Nedd9 Protein, a Cas-L Homologue, Is Upregulated After Transient Global Ischemia in Rats

Possible Involvement of Nedd9 in the Differentiation of Neurons After Ischemia

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Background and Purpose—Some proteins involved in self-repair after stroke in the adult brain are primarily expressed during embryonic development and strongly down-regulated during the early postnatal phase. Neuronal precursor cell-expressed, developmentally down-regulated gene (Nedd) 9 was recognized to be identical to Crk-associated substrate lymphocyte type (Cas-L), a docking protein that associates with a variety of signaling molecules, such as focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2), and Crk. We investigated the involvement of these proteins in the pathophysiology of global cerebral ischemia.

Methods—The mouse Cas-L/Nedd9 cDNAs were cloned. The expression and function of Cas-L/Nedd9 protein in the pathogenesis of global ischemia in rats was investigated by RT-PCR, Western blot analysis, and immunohistochemistry. The neurite outgrowth of the transfectants of Nedd9 deletion mutants in PC-12 cells was also assessed to clarify the function of the Nedd9 protein.

Results—Nedd9 was a splicing variant of Cas-L and was selectively induced in neurons of the cerebral cortex and hippocampus 1 to 14 days after the ischemia. Induced Nedd9 protein was tyrosine phosphorylated and was bound to FAK in dendrite and soma of neurons after the ischemia. Finally, it was demonstrated that Nedd9 promoted neurite outgrowth of PC-12 cells.

Conclusions—Our study may support the potential of Nedd9 for participation in the differentiation of neurons after global ischemia in rats. (Stroke. 2005;36:2457-2462.)

Key Words: cerebral ischemia • global • rats • neural differentiation

Identification of an endogenous protein involved in self-repair after stroke in adult brain can potentially widen the therapeutic time window. Ischemia is a powerful reformatting and reprogramming stimulus for the brain, which induces endogenous proteins related to the pathophysiology of the injured brain.1 Some of these proteins, such as neurocan,2 Nedd2/Caspase2,3 and GAP43,4 are primarily expressed by neurons or glia during embryonic development and are strongly down-regulated during the early postnatal phase.

Nedd9 was initially identified as a neuronal precursor cell (NPC)-expressed, developmentally down-regulated gene in the mouse central nervous system. Gene expression of Nedd9 is detected in the embryonic brain of embryonal day (E) 10 and 14 and disappears in the adult mouse brain.5 The product of Nedd9 was subsequently reported to be identical to the mouse Crk-associated substrate lymphocyte-type (Cas-L) according to the homology database (http://www.ncbi.nlm.nih.gov/), which is also known as human enhancer of filaments 1 cloned by another group.6 Human Cas-L was first identified by our group as a 105-kDa protein predominantly tyrosine phosphorylated by the ligation of β1 integrins in human leukemia H9 cells.7 The major biological functions of Cas-L are the restoration of interleukin-2 production by costimulation with β1 integrins and T-cell receptor complex8 and the enhancement of cell migration by the engagement of β1 integrins and T-cell receptor complex or β1 integrins alone.9 To exert these functions, it is necessary that Cas-L is associated with focal adhesion kinase (FAK) or proline-rich tyrosine kinase 2 (Pyk2) and is tyrosine phosphorylated by these kinases.10 Cas-L is a hematopoietic variant of p130Cas,7

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which was identified as a 130-kDa protein that is highly tyrosine phosphorylated in v-Src-11 and v-Crk-transformed cells.12 These proteins and Efs/Sin compose the Cas family, which has a conserved secondary structure with numerous protein-protein interactions, such as Src-homology 3 (SH3) domain, substrate domain, serine-rich domain, coiled-coil regions, helix-loop-helix domain, and COOH-terminal domain.7,13 These structures feature a docking molecule, which interacts with a variety of signaling molecules, including FAK and Pyk2. The function of Cas-L/Nedd9 in relation to the pathogenesis of brain ischemia, as well as the expression in adult brain, remains unknown.

In the present study, we cloned the cDNAs of mouse Cas-L/Nedd9 and assessed the temporal profile of Cas-L/Nedd9, as well as its related molecules, such as p130Cas, FAK, and Pyk2, in the brain of rats with transient global ischemia. We also investigated its physiological function by using PC-12 cells transfected with Nedd9.

Methods

Antibodies and Reagents
Monoclonal antibodies (mAbs) against FAK, p130Cas, and Pyk2 were obtained from Transduction Laboratories. Antiphosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Inc. A mAb to neuron-specific nuclear protein (NeuN) was from Chemicon International, and horseradish peroxidase-conjugated goat anti-mouse and anti-goat anti-rabbit antibodies were from Promega. Antibodies and reagents for RT-PCR were from Pharmacia Biotech. All of the other reagents were purchased from Sigma-Aldrich unless otherwise stated.

cDNA Cloning of Murine Cas-L/Nedd9
A Agt11 human placenta cDNA library (Clontech Laboratories) was screened by hybridization with a 32P-labeled probe for human Cas-L cDNA, which was labeled with [α-32P] dCTP by the random primer labeling method, for 16 hours at 50°C in 0.5 mol/L Tris-HCl, pH 7.5, 1 mol/L NaCl, 1% SDS, and 100 μg/mL sonicated salmon testis DNA, and then washed at 65°C in 0.1× SSC containing 1% SDS.15 The hybridization-positive clones were sequenced by an ABI Dideoxy Terminator Cycle Sequencing kit.

Animals
Male Sprague-Dawley rats weighing 250 to 350 g were anesthetized by an IP injection of pentobarbital sodium (40 mg/kg). Twenty-one minutes of global ischemia were induced by occlusion of both common arteries with systemic hypotension. Male Sprague-Dawley rats weighing 250 to 350 g were anesthetized by an IP injection of pentobarbital sodium (40 mg/kg). Twenty-one minutes of global ischemia were induced by occlusion of both common arteries with systemic hypotension. Male Sprague-Dawley rats weighing 250 to 350 g were anesthetized by an IP injection of pentobarbital sodium (40 mg/kg). Twenty-one minutes of global ischemia were induced by occlusion of both common arteries with systemic hypotension. Male Sprague-Dawley rats weighing 250 to 350 g were anesthetized by an IP injection of pentobarbital sodium (40 mg/kg). Twenty-one minutes of global ischemia were induced by occlusion of both common arteries with systemic hypotension.

RT-PCR
RT-PCR was performed using ISOGEN agent and BcaBEST RNA PCR kit (Takara Bio, Inc) with the following primers: forward 5′-AAATTGAGGCTTGAAGAAT-3′ for rat Nedd9, 5′-AGGCTCAGCTGACAC-3′ for rat Cas-L, and reverse 5′-TGACTGAGGGCTCTTGG-3′ for both cDNAs. PCR cycles were as follows: 94°C, 1 minute; 55.6°C, 1 minute; and 72°C, 2 minutes (30 cycles).

Immunoprecipitation and Immunoblotting
Tissues of cerebral cortex and hippocampus were homogenized in suspension buffer (20 mmol/L HEPES-KOH, pH 7.5, 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonfluoride, 2 μg/mL aprotinin, 10 μg/mL leupeptin, and 5 μg/mL pepstatin A). The lysates were immunoprecipitated with anti–Cas-L polyclonal antibody and protein A sepharose beads. The samples were separated by 8% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore). For immunoblotting, the primary antibodies and their dilutions were as follows: anti–Cas-L polyclonal antibody (1:2500), anti-Pyk2 mAb (1:2500), anti-FAK mAb (1:2000), anti p130Cas mAb (1:2500), and anti–β-actin mAb (1:5000).

Histological Examinations
Anesthetized rats were perfused transcardially with heparinized saline followed by 4% paraformaldehyde/PBS for tissue fixation, and paraffinized sections were made. The paraffinized sections (10 μm) were dewaxed and permeabilized with 0.1% Triton X-100/PBS and then blocked in 4% FBS/PBS. The primary antibodies and their dilutions were as follows: anti–Cas-L rabbit polyclonal antibody, 1:200; anti–FAK mAb, 1:200; and NeuN, 1:200. The sections were finally exposed to 0.025% diaminobenzidine and 0.075% H2O2 in PBS for 1 minute. For immunofluorescence staining, fluorescein isothiocyanate or Texas Red-conjugated anti-rabbit or anti-mouse IgG antibodies at 1:100 were used as the secondary antibody.

Gene Transfer Using Retrovirus
Retroviral gene transfer was carried out using the ping-pong infection method using Plat-E cells,17 PT-67 cells (Clontech), and Fugene6 reagent (Roche Diagnostics) with pMX-Nedd9 wild type–IRES-GFP, pMX-Nedd9 SH3-IRES-GFP, pMX-Nedd9 F-IRES-GFP, pMX-Nedd9 δSH3-IRES-GFP, pMX-Nedd9 δSD-IRES-GFP, or pMX-IRES-GFP. The supernatants of PT-67 infected with each retrovirus were used to infect PC-12 cells.

Assessment of Neurite Outgrowth of PC-12 Cells
Infected PC-12 cells were cultured in DMEM plus 10% fetal calf serum and 5% horse serum. For the examination of neuritogenesis, 2.5×104 cells were seeded per 35-mm plate. grown overnight, and starved (0.5% FCS plus 0.5% horse serum) for 16 to 20 hours, then 50 ng/mL nerve growth factor (NGF) was added. After 6 days of stimulation, neurite length was measured on photographed fields containing 50 to 100 cells. Data were expressed in 2 ways: first, as the neurite length averaged over diameter of soma (bar diagrams; y axis=cell diameters); and second, as the number of neurites per cell. The average neurite length was measured by an automated image analysis system.

Results
Cloning of Mouse Cas-L Revealed That Cas-L Was a Splicing Variant of Nedd9 With Identical Functional Domains
Using the cloned mouse Cas-L–related cDNAs, a sequence homology search was performed against the cDNA and genomic database. Nedd9 was found to share high degrees of homology with mouse Cas-L: 98.9% in nucleotide sequence and 98.6% in amino acid sequence (Figure 1a). An analysis of mouse genomic database revealed that Cas-L (MKYK-) was a splicing variant of Nedd9 (MWAR–), and the functional domains of these proteins were identical to each other. A homology search against a rat genomic database showed that the deduced amino acid sequences of mouse and rat Nedd9 were highly conserved between these species: 92.7% in the
nucleotide sequence and 93.3% in the amino acid sequence, respectively. Rat Nedd9 shared high degrees of homology with rat Cas-L, 99.8% in nucleotide sequence and 99.8% in amino acid sequence (Figure 1a and 1b).

Nedd9, Not Cas-L, Was Transcriptionally Upregulated and Tyrosine Phosphorylated, Along With FAK in Cerebral Cortex and Hippocampus After Transient Global Ischemia

The Table shows the physiological parameters just before the induction of ischemia. There were no significant differences in the parameters among the groups, demonstrating that all of the rats used in the ischemia study had no significant difference in their condition. Expression of Nedd9 and Cas-L mRNAs in rats with global ischemia was analyzed by RT-PCR at 1, 3, 5, 7, or 14 days after 21-minute ischemia or at 1 hour after the sham operation. The RT-PCR assay was performed using a set of primers that amplifies the fragments of 203 and 225 bp from rat Nedd9 mRNA and Cas-L mRNA, respectively (Figure 1b). The expression of Nedd9 mRNA was assessed by real-time PCR at 1, 3, 5, 7, or 14 days after 21-minute ischemia or at 1 hour after the sham operation. The expression of Cas-L mRNA was assessed by real-time PCR at 1, 3, 5, 7, or 14 days after 21-minute ischemia or at 1 hour after the sham operation.

Nedd9 Protein Is Upregulated After Ischemia

**Figure 1.** Nedd9 is a splicing variant of Cas-L. a, Comparison of the deduced amino acid sequences of Nedd9 and Cas-L from mouse and rat. b, Proposed model for the generation of Nedd9 and Cas-L mRNA variants and the multiple domain structure of Cas-L/Nedd9. Top, Organization of the Cas-L/Nedd9 gene. Exons are indicated by black boxes and numbered. Middle, The presence of exons in the different mRNAs represented by boxes linked by connecting lines. Translation initiation sites in exons 2A and 2B and the translation stop site in exon 8 are indicated. Arrows indicate the primer sites for PCR amplification designed to give products from Nedd9 mRNA and Cas-L mRNA, respectively. Bottom, The domain structure of Cas-L/Nedd9 is demonstrated. Bar indicates the responsible domain to be recognized by anti-Cas-L antibody used in this study. CC indicates coiled-coil regions; HLH, helix-loop-helix domain; CT, COOH-terminal region.
was upregulated in both the cerebral cortex and hippocampus 1 to 14 days after ischemia, whereas the expression of Cas-L mRNA was not observed (Figure 2a).

In order to address the Cas-L/Nedd9-mediated signal transduction pathway, we examined the expression of other proteins, such as FAK, Pyk2, and p130Cas. Expression of Nedd9 protein and FAK was upregulated in a similar time course, whereas others did not show significant changes (Figure 2b). Furthermore, Nedd9 and FAK were coimmunoprecipitated at 3, 7, or 14 days after transient global ischemia. The immunoprecipitates were also blotted with the antiphosphotyrosine antibody, because activated Cas-L/Nedd9 is known to be tyrosine phosphorylated. Hence, our data demonstrated that Nedd9 was tyrosine-phosphorylated with similar kinetics as observed with protein levels of Nedd9.

**Induced Nedd9 Was Localized in Dendrite-Like Structure and Cytosol of Neurons in Cerebral Cortex and Hippocampus**

Nedd9- and FAK-immunoreactive cells were detected at the cerebral cortex and hippocampus 3 and 7 days after the ischemic insult but not detected at those of sham-operated rats. The expression pattern of these proteins 3 and 7 days after the ischemia were identical to each other (Figure 3a; data not shown). The rat cerebral cortex and hippocampal...
Nedd9 Promoted Neuronal Outgrowth of PC-12 Cells in the Presence or Absence of NGF

In order to clarify the physiological role of Nedd9, we examined the effect of Nedd9 on neurite outgrowth in PC-12 cells. Because we preliminarily found that the Nedd9 protein was expressed in neurons undergoing differentiation in the mouse embryonic neuroseptum and neurosphere (T. Sasaki, MD, PhD, unpublished data, 2003), we hypothesized that Nedd9 might play a role in neuronal differentiation. The rat pheochromocytoma PC-12 cells have been used for molecular analysis of the signaling pathways that lead to differentiation of peripheral nervous system neurons. Recently, this cell line was widely used for assays of regulatory factors expressed during central nervous system development.18 PC-12 cells were infected with recombinant retrovirus to express myc-tagged Nedd9 and its mutants (Figure 4a). In contrast to cells infected with empty vector and mutants such as δSH3, SH3, and δSD, those expressing wild-type Nedd9 and mutant F showed promotion of neurite genesis in the presence or absence of NGF after 6 days of the addition of NGF or vehicle (Figure 4b). These findings suggest that Nedd9 may contribute to the promotion of neuronal differentiation of PC-12 cells.

Discussion

In this study, we have shown that Nedd9 is a splicing variant of Cas-L and is transcriptionally upregulated in the dendrites and cytosol of neurons in the cerebral cortex and hippocampus from 1 to 14 days after global ischemia in rats. We have also found that Nedd9 promotes neurite outgrowth of PC-12 cells. These data demonstrate that Nedd9, which is upregulated transiently during neuronal development and disappears in the adult brain, is upregulated for neuronal differentiation after ischemia.

This is the first report to demonstrate that Cas-L and Nedd9 are splicing variants. The genetic difference between Cas-L and Nedd9 involves just 3 amino acids in the NH2-terminus (Figure 1a): MKYK and MWAR, respectively. The fact that only Nedd9, but not Cas-L, expression is induced in response to ischemia recapitulates the physiological role of Nedd9, because it is expressed in NPCs during brain development (Figure 2a). The difference in expression between Nedd9 and Cas-L in the brain may be because of the transactivation of the Nedd9 gene mediated by nuclear factor κB, heat shock factor 1, or other related transcription factors known to be induced gradually after ischemia, because their binding sites exist in the upstream region of the rat Nedd9 gene. The molecular mechanism of Nedd9 gene regulation in these neurons will be an important subject for additional studies.

This is the first report to identify the expression of Nedd9 in the adult brain. In the focal ischemia model of mice, upregulation of several genes has been investigated and grouped as temporal episodes or “waves” of expression of different groups of genes.1 Later waves of new gene expression, like Nedd9, include mediators that appear to be important in tissue remodeling and recovery of functions,19 such as TGF-β20 and osteopontin.21 In this study, Nedd9 was selectively induced and tyrosine-phosphorylated in neurons of the cerebral cortex and hippocampus 1 to 14 days after transient global ischemia (Figures 2b and 2c and 3a and 3b). The regional difference of temporal expression of those proteins may be because of the different response to ischemic insult between the cerebral cortex and hippocampus. Those results led us to postulate that Nedd9 is required for neuronal differentiation, being primarily expressed in the embryonic brain but not in the fully differentiated adult neurons. Recently, it has been reported that stroke increases cell proliferation, with stroke-generated new neurons, as well as neuroblasts already formed before the insult, migrating into the damaged area.22 These cells undergo differentiation and repair the neuronal network as short, remodeled neurons. The upregulation of Nedd9 in the adult brain appears to play a role in differentiation of neurons after ischemia.

To test the above hypothesis, we investigated the physiological function of Nedd9 in neurite outgrowth of PC-12 cells. We demonstrated that Nedd9 had a great impact on neurite outgrowth in the presence or absence of NGF when overexpressed in PC-12 cells. The fact that expression of δSH3, SH3, and δSD (substrate domain) inhibited neurite outgrowth is compatible with the role of the functional components of Nedd9, because Cas-L/Nedd9 bind to FAK through their SH3 domains,8 and substrate domain is required for interaction with downstream SH2-containing proteins.
such as Crk, Nck, and SH-PTP2. Crk has been reported to induce differentiation of PC-12 in an NGF-independent manner. These results, thus, confirm the hypothesis that delayed expression of Nedd9 and FAK may contribute to differentiation of neurons after ischemic injury in brain. We need to additionally investigate the role of Nedd9 in vivo, because PC-12 cells are not terminally differentiated in the setting used in the present study.

The source of Nedd9-positive neurons in ischemic brain should be investigated in future studies for its potential application to clinical treatment. It has been shown that some pathological conditions, such as ischemia, induce neurogenesis in the adult mammalian brain. Even if Nedd9-positive neurons are derived from NPCs or have existed since the occurrence of ischemic insult, Nedd9 may be required to facilitate these regenerative or differentiating processes. The overexpression of Nedd9 may lead to a widening of the therapeutic time window for cerebral ischemia, particularly in the later phase of a stroke. In conclusion, our study may support the potential of Nedd9 for participation in the differentiation of neurons after global ischemia in rats.

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