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CLP36 interacts with palladin in dorsal root ganglion neurons

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ABSTRACT

CLP36, a member of the α-actinin-associated LIM protein (ALP)/enigma protein family, plays a role in neurite outgrowth in the peripheral nervous system. However, the underlying molecular mechanisms are not known. In this study, we performed yeast two-hybrid screening of an E18 mouse whole-body cDNA library with CLP36 as the bait and isolated palladin as a CLP36-binding protein. Palladin is an actin-binding protein and it was shown to have a role in the extension of cortical neurons. A coimmunoprecipitation study showed that CLP36 and palladin formed a complex in the dorsal root ganglion (DRG). In addition, CLP36 and palladin were colocalized in the neurites and cell bodies of primary DRG neurons. Furthermore, sciatic nerve transection increased the expression of both CLP36 and palladin mRNAs in DRG neurons, with the increase in CLP36 mRNA being more prominent. This implies that CLP36 has a more specific role in nerve regeneration than palladin. Our results suggest that CLP36 may interact with palladin to influence neurite outgrowth during sciatic nerve regeneration.

Peripheral nerves have the ability to regenerate after injury. After a peripheral nerve is injured, the proximal stump undergoes transformation into a growth cone and the nerve begins to regenerate. Growth cones are motile structures and their formation and movement are based on rearrangement of the actin cytoskeleton. To date, much research has been done in attempts to clarify the mechanism by which the actin cytoskeleton is regulated in growth cones. While these studies have suggested that a large number of actin-binding proteins (ABPs) may be involved, their actual roles in the growth cone are not well understood [4].

CLP36 is a member of the ALP/enigma family of proteins that have a PDZ domain at the N-terminal and 1–3 LIM domains at the C-terminal. Because ALP/enigma proteins interact with ABPs such as α-actinin [1,11,17,19,21,22] and β-tropomyosin [7], these proteins are thought to modulate the structure and/or dynamics of the actin cytoskeleton. Recently, we reported that CLP36 forms a complex with α-actinin in DRG and PC12 cells [13]. CLP36 is localized to growth cones and its expression is markedly increased in peripheral sensory and motor neurons after nerve injury. In addition, knockdown of CLP36 expression was shown to promote neuritogenesis with enhancement of growth cone movement in nerve growth factor (NGF)-treated PC12 cells. These findings suggest that CLP36 may play an important role in rearrangement of the actin cytoskeleton during peripheral nerve regeneration. However, there is no information available about the molecular mechanisms underlying this function. To further investigate the role of CLP36 in neurite outgrowth, including the functional significance of the CLP36-α-actinin interaction, we considered that it was necessary to identify other proteins that bind with CLP36 in neurons.

Yeast two-hybrid screening was carried out with the DupLex-A hybrid system kit (Origene Technologies). The cDNA fragment encoding CLP36 (a kind gift from H. Betz, Frankfurt, Germany) was subcloned in frame within the inducible pGilda bait vector. Then this bait construct was used to screen 10^8 independent recombinant clones of a mouse E18 whole-body cDNA library in pJG 4-5 transformed into yeast strain EGY48. We obtained 100 blue colonies that grew on the original galactose-inducible selection plates and analyzed the cDNA fragments contained within these colonies.

For mapping of the sites involved in binding between CLP36 and palladin, yeast two-hybrid assays were performed. A deletion mutant of palladin lacking the last three amino acids (palladin ΔEDL) was amplified by the polymerase chain reaction (PCR) and was cloned into the pJG 4-5 prey vector. Then five CLP36 deletion mutant fragments (PDZ + MID amino acids 2–262, MID + LIM 8–327, PDZ alone 2–89, MID alone 80–262, and LIM alone 253–327) were amplified by PCR and cloned into the pGilda vector. To test the interaction of palladin with RIL and mystique, as well as that of CLP36 with Pick1, syntenin, and PSD-95, cDNA fragments for RIL and mystique were amplified by reverse transcriptase (RT)-PCR from rat brain total RNA and cloned into the pGilda vector. Similarly, pJG 4-5 Pick1, syntenin, and PSD-95 cDNA fragments were obtained

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by RT-PCR and cloned into the pJG 4-5 vectors. Yeast two-hybrid assays were employed to test the above-mentioned interactions.

F2408 fibroblast cells were provided by the Health Science Research Resources Bank (Osaka, Japan). Primary DRG neurons were prepared from adult male Wistar rats (200 g), as described previously [15]. For immunofluorescence, cells were fixed with 4% formaldehyde for 20 min, permeabilized with 0.2% Triton X-100 and 4% normal goat serum in PBS for 20 min, and then were reacted with anti-CLP36 mAb (BD Transduction Laboratories, Biosciences, 1:200) and anti-palladin polyclonal Ab (Proteintech, 1:200) for 3 h at RT. Primary antibodies were detected by incubation with anti-mouse or anti-rabbit Arexa 488- or 594-conjugated secondary IgG antibodies (Molecular Probes, 1:500) for 1 h at RT. For visualization of actin filaments, permeabilized cells were reacted with Alexa 594-conjugated phalloidin (Molecular Probes, 1:200). Immunofluorescence images were captured with a BZ-8000 (Keyence) and were processed using the BZ-analyzer software (Keyence).

For coimmunoprecipitation experiments, the Triton X-100 soluble fraction extracted from F2408 cells or rat dorsal root ganglia was incubated for 3 h at 4 °C with anti-CLP36 mAb (0.75 μg) or control mouse IgG conjugated with protein G sepharose (Amersham Biosciences). After washing, the reaction products were subjected to western blot analysis. The primary and secondary antibodies were as follows: anti-CLP36 mAb (1:500); anti-palladin polyclonal Ab (1:1000); anti-α-actinin mAb (Sigma, 1:400); anti-β-actin mAb (Sigma, 1:2000); horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (Nakalai Tesque, 1: 5000). For the absorption test, GST-palladin (Proteintech) and GST were bound to glutathione-sepharose beads (Amersham Biosciences) and then incubated overnight with an anti-palladin polyclonal antibody. After centrifugation, the supernatants were used for western blot analysis. GST protein was purified according the manufacturer's protocol (Amersham Biosciences).

Sciatic nerve transection was performed in adult male Wistar rats (200 g). The animals were deeply anesthetized with sodium pentobarbitol (50 mg/kg i.p.), and sciatic nerve transection and control surgery were performed as described previously [13]. At 3 days after surgery, the rats were anesthetized again and exsanguinated by cutting the carotid vessels, after which the L3–L5 DRGs were immediately removed from the control and operated sides. Frozen sections (18 μm thick) were cut on a cryostat. The sequences of the probes for *in situ* hybridization were checked in a database search to exclude significant homology with other genes. For CLP36 mRNA, we used probes with previously published sequences [13]. For palladin mRNA, a specific oligonucleotide probe (BC127081: nucleotides 2267–2306) was employed. These probes were labeled with [^{35}S]dATP using terminal deoxynucleotidyl transferase. Sections incubated with control sense probes for CLP36 and palladin did not display any signals (data not shown). For quantitative analysis, DRG neurons of a similar size ($n = 30$) were selected on both the operated and control sides, and the number of silver grains localized on these neurons was counted. Statistical evaluation was performed using Student's *t*-test. For immunohistochemistry, frozen sections were fixed with 4% paraformaldehyde and pretreated with 0.35% hydrogen peroxidase. They were reacted with anti-CLP36 polyclonal Ab (1:200, Proteintech) or anti-palladin polyclonal Ab (1:200), then with polymer-horseradish peroxidase-labeled anti-rabbit secondary Ab (Dako), and were finally visualized with diaminobenzidine and hydrogen peroxidase. The specificity of staining was confirmed by absorption of the antibodies with GST-CLP36 [13] or GST-palladin.

Real-time PCR was performed with SYBR Premix ExTaq (Takara) and a Thermal Cycler Dice Real Time System (Takara). Total RNAs from L3 to L5 DRGs were isolated with an RNeasy mini kit (Quiagen) and converted to cDNA by reverse transcription. Primer sequences used for PCR analysis were listed in Supplemental Table 1. Levels of CLP36, palladin, mystique, and RIL mRNAs were normalized to that of GAPDH mRNA and were shown as ratios to the value of control CLP36 mRNA. PCR-amplified products were also electrophoresed on 1% agarose gels to confirm that single bands were amplified.

By screening the E18 mouse whole-body cDNA library, we isolated three clones encoding the in-frame C-terminal region of palladin cDNA, two clones encoding the in-frame full-length γ-filamin, α-actinin, telethonin binding protein (FATZ) cDNA, and one clone each encoding in-frame actinin-2, actinin-3, and actinin-4 cDNA fragments. The yeast two-hybrid assay confirmed the specificity of the interaction of these clones with full-length CLP36 (data not shown). Because FATZ is exclusively expressed in muscle tissue [5], further investigation was focused on the relationship between CLP36 and palladin. CLP36 has a PDZ domain at its N-terminal region and palladin carries a putative PDZ-binding motif (EDL) at the end of its C-terminal region. Thus, we speculated that the interaction between CLP36 and palladin involved the PDZ domain of CLP36 and the C-terminus of palladin. To test this hypothesis, we generated deletion mutants of CLP36 and examined their binding to the C-terminal region of palladin by the yeast two-hybrid assay (Fig. 1A). The results showed that all of CLP36 mutants that involve its PDZ domain could bind to the C-terminal region of palladin. However, a palladin mutant lacking the last three amino acids (EDL) was unable to bind with CLP36 (Fig. 1B). These findings supported our hypothesis and indicated that the C-terminus of palladin binds with the PDZ domain of CLP36. To examine the specificity of this interaction, we tested whether the C-terminus of palladin could bind with the PDZ domains of other proteins. We found that the C-terminus of palladin did not interact with the PDZ domain of PSD-95, Pick1, or syntenin (Fig. 1C). However, it interacted with mystique and RIL, which are from the ALP protein family along with CLP36 (Fig. 1D). These results suggested that the C-terminus of palladin preferentially binds to the PDZ domain of proteins from the ALP family.

We next investigated whether CLP36 and palladin could form an intracellular protein complex. Initially, we searched for a CLP36–palladin complex in fibroblasts (F2408 cells) because both CLP36 and palladin have been shown to play an important role in stress fiber formation. In F2408 cell lysates, anti-palladin antibody reacted with a major band of ~90 kDa and also recognized bands of 50 and 70 kDa. These bands coincided with palladin isoforms that have been reported previously [6]. Furthermore, two additional bands were observed at 90–100 kDa. All of these bands disappeared after preadsorption with the antigenic peptide (Fig. 2A, left). A coimmunoprecipitation study demonstrated that palladin was precipitated with anti-CLP36 antibody but not with control IgG (Fig. 2A, right). Interestingly, among several palladin isoforms, the high molecular weight 90 kDa isoform was only precipitated with anti-CLP36 antibody (asterisk). In addition, α-actinin was found in the precipitates. To compare the subcellular localization of palladin and CLP36, we stained F2408 cells with an anti-palladin antibody (Fig. 2B, upper) and an anti-CLP36 antibody (Fig. 2B, lower). We found that palladin and CLP36 were localized at stress fibers, with both molecules being detected at the same sites (Fig. 2B, right panel, arrowheads). These findings indicate that CLP36 and palladin are associated with each other in F2408 cells. Similarly, endogenous palladin and α-actinin were precipitated from DRG tissue specimens with anti-CLP36 antibody. As seen in F2408 cells, several palladin isoforms were also expressed in DRG tissues and a high molecular weight 90 kDa isoform was only precipitated with anti-CLP36 antibody (Fig. 2C). Primary DRG neurons showed intense staining for CLP36 and palladin in the cell body, and staining was also observed along neurites including the growth cones (Fig. 2D).

It has been shown that both CLP36 and palladin play a role in neurite outgrowth [2,13]. It is known that neurons increase the synthesis of proteins required for regeneration after nerve injury.