Non-canonical YXXGΦ endocytic motifs: recognition by AP2 and preferential utilization in P2X4 receptors

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Summary

During clathrin-mediated endocytosis, proteins on the cell surface are selected for inclusion in clathrin-coated vesicles by clathrin adaptors, mainly the adaptor complex AP2. The P2X4 subtype of ATP-gated ion channel has in its C-terminus two putative endocytic motifs: a canonical YXXΦ motif and a non-canonical YXXGΦ motif (YEQGL). We demonstrate that endocytosis of P2X4 receptors is mediated preferentially by the YXXGΦ motif because the YXXΦ motif is inaccessible to AP2 owing to the structure of the channel. The crystal structure of a complex between residues 160-435 of the μ2 subunit of AP2 and a P2X4 C-terminal peptide showed that the YEQGL motif binds to μ2 at the same site as YXXΦ motifs. Y and Φ residues are accommodated in the same hydrophobic pockets in μ2 with the extra residue between them being accommodated by changes in the peptide's backbone configuration, when compared to YXXΦ motifs. These data demonstrate that the family of potential tyrosine-based endocytic signals must be expanded to include motifs with an additional glycine at Y+3 (YXXGΦ).

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Introduction

Cells can control the density of receptors at the cell surface in order to influence the response to released extracellular ligand. P2X receptors bind extracellularly released ATP and contain an integral channel for the passage of cations (North, 2002). P2X receptors that contain P2X4 subunits, undergo rapid constitutive cycling between the plasma membrane and intracellular compartments (Bobanović et al., 2002; Royle et al., 2002). Changes in the relative rates of receptor removal or insertion are predicted to cause rapid changes in the density of receptors at the cell surface, and therefore alter the magnitude of response to ligand-binding (Royle and Murrell-Lagnado, 2003).

Transmembrane proteins that undergo such constitutive cycling are internalized through clathrin-mediated endocytosis (CME) (Brodsky et al., 2001). These cargo proteins often contain specific peptide motifs that are recognized by the AP2 clathrin adaptor protein complex and thus cargo proteins become clustered and subsequently included into clathrin-coated vesicles (Bonifacino and Traub, 2003). The AP2 complex is a compact heterotetrameric assembly comprising the large α and β2 subunits, a μ2 (medium chain) subunit and a σ2 (small chain) subunit (Collins et al., 2002). The homologous α and β2 subunits can be divided into N-terminal trunk domains composed of stacked α-helices and C-terminal bilobal appendage domains that are attached by flexible linkers (Owen et al., 2004). The β2 subunit interacts with clathrin through a LΦXΦ[DE] 'clathrin box' motif in its linker region and induces the assembly of a polymeric clathrin lattice at the plasma membrane (Brodsky et al., 2001). The μ2 subunit is involved in recognizing tyrosine-based sorting motifs within transmembrane proteins (Bonifacino and Traub, 2003). These motifs are of the form YXXΦ (where Φ is an amino acid with a bulky, hydrophobic side chain and X is any amino acid). Other endocytic signals, such as the [DE]XXXL[LI] and FXNPXY motifs, either bind to distinct sites on AP2 or to alternative clathrin adaptors, respectively (Bonifacino and Traub, 2003; Traub, 2003). Finally, polybasic motifs found in synaptotagmin (Grass et al., 2004) and in the AMPA-receptor subunit GluR2 (Lee et al., 2002) appear to act as AP2-association sequences; however, the precise binding site is unclear.

P2X4 receptors are ATP-gated ion channels with an extensive pattern of distribution throughout the CNS and periphery (Bo et al., 2003; Buell et al., 1996; Rubio and Soto, 2001; Tsuda et al., 2003). It has recently been reported that P2X4 receptors are upregulated following nerve injury and contribute to hypersensitivity to sensory stimuli (Tsuda et al., 2003). For the P2X4 receptor, we have previously proposed the existence of a non-canonical type of tyrosine-based endocytic motif with the sequence YXXGL, which mediates its constitutive cycling (Royle et al., 2002). This result was unexpected for two reasons: first, a canonical YXXΦ motif was present upstream of the YXXGL motif but was apparently not utilized and second, structural studies had indicated that spacing between the Y and the Φ residues is crucial for binding (Owen and Evans, 1998).

Here, we examine in vivo the roles of both the canonical motif (starting at Tyr372) and the non-canonical motif (starting at Tyr378) in the internalization of homomeric P2X4 receptors and a CD8-P2X4 cytoplasmic tail chimera. There were a number of questions that we wanted to address including: why is endocytosis in homomeric P2X4 receptors mediated through YXXGL in preference to YXXV, and also how do the YXXGΦ motifs bind to μ2 compared with YXXΦ motifs? We demonstrate that in the P2X4 receptor the canonical YXXV motif at Tyr372 is buried, presumably by interactions that allow formation of the conductance pore in the trimeric receptor, and so is inaccessible to AP2. By contrast, in the chimeric CD8 construct, in which the cytoplasmic tail is unstructured, the YXXV motif is preferentially utilized. Structure determination of a complex between μ2 residues 160-435 and the cytoplasmic C-terminal tail of P2X4 showed that YXXGΦ motifs bind to μ2 in a manner similar to canonical YXXΦ motifs, with Y and Φ residues able to bind in the same pockets in both cases. This implies that the definition of tyrosine-based endocytic motifs should be expanded to include YXXGΦ sequences.

Materials and Methods

Protein crystallography

Rat P2X4 C-terminus peptide (residues 375-384 VEDYEQGLSG) was synthesized and purified by HPLC at the Medical Research Council, Cambridge, UK. Signal-binding domain of μ2 (residues 160-435) was expressed and purified as described previously (Owen and Evans, 1998). Co-crystallization was carried out as described previously (Owen et al., 2001). The coordinates and structure factors have been deposited in the Protein Data Bank with the accession code 2BP5.

DNA constructs

The construction of epitope (AU5)-tagged and mutant P2X4 receptors has been described previously (Bobanović et al., 2002; Royle et al., 2002) and all new mutants for this study were made in a similar way. For construction of the CD8-4C chimera, the P2X4 C-terminus was amplified by PCR introducing an *Afl*II site in front of Tyr359 and a *Not*I site after the stop codon. Human CD8 α-chain cDNA in pBluescript II SK(–) (a kind gift of G. Ihrke, Cambridge Institute for Medical Research, UK) with an *Afl*II site after the transmembrane domain (Ihrke et al., 2000) and PCR product were digested with *Afl*II and *Hin*dIII. The product was amplified, and subcloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA) using *Hin*dIII and *Not*I sites which excised the coding sequence of GFP; CD8 constructs were therefore expressed under a cytomegalovirus (CMV) promoter. Point mutations in CD8 chimeras were made using the P2X4 receptor sequence or previously constructed mutants as templates. The sequences of all amplified regions were verified using automated DNA sequencing (Department of Genetics, University of Cambridge and MRC GeneService, UK).

Cell culture

Normal rat kidney (NRK) cells (gift from G. Ihrke) or human embryonic kidney (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 100 units/ml penicillin-streptomycin at 37°C and 5% CO_2. Cells were plated onto poly-L-lysine-coated cover slips and transiently transfected 12 hours later by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions or with calcium phosphate-mediated transfection (Royle et al., 2002).

Live-labeling immunofluorescence protocols

The basic protocol for live-labeling of AU5-tagged P2X4 receptors or CD8 chimeras is as follows. NRK cells were used 24 hours post-transfection. Anti-AU5 or anti-CD8 primary antibodies were applied in serum-free DMEM for 30 minutes at 37°C. Cells were washed five times with DMEM and then cooled to 12°C to prevent further trafficking of receptors. To detect surface and internalized receptors, labeled receptors at the cell surface were stained with a Cy3 (indocarbocyanine)-conjugated anti-mouse secondary antibody in DMEM for 2 hours at 12°C. Cells were then washed five times with DMEM at 12°C, fixed, permeabilized and stained with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody for 2 hours at RT to visualize pre-labeled internalized receptors.

To visualize P2X4-GFP constructs, HEK293 cells were fixed in 3% paraformaldehyde and 4% sucrose in PBS for 10 minutes at 4°C, incubated with TOPRO-3 for 10 minutes at RT, washed twice with PBS, and mounted onto slides with ProLong anti-fade (Molecular Probes) as a mounting medium.

Image collection and analysis

All non-quantitative imaging of GFP-tagged P2X4 receptors was done on a BioRad Radiance2000 confocal microscope with a 60× or 100× oil immersion objective. For live-labeling experiments, fluorescence was visualized using a Zeiss Axiovert LSM510 confocal microscope with a 63× oil immersion objective. For FITC-Cy3 double labeling, FITC and Cy3 were excited at 2% and 60% of 488 and 543 nm laser power, respectively. Images for analysis were taken where the confocal plane was focused on the middle of the cell (0.8 μm thickness), and at 0.14 μm/pixel at a depth of 8-bit. Identical acquisition-parameters were used for image capture of individual experiments. Images were imported into NIH image Version 1.62, cells were outlined and mean pixel values for each channel were obtained for a region over the cell soma. Experiments were repeated three times, and each time, data were analyzed for cells from two separate cover slips. The *n* values refer to the number of cells analyzed. All data are presented as the mean ± s.e.m. Data were processed in Microsoft Excel and Igor Pro Carbon (Wavemetrics). Statistical analysis was performed by Student's unpaired *t*-test or ANOVA with Dunnet's post-hoc test.

Electrophysiology

Standard whole-cell recordings from HEK293 cells expressing untagged P2X4 receptors were performed as previously described (Bobanović et al., 2002).

Antibodies and reagents

The following mouse monoclonal primary antibodies were used: affinity-purified anti-AU5 (5 μg/ml; Babco) and anti-CD8 (1:7000; BD Pharmingen). FITC- and Cy3-conjugated goat anti-mouse IgG were used as secondary antibodies (1:250; Jackson ImmunoResearch). Nucleic acids were stained using the carbocyanine monomer TOPRO®-3 iodide (Molecular Probes).

Results

P2X4 receptors have two tyrosine-based endocytic motifs, but only one is utilized

Homomeric P2X4 receptors are probably assembled from three P2X4 subunits (Aschrafi et al., 2004; Barrera et al., 2005; Jiang et al., 2003; Nicke et al., 1998). The topology of a P2X4 receptor and an individual subunit is shown in Fig. 1. There are two potential tyrosine-based endocytic motifs in the C-terminus of P2X4: a canonical motif (YXXV) at Tyr372 and a

non-canonical motif (YXXGL) at Tyr378 (Fig. 1C). If both motifs were equally able to bind μ2, then either motif alone would be sufficient to mediate endocytosis of the receptor. This is not the case however, because mutation of the non-canonical motif (Tyr378, Gly381 or Leu382) but not the canonical motif (Y372F or V375A), caused significant inhibition of P2X4 receptor endocytosis (Royle et al., 2002). In other words, the canonical YXXV motif cannot effectively substitute for the non-canonical YXXGL motif.

Why is the canonical YXXV motif within the C-terminus of P2X4 not utilized?

There are at least two potential reasons why the canonical motif is not efficiently utilized for endocytosis in the homomeric P2X4 receptor. First, Tyr372 could be inaccessible to μ2. This inaccessibility might result from the folding of this region or from the proximity of the YXXV motif to TM2. Second, the canonical motif might have unfavorable amino acids surrounding Tyr372. There are three positively-charged lysines surrounding Tyr372 (Lys370, Lys371 and Lys373), whereas three negatively-charged amino acids (Glu376, Asp377, and Glu379) surround Tyr378 (Fig. 1C). This might directly influence binding to AP2 through long-range electrostatic effects, or involve an interaction with negatively-charged phospholipids.

To help distinguish between these possibilities, we generated a chimeric protein, which had the C-terminus of P2X4 fused downstream of the transmembrane segment of CD8 (CD8-4C, Fig. 1). This produced a reporter construct, in which the role of residues within the P2X4 C-terminal tail can be studied in isolation, i.e. without potential interactions with the P2X4 N-terminal tail and transmembrane regions in the context of a trimer. Circular dichroism (CD) measurements of a peptide corresponding to the cytoplasmic C-terminal tail of P2X4 confirmed that it was, as expected, unstructured in aqueous solution (data not shown). Internalization was assayed using an antibody to the extracellular N-terminus of CD8. When

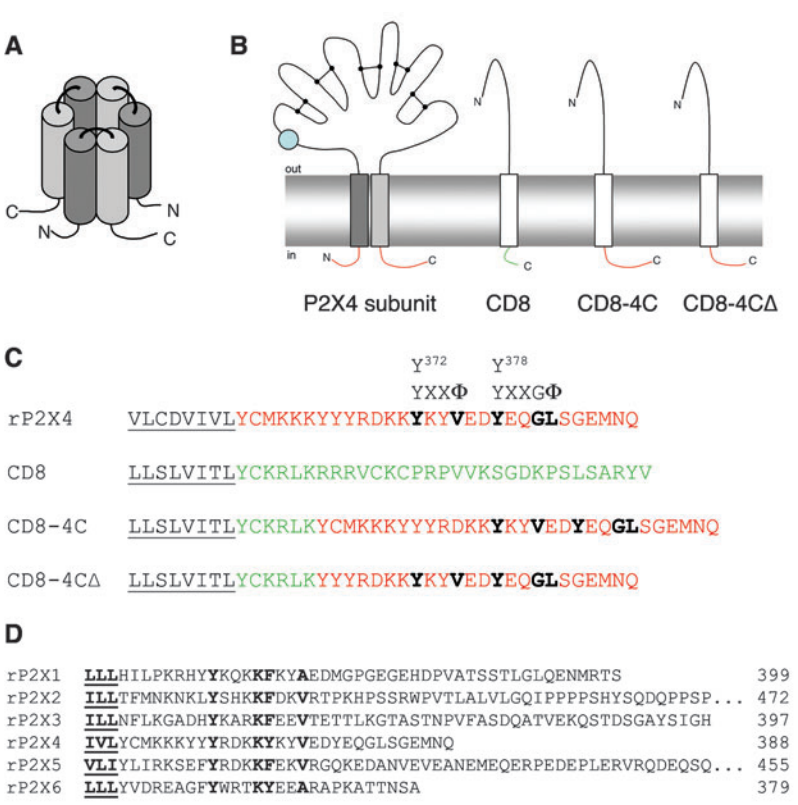

Fig. 1. Schematic diagram of constructs used in this study. (A) P2X receptors are thought to assemble as homo- or hetero-trimers. Extracellular domains are not shown to aid visualization. (B) A single P2X4 subunit has intracellular N- and C-termini and two transmembrane domains. The position of epitope AU5 at residue 76 is shown as a blue circle. Diagrams of CD8 chimeras, CD8 (left), CD8-4C (middle) and CD8-4CΔ (right). (C) Primary sequences of the C-termini of the constructs shown in B. (D) Sequence alignment of C-terminal regions of P2X subunits 1-6 from *Rattus norvegicus*, full or partially conserved residues are shown in bold. Total number of residues of each subunit is shown to the right, P2X2 and P2X5 have been truncated. Transmembrane helices in C and D are indicated by underlined regions. P2X4 is the only P2X family member that has a YXXGΦ motif in its C-terminus. Other receptors and channels with a YXXGΦ motif include the inward rectifier K^+ channel Kir2.3, the adenosine receptor A2a and the cannabinoid receptor CB-2 (Royle et al., 2002).

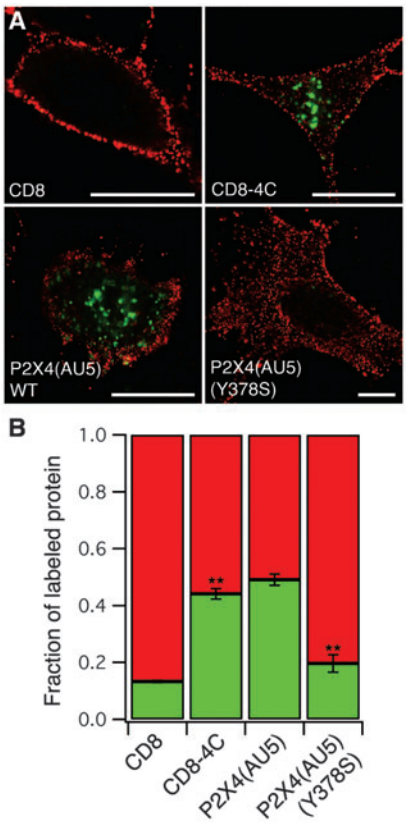

Fig. 2. The C-terminus of P2X4 alone can mediate internalization from the plasma membrane. (A) Representative confocal images of NRK cells transfected with either CD8-reporter or CD8-4C-reporter constructs, P2X4(AU5) wild-type, or Y378S-mutant receptors. Cells were live-labeled with anti-CD8 or anti-AU5 for 30 minutes at 37°C. Cell surface (red) and internalized (green) receptors were visualized using Cy3- and FITC-conjugated secondary antibodies, before and after permeabilization, respectively. Bars, 10 μm. (B) Histogram, showing the proportion of protein detected at the cell surface (red) or in internal compartments (green) after labeling (n=18-54). **, P<0.01.

expressed in NRK cells, wild-type CD8 was targeted to the plasma membrane and showed little internalization during a 30-minute period (Fig. 2). By contrast, there was considerable internalization of the CD8-4C chimera and surface expression was much lower. As replacement of the C-terminus with that of P2X4 was sufficient to confer internalization to CD8, this indicated that all the determinants for internalization are contained within the C-terminal region. In fact, the amount of internalization seen in CD8-4C was similar to that seen for the intact P2X4 receptor (Fig. 2B). The CD8-4C chimera, however, exhibited a key difference compared with the full-length P2X4 receptor. In intact P2X4 receptors, a single mutation in the YXXGL motif (Y378S) substantially inhibited internalization (Fig. 2), whereas CD8-4C(Y378S) produced only a modest decrease in its internalization (Fig. 3). This suggests that in CD8-4C(Y378S), endocytosis is mediated by another motif. In support of this, we found that mutations at Tyr372 (Y372A or Y372F) markedly reduced the internalization of CD8-4C, indicating that in the CD8-4C chimera, the YXXV motif is operational. In our assay, double mutations of Tyr372 and Tyr378 produced no further inhibition of endocytosis as compared with the effect of a single Tyr372 mutation (Fig. 3). Thus, within this chimera, both tyrosine-based motifs could mediate endocytosis and there was actually a preference for YXXV over YXXGL. The internalization of CD8-4C by the YXXV motif was presumably through an interaction with AP2, which was confirmed by the observation that a short peptide corresponding to this region (KYKYVE) bound and co-crystallized with the signal-binding domain of μ2 (data not shown). In addition, because both motifs were operational in CD8-4C, this argues against the idea that the charged environment surrounding each motif could influence their interaction with AP2.

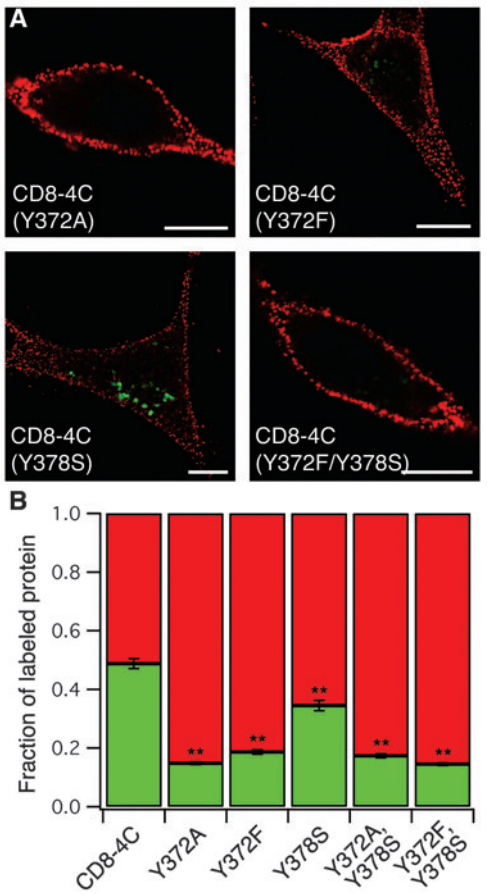

Fig. 3. In the CD8-4C chimera, both YXXV and YXXGL motifs are functional. (A) Representative confocal images of CD8-4C chimeras with point mutations in the intracellular domain following live-labeling with anti-CD8 as described in Materials and Methods. Bars, 10 μm. (B) Histogram, showing the proportion of protein detected at the cell surface (red) or in internal compartments (green) after labeling (n=61-76). **, P<0.01.

We next considered the possibility that distance from the transmembrane region is crucial for recognition of the motifs. In the CD8-4C chimera, several extra residues are predicted to be between the end of the TM segment and Tyr372 as compared with the receptor (Fig. 1). Thus, it is possible that in the receptor, Tyr372 is too close to the transmembrane region to bind μ2. Unfortunately, we were unable to study the trafficking of a chimeric construct of the CD8 extracellular domain and the TM2 and C-terminus of P2X4, because this protein was retained in the ER and was not delivered to the cell surface, most probably owing to aggregation of the construct caused by the absence of TM1 of P2X4. We therefore generated an additional CD8 chimeric construct that had six amino acids deleted at the start of the P2X4 C-terminus (CD8-4CΔ). This time, the individual Y372A and Y378S mutations produced only slight reductions in internalization but together they produced a greater inhibition (see supplementary material, Fig. S1). Thus, in CD8-4CΔ, both motifs were again operational but there was no clear preference for the Tyr372- or Tyr378-based motifs. Because each motif mediated internalization, then in the full-length receptor the proximity of Tyr372 to TM2 and the membrane surface is unlikely to prevent binding to μ2.

In P2X4 receptors, Tyr372 is necessary for receptor structure and function but not for endocytosis

The possibility remained that in the context of the intact homomeric P2X4 receptor, utilization of the YXXV motif is inhibited because the C-terminus is folded in a way that makes Tyr372 inaccessible to the AP2 complex. Such folding might be owing to the involvement of the membrane proximal region of the C-terminal tail, possibly in conjunction with the N-terminal tail, in formation of the conductance pore and would therefore not occur in the CD8 reporter construct. We reasoned that, if Tyr372 were buried rather than exposed to the cytosol, non-conservative substitutions are likely to disrupt the folding of this region in the full-length receptor. Fig. 4 shows a comparison of the steady-state subcellular distribution of P2X4-GFP and related mutants expressed in HEK293 cells. For wild-type P2X4-GFP very little fluorescence was at the plasma membrane and the majority of receptors were found intracellularly (Bobanović et al., 2002; Royle et al., 2002). At steady state, mutants that are internalization-deficient show greater surface expression. This is seen as a distinctive fluorescence at the plasma membrane and in filopodia that is absent from the wild-type receptor. As observed in neurons (Royle et al., 2002), mutation of Tyr378 in the homomeric P2X4 receptor to either alanine or phenylalanine caused an increased amount of fluorescence at the cell surface (Fig. 4).

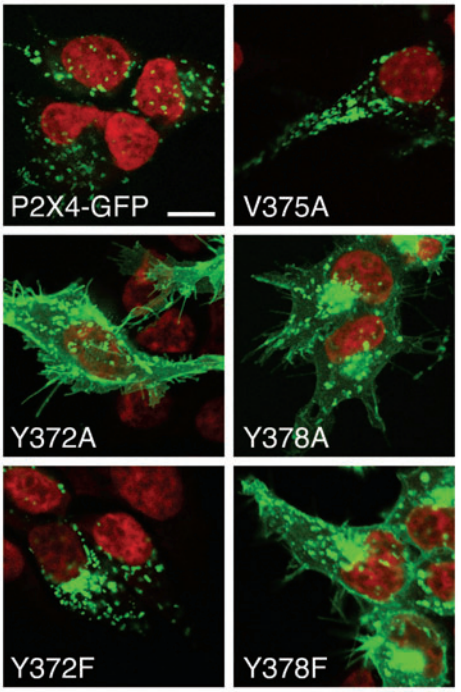

Fig. 4. P2X4-GFP mutants Y372A, Y378A and Y378F, but not Y372F or V375A, show internalization defects. Representative confocal micrographs of P2X4-GFP and related mutants (Y372A, Y372F, V375A, Y378A and Y378F) expressed in HEK293 cells. Nucleic acids are shown in red. Internalization-deficient mutants (Y372A, Y378A, Y378F) have greater plasma membrane expression. Bar, 10 μm.

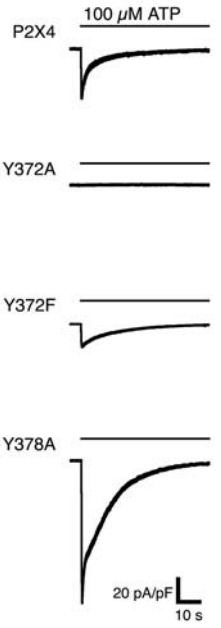

Fig. 5. Tyr372 is essential for function. Typical inward currents elicited by application of ATP (100 μM) at untagged P2X4 receptors and related mutants (Y372A, Y372F, Y378A) expressed in HEK293 cells. Y372F is functional, but Y372A is not. Note the large response of Y378A mutant, reflecting the abundant surface distribution.

Interestingly for Tyr372 mutants of the canonical YXXΦ motif, Y372A had pronounced surface expression whereas Y372F was distributed similarly to wild-type, i.e. it underwent normal CME. Mutation of the Φ position (V375A) also had no effect on the internalization of intact receptor, again suggesting that this motif is not used by the homomeric P2X4 receptor for endocytosis. These results pose the question: how can only one of three mutations in the same motif affect internalization when all should prevent interaction with μ2 subunit of AP2?

One possibility is that the Y372A mutation actually causes a change in receptor conformation and/or multimericity that might manifest itself in a change in functionality of the P2X4 receptor. Hence, we tested the effect of these mutations on the function of the untagged receptor. Using whole-cell patch-clamp recording, we measured the current flow through the channels in response to binding of 100 μM ATP. Wild-type P2X4 receptors exhibited large inward currents upon ATP application. All point mutants were functional, with the exception of Y372A (Fig. 5). Note that Y378A had very large currents per unit-area of membrane, reflecting the abundant surface distribution resulting from impaired endocytosis. Despite Y372A having an abundant surface expression, comparable to Y378A, no current was elicited at this mutant by ATP, indicating that the mutation had abolished function. The different types of P2X-receptor subunits (P2X1-6) all have either a tyrosine or phenylalanine at this position (Fig. 1D), suggesting that a bulky, aromatic residue near the intracellular pore is essential for channel activity in P2X receptors. The non-conservative Y372A mutation appeared to cause a conformational change that resulted in a non-functional receptor such as trapping the receptor in a closed state. This is supported by the observation that, unlike all other mutants studied, the Y372A mutant could not be 'live-labeled' with anti-AU5 despite having a high surface expression (data not shown). The AU5 epitope in this construct was present, however, because in formaldehyde-fixed permeabilized cells the mutant could be detected by immunolabeling with anti-AU5 (data not shown). Taken together, the non-functionality of the receptor and the unavailability of the AU5 epitope, caused by mutating Tyr372 to Ala, suggest that a change in receptor structure has occurred. If this scenario were true, inhibition of receptor endocytosis in the Y372A mutant would result from the YXXGL at Tyr378 no longer being accessible to AP2 owing to the conformational change induced by the Y372A mutation.

The intolerance of alanine at position 372 is consistent with Tyr372 being at least partially buried within the receptor. We therefore conclude that in the intact P2X4 receptor, the canonical YXXΦ motif cannot be efficiently utilized because it is inaccessible to AP2. This inaccessibility is probably owing to the structure of the functional trimeric channel.

Mechanism of non-canonical YXXGΦ motif binding to μ2

To determine the molecular nature of the interaction between YXXGΦ motifs and μ2, the structure of a complex between the two was determined by protein X-ray crystallography. A synthetic peptide comprising amino acids 375-384 (VEDYEQGLSG) from P2X4 was co-crystallized with the signal-binding domain of μ2 and the structure solved to a resolution of 2.8 Å (Fig. 6A; data collection and refinement statistics are shown in Table 2). Good electron density was achieved for residues Y-3 to Y+5 (Tyr378 is considered as position Y0, Fig. 6B). The structure shows that, in many ways, the binding of the YXXGL motif is similar in concept to the binding of a YXXΦ motif; Tyr378 (Y0) and Leu382 (Y+4) of

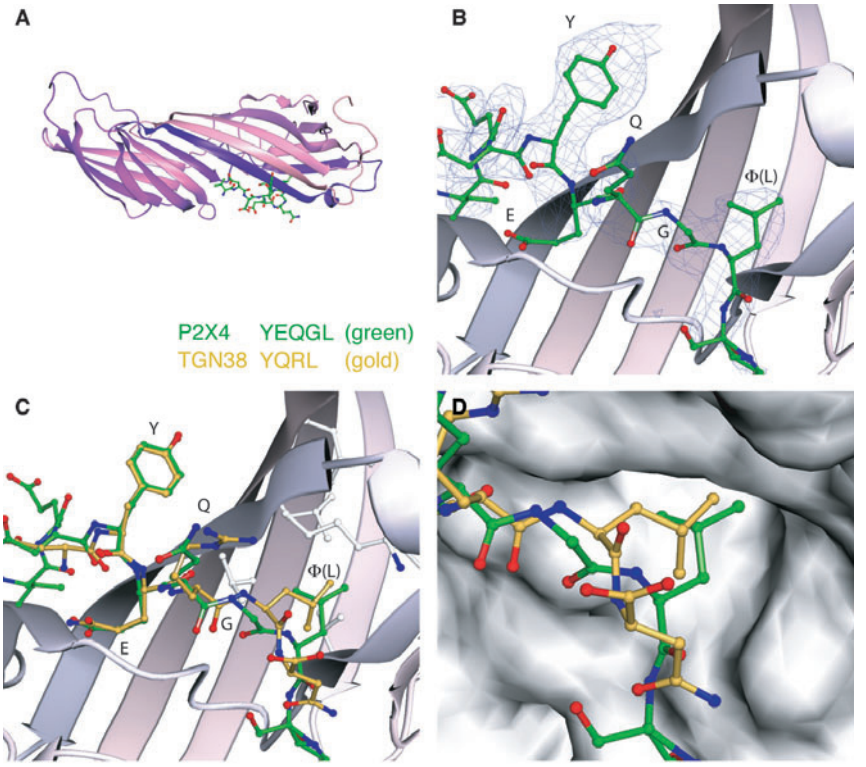

Fig. 6. Interaction of a non-canonical tyrosine-based endocytic motif with μ2-adaptin. (A) Structure of the signal-binding domain of μ2 complexed with a peptide corresponding to P2X4 amino acids 375-384. (B) Electron density (mFo-DFc) map of the P2X4 peptide bound to μ2; σ level is 2.56. (C) Overlay of the P2X4 endocytic motif (YXXGL, green) with that of TGN38 (YXXL, gold), to compare binding of non-canonical and canonical tyrosine-based endocytic motifs. (D) Close-up view of the second hydrophobic pocket of μ2 to compare the entry and co-ordination of the Y+4 and Y+3 leucine residues of YXXGL and YXXL, respectively.

P2X4 bind within the same two pockets on the surface of μ2 occupied by the Y0 tyrosine and Y+3 Φ residues from all YXXΦ complexes determined to date. When the new structure is superimposed on the TGN38 crystal (PDB code 1BXX), it can be seen that both tyrosine residues occupy virtually the same position (Fig. 6C). The binding of Tyr378 in the first hydrophobic pocket is supported by results from live cells stably expressing dominant-negative μ2 (D176A,W421A) (Nesterov et al., 1999), in which there was an approximately 3-fold reduction in P2X4-receptor internalization (Royle et al., 2002). As shown in Fig. 6D, the terminal aliphatic portions of the Φ residue (a leucine) at Y+3 in TGN38 and the Φ residue (again a leucine) at Y+4 in P2X4 occupy the same hydrophobic pocket, although both the orientation of the side chain and the point of entry of the side chain into the pocket are completely different. As there is no change in the overall structure of the signal-binding domain of μ2 and the hydrophobic pockets remain in the same place, i.e. it is a rigid template, how can the Y+4 leucine of P2X4 (Leu382) fit into the Φ pocket? Akin to YXXΦ peptides, the backbone of the YXXGL peptide again adopts an extended conformation that allows the formation of standard β-sheet H-bonds to the main chain of μ2. The extra residue between Y+2 and Φ is accommodated by a 'kink' in the peptide backbone that causes a relative 2.2 Å movement of the Φ residue α-carbon and thus the Φ side chain enters the pocket from the opposite side compared to YXXΦ. A glycine at Y+3 in YXXGΦ (Gly381) affords sufficient flexibility to the main chain to allow it to adopt a conformation that would be unfavorable for a residue with a side chain (Fig. 6D). In fact the phi-psi angles (+84,+142) of the main chain are such that only a glycine is likely to be allowed in this conformation (Ramachandran and Sasisekharan, 1968). This explains mutant screening data (summarized in Table 1), in which mutation of Tyr378, Leu382 or Gly381 all caused a significant decrease in receptor internalization.

The structural data presented here indicate that non-canonical tyrosine-based endocytic motifs (YXXGΦ) bind to μ2 in a similar way as canonical YXXΦ motifs and are therefore functionally interchangeable. This was also demonstrated in vivo, where the subcellular distribution of a GFP-tagged P2X4 receptor, in which the endogenous YXXGΦ motif had been replaced with the YXXΦ motif from TGN38 (YQRL), was indistinguishable from wild type (Fig. 7). Moreover, we confirmed that the substituted YQRL motif in this mutant actually mediates endocytosis, because replacing YXXGΦ with AQRL ablated internalization (Fig. 7). We conclude that, in the case of P2X4, the selectivity of AP2 for

Table 1. Summary of live-labeling experiments showing an inhibition of P2X4 receptor endocytosis with mutations in the YXXGL motif

P2X4(AU5) construct	Mean internalized fraction	Mean surface fraction	s.e.m.	Sequence (aa 372-383)	Different from wild type
WT	0.420	0.580	0.020	YKYVEDYEQGLS	–
V375A	0.429	0.571	0.028	YKYAEDYEQGLS	n.s.
Y378A	0.160	0.840	0.016	YKYVEDAEQGLS	**
Y378F	0.148	0.852	0.009	YKYVEDFEQGLS	**
G381A	0.248	0.752	0.022	YKYVEDYEQALS	**
L382A	0.153	0.847	0.013	YKYVEDYEQGAS	**

P2X4(AU5) receptors with or without point mutations were expressed in neurons. Cells were incubated with anti-AU5 for 30 minutes at 37°C. Surface and internalized receptors were detected with Cy3- and FITC-conjugated secondary antibodies, before and after permeabilization, respectively. Surface or internalized signal is expressed as a fraction of the total AU5 label (surface + internalized). **, $P<0.01$.

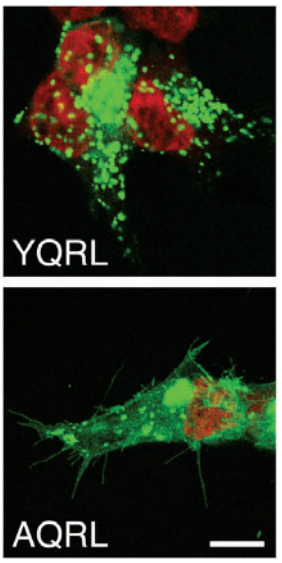

Fig. 7. YXXGΦ and YXXΦ motifs are interchangeable in the P2X4 receptor. P2X4-GFP with residues YEQGL replaced by YQRLN (YQRL, above) was indistinguishable from wild type (see Fig. 5, WT). Mutation of Y378A abolished internalization (AQRL, below). Cells were co-stained for DNA (red). Bar, 10 μm.

Table 2. X-ray data collection statistics

Data collection	
Resolution (Å) (outer bin range)	24 – 2.8 (2.95-2.8)
Completeness	0.998 (1.0)
Multiplicity	17.5 (15.4)
Wilson plot B ($Å^2$)	92
R_{merge}*	0.072 (0.751)
R_{meas}†	0.074 (0.777)
<<I>/<σ>>	36.0 (3.7)
Refinement statistics	
R factor‡ (working set)	0.193
R_{free}	0.246
<B> ($Å^2$) (mean, overall)	68
$N_{reflections}$ (N_{free})	15655 (825)
N_{atoms} (N_{water})	2182 (69)
R_{msd} bond length (Å)	0.023

Values in parentheses apply to the highest resolution shell.
*$R_{merge}=\Sigma\Sigma_i\ |I_h–I_{hi}|\ /\ \Sigma\Sigma_i\ I_{hi}$, where I_h is the mean intensity for reflection h.
†$R_{meas}=\Sigma_\sqrt{(n/n–1)}\Sigma_i\ |I_h–I_{hi}|\ /\ \Sigma\Sigma_i\ I_{hi}$, the multiplicity weighted R_{merge}.
‡$R=S\ |F_P–F_{calc}|\ /\ F_P$.

YXXGΦ over YXXΦ is simply owing to dormancy, i.e. conformational masking of the YXXΦ motif.

Discussion

Within the P2X4 C-terminus both canonical and non-canonical tyrosine-based endocytic motifs are present and in close proximity to one another. In vivo, internalization of homomeric P2X4 receptors is mediated by YXXGL in preference to YXXV. By mutational analysis of a chimeric CD8 reporter protein, we show that the YXXV motif could be used outside the context of the receptor but still within the context of the P2X4-C-terminus. Within the homomeric P2X4 receptor, presumably owing to its native structure, the YXXV motif is dormant. Consistent with the notion that Tyr372 is at least partially buried, the non-conservative alanine substitution had a profound effect on the function and trafficking of the receptor, whereas a more conservative phenylalanine substitution had little effect on either. But how is the proximal part of the P2X4 C-terminus folded so as to bury Tyr372? This residue could be involved in the trimerization of P2X4 to form a functional channel and/or it might actually contribute to the structure of the channel itself. So far, studies on residues important for conductance have concentrated on residues believed, on the basis of secondary structure prediction, to be part of TM2 (Egan et al., 1998; Li et al., 2004; Migita et al., 2001). It is possible that the pore possesses a small cytoplasmic domain or 'vestibule' as proposed for other channels (Jiang et al., 2002; Nishida and MacKinnon, 2002) and consistent with this concept, mutation of Y367A or K371A in P2X4, which are C-terminal to the proposed TM2 boundary, leads to non-functional channels (Chaumont et al., 2004). Tyr372 might also participate in interactions between the cytoplasmic C-terminal and N-terminal regions of the same or different P2X4 molecules, resulting in AP2 inaccessibility. Another possibility is that Tyr372 is actually embedded in the plasma membrane as a short helical region. Such a structure has been described for regions adjacent to transmembrane segments of the SNARE protein VAMP-2 (Kweon et al., 2003) and is so able to interact directly with phospholipids in the plasma membrane. The primary structure of this region (Fig. 1) contains a PtdIns(4,5)P_2-binding signature [KXK(HY)] found in plasma membrane clathrin adaptors including AP180 (Ford et al., 2001) and α-adaptin (Collins et al., 2002), in which this sequence adopts a helical conformation in order to bind the PtdIns(4,5)P_2 headgroup. However, CD measurements of the cytoplasmic C-terminal domain of P2X4 in isolation from the N-terminus indicated that it is unfolded in solution, and that addition of Ins(1,4,5)P_3 (i.e. the PtdIns(4,5)P_2 headgroup) caused no increase in the helical content of this unstructured polypeptide (data not shown). Indeed by isothermal titration calorimetry (ITC), no interaction between the P2X4 C-terminal tail and Ins(1,4,5)P_3 was detected (data not shown). This suggests that this membrane-embedding–phospholipid-binding possibility might be incorrect. Although, when isolated, the P2X4 C-terminus is unstructured, the identification of conformational masking of Tyr372 in the intact multimeric receptor, provides an indication that the membrane proximal segment of P2X4 is structured, whereas the remainder of the C-terminus, perhaps from Val375 onwards, is unstructured and therefore free to interact with AP2.

The structure of a complex between the signal binding domain of μ2 (residues 160-435) and the non-canonical YXXGΦ motif from P2X4 shows that binding of YXXGΦ and canonical YXXΦ motifs occur in a similar manner at the same site. In both cases Y and Φ side chains (in both cases leucine residues) sit in the same chemically-compatible pockets, although the orientation of the two leucine side chains in the Φ pocket differ. The extra residue between the Y- and Φ-specificity-defining residues in the YXXGΦ motif is accommodated by a bulge in the backbone of the motif. This conformation is only energetically favorable when the 'extra' residue is a glycine, owing to steric clashes that would be made by side chains in the case of a non-glycine. Thus the possible range of tyrosine-based endocytic motifs must be extended to include YXXGΦ sequences.

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