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The carboxyl-terminal region of Crtac1B/LOTUS acts as a functional domain in endogenous antagonism to Nogo receptor-1

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ABSTRACT

Myelin-derived axon growth inhibitors, such as Nogo, bind to Nogo receptor-1 (NgR1) and thereby limit the action of axonal regeneration after injury in the adult central nervous system. Recently, we have found that cartilage acidic protein-1B (Crtac1B)/lateral olfactory tract usher substance (LOTUS) binds to NgR1 and functions as an endogenous NgR1 antagonist. To examine the functional domain of LOTUS in the antagonism to NgR1, analysis using the deletion mutants of LOTUS was performed and revealed that the carboxyl-terminal region (UA/EC domain) of LOTUS bound to NgR1. The UA/EC fragment of LOTUS overexpressed together with NgR1 in COS7 cells abolished the binding of Nogo66 to NgR1. Overexpression of the UA/EC fragment in cultured chick dorsal root ganglion neurons suppressed Nogo66-induced growth cone collapse. These findings suggest that the UA/EC region is a functional domain of LOTUS serving for an antagonistic action to NgR1.

1. Introduction

It has been widely accepted that neurons in the adult central nervous system (CNS) limit axonal regeneration after injury. This limitation can be attributed to inhibitory environmental factors such as axon growth inhibitors derived from myelin. Nogo proteins, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) are well known as myelin-derived axon growth inhibitors [1,2]. Nogo receptor-1 (NgR1) is a glycosylphosphatidylinositide (GPI)-anchored protein and a common receptor for at least these three myelin components [1,2]. Accumulating lines of evidence have shown that neutralization of these inhibitors' function by antibodies [3], soluble NgR1 peptides [4], an NgR1 antagonist [5] or gene deletion [6,7] improves axonal regeneration after injury.

Recently, we identified cartilage acidic protein-1B (Crtac1B)/lateral olfactory tract usher substance (LOTUS), which binds specifically to NgR1, and found that LOTUS functions in lateral olfactory tract development through an antagonism to NgR1 [8]. LOTUS is a 646 amino acid protein and consists of a signal peptide sequence, four phenylalanyl–glycyl and glycyl–alanyl–prolyl (FG-GAP) domains, a UnbV/ASPIC (UA) domain, an epidermal growth factor-like calcium binding (EC) domain and a transmembrane (TM) domain that is located at the carboxyl-terminus [8–10]. However, the region of LOTUS involved in the antagonistic action to NgR1 has not been determined. In this study, we identified the region of LOTUS that binds to NgR1 by using the deletion mutants of LOTUS, and we examined whether the domain exerts antagonism to NgR1. Our results demonstrate that two carboxyl-terminal domains of LOTUS bind to NgR1 and act as a functional domain in antagonism to NgR1.

2. Materials and methods

2.1. Animals

Fertilized White Leghorn eggs were purchased from Yamagishi (Gumma, Japan) and incubated at 37 °C in a standard egg incubator. Dorsal root ganglions (DRGs) were dissected from chick embryos of indeterminate sex at embryonic day 13 (E13). Throughout the experimental procedures, all efforts were made

Abbreviations: AP, alkaline phosphatase; CNS, central nervous system; Crtac1B, cartilage acidic protein-1B; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; EGF, epidermal growth factor; GPI, glycosylphosphatidylinositide; HSV, herpes simplex virus; LOTUS, lateral olfactory tract usher substance; LRR, leucine-rich repeat; MAG, myelin-associated glycoprotein; NgR1, Nogo receptor-1; OMgp, oligodendrocyte myelin glycoprotein; TM, transmembrane.

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to minimize the number of animals used and their suffering. The experimental procedures were approved by the institutional animal care and use ethical committee of the Yokohama City University School of Medicine.

2.2. Antibodies and reagents

Affinity-purified polyclonal rabbit antibodies against LOTUS were obtained from Medical and Biological Laboratories (MBL, Aichi, Japan). Goat anti-human Nogo receptor polyclonal antibody (R&D Systems, Minneapolis, MN, USA), biotinylated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, USA), Alexa594-labeled donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA), Alexa488-labeled donkey anti-rabbit IgG (Invitrogen), Alexa594-labeled goat anti-rabbit IgG (Invitrogen), Alexa488-conjugated phalloidin (Invitrogen) and purified peptides of rat Nogo66-mouse Fc (R&D Systems) were obtained commercially.

2.3. Expression vectors and protein purification

The plasmids encoding the signal sequence His6-human placental alkaline phosphatase (AP)-fused mouse Nogo66 (AP-Nogo66) or mouse LOTUS (AP-LOTUS) were generated as previously described [8]. Each sequence consisting of a single domain of mouse LOTUS was amplified by PCR and ligated into the pcDNA3.1AP6.str vector to generate a plasmid encoding the signal sequence His6-human placental AP-fused each deletion mutant of mouse LOTUS [11]. These plasmids were transfected by the lipofection method with FuGENE 6 (Roche, Basel, Switzerland) into HEK293T cells cultured in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose with L-glutamine and sodium pyruvate; Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS, Biowest, Nuaillé, France). Human placental His6-AP-tagged fusion proteins were then purified from the cultured medium with an affinity column.

2.4. Binding assay by AP-fused proteins and immunocytochemistry

The plasmid encoding the full length of mouse NgR1 was generated as previously described [8]. The sequence coding the UA/EC domain of mouse LOTUS was amplified by PCR and ligated into the vector encoding the signal sequence Myc at the amino-terminus to generate a plasmid encoding the signal sequence Myc-UA/EC domain (Myc-UA/EC). The plasmids encoding the full-length NgR1 and/or Myc-UA/EC were transfected by the lipofection method with FuGENE 6 (Roche) into COS7 cells cultured in DMEM (4.5 g/l glucose with L-glutamine and sodium pyruvate; Nacalai Tesque) containing 10% FBS (Biowest).

The binding assays of AP-fused proteins (AP-Nogo66, AP-LOTUS or each AP-fused deletion mutant of LOTUS) to the DMEM-cultured COS7 cells were performed as previously described [8,12,13]. Unfixed cells were incubated with the AP-fusion proteins (2–50 nM) in the DMEM solution for 1 h at 4 °C or 37 °C, fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) containing 2 mM $MgCl_2$ for 1 h at room temperature (RT) and heated for 1 h at 67 °C to inactivate endogenous AP activity. The binding of the AP-fused proteins was visualized by the color reaction of nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) or detected quantitatively with *p*-nitrophenyl phosphate (*p*NPP) as previously described [8]. Bound heat-stable AP activity was histologically detected by the deposition of an insoluble reaction product after incubation with NBT/BCIP. AP color reactions were terminated when deposition of the reaction product was visible (2–12 h), but not saturated. The digital images were taken with a microscope equipped with 10× or 20× objective lenses (Olympus IX70, Olympus, Tokyo, Japan) and a CCD camera (SPOT-2e, SPOT Imaging Solutions, Sterling Heights, MI, USA). The intensity of the AP color reactions was determined from digital images, using an image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA). For biochemical quantitative detection of AP binding, the intensity of the AP color reaction after incubation with *p*NPP was measured by a plate reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA) with absorbance at 405 nm wavelength.

The cell surface expression of NgR1 and/or Myc-UA/EC was confirmed by immunocytochemistry under nonpermeabilizing conditions. Unfixed cells were incubated with anti-NgR1 (1 μg/ml, R&D Systems) and/or anti-LOTUS (1 μg/ml, MBL) antibodies in the described DMEM solution for 1 h at 37 °C and fixed with 4% PFA in PBS for 1 h at RT. The samples were then incubated with Alexa594-labeled donkey anti-goat IgG (1:500 dilution, Invitrogen) and Alexa488-labeled donkey anti-rabbit IgG (1:200 dilution, Invitrogen) antibodies in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 2 h at RT. The digital images were taken as described earlier. For immunocytochemistry with AP color reaction, unfixed cells were incubated with anti-NgR1 (1 μg/ml, R&D Systems) antibody for 1 h at 37 °C, fixed with 4% PFA in PBS containing 2 mM $MgCl_2$ for 1 h at RT and heated for 1 h at 67 °C to inactivate endogenous AP activity. The treated cells were then incubated with biotinylated donkey anti-goat IgG (1:5000 dilution, Jackson ImmunoResearch) antibody in TBS containing 0.1% Tween 20 for 1 h at RT, followed by incubation with VECTASTAIN ABC-AP solution (1:5000 dilution, Vector Labs, Burlingame, CA, USA) in TBS containing 0.1% Tween 20 for 1 h at RT. The intensity of the AP color reaction after incubation with *p*NPP was measured as described earlier.

2.5. Virus purification

Recombinant herpes simplex virus (HSV) was prepared as previously described [13]. The sequence coding the signal sequence Myc-UA/EC domain (Myc-UA/EC) was inserted into an amplicon plasmid, pHSV-PrpUC vector. This plasmid was transfected into the HSV packing cell line 2–2 cells by the lipofection method with Lipofectamine LTX (Invitrogen), and then the treated cells were superinfected with 5dl5 HSV helper virus after 1 day of transfection. The recombinant virus was amplified by three sequential rounds of infection, concentrated with sucrose density gradient centrifugation and stored at −80 °C.

2.6. Primary culture and Myc-UA/EC overexpression in DRG neurons

Dissected chick E13 DRGs were directly plated on glass bottom dishes (Asahi Glass, Tokyo, Japan) coated with poly-L-lysine (100 μg/ml, Wako, Osaka, Japan) and mouse laminin (10 μg/ml, Invitrogen), and then grown in Ham's F-12 medium (Wako) containing 10% FBS (Biowest) and nerve growth factor (10 ng/ml, Wako). The explants exposed to HSV-Mock or HSV-Myc-UA/EC were cultured for 24 h at 37 °C. The cultured explants were infected with the recombinant HSV. The percentage of neurons expressing Myc-UA/EC ranged from 50% to 60% in infected cultures. The expression of recombinant Myc-UA/EC was confirmed by immunocytochemistry as previously described [14]. After 1 day of incubation, the cultured DRG explants were fixed with warmed 4% PFA in culture medium for 10 min at 37 °C and then for 10 min at RT. The samples were permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT, blocked with 1% bovine serum albumin in the permeabilizing solution for 1 h at RT. Immunostaining was performed with anti-LOTUS (1 μg/ml, MBL) antibody in the blocking solution for 1 h at RT, followed by incubation with Alexa594-labeled goat anti-rabbit IgG (1:500 dilution, Invitrogen) for 1 h at RT. The digital images were taken with a microscope equipped with 40× objective lenses (Olympus IX71, Olympus) and a CCD camera (CoolSnap HQ, Roper Scientific, Tucson, AZ, USA).