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A proteomic screen reveals novel Fas ligand interacting proteins within nervous system Schwann cells

Peter B. Thornhill[a], Jason B. Cohn[b,c], Gillian Drury[a], William L. Stanford[e], Alan Bernstein[b,c,d], Julie Desbarats[a,*]

[a] *Department of Physiology, McGill University, 3655 Promenade Sir William Osler, Montréal, Québec, Canada H3G 1Y6*
[b] *Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, ON, Canada M5G 1X5*
[c] *Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada M5S 1A8*
[d] *Canadian Institutes of Health Research, 410 Laurier Ave. W., Ottawa, ON, Canada K1A 0W9*
[e] *Institute for Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, ON, Canada M5S 3G9*

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Abstract Fas ligand (FasL) binds Fas (CD95) to induce apoptosis or activate other signaling pathways. In addition, FasL transduces bidirectional or 'reverse signals'. The intracellular domain of FasL contains consensus sequences for phosphorylation and an extended proline rich region, which regulate its surface expression through undetermined mechanism(s). Here, we used a proteomics approach to identify novel FasL interacting proteins in Schwann cells to investigate signaling through and trafficking of this protein in the nervous system. We identified two novel FasL interacting proteins, sorting nexin 18 and adaptin β, as well as two proteins previously identified as FasL interacting proteins in T cells, PACSIN2 and PACSIN3. These proteins are all associated with endocytosis and trafficking, highlighting the tight regulation of cell surface expression of FasL in the nervous system.
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1. Introduction

Fas ligand (FasL, CD95L, CD178), a member of the tumor necrosis factor (TNF) superfamily, is a type II, homotrimeric protein with cell surface and secreted isoforms. FasL is inducibly expressed by cells of the immune system upon activation and in the nervous system after injury, and is constitutively expressed in "immune privileged" tissues, such as the eye and reproductive system [1,2]. Traditionally, FasL has been viewed exclusively as a ligand for the Fas receptor, stimulating formation of a death inducing signaling complex (DISC) and activation of a caspase cascade, ultimately culminating in apoptosis [3]. The apoptotic effector function of FasL is particularly well documented in the immune system, where FasL is an effector of activation induced lymphocyte cell death (AICD), and of target cell killing by cytotoxic T lymphocytes [4]. In addition to the effector function of FasL as a ligand for Fas, increasing evidence has suggested that FasL and other membrane-bound TNF superfamily ligands can also transduce "reverse signals" and thus participate in bidirectional signaling [5].

The FasL intracellular domain (ICD) contains a modular architecture consistent with its role as a signal transducing molecule. It contains a 26 amino acid proline rich region which interacts with multiple Src homology 3 (SH3) and WW domain containing proteins in T cells, including several members of the Src tyrosine kinase family [6,7]. In T cells, stimulation of FasL with agonistic antibodies or Fas fusion proteins increases its association with specific SH3 domain containing proteins and results in activation of the extracellular-signal regulated kinase (ERK) mitogen activated protein kinase (MAPK) pathway [8,9]. In addition to its potential role in signal transduction, the FasL proline rich region has also been implicated in sorting of the molecule to different cellular compartments [10], which may be important for regulating both FasL effector and reverse signaling activities. The FasL ICD also contains a conserved, cytosolic casein kinase I (CKI) motif, SXXS, that has been implicated in reverse signaling in other TNF family members [5,11]. Recently, Sun et al. have documented changes in FasL serine phosphorylation following treatment of cultured cells with a Fas fusion protein [9].

Although FasL has been extensively studied in the immune system, FasL trafficking and reverse signaling have never been studied in the nervous system. FasL expression is induced on nervous system glial cells, including Schwann cells, following injury [12]. Fas is also expressed in the nervous system and is upregulated after injury, suggesting that Fas/FasL interactions may play a role in the injured nervous system [13]. We have previously found that expression of Fas and FasL influenced peripheral nerve regeneration after crush injury [14].

Here, we used a proteomic approach to identify novel FasL interacting proteins in Schwann cells, the glial cells of the peripheral nervous system, to gain insight into mechanisms of reverse signaling and trafficking of FasL in the nervous system. Using liquid chromatography-tandem mass spectrometry

*Corresponding author. Fax: +1 514 398 7452.
E-mail address: julie.desbarats@mcgill.ca (J. Desbarats).

Abbreviations: AICD, activation induced cell death; DISC, death inducing signaling complex; SH3, Src homology 3; ICD, intracellular domain; LC-MSMS, liquid chromatography coupled tandem mass spectrometry; IRES, internal ribosomal entry site; GST, glutathione-S-transferase; PX, Phox homology domain; CC, coiled coil domain; MAPK, mitogen activated protein kinase; ERK, extracellular-signal regulated kinase; AP, adaptor protein; EGFP, enhanced green fluorescent protein; TNF, tumor necrosis factor

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(LC-MSMS) for identification of FasL co-immunoprecipitating proteins, we identified Sorting Nexin 18 (SNX18), adaptin β, Protein Kinase C and Casein Kinase Substrate in Neurons 2 (PACSIN2), and PACSIN3 as FasL interacting proteins. The binding of FasL to multiple proteins implicated in trafficking suggests tight regulation of the reverse signaling and effector functions of FasL within the nervous system.

2. Materials and methods

2.1. Cells

Mouse Schwann cells were isolated from sciatic nerves of neonatal (P1 to P3) C3H/HeJ mice. Nerves were digested in collagenase (Sigma) then in trypsin/DNase (Worthington Biochemicals). Cells were grown in DMEM plus 10% fetal calf serum (FCS, Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 0.1 mM non-essential amino acids (Gibco), and 10 μM cytosine-β-D-arabinofuranoside (Ara-C, Sigma). Schwann cell cultures were treated with anti-mouse Thy1.2 (CD90.2) antibody-containing supernatant derived from the HO-13-4 hybridoma (American Type Culture Collection) and rabbit LowTox™ complement (Cedarlane) to remove residual fibroblasts. To verify the purity of the cultures, cells were examined using flow cytometry as follows: cells were trypsinized from cell culture plates, then either permeabilized and stained using the Cytofix/Cytoperm™ fixation/permeabilization solution kit (BD Biosciences) for intracellular proteins according to the manufacturer's recommendations, or stained in PBS/2% FCS for surface markers. Labelling was performed on ice for 25 min with the indicated primary antibodies or isotype control antibodies. After two wash cycles all samples were stained with phycoerythrin conjugated secondary antibodies. Cells were washed twice more and analyzed using a FACSAria™ cell analyzer (BD Biosciences).

2.2. Construction of FasL deletion mutants

Human Fas Ligand (huFasL) cDNA was purchased from Open Biosystems. FasL deletion mutants, HuFasLΔ 2–33-FLAG and HuFasLΔ2–70-FLAG, were created using wild-type FasL cDNA as a template to produce PCR products missing base pairs corresponding to N-terminal amino acids 2–33 and 2–70, respectively, but still containing the initiator methionine (AUG) base pairs. The huFasL and huFasLΔ2–33 coding sequences were PCR amplified using forward primers 5′-CACCTGCAGCCATGCAGC-3′ and 5′-GGGCTGCAGCCATGACCTCTGTGCCCAGAAGGC-3′, respectively, paired with the reverse 5′-CTCTTAAAGCTTATATAAGCCG-3′ primer and PFUltra™ DNA polymerase (Stratagene). The resulting PCR products were cloned in frame into pCMV-Tag4A (Stratagene) with PstI (Invitrogen) and HindIII (New England Biolabs) to generate C-terminal FLAG tagged proteins. HuFasL-FLAG and huFasLΔ 2–33-FLAG were then excised with PstI and Acc65I (Fermentas), and subcloned into the pIRES2-EGFP (Clontech) SmaI (New England Biolabs) site after fill-in reactions using the Klenow fragment (Invitrogen). HuFasLΔ 2-70-FLAG was created by PCR amplification with primers 5′-GGGCTGCAGCCATGCTGAAGAAGAGAGGGAACCACAGC-3′ (forward) and 5′-CTACTTATCGTCGTCATCCTTG-3′ (reverse) using huFasL-FLAG as a template and cloned into the pIRES2-EGFP SmaI site. All vectors were fully sequenced before transfecting into cells to ensure the absence of mutations/frameshifts created during PCR amplification.

2.3. Creation of stably transfected cell lines

Mouse Schwann cells were transfected with huFasL-FLAG, huFasLΔ2-33-FLAG, huFasLΔ2-70-FLAG, or empty pIRES2-EGFP vector as a control using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's recommendations. EGFP positive cells were single cell sorted into 96 well plates using a FACSAria™ fluorescence activated cell sorter (BD Biosciences). Cells were expanded in DMEM with 10% FCS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (Gibco) and 400 μg/ml G418 (Wisent Bioproducts). Clones were screened for expression of FasL constructs and EGFP by immunoblotting cellular lysates.

2.4. RNA extraction and RT-PCR

RNA extraction was performed on 2×10^5 cells using the ChargeSwitch total RNA kit (Invitrogen) according to the manufacturer's instructions. RNA was quantified using the Quant-iT Ribogreen RNA assay kit (Invitrogen) according to the manufacturer's protocol. Reverse transcription reactions were primed with 5 μM random decamers (Ambion), and amplified with 4U Sensicript reverse transcriptase (Qiagen). Primers for PCR amplification were as follows: 18S rRNA sense: 5′TGAGAAACGGCTACCACATCC3′, 18S rRNA antisense: 5′TCGCTCTGGTCCGTCTTGC3′, FasL sense: 5′CTGGTTGCCTTGGTAGGATTGGG3′, FasL antisense: 5′GGACCTTGAGTTGGACTTGCCTG3′, EGFP sense: 5′GCCACAAGTTCAGCGTGCTCG3′, EGFP antisense: 5′TCTTCTGCTTGTCGGCCATGATAT3′ (Invitrogen). PCR reactions were performed using HotstarTaq (Qiagen) in a Techne Touchgene Gradient thermocycler as follows: 18S rRNA: 15 min at 95 °C, cycled 13 × 60 s at 94 °C, 40 s at 66.2 °C, 30 s at 72 °C, then final elongation 10 min at 72 °C. EGFP and FasL: 15 min at 95 °C, cycled 25 × 60 s at 94 °C, 20 s at 65.2 °C, 40 s at 72 °C, then final elongation 10 min at 72 °C. PCR products were visualized on 1.2% agarose gel (Invitrogen).

2.5. Immunoprecipitation (IP) and immunoblotting

For IP, mouse Schwann cells were lysed in buffer containing 0.5% Nonidet P-40 (Bioshop Canada), 25 mM HEPES, pH 7.4, 115 mM potassium acetate, 5 mM magnesium chloride, 1 mM sodium orthovanadate (all from Sigma), and Complete Mini™ protease inhibitor cocktail (Roche). Lysates remained on ice for 15 min before centrifugation at 15000 rpm for 10 min at 4 °C. Supernatants were then precleared against unconjugated Sepharose 4B (Sigma) for 1.5 h at 4 °C. For validation experiments, supernatants were precleared with Protein G-Sepharose and isotype control antibodies. Beads were then pelleted, and precleared supernatants were incubated for at least 2 h at 4 °C with anti-FLAG or isotype control antibodies (Sigma) covalently cross-linked to Protein G-Sepharose (GE Healthcare) using dimethyl pimelimidate (Sigma). The beads were pelleted, washed three times in cold lysis buffer, boiled in Laemli buffer containing β-mercaptoethanol, and electrophoresed on SDS–polyacrylamide gels. SDS gels were then either transferred to polyvinylidene difluoride membranes (PVDF, BioRad) and subjected to immunoblotting or stained with colloidal Coomassie blue (Invitrogen) followed by tandem mass spectrometry of selected gel bands. PVDF membranes were blocked overnight at 4 °C in 5% non-fat dry milk powder (w/v) in Tris-buffered saline/0.1% Tween 20 (Fisher Scientific, TBST) and blotted with the indicated primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies. Bands were visualized using ECL PLUS™ reagents (GE Healthcare) and film from Molecular Technologies Inc.

2.6. Mass spectrometry of selected gel bands

Coomassie stained gel bands detected at higher intensity in the FLAG IP than in the isotype control IP were excised and minced manually with a scalpel, washed with water and dehydrated in acetonitrile (Sigma-Aldrich). The corresponding gel area in the isotype control lane was also excised for mass spectrometric analysis to separate legitimate interacting proteins from those highly prevalent at the same molecular weight. Gel pieces were spun down and dried using a vacuum centrifuge, followed by reduction in 10 mM dithiotreitol/0.1 M ammonium bicarbonate (Sigma-Aldrich), alkylation in 55 mM iodoacetamide (Sigma-Aldrich)/0.1 M ammonium bicarbonate, followed by extensive washing in 1:1 (v/v) 0.1 M ammonium bicarbonate/acetonitrile to remove residual Coomassie stain. Digestion was performed with 12.5 ng/μl trypsin (Promega) in 50 mM ammonium bicarbonate, 5 mM calcium chloride (Sigma-Aldrich). Peptides remaining in the gel were then extracted twice in 1:1 (v/v) 25 mM ammonium bicarbonate/acetonitrile and 1:1 (v/v) 5% formic acid/acetonitrile, respectively. Extracted samples were pooled and dried using a vacuum centrifuge for mass spectrometry at the Institute for Research in Immunology and Cancer proteomic center at the Université de Montréal. The dried peptides were resuspended in 5 μl of 0.2% formic acid and injected on a NanoACQUITY HPLC system (Waters Limitée). Peptides were first trapped by a reversed phase C18 precolumn (300 μm i.d. × 5 mm length) and subsequently separated on a C18 analytical column (150 μm i.d. × 10 cm, particle size 3 μm). NanoLC separation was achieved using a 56-min linear gradient from 5% to 60% aqueous acetonitrile (0.2% FA) at a flow rate of 600 nl/min. Mass spectrometric

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