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The latrophilin family: multiply spliced G protein-coupled receptors with differential tissue distribution

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Abstract Latrophilin is a brain-specific Ca^{2+}-independent receptor of α-latrotoxin, a potent presynaptic neurotoxin. We now report the finding of two novel latrophilin homologues. All three latrophilins are unusual G protein-coupled receptors. They exhibit strong similarities within their lectin, olfactomedin and transmembrane domains but possess variable C-termini. Latrophilins have up to seven sites of alternative splicing; some splice variants contain an altered third cytoplasmic loop or a truncated cytoplasmic tail. Only latrophilin-1 binds α-latrotoxin; it is abundant in brain and is present in endocrine cells. Latrophilin-3 is also brain-specific, whereas latrophilin-2 is ubiquitous. Together, latrophilins form a novel family of heterogeneous G protein-coupled receptors with distinct tissue distribution and functions.

Key words: α-Latrotoxin; Latrophilin; Homolog; Splicing; Tissue distribution

1. Introduction

α-Latrotoxin (LTX), a potent activatory presynaptic toxin from the black widow spider venom, is widely used as a tool to study exocytosis [1]. The toxin binds to its receptors on plasma membrane and stimulates massive release of neurotransmitters from neuronal, neuroendocrine and endocrine cells [2–5]. In order to understand the functions of these receptors in LTX action and in the normal secretory process, it is important to thoroughly investigate these proteins.

Two neuronal proteins are known to bind the toxin with high affinity, neurexin Ia [6,7] and the recently isolated latrophilin (also called CIRL for Ca^{2+}-independent receptor of α-latrotoxin) [8–11]. Neurexins are a family of neuronal cell-surface proteins with one transmembrane domain, ubiquitous distribution within the nervous system and an uncertain signalling mechanism [7,12,13]. Neurexin Iα binds LTX only in the presence of Ca^{2+} and, therefore, cannot mediate the toxin action in the absence of this cation [14]. Latrophilin is a G protein-coupled receptor (GPCR) with an unusual structure. Its physical and functional coupling to G proteins [11,15] makes it suited for signal transduction into the cell. Latrophilin, which is expressed in nerve terminals of neurones and in endocrine cells, binds LTX both in the presence and in the absence of Ca^{2+} ([8,11,14,16]; Ushkaryov, unpublished).

In the process of cloning and sequencing of latrophilin [11], we found several homologous proteins. Herein, we report the complete structure of bovine latrophilin (LPH1) and two of its homologues, termed latrophilin-2 and latrophilin-3 (LPH2 and LPH3). Our results reveal that all latrophilins possess the same architecture as unusual GPCRs, whilst multiple alternative splicing makes these proteins highly variable. Differential tissue distribution of latrophilins and the ability of only LPH1 to bind LTX suggest distinct functions for the members of this family of receptors.

2. Materials and methods

The full size insert from the rat latrophilin clone R9-15 [11] was used for conventional screening of a bovine brain oligo(dT)-primed cDNA library in λZAP II (Stratagene). Forty-nine independent clones were isolated in three initial rounds of screening. As none of these clones contained the 5′-end of the corresponding cDNAs, a randomly primed bovine brain cDNA library in λgt10 (Clontech) was hybridised using latrophilin type-specific probes. A further five rounds of screening yielded the total of 27 LPH1 clones, 35 LPH2 clones, and 19 LPH3 clones. Partial or complete sequences of all inserts were determined on an ABI PRISM automated sequencer, using synthetic oligonucleotides as primers and the ABI Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase according to the manufacturer's instructions (Perkin-Elmer). Sequences were analysed using the Lasergene software package (DNAStar). Current database searches were conducted on-line using the BLAST program; PEST motifs were identified with the help of PEST-FIND software. Nucleotide sequences of bovine LPHs and their splice variants determined in this work have been submitted to GenBank under accession numbers AF111069–AF111099.

Hybridisation of rat multiple tissue RNA blots was carried out as suggested by the manufacturer (Clontech). The longest inserts from rat or bovine latrophilin cDNA clones were used as probes. Affinity chromatography of brain and liver membranes and Western blotting of protein fractions was performed as described [8].

3. Results and discussion

3.1. Molecular cloning of bovine latrophilin and its homologues

Using affinity chromatography of solubilised bovine brain membranes, we purified latrophilin and determined the structure of four peptides isolated from this protein after its trypsinolysis [11]. Sequences of these peptides (YDLRTRIK, SGETVINTANYHDT, SGENAANIASELAR, and LAGEAGSGG, in the single-letter amino acid code) were used to verify the identity of clones isolated from the bovine brain cDNA library. Among these, we found two groups of clones that contained sequences which were distantly homologous to rat LPH [11] but which did not match exactly the peptides from the bovine brain protein. Proteins encoded by these cDNAs were termed bovine LPH2 and LPH3, whilst latrophilin proper was designated LPH1. Complete nucleotide sequences of all three LPHs were determined from corresponding overlapping clones. The location of initiation codons in all bovine cDNAs was facilitated by the fact that, in contrast to the rat sequence, stop codons are found just upstream of the

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start codons. The deduced amino acid sequences of the three bovine LPHs aligned with rat LPH1 are shown in Fig. 1A.

3.2. Domain structure and sequence homologies of latrophilins

All LPH proteins have the same architecture (Fig. 1A). The large extracellular domain begins with a well-defined signal peptide (*SP*) attached to a cysteine-rich domain (*Lectin*) that resembles some carbohydrate-binding proteins: galactose-specific lectin from sea urchin (SwissProt: P22031) and β-galactosidase (P45582). The *Lectin* domain is connected to an extended sequence (*Olf*) homologous to olfactomedin (Q07081), neuronal olfactomedin-related protein (Q62609, Q99784) and to myocilin (Q99972). Olfactomedin is found in olfactory epithelium and has been implicated in odorant binding [17]. Myocilin is predominantly expressed in retina; mutations in its functionally important region, which is homologous to olfactomedin and LPH, are associated with glaucoma [18]. The olfactomedin domain *Olf* is followed by a proline/threonine-rich sequence (*Pro/Thr*) and a *Linker* domain, which both have no substantial (above 20%) sequence homology to proteins in databases. A short stretch of residues at the borderline between the *Pro/Thr* and the *Linker* (termed *Signature*) can also be found in receptors of corticotropin-releasing factor, growth hormone-releasing factor, vasoactive intestinal peptide, secretin, and parathyroid hormone. These receptors, together with LPH, belong to the secretin/calcitonin family of GPCRs. The *Pro/Thr* and the *Linker* connect the olfactomedin domain with a region (*Long*) that lies immediately upstream of the transmembrane domains and is distantly similar to such 'long' GPCRs as CD97 (P48960) and EMR1 (Q61549). At the C-terminal part, just next to the membrane, the *Long* contains a conserved pattern (*4Cys*) with four cysteines residues CxFW....GxWxxxGC.....CxCxHLTxFA(I/V)LM (where x denotes any amino acid and dots represent variable number of residues). This pattern is present in a similar, near-membrane position in many GPCRs and could determine the site of proteolytic degradation that may occur in LPH1 under certain conditions [10]. The seven transmembrane regions (*TMRs*) bear substantial homology to all GPCRs of the secretin/calcitonin family. These receptors have been shown to participate in various secretory processes mediated by G proteins. The C-terminal cytoplasmic sequence (*Cytoplasm*) does not exhibit any distinct domain structure, except that it contains multiple potential sites of phosphorylation and palmitoylation and several *PEST* regions. PEST regions (rich in prolines, glutamic acids, serines and threonines) confer susceptibility to rapid proteolysis [19–21], and many proteins that contain such a motif may be extraordinarily short-lived. There are three possible *PEST* regions in LPH1, two in LPH2 and one in LPH3 (Fig. 1), suggesting that the C-terminus of LPH1 may be prone to fast degradation consistent with our observation of its very labile character (unpublished).

At the amino acid level, bovine LPH1 is highly homologous to the rat protein (98.3% of identical amino acid residues). Furthermore, the LPH1 extracellular domains are almost identical in the two species (99.4%). The overall similarity between all three bovine LPH homologues is also substantial and ranges from 70 to 75%. Thus, LPH proteins of the same type from different animal species are much more alike than different LPH types within the same species, indicating a high degree of specialisation and distinct functions. The distribution of evolutionarily invariable residues is uneven along the molecule. The highest sequence conservation is found in the *Lectin* domains (80–87%), suggesting that this LPH region is important for receptor functioning, e.g. it could interact with some glycosylated proteins. The *Olf* domains, *Linkers*, *Long* regions, *TMRs* and the upstream thirds of the cytoplasmic domains all display 60–75% identity. In contrast, the *Pro/Thr* domains as well as the large distal parts of the C-termini of LPHs are much more divergent (20–40% homology) and may have evolved to carry out diverse functions.

3.3. Extensive alternative splicing

One of the interesting features of LPHs is the presence of multiple splice sites that can alter the proteins by introducing insertions or deletions in both the extracellular and the cytoplasmic domains. Positions of some splice sites seem to be conserved among LPHs; based on this observation and on partial genomic sequences of these proteins, we postulate seven potential sites of alternative splicing (Fig. 1B). Considering a very elaborate intron-exon structure of the LPH genes (unpublished), it is likely that other splice sites also exist that have not been identified in this work. Of the seven splice sites, four are located in the extracellular domain, at the borders of the *Lectin* and *Olf* domains. These sites have a simplified repertoire of alterations: they usually bring about either a deletion or an insert, which sometimes may be large (Fig. 1A). The splicing pattern in the cytoplasmic parts of LPH is usually more elaborate. Thus, up to three different variants of inserts can be introduced in the third cytoplasmic loop of LPH2 (splice site 5). These are likely to substantially modify the coupling and G protein-mediated signalling of this GPCR. The most striking, however, is the splice site 7. We found that some of the inserts present at this position in LPH2 and 3 cause a frame shift and occurrence of stop codons soon after the insert and long before the usual end of the sequence. This results in the translation of a C-terminally truncated protein and may significantly affect the protein's stability, targeting and/or signalling. Not all possible alternative splicing is realised in each gene. In contrast to LPH2, LPH3 and, especially, LPH1 are conserved. In LPH1, we were able to find only one position in the extracellular domain (splice site 2) where an insert of just five amino acids may be spliced in or out. In LPH3, in addition to a similar five-residue insert at splice site 2, sixty-seven amino acid residues can be introduced (or removed) at splice site 1, immediately downstream of the signal peptide (Fig. 1A). The conservation of the extracellular sequences of LPH1 and 3 could mean that they have important and specialised functions, different from those of LPH2, and

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Fig. 1. The structure of latrophilins. A: Alignment of deduced amino acid sequences of rat LPH1 [11] and bovine LPH1, 2 and 3. Residues identical to those of bovine LPH1 are shaded; different shades of grey are used to distinguish the LPH domains, which are labelled above the sequences (see text). Residues that can be spliced out at indicated sites are shown in bold and crossed out. Asterisks denote stop codons, putative PEST regions are underscored. B: Multiple alternative splicing of latrophilins. LPH proteins are schematically presented with the structural domains shaded as in A. Triangles represent inserts of variable sizes found in respective positions in several independent clones.


A
SP
SPLICE SITE 1
Lectin
SPLICE SITE 2
Olf
Olf
Pro/Thr
SPLICE SITE 3
Signature
Linker
SPLICE SITE 4
Long
Long
4Cys
TMR1
TMR2
TMR3
TMR4
TMR5
SPLICE SITE 5
TMR6
TMR7
Cytoplasm >
SPLICE SITE 6
SPLICE SITE 7(1,2)
SPLICE SITE 7(3)
PEST
PEST
PESTs
LPH1 RAT
LPH1 BOV
LPH1varC
LPH2 BOV
LPH3 BOV
LPH2varD
LPH3varG
LPH3varH
B
SP Lectin
Olf
Pro/Thr
Linker
Long
4Cys
TMRs
Cytoplasm
PESTs
LPH1
LPH2
LPH3

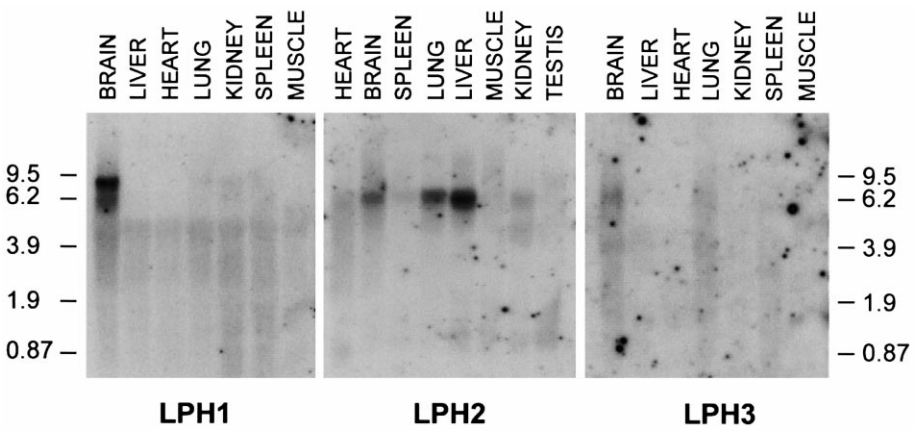

Fig. 2. Northern blot analysis of latrophilins. Rat multi-tissue RNA blots were hybridised to radiolabelled bovine cDNA probes specific for LPH1, 2 or 3, as indicated. Positions and sizes (in kbp) of RNA standards are shown on the sides.

are under evolutionary pressure to keep their structure unchanged. Another striking observation is the presence of a stop codon in the rare insert C at splice site 2 of LPH1 (Fig. 1A). The N-terminal *Lectin* domain in this case must be released and could function as a soluble ligand of some receptors.

3.4. *Distribution of latrophilins and their interaction with α-latrotoxin*

Northern blotting of RNA isolated from different rat tissues with probes specific for LPH1, 2 and 3, revealed that both LPH1 and 3 are almost exclusively brain-specific, although LPH3 is in general much more scarce (Fig. 2). Exceedingly low amounts of LPH1 were found also in kidney, lung and spleen, but not in muscle, liver or duodenum. This may be due to the presence of neuronal or endocrine cells in these preparations. Indeed, LTX is known to stimulate latrophilin-mediated secretion from adrenal chromaffin cells and pancreatic β-cells [5,16]. Alternatively, since LPH1 has been implicated in secretion [10,11,22], very low levels of LPH1 expression may be needed for many cells to regulate their exo- or endocytic functions. Curiously, another LTX receptor, neurexin is expressed in appreciable amounts not only in brain but also in other tissues, mostly in lung (data not shown). LTX, however, does not appreciably interact with non-neuronal or non-endocrine tissues, nor does it bind to COS cells that are derived from kidney cells. Probably, the toxin requires a certain concentration of receptors (LPH and/or neurexin) to bind to the cell surface. It is also possible that in non-neuronal tissues that have a very low level of LPH1 mRNA, the protein is not translated or not properly modified and/or targeted. Considering that LPH1 is at least 50 fold more abundant in brain than in any other tissue and that its localisation in the nervous system is restricted to nerve terminals (to be published), it may have a highly specialised function. LPH3, too, is evident outside brain, namely in lung and spleen. In contrast, the distribution of LPH2 is radically different (Fig. 2). This ubiquitous protein appears to be prevalent in liver but is also abundant in lung and brain and is expressed to variable extent in all tissues tested.

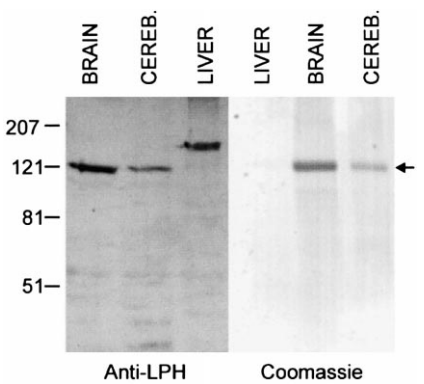

Fig. 3. Left: Immunostaining of LPH1 and LPH2 in the plasma membranes from bovine brain, cerebellum and liver. Membrane proteins were separated by SDS-electrophoresis and electroblotted and stained with an anti-LPH1 antibody. Right: Affinity chromatography of solubilised membranes from the same tissues on immobilised LTX. Membranes from bovine tissues were solubilised and passed through the LTX column [11]; eluted proteins were analysed by SDS-electrophoresis with Coomassie blue detection. Positions and molecular masses (in kDa) of standard proteins are shown on the left; the arrow indicates LPH1.

In order to extend the observations made at the RNA level to the level of proteins, we performed immunoblotting of brain and liver membranes, using an antibody against the extracellular domain of LPH1. As demonstrated in Fig. 3, this antibody recognised both LPH1 and LPH2, which are abundant in brain and liver, respectively. We were able to distinguish between the two proteins because of the difference in their molecular masses, which is most likely due to differential proteolysis. No LPH1 was detected in liver and, reciprocally, no LPH2 could be seen in brain (Fig. 3). In spite of the similar primary and domain structures of LPH1 and 2, LTX affinity chromatography of brain and liver membranes (Fig. 3) produced only LPH1 and only from brain but not from liver. This indicates that although LPH2 is abundant in liver, it cannot serve as LTX receptor. Vice versa, although the toxin strongly binds to the receptor, it is unable to concentrate any detectable amount of LPH1 from liver. As a control of specificity of the chromatographic procedure, another tissue (cerebellum) that contains substantial amounts of LPH1 was used. In full agreement with the toxin binding data, the concentration of LPH1 in cerebellum was three to four fold lower than in brain; this ratio was also preserved in the course of affinity chromatography (Fig. 3). Based on the absence of LPH3 sequences among the 18 peptides isolated

from the receptor purified from brain, LPH3 is also unlikely to bind the toxin.

In conclusion, the experiments reported in this paper describe a novel family of latrophilin-related proteins. These proteins display fascinating common structural features: complex domain structure, potential to couple to G proteins and multiple alternative splicing. However, their differential distribution in the organism, type-specific evolutionary preservation and binding of LTX by only one member of the family suggest that LPH proteins have distinct functions. Despite their similarities, LPHs are likely to interact with different ligands. Future work will concentrate on the functions of these interesting receptors and their endogenous ligands.

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