are representative of more than 10 experiments.

synthetic ATP derivative that is used to activate P2X₇, and the YO-PRO-1 dye uptake assay is a method of assessing the ability of P2X₇ to stimulate the formation of a pore (20). To demonstrate that exogenously expressed P2X₇ in HEK293 cells is functional, BzATP-induced ERK1/2 phosphorylation tests were employed (Figure 4E). ERK1 and ERK2 are phosphorylated/activated by BzATP when wild-type P2X₇ is expressed but not when the cells are transfected with the empty vector control.

Mutation of Human P2X₇ at N187, N202, N213, N241, and N284 Results in Increased Electrophoretic Mobility. To identify the residues that likely contain a glycosylation modification, the five predicted N-linked glycosylation sites were mutated to alanine either individually or doubly, and their electrophoretic mobility was examined (Figure 5). All of the Asn to Ala single and double mutants migrated faster than wild-type P2X₇. To illustrate these migratory differences were likely due to an altered glycosylation profile and not protein degradation, lysates were treated with EndoH or PNGase F and immunoblotted for P2X₇. After cleavage with either glycosidase, the mutants had apparent molecular weights comparable to that of the enzyme-treated wild-type receptor (Figure 5B,C). In addition, we observed that P2X₇ N187A appears to have a slightly higher molecular weight than receptors containing the N202A, N213A, N241A, or N284A mutation (Figure 5A,D) as assessed by SDS–polyacrylamide gel electrophoresis. It is possible that this site (N187) is differentially glycosylated compared to the other sites and/or that the protein is altered in its folding and interaction with SDS.

Treatment of RAW 264.7 Macrophages with Tunicamycin Results in Attenuated BzATP-Induced ERK1/2 Activation. To determine whether N-linked glycosylation is required for P2X₇ agonist-induced ERK1/2 phosphorylation/activation, RAW 264.7 macrophages were treated with the N-linked glycosylation synthesis inhibitor tunicamycin for 42 h, and the cells were stimulated with either BzATP or phorbol 12-myristate 13-acetate (PMA) for 5 min to activate ERK1/2. As shown in Figure 6, treatment of cells with 5 μg/mL tunicamycin decreases the level of BzATP- but not PMA-stimulated ERK1/2 phosphorylation. Treatment with tunicamycin results in the formation of faster migratory P2X₇ proteins, which are presumably newly synthesized P2X₇ receptors that do not contain glycosylation modifications. In addition, less full-length receptor is observed in the cells treated with the inhibitor, providing further evidence that a smaller pool of P2X₇ is N-linked glycosylated in these samples (Figure 6A). It should be noted that a cell viability assay was conducted to determine whether treatment with 5 μg/mL tunicamycin for 42 h results in significant cell death, and we found 85% of the tunicamycin-treated cells were metabolically active in comparison to the methanol control-treated cells (data not shown), indicating that decreases observed after tunicamycin treatment were not due to cytotoxicity. The change in the level of ERK1/2 phosphorylation between tunicamycin-treated and buffer control-treated cells is depicted in Figure 6B. Treatment with tunicamycin results in an approximately 30% reduction in the level of BzATP-induced ERK1/2 phosphorylation but no significant change in PMA-stimulated ERK1/2 phosphorylation.

Mutation of P2X₇ N-Linked Glycosylation Sites Results in Attenuated BzATP-Induced Signaling and Pore Formation. To determine which N-linked glycosylation sites of P2X₇ are required for its activity, we examined BzATP-induced ERK1/2, CREB, and p90RSK phosphorylation/activation in cells stably expressing the wild-type receptor or the Asn to Ala mutants. The phosphorylation of CREB was assessed by immunoblotting cell lysates with an antibody that detects phosphorylated and/or activated CREB and by analyzing mobility shifts on an anti-CREB antibody immunoblot. Cells expressing P2X₇ N187A do not detectably exhibit ERK1/2, CREB, or p90RSK phosphorylation in the presence of the P2X₇ agonist BzATP (Figure 7). Mutation of P2X₇ N213, N241, or N284 also resulted in an apparent reduction in BzATP-induced ERK1/2 phosphorylation,

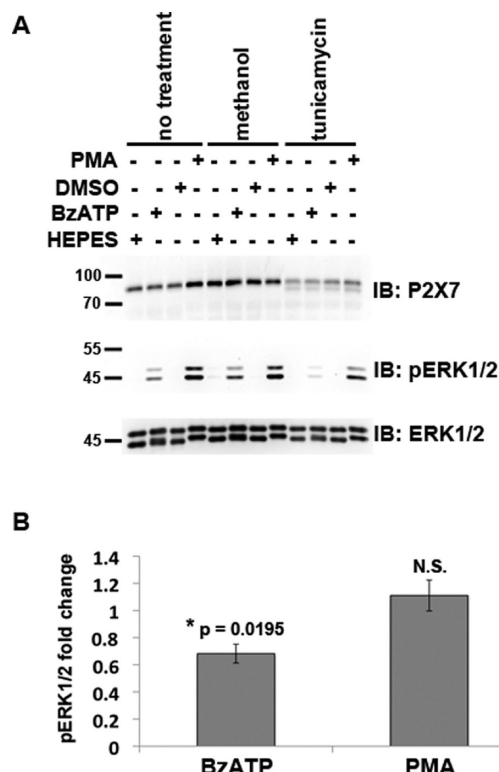


FIGURE 6: Treatment of RAW 264.7 macrophages with tunicamycin results in attenuated BzATP-induced ERK1/2 activation. (A) RAW 264.7 macrophages were treated with the N-linked glycosylation synthesis inhibitor tunicamycin (5 μ g/mL) for 42 h, and the cells were stimulated with either 250 μ M BzATP or 1 μ g/mL PMA for 5 min. Cell lysates were then immunoblotted with anti-P2X₇, anti-pERK1/2, and anti-ERK1/2 antibodies. Methanol is the vehicle control for tunicamycin; HEPES is the buffer control for BzATP, and DMSO is the vehicle control for PMA. These data are representative of four experiments. (B) Changes between tunicamycin-treated cells stimulated with BzATP or PMA and tunicamycin-treated cells stimulated with buffer control were quantified and graphed. The band intensities of the phospho-ERK1/2 bands were normalized to total ERK1/2, and the percent activation of ERK1/2 with either BzATP or PMA in comparison to the appropriate buffer control was calculated. Student's *t* tests were performed to determine the *p* values. N.S. means not significant.

but not to the same extent as the P2X₇ N187A mutation. Conversely, mutation of P2X₇ N202 to Ala did not result in attenuated ligand-stimulated ERK1/2, CREB, or p90RSK phosphorylation in either the stably expressing cells (Figure 7) or transient transfection experiments (data not shown). The data from three separate experiments revealed that the change in ERK1/2 phosphorylation following a 10 min BzATP treatment in wild-type P2X₇-expressing cells ranged from 8.2- to 29-fold. In contrast, the change in the level of BzATP-induced ERK1/2 activation ranged from 1.5- to 4.8-fold for P2X₇ N213A, from 3.4- to 11-fold for P2X₇ N241A, and from 3.8- to 13-fold for P2X₇ N284A. In addition, attenuated BzATP-induced ERK1/2 activation was also observed in HEK293 cells transiently expressing N213A, N241A, and N284A mutations (data not shown). Similar trends in CREB activation were also obtained, but smaller changes were achieved in comparison to ERK1/2 (data not shown). For example, in three separate experiments, the average increase in the level of CREB activation in BzATP-treated cells expressing wild-type P2X₇ was 2.0-fold. These decreases in BzATP-stimulated responses observed in cells expressing a mutant P2X₇ in which the Asn at position 284 has been altered to alanine complement a

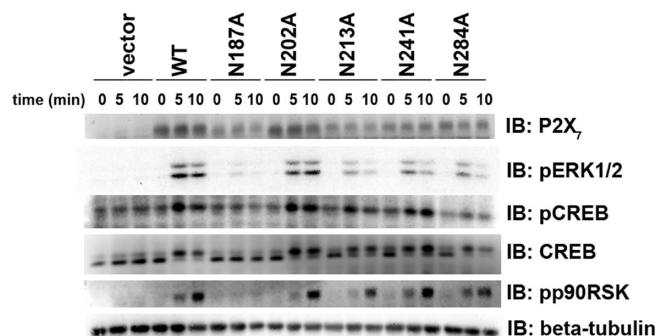


FIGURE 7: Mutation of P2X₇ N-linked glycosylation sites results in attenuated BzATP-induced signaling. Wild-type P2X₇ and the Asn to Ala mutants were stably expressed in HEK293 cells by selecting G418 sulfate-resistant populations. The cells were treated with 250 μ M BzATP for 5 or 10 min, and cell lysates were prepared and immunoblotted with anti-P2X₇, anti-pERK1/2, anti-CREB, anti-pCREB, anti-pp90RSK, and anti- β -tubulin antibodies. The β -tubulin immunoblot is the loading control for the anti-pERK1/2 immunoblot. These data are representative of three experiments.

prior report demonstrating that the introduction of an Asn residue at the equivalent residue in mouse P2X₇ (D284) confers increased sensitivity to BzATP and ATP (27). These data support the idea that P2X₇ N284 may also be important for human P2X₇ function.

Because P2X₇ N187A generally exhibited lower expression levels than other Asn to Ala mutants, we increased the amount of P2X₇ N187A/pCMV-Myc DNA used in transient transfections in an effort to drive its expression to a level that is comparable to that of the wild-type receptor. Even when P2X₇ N187A is expressed at a level similar to or higher than the level of the wild-type receptor, the mutant receptor does not exhibit BzATP-induced ERK1/2 activation (Figure 8A). In addition, COS7 cells transiently expressing P2X₇ N187A exhibit attenuated BzATP-induced YO-PRO-1 uptake in comparison to cells expressing wild-type receptor (Figure 8B), providing further evidence that this residue is important for P2X₇ function. To further verify that residue N187 is important for receptor activity and that our observations with an alanine substitution at this position are not restricted to the specific chemistry (hydrophobicity) of alanine, we also mutated P2X₇ residue N187 to the more polar residue histidine, as well as to a residue structurally related to the asparagine normally located at this site, i.e., glutamine. As shown in panels C and D of Figure 8, mutation of P2X₇ N187 to His or Gln also results in attenuated BzATP-induced ERK1/2 phosphorylation, thereby supporting the specific importance of asparagine and/or glycosylation at this site.

Wild-Type P2X₇ and Mutant P2X₇ Display Similar Localization Patterns. To delineate the mechanism by which P2X₇ N187A exhibits attenuated activity, we considered the possibilities that it may be mislocalized or not efficiently trafficked to the plasma membrane. To test these ideas, we expressed wild-type P2X₇ and the Asn to Ala mutants in COS7 cells and examined their overall localization patterns by immunofluorescence. As shown in Figure 9, wild-type P2X₇ and the glycosylation mutants, including N187A, all display a perinuclear, endoplasmic reticulum-like pattern. Even though P2X₇ N187A has diminished activity, it appears to localize in a pattern comparable to that of wild-type P2X₇ and to the other Asn to Ala mutants. These data support the notion that the diminished activity of P2X₇ N187A is not likely the result of gross mislocalization of the receptor.

P2X₇ N187A Cell Surface Expression Is Altered. Because we did not detect any major differences in the localization between

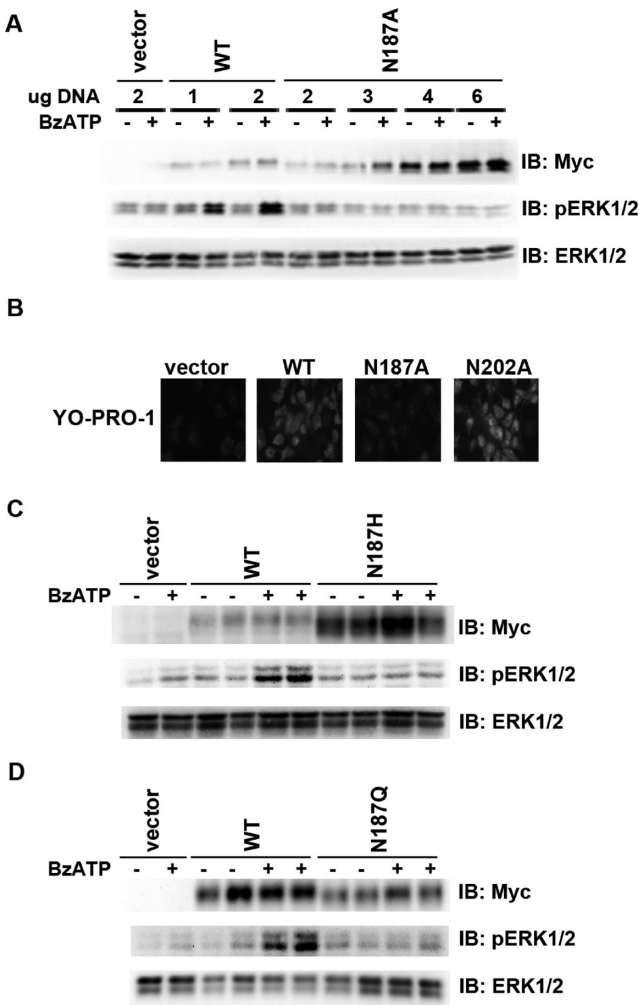


FIGURE 8: Mutation of P2X₇ N187 results in a decreased level of BzATP-induced ERK1/2 activation and pore formation. (A) HEK293 cells were transfected with the indicated amounts of pCMV-Myc vector control, P2X₇ WT/pCMV-Myc, or P2X₇ N187A/pCMV-Myc. After 24 h, the cells were treated with 250 μ M BzATP for 5 min, and cell lysates were prepared and immunoblotted with anti-Myc, anti-pERK1/2, and anti-ERK1/2 antibodies. These data are representative of two independent experiments. (B) COS7 cells were transfected with pCMV-Myc, P2X₇ WT/pCMV-Myc, P2X₇ N187A/pCMV-Myc, or P2X₇ N202A/pCMV-Myc. After 24 h, a YO-PRO-1 dye uptake assay was performed. Similar data were obtained in two separate experiments. (C) HEK293 cells were transfected with pCMV-Myc vector control, P2X₇ WT/pCMV-Myc, or P2X₇ N187H/pCMV-Myc. After 24 h, the cells were treated with 250 μ M BzATP for 5 min, and cell lysates were prepared and immunoblotted with anti-Myc, anti-pERK1/2, and anti-ERK1/2 antibodies. These data are representative of three independent experiments. (D) HEK293 cells were transfected with pCMV-Myc vector control, P2X₇ WT/pCMV-Myc, or P2X₇ N187Q/pCMV-Myc. After 24 h, the cells were treated with 250 μ M BzATP for 5 min, and cell lysates were prepared and immunoblotted with anti-Myc, anti-pERK1/2, and anti-ERK1/2 antibodies. These data are representative of three independent experiments.

wild-type and N187A P2X₇ in fixed cells as assessed by immunostaining, we used a live cell proteinase K digestion assay to analyze the plasma membrane expression of P2X₇ over a given period of time and to test the hypothesis that P2X₇ N187A is not transported as efficiently to the plasma membrane as wild-type P2X₇. When the receptor is expressed on the surface of live cells, the extracellular domain is digested with proteinase K, liberating the C-terminal tail. In this assay, we treated cells with protease for 90 min, allowing the enzyme to digest proteins that become

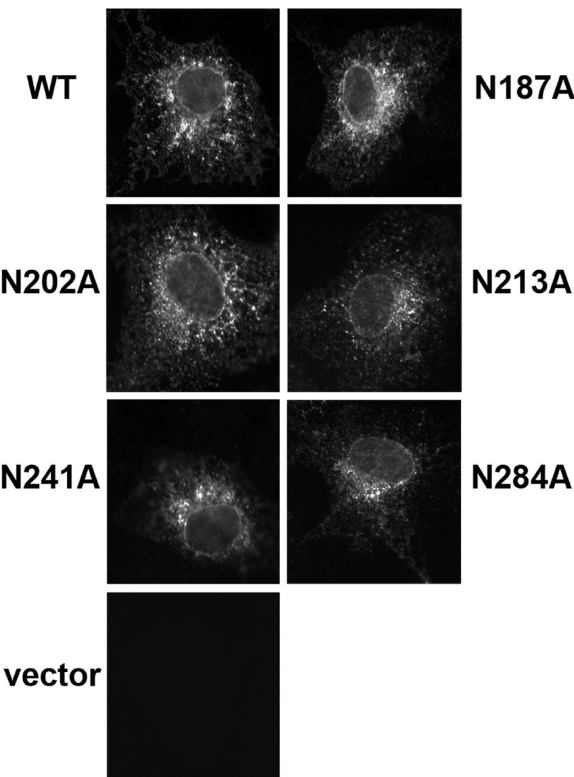


FIGURE 9: Wild-type and mutant P2X₇ display similar localization patterns. Wild-type P2X₇, the Asn to Ala mutants, and the vector control were expressed in COS7 cells. Approximately 24 h after the cells were transfected, they were fixed with paraformaldehyde, stained with an anti-P2X₇ antibody, and imaged with a Zeiss Axioplan 2 microscope. Similar data were obtained in two separate experiments.

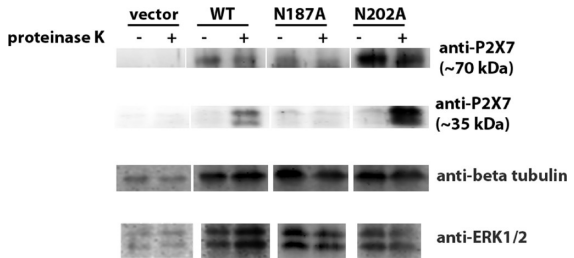


FIGURE 10: P2X₇ N187A expression on the cell surface is altered. HEK293 cells stably expressing pcDNA3 vector control, P2X₇ WT, P2X₇ N187A, and P2X₇ N202A were treated with 333 μ g/mL proteinase K for 90 min as described in Materials and Methods. The cells were then boiled for 10 min to inactivate the enzyme, diluted in 2 \times sample buffer, sonicated, and immunoblotted with anti-P2X₇, anti-ERK1/2, and anti- β -tubulin antibodies. These data are representative of three or more experiments.

exposed on the cell surface at any point during the incubation period. A key advantage of this approach is that it represents an accumulation of events that occur over 90 min versus the single “snapshot” in time represented by the immunostaining approach. We predicted that treatment of live cells with proteinase K would result in the cleavage of full-length wild-type P2X₇, but not the N187A mutant, yielding a fragment containing only the intracellular domain. As depicted in Figure 1, human P2X₇ contains an intracellular C-terminal domain that contains more than 200 amino acids. Digestion of HEK293 cells stably expressing wild-type P2X₇ or N202A but not the N187A receptor resulted in the formation of ~35 kDa proteins that are immunoreactive with an anti-P2X₇ antibody that was raised against the C-terminus of

the protein (Figure 10). These immunoreactive proteins are likely the C-terminal tail of full-length plasma membrane-localized P2X₇. The predicted size of the C-terminal tail, including the second transmembrane domain, is ~30 kDa. The ~35 kDa proteins were also immunoreactive with a different anti-P2X₇ antibody that was also raised against the intracellular domain of the protein (data not shown). The samples were immunoblotted with anti-ERK1/2 and anti- β -tubulin antibodies to demonstrate the protease did not digest intracellular proteins. These data are evidence that P2X₇ N187A is not efficiently transported to the cell surface, providing a plausible mechanism for why this mutant displays attenuated activity.

DISCUSSION

We have presented the first evidence that the nucleotide receptor P2X₇ appears glycosylated on five asparagine residues and that residue N187, which is an amino acid that is conserved among six of the seven P2X family members, is important for receptor-stimulated function and signaling. Mutation of residues N213, N241, and N284 also results in a modest attenuation of BzATP-induced ERK1/2 phosphorylation, but not to the same extent as mutation of N187. In addition, we have provided evidence that the P2X₇ N187A point mutant displays reduced activity, at least in part, because it is not properly transported to the plasma membrane when compared to wild-type receptor.

There are several potential explanations for how mutation of P2X₇ N187 attenuates receptor function. One possibility is that the proper folding and assembly of P2X₇ monomers into oligomers requires glycosylation at this site (4). Cell surface-bound proteins that are not folded properly in the ER are often degraded during the unfolded protein response (28). This process may contribute to the observations that it was often difficult to express P2X₇ N187A at levels that were similar to that of the wild type receptor and that P2X₇ Asn to Ala double mutants containing N187A were generally expressed at lower levels than the single mutants (Figure 8 and data not shown). PredictProtein was used to determine where predicted secondary structures in human P2X₇ are located. This analysis predicted that residue N187 is located in a short loop between an α helix and a β sheet. Thus, it is possible that mutation at this residue disrupts these secondary structures, altering tertiary protein structure.

Another possible explanation for why the P2X₇ N187 point mutants exhibit decreased activity is that glycosylation at this residue is required for the trafficking of P2X₇ to the plasma membrane. Assessing P2X₇ cell surface expression at a given time point has been challenging in the cell lines commonly used for P2X₇ analysis. It has been reported by others that P2X₇ is naturally highly represented in intracellular compartments in several cell types (29). Similarly, we have noted that endogenous P2X₇ in RAW 264.7 macrophages and overexpressed P2X₇ in COS7 cells appear to be predominantly localized at intracellular sites, as assessed by immunofluorescent staining (Figures 4 and 9). It is plausible that the trafficking of P2X₇ to and from the cell surface occurs rapidly in cells naturally expressing the receptor as well as in those transfected with the exogenous receptor. Therefore, visualizing P2X₇ localization at the plasma membrane at any single time point is likely to be limited by the possibility that only a small percentage of the receptor is present at the surface at any given time. In agreement with this concept, we have found that P2X₇ levels on the cell surface appear low using other methods, including flow cytometry (data not shown) and EndoH resistance assays (Figures 3 and 5). Accordingly, the live cell

protease K digestion assay described herein appears to be a valuable tool for examining the plasma membrane localization of P2X₇ over 1 h. This assay has been useful in identifying P2X₇ mutants that exhibit a major defect in transport to the cell membrane.

Interestingly, a recent report by Roger et al. has shown that two P2X₇ SNPs located near N187, E186K and L191P, exhibit decreased ion channel and pore activity, providing further evidence this extracellular region of the receptor is critical for function (17). The authors propose the E186K and L191P mutations affect ATP binding because they are in the proximity of the ATP-binding amino acids. As discussed above, our data support two alternative explanations for why P2X₇ exhibits attenuated activity when residues in that region are mutated. We propose that the region of the receptor encompassing residue N187 is important for protein folding and/or transport to the cell surface and that any ATP binding issues may be secondary to the trafficking defect. Nonetheless, this report and that of Roger et al. demonstrate that the region surrounding N187 is important for P2X₇ function, and this information should help in improving our understanding of how naturally occurring genetic variations in P2X₇ lead to altered physiology.

As discussed previously, we and others have provided evidence that residue N284 may also be an important determinant of P2X₇ function. For example, we have shown herein that mutation of N284 to Ala in the P2X₇ receptor results in a modest decrease in the level of BzATP-induced ERK1/2 activation (Figure 7). Interestingly, Young et al. have demonstrated that introduction of Asn at the equivalent residue in mouse P2X₇ (D284), which leads to an increased level of receptor glycosylation, results in increased sensitivity to BzATP- and ATP-induced responses, and that mutation of the preceding amino acid in rat P2X₇ (T283) results in attenuated channel and pore activities (27, 30). This threonine residue is conserved among the human, mouse, and rat sequences (27, 30). It is possible that we observed an only modest decrease in P2X₇ N284A activity in response to BzATP because this region of the protein may be less critical for the interaction of BzATP with the receptor when compared to other P2X₇ ligands such as ATP. In support of this idea, introduction of Asn at mouse P2X₇ D284 promoted a greater sensitivity to ATP than BzATP (27).

In summary, this work highlights the importance of N-linked glycosylation in the regulation of the immunomodulatory critical protein P2X₇. We have demonstrated that both mutation of the conserved glycosylation site N187 and treatment of cells with the glycosylation synthesis inhibitor tunicamycin result in attenuated receptor activity, supporting the idea that N-linked glycosylation is essential for P2X₇ function.

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