The Rai (Shc C) adaptor protein regulates the neuronal stress response and protects against cerebral ischemia

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Rai (Shc C or N-Shc) is a neuron-specific member of the family of Shc-like adaptor proteins. Rai functions in the cytoplasmic propagation of Ret-dependent survival signals and regulates, in vivo, the number of sympathetic neurons. We report here a function of Rai, i.e., the regulation of the neuronal adaptive response to environmental stresses. We demonstrate that (i) primary cultures of cortical neurons from Rai−/− mice are more sensitive to apoptosis induced by hypoxia or oxidative stress; (ii) in Rai−/− mice, ischemia/reperfusion injury induces severe neurological deficits, increased apoptosis and size of the infarct area, and significantly higher mortality; and (iii) Rai functions as a stress-response gene that increases phosphatidylinositol 3-kinase activation and Akt phosphorylation after hypoxic or oxidant insults. These data suggest that Rai has a functional neuroprotective role in brain injury, with possible implications in the treatment of stroke.

The Shc protein family is characterized by the (CH2)-PTB-CH1-SH2 modularity. Its complexity increased during evolution from one locus in Drosophila (dShc) to three loci in mammals (shc, rai, and sli). Because of alternative initiation codon usage and splicing pattern, the three mammalian loci encode at least six Shc-like proteins. These proteins share an amino-terminal phosphotyrosine-binding (PTB) domain, a central collagen homology region rich in proline and glycine (CH1), and a carboxyl-terminal Src homology 2 (SH2) domain and differ at their amino termini. A second amino-terminal collagen homology region (CH2) is present in p66Shc and p64Rai (1).

Despite their structural similarity, there is emerging evidence that the Shc proteins are not functionally redundant and regulate functions as diverse as growth (p52/p46Shc), apoptosis (p66Shc), and life span (p66Shc). P52Shc and p66Shc are implicated in distinct signal transduction pathways. After growth factor stimulation, p52Shc is recruited to the activated receptor, is phosphorylated on tyrosine residues, and associates with the Grb2/SOS complex. We therefore propose a model wherein this Shc isoform participates in receptor tyrosine kinase-dependent Ras activation. On the contrary, p66Shc, which is also phosphorylated on tyrosine residues and binds Grb2 upon growth factor stimulation, is not able to activate Ras (2, 3), suggesting its involvement in different pathways. Indeed, it has been recently demonstrated that p66Shc regulates intracellular levels of reactive oxygen species and reactive oxygen species-dependent apoptosis (4, 5).

Rai (Shc C or N-Shc) is a more recently identified member of the Shc-like proteins family (1). Its locus encodes for two proteins with predicted molecular masses of 64 (p64Rai) and 52 kDa (p52Rai). Unlike p52/p46Shc, which is expressed in different tissues, Rai expression is restricted to the developing and adult central nervous system (6–10). We have recently demonstrated that Rai is a physiological substrate of the receptor tyrosine kinase Ret and that it stimulates neuronal cell survival by inhibiting apoptosis in conditions of limited availability of the Ret ligand (11). After Ret stimulation, ectopic Rai expression increases phosphatidylinositol 3 (PI3)-kinase activation and induces hyperphosphorylation of Akt. Akt is a serine–threonine kinase, which is known to regulate survival in different cell types; activated Akt phosphorylates several substrates and transduces signals that regulate multiple biological functions, including suppression of apoptosis (12). Because mutations of Rai (13) or Ret (14) in mouse result in the loss of sympathetic neurons, it is proposed that one of the physiological functions of Rai is the cytoplasmic propagation of Ret-dependent survival signals in specific neuronal subpopulations.

Overexpression of Rai, however, results in increased survival of serum-starved neuronal cell lines, suggesting that Rai exerts an inhibitory effect on apoptosis also in the absence of receptor activation (11). We have further investigated the Ret-independent effect of Rai on survival and found that it is involved in the cellular response of mature neurons to environmental stresses. In particular, we found that Rai expression is neuroprotective in a mouse model of ischemia/reperfusion brain injury and exerts this function by activating the PI3-kinase/Akt pathway.

Methods


Cell Culture. Cultures of cortical neurons were prepared from 129S2/SvHsd (Harlan Italy, Indianapolis) mouse embryos (embryonic day 14.5). After mechanical dissociation, dispersed cortical neurons were plated in defined medium (neurobasal medium supplemented with B27) onto poly(D-lysine)-coated 96-, 12-, or 6-well plates, depending on the experimental protocol, and were maintained in a humidified atmosphere (37°C) with 5% CO2. Culture was treated with antimotic agent (cytosine arabinoside) on day 3, and the experiments were performed on day 8, when the culture contained 95% neurons. SK-N-MC-Rai and SK-N-MC-pinco (Ctrl) cells were obtained by infection with the Rai-GFP-PINCO retroviral expression vector or the empty vector (11). Transfection of the Phoenix

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Abbreviation: PI3, phosphatidylinositol 3.

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packaging cell line and infection of target cells were performed as described in ref. 15. CoCl₂ or H₂O₂ were given in complete medium.

**Western Blotting.** Brain homogenates of animals subjected to middle cerebral artery occlusion and lysates of neuronal cultures were obtained as described in refs. 8 and 11. SDS/PAGE and Western blotting were performed according to established procedures. The following antibodies were used: α-vinculin (Sigma) and α-Shc C (Transduction Laboratories, Lexington, KY) monoclonal antibodies; and α-CH1Rai, α-AktP (Ser-437), α-Akt, and α-elevated caspase-3 (all from Cell Signaling Technology, Beverly, MA) polyclonal antibodies.

**Metabolic Labeling.** PFSK1 cells were plated at a density of 10⁴ per 100-mm dish. After 2 h of incubation in a PO₄-deficient medium, 0.5 mM CI/m (1 Ci = 37 GBq) [³²P]orthophosphate (20 mM/m; product code PBS43-20MCI, Amersham Biosciences) was added for 90 min. After 15 min or 3 h of H₂O₂ and CoCl₂ treatments, the cells were lysed, and equal amounts of protein were immunoprecipitated with α-Rai antibody. The samples were loaded on a 10% SDS/PAGE, and [³²P]-labeled bands were detected by autoradiography.

**Cerebral Transient Focal Ischemia.** Male Rai⁻/⁻ (13) and WT mice with 26–28 g of body weight were used. Transient focal cerebral ischemia was achieved by middle cerebral artery occlusion as described in refs. 16 and 17. Anesthesia was induced by i.p. ketamine (100 mg/kg), and xylazine (5 mg/kg). To confirm the adequacy of the vascular occlusion in each animal, blood flow was measured by laser Doppler flowmetry (BLF21 series, Transonic Systems, Ithaca, NY) by using a flexible 0.5-mm fiber optic probe (type M, 0.5-mm diameter, Transonic Systems) positioned on the brain surface and secured with impression material on the skull at the following coordinates: anteroposterior, −1 mm; lateral, −3.5 mm (18). Briefly, a 5.0 monofilament nylon suture, coated in poly(L-lysine), was introduced into the internal carotid artery through the external carotid artery stump and advanced so as to reach the middle cerebral artery origin. A >70% reduction of blood flow, compared with preischemic baseline, was considered an indication of successful ischemia. At the end of a 15-min ischemic period, blood flow was restored by carefully removing the nylon filament.

For lesion size determination, 40-μm coronal brain sections were cut serially at 320-μm intervals and stained with neutral red (neutral red Gurr Certistain, BDH) (16). On each slice, infarcted areas were assessed blindly and delineated by the relative paleness of histological staining. Infarct volumes were calculated areas were assessed blindly and delineated by the relative paleness of histological staining. Infarct volumes were calculated as described in refs. 16 and 17.

**Neurological Deficits Evaluation.** Twenty-four hours after ischemia, each mouse was rated on two neurological function scales unique to the mouse (16, 19). For both scales, mice were scored from 0 (healthy mouse) to 28. The total score resulting from the investigation is the sum of the results of all categories for each scale. The general deficit scale evaluates hair, ears, eyes, posture, spontaneous activity, and epileptic behavior. The focal deficit scale evaluates body symmetry, gait, climbing on a surface held at 45°, circling behavior, front limb symmetry, compulsory circling, and whisker response to a light touch. A trained investigator blinded to the experimental conditions ran all of the experiments. Data are expressed as median and 25th–75th percentiles, because intervals between scores are not equal.

**RNA Isolation, cDNA Synthesis, and Real-Time PCR.** Real-time PCR was performed with a GeneAmp 5700 sequence detection system (Applied Biosystems). RNA was isolated from lesioned side cortex according to the acid guanidium-phenol-chloroform method shown in ref. 20 and reverse-transcribed into cDNA. Primers were designed by using PRIMER EXPRESS software (Applied Biosystems) based on the following GenBank accession numbers: NM007393 (β-actin), NM007527 (Bax), and NM009810 (procaspase-3).

**Cell Viability Assay.** The viability of cultured cells was evaluated by using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay. SK-N-MC cells (1 × 10⁴ per well) or primary neurons (8 × 10⁵ per well) were cultured in 96-well culture plates and treated as indicated. Briefly, after treatment with H₂O₂ and CoCl₂, cultures were incubated for 4 h (37°) with culture medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After aspiration of the medium, the dark blue crystals formed were dissolved by adding 100 μl per well of DMSO. Plates were shaken at room temperature to ensure that all crystals were dissolved before taking absorbance readings on a Micro ELISA plate reader (test wavelength, 570 nm).

Results are presented as a percentage of survival, taking control as 100%.

**Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling.** For *in vitro* (adherent cells cultured on chamber slides) and *in vivo* (paraffin-embedded tissue sections) detection and quantification of apoptosis at the single-cell level, the *in situ* cell death detection kit Fluorescein (Roche Molecular Biochemicals) was used according to the manufacturer’s suggestions. The analysis was performed by fluorescence microscopy. At least 300 cells were scored for each experimental point by a fluorescence microscope. The experiment was repeated three times.

**In Vitro Dephosphorylation Assay.** Cellular lysates obtained from brain tissue or PFSK-1 cells were immunoprecipitated with α-Rai antibody coupled to protein A protein–Sepharose (Amersham Pharmacia) beads for 2 h at 4°C. The beads were washed three times with lysis buffer, followed by incubation with calf intestine alkaline phosphatase (Boehringer Mannheim) or protein tyrosine phosphatase (T cell protein tyrosine phosphatase, New England Biolabs) for 30 min at 30°C. The reaction was stopped by boiling in SDS sample buffer, and Rai protein was detected by Western blotting with anti-Rai antibodies.

**In Vitro P13-Kinase Assay.** Lysate (300 μg) was immunoprecipitated with α-p85 antibodies. The kinase reaction was performed for 30 min at room temperature in the P13-kinase buffer (20 mM Tris-HCl, pH 7.5/100 mM NaCl/0.5 mM EGTA, pH 8/25 mM MgCl₂/10 μg of [³²P]yATP/1 μg of phosphatidylinositol) and terminated by the addition of HCl. TLC was conducted on Whatman silica gel plates in a closed TLC tank by using methanol/chloroform/water/ammonia (47:60:11.3:2: vol/vol) as buffer. The plates were processed for autoradiography, and the level of incorporation of radioactive ATP was quantified by using a PhosphoImager (Molecular Dynamics).

**Statistical Analysis.** Data are presented as average ± SD unless specified otherwise. Statistical comparisons were made by using Student’s *t* test, and *P* < 0.05 was considered statistically significant.

**Results**

**Rai Expression Decreases Stress-Induced Apoptosis.** We investigated the effect of Rai expression on the cellular response to different stresses by using the neuroepithelioma SK-N-MC cells, which are negative for Rai expression, and primary cultures of cortical neurons from WT or Rai⁻/⁻ mouse embryos (embryonic day 15). Cells were treated with different concentrations of H₂O₂ as inducer of oxidative stress, or CoCl₂, which induces intracellular hypoxia (21). H₂O₂ or CoCl₂ induces a dose-dependent reduc-
Rai expression stimulates survival of neuronal cells after subjecting to different stress conditions. Evaluation of the percentage of surviving cells after 24 h of treatment with different types of stress. H$_2$O$_2$ and CoCl$_2$ in SK-N-MC cells stably expressing Rai or in control cells infected with the empty vector (Ctrl) (a) and in cortical primary neurons derived from Rai WT and genetically deleted Rai (Rai$^{-/-}$) mice (c). Evaluation of the percentage of apoptotic cells by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining is shown for SK-N-MC cells treated with 100 $\mu$M H$_2$O$_2$ and 150 $\mu$M CoCl$_2$ for 24 h (b) and for cortical primary neurons derived from Rai WT and Rai$^{-/-}$ mice (d). Values in the graphs are the average ($\pm$ SD) from three independent experiments, each performed in triplicate.
protein tyrosine phosphatase), induced a similar acceleration of the Rai polypeptide gel migration, suggesting that the different stress conditions induced serine–threonine dephosphorylation of Rai (Fig. 3d). As positive control for protein–tyrosine–phosphatase activity, we used lysates from epidermal growth factor-stimulated HeLa cells immunoprecipitated with epidermal growth factor receptor antibodies (data not shown). Lysates were analyzed by Western blotting by using α-P-tyrosine antibodies (Upstate Biotechnology, Lake Placid, NY). We did not detect tyrosine-phosphorylated epidermal growth factor receptor in the lysates treated with alkaline phosphatase or T cell protein tyrosine phosphatase (data not shown).

To directly demonstrate that the gel-mobility shift of Rai after stress is due to protein dephosphorylation, we performed the 32P labeling of PFSK1 cells treated with H2O2 or CoCl2. Autoradiography of α-Rai immunoprecipitates revealed a dramatic reduction of 32P incorporation after stress treatments (Fig. 3e), confirming that Rai undergoes dephosphorylation.

Finally, by using specific proteosome inhibitors, we excluded proteolysis as responsible of the Rai protein downshift (data not shown).

Rai Expression Increases Stress-Induced Activation of the PI3-Kinase/Akt Pathway. To investigate the downstream mechanisms of the antipoptotic function of stress-activated Rai, we analyzed its effect on the PI3-kinase/Akt pathway. Activated receptors promote membrane recruitment of PI3-kinase, which then leads to the local formation of PI(3,4)P2 and PI(3,4,5)P3 (24). Akt is a kinase that, upon binding to PIP2 and PIP3, is phosphorylated by other serine/threonine kinases (phosphoinositide-dependent protein kinases 1 and 2) and phosphorylates several substrates involved in the suppression of apoptosis (12). Rai is constitutively complexed to the p85 subunit of PI3-kinase and, after Ret activation, is recruited to the activated receptor and favors hyperphosphorylation of Akt (11). To evaluate whether Rai regulates Akt upon stress, we measured the levels of Akt phosphorylation in primary cultures of cortical neurons from WT and Rai−/− mice after H2O2 treatment or after occlusion of the middle cerebral artery in the cortex or in the hippocampus of WT and knockout mice. H2O2 treatment of primary cortical neurons derived from WT mice induces a rapid (5-min) Akt phosphorylation, which continued up to 2 h after the challenge. Instead, in the Rai−/− cultured neurons, the extent of Akt phosphorylation was significantly reduced, and its duration was limited to the first 10 min (Fig. 4a). In both the cortex and the hippocampus of ischemic WT mice, we observed a transient decrease of Akt phosphorylation (5–30 min) that returned to the
We report here that Rai stress conditions (oxidative stress and hypoxia). PI3-kinase indicates that expression of Rai favors the activation of the cell samples, by using phosphatidylinositol as a substrate. H2O2-dependent pathways (12), we then measured the enzymatic Akt can be activated through either PI3-kinase-independent or neuroepithelioma cells resulted in increased Akt activation after stress. Consistently, ectopic expression of Rai in SK-N-MC it seems, therefore, that Rai expression regulates Akt activation of Akt phosphorylation were lower at every time point (Fig. 4). instead, in the ischemic Rai/− mice, the levels of Akt phosphorylation were lower at every time point (Fig. 4b). It seems, therefore, that Rai expression regulates Akt activation after stress. Consistently, ectopic expression of Rai in SK-N-MC neuroepithelioma cells resulted in increased Akt activation after treatment with low doses of H2O2 or CoCl2 (Fig. 4c). Because Akt can be activated through either PI3-kinase-independent or-dependent pathways (12), we then measured the enzymatic activity of anti-PI3-kinase immunoprecipitates from the same cell samples, by using phosphatidylinositol as a substrate. H2O2 treatment failed to induce a detectable increase of PI3-kinase activity in SK-N-MC cells, which became instead evident after Rai expression (Fig. 5a). This effect of Rai expression on PI3-kinase activity is relevant to the effect of Rai on Akt and neuronal survival. Indeed, treatment of SK-N-MC cells with the PI3-kinase inhibitor wortmannin (i) prevented H2O2-induced Akt phosphorylation in both control and Rai-expressing cells (data not shown) and (ii) abrogated the survival-promoting effect of Rai expression after H2O2 (Fig. 5b). These results indicate that expression of Rai favors the activation of the PI3-kinase/Akt-survival pathway in neuronal cells exposed to stress conditions (oxidative stress and hypoxia).

Discussion

We report here that Rai−/− primary cortical neurons are less resistant to H2O2 treatment and hypoxia and that Rai−/− ischemic mice have significantly higher mortality, more severe neurological deficits, and increased apoptosis and size of the infarct area, compared with WT mice. These data demonstrate a function of Rai as a stress response gene that counteracts stress-induced apoptosis of neuronal cells.

Rai Is a Neuron-Specific Antistress Gene. In cultured neural cells and in different areas of the brain, Rai proteins undergo serine–threonine dephosphorylation after being subjected to different stress conditions, including oxidative stress, hypoxia, and ischemia. In the same cells, overexpression of Rai increases survival after they undergo the same toxic stimuli. Rai expression might, as a consequence, function to protect neuronal cells from stress-induced apoptosis. Apoptosis plays a major role in the pathogenesis of acute brain injury. Although it is not the only mechanism, morphological and biochemical evidence of apoptosis has been documented in the pathological lesions of experimental animal models with ischemic brain injuries (26). However, how apoptosis is triggered in this pathological condition remains unclear. Oxidative stress-induced apoptosis might, therefore, represent one of the pathogenic steps in the brain ischemic injuries, and Rai, which is mainly expressed in the adult brain, may function as a neuron-specific antistress gene.

Functional Role of Rai in Cerebral Ischemia. We also provide evidence that Rai expression protects against brain damage after transient occlusion of the middle cerebral artery, through stimulation of the PI3-kinase–Akt pathway and inhibition of apoptosis induced by ischemia/reperfusion of the involved brain area. As a matter of fact, Rai−/− mice were markedly more susceptible to ischemia/reperfusion injury, in terms of mortality, apoptosis, and size of the injured brain area. Consistently, Rai−/− mice showed a significant exacerbation of both general and focal neurological deficits (worse appearance, motor performance, reactivity, and response to stimuli). Our data suggest that Rai
might then represent a suitable target for antistroke strategies. Notably, Rai is dephosphorylated during the ischemic/reperfusion lesion, suggesting that phosphorylation/dephosphorylation of Rai is critical to the activation of its signaling potential. Investigation of the molecular mechanisms involved in Rai activation by stress might therefore lead to the development of new drugs for antistroke treatment.

**Rai Increases PI3-Kinase/Akt Activity in Vitro After Oxidative Stress and Hypoxia and in Vivo After Middle Cerebral Artery Occlusion.** The PI3-kinase/Akt pathway is an important regulator of neuronal survival. Rai expression increases the activation of the PI3-kinase/Akt pathway in neuronal cells exposed to different stress conditions, suggesting that Rai promotes neuronal survival by activating PI3-kinase. In brain areas of Rai−/− mice subjected to cerebral ischemia, damage correlated with prevention of an increase in c-Akt activation. The mechanism by which Rai activates PI3-kinase/Akt pathway after stress is still not known. As we have previously demonstrated, Rai forms a constitutive complex with the regulatory subunit of PI3-kinase in vivo, and treatment of cells with a PI3-kinase inhibitor blocks its prosurvival effect (11). A critical step in the activation of PI3-kinase after stress might be its translocation from the cytosol to the plasma membrane, where its substrate lipids are located. Notably, Rai is a cytoplasmic protein that localizes within different membranous compartment fractions (immunofluorescence and cell fractionation experiments; unpublished results). However, we did not notice an enrichment of membrane-bound Rai proteins after treatment of neuronal cells with various stress stimuli.

Regardless of its mechanism, therefore, Rai seems to be a positive regulator of PI3-kinase/Akt pathway and is part of a general adaptive response of neuronal cells to environmental stresses, adding a further level of complexity to the functions of the family of Shc-like proteins.

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