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Two cytokine signaling molecules co-operate to promote axonal transport and growth

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ABSTRACT

The neuropoietic cytokines and their cytoplasmic signaling molecules contribute to axotomy-induced events in the nerve cell body that are beneficial to axonal regeneration. Previous studies have revealed a paradox in that, in vivo, suppressor of cytokine signaling (SOCS3) is induced in axotomized primary sensory neurons which are in a growth mode but, in vitro, SOCS3 strongly inhibits neurite growth from the same neurons. The present studies in cell lines with immuno-precipitation and western blotting, and Förstner resonance energy transfer showed that SOCS3 binds to the C terminus of C-Jun N-terminal kinase-interacting protein-1 (JIP1), increases its serine phosphorylation, and increases its binding to kinesin. Axonal transport was studied in vitro in adult rat primary sensory neurons by analyses of recovery of fluorescence after photobleaching and of the velocity and direction of movement of organelles. Over-expression of SOCS3 in addition to JIP1 had two consequences. First, recovery of fluorescence after photobleaching was more rapid and, second, JIP1-containing organelles moved more quickly and more frequently in retrograde direction. With respect to neurite outgrowth, SOCS3 alone was, as expected, strongly inhibitory but, in the presence of excess JIP1 augmented the stimulatory activity of the latter. The observations indicate that interactions between JIP1 and SOCS3 influence favorably axonal transport and growth *in vitro*.

Introduction

Anterograde and retrograde transport of molecules along microtubules within axons are mediated by kinesins and dyneins, which are molecular motors deriving energy from ATP (Hirokawa and Takemura, 2005; Schliwa and Woehlke, 2003; Vale, 2003). In the past decade, molecules that link kinesin to its molecular cargos have been identified. The prototypic cargo linker, JIP-1 was identified first as a scaffolding protein approximating C-Jun N-terminal kinase (JNK) with its upstream kinases (Dickens et al., 1997) and subsequently as a binding partner for KLC (kinesin light chain) (Verhey et al., 2001). Axonal transport incorporates mechanisms for the coupling and uncoupling of linker molecules to kinesin in cell bodies, at axon terminals (Blasius et al., 2007; Cavalli et al., 2005; Guillaud et al., 2008), and even along microtubules in axons where linkers may shift between dynein and kinesin (Muller et al., 2008). As one specific example of regulation of transport, JNK and its upstream kinases modify the affinity of binding of JIP-1 to kinesin (Horiuchi et al., 2007): in other words, JNK may regulate its own provision in an active form to axon terminals. Nevertheless, knowledge of the regulation of JIP1-to-kinesin binding during axonal transport remains incomplete.

Peripheral nerve injury causes molecular changes in the nerve cell body which promote axonal regeneration (Richardson and Issa, 1984; Neumann and Woolf, 1999), presumably through greater provision to the axon tip of molecules that stimulate cytoskeletal re-modeling (Arimura and Kaibuchi, 2007). The neuropoietic cytokines and their signaling pathway (gp130/JAK, [Janus kinase]/STAT3, (signal transducer and activator of transcription) have been convincingly implicated in the induction of regenerative responses in DRG (dorsal root ganglion) neurons (Liu and Snider, 2001; Qiu et al., 2005). Our studies of the functions of SOCS3 (suppressor of cytokine signaling), a feedback inhibitor of STAT3, revealed an apparent paradox: SOCS3 mRNA is increased in concentration in axotomized DRG neurons which exhibit strong regenerative propensity in vivo yet over-expression of the SOCS3 gene in the same neurons in vitro inhibits axonal extension (Miao et al.,

Abbreviations: CNTF, ciliary neurotrophic factor; ChFP, cherry fluorescent protein; CyFP, cyan fluorescent protein; DRG, dorsal root ganglion; FRAP, fluorescence recovery after photobleaching; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; JIP1, C-Jun N-terminal kinase interacting protein-1; KLC, kinecin light chain; JNK, C-Jun N-terminal kinase; JAK, Janus kinase; SOCS3, suppressor of cytokine signaling-3; STAT3, signal transducer and activator of transcription-3; YFP, yellow fluorescent protein.

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2006). Further investigation of relevant molecular actions of SOCS3 (Miao et al., 2008) revealed that it suppresses AP-1 (activator protein-1) transcriptional activity, inhibits JNK, and binds to JIP1. Such non-canonical actions of SOCS3 may provide an explanation for its disparate actions on axonal growth.

The experiments to be reported here provide further information on the binding of SOCS3 and JIP1, show that SOCS3 can enhance axonal transport of JIP1, and reveal a beneficial effect of the combination of SOCS3 and JIP1 on axonal growth in vitro.

Materials and methods

Animal surgery and RT-PCR

Animal care and procedures were performed under United Kingdom Home Office protocols and guidelines in agreement with the UK Animal Act 1986. Under sterile conditions, female Sprague–Dawley rats, weighing approximately 200 g were anesthetized, and the left sciatic nerve was exposed and transected in the mid-thigh. In control experiments, the contralateral sciatic nerve was exposed but not transected. Rats were allowed to live for 0–14 days after nerve injury.

L4 and L5 (fourth and fifth lumbar) dorsal root ganglia (DRG) from rats after killing by carbon dioxide, were quickly removed and frozen in liquid nitrogen for RNA extraction. Total RNA was extracted with TRIzol® reagent (Invitrogen, Paisley, UK) from L4 and L5 DRG pooled from 3 rats subjected to either nerve injury or sham surgery. cDNA was reverse transcribed from 1 μg of total RNA with random hexanucleotide primers using Superscript III® reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed according to the protocol of the Qiagen QuantiTect™ SYBR® Green PCR kit (Qiagen GmbH, Hilden, Germany) in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). Each sample was tested in triplicate. The concentration of each JIP family mRNA was normalized to the concentration of 18 S RNA and this ratio compared to the same ratio in contralateral DRG. The primers used for RT-PCR were, CTCTGAA-GACAGGGGAGC, and:ATTCGATCTCGATGGGAG corresponding to bp 712–729 and 902–919 for JIP1(GenBank Accession Number AF108959); AAGAGCTACCAGCCCCTCTC and :GACCTTCATGGCCTC-CACTA corresponding to bp 1244–1263 and 1413–1432 for JIP2 (GenBank Accession Number XM_235565); TCACACCACTCAACGA-GAGC and GTCCTGAACATCGGACCACT corresponding to bp 857–876 and 1013–1032 for JIP3 (GenBank Accession Number XM_220232); CGGCTACCACATCCAAGGAA and TGCTGGCACCAGACTTGCCCTC for 18 S RNA (GenBank Accession Number M11188).

Generation of constructs

Mouse SOCS3 constructs, each with a C-terminal Myc tag, were described previously (Miao et al., 2006). Rat JIP1 was cloned from rat brain cDNA by PCR and tagged with a Flag peptide at the N-terminus for detection. Generation of full length rat JIP1 was completed by amplification of N-terminal JIP1 with an N-terminal primer including a Flag tag, Kozak consensus sequence and Nhe1 restriction site and a C-terminal primer with an EcoR1 restriction site. Mutant JIP1 (Y713A) was derived by site-directed mutagenesis PCR. JIP1(493–715) truncated from the N terminus to contain SH3 and PTB domains only and JIP1 (493–553), the SH3 domain alone, were prepared by PCR and re-ligation. All constructs were ligated into the pcDNA3.1 expression vector engineered to contain IRES GFP (internal ribosome entry site green fluorescence protein) and confirmed by sequencing. For fluorescence studies, cyan fluorescent protein (CyFP) or cherry fluorescent protein (ChFP) was introduced at the C-terminus of SOCS3, yellow fluorescent protein (YFP) was introduced at the N-terminus of JIP1(493–715), and GFP at the N-terminus of full length JIP1. Each terminus for tagging was chosen to minimize interference of function by the fluorescent tag.

Lentivirus vectors were prepared as previously described (Miao et al., 2006). GFP, SOCS3-ChFP, and GFP-JIP1 were cloned into the lentivirus transfer vector pRRL-MCS + (Ruitenberg et al., 2002). Non-replicative lentiviruses were generated by co-transfection into HEK 293 T cells of the transfer vector, the viral core packaging construct VdeltaR8.74, and the vesicular stomatitis virus G envelope protein vector pMD.G.2. Viruses were concentrated by ultracentrifugation. Titers in HEK cells were 10^9–10^{11} transducing units per ml.

The constructs are depicted in Fig. 1.

Cell culture and transduction

Cells from the HEK293 human embryonic kidney cells and SH-SY5Y neuroblastoma cell line were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 μg/ml ampicillin and 50 μg/ml streptomycin. Medium was changed every 2 days and cells were passaged once a week.

Neurons from adult rat DRG were harvested, dissociated, and maintained in culture as previously described (Miao et al., 2006). In brief, neurons were dissociated in 0.125% collagenase type IX, enriched by centrifugation through albumin, suspended in serum-free N2 medium and infected with lentivirus constructs for 20 h. For analysis of neurite growth, approximately 1000 neurons were plated on each laminin-coated well in an 8-well culture plate and maintained for a further 44 h.

HEK 293 cells were transfected by a standard calcium phosphate precipitation method and harvested for protein preparation 48 h later.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting were performed as described previously (Miao et al., 2008) with antibodies to Flag, Myc, c-Jun, phosphotyrosine and phosphoserine (all from Cell Signaling, Boston, MA), or KLC (kinesin light chain (Abcam, Cambridge, UK)).

Immunocytochemistry and image analysis of immuno-reactivity

Immunocytochemistry and image analysis of immuno-reactivity were performed as described previously (Miao et al., 2006). Because green and red fluorescent molecules were used for JIP1 and SOCS3 respectively,

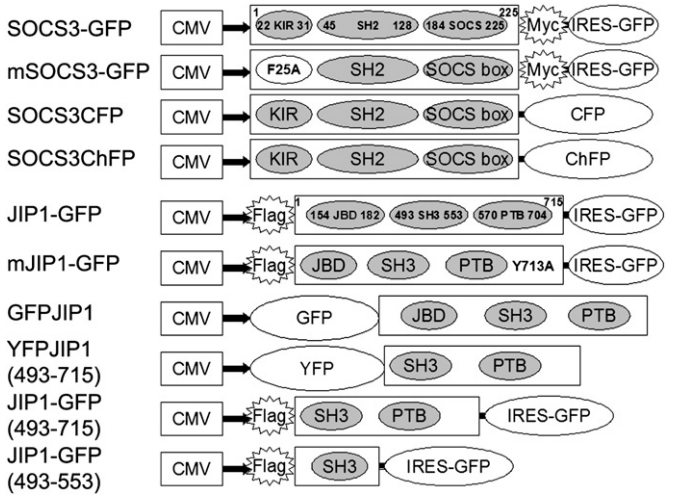

Fig. 1. Schematic diagram of constructs. Most constructs were generated in pcDNA3.1 IRES-GFP vector backbone, driven by CMV promoter and then engineered into lentivirus transfer vector if necessary. The numbers indicate the positions of amino acids, main domains are shown as abbreviated words. Proteins with fused expression of fluorescence proteins are shown in constructs without IRES and proteins with separated expression of fluorescence proteins are shown in constructs with IRES.