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Expression of Full-Length and Truncated Recombinant Human Brain Type I Inositol 1,4,5-Trisphosphate Receptors in Mammalian and Insect Cells

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Intracellular inositol 1,4,5-trisphosphate receptors (IP_3Rs) form tetrameric Ca^{2+}-release channels that are crucial for Ca^{2+} signalling in many eukaryotic cells. IP_3R subunits contain an N-terminal, cytoplasmic, ligand binding domain linked by a modulatory domain to a channel-forming, hydrophobic C-terminal domain. We assembled and sequenced cDNAs encoding the $SI^-/SII^+/SIII^+$ splice variant of the human brain type I IP_3R, and functionally expressed the full-length receptor, and a C-terminally truncated receptor lacking the final 20% of the protein, in mammalian and insect cells. Both proteins were insoluble, consistent with *in vivo* immunofluorescence and ligand binding studies. This contrasted with the behaviour of recombinant FKBP12 (a soluble control protein). The truncated receptor also fractionated with the "membrane" pellet after alkaline carbonate treatment. We conclude that the human type I IP_3R forms high MW aggregates or complexes in cells when expressed without the C-terminal hydrophobic domain. This behaviour should be considered when expressing and refolding "soluble" human type I IP_3R domains for structural studies.

Key Words: **baculovirus; Ca^{2+}-release channel; HEK-293 cells; intracellular Ca^{2+} channel; IP_3R.**

Ca^{2+} mobilisation from intracellular stores by the second messenger inositol 1,4,5-trisphosphate (IP_3) controls a wide range of cellular events, extending from fertilisation to cell death [1, 2]. Ca^{2+}-release is mediated by two families of proteins: ryanodine receptors (reviewed by Shoshan-Barmatz and Ashley, [3]) and IP_3 receptors (IP_3Rs). Both proteins form tetrameric ion channels, and there is currently substantial interest in determining whether certain regions of their subunits can be expressed as refoldable, soluble domains for crystallisation studies.

Three highly homologous mammalian *IP_3R* genes (*IP_3RI-III*) have been cloned [4–12], and (where examined) the corresponding proteins form homo- [13] and heterotetrameric [14, 15] assemblies. Although RT-PCR studies have suggested there may be two additional genes, *IP_3RIV* [16, 17] and *IP_3RV* [17], both mRNAs may be variants of *IP_3RII* [16, 18, 19]. All the encoded proteins, including the human type I IP_3R (the major IP_3R isoform), have a predicted subunit molecular weight (MW) close to 300 kDa, and an apparent MW from SDS–PAGE of ~260 kDa. Each subunit can be subdivided into three major regions: an N-terminal "IP_3-binding domain," an intervening "regulatory domain," and a C-terminal "hydrophobic domain."

Experiments using deletion mutants [20, 21] showed that IP_3 binds to a site formed by the N-terminal 650 amino acids, independently of tetramer formation. Further deletion analysis has defined amino acid residues 226–578 as the minimal region required for high affinity IP_3 binding [22]. The IP_3-binding pocket consists of two closely associated structural domains, each of which has a discrete function. Residues 341–604 form a domain with an intrinsically low affinity for IP_3, and while the domain formed by residues 1–343 is unable to bind IP3 on its own, it potentiates IP_3 binding by its neighbour [23].

The central signal-transducing domain of the IP_3R couples IP_3 binding to channel opening [20]. It contains several Ca^{2+}-binding sites [24, 25], at least two ATP-binding sites, and sites for phosphorylation by different protein kinases [4]. It also contains sites for interaction with a number of accessory proteins. For example, the Ca^{2+}-dependent phosphatase, calcineurin, is anchored to the IP_3R by the FK-506 binding protein FKBP12 [26, 27]. FKBP12 itself binds to the IP_3R at residues 1400–

Abbreviations used: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; FKBP, FK-506 binding protein; IP_3R, inositol 1,4,5-trisphosphate receptor; PEG, polyethylene glycol; PKA(C), protein kinase A (C); RT-PCR, reverse transcription polymerase chain reaction.

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1401, a leucine-proline dipeptide structurally similar to FK-506. The hydrophobic C-terminal domain of the IP_3R is thought to contain all the membrane-spanning segments [28].

IP_3RI is spliced at three regions, termed SI [29], SII [29, 30], and SIII [12]. S1 splicing occurs near the middle of the ligand-binding domain. Alternative SII splicing [29, 31] occurs between two PKA phosphorylation sites in the regulatory domain, and may affect PKA phosphorylation kinetics. A novel SIII splice in the brain receptor encodes 9 additional residues that contain a potential PKC phosphorylation site [12]. In this study, we set out to functionally express the human type I $SI^-SII^+SIII^+$ IP_3R in both human and insect cells, and to test the idea that a truncated receptor lacking the hydrophobic C-terminus, thought to include all the transmembrane domains, would be expressed as a soluble protein, like the truncated rat IP_3R [5, 32], and FKBP12 [26, 27].

MATERIALS AND METHODS

Cloning and sequencing of human IP_3RI and FKBP12 cDNAs. Human *FKBP12* cDNA (GenBank AH002818) was kindly provided by M.D. Walkinshaw. Full-length and truncated *IP_3R* cDNAS were assembled from partial-length cDNAs kindly provided by C.A. Ross [12]. Each insert was cloned into both pcDNA3.1 and pBlueBac (Invitrogen) using standard recombinant DNA protocols, and re-sequenced [33].

In vitro expression. *In vitro* transcription/translation reactions were carried out on a 25 μl scale using Coupled Reticulocyte Lysate Systems (Promega) in the presence of [^{35}S]-labelled methionine (ICN).

Cell culture and transfection. COS-7 and HEK-293 cells were cultured in DMEM (Gibco) supplemented with 10% (v/v) foetal calf serum (Gibco), 2 mM L-glutamine, 2 mM sodium pyruvate, 100 units penicillin and 100 μg/ml streptomycin, and transfected using Lipofectamine (Gibco). Sf9 and Sf21 cells were cultured in Grace's medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco), or non-supplemented Sf-900 II medium (Gibco), respectively, each containing 2.5 μg/ml Fungizone, 100 units penicillin, and 100 μg/ml streptomycin. Insect cells were transfected using the MaxBac 2.0 Transfection Kit (Invitrogen).

Isolation and amplification of recombinant baculovirus. About 90% of the insect cells co-transfected with recombinant transfer plasmid and Bac-N-Blue viral DNA (Invitrogen) lysed after 4–5 days. The medium was then screened for the presence of recombinant virus by plaque assay, and "positive" plaques were further screened by PCR to rule out any that contained a mixture of recombinant and non-recombinant viruses. Briefly, one set of primers (Bac-For: 5′-TTTACTGTTTTCGTAACAGTTTTG-3′; Bac-Rev: 5′-CAACAACGCACAGAATCTAGC-3′) was designed to flank the polyhedrin gene (839 bp) disrupted during homologous recombination in this system, and another set of primers (IP_3R-For: 5′-AGGCGCTCAGGCAAGTTC-3′; IP_3R-Rev: 5′-GGCATTGTTCTTCAGTTC TAA-3′) was designed to produce a 1076 bp fragment corresponding to positions 5180 to 6255 of the human *IP_3RI* cDNA. Amplification of pure recombinant viral DNA produced a single band corresponding to 1076 bp, whereas virus stock containing both wild-type and recombinant virus produced bands at 1076 bp and 839 bp. Pure recombinant virus was amplified in cells to produce high titre virus suitable for protein expression. Virus titres were measured by end-point dilution assays, and maximal protein expression was determined by immunoblotting. Infection times of 24 h and 48 h showed similar results, but receptor expression declined after 72 h. This may have been due to IP_3RI degradation, because many infected cells lysed to release proteases from intracellular compartments by this time point. High titre viral stocks were stored at 4° for up to 6 months without significant loss of titre.

Indirect immunofluorescence. Clonal mammalian cells were cultured on glass coverslips for up to 48 h following transfection, fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS, pH 7.0) at room temperature for 10 min, then permeabilised with 0.1% (v/v) Triton X-100 in PBS. Following inversion onto 100 μl of primary antibody (rabbit anti-human IP_3R N-terminal antibody, Calbiochem, diluted 1:100 in 0.2% (v/v) fish skin gelatin in PBS), and incubation at room temperature for 1 h, the cells were washed extensively in 0.2% (v/v) fish skin gelatin in PBS and in PBS alone. The cells were finally inverted onto 100 μl of 1:400 rhodamine-conjugated secondary antibody for 1 h, and the inverted coverslips were mounted on glass slides (Aquamount, BDH) and examined using a Leica TCS NT confocal fluorescence microscope.

Cell harvesting and immunoblotting. We followed the procedures described by Yoneshima *et al.* [34], with minor modifications. Briefly, transfected or control cells from 3 × 90 mm plates were suspended in 3 ml ice cold homogenisation buffer (0.32 M sucrose, 5 mM Tris–HCl, pH 7.4) containing a mixture of antiproteases: 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, 10 μg/ml trypsin inhibitor and 150 μg/ml benzamidine. After 30 strokes of a tight-fitting teflon-glass homogeniser, the homogenates were centrifuged in a TLA-100.3 rotor at 3000*g* for 10 min at 4°C using a TL-100 centrifuge (Beckman). The supernatant was re-centrifuged at 300,000*g* for 20 min at 4°C (i.e. 100,000*g* × min). The final "P2" membrane pellet was resuspended in homogenisation buffer and the supernatants and pellets were either processed immediately or frozen in liquid nitrogen and stored at −70°C.

Proteins were size-fractionated using 5% (w/v) SDS–PAGE and transferred to Hybond C-Pure nylon membranes (Amersham). The membranes were incubated with blocking buffer (5% (w/v) skimmed milk in PBS) overnight at 4°C and probed with rabbit anti-human IP_3R N-terminal antibody (Calbiochem) or goat anti-human FKBP12 C-terminal polyclonal antibody (Santa Cruz), followed by anti-rabbit or anti-goat peroxidase conjugated secondary antibody at 1:1000 dilution in blocking buffer. The labelled bands were detected by ECL (Amersham) using autoradiography film or, for quantitative analysis, a Phosphorimager (Fujifilm LAS-1000).

IP_3 binding assay. Protein samples were incubated with 10 nM [^{3}H]IP_3 (Amersham) in binding buffer (50 mM Tris–HCl, pH 8.8, 1 mM EDTA, 1 mM DTT, 100 mM NaCl) for 10 min at 4°C. The IP_3/protein mixture was then added to 4 μl γ-globulin solution (50 mg/ml) and 100 μl polyethylene glycol (PEG) precipitation buffer (30% (w/v) PEG 6000, 1 mM EDTA, 50 mM Tris–HCl, pH 8.8), and incubated for 5 min at 4°C. Protein-PEG complexes were pelleted by centrifugation at 18,000*g* for 10 min at 4°C, and the pellet was rapidly washed with 100 μl binding buffer and resuspended in 200 μl H_2O. The radioactivity of the samples was measured by adding 3.5 ml Ultima Gold (Packard) followed by standard liquid scintillation counting. Specific binding was defined as total binding minus non-specific binding measured in the presence of 25 μM unlabeled IP_3.

RESULTS

Cloning and in Vitro expression of cDNAs

Human *FKBP12* was cloned into pcDNA3.1 to express FKBP in mammalian cells as a "control" cytoplasmic protein. Three overlapping human cDNAs encoding the entire human *IP_3RI* ($SI^-/SII^+/SIII^+$) [12]

were subcloned to assemble two *IP_3R* cDNAs, one encoding all 2744 residues of the corresponding human type I IP_3R, referred to as the full-length protein, and one encoding only the first 2151 residues (78.4%) of the type I IP_3R, referred to as the truncated protein.

The cloning sites were checked by dideoxy sequencing, and fully assembled *IP_3RI* cDNAs were subjected to additional single-stranded automated sequencing (Oswel DNA). Sequences found to be ambiguous compared to the published sequence [12] were resequenced using a new reverse primer. After a total of 19 rounds of sequencing, 8 nucleotides were confirmed to be different from the published sequence (G18T, T21C, A79G, A679G, G682A, A721T, C1290T, and G7297T), resulting in 5 residue reassignments (I27V, S227G, D228N, R241W, and A2433S). *In vitro* coupled transcription/translation confirmed expression of proteins of the expected sizes, ~260 kDa and ~200 kDa (not shown).

FKBP12 and IP_3RI Expression in Mammalian Cells

FKBP12 cDNA was transfected into COS-7 and HEK-293 cells using a DNA/Lipofectamine ratio of 7 μg/35 μl per 100 mm culture dish, with similar results. 48 h after transfection, cells from 3 dishes were combined for each experiment, homogenised in 3 ml of homogenisation buffer, and centrifuged at 1,000*g* for 10 min at 4°C. Supernatants and pellets were subjected to immunoblotting to confirm that FKBP12 was only present in the soluble, cytoplasmic fraction (data not shown).

Truncated and full-length IP_3Rs were expressed in both COS-7 and HEK-293 cells using Lipofectamine-mediated transfection (DNA/Lipofectamine ratio: 7 μg/35 μl per 100 mm culture dish). Cytosolic and membrane fractions prepared 48 h after transfection (see Materials and Methods) were resolved using 5% (w/v) SDS–PAGE, and typical immunoblot results from HEK-293 cells are shown in Fig. 1a. Both the full-length IP_3RI and the truncated IP_3RI were found in the insoluble "membrane" fraction, and equivalent amounts of the cytosolic protein fraction showed no detectable signal. Indirect immunofluorescence localisation of the truncated and full-length IP_3Rs (Fig. 1b) showed discrete, non-cytoplasmic distributions for both proteins.

Expression of IP_3RI in Insect Cells

In order to obtain larger amounts of IP_3R protein for binding studies, and to determine how the proteins behaved in a different cell system, full-length and truncated IP_3Rs were expressed in Sf9 and Sf21 cells, as described. Expression levels in Sf21 cells were slightly higher than in Sf9 cells (not shown). As in mammalian cells, both the full-length receptor and the truncated IP_3R (~200 kDa) were localised to the insoluble fraction (Fig. 2a). We also noted minor expression of endogenous full-length receptor.

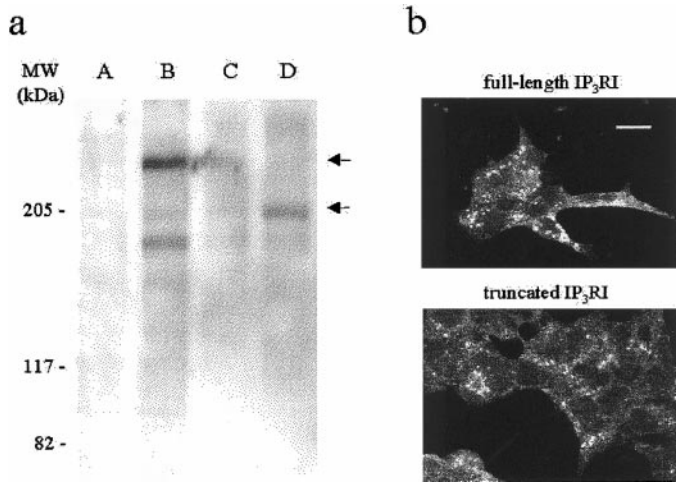

FIG. 1. Expression of human IP_3RI in mammalian cells. (a) HEK-293 cells were transfected with cDNA constructs expressing either full-length or truncated IP_3Rs, and subjected to subcellular fractionation and anti-IP_3R immunoblotting. Lane A contains mock-transfected cell membranes, lane B contains the membrane fraction of cells expressing full-length receptor, and lanes C and D contain cytosol and membranes, respectively, from cells expressing truncated receptor. The upper and lower arrows indicate the full-length and truncated protein bands, respectively, and the positions of MW markers are shown. (b) Both IP_3R proteins are immunocytochemically localised to discrete regions of fluorescence (bright areas) in transfected HEK-293 cells (mock-transfected cells showed no signal, not shown). Scale bar, 10 μm.

We carried out IP_3 binding assays to confirm the location of the truncated human IP_3RI. The insoluble ("membrane") fractions of both Sf9 and Sf21 cells infected with virus encoding the truncated IP_3R had relatively high IP_3 binding activities of ~3 pmol/mg of microsomal protein, whereas the cytosolic fraction displayed negligible IP_3 binding activity (Fig. 2b). To check that the IP_3 binding activities of truncated receptors in the insoluble fractions from different experiments were similar, the binding in four independent experiments was analysed under the same conditions and normalised to immunoblot density to allow for variations in the efficiency of expression between different infections. This gave a reasonably consistent value of 22 ± 6 fmol per unit of absorbance (mean ± SD, $n = 4$).

The microsomal fraction of Sf21 cells infected with recombinant virus encoding the truncated IP_3RI was suspended in 100 mM Na_2CO_3 at a pH of 11.4 and incubated at a protein concentration of 1.5 mg/ml at 4°C for 30 min. The mixture was then centrifuged at 300,000*g* for 20 min (equivalent to 100,000*g* × min), and the pellet and supernatant fractions were collected and resolved on 5% (w/v) SDS–PAGE, followed by immunoblotting. Under these conditions, the truncated IP_3RI continued to sediment with the

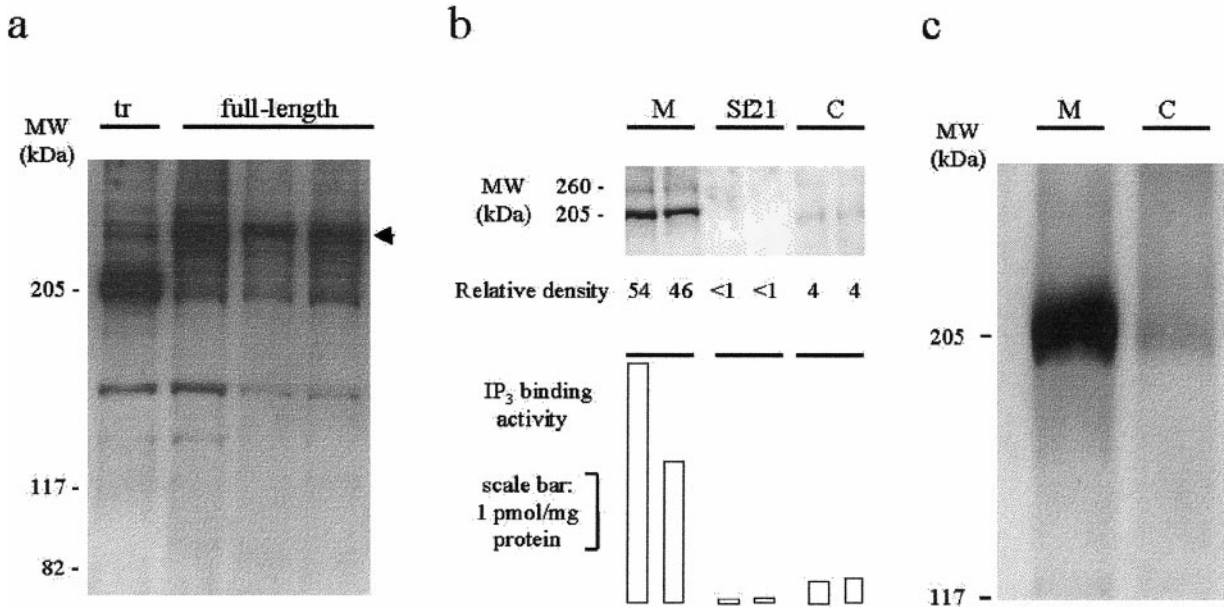

FIG. 2. Expression of human IP_3RI in insect cells. (a) Immunoblots of cell membranes prepared from cells expressing truncated (tr) and full-length receptors (the 3 lanes represent different virus titres). The arrowhead indicates the position of the full-length receptor band. (b) Immunoblots of membrane (M) and cytosolic (C) fractions of cells expressing the truncated receptor are compared to immunoblots of the membrane fraction of control cells (Sf21). The samples were analysed together, and the relative densities of the major bands are indicated. The lower part of b summarises the IP_3 binding activity of each sample. In an immunoblot carried out after alkaline carbonate treatment (c), the pelleted "membrane" (M) fraction from Sf21 cells expressing the truncated receptor is compared to cytosol (C) from the same cells.

"membrane" pellet (Fig. 2c). Other bands, possibly representing non-specific cross-reacting proteins, were removed.

DISCUSSION

We assembled a full-length human *IP_3RI* encoding 2744 residues and containing the SII and SIII splice sites [12] from cDNAs cloned from human brain. The novel SIII splice site [12] contains nine amino acids, and is located near the IP_3 binding core [35]. We confirmed 8 nucleotide differences from the previously published sequence [12], requiring 5 residue reassignments, confined mainly to the IP_3 binding domain. We functionally expressed both the full-length protein, and also a truncated protein lacking the hydrophobic C-terminal domain.

We expected to find that elimination of the C-terminal domain would result in the expression of a soluble protein. In previous work, eliminating residues 2205–2225 of the rat type I (SI^-/SII^+) IP_3R (the full-length receptor contains 2734 residues) caused the expressed protein to be located in the cytoplasm [5]. More recently, Galvan *et al.* [32] showed that truncating the same receptor after residue 2210 also results in a soluble protein. Using the human *IP_3RI* homologue encoding an additional SIII spliced region, we show in this study that recombinant human IP_3RI protein pellets with the "membrane" fraction even after being truncated at residue 2151. The same behaviour was observed in each of two different clonal cell lines of both human and insect cells, using three different approaches: subcellular fractionation and immunoblotting, immunocytochemistry, and an IP_3 binding assay. The behaviour of the truncated IP_3R contrasted with that of soluble recombinant FKBP12, and was not altered by alkaline carbonate treatment of the Sf21 microsomal membrane pellet. These results suggest that the truncated receptor may be self-associating into high-MW complexes, or associating with other high-MW proteins (e.g., the cytoskeleton). The results after alkaline carbonate treatment suggested the IP_3R was not bound to membranes as an extrinsic membrane protein.

The C-terminal domain of the IP_3R has been shown to play a vital role in membrane targeting and oligomer formation [32]. Further work may confirm whether the truncated human type I IP_3R, lacking this domain, forms oligomers, or heterooligomerises with native receptor proteins. However, while functional expression was possible in two different cellular systems, our results suggest that the cytoplasmic domain of the human type I IP_3R is not soluble in mammalian and insect cells, and may not be a good candidate for overexpression and refolding in heterologous systems, including bacteria.

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