Epithelial Membrane Proteins Induce Membrane Blebbing and Interact with the P2X$_7$ Receptor C Terminus*

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The binding of extracellular ATP to the P2X$_7$ receptor opens an integral cation-permeable channel; it also leads to membrane blebbing and, in certain immune cells, interleukin-1β secretion and eventual death. The latter three effects are unique to the P2X$_7$ receptor; also unique among P2X receptors is the long intracellular C terminus of the protein. We have shown that the C-terminal domain of the P2X$_7$ receptor is responsible for the cell blebbing phenotype. A screen for proteins that associate with the C-terminal domain of the P2X$_7$ receptor and might mediate the blebbing phenotype, identified epithelial membrane protein 2 (EMP-2). The interaction between EMP-2 and P2X$_7$ was confirmed biochemically by co-immunoprecipitation, co-purification, and glutathione S-transferase pull-down assays, and this interaction was entirely dependent on the C-terminal domain of P2X$_7$. The P2X$_7$ receptor also interacted with the other members of the epithelial membrane protein family (EMP-1, EMP-3, and PMP-22). All four EMPs were found to be expressed in HEK-293 cells and in THP-1 monocytes, which express P2X$_7$ receptors. Interestingly, the constitutive overexpression of any of the EMPs in HEK-293 cells led to cell blebbing, annexin V binding, and cell death, by a caspase-dependent pathway. These findings suggest that the P2X$_7$, C-terminal domain associates with EMPs, and this interaction may mediate some aspects of the downstream signaling following P2X$_7$ receptor activation.

P2X$_7$ receptors belong to a family of ion channels gated by extracellular ATP (1), all with the same predicted topology of two transmembrane domains and intracellular N and C termini (1). The P2X$_7$ receptor shares 40–45% amino acid identity with the other P2X proteins, but it is structurally distinct at the C terminus, extending for an additional 100–200 amino acids (2). P2X$_7$-α receptors are widely distributed in both neuronal and non-neuronal cells, whereas P2X$_7$ receptors are most highly expressed in immune and epithelial cells (3, 4). Brief stimulation (10–30 s) of the P2X$_7$ receptor leads to the formation of a channel permeable to large cations, as is also seen for some of the other P2X receptors (2, 5). However, more prolonged activation of the P2X$_7$ receptor results in extensive membrane blebbing from within seconds to minutes (5), and eventual cell death (5–7), although such responses are not observed with other P2X receptors. We hypothesized that the C terminus of the P2X$_7$ receptor might engage other cellular proteins to mediate its distinct responses of membrane blebbing and cell death. We tested this hypothesis by using a construct containing the soluble C-terminal domain (Asn$_{356}$–Tyr$_{395}$) of the rat P2X$_7$ protein as bait in a yeast two-hybrid screen. Because HEK-293 cells expressing P2X$_7$ receptors exhibit pronounced blebbing and eventual cell death when stimulated with ATP (5), we sought interacting proteins using a HEK-293 cell library.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The full-length cDNA encoding epithelial membrane protein EMP-2 was isolated using PCR from the pACT-2 library and subcloned into pDNA3.1-Myct/HisA vector, hence incorporating a Myc epitope and His tag at the C terminus of the expressed protein. Full-length EMP-1, EMP-3, and peripheral myelin protein-22 (PMP-22) were isolated using RT-PCR (see below) from HEK-293 cell mRNA and subcloned into pDNA3.1-Myct/HisA. Full-length rat P2X$_7$, cDNA was expressed with a C terminus epitope tag (EYTMPMEE[P2X7,EE]), in pDNA3. cDNAs encoding truncated rP2X$_7$ receptors, to residue 418 ([418] and an EE-tagged truncated receptor to residue 392 ([ΔCTP2X7][EE]) were also subcloned into the pDNA3.1+ plasmid.

Cell Imaging—Fluorescence was measured using a Zeiss Axiovert 100 and Fluor 20x objective with Photonics monochrometer imaging (Photonics, Graefelfing, Germany). HEK-293 cells were loaded for 40 min at 37 °C with 2 μM Fluo-4AM (Molecular Probes). Images were captured at 0.5 Hz. Calcium concentrations were calibrated with standards, where free Ca$^{2+}$ was calculated using the EQCAL program, in 500 nM ionomycin to allow free exchange between cells and the extracellular medium.

Yeast Two-hybrid Screen—A bait construct was made (encoding Asn$_{356}$–Tyr$_{395}$ of rat P2X$_7$) by amplification of the rat P2X$_7$, cDNA (primers: 5′-GGCCGGGAATTCACAGATGACGATGCATGCAGGATCTTGAGG AG C3′- and 5′-GCGGCGCTGAGTCGAGTACCTGACGC CGC C3′). The PCR product was subcloned into pAS2–1 using EcoRI and SalI (XhoI on PCR product) restriction sites, thereby generating a C-terminal fusion to the binding domain of Gal-4. The HEK-293 cell library (CLONTECH) in pACT2 as a fusion with the activation domain of Gal-4, was amplified according to the manufacturer’s instructions (CLONTECH). The Matchmaker Two-hybrid System (CLONTECH) was used for screening.

RT-PCR—Messenger RNA was isolated from HEK-293 cells using the Poly(A)Pure mRNA isolation kit (Ambion), followed by reverse transcription using SUPERSCRIPT™ II (Invitrogen) RNase H-reverse transcriptase. The following primers were used: actin, 5′-GGCTTCTCTCC AGGTCTTCCTTCCTCG-3′ and 5′-ACAGAGATCTGTCGCTCACGGAGG3′ (product predicted from cDNA 241 bp; product predicted from genomic source 400 bp); PMP-22, 5′-GGCGGAAGCTTGGACACATGCTCTCCTCTTGCTGATAGATC3′ and 5′-GGCGCGCGTGGATTGGTCCTCCCGAGAATCATCAC-ATAGATGCAC3′; EMP-1, 5′-GGCGGAAAGCTCTGACCGACATGCTGTA TGGATGATC3′; and EMP-2, 5′-GCGCGGGAATTCACAGATGACGATGCATGCAGGATCTTGAGGAG C3′.
Fig. 1. Truncation of the P2X₇ receptor prevents ATP-mediated cell blebbing. A, immunocytochemistry of the rP2X₇ receptor (WT) and C-terminal truncation (∆418), expressed in HEK-293 cells. Bz-ATP (30 μM) was applied for 2 min (WT cells), or Bz-ATP (200 μM) for 5 min (∆418), prior to fixation and staining. The receptor was visualized using the antibody against the rP2X₇ receptor ectodomain. B, left, video images of cells were captured after stimulation with Bz-ATP (100 μM) for the times indicated. Transfected cells were identified by expression of cotransfected GFP. Right, cells imaged for Ca²⁺ uptake in response to Bz-ATP (100 μM) for WT and ∆418-truncated rP2X₇ receptor. C, pooled results for blebbing (WT, n = 120 cells; ∆418, n = 241; mock-transfected, n = 27) and Ca²⁺ responses (WT, n = 53 cells; ∆418, n = 53; mock-transfected, n = 27); results are shown as percent of cells responding.

CTGGCTGGTATC-3′ and 5′-CGGCCCTCGAGTTTGCGCTTCCTCAGTATCA-GATAGAG-3′. EMP-2, 5′-GCGGAAGCTGCACTGTTCTTCCAGGAGCTTCTCGCTTCTC-5′. EMP-3, 5′-GCGGAAGCTGCACTGTTCTTCCAGGAGCTTCTTCTC-5′. EMP-4, 5′-CGGCCCTCGAGTTTGCGCTTCCTCAGTATCA-GATAGAG-3′. Subcloned products were confirmed by sequencing (ABI BigDye® or Beckman-Coulter CEQ 2000 Dye Terminator).

Co-purification on Affinity Resin—HEK-293 cells were co-transfected with 1 μg of plasmid encoding EMP-2myc/His (or other family members) and 1 μg of plasmid encoding P2X₇-EE, using LipofectAMINE 2000, according to the manufacturer’s instructions (Invitrogen). After 24–36 h, cells were harvested by washing with phosphate-buffered saline (PBS) and then solubilized in extraction buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 5 mM imidazole, 1% Nonidet P-40) for 15 min on ice. Particulate matter was removed by two centrifugations at 16,000 × g for 15 min at 4 °C. TALON® resin (Cobalt affinity resin, CLONTECH) was washed in extraction buffer, and cell extracts were added and incubated by rotating at 4 °C for 1 h. Unbound protein was removed, and the resin was washed four times in extraction buffer for 5 min per wash by rotating. The bound protein was eluted in 30 μl of elution buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 200 mM imidazole, 1% Nonidet P-40) by heating to 92 °C for 15 min. Eluted proteins were analyzed by using SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Samples were tested for the presence of P2X₇ receptor using a C-terminal polyclonal antibody (Alomone). Samples from original crude cell extracts that had not been TALON®-purified were tested for the presence of P2X₇ and EMPs by immunoblotting using the C-terminal polyclonal antibody (Alomone) or c-Myc antibody (9E10 clone from Santa Cruz Biotechnology), respectively. Blots were then reacted with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Vector Laboratories) and detected using the ECL-Plus system (Amersham Biosciences). All antibody incubations were carried out in PBS-Tween (0.1%) plus 2% nonfat milk, and washes of the blots were performed in PBS-Tween (0.1%),

Co-immunoprecipitation—HEK-293 cells were transfected with EMP-2myc/His (or other family members) plus P2X₇-EE as described above. Controls were used for EMP-2myc/His co-transfected with Ref 2–1EE, a 25-kDa unrelated protein (8). Cells were washed with PBS and then treated with solubilization buffer (PBS, Nonidet P-40 (1%), and Calbiochem Protease Inhibitor Mixture Set III) for 15 min at 4 °C. Particulate matter was removed by two centrifugations at 16,000 × g for 15 min at 4 °C. For the pre-clearing step, cell extracts were added to protein G-Sepharose (Amersham Biosciences), pre-washed in solubilization buffer, and incubated by rotating at 4 °C for 2 h. Unbound protein was removed and incubated with a monoclonal anti-EE antibody (Covalent Affinity), or Bz-ATP (200 μM) for WT cells and 5 μM for ∆418, for 15 min at 4 °C. This reaction product was then added to a new aliquot of protein G-Sepharose (Amersham Biosciences), pre-washed in solubilization buffer, and incubated by rotating at 4 °C for 2 h. Unbound protein was removed and incubated with a monoclonal anti-EE antibody (Covalent Affinity) or Ref 2–1EE (lane 4). B, immunoprecipitation of cell extracts expressing P2X₇-EE or Ref 2–1EE with EMP-2myc/His with anti-EE results in coprecipitation of EMP-2 with P2X₇ (lane 4) but not with Ref 2–1EE (lane 3). EMP-2 was detected by immunoblotting with the c-Myc antibody.

by incubating at room temperature for 15 min. Eluted proteins were analyzed by using SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Samples were tested for the presence of P2X₇ receptor using a C-terminal polyclonal antibody (Alomone). Samples from original crude cell extracts that had not been TALON®-purified were tested for the presence of P2X₇ and EMPs by immunoblotting using the C-terminal polyclonal antibody (Alomone) or c-Myc antibody (9E10 clone from Santa Cruz Biotechnology), respectively. Blots were then reacted with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Vector Laboratories) and detected using the ECL-Plus system (Amersham Biosciences). All antibody incubations were carried out in PBS-Tween (0.1%) plus 2% nonfat milk, and washes of the blots were performed in PBS-Tween (0.1%).

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Supernatants were analyzed for lactose dehydrogenase (LDH) activity using the LDH-optimized kit (Sigma) and compared with control extracts treated with 1% Triton X-100 as the maximal activity. At 48 h after treatment, cells were harvested and cell viability was determined by trypan blue exclusion.

Annexin V Binding — Phosphatidylserine exposure on the extracellular leaflet of the membrane was measured by annexin V binding (9). HEK-293 cells were transfected as described, with plasmids encoding EMP-1, EMP-2, EMP-3, PMP-22, and P2X2 proteins. Cells were washed and provided with fresh medium 24 h post-transfection and harvested at 48 h. The binding of annexin V conjugated to fluorescein isothiocyanate (Molecular Probes) was measured by using flow cytometry. Cells were also gated by propidium iodide, used in this case as a marker of non-viable, necrotic cells. The number of cells binding annexin but not propidium was normalized to that observed with staurosporine (Molecular Probes) at 48 h. The binding of annexin V conjugated to fluorescein isothiocyanate (Molecular Probes) was measured by using flow cytometry. Cells were also gated by propidium iodide, used in this case as a marker of non-viable, necrotic cells. The number of cells binding annexin but not propidium was normalized to that observed with staurosporine (Molecular Probes) at 48 h.

Immunocytochemistry — HEK-293 cells were washed in PBS, fixed in Zambonii’s fixative (20 min), and blocked in 5% goat serum in PBS with 2% Triton X-100 (30 min), and then incubated with primary antibody (anti-c-Myc, anti-EE, anti-P2X7, ectodomain (11)) for 1–2 h at room temperature, or overnight at 4°C. Cells were washed, and secondary antibody was added for 1 h at room temperature.
FIG. 5. Overexpression of epithelial membrane proteins induces cell death. Plasmids encoding each epithelial membrane protein were separately transfected into HEK-293 cells. At 12 h after transfection, cells were incubated in 1% serum supplemented with either 0.5% Me$_2$SO or 30 μM z-VAD in 0.5% Me$_2$SO. A, after 36 h, a sample of supernatant was withdrawn and assayed for LDH content (Sigma kit). An equivalent HEK-293 cell sample was treated with 1% Triton X-100 and assayed for LDH content, which was taken as the 100% maximal release. B, at 48 h after treatment, cells were harvested and viable vs. nonviable cells were assayed by trypan blue exclusion. C, HEK-293 cells overexpressing each EMP family member were tested for annexin V binding by flow cytometry, using propidium iodide (PI) as a cell-dead stain. For each sample, a cell count of 2,500 cells was taken, and cells were gated into four quadrants where Q1 is the proportion of nonviable (PI-positive), non-annexin V-bound cells; Q2 is PI-negative viable annexin V-positive cells; Q3 is PI-negative (viable) annexin V-negative cells; and Q4 represents annexin V-positive viable (PI-negative) cells. Typical flow cytometry traces are shown for mock-transfected, staurosporine-treated, and EMP-2 overexpressing HEK-293 cells. D, annexin V binding measured in the Q4 gating. Each column shows the percentage of annexin-positive/propidium-negative cells. In each experiment, 100% was assigned to the number of annexin-positive/propidium-negative cells observed with staurosporin (1 μM) treatment ($n = 4$ in each case).
RESULTS

Previous studies on the P2X7 receptor have shown that truncation of the receptor by removal of most of the intracellular C terminus reduces YOPRO-1 dye uptake and channel dilatation (2). We have now verified that the C terminus is required for the P2X7 receptor-mediated cell blebbing. In HEK-293 cells expressing the wild-type (WT) rP2X7 receptor, cells underwent membrane disruption within 1 min of activation with 100 μM Bz-ATP, whereas cells transfected with the truncated (Δ418) rP2X7 receptor did not show any significant membrane disruption over a 4-min stimulation with 100 μM Bz-ATP (Fig. 1, A–C). Both the WT rP2X7 receptor and the truncated receptor were expressed on the cell surface as determined by immunocytochemistry (Fig. 1A). In agreement with previous electrophysiological recordings (2), the Δ418 truncated rP2X7 receptor was shown to form a functional channel, because cells loaded with the calcium indicator Fluo-4AM displayed a prolonged Ca²⁺ rise after stimulation with 100 μM Bz-ATP (Fig. 1B). Therefore, the P2X7 receptor C terminus is required for ATP-mediated cell blebbing. For this reason we used this intracellular domain to probe for interacting proteins, which might mediate this response.

EMP-2 was identified as a strong positive hit in the yeast two-hybrid screen of a HEK-293 cell cDNA library with the P2X7 receptor C terminus (P2X7CT). The interaction was confirmed by isolation of the EMP-2 Gal-4 activation domain fused to mRNAs (Fig. 3C). Each of the EMP cDNAs (Myc/His tagged) showed predominant expression at the cell surface in a similar pattern to the WT P2X7 receptor (Fig. 4D). This indicates that EMPs specifically interact with the C terminus of the P2X7 receptor.

Overexpression of EMP-2 and EMP-2 in NIH-3T3 cells has previously been shown to cause cell rounding, blebbing, and a reduction in survival rate (13–15). We therefore sought to determine whether overexpression of all four members of the family mediate similar effects in HEK-293 cells. Overexpression of each protein led to a significant increase in cell death. A significantly higher release of LDH occurred in EMP-transfected cells after 36 h of treatment in low serum (Fig. 5A). In addition, an increase in trypan blue uptake was observed as determined by cell counting (Fig. 5B), compared with control mock-transfected cells also maintained in low serum. Fluorescence-activated cell sorter analysis of HEK-293 cells 48 h after transfection showed that each member of the EMP family resulted in a significant proportion of annexin-positive cells (Fig. 5, C and D). Mock-transfected cells and cells transfected with P2X7 receptor cDNAs showed no significant annexin binding. The high percentage of propidium-positive cells in the mock-transfected control (Fig. 5C) is likely caused by the addition of transfection reagent. The LDH release and trypan blue uptake in EMP-overexpressing cells was significantly inhibited by the addition of the caspase inhibitor, z-VAD (Fig. 5, A and B).

Immunocytochemistry of HEK-293 cells transfected with each of the EMP cDNAs (Myc/His tagged) showed predominant staining at the cell surface (Fig. 6). All cells transfected with PMP-22, EMP-1, EMP-2, or EMP-3 exhibited several large membrane blebs on their surfaces (Fig. 6).

DISCUSSION

The P2X7 receptor is functionally distinct compared with the other members of the ATP-gated ion channel family because its activation leads to membrane blebbing (5). Here we have demonstrated that the unique intracellular C terminus of the P2X7 receptor is required for this response. The removal of the
The C-terminal domain of P2X<sub>7</sub> also greatly reduces the uptake of dyes such as YOPRO-1, but it does not grossly alter the ATP-gated cation channel function (2).

The search for proteins that interact with the C-terminal domain of P2X<sub>7</sub>, and may be involved in the membrane-blebbing phenotype, led to the identification of EMP-2. EMP-2 consists of 167 amino acids containing four predicted transmembrane domains (16) and exhibits 40% amino acid identity to PMP-22. In addition, two other related proteins have been identified by random sequencing of a mouse intestine library (EMP-1 (17)) and from related expressed sequence tag (EST) sequences (EMP-3 (16)). Direct interaction of EMP-2 and the C terminus of the P2X7 receptor was verified by using biochemical approaches—copurification, coimmunoprecipitation, and GST pull-down assays. In addition, the related proteins EMP-1, EMP-3, and PMP-22 were also found to interact with the P2X<sub>7</sub>CT using these same techniques. Notably these interactions did not depend on activation of the P2X<sub>7</sub> receptor. Consistent with a role in the membrane-blebbing phenotype of P2X7 and not its channel functions, overexpression of EMP-2 in HEK-293 cells had no significant effect on P2X<sub>7</sub>-activated cation currents, YOPRO-1 uptake, or a significant shift in the EC<sub>50</sub> value for Bz-ATP (data not shown).

P2X7 has previously been found to associate with a multi-protein complex including cytoskeletal proteins, heat shock proteins, and two integral membrane proteins, receptor-phosphotyrosine phosphatase-β/H<sub>9252</sub> (RPTP<sub>β</sub>/H<sub>9252</sub>) and integrin β<sub>2</sub>, although the domains of the P2X<sub>7</sub> receptor responsible for most of these interactions have not yet been determined (18). The C terminus of the P2X<sub>7</sub> receptor is generally thought to be entirely intracellular.

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**Fig. 7. EMPs share homology to the γ subunits of voltage-activated Ca<sup>2+</sup> channels.** Alignment of the human five exon γ5 and γ7 to human PMP-22, EMP-1, EMP-2, and EMP-3 using the ClustalW algorithm. Bars above the sequence represent predicted transmembrane spanning domains; solid triangles (above sequence) show exon–intron boundaries for PMP-22, EMP-1, EMP-2, and EMP-3; open triangles (below sequence) show exon–intron boundaries for γ5 and γ7 subunits. The lower line provides the consensus sequence for the alignment.

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cellular, whereas EMPs have a predominantly intramembrane disposition with only a small part of the protein likely to be intracellular (amino acids 86–94 in EMP-2), although this domain may be sufficient for the observed interactions. Alternatively, there is a significantly hydrophobic segment in the P2X7 receptor C terminus (amino acids 512–536), raising the possibility that the main interaction occurs between hydrophobic regions within the membrane.

The EMPs share 22–25% similarity to the “stargazin” or voltage-gated channel α subunits, with common exon–intron boundaries and alignment of the four transmembrane domains (Fig. 7). This is of interest, as the y subunits (γ1–γ8) have been shown to interact with neuronal voltage-dependent calcium channels (19, 20), indicating that the P2X7 receptor–EMP interaction is not unique, and a number of ion channels may form complexes with tetra-span membrane proteins.

The normal physiological role of EMPs is not well understood. Genetic studies and the generation of PMP-22-deficient mice have established that it is responsible for a set of inherited peripheral neuropathies in mice and humans. One type of Charcot-Marie Tooth disease (CMT1A) results from a mutation in PMP-22 (21), and Dejerine-Sottas syndrome, which also involves impaired nerve conduction, is a product of a deletion within the PMP-22 gene (22). In rodents, point mutations of PMP-22 are associated with the “Trembler” phenotype due to impaired conduction in peripheral nerves (23). However, the wide tissue distribution of PMP-22 suggests that it may play a more general role than in myelin formation. PMP-22 was originally isolated from NIH-3T3 cells and was first named gas-3 for its growth arrest-dependent expression (24). Northern analysis has shown that each member of the EMP family, including PMP-22, is widely expressed (16, 24), and there is considerable overlap with the tissue distribution of P2X, receptors. For example, many epithelial cells express both EMPs and P2X7 receptors (4). PMP-22 is highly expressed in Schwann cells (25, 26), where there is also good functional evidence for P2X7 receptors (27, 28).

The RT-PCR analysis confirmed that mRNAs encoding all members of the EMP family are present in both HEK-293 cells and in THP-1 monocytes. P2X7 receptors are strongly expressed in THP-1 monocytes, and their activation is an important trigger for interleukin-1β release (29). After prolonged stimulation, P2X7 receptors mediate ATP-induced killing in human macrophages (30, 31). In NIH-3T3 cells, overexpression of PMP-22 also causes cell rounding, blebbing, and a reduction in the survival rate (13, 14), and recently bleb formation has been reported following overexpression of EMP-2 (15). We have now extended these observations, and shown that membrane blebbing is induced and cell survival is decreased when any one of the EMPs is overexpressed in HEK-293 cells, as evidenced by increased LDH release, trypan blue uptake, annexin V binding, and immunofluorescence. The cell death could be inhibited by the addition of the caspase inhibitor, z-VAD, indicating that overexpression of EMP-1, EMP-2, EMP-3, or PMP-22 results in a caspase-dependent apoptotic-like phenotype.

Although overexpression of EMPs leads to constitutive cell blebbing, the normal cellular trigger for an EMP to manifest this response is currently unknown. This study indicates that EMPs can associate with other integral membrane proteins such as the P2X7 receptor, which under the appropriate conditions (i.e. ATP stimulation), can provide that trigger and promote blebbing, although the mechanism by which the P2X7–EMP complex could promote cell blebbing is not understood at present. Because EMPs are widely expressed, even in cells lacking P2X7, this would suggest they may have additional integral membrane protein partners that link EMP-like proteins to apoptosis pathways. Identification of such proteins will be an important goal for the future together with understanding the mechanisms involved in the constitutive cell blebbing observed when EMP proteins are overexpressed.

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