Human inositol 1,4,5-trisphosphate type-1 receptor, $\text{InsP}_3\text{R1}$: structure, function, regulation of expression and chromosomal localization

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We have isolated cDNA clones encoding an inositol 1,4,5-trisphosphate receptor type 1 ($\text{InsP}_3\text{R1}$) from human uteri and a leukaemic cell line, HL-60. Northern-blot analysis showed that approx. 10 kb of $\text{InsP}_3\text{R1}$ mRNA is expressed in human uteri, oviducts and HL-60 cells. The predicted amino acid sequence of human $\text{InsP}_3\text{R1}$ (2695 amino acids) has 99% identity with that of the mouse SI^-/SII^- -splicing counterpart. Western-blot analysis with anti-(mouse $\text{InsP}_3\text{R1}$) antibodies showed that $\text{InsP}_3\text{R1}$ protein of human uteri and oviducts of approx 220 kDa is immunostained. Northern-blot analysis of HL-60 cell differentia-

tion along the neutrophilic lineage induced by retinoic acid or dimethylsulphoxide showed an accompanying enhanced expression of $\text{InsP}_3\text{R1}$ mRNA. Immunohistochemical analysis of the cerebella of spinocerebellar degeneration patients showed a variable loss of Purkinje cells with an altered pattern of immunostaining. The $\text{InsP}_3\text{R1}$ gene (*Insp3r1*) was localized to the 3P25-26 region of human chromosome 3. The data presented here clearly show that $\text{InsP}_3\text{R1}$ exists widely in human tissues and may play critical roles in various kinds of cellular functions.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) mediates the effect of receptors linked to polyphosphoinositide (PI) hydrolysis on intracellular Ca^{2+} mobilization in a variety of cells [1]. Ca^{2+} is a modulator of the physiological functions of the cells. InsP_3 selectively binds to a receptor that incorporates a Ca^{2+} -release channel and is localized to the endoplasmic reticulum, which is considered to be an intracellular Ca^{2+} store [2]. Previously, our group and others purified the InsP_3 receptors (InsP_3Rs) from rodent cerebella, and determined their primary structures by cDNA cloning [2–5]. The InsP_3 binding sites and channel domains were shown to be in the N-terminal and C-terminal regions respectively [6,7]. The remaining part of the receptor has phosphorylation sites recognized by protein kinase A (PKA) [8,9], and binding sites for ATP [3,5,10,11]. Protein kinase C [12] and Ca^{2+} /calmodulin-dependent protein kinase II [9,12] also phosphorylate the InsP_3R *in vitro*. Binding sites for calmodulin [10,12] and Ca^{2+} [13] have also been reported. Alternative splicing [6,14,15] has been considered to confer further functional complexity. These studies suggest that InsP_3R is a key molecule into which various kinds of signal pathways converge. Recently, cDNAs of distinct types of InsP_3Rs have been cloned [16–18]. Thus the original receptor is now named the type-1 receptor ($\text{InsP}_3\text{R1}$) and is widely distributed in various tissues.

We also isolated the cDNA of InsP_3R from *Drosophila*

melanogaster [19] and *Xenopus laevis* [20], and showed good preservation of this receptor among the species. Thus we assumed that a similar InsP_3R exists in human cells. There are indeed studies showing that rapid formation of InsP_3 and subsequent Ca^{2+} release occur in response to extracellular stimuli in human cells, such as smooth muscle cells [21], T-cells [22] and platelets [23]. Furthermore, PI- Ca^{2+} signalling could turn out to be linked with some specific human pathologies. Recently, the putative pathogenic gene product of the oculocerebrorenal syndrome of Lowe (OCRL) was shown to have strong homology with a soluble 75 kDa inositol polyphosphate-5-phosphatase [24], suggesting that an altered metabolism of InsP_3 affects Ca^{2+} signalling in OCRL patients [25]. Manic-depressive psychosis, characterized by swings in moods, is known to be well controlled by Li^+ administration, probably through its inhibitory action on inositol phosphate metabolism [26,27]. In these situations, characterization of human InsP_3R would be informative.

Our previous study showed that InsP_3R mRNA is present in the mouse uterus [28]. In the present study, we first showed that anti-(mouse $\text{InsP}_3\text{R1}$) monoclonal antibodies (mAbs) could cross-react well with the putative $\text{InsP}_3\text{R1}$ in the human uterus. Subsequently, we isolated cDNA of human $\text{InsP}_3\text{R1}$ from uterine libraries first by immunoscreening, followed by hybridization. The amino acid sequence deduced from the cloned cDNA showed very strong homology with that of the rodent $\text{InsP}_3\text{R1}$. For preliminary characterization of human $\text{InsP}_3\text{R1}$, we studied two

Abbreviations used: InsP_3 , inositol 1,4,5-trisphosphate; InsP_3R , InsP_3 receptor; $\text{InsP}_3\text{R1}$, type-1 InsP_3R ; $\text{InsP}_3\text{R2}$, type-2 InsP_3R ; $\text{InsP}_3\text{R3}$, type 3 InsP_3R ; RyR, ryanodine receptor; RyR1, skeletal muscle RyR; PKA, protein kinase A; PI, phosphoinositide; OCRL, oculocerebrorenal syndrome of Lowe; mAb, monoclonal antibody; RA, retinoic acid; DMSO, dimethylsulphoxide; ECL, enhanced chemiluminescence detection system; OPCA, olivopontocerebellar atrophy; ABC, avidin-biotin-peroxidase complex.

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The nucleotide sequence reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI Nucleotide Sequence Databases under the accession number D26070.

systems for $\text{InsP}_3/\text{Ca}^{2+}$ signalling. We first chose HL-60 cells, originally derived from a patient with acute promyelocytic leukaemia [29], to examine the transcriptional regulation of $\text{InsP}_3\text{R1}$ during differentiation along the neutrophilic lineage. Secondly, we focused on cerebellar Purkinje cells which are abundant in $\text{InsP}_3\text{R1}$ in both mouse [30] and human [31]. Immunohistochemical analysis with the anti-(mouse $\text{InsP}_3\text{R1}$) mAb reveals that immunoreactive patterns of Purkinje cells have changed significantly in spinocerebellar degeneration. In addition, we mapped the human $\text{InsP}_3\text{R1}$ gene (*Insp3r1*) to the specific locus of human chromosome 3 and discuss its relevance to already mapped diseases and genes.

MATERIALS AND METHODS

Human tissue preparation

Normal parts of myometrium were obtained from pathological samples of myoma uteri. Oviducts were also obtained from them. The patients were in either their 30s or 40s without complications. These tissues were cut into pieces and kept at -80°C until use.

Preparation of membrane functions and immunoblot analysis

The microsomal fraction (P3) of mouse cerebella was prepared as described [30]. Crude membrane protein fractions (P2+P3) of human uteri and oviducts were prepared as described [5]. After SDS/PAGE (5–10 $\mu\text{g}/\text{lane}$) [32], the proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham), and

analysed by the Enhanced Chemiluminescence detection system (ECL kit; Amersham) using the anti-(mouse $\text{InsP}_3\text{R1}$) mAbs 4C11, 10A6 and 18A10 [30,33] as primary antibodies.

cDNA cloning and sequencing

All bacterial manipulations and cloning procedures were performed by standard methods [34] unless otherwise mentioned. Total RNA from human uteri and oviducts was purified into Poly(A)⁺ RNA by oligo(dT)–cellulose chromatography or oligo(dT)–Latex [35]. The cDNA was prepared using random hexamers as primers and ligated into the λgt11 vector (Stratagene) or the $\lambda\text{ZAP II}$ vector (Stratagene). We synthesized two oligonucleotide primers corresponding to the upstream portion of human clones 6Y and R62 (Figure 1a) by the phosphoramidite method using a DNA synthesizer (model 382A; Applied Biosystems). The cDNA was then synthesized by using these primers and a You-Prime cDNA Synthesis Kit (Pharmacia LKB), and ligated into the λgt10 vector (Stratagene). The library in λgt11 was first immunoscreened [36] with mAbs 4C11, 10A6 and 18A10 as described [3]. All the libraries were also screened by plaque hybridization using the ^{32}P -labelled cDNA inserts of mouse $\text{InsP}_3\text{R1}$ clones [3] and putative human InsP_3R clones (5×10^5 c.p.m. per ml). Plaque hybridization and washing procedures using the mouse probes were performed at 50 – 60°C . In the case of the human probes, those were performed at 65°C instead. The sequences of positive cDNA inserts were determined from both strands as described [19]. Some parts of the human $\text{InsP}_3\text{R1}$ cDNA were also isolated from a library derived from dimethylsulphoxide (DMSO)-treated HL-60 cells.

Northern-blot analysis of human uteri and oviducts

Poly(A)⁺ RNAs (5 μg) from human uteri and oviducts and total RNA (5 μg) from the cerebella of ICR mice (Japan SLC) were electrophoresed and blotted on to nylon membrane filters. Northern blots were probed by the ^{32}P -labelled human clone 6Y (Figure 1a) as described [3].

Expression of the ligand-binding site of human $\text{InsP}_3\text{R1}$

The 3728-bp cDNA fragment 81SB1 (Figure 1a) encoding the 1190 N-terminal amino acids was ligated into a mammalian expression vector pBactS as described [7]. A neuroblastoma/glioma hybrid cell line NG108-15 was transfected with the resultant plasmid pB81SB DNA by the calcium phosphate precipitation technique [37]. The expressed protein was expected to appear in the cytosol. The preparation of the cytosolic proteins and Western-blot analysis using mAb 4C11 were performed as described [7,19].

InsP_3 -binding assay

The assay were performed as described previously [7].

Northern-blot analysis of differentiated HL-60 cells

HL-60 cells were cultured as described [38]. 1.25% DMSO or 1 μM retinoic acid (RA) were added to the culture medium for 1–3 days to induce granulocytic differentiation. RNA was harvested with a Fast Track Kit (Invitrogen) and Northern blots were probed by a ^{32}P -labelled $\text{InsP}_3\text{R1}$ cDNA of the HL-60 clones [34]. The control probe was human β -actin cDNA (Clontech). After autoradiography, relative quantification was done by scanning densitometry (CS-9000U; Shimadzu).

Figure 1 Human $\text{InsP}_3\text{R1}$ cDNA clones, functional domains and hydrophilicity of the deduced protein

(a) Schematic representation of human $\text{InsP}_3\text{R1}$ cDNA clones from uteri. The open box represents the protein coding region. Nucleotide residues (shown at the top) are numbered from the first residue of the 5' untranslated sequence. Solid bars indicate the clones used for the sequencing. (b) Functional domains and cysteine residues conserved among the InsP_3R family. Human $\text{InsP}_3\text{R1}$ consists of three main domains: an InsP_3 -binding domain, a modulatory domain (▼, PKA phosphorylation consensus sites; ●, putative ATP-binding sites), and a putative Ca^{2+} -channel domain (six vertical thick bars, membrane-spanning segments M1–M6). Vertical lines beneath the open box indicate the conserved cysteine residues among the InsP_3R family. The longer lines indicate cysteine residues which are conserved even in the RyR. SI and SII denote the sites alternatively spliced out in the mouse $\text{InsP}_3\text{R1}$. (▽) show putative Asn-linked glycosylation sites. (c) Hydrophilicity plot (window size: 17) of the deduced human $\text{InsP}_3\text{R1}$ according to the method of Kyte and Doolittle [66].

Figure 2 Western-blot analysis of InsP_3R in human tissues

Membrane proteins from human uteri (15 $\mu\text{g}/\text{lane}$), human oviducts (10 $\mu\text{g}/\text{lane}$), and mouse cerebella (1 $\mu\text{g}/\text{lane}$) were subjected to SDS/5% PAGE and were detected by immunoblot analysis with anti-(mouse $\text{InsP}_3\text{R1}$) mAb 10A6 and an ECL kit. The other two mAbs, 4C11 and 18A10, gave essentially the same results (results not shown). The positions of molecular-mass markers (in kDa) are shown at the left.

Immunohistochemical analysis of the cerebella of the spinocerebellar-degeneration patients

Samples from 17 histologically normal cerebella of humans (20–65 years old) and 16 cases of spinocerebellar degeneration [14 cases of olivopontocerebellar atrophy (OPCA) and two cases of hereditary cerebellar atrophy of Holmes type] were examined. All the cerebella were obtained at autopsy within 6 h of death, fixed with 10% neutral formalin or 4% formaldehyde in 0.1 M PBS, pH 7.4, and embedded in paraffin. Sagittal and parasagittal sections (3 μm thick) were initially stained by the haematoxylin–eosin, Klüver–Berra and Bodian methods, and the subsequent sections were examined by the avidin–biotin–peroxidase complex (ABC) method [39] using mAb 4C11 [30]. For control sections, the primary antibody was replaced with PBS.

Chromosomal mapping

Chromosome spreads were obtained from phytohaemagglutinin-stimulated human lymphocytes cultured for 72 h. 5-Bromodeoxyuridine was added for the final 7 h of culture (60 $\mu\text{g}/\text{ml}$ of medium), to ensure post hybridization chromosomal banding of good quality. The mouse $\text{InsP}_3\text{R1}$ clone, p23-2 [3], was ^3H -labelled (2×10^8 d.p.m./ μg) and used as a probe. The probe was hybridized to metaphase spreads at a final concentration of 50 ng/ml of hybridization solution as described [40]. After coating with nuclear track emulsion (NTB2; Kodak), the slides were exposed for 20 days at 4 $^{\circ}\text{C}$, and then developed. To avoid slipping of silver grains, chromosome spreads were first

Figure 3 Northern-blot analysis of $\text{InsP}_3\text{R1}$ mRNA

Lanes 1 and 2 contain 5 μg of poly(A)⁺ RNA from human uteri and oviducts respectively. Lane 3 contains 5 μg of total cellular RNA from ICR mouse cerebella as a positive control. The arrow indicates a putative $\text{InsP}_3\text{R1}$ mRNA of approx. 10 kb.

stained with buffered Giemsa solution and metaphases were photographed. R-banding was then performed by the fluorochrome–photolysis–Giemsa method and metaphases re-photographed.

RESULTS AND DISCUSSION

Western-blot analysis of human tissues

We first determined whether human tissues possess proteins immunoreactive with the anti-(mouse $\text{InsP}_3\text{R1}$) mAbs. Previously, we showed that $\text{InsP}_3\text{R1}$ is located in smooth-muscle-enriched tissues of mice such as myometrium [28]. The crude membrane fraction of both human uteri and oviducts contained a 220 kDa putative $\text{InsP}_3\text{R1}$ protein which was immunoreactive with all three mAbs (Figure 2). The protein has a slightly lower molecular mass than that of the mouse cerebellar $\text{InsP}_3\text{R1}$ (250 kDa), probably due to tissue-specific alternative splicing [15].

Isolation of human InsP_3R cDNA

To isolate human InsP_3R cDNA from uterus, we constructed three human uterine cDNA libraries (see the Materials and methods section). We first immunoscreened the expression library by mAb 18A10, and isolated several positive clones including clone R62 (Figure 1a). Subsequently, we obtained a full-length clone of putative human $\text{InsP}_3\text{R1}$ cDNA by continual screenings of the libraries with parts of the mouse $\text{InsP}_3\text{R1}$ [3] and the

HUM-1	1	MSDKM---SSFLHIGDICSLEYAEGSTNGFISTLGLVDDRCVVPETGDLNPPKFRDCLFKLCPMNRYSAQKQFWKA--AKPGANSTIDAVLLNKLHHAADLEKKQNETENRKLGLTVI
MUS-1	1	A.....
RAT-1	1	A.....
XEL-1	1	V.....N.....S.....DA.....R.....S.....T.....
DRO	1	..G..NIIGSA..L..V..VC..L..T..C..A..SC..I..I..Q..S..S..N..PN..KR..EI..K..S..
RAT-2	1	Y.....V.....V.....H..A..T.....V.....Y..KQ..Q..H..EA..K..Q..E..Q..S.....EIV
RAT-3	1	..NE.....V.....V.....E..AA..D.....V.....Y..KQT..QDKEIA..V..Q..Q..QM..Q..D..K..VH..D..V
HUM-1	116	QYGN-VIQLLHLKSNKYLTVNKRPLALLEKNAMRVTLDEAGNEGSWFYIQPFYKLSIGD-SVVGDKVVLNPNVNAQP--LHASSHLQVDNPGCNEVNSVNCNTSWKIVLFPMKWSDNKD
MUS-1	116	
RAT-1	116	
XEL-1	116	Q..S.....
DRO	119	RA..V.....S.....Y..AN.....K.....YV..V..I..S..D..QNLHV..ANYE..P.....K..VL..SS.....S..EHKE..QE
RAT-2	116	..K..SK.....I.....S..A.....H..W..E..NI..V..M.....NVE..L.....K..A.....T.....F..SYRE
RAT-3	117	..K..S.....M.....AT.....LF..W..N..N..V..I.....NYE..S..V..K.....N..QFR..HLE
		SI
HUM-1	232	DILKGGDVVRLFHAQEKFLTCDEHP-KKQHVFLRTTGRQSATSATSSKALWEVEVVQHPDPCRGAGYWNLSFRFKHLATGHYLAEE-----VDPDQ--DAS-----
MUS-1	232	VDPDFEECELEFQPS.....
RAT-1	232	VDPDFEECELEFQPS.....
XEL-1	232	VDPDFEECELEFQPS.....
DRO	239	H.....M..YK..QY.....T..A.....I.....S.....D.....Y.....AEI..VSAG..MSATSASGH
RAT-2	232	..V.....DYE.....I.....L.....I.....H.....Q.....N.....LN..Y..R..QNEKTVRD
RAT-3	232	EV.....Y..G..LQ.....L.....N.....H.....H..G..Y.....N.....EN..SYKG..V..DPKPAAGP-
HUM-1	326	-----RSRLRN-AQEKMYSLVSVPEGNDISSIFELDPTTLRGGDSLVPRNSVVRHLCTNIWVHSTNIPIDKKEEPVMLKIGTSPVKDEKAFAIVPVSPAIEVR
MUS-1	341	L.....
RAT-1	341	L.....
XEL-1	326	-----NA..MQV..VP..MCT..I.....S.....D.....R.....
DRO	343	DLHLGDCSKDSGLSCSTMN..TIND-KPKGKQ..R.....YSA..A..V..V..A..MARP.....QS.....Q..I..S.....A..S.....ADDD.....SMVCC..I.....LI.....V..
RAT-2	335	GELP-----T..KKKHQ..G..IM..T.....H.....A..L.....A..QRA..C.....T..S.....T..R.....CQT.....C..PLS..
RAT-3	336	GAQS-----TGR.....G..IK..R..A..H.....A..L.....A..QKT..F.....IQ..A..V..R..R..ML..C..T.....C..PVS..I..
HUM-1	427	DLDFANDASKVLGSIAGKLEKGTITQNERRSVTKLLEDLVYFVTGGTNSGQDV--LEVVSFKPNRERQKLMREQNILKQIFKLLQAPFTD--CGDGPMLRLEELGDQRPAPFHICRLCY
MUS-1	442	
RAT-1	442	
XEL-1	427	S.....A.....E.....LV..H..I.....LVN.....S.....
DRO	462	C..ATVTS..DN..S..SI..ALIS..Q..I..IA..ME..EQNKTKA..FTIKN..I..D.....L..Y.....L..I..G..QHTA.....F..D..S..PKNS..YKN..F...
RAT-2	441	N..ATTVK.....N..S..F.....F.....IP..ADV..N.....D..IT.....A..V..GI..K..KEK-A..E..S.....D.....Y..YKVMF...
RAT-3	441	SM..A..AVE..NE..F..S..D..F..IQ.....F..SDVP..N..N.....DIMVT.....DE.....GI..K..R..K..G..E..LV.....S..KN..YQYMF...
HUM-1	543	RVLHRSQQDYRNKQYIAKQFGMKQIGYDVLAEDTITALLHNNRKLLEKHITAAEIDTVPVSLVRKNR--EPRLFYLSDLCSVMNKSIPVTQELICKAVLNPTNADILIEKTLVLSR
MUS-1	558	
RAT-1	558	
XEL-1	543	A.....
DRO	582	..I..L.....H..L.....I.....E.....G.....MHNWDS.....NR..A..A.....S..SDK..K.....QVKAL..
RAT-2	558	N..CV..S.....I.....K..E.....L..R.....NSTA.....FM..S..G.....Q.....SMQ
RAT-3	558	E.....H.....M..S.....I.....KT..VE.....NRIA.....C..D..K..S.....Q..E..RPVK
HUM-1	660	FEFEGV-----SSTGENA-LEAGEDEEVWLFWRDSNKEIRSK-----SVRELAQDAK-EQKEDRDVLSYYRQNLNFAARMCLDRQYLAINIS
MUS-1	675	I.....
RAT-1	675	
XEL-1	660	-----A..S..SS.....KA.....R.....Q..N.....
DRO	702	TGSGP..RCYKNSDENVCLATLAEDPGDDEDSVQST..TTT-WDS..SLN..DGTSTPGT..K..Y..HL..WTGQPTS..SM..DL..SCD..GELEAAI..N..H.....SN..N.....L..L..
RAT-2	675	V-----N..PM..SSI..PDDI..D.....Y..I.....PHG.....AI..H.....E..R.....T..A..LE..T.....Q..
RAT-3	675	EM-----AQSH..Y--SIEYS.....T..T..R..N..HHE.....Q.....E..R..A..NAH..EN.....K.....D..
HUM-1	743	GQLDVLILRCMSDENLPPYDLRASFCRLMLHMHVDRDPQEQVTVPKYARLWSEIPSEIADDDYS-----S-GASKDEIKERFAQTMFVEEYLRDVCQRPFPSPDEKNKLTPEVNVLAR
MUS-1	757	T.....
RAT-1	757	
XEL-1	742	F.....T..R..D.....G.....A.....
DRO	820	PR..I.....K.....TM..E.....L.....P.....KMS..Q.....GKNQOP..DON..QACRAK..NT..IA.....N..CN..ATKVM..T..Q..Q.....K..
RAT-2	754	T..S.....V..S..F.....S..V..R.....T.....TK..T..HE.....ITDS..RNDM..RK..L.....KE..N..P..G.....H..
RAT-3	753	K..G..E..LFL..A..M..F.....H.....V.....L.....F.....T..TA..T..K.....NLN..R..DK..NK..S.....D..NN..GEAV..AND.....I.....S..H
HUM-1	858	NLIYFGFYNPSDLLRLTKILLAILDCVHVTTI--FPISKMAKGEENK--GS-NVMRSIHGVGELMTQVVL-RGGG-FLPMTMAAAPE-GNVK-QABP-EKEDIMVMDTKLKIIEILQF
MUS-1	872	T.....
RAT-1	872	T.....
XEL-1	857	V.....LIAN--..SG..LG.....S..G.....T..A..R.....L.....
DRO	939	D.....S.....T..S.....SD..SSGE..ASTDIDSV..ETNAEAGS..L..GDINTV..SLA--..SVGQAIAAPTISLQQRKSVS--..LM..YPL.....
RAT-2	870	S..E.....RT.....I..QAPMSSV..ERLSKFPD--..N..T.....M..M..S..SI..PVSW..D..Q..SVHPS--..S..G..Q..VT.....V..
RAT-3	869	S..E.....RT.....I..QAPAAVLQA-----Y..P--..GK..R..Q.....HM..STM..S..KQSV..GASSLPTGVVPEQLDRSK-FEDN..HTV..E.....L.....
HUM-1	967	ILNVRLDYRISCLLCIFKREFDENSQ-----TSETSGNSSQEGPSNVPGALDFEHIIEQAEIGFGGSEENTPLDLDDHGGRFTLRLVL
MUS-1	981	S.....
RAT-1	981	S.....
XEL-1	967	S.....S..G..T--..AITV..T.....G.....D.....
DRO	1053	D.....S.....EVPLRPLAMRQVSSSRNNRNRPLAAMPRLIPSTVPSLWPPRCAA--AATTARQKNI..L..S..GV.....DCERTPAN.....Q.....
RAT-2	980	S.....YM..S..Y..K..G..ND-----GNGDPSA..TPETLL..ALVPDI..-..AA..AM..A..RK..K..VQ.....E.....
RAT-3	978	Y..SV..K..V..VF-----PMQD..GADGTAPEAFDSSTANMLDR..G.....TM..VGTSSM..EV..E.....M.....
		Area 1
HUM-1	1051	LHLTMHDYPLVSGALQLLFRHFSQRQEVLAQFKQVQLLVTSQDVNRYKIQDLQDLRSIVEKSELVYVK-QGQPDETMDGASGENEHKKEEGN-----NKPQKHESTSYNNRV
MUS-1	1065	P.....T-----S..L.....
RAT-1	1065	P.....S..L.....
XEL-1	1046	H.....S..E..VTAQA..GAD--KG..TP-----G..AK..S.....
DRO	1168	I..A.....H.....K.....R.....SDS..ES.....S..I..QS.....AKAT..LGATDA..GDA--VSELY-----AALSQ..QRN--E..K
RAT-2	1062	I..I.....A..L.....K.....A.....SN.....A.....LT.....E..S..SYENGD..GEGQAKGGEAN..S..LLSPVQGA..TPQID..NKGN.....E..
RAT-3	1060	K.....AMHT.....ISA..E..V..SE..R..TM.....D..K..SVKG..EGEAGASKDKKRPSEDE-----GFL..P..GK.....E..I
HUM-1	1162	VKEILIRLSKLCVQESASVR--KSRKQQQLRLRNMGAAHVLELLQIPYEAEDTKMQEIMRIHAHEFLQNFCAQNOQALLHKKHNLNLPNGILEAVTMQHFMNNQQLCSEINERVVQ
MUS-1	1176	K.....
RAT-1	1176	
XEL-1	1154	L.....NTTG--RN.....S.....T..R.....KI.....T.....
DRO	1274	MN..F..TA..GPGS/V..P..HE.....V..V..T..D..N..DEKD..EL..K..L..C.....L.....V..N..LD.....K..VCA..KD..LA..N..VTDK..
RAT-2	1182	NK-----C..N..H.....K.....S..D.....T..E..N..V..D..T.....R..P..V..L.....T..L..E..R.....YH..N..S.....
RAT-3	1172	G..E..N..M..GVGE-----QM..K.....K..D..K..M..D.....D..N..N..M..L..YT..Q.....K.....PG.....LQ.....T..L..E.....Y.....S..P..L
HUM-1	1280	HFVHCIEHGRNVQYIKFLQITVKAEGKFKKKQDMVMAELVNSGEDVLVFNDRASFQTLIQMMRS--ERDRMDENSPL-MYHIHLVBLAVCTEGKNVYTEIKCNLLPLDDIVRVV
MUS-1	1294	
RAT-1	1294	F.....
XEL-1	1272	A.....RY.....I.....V.....
DRO	1393	I.....H..A..LQ.....V..A..NQ..RP.....Q..I.....KG..NHV.....QQQMLMEKLSDD..-K..VE..K..C..M.....N..S.....TII
RAT-2	1295	H..E..LR.....D..YV.....T..I..G.....I.....PI..LN..C--..A..G..SG..-A..T.....S..D.....A..A.....T.....E..V..S..
RAT-3	1285	WP.....H.....LD.....H..VI.....YV.....I..T..T..A..D..V.....K..LAH..LD..KA--A..GVEDH.....S..D.....A..A.....T.....E..V..S..

Figure 4 Alignment of the deduced amino acid sequence of human *InsP₃R1* with those of the mouse, rat, *Xenopus laevis* and *Drosophila melanogaster*

The predicted amino acid sequence of human *InsP₃R1* (HUM-1) is shown in comparison with the published mouse type-1 (MUS-1) [3], rat type-1 (RAT-1) [4], *Xenopus* type-1 (XEL-1) [20], *Drosophila* (DRO) [19], rat type-2 (RAT-2) [16], and rat type-3 (RAT-3) [17] sequences. Identical amino acids are indicated by dots. Dashes indicate gaps (deletions or insertions) for maximal homology.

HUM-1	1396	THEDCIPEVKIAYINFLNHCYVDTEVEMKEIYTSNNHMKLFE-NFLVDICRACNNSTDRKHADSILEKYVTEIVMSIVITFFSPPSDQSTTLQ-----TRQPVFVQLLQGVFRVY
MUS-1	1410	V.....ASKLAVVHQ.....AI.....
RAT-1	1411	V.....ASKLAVVHQ.....AI.....
XEL-1	1388	V.....ASKLAVVHQ.....AI.....
DRO	1512	C.PL.M...E.VD...I...A.G...S...KS...NQLIT.PAA--ASNKT.QA.LNG.TNLGS.A...AIV...S.LI...AAH.IT
RAT-2	1411	D...V...I...MA.V...T...TF.RC...S.N.SG.N...N.S...H...I...SA...I...
RAT-3	1401	T...M.V.V...I.T...TL.MALV...KREKRLS.PT...LTV.LDTISA...EN.S...H.TIV...STT.LL
HUM-1	1506	HCNWLMPQSQKASVESCIRVLSDVAKSRAIAIPVLDSDQVNNFLKSH-SIVQKTMNNWRLSARNAAR--RDSVLAASRDYRNIIERLQDIVSALEDRLRPLVQAEISVLVDVLRPELL
MUS-1	1520	N.....S.M.V...T...S.....
RAT-1	1521	N.....S.M.V...T...S.....
XEL-1	1507	L.....T.....N.....S.M.V...T...S.....
DRO	1621	Q.R...SLGDRFN...N...T.TES...M.S...L.PE.EQ...ATMSS.T-AMLTQ.TKWLLA.KQPKYE--AQQAASLM.WD.S...G...L...Q.K.V.E...L...I.Y.S...
RAT-2	1521	N.T.PN.A...A.AE...N.G...T.M.N.S.T.RA...G...SGP-F-KEALGGPAW...K...V.AS.QQS.MM...F...YS...
RAT-3	1510	E.P...QQHG...A.V.T.AM...LL.M...AHMSA.LSSGGS--CSAA.QRSAANYKT.T.TFP.VIPT.NQW...K...K...IT...E.K...M.W...
SII		
HUM-1	1622	HPENTDARRKCESGGFICKLIKHTKQLEENEEKLCIKVLQTLREMMTKDRGYGK-----GEALRQVLNRYGYNVRPSGRRES
MUS-1	1636	P...S...P...T...QISIDSENAELPQAPAEENSTEQBLESPPLRQLEDHHR.....I.....I.....
RAT-1	1637	P...S...P...T...QISIDSENAELPQAPAEENSTE-ELESPPLRQLEDHHR.....I.....I.....
XEL-1	1623	K...Q...R...L...A...F.D...VI...I...A...
DRO	1737	AG.E...KR...R...EK...K.RM.V...R...AI.VN...D...T.LL...FQTKSTPRLP.D
RAT-2	1639	GS...IR...A.MS...N...K.M-K...I...LE.KDSFM.E...SST...KI.L...FKGDHVS.V--
RAT-3	1628	L.GSE.YQR...LS...R...G.M-S...V...R...QQ.LQ.KSK...DR...NQ...KM.LQN.LQ...K.P--
Area 2		
HUM-1	1702	LTSFGNGPLSAGGPKGGGGG-SGSSSMRGEMSLAEVQCHLDKEGASNLVIDL-IMNASSDRVFHESILLAILLEGNTTIQHSFFCRLTEDKKSEKFFKVFYDRMKVAQGEIKAT
MUS-1	1756	P...S...P...T...S.....T.....F.....
RAT-1	1756	P...S...P...T...S.....T.....F.....
XEL-1	1703	G.SS...S...S-I...G.L.S...SD...Q...D...T.....PI...KGM.QKFLS.DLNQA...FEK...D...S...
DRO	1817	EVPLLA...MDPAKQN.HLVTH...P.AKYLQ.AGKT.H.M.N...R...D...VE.V.KSVH.PNI.V.AVE.G...PI...KGM.QKFLS.DLNQA...FEK...D...S...
RAT-2	1713	I...S...REDS.LM--ALGP.MRVDRSSLHLK.GMKG.T...S...S.YCVY...M...I.TMC.QEA...GT.N.YQQ.H.QQ...L...A.A.K.RS...
RAT-3	1704	E...TD.T.S.VD-QDW--AI.AT.R...TK.C...TSTNKEKI.Q...G...R...D...E.K.YNLM.S...R...LH...R...T.S...
HUM-1	1820	VTVNTSDLGKNNKDDVD-R---DAPSRKKAKEPTTQITEVPRDQLEASAAATKRAFTTFRREADPD-DHYQPGEGT-----QATADKAKDDL-EMSAVITIMQPIRLFLQLLCE
MUS-1	1874	S.....T.....
RAT-1	1874	S.....T.....
XEL-1	1819	S...R.E.QSE---ET.HQQRVR...SG...AKE...I...V...K...YYS...FSL...V---M.V.E.GR.E...L...V.V...
DRO	1935	T.IAA.AHEHQQ.TNLELDKIARKHGL.SNGVV...LKRE.HN.GL...AR.YGNA.NIHSGEASSIAVNSPLEDILAEKLEKHKDSR.QRNLQ.NKVLV...
RAT-2	1825	I...S...REDS.LM--ALGP.MRVDRSSLHLK.GMKG.T...S...S.YCVY...M...I.TMC.QEA...GT.N.YQQ.H.QQ...L...A.A.K.RS...
RAT-3	1804	A...M...SQPRE---EP-AD.TT.GRVSSFS---MP--SSRYSLGPGH.GH.VS-ERA.NN---GS.EEKS.EEV-T...PA...R...GTSVL...R...
HUM-1	1925	HNRLQNLRLCQNNKTNVNLVCTQLQFLDCICGSTTGGLGLGLYINEKNVALINQTLLESLEYCQSPCHENQNCIATHESNGIDIITALILNDINPLGKKRMDVLLEKNNASKLLAI
MUS-1	1979	S.....R.....EN.....
RAT-1	1979	S.....R.....EN.....
XEL-1	1925	S.....R.....EN.....
DRO	2055	P.M...L...N...N...S...M...H.L...A...L...N...EN...
RAT-2	1932	P...M...L...N...N...S...M...H.L...A...L...N...EN...
RAT-3	1883	IM...D...G.VI...T...T...V...S...C.Y...Q...D...L...
HUM-1	2045	MESRHDSENAERILYNMRPKELVEIKKAYMQGE-----VEFEDGEEDGAASPRNVGNIYILAHQARHNKELQSMKPKGGQ-----V
MUS-1	2099	T...G-----
RAT-1	2099	T...G-----
XEL-1	2045	L...S...Y...T...H...V...T...G-----
DRO	2175	G...N...Q...AC...H.E.LIDEQDDGD.PDA.SDDP.ATV...E...C...Q...AGL.ASEDPQASFD-----
RAT-2	2052	F...D.M.N.N--L---ECNHGD.E.G.DCV...KD...L...Q...SDP-----
RAT-3	2003	ISL...Q...D...L.E-----R-----NSEV...E...L...S...Q...HL...VKRIQEEBAEGISSMLSLNNKLSQMLKSSAPAQE
M1		
HUM-1	2126	DGDEALEFYAKHTAQIEIVRLDRTMEQIVFPVPSICEFLTKESKLRIYYTTERDEQGSKINDFLRSEDLFNEMNQKLRQPVLYWCARNMSFWSSISFNLAIVMLNLLVAFFYFP-KG
MUS-1	2180	L.....
RAT-1	2180	L.....
XEL-1	2127	L...Q...S...T...V-N
DRO	2271	KTSQ...MY...T...N...L...I.E...Y...TDT.IK.LN.A...D...L...H...Q...S...L.F.ISSY...L.N.L...CV.VI.MI...DN
RAT-2	2134	E...KY...N...H...N...R...Y.VFN...V...QQT...Y...K...I.NN.A.F.FS.HI.L.G...FI...A...L...GDD
RAT-3	2108	EEEDP.AY.EN...S...Q.S...A.Q...E.T.H.LFT...Q...VS...DQ.SF.H...E.RR...SM.LI...FS.R.TL.G...FI.III...YVE.
M2		
HUM-1	2245	VRGGTL-EPHWSGLL-WTAMLSLALVIALPKPHGIRALIASTILRLIFSVGLQPTFLLLGAFNVNCKIIFLMSFVGNCGTFTGRGYRAMVLDVEFLYHLLVLYVICAMGLFVHEFFYSLLL
MUS-1	2299	L.....
RAT-1	2299	L.....
XEL-1	2246	H...DSRL...V...V...I...M...L...G...L...V...T...G-----
DRO	2390	T-VPE--SS.I...F.IITIF.V...L...RES...TF.G.V...P...LL.PES...C...VVT.TL.SVHIV...M...K...LEKQLIKIT.FSTYTA.YSVLL--LR.IF.P...
RAT-2	2254	GDE...S.LF.A...V.VA.CTSMLEFFS...V...PFLV.IM...S.YTI...G...I...A...L...V...V...R...I...K...M...V.VV...VG.ILTSLV...A...L...I...
RAT-3	2228	AST.V.GS.LI...F.ILICF.I.ALF--T.HYSV.P.VALV...S.YYL...G...NI...L.LT...V.VV...R...I...K...M...V.VV...VG.ILTSLV...A...L...I...
M3		
M4		
M5		
Area 3		
HUM-1	2363	FDLVYREETLLNVIKSVTRNGRSIILTAVALILVYLFISIVGYLFFKDDFILEVDRLPN--ETAVPETGESLASEFLSDVCRVSG-ENCSSAPPEELVPAETEQDK-EHTCETLLM
MUS-1	2417	ND...Y...T...T...K...L...T...K...L...V...T...K...L...V...T...K...L...V...T...K...L...V...T...K...L...V...
RAT-1	2417	ND...Y...T...T...K...L...T...K...L...V...T...K...L...V...T...K...L...V...T...K...L...V...
XEL-1	2364	Q...IF.N.GTMT...L.YPE...G.D-T...TH--E.LAQVT...E.EE...
DRO	2505	V...V...R...V...I...M...LVS...FEEQ--DN.P.PSVPLTL.VPVS.G.S.SAPDDLQ.QAA--K.VAP.SAGGGEV--RS.DS.V
RAT-2	2372	I.F.L...TM...K--R.P.TGNDGVPT-MT.T.MLGTCPK--TIPSSNA--G.GGE.GI.R...D...
RAT-3	2345	I...F...L...L...F.L...GNHSR.STLGMPHG.AT.M--GT.SGDK--MD.V.EVSV.P.IL-E.DE.L.ST.RA.D...
M6		
HUM-1	2479	CIVTVLSHGLRSGGGVGDVLRKPSKEEPLFAARVIYDILLFFFMVIIIVLNLIFGVIIDTADLRSEKQKKEEILKTTCTFIOGLERDKPDNKTTFTEHKEEHNMMHYLCFIVLVKVKDS
MUS-1	2533	V.....A...F...F...
RAT-1	2533	V.....A...F...F...
XEL-1	2477	V.....A...F...F...
DRO	2620	T.NQ...N...I...A...SK.G.V...I...Q.A...S.N.SA...S...S...Y...P
RAT-2	2485	NQ...N...R...D...V...I...K...S...S...S...Y...P
RAT-3	2460	MN...N...I...D.S.P...V...I...I...S...S...L...N...Y...R.NK
HUM-1	2599	TEYTGPSYVAEMIKERNLDWFPNRAMSLVSSDSEGEQNELRLNQBKLESTMKLVNLSGQLSELKDQMTBQRKQKRIIGLGHPPHNNVNPQQA
MUS-1	2653	R...L.....
RAT-1	2653	R...L.....
XEL-1	2597	D...R...Q.A.L.I...
DRO	2740	F...YA.V.AGI.E...L...AAV.AD...I...SM.AQ.LD.QL.IKF...T...H...H...L...NTANSLLPE
RAT-2	2605	Q.T.K...NEGDS...I...S...KQ...A...E...N...L.F...SMTPHNHMP.H
RAT-3	2580	D...Q...NK...GE3...I...I...G...SH.TA...N...E...RR...L.FVDVQNC.SR

The predicted transmembrane regions are marked by solid lines and denoted as M1–M6. Consensus ATP-binding sites are marked with bold broken lines. Bold lines and filled triangles represent consensus sequences for PKA phosphorylation and possible phosphorylated Ser residues respectively. SI and SII are regions that could be deleted by alternative splicing in the mouse *InsP₃R1* [15]. Large filled circles represent putative Asn-linked glycosylation sites. Areas 1–3 are where sequence diversity among the *InsP₃R* family is prominent.

putative human $\text{InsP}_3\text{R1}$ cDNAs. One cDNA clone was less homologous with the mouse $\text{InsP}_3\text{R1}$, and corresponded to the type-2 InsP_3R ($\text{InsP}_3\text{R2}$) [16].

The complete cDNA sequence (9076 nucleotides) was determined by sequencing the six uterine clones shown in Figure 1(a), and included a single long open reading frame (nucleotide positions 257–8341: an open box in Figures 1a and 1b) preceded by an untranslated leader sequence of at least 256 nucleotides. The surrounding sequence of a putative initiation codon, GAGATGT, was in relatively good accordance with Kozak's optimal sequence for translation initiation [41], although another in-frame methionine-coding codon (nucleotides 269–271) might be used as a translation initiation signal. We could not find any poly(A) tracts in uterus-derived clones. There was an adenylation control element (UUUUUAU) assumed to be involved in the control of translation [42] 89 nucleotides upstream of the polyadenylation signal. We also isolated six cDNA clones for the $\text{InsP}_3\text{R1}$ from a cDNA library of DMSO-treated HL-60 cells. From these clones, we could determine the sequence of 4387 bases including the complete 3' untranslated region and a poly(A) tract. The 5' 3932 base sequence of the cloned myeloid cDNA completely matched with the 3'-terminal sequence of the uterine cDNA. Thus the total length of cDNA isolated was 9531 nucleotides long. The complete 3' untranslated region of HL-60 cells contained one polyadenylation signal, while that of the mouse $\text{InsP}_3\text{R1}$ [3] had two. Northern-blot analysis (Figure 3) of human uteri and oviducts showed that the cloned cDNA could hybridize with single RNA of approx. 10 kb, which agreed with that of the mouse $\text{InsP}_3\text{R1}$ mRNA [3].

Sequence comparison among the InsP_3R family

The human InsP_3R deduced from the cDNA sequence was composed of 2695 amino acids with a calculated molecular mass of 307 kDa. The amino acid sequence of the human InsP_3R has 99% identity with the sequences of both mouse and rat $\text{InsP}_3\text{R1s}$. Mouse and rat $\text{InsP}_3\text{R1s}$ share approx. 70% and 62% identity with the rat type-2 [16] and type-3 [17] receptors respectively. Therefore, our human clones could be $\text{InsP}_3\text{R1}$. Furthermore, the present human receptor was categorized as one of the alternative-splicing subtypes of mouse $\text{InsP}_3\text{R1}$ [15], lacking two splicing segments SI and SII (SI⁻/SII⁻ subtype) (Figure 4). Our sequence data on SII splicing was consistent with the previous observation that the SII⁻ subtype exists exclusively in neuronal tissues [14,15].

It should be noted that Ser-666 is a human-specific insertion, and is not found in the corresponding site of $\text{InsP}_3\text{R1s}$ from other species (Figure 4). Among 32 amino acid replacements between human and mouse $\text{InsP}_3\text{R1}$, 17 replacements concentrate in three areas. Four replacements concentrate in Area 1 (Thr-1128–Gln-1149: 22 amino acids), six in Area 2 (Val-1684–Met-1730: 47 amino acids), and seven in Area 3 (Ser-2434–Val-2460: 27 amino acids). These areas are also consistent with the variable regions among the InsP_3R family (Figure 4). The sequence similarity between the mouse $\text{InsP}_3\text{R1}$ [3] and the rabbit ryanodine receptor (RyR1) [43] is also observed in human $\text{InsP}_3\text{R1}$ (e.g. eight conserved Cys residues: Cys-623, -1231, -1922, -1993, -2473, -2479, -2556, -2559 in human $\text{InsP}_3\text{R1}$, Figure 1b).

Transmembrane topology and the putative Ca^{2+} -release channel region

Hydrophobicity analysis of the human $\text{InsP}_3\text{R1}$ protein (Figure 1c) revealed several hydrophobicity stretches which might represent multiple membrane-spanning sequences (residues

2222–2535). These putative membrane-spanning sequences cluster around the C-terminus (Figure 1b) and, together with the neighbouring sequence, might constitute a Ca^{2+} -release channel. From previous studies [3,19,44], we have proposed that InsP_3R traverses the membrane six times. Therefore, we assigned the names M1–M6 to these six sequences (M1, residues 2222–2240; M2, 2254–2272; M3, 2298–2317; M4, 2337–2353; M5, 2386–2408; and M6, 2516–2535). M1–M4 are less conserved among the whole InsP_3R family than M5 and M6 (Figure 4), which are homologous even with the RyR family. Of eight cysteine residues conserved in InsP_3Rs and RyR1 described above, four cysteine residues are present in this region (Figure 1b), suggesting the existence of a common mechanism to maintain or regulate the receptor-channel conformation. In the N-terminal half of the putative third intraluminal loop (between M5 and M6), there are many acidic residues and putative N-glycosylation sites (NET, 2421–2423; NCS, 2449–2451) in addition to Area 3 (Figure 4). We recently confirmed that the mouse $\text{InsP}_3\text{R1}$ is glycosylated at these corresponding positions in an Asn-linked manner [44a]. Interestingly, human $\text{InsP}_3\text{R2}$ has two consensus sites in these corresponding positions, while human $\text{InsP}_3\text{R3}$ has one [44b]. The function of these sugar chains of the InsP_3R family remains obscure. The cluster of acidic residues seems to be involved in efficient Ca^{2+} permeation by concentrating Ca^{2+} near the putative channel pore [19]. In spite of the sequence variability in Area 3, these acidic residues (Asp and Glu) are strictly conserved among the $\text{InsP}_3\text{R1s}$. Thus the stretch could constitute an integral part of this type of Ca^{2+} channel. The sequence divergence in the regions such as Area 3 in turn may be related to the distinct channel properties among the family.

Putative InsP_3 -binding region

Previously, we showed that the 650 N-terminal amino acids are a prerequisite for InsP_3 binding [7]. The amino acid sequence of the corresponding region of human $\text{InsP}_3\text{R1}$ (Met-1–Glu-635) is almost identical with that of mouse, except for SI deletion and two conservative amino acid substitutions (human/mouse, Thr-43/Ala-43; Val-408/Leu-423). In and near this region, 11 Cys residues are conserved among the InsP_3R family (Figure 1b: Cys-56, -61, -206, -292, -379, -541, -623, -638, -730, -753, and -768). In addition, it was reported that mutation of Arg-615 of RyR1 to Cys has been identified as the possible cause of pig malignant hyperthermia characterized by hypersensitive gating of RyR1 [45]. This mutation of RyR1 is thought to lead to abnormal regulation of the channel gating by its ligands and modulators. There is 31% sequence identity between the 30 amino acids surrounding Arg-615 of RyR1 and human $\text{InsP}_3\text{R1}$ (note that Cys-623 in the corresponding region of human $\text{InsP}_3\text{R1}$ is also conserved in RyR1: Figure 1b). This homologous region is therefore a candidate site concerned with the fundamental architecture for the binding of analogous ligands and/or for the coupling of ligand binding to channel opening.

Modulatory region

The vast region between the putative InsP_3 -binding region and the transmembrane region, has been designated as the modulatory region. Two consensus sites for PKA phosphorylation in rodent $\text{InsP}_3\text{R1s}$ [3,4] are conserved in human forms also (RRDS/1571–1574, RRES/1698–1701). The rodent $\text{InsP}_3\text{R1}$ has the neuronal tissue-specific SII splicing segment (40 amino acids) between these two potential phosphorylation sites [15]. The human $\text{InsP}_3\text{R1}$ cloned from uterus and HL-60 cells does not have this SII segment at the corresponding position (between

residues Lys-1677 and Gly-1678), although the neuronal tissue-specific SII⁺ subtype has not yet been found in human. Thus the present human SII⁻ subtype could be phosphorylated mainly at Ser-1574, according to previous work which showed that the splicing segment affects the alternative of two PKA phosphorylation sites in rat [14]. Two potential ATP-binding sites (GXGXXG) have been found in rodent InsP₃R1 [3,4]. One is well-conserved among the InsP₃R family (GLGLLG; 1962–1967 in human InsP₃R1). The other site is in Area 2 where the amino acid sequence is divergent even among InsP₃R1s, and is composed of two overlapping consensus sequences in rodent forms. A replacement (human/mouse, Gly-1716/Ser-1770) of this area adds one more overlapping consensus sequences (GPGKPGGGGGGSG; 1714–1726) to human InsP₃R1. Because of the amino acid sequence diversity, the later consensus sequences are absent in *Drosophila* InsP₃R, and rat InsP₃R2 and InsP₃R3. Removal of the SII segment creates an additional consensus sequence for ATP binding in rodent [46] and human SII⁻ InsP₃R1 (GYGEKG; 1673–1678). Thus the alternative splicing might control patterns of ATP binding as well as PKA phosphorylation of InsP₃R1, resulting in modulation of channel activity [10,11,47,48,48a]. It is known that the ATP binding to the purified cerebellar InsP₃R1, which is predominantly the SII⁺ subtype [15] without the additional ATP-binding consensus site, has a stoichiometry of one [10]. Therefore, the actual ATP-binding site and its physiological role may vary depending upon cell types. There is a cluster of 13 cysteine residues (Cys-1173, -1231, -1270, -1284, -1312, -1370, -1382, -1400, -1415, -1507, -1521, -1632, -1657) in 484 residues (1173–1657) which is strictly conserved in the InsP₃R family (Figures 1b and 4). Only Cys-1231 is conserved in RyR1. Around this region, there is the longest stretch of non-homologous sequence (Cys-1405–Ser-1736 in human InsP₃R1) between InsP₃R and RyR [3]. It was recently proposed that InsP₃R1 has distinct classes of cysteine residues with different sensitivities to thiomersal, and that modification of specific cysteine residues by the reagent can alter InsP₃-induced Ca²⁺ release without affecting Ca²⁺-binding activity [49]. Thus some of the cystine residues in the modulatory and channel regions may take part in the regulation of Ca²⁺-channel activity of InsP₃R1.

InsP₃-binding activity of expressed receptor proteins

To confirm the InsP₃-binding property of our cloned human InsP₃R1, we transiently expressed the clone 81SB1 (Figure 1a) encoding the 1190 N-terminal amino acids in a neuroblastoma/glioma hybrid cell line, NG108-15. We first confirmed its expression by Western-blot analysis on cytosolic proteins using mAb 4C11. The cDNA-transfected cells produced a 130 kDa immunopositive protein as expected (Figure 5a). The expression of the truncated human InsP₃R1 was apparently coupled with the elevation of [³H]InsP₃-binding activity (Figure 5b). The vector-transfected NG108-15 cells exhibited almost no increase in InsP₃-binding activity. Therefore, our cloned human uterine InsP₃R1 has InsP₃-binding activity in the N-terminal putative large cytoplasmic region.

Northern-blot analysis of differentiated HL-60 cells

To evaluate the expression pattern of InsP₃R1 gene during cell differentiation, we used HL-60 cells whose differentiation toward the neutrophilic lineage is induced by RA or DMSO [50]. In undifferentiated HL-60 cells, a probe for human InsP₃R1 detected a single 10-kb mRNA species in Northern-blot analysis (Figure

Figure 5 Analysis of expressed human InsP₃R1 cDNA clone, 81SB1

(a) Immunoblot analysis of truncated protein of human InsP₃R1 expressed in NG108-15 cells. Cytosolic proteins from NG108-15 cells transfected with the truncated construct DNA pB81SB (10 µg/lane) and vector DNA pBactS (10 µg/lane) as control and mouse cerebellar microsomal fraction proteins (1 µg/lane) were analysed by immunoblotting using mAb 4C11 and an ECL kit. The positions of molecular-mass markers (in kDa) are shown at the left. (b) Specific binding of [³H]InsP₃ to the expressed protein. 81SB1, soluble proteins from NG108-15 cells transfected with the truncated construct of human InsP₃R1. Vec, soluble proteins from NG108-15 cells transfected with vector DNA as control. *N*, number of samples. The data are the means of *n* samples. Specific binding was defined as the total binding minus non-specific binding as described [3,7].

Figure 6 Northern-blot analysis of InsP₃R1 mRNA in HL-60 cells before and after differentiation

Poly(A)⁺ RNA was harvested from undifferentiated cells (left hand lane) or from cells exposed to either 1.25% DMSO (D) or 1 µM RA (R) for either 1 or 3 days as indicated. The InsP₃R1 and β-actin probes hybridized to 10- and 2-kb species respectively. Relative InsP₃R1 expression is based on scanning densitometry and is corrected for the actin signal in each lane.

6). In HL-60 cells, induced to differentiate toward the neutrophilic lineage by either RA or DMSO, there was a substantial increase in the level of InsP₃R1 mRNA. When normalized by an actin standard, the range of the increase in InsP₃R1 mRNA relative to undifferentiated cells was 2.3- to 4.4-fold. Sequence analysis revealed that the transcript might be that of InsP₃R1, although other types can co-exist. The increase in InsP₃R1 mRNA goes along with the acquisition of functions such as phagocytosis, degranulation and enhanced oxidative metabolism which are known to be associated with InsP₃/Ca²⁺ signalling. The extent of this upregulation was somewhat greater and its time course more extended with RA than with DMSO. The difference may reflect their distinctive ways of inducing differentiation [51]. Bradford et al. [52,53] recently reported transcriptional upregulation of the

Figure 7 Immunohistochemistry of cerebella of normal humans and spinocerebellar-degeneration patients with anti-InsP₃R1 mAb 4C11

Original magnification $\times 50$ for all sections. The nuclei of the granule cells which are not immunopositive are counterstained with haematoxylin–eosin to enhance photographic contrast. (a) Purkinje cells in a 64-year-old man with a histologically normal cerebellum showing specifically immunostained cell bodies, dendritic arborization and axons (arrowheads). (b) Purkinje cells in a 66-year-old man with cerebellar atrophy of Holmes type, showing unstained spiny branchlets and intensely stained cell bodies and main dendritic trunks. (c) Cerebellar cortex from the same case as (b) showing completely unstained Purkinje cell bodies (arrows) and an intensely stained cell body and main dendritic trunks. (d) Cerebellar cortex in a 64-year-old-man with OPCA showing a weakly stained Purkinje cell body on the right and an intensely stained torpedo (arrow).

InsP₃R gene in HL-60 cells exposed to either RA or 1,25-dihydroxyvitamin D3 by using a mouse InsP₃R1 probe. A previous study detected an increase in functional InsP₃ binding in cells exposed to either RA or DMSO [51]. Our data obtained with human InsP₃R1 probes are consistent with those results and suggest that the complex regulatory mechanism of InsP₃R1 mRNA expression could exist in the cells. In addition, it was recently shown that InsP₃-sensitive intracellular Ca²⁺ stores regulate Ca²⁺ influx in HL-60 cells [54,55] and that the capacity of the Ca²⁺ signalling system composed of Ca²⁺ release and influx appeared to increase during cellular differentiation [55]. Thus the upregulation of InsP₃R1 transcription in the neutrophilic differentiation may reflect the increase in the overall capacity of the Ca²⁺-signalling system.

Immunohistochemistry of human cerebella

In rodents, InsP₃R1 is located predominantly in cerebellar Purkinje cells [3,56,57]. It is known that there are various mouse cerebellar mutations like *Purkinje cell degeneration*, *nervous*, *Lurcher* and *staggerer* that show massive degeneration or marked underdevelopment of Purkinje cells [58], causing the reduction in InsP₃R1 expression [3]. InsP₃R1 mRNA is also observed in human cerebella by Northern blotting (results not shown). To establish the localization of InsP₃R1 in human cerebella, we performed immunohistochemical analysis of normal and ab-

normal cerebella of humans using mAb 4C11, which reacts with human uterus and oviduct InsP₃R1 (Figure 2). In normal cerebella, Purkinje cells were predominantly immunostained. The cell bodies, dendrites and axons of the cells were specifically immunostained (Figure 7a). The dendrites were clearly visualized, not only their main dendritic trunks but also the spiny branchlets studded with numerous spines. The axons of the Purkinje cells were traceable to the dentate nucleus, where the presynaptic terminals of the axons were seen as immunopositive products around the immunonegative cell bodies and dendrites of the dentate nucleus neurons. In the cerebella of the spinocerebellar-degeneration patients, the most striking feature was that spiny branchlets of the Purkinje cell dendrites were not stained, although atrophic cell bodies and main dendritic trunks were intensely stained (Figure 7b). There were some Purkinje cells whose cell bodies were completely negative for the InsP₃R1 (Figure 7c). Purkinje cell axons and torpedoes were more intensely stained than the cell bodies (Figure 7d). Our previous study suggested that the mRNA of InsP₃R1 might well exist in the dendrites of Purkinje cells [56]. A limited number of other mRNAs were shown to be present in dendrites [59,60]. The dendritic localization may permit the local regulation of their translation, probably including regulation by synaptic activity. Therefore, the patchy distribution of InsP₃R1 protein observed in the cerebella of those patients may reflect the loss of such local regulation. These findings demonstrate that InsP₃R1 is

Figure 8 Chromosomal mapping of human *InsP₃R1* gene (*Insp3r1*)

(a) Two partial human metaphases showing the specific site of hybridization to chromosome 3. Upper panels: arrowheads indicate silver grains on Giemsa-stained chromosomes, after autoradiography. Lower panels: the chromosomes were subsequently identified by R-banding. Arrows indicate chromosome 3. (b) Idiogram of human G-banded chromosome 3 illustrating the distribution of the *InsP₃R1* probe.

potentially important in normal brain physiology, such as long-term depression in cerebellum [61] and in aetiology of spinocerebellar degenerations such as OPCA and hereditary cerebellar atrophy of Holmes type.

The *InsP₃R1* gene (*Insp3r1*) maps to human chromosome 3

In the 120 metaphase cells examined, there were 254 silver grains associated with chromosomes and 71 of these were located on chromosome 3. A large percentage (78.8%) of them were mapped to the p25-p26 region of the chromosome 3, with the maximum

in the 3p26 band (Figure 8). These results allow us to map the *InsP₃R1* gene (*Insp3r1*) to the 3p25-p26 bands of the human genome. Comparison with chromosomal mapping of the mouse [56] reveals that the *c-raf-1* gene (3p25 in the human genome [62]) exists in the vicinity of the *InsP₃R1* gene in both species. The short arm of chromosome 3 is one of four regions of human chromosomes where loss of heterozygosity often accompanies many types of tumours, suggesting the existence of tumour suppressor genes in the regions [63]. The genetic locus (3p25-26) is also coincidental with that of von Hippel-Lindau disease [64], the disease gene of which behaves as a typical tumour suppressor. Recently, a putative tumour-suppressor gene of the disease was isolated and partially sequenced [65]. We compared the deduced amino acid sequence of that gene with that of human *InsP₃R1*, and could not detect any homology between the two genes.

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