A Tripartite Nuclear Localization Signal in the PDZ-domain Protein L-periaxin*

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The murine Periaxin gene encodes two PDZ-domain proteins in myelin-forming Schwann cells of the vertebrate peripheral nervous system (Dytrech, L., Sherman, D. L., Gillespie, C. S., and Brophy, P. J. (1998) J. Biol. Chem. 273, 5794–5800). Here we show that L-periaxin is targeted to the nucleus of embryonic Schwann cells. Subsequently, the protein redistributes to the plasma membrane processes of the myelinating Schwann cell where it is believed to function in a signaling complex. In contrast, L-periaxin remains in the nucleus when expressed ectopically in oligodendrocytes, the myelinating glia of the central nervous system. The nuclear localization signal (NLS) is basic and tripartite and comprises three signals that act synergistically. Nuclear targeting of L-periaxin is energy-dependent and is inhibited by cell-cell contact. These data show that L-periaxin is a member of a growing family of proteins that can shuttle between the nucleus and cortical signaling/adherence complexes.

L-periaxin was identified in a screen for cytoskeleton-associated proteins in myelinating Schwann cells of the peripheral nervous system (PNS)† (1). Subsequently it was shown that the murine Periaxin (Prx) gene encodes L-periaxin and a truncated isoform, S-periaxin (2). Both proteins have N-terminal PDZ protein-binding domains (2), a motif which was named after the three proteins in which it was first identified, namely post-synaptic density protein PSD-95, Drosophila discs large (dlg) tumor suppressor gene and the tight junction-associated protein ZO-1. This motif consists of an approximately 90-amino acid protein-binding module found in a growing family of proteins that are believed to have an organizing and signaling function at sites of cell-cell contact (3).

Some PDZ-domain proteins such as ZO-1, MAGI-1c, and LIM-kinase 1 can redistribute between the cell cortex and the nucleus which is believed to reflect their role in transmitting regulatory signals between the cell surface and the nucleus (4–6). Nuclear targeting of MAGI-1c is thought to be regulated by a nuclear localization signal (NLS) (4). NLSs are relatively short sequences which may be identified by their capacity to transport heterologous proteins into the nucleus (7). Classical NLSs comprise either one or two (mono- or bipartite) basic sequences (7), although new types of non-basic targeting domains seem to function as NLSs in nuclear ribonucleoprotein particle proteins (8). In the case of the LIM-kinase 1, the PDZ domain itself has a direct role in regulating the nuclear export whereas a short, basic NLS appears to direct import (6). Classical NLS functions by binding to the importin/karyopherin complex prior to Ran-mediated nuclear uptake (7).

Here we show that although L-periaxin has a PDZ domain, which is believed to recruit proteins to a cortical structure involved in transmembrane signaling (1, 9), it also possesses an unusual tripartite NLS. Localization of L-periaxin to the Schwann cell nucleus when it is first expressed in the embryonic PNS indicates that the NLS is functional in vivo.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—The Schwann cell line, 33B, was a generous gift from Dr. Eric Blair, Leeds University, and was maintained in complete medium, 10% fetal calf serum (FCS), Dulbecco’s modified Eagle’s medium with penicillin (100 IU/ml), and streptomycin (100 μg/ml)/Sigma). Schwann cells were isolated from the sciatic nerves of P5 rats and placed in a 35-mm Petri dish containing pre-warmed L15 (Sigma). The ependyma was removed by dissection with fine forceps and the nerve fibers were cut into small pieces and digested with collagenase (0.24 mg/ml) for 45 min at 37 °C before the addition of trypsin (0.6 mg/ml) for 10 min. Trypsinization was terminated by the addition of complete medium. After trituration, the cells were centrifuged for 5 min at 1000 rpm, resuspended in complete medium and cultured on flasks pre-coated with poly-L-lysine (0.1 mg/ml). After exposure to 10 μM cytosine arabinoside (Ara C, Sigma) in complete medium for 7 days, fibroblasts were killed by complement lysis using Thy-1.1 monoclonal antibody diluted 1:10 (generous gift of Dr. A. F. Williams, Oxford University) and baby rabbit complement (Cedarlane Laboratories Ltd., Haltham, NY). The complement-mediated lysis was repeated. The cultures were then expanded in complete medium with 2 μM forskolin and seeded on coverslips coated with poly-L-lysine and laminin (Sigma). A modified protocol was used for embryonic Schwann cells. After collagenase treatment at 37 °C for 40 min, hyaluronidase (1 mg/ml, Sigma) was added to the nerve segments for 15 min after which an additional amount of collagenase was added. Cells were transferred to collagen-coated 6-cm dishes. Cells were plated on laminin-coated coverslips that had been pre-coated with poly-L-lysine. The cultures were incubated for 6.5 h before fixation.

**Mammalian Expression Vectors and Constructs**—L-periaxin cDNA (1) was directionally subcloned into pcCB6 expression vector (a gift from Dr. David Russell, Southwestern Medical Center, Dallas, TX) which includes a neomycin-resistance gene for selection. BD-GFP, in which the basic domain (244 bp) of L-periaxin (residues 118–141) was fused to green fluorescent protein (GFP), was generated by PCR. The enzyme Pfu polymerase (Stratagene) was used for all the PCR reactions. The forward primer was designed with an EcoRI site followed by an initiation codon with a strong Kozak consensus sequence. The reverse primer contained a BamHI site. The PCR product was digested with EcoRI and BamHI and cloned into the expression vector pEGFP-N1 (CLONTECH). This vector encodes a GFP which fluoresces with an enhanced quantum yield compared with wild-type GFP. The basic domain of L-periaxin was then expressed as an N-terminal fusion with GFP. DNA for transfection was prepared by Qiagen Midi-prep. A similar strategy was adopted to generate GFP fusion constructs with the basic sub-domains BD1 (residues 116–145, BD1-GFP), BD2 (residues 140–150, BD2-GFP), BD1 and BD2 (residues 116–176, BD1.2-GFP), and BD2 and BD3 (residues 140–196, BD2.3-GFP). The basic domain of L-periaxin cDNA (residues 118–196) was deleted using a PCR-ligation-PCR technique to generate Prx3BD (10). The template for the PCR was wild-type L-periaxin cDNA in pSPORT (Life Technologies,
Inc.), and the final product was subcloned into the expression vector pSVSPORT (Life Technologies, Inc.).

**Transfection**—33B cells grown in 100-mm plates were transfected with DNA (24 μg) and LipofectAmine (64 μg) (Life Technologies, Inc.) in OptiMEM (Life Technologies, Inc.) for 4 h at 37 °C in a humidified CO₂ incubator. They were then washed and complete medium was added. To prepare permanent L-periaxin transfectants, 600 μg/ml G418 was added to the medium, and the cultures were maintained until single G418-resistant colonies appeared. These colonies were cloned out. Schwann cells were seeded on poly-d-lysine-coated coverslips in 35-mm dishes, and plasmid DNA (3 μg) with LipofectAmine was added to the cells for 4 h after which they were grown for 24 to 48 h in DMEM with 10% FCS. The cells were either processed for immunocytocchemistry or they were viewed directly for GFP fluorescence after brief fixation with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature.

**Antibodies**—Rabbit anti-periaxin (anti-170pep1) has been described (1) and was diluted 1:3000 for immunofluorescence, and affinity-purified anti-170pep1 was used at 1:50 for immunoelectron microscopy. Anti-CNP (Chemicon) was used for immunocytocchemistry at a dilution of 1:200.

**Immunofluorescence Microscopy and Western Blotting**—Sciatic nerves from mice at embryonic ages were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 2 h. The nerves were washed in three changes of 0.1 M phosphate buffer for 10 min each and prepared for immunofluorescence microscopy as described previously (2). Cultured cells on 13-mm glass coverslips (BDH) were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 30 min at room temperature. The cells were washed four times for 5 min each with PBS and blocked with 4% goat serum, 0.2% gelatin, 0.1% Triton X-100 in phosphate-buffered saline for 30 min. For both sections and cultured cells, immunofluorescence labeling was as described previously (2). In some experiments, propidium iodide staining was combined with immunolabeling. Sections were incubated with propidium iodide (100 μg/ml; Sigma) for 2 min after immunolabeling was completed. The slides were viewed on an Olympus BX60 microscope or a Leica TCS 4D scanning confocal microscope when a maximum projection was made from a series of optical scans through the z axis of the tissue. The digitized images were processed using Adobe Photoshop™ 3.0 software on a Power Macintosh 8600/200. Western blotting was performed as described previously (1).

**Post-embedding Immunoelectron Microscopy**—P1 rat sciatic nerves were removed and fixed by immersion in 4% formaldehyde (freshly prepared from paraformaldehyde) in a 0.01 M sodium periodate, 0.075 M lysine, 0.1 M phosphate buffer containing 3% sucrose at pH 7.4 (11) for 2 h at room temperature. Tissue was processed, infiltrated with LR Gold (Agar Scientific Ltd., Stanstead, Essex, UK) containing 0.5% benzoin methyl ether, and then embedded in gelatin capsules as described previously (1, 12). Sections on formvar-carbon-coated nickel grids were block stained with 1% bovine serum albumin, 0.5% fish skin gelatin, 0.05% Tween 20, 10 mM Tris, 500 mM NaCl, pH 7.4 for 30 min at room temperature, and incubated with affinity-purified anti-170pep1 antibody in the same buffer overnight at 4 °C. Grids were washed with the same buffer and incubated for 1 h with goat anti-rabbit IgG conjugated to 10 nm gold (1:20, Amersham Pharmacia Biotech). The grids were then processed for electron microscopy as described (1) and examined at 80 kV in a Phillips 400 electron microscope.

L-periaxin Expression in Transgenic Mouse Oligodendrocytes—Rat L-periaxin cDNA was ligated downstream of the 1.9-kilobase myelin basic protein (MBP) promoter (generous gift of Drs. A. Gow and R. A. Lazzarini, Mt. Sinai School of Medicine, NY) at a BamHI site in the pMG2 vector (13). Purified DNA was diluted to a concentration of 2.5 μg/ml and introduced into the C57BL6 mouse genome by standard pronuclear microinjection of fertilized eggs from superovulated F1 (C57BL6 x CBA hybrid mice) hybrids (14). A total of 13 Founders were identified by PCR of tail-tip DNA using forward and reverse primers specific to the MBP promoter and L-periaxin cDNA, respectively.

**Energy Depletion**—Schwann cells were transiently transfected with BD-GFP. After 24 h, the cells were washed twice with glucose-free minimal Eagle's medium (Life Technologies, Inc.) containing 10% dialyzed FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml), and 2 mM glutamine and then incubated with 6 mM 2-deoxyglucose (Sigma) and 10 mM sodium azide in glucose-free medium containing dialyzed FCS for 2 h. Some cultures were allowed to recover for 2 h in complete medium at 37 °C. At the end of the incubation times, the cells were fixed with 4% paraformaldehyde, and images were captured on a Leica TCS 4D scanning confocal microscope.

**RESULTS**

**L-periaxin Is Targeted to the Nuclei of Embryonic Schwann Cells**—Schwann cells are first generated in mouse peripheral nerve between embryonic days 13.5 and 14.5 (E13.5 and E14.5) (15). This is the earliest time at which L-periaxin is detectable by immunocytocchemistry in Schwann cells of the mouse PNS, but, surprisingly, the protein is localized to the nucleus of these cells (Fig. 1A) and this nuclear expression is also evident in freshly isolated Schwann cells in vitro (Fig. 1B). Western blotting of embryonic peripheral nerve extracts confirmed that the protein recognized by the antibody was indeed L-periaxin (data not shown). Subsequently, relocation of L-periaxin to the processes of myelin-forming Schwann cells of murine sciatic nerve (Fig. 1C) and of rat sciatic nerve (Fig. 1D) presages its mature association with the plasma membrane and abaxonal myelin lamella (1). The redistribution of L-periaxin to the plasma membrane appears to be specific to Schwann cells because it is confined to the nucleus when ectopically expressed by transgenesis in the myelin-forming glia of the central nervous system (CNS) (Fig. 2).

**A Basic Domain Targets L-periaxin to the Nucleus**—L-periaxin has a highly basic domain between amino acids 118 and 196 (Fig. 3) (1). Because basic domains are well known to function as nuclear targeting signals, we wished to determine whether this basic region was an NLS. Because L-periaxin expression in Schwann cells gradually declines below detectable levels in the absence of axonal contact (9), to study the targeting of L-periaxin in cultured Schwann cells they were transfected with a variety of cDNA constructs. When Schwann cells were transfected with wild-type L-periaxin cDNA, the protein was concentrated appropriately in the nucleus (Fig. 4A). We next asked if deletion of this domain would abrogate the nuclear localization of L-periaxin in Schwann cells; this was indeed the case (Fig. 4B). Further evidence that the basic domain was an NLS was provided by the targeting of a green fluorescent protein (GFP)–L-periaxin basic domain fusion protein to the Schwann cell nucleus (Fig. 4, C and D). The same construct was used to test the energy dependence of nuclear targeting. Energy depletion of Schwann cells abolished nuclear localization of this GFP–L-periaxin fusion protein (Fig. 4D).

**FIG. 1.** Redistribution of L-periaxin from the nucleus to myelinating processes during myelination. Immunofluorescence localization of L-periaxin in the nuclei of murine embryonic Schwann cells both in vivo at an embryonic age of 14.5 days (E14.5) (A) and E16.5 in vitro (B). By E17.5, Schwann cells in the sciatic nerve no longer concentrate the protein in the nucleus (C). Immunoelectron microscopy using secondary antibodies coupled to colloidal gold reveals that L-periaxin is restricted to myelin processes once myelination is initiated at post-natal day 1 in the rat sciatic nerve (P1) (D) (arrow). Immunostaining with an anti-L-periaxin antibody was performed as described under "Experimental Procedures." Scale bars A-C, 20 μm, and D, 0.2 μm.
uptake, indicating that nuclear translocation was an active process (Fig. 4E). When cells depleted of energy were allowed to recover, they regained their ability to utilize the NLS of L-periaxin (Fig. 4F).

Nuclear Translocation of L-periaxin Is Influenced by Cell-Cell Contact—Because the nuclear uptake of the tight junction PDZ domain protein ZO-1 is inhibited by cell-contact, we were interested to determine whether the degree of cell confluency would affect the nucleocytoplasmic localization of L-periaxin. In sub-confluent monolayers of 33B cells permanently expressing L-periaxin, the protein was predominantly targeted to the nucleus (Fig. 5A). However, in denser cultures this nuclear accumulation was lost (Fig. 5B). The reversibility of this nucleocytoplasmic distribution was demonstrated in confluent cultures by wounding the monolayer with a pipette tip (Fig. 5C). At the edges of the wound, where the cell density is reduced, there is a clear increase in the number of cells that demonstrate nuclear labeling (Fig. 5C).

Domains of L-periaxin

| PDZ | Basic |
|-----|-------|
| BD1 | 118-KGPRAKVAKLNIQSLSPVKKKMVIGTL |
| BD2 | GTPADLPDVDEFSPFKLRLVRRLGKADAVK |
| BD3 | GPVPAAPARRLDPLRVR-196 |

BD-GFP (116-196)
BD1-GFP (116-145)
BD1,2-GFP (116-176)
BD2,3-GFP (140-198)

Fig. 3. The basic domain of L-periaxin. The basic domain comprises three highly basic sub-domains which are outlined in gray. GFP fusion constructs are shown below (see "Experimental Procedures").

Cell-cell contact influences nuclear localization. Immunofluorescence labeling of L-periaxin in permanently transfected 33B cells at low (A) and high cell density (B). The reversibility of the cell density effect on nuclear localization is seen in a wounded confluent culture in which a number of cells that border the scrape area have resumed the accumulation of L-periaxin in the nucleus (arrows, C). Immunostaining for L-periaxin was performed as described under "Experimental Procedures." Scale bars A and B, 20 μm, and C, 50 μm.

The L-periaxin NLS Is Tripartite—Three strongly basic sub-domains are evident within the NLS (Fig. 3). NLSs are normally mono- or bipartite. Hence we predicted that at least one of these sub-domains would be redundant for nuclear targeting. Surprisingly, we found that all three domains were required for nuclear localization when they were fused to the GFP reporter (Table I). Predominantly nuclear localization was as observed in Fig. 4D. The first sub-domain yielded significant levels of nuclear labeling of the reporter protein (61%) which were enhanced when the second sub-domain was included (83%). In-
The first sub-domain was absolutely required for nuclear translocation, indicating that the other two sub-domains are weaker components of the NLS. The well characterized bipartite NLS of nucleoplasmin contains an essential KKKK sequence. However, mutation of the second and third lysine residues in the equivalent sequence in the first sub-domain of L-periaxin (Fig. 3) did not affect the function of the NLS (data not shown) which corroborates the distinctiveness of the NLS in L-periaxin.

**DISCUSSION**

We have shown that L-periaxin has a functional NLS of an unusual tripartite type. The classical NLS is typified by the single signal of the SV40 large T antigen (PKKKRKV) or the bipartite NLS of nucleoplasmin (KRPAAIKKAGQAKKKK) (17). These signals interact with the importin/karyopherin complex which is subsequently translocated into the nucleus in an energy-dependent fashion by a mechanism that requires, among other things, GTP and the GTPase, Ran (18). The existence of many α-importin-related proteins in higher eukaryotic cells has prompted the suggestion that this diversity might reflect the need for specificity in NLS recognition (19).

The stimulus that influences the nucleocytoplasmic distribution of the PDZ domain protein ZO-1 is cell-cell contact (5), whereas zyxin, a LIM domain protein, appears to shuttle between the nucleus and focal contacts in response to cell-substrate interaction (20). Recent data suggests that the nucleocytoplasmic distribution of several proteins that undergo active nuclear uptake is affected by cell-cell contact (21). Although it is not yet clear what the nuclear function of L-periaxin might be, it joins a growing list of proteins that redistribute between the cytoplasmic distribution of several proteins that undergo active transport through the classical active route even though it does not utilize a classical NLS. Thus far, little is known of how a tripartite signal might be recognized by the import machinery. However, it is known that more than one copy of a basic NLS can increase the efficiency of nuclear uptake (23, 24). The closest precedent to the tripartite NLS of L-periaxin is that of the maize R protein which has three basic sequences, any two of which are required to function as a strong NLS (25).

The developmentally regulated nucleocytoplasmic redistribution of L-periaxin in embryonic Schwann cells is the first such example for a PDZ-domain protein. The appearance of appropriate binding partners at the cell surface of the Schwann cell may be the stimulus for the translocation of L-periaxin from the nucleus to myelinating processes as they ensheathe the axon. It seems unlikely that the same partners are expressed in the myelin-forming glia of the CNS because no such redistribution from the nucleus occurs when L-periaxin is ectopically expressed in the myelinating oligodendrocytes of transgenic mice. Some PDZ motifs such as the third PDZ domain of the synaptic protein PSD-95 have been shown to have the potential to bind to multiple target sequences (26–29). Hence, we speculate that nuclear targeting of L-periaxin in embryonic Schwann cells may sequester the PDZ domain from inappropriate interactions in the cytoplasm until the correct ligand becomes available at the cell cortex of the maturing myelin-forming Schwann cell.

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