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

Takahiro Sasaki, MD, PhD; Satoshi Iwata, MD, PhD; Hirotaka James Okano, MD, PhD; Yasuyo Urasaki, MS; Junichi Hamada, MD, PhD; Hirotoshi Tanaka, MD, PhD; Nam H. Dang, MD, PhD; Hideyuki Okano, MD, PhD; Chikao Morimoto, MD, PhD

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. Some of these proteins, such as neurocan, Nedd2/Caspase2, and GAP43, 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. 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 filamentation 1 cloned by another group. 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. The major biological functions of Cas-L are the restoration of interleukin-2 production by costimulation with β1 integrins and T-cell receptor complex and the enhancement of cell migration by the engagement of β1 integrins and T-cell receptor complex or β1 integrins alone. 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. Cas-L is a hematopoietic variant of p130Cas.
which was identified as a 130-kDa protein that is highly tyrosine phosphorylated in v-Src- and v-Crk-transformed cells. 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. 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. Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Inc. A mAb to neuron-specific nuclear protein (NeuN) was 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 a solution of 50 mmol/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. 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 <50 mm Hg. The rectal temperature was continuously monitored and maintained at 37.0°C to 37.5°C with a thermostatically controlled heating pad. The experimental committee of Keio University approved the experimental protocol as meeting the experimental animal guidelines of Keio University School of Medicine.

RT-PCR
RT-PCR was performed using ISOGEN agent and BcaBEST RNA PCR kit (Takara Bio, Inc) with the following primers: forward 5'-ATAATCCTGGCAGAAT-3' for rat Nedd9, 5'-AGGCTCATCTGACAC-3' for rat Cas-L, and reverse 5'-TGACTGGAGGGCTTCTG-3' for both cDNAs. PCR cycles were as follows: 94°C, 1 minute; 55°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 phenylmethylsulfonyl fluoride, 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 electrotransferred 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 (1:200), 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 dehydrated 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, 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 level of statistical significance was assessed by ANOVA followed by Scheffe’s post hoc test. Statistical significance was set at P<0.01.

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

<table>
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<tr>
<th>Day of Sacrifice</th>
<th>pH</th>
<th>Pao2, mm Hg</th>
<th>Paco2, mm Hg</th>
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</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>7.41±0.022</td>
<td>73.1±2.2</td>
<td>35.8±2.9</td>
</tr>
<tr>
<td>1 day after ischemia</td>
<td>7.445±0.011</td>
<td>84.7±3.8</td>
<td>38.3±3.0</td>
</tr>
<tr>
<td>3 days after ischemia</td>
<td>7.442±0.003</td>
<td>77.0±3.1</td>
<td>39.0±1.9</td>
</tr>
<tr>
<td>5 days after ischemia</td>
<td>7.408±0.014</td>
<td>84.9±2.4</td>
<td>38.9±2.6</td>
</tr>
<tr>
<td>7 days after ischemia</td>
<td>7.437±0.023</td>
<td>90.7±4.7</td>
<td>37.9±4.6</td>
</tr>
<tr>
<td>14 days after ischemia</td>
<td>7.449±0.019</td>
<td>84.8±6.1</td>
<td>40.2±1.8</td>
</tr>
</tbody>
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Mean±SEM. Physiological parameters did not differ among rats before induction of transient global ischemia. N=5.
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

Figure 2. Nedd9 is preferentially upregulated with FAK and tyrosine-phosphorylated after transient global ischemia. a, A representative result of RT-PCR discriminating the 2 splicing variants of Cas-L/Nedd9 with β-actin as an internal control. Spleen of rats serves as positive control of Cas-L. b, The expression of proteins related to Cas-L/Nedd9 by immunoblotting, c, Nedd9 was immunoprecipitated at the indicated days after ischemia. Statistical significance is tested with respect to sham operated controls (*P<0.001).

Figure 3. Induced Nedd9 protein is localized in dendrite-like structure and cytosol of neurons in the cerebral cortex and hippocampus. a, Representative figures of Nedd9 and FAK-immunoreactive cells detected at the cerebral cortex and hippocampus 7 days after transient global ischemia. Rat cerebral cortex and hippocampal coronal slices reveal intense Nedd9 and FAK immunoreactivity in the neuronal dendrites. In contrast, the neuronal cell nucleus appeared devoid of Nedd9 and FAK staining, A, C, E, G, and I, cerebral cortex; B, D, F, H, and J, hippocampus; A through D and G through J, ischemia (∼100); E and F, control; G and H, anti-FAK mAb; I and J, control; A and B, ischemia (∼400); C through F and G through J, ischemia (∼400); b, colocalization of Nedd9 and NeuN or FAK (∼200).
Nedd9 Protein Is Upregulated After Ischemia

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. Later waves of new gene expression, like Nedd9, include mediators that appear to be important in tissue remodeling and recovery of functions, 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 neural blastoids already formed before the insult, migrating into the damaged area. 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, 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.23 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.24,25 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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