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Expression, purification, electron microscopy, N-glycosylation mutagenesis and molecular modeling of human P2X4 and *Dictyostelium discoideum* P2XA

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ABSTRACT

The recent publication of the *apo*-, closed-state 3D crystal structure of zebrafish (zf) P2X4.1 has not only revolutionized the P2X research field, but also highlighted the need for further crystal structures, of receptors in different activation states, so that we can gain a complete molecular understanding of ion channel function. zfP2X4.1 was selected as a 3D-crystallization candidate because of its ability to form stable trimers in detergent solution, and purified from over-expression in baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cells. In this work, we have used a similar approach to express both human P2X4 (hP2X4) and *Dictyostelium discoideum* P2XA (DdP2XA) in Sf9 cells. Although hP2X4 did not form stable trimers in detergent solution, both receptors bound to ATP-coupled resins, indicating that their extracellular domains were folded correctly. DdP2XA formed strong trimers in detergent solution, and we were able to selectively purify trimers using preparative electrophoresis, and build a 21 Å-resolution 3D structure using transmission electron microscopy and single particle analysis. Although the structure of DdP2XA possessed similar dimensions to those of the previously determined low-resolution hP2X4 structure and the zfP2X4.1 crystal structure, N-glycosylation mutagenesis and molecular modeling indicated differences between N-glycan usage and predicted accessibility in models of DdP2XA based on the zfP2X4.1 crystal structure. Our data demonstrate that DdP2XA expressed in insect cells retains ATP-binding capacity after detergent solubilization, is an ideal candidate for structural study, and possesses a significantly different 3D structure to that of both hP2X4 and zfP2X4.1.

1. Introduction

P2X receptors are trimeric cation channels gated by extracellular ATP. There are seven subtypes in mammals, which display differential agonist/antagonist selectivity and ion channel properties [1], and play roles in physiological processes such as nerve transmission, pain sensation, control of vascular tone and inflammation, making them key drug targets [2,3]. In addition, P2X receptors are found in zebrafish (zf) [4], and several lower eukaryotes including the slime mold *Dictyostelium discoideum*[5]. The publication in 2009 of the first crystal structure of a P2X receptor, *apo*-zfP2X4.1 at 3.1 Å resolution [6] represents a major advance. Success with zfP2X4.1 was dependent upon three main factors: (i) screening of many different receptor constructs to find those which formed the most stable trimers in detergent solution [7]; (ii) use of the Sf9 insect cell expression system; and (iii) significant construct modification to achieve well-diffracting crystals. The extensive construct modification altered ion channel function; peak currents were reduced, channel closure after removal of agonist was delayed, and agonist potency was increased [6], implying that the structure may not represent a native conformation, but rather an artificially stabilized, closed state. Nevertheless, it has enabled the interpretation of a large quantity of previous experimental data relating to transmembrane domain orientation, ligand-binding and channel opening in molecular detail [8–10], and serves as an excellent template from which to model other P2X receptors.

To fully understand the molecular basis of agonist/antagonist binding, channel activation and subtype selectivity, further P2X receptor structures, including the ligand-bound state (in closed and open conformation), and structures of different receptor subtypes (particularly the mammalian receptors) are needed. Obtaining these structures represents a huge challenge because it is difficult to produce stable, folded P2X receptors in the milligram-quantities required for 3D crystal trials. Thus far, successful over-expression of P2X receptors for structural study has been achieved in mammalian cell lines

Abbreviations: β-OG, n-octyl-β-D-glucoside; $C_{12}E_8$, octaethylene glycol monododecyl ether; DDM, n-dodecyl-β-D-maltoside; FC-12, n-dodecylphosphocholine; LDAO, n-dodecyl-N,N-dimethylamine-N-oxide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Sf9 cells, *Spodoptera frugiperda* cell-line derived from pupal ovaries; TEM, transmission electron microscopy, TX-100, Triton-X-100; zf, zebrafish

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for rat P2X2, rat P2X6 [11] and human P2X4 [12,13], and in baculovirus-infected Sf9 insect cells for rat P2X2 [14,15] and zfP2X4.1 [6]. However, in most cases, the yield of protein has been too low to permit 3D crystallography, and structural data have been limited to low-resolution studies using atomic force microscopy [11,12,15] and single particle analysis [13,14,16], which require significantly lower quantities of protein. By far the most successful approach has been that of Kawate et al., determining the construct most likely to produce a high yield of stable trimers, and expressing this in Sf9 cells [6].

To exploit and extend this recent advance, we have employed the Sf9 insect cell system to express both human P2X4 (hP2X4) and *D. discoideum* P2XA (DdP2XA). hP2X4 is the nearest human homologue to zfP2X4.1, sharing 57% amino acid identity. It has also been shown to form a significant proportion of stable trimers when over-expressed in human cells [13]. DdP2XA is more distantly related to zfP2X4.1, sharing only 16% amino acid identity. However, this protein has been shown to form highly stable trimers when expressed in human cells [5], which was an important indicator of success with zfP2X4.1 [6].

We assessed the degree of trimer formation of each receptor in a variety of detergents using non-denaturing perfluorooctanoic acid (PFO)–PAGE, and found that while hP2X4 did not form stable trimers in any of the detergents tested, DdP2XA was very stable in several detergents, including n-dodecyl-β-D-maltoside (DDM). We also assessed whether or not the receptors were folded by performing pull-down assays with ATP-coupled sepharose beads; both receptors bound to beads coupled to ATP at the γ-phosphate- or 6-amino-position, but not at the 8-position, which may give some indication of how ATP is positioned within the binding site. Following selective purification of DdP2XA trimers using preparative PFO–PAGE, electron microscopy and single particle analysis were used to build a low-resolution 3D structure of the receptor, which possessed pronounced extracellular-domain propellers compared to the previously determined hP2X4. Finally, we generated molecular models of both hP2X4 and DdP2XA using the zfP2X4.1 crystal structure as the template, and probed their accuracy using N-glycosylation mutagenesis, finding several discrepancies between actual N-glycan usage in DdP2XA and predicted residue accessibility.

Our data show that (i) wild-type hP2X4 expressed in insect cells does not form stable trimers in detergent solution, but is still capable of binding ATP; and (ii) wild-type DdP2XA forms stable trimers in detergent, is an excellent candidate for over-expression in Sf9 cells for structure determination, and that its overall 3D-structure appears to be significantly different to that predicted from molecular modeling studies.

2. Experimental

2.1. cDNA constructs, mutagenesis, expression in HEK cells and Western blotting

cDNA constructs corresponding to human P2X4-$(His)_{10}$ and human codon-optimized DdP2XA-$(His)_6$ have been described previously [5,13]. Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene) according to manufacturers' instructions. HEK-293 cells were cultured to approximately 80% confluency in 35-mm dishes, and transfected with 1 μg cDNA using 3 μl Lipofectamine (Invitrogen) according to manufacturers' instructions. 24 hours post-transfection, cells were washed twice in ice-cold phosphate-buffered saline (PBS, Sigma) and pelleted by centrifugation at 4000*g* for 3 minutes. Total protein samples were prepared by solubilizing the cells from one 35-mm dish in 50 μl PBS containing 1% (w/v) n-dodecyl-β-D-maltoside (DDM) and protease inhibitors (Roche Complete—EDTA). Solubilized protein concentrations were determined using Bradford assay, protein samples were denatured by boiling for 2 minutes at 100 °C, separated by SDS–PAGE on 10% polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes. Membranes were blocked with 3% bovine serum albumin (BSA) for 1 hour at room temperature and incubated in blocking buffer with mouse monoclonal anti-His primary antibody (Qiagen; 1:2000 dilution) overnight at 4 °C. After washing in 3 changes of PBS containing 0.1% Tween-20 (PBS-T, Bio-Rad), membranes were incubated with either alkaline phosphatase- or HRP-coupled secondary antibody (Sigma or DAKO Cytomation; 1:2000) for 1 hour at room temperature and washed 4× in PBS-T. Blots were developed using either BCIP/NBT tablets (Sigma) or the ECL-Plus kit (GE Healthcare) according to manufacturers' instructions.

2.2. Generating recombinant bacmid for Sf9 expression

Our starting point was the above-mentioned clones which were used as templates for PCR. cDNA encoding human P2X4-$(His)_{10}$ and human codon-optimized DdP2XA-$(His)_6$ was amplified from these templates to include a CACC sequence in place of the initiation codon at the 5′ end and a termination codon at the 3′ end. The PCR products were subcloned into an insect cell expression vector using the Gateway system (Invitrogen, Carlsbad, CA). Briefly, cDNAs were subcloned into the "entry vector" pENTR–D-TOPO and recombined into the "destination vector" pDEST8 according to the manufacturer's recommendations. The sequence of pDEST8 clones was confirmed by the Keck DNA sequencing facility (New Haven, CT). pDEST8 clones were transformed into DH10Bac cells (Invitrogen) which generated recombinant bacmid that was isolated and purified from the DH10Bac cells according to manufacturer's recommendations. The presence of P2X cDNA in the bacmid was confirmed by PCR. 1 μg of the recombinant bacmid DNA was used to transfect Sf9 cells.

2.3. Culture, transfection, and harvesting of Sf9 cells

Serum-free medium (SFM) adapted Sf9 cells (Invitrogen) were seeded at a density of 9×10^5 cells per well in a 6-well plate that contained 2 ml of Sf-900 II SFM (Invitrogen). Cells were allowed to attach for 1 hour at 27 °C, the medium was removed and cells were transfected by drop-wise addition of transfection mixture (1 μg bacmid, 6 μl cellfectin, 1 ml Sf-900 II SFM). Cells were incubated with the transfection mixture for 5 hours at 27 °C, after which time the transfection mixture was aspirated and replaced with 2 ml of Sf-900 II SFM. Transfected cells were incubated for 3 days at 27 °C and the supernatant (presumed to contain a low-titer of baculovirus particles) was harvested. 60 μl of the supernatant was used to infect further insect cells in a 6-well plate to produce 2 ml of high-titer viral stock. The viral titer was determined to be 2×10^7 using the BacPAK baculovirus rapid titer kit (Clontech, Mountain View, CA) and expression of P2X in these cells was confirmed by Western blotting using an anti-His antibody (GE Healthcare, Waukesha, WI). This high-titer viral stock was used to infect progressively larger volumes of Sf9 cells that were cultured in suspension in ProCulture spinner flasks (Corning Inc., Corning, NY), which in turn generated progressively larger volumes of high-titer viral stock. Finally, 1 L of Sf9 cells grown in suspension culture to a density of 1×10^6 cells/ml in Sf-900 II SFM were infected with viral particles at a multiplicity of infection of 0.5 (viral particles per cell). After 3 days, we harvested cell pellets by centrifugation at 500*g* for 15 minutes. Cell pellets were stored at −80 °C.

2.4. Sf9 cell membrane preparation, detergent screening and PFO–PAGE

All steps were performed on ice and protease inhibitors were present throughout. Sf9 cells from a 5-litre culture were resuspended in 50 ml phosphate-buffered saline (PBS) and disrupted by sonication for 6×5 seconds at 40% amplitude, with 10 seconds rest between pulses. Lysis was judged to be complete by light microscopy. Cell lysates were centrifuged at 2000*g* for 2×5 minutes to pellet cell