The Calcium-independent Receptor for α-Latrotoxin from Human and Rodent Brains Interacts with Members of the ProSAP/SSTRIP/Shank Family of Multidomain Proteins

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Subtypes of the calcium-independent receptors for α–latrotoxin (CIRL1–3) define a distinct subgroup within the large family of the seven-transmembrane region cell surface receptors. The physiological function of CIRLs is unknown because neither extracellular ligands nor intracellular coupling proteins (G-proteins) have been identified. Using yeast two-hybrid screening, we identified a novel interaction between the C termini of CIRLs and -2 and the PSD-95/discs large/ZO-1 (PDZ) domains of a recently discovered multidomain protein family (ProSAP/SSTRIP/Shank2) present in human and rat brain. In vitro, CIRL1 and CIRL2 interacted strongly with the PDZ domain of ProSAP1. The specificity of this interaction has been verified by in vivo experiments using solubilized rat brain membrane fractions and ProSAP1 antibodies; only CIRL1 but not CIRL2, was co-immunoprecipitated with ProSAP1. In situ hybridization revealed that ProSAP1 and CIRL1 are co-expressed in the cortex, hippocampus, and cerebellum. Colocalization was also observed at the subcellular level, as both CIRL1 and ProSAP1 are enriched in the postsynaptic density fraction from rat brain. Expression of all three CIRL isoforms is highly regulated during postnatal brain development, with CIRL3 exhibiting its highest expression levels immediately after birth, followed by CIRL2 and finally CIRL1 in aged rats.

Most neuronal cell surface receptors are attached to intracellular proteins via specific protein/protein interactions; this has been demonstrated in a number of cases involving ligand-gated ion channels and G-protein-coupled receptors, as well as cell adhesion molecules (e.g. Refs. 1–3). A protein module frequently involved in this type of interaction is the PDZ domain, which can specifically contact the intracellular C terminus of distinct membrane proteins (e.g. Ref. 1). It is generally believed that this type of interaction serves a function in anchoring receptors to the cytoskeleton, thus leading to the targeting of receptors to their presumed site of action, e.g. in the postsynaptic specializations of neurons. In addition, the interaction with PDZ domains may be important for the recruitment of receptors to specific signal transduction pathways, as exemplified by the interaction of β-adrenergic receptors with the Na+-H+ exchanger regulatory protein (11).

Recently, a novel group of cell surface receptors has been described that resembles the G-protein-coupled receptor superfamily because it shares the typical pattern of seven hydrophobic transmembrane regions. A characteristic feature of this new family is the very large N-terminal, extracellular part, which may contain several domains usually found in cell adhesion molecules. A prototype of this group of receptors is the calcium-independent receptor for α-latrotoxin (CIRL). Together with neurexin 1a, CIRL is present on axon terminals, where it is thought to tether the toxin from black widow spider venom to presynaptic structures; this facilitates insertion of α-latrotoxin into the plasma membrane which then leads to massive exocytosis of neurotransmitter (12, 13). Three isoforms of CIRL have been identified by molecular cloning (latrophlin 1–3/CIRL1–3 (14–16)). CIRLs have been shown to contain two subunits, derived from a common precursor by proteolytic processing and linked together by disulfide bonds (17, 18). Together with the brain angiogenesis inhibitor proteins (19), the Emr1 protein, CD97, and the HE-6 protein, about 15 members of this receptor family can be identified by data base searches (see Ref. 14). However, the physiological function of CIRLs remains enigmatic, particularly because neither the physiological ligands nor intracellular signal transducing proteins are known. To shed more light on the function of CIRLs, it will be necessary to determine their cellular and subcellular distribution. In addition, the identification of intracellularly associated proteins may help to delineate the signal transduction pathway in which CIRLs are involved.

Here, we show that the intracellular C terminus of CIRLs binds with high affinity to the PDZ domains of the ProSAP/SSTRIP/Shank family of multidomain proteins. Recently, it has been shown by several groups that ProSAP/SSTRIP/Shank proteins are important structural constituents of synaptic

1 The abbreviations used are: PDZ, PSD-95/discs large/ZO-1; BAI, brain angiogenesis inhibitor; CIRL, calcium-independent receptors for α-latrotoxin; CA1–3, Cornu ammonis regions 1–3; GST, glutathione S-transferase; ProSAP, proline-rich synapse-associated protein; PSD, postsynaptic density; RIPA, radioimmune precipitation; SAP, synapse-associated protein; SSTR, somatostatin receptor; SSTRIP, SSTR-interacting protein; bp, base pair(s).

2 Because of the fact that the ProSAP/SSTRIP/Shank protein family has been discovered independently by several groups, the terminology for the three members existing thus far varies as follows: SSTRIP/Shank1/Synapom (4–6), ProSAP1/CortBP1/Shank2 (5, 7–9), and ProSAP2/Shank3 (7, 8, 10).
structures in human and rodent brain (4–8, 10). ProSAP/SSTRIP/Shank have been shown to interact with the C terminus of the somatostatin receptor subtype-2 (3, 4) and with members of a PSD protein family termed SAPAP/GKAP (SAP90/PSD95-associated protein/guanylate kinase-associated protein) (5, 6, 8, 10). The latter interact directly with postsynaptic scaffold proteins (20–25). The data reported here suggest that members of the ProSAP/SSTRIP/Shank protein family are also involved in the synaptic targeting of CIRLs and thus may contribute to signal transduction events mediated by the so far unknown physiological ligands of these receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit antiserum against the N-terminal regions of CIRL1 and CIRL2 were generously supplied by Drs. V. Krasnoperov and A. Petrenko (New York University School of Medicine, New York). The antiserum directed against the C terminus of ProSAP1 was described previously (7).

**Yeast Two-hybrid Screening**—The PDZ domain of human STRIP cloned into pSB1-Cih2 (CLONTECH Laboratories Inc., Palo Alto, CA) and the PDZ domain of rat ProSAP1 cloned into pAS2 were used as bait for screening rat brain and rat brain Matchmaker two-hybrid cDNA libraries (CLONTECH), respectively, as described (3, 7, 8).

**In Situ Hybridization**—Rat brains were cut with a cryostat in horizontal sections (18 μm). CIRL mRNAs were detected with CDNA antisense oligonucleotides purchased from MWG-Biotech (Ebersberg, Germany); CIRL1 (GenBank® accession no. U72457), 5′-TCC-AGA-CTG-CTG-CAG-CCA-AGC-GGC-CCA-TGG-CAA-A3′ (bp 112–78); CIRL2 (GenBank® accession no. AF063102), 5′-GCC-TCC-TCT-CTG-GAA-ACC-CTC-GTG-ATT-CGA-GGA-3′ (bp 113–79) and 5′-TCA-GGA-CTG-TGC-ACG-GGT-GTG-GGG-GAC-CGG-GAA-3′ (bp 364–3612); and CIRL3 (GenBank® accession no. AF063103), 5′-AGG-GCC-TCC-TGG-GCTGTG-CTG-ATG-CTAG-TGG-GAA-3′ (bp 230–197). Hybridization was performed as described previously (7).

**Fractionation of Rat Brain Membranes**—Crude synaptosomal and PSD fractions were prepared from adult rat brain as described (26). The PSD/One Triton fraction was used for Western blotting experiments as previously described (4).

**Immunoprecipitation from Rat Brain Membranes**—ProSAP1 antiserum (5 μl) was coupled to NHS-activated Sepharose (Amersham Pharmacia Biotech) following the manufacturer's instructions. Crude membranes derived from rat brain (2 mg total protein) were solubilized in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) at 37 °C for 30 min followed by incubation on ice for 30 min. Insoluble matter was removed by centrifugation at 20,800 × g for 30 min, and the supernatant fractions were incubated with the immobilized antiserum overnight at 4 °C. After washing extensively in RIPA buffer, the precipitates were eluted with Laemmli sample buffer containing 8 M urea, separated on 8% polyacrylamide gels containing 8 M urea, and electoblotted onto nitrocellulose. CIRL proteins were detected using CIRL1- and CIRL2-specific antisera followed by alkaline phosphatase detection.

**RESULTS**

**Interaction between the PDZ Domain of Human STRIP/Shank 1 and the C Terminus of CIRLs**—To identify potential candidates interacting with members of the STRIP/Shank 1 family, the PDZ domain of human STRIP was used as bait in the yeast two-hybrid system. By screening a human brain cDNA library, we observed a specific interaction with a clone containing the C-terminal part of latrophilin-2, the human homologue of CIRL2 (27) and four clones containing the C-terminal part of brain angiogenesis inhibitor-2 (BAI-2; Ref. 19). In an independent yeast two-hybrid experiment, the highly homologous PDZ domain of ProSAP1 was used to screen rat brain cDNA library. In this case, four independent specifically interacting clones all coding for the C terminus of the rat CIRL1 protein were obtained (Table I). Both the CIRL and the BAI proteins have recently been identified as seven-transmembrane proteins, characterized by an exceptionally large extracellular domain. The C-terminal sequences of CIRL1 and CIRL2 are very similar to each other and contain typical consensus sequences for the interaction with PDZ domains, i.e. the sequence motif SS/T-X-ϕ; where ϕ is a large, hydrophobic amino acid residue. This motif is also shared by BAI-2, suggesting that all three receptors identified here (i.e. CIRL1, CIRL2, and BAI-2) are potential interaction partners for the PDZ domains of the STRIP/Shank 1 protein family.

The CIRL-STRIP/ProSAP interactions detected by the yeast two-hybrid system were further verified by overlay assays using GST fusion proteins of the PDZ domains of rat ProSAP1 and human STRIP. The biotinylated fusion proteins of the C termini of the CIRL and BAI proteins were used as probes (3). As depicted in Fig. 1, the overlay assays revealed strong interactions of the PDZ domain of rat ProSAP1 with both CIRLs but not with BAI-2. Using the PDZ domain of human STRIP, an interaction with the C terminus of CIRL1, but neither CIRL2 nor BAI-2, was observed, suggesting that the interaction of the PDZ domain of ProSAP1 and the C termini of CIRL1 and -2 exhibits a higher affinity than that between STRIP and the two CIRLs. Interactions of ProSAP1 and STRIP with BAI-2 were sufficient in the yeast two-hybrid system but were absent in the overlay assay, indicating that they may be of little physiological significance. Therefore, we have focused our further studies on the interaction of STRIP/ProSAP1 with the α-latrotoxin receptor isoforms.

To verify our findings in vivo, we performed co-immunoprecipitation experiments using plasma membrane fractions of rat brain and specific antibodies raised against the two proteins. Initial attempts with membrane fractions solubilized by boiling in Laemmli buffer did not yield CIRL1 or CIRL2 immunoreactivity (CIRL antibodies were directed against the N-terminal extracellular regions) in Western blotting experiments. Significant bands at the anticipated molecular weight of 120 kDa for CIRL1 and at ~140 kDa for CIRL2 were detected only when solubilization of the membranes was performed in the presence of 8 M urea at 37 °C (Fig. 2A). As ProSAPs are not easily soluble in nondenaturing detergents (7, 8), as well as to compromise between the different solubility characteristics of ProSAPs and CIRLs, the brain membrane fractions were treated with RIPA buffer containing 0.1% SDS at 37 °C. Under these conditions, ProSAP1 was efficiently immunoprecipitated from solubilized rat brain membranes using an antibody directed against the C terminus. In this reaction, CIRL1 but not CIRL2 was coprecipitated (Fig. 2B). Thus, although in vitro both the C termini of CIRL1 and CIRL2 interact with the PDZ domain of ProSAP1, in vivo a physiological protein/protein interaction appears to occur only between CIRL1 and ProSAP1.

**Coproexpression of ProSAP1 and CIRL1**—As CIRLs are involved in the presynaptic action of α-latrotoxin, whereas the

| C terminus | Interacting partner |
|------------|---------------------|
| Human latrophilin/CIRL2 | Q L V T S L * |
| Human BAI-2 | Q V T S L * |
| Rat CIRL1 | Q V T S L * |
| Rat ProSAP1 | Q V T S L * |
| SAPAP/GKAP | E A Q T R L * |
| SSTRIP2 | D L Q T S I |
| mGluR5 | Q S S S L * |

* The last six amino acid residues (identical ones are in bold letters) at the C terminus of the targeted proteins are depicted. The respective references are given in parentheses. +, stop codon; SAPAP, SAP90/PSD95-associated protein; GKAP, guanylate kinase-associated protein; mGluR5, metabotropic glutamate receptor 5.
ProSAP/SSTRIP/Shank proteins are constituents of postsynaptic structures (4, 5, 7, 8), we investigated whether CIRLs might also be present in postsynaptic membrane fractions. By Western blotting, we detected enrichment of the CIRL1 protein in the PSD fraction from rat brain confirming that the specific interaction between ProSAP1 and CIRL1 correlates with the presence of both proteins in the PSD. In contrast, CIRL2 was present only in crude and synaptosomal fractions but not in the PSD fraction (Fig. 2C).

As no data on the temporal and regional patterns of expression of the CIRL proteins in brain were available, we compared the distribution of mRNAs coding for ProSAP1 and CIRL1–3 by in situ hybridization experiments using specific oligonucleotide probes on rat brain sections (Fig. 3A). In agreement with previous observations, ProSAP1 is expressed very early, during postnatal development, and is widely distributed in the central nervous system of rats. Particularly high levels are detected in the cortex, the hippocampus (CA1–CA3 regions and the dentate gyrus), and the Purkinje cell layer of the cerebellum. CIRL1 mRNA is present during early development. However, the highest expression levels are detected at the age of 2–3 weeks, with a moderate decline toward adulthood. CIRL1 was found mainly in the cortex, the CA1–CA3 regions of the hippocampus, and the cerebellum in a pattern largely overlapping that of ProSAP1. Lower levels of expression were detected also in the caudate putamen. In contrast to CIRL1, expression levels of CIRL2 peaked during early postnatal development and declined during adult life, with an almost complete loss of expression in the 1-year-old rat. The regional distribution of CIRL2 was also strikingly different when compared with CIRL1. In particular, expression is strong in the CA1 region of the hippocampus but is absent in CA2 and CA3 as well as in the dentate gyrus. Strong labeling was also observed in the thalamus and in caudate putamen. CIRL3 exhibited its highest expression level immediately after birth, thus preceding both CIRL1 and CIRL2. Whereas expression in the forebrain is strongly reduced in the adult stages, expression
of CIRL3 is maintained in the cerebellum of adult rats. When comparing the expression profiles of ProSAP1 and CIRL1, both are largely identical, again supporting the notion that CIRL1 and ProSAP1 are physiologically interacting partners (Fig. 3B).

**DISCUSSION**

The data shown here provide evidence for a novel interaction between the PDZ domain of ProSAP/SSTRIP/Shank proteins and the seven transmembrane-spanning, putative G-protein-coupled receptors for α-latrotoxin, CIRL1. The interaction is presumably driven by the very C terminus of the CIRL protein, which carries a typical recognition motif for the PDZ domain. Data previously obtained with the PDZ domains of ProSAP/SSTRIP/Shank (4, 5) and those presented here may help to delineate the structural constraints that determine the specificity in the interaction of ProSAP/SSTRIP/Shank with proteins carrying the PDZ recognition motif. Thus, a larger hydrophobic residue (isoleucine or leucine) in the last position of the consensus sequence is required for binding (see Fig. 1), whereas valine (which is required for the NMDA (N-methyl-D-aspartate) receptor/PSD-95/SAP102 interactions (1)) is not present in this position in any of the interacting proteins. In agreement with the data obtained by Naisbitt et al. (5), a negative charge is not tolerated in the −2 position, as seen here for BA1-2, which interacts only with low affinity (see Table I and Fig. 1). On the other hand, specificity may not exclusively be determined by the PDZ recognition motif; CIRL1–3 carry identical PDZ consensus sequences at their C termini (last five amino acid residues), and yet only CIRL1 has been found to interact specifically in vitro and in vivo with ProSAP1.

An important question remains as to the actual location in neurons where CIRL1 and ProSAP1 interact. Because of the toxic principle of α-latrotoxin, which induces neurotransmitter release after binding to CIRL1 and neurexin (12), CIRL has been presumed to be a presynaptic receptor. ProSAP1 and other members of this protein family, however, have been shown to be highly enriched in the postsynaptic density of neurons (4, 5, 7, 8). The CIRL antibodies available to us were not suitable for ultrastructural analysis,3 which could ultimately solve this question. We show here the presence of CIRL1 in the postsynaptic membrane fraction derived from rat brain. Additionally, it appears possible that isoforms of the ProSAP/SSTRIP/Shank family are also present in presynaptic compartments of neurons. Because of the unique extracellular domain structure of CIRL, which includes several motifs otherwise found in cell adhesion molecules, it has been suggested that CIRLs and related receptors function as such and not as G-protein-coupled receptors that bind to soluble, secreted ligands. In this respect, our data indicate that CIRLs are involved in neuronal cell adhesion events, possibly during synaptogenesis, by acting on presynaptic, axonal elements as well as the postsynaptic, dendritic structures. In agreement with a role in synaptogenesis, CIRL expression is highly regulated during development. CIRL3 expression peaks very early in development, with the highest levels observed immediately after birth. CIRL2 mRNA is also present during early postnatal development, reaching its peak expression level a few days later. During later stages, the roles of CIRL2 and -3 may be taken over by CIRL1, which shows an increase in expression concomitant with the loss of CIRL3 and especially CIRL2, which is hardly detectable in the aged rat. CIRL3 expression remains strong in the cerebellum, where it partially matches that of ProSAP1. It appears possible that an interaction occurs between these two proteins in the cerebellum, as the C-terminal sequence of CIRL3 (HLVTSL-Stop) is similar to that of the other CIRLs. However, as no CIRL3 clone was detected in our yeast two-hybrid screen, and because no CIRL3 specific antibodies were available to us, we were unable to verify this possibility.

Taken together, the presence of CIRL on pre- and postsynaptic elements of synapses, the highly regulated pattern of CIRL isofrom expression, and the domain structure of its extracellular region suggest a role for CIRL proteins as cell adhesion molecules during synaptogenesis, which may be mediated in part through interactions of the C terminus with synaptic scaffolding molecules of the ProSAP/SSTRIP/Shank multidomain protein family.

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