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Cloning of a Putative Vesicle Transport-related Protein, RA410, from Cultured Rat Astrocytes and Its Expression in Ischemic Rat Brain*

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To elucidate the role of astrocytes in the stress response of the central nervous system to ischemia, early gene expression was evaluated in cultured rat astrocytes subjected to hypoxia/reoxygenation. Using differential display, a novel putative vesicle transport-related factor (RA410) was cloned from reoxygenated astrocytes. Analysis of the deduced amino acid sequence showed RA410 to be composed of domains common to vesicle transport-related proteins of the Sec1/Unc18 family, including Sly1p and Sec1p (yeast), Rop (*Drosophila*), Unc18 (*Caenorhabditis elegans*), and Munc18 (mammalian), suggesting its possible role in vesicular transport. Northern analysis of normal rat tissues showed the highest expression of *RA410* transcripts in testis. When astrocyte cultures were subjected to a period of hypoxia followed by reoxygenation, induction of *RA410* mRNA was observed within 15 min of reoxygenation, reaching a maximum by 60 min. At the start of reoxygenation, the addition of diphenyl iodonium, an NADPH oxidase inhibitor, blocked in parallel astrocyte generation of reactive oxygen intermediates and expression of *RA410* message. In contrast, cycloheximide did not affect *RA410* mRNA levels, indicating that *RA410* is an immediate-early gene in the setting of reoxygenation. Using polyclonal antibody raised against an RA410-derived synthetic peptide, Western blotting of lysates from reoxygenated astrocytes displayed an immunoreactive band of ≈70 kDa, the expression of which followed induction of the mRNA. Fractionation of astrocyte lysates on sucrose gradients showed RA410 antigen to be predominantly in the plasma membrane. Immunoelectron microscopic analysis demonstrated RA410 in large vesicles associated with the Golgi, but not in the Golgi apparatus itself, consistent with its participation in post-Golgi transport. Consistent with these *in vitro* data, RA410 expression was observed in rat brain astrocytes following transient occlusion of the middle cerebral artery. These data provide insight into a new protein (RA410) that participates in the ischemia-related stress response in astrocytes.

Changes in the cellular microenvironment, especially those that challenge cell viability, such as glucose deprivation, hypoxia, and accumulation of toxic metabolites, are major contributors to tissue damage consequent to cerebral ischemia (1). Adaptation to such circumstances leads to a change in the cellular phenotype due, at least in part, to redirection of biosynthetic properties with expression of stress proteins. The latter are central to the cellular stress response; global inhibition of their expression results in cell death in the altered environment (2).

Because of their abundance and ability to sustain environmental perturbations, astrocytes have an important role in maintaining neuronal function under homeostatic and pathologic conditions (3). For example, astrocytes subjected to hypoxia, a major component of the ischemic milieu, express stress proteins such as the 78-kDa glucose-regulated protein (GRP78) (4) and the 150-kDa oxygen-regulated protein (ORP150) (5). Induction of these stress proteins in response to hypoxia is indicative of the capacity of astrocytes to maintain biosynthetic processes, albeit redirected toward different products, in the setting of oxygen deprivation.

In contrast to oxygen deprivation, reoxygenation, an abrupt restoration of ambient oxygen tension in the cellular environment after a period of hypoxia, triggers a quite different cellular response. Since the period of reoxygenation is that most closely associated with damage to parenchymal cells (6), we reasoned that astrocytes would rise to the challenge of ischemia/reperfusion by redirecting cellular activities in support of the more vulnerable population of neurons (7). Furthermore, inhibition of protein synthesis at the time of reoxygenation, even though it transiently increases the pool of high energy phosphate compounds, ultimately results in failure to adapt to the oxygen-rich environment and subsequent cell death (8).

We have previously described the cloning of a novel RNA-binding protein expressed by reoxygenated astrocytes, suggesting that changes in RNA processing/editing may be important in the cellular response to ischemia (9). Here, we have identified another novel polypeptide (RA410), a vesicle transport-related protein, also induced in reoxygenated astrocytes. Our

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI/DDBJ Data Banks with accession number(s) D79221.

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data demonstrate that RA410 is expressed intensely in astroglia in ischemic brain, and its subcellular localization suggests a role in post-Golgi protein processing.

MATERIALS AND METHODS

Cell Culture and Exposure to Hypoxia/Reoxygenation—Rat primary astrocytes were obtained from neonatal rats by a modification of a previously described method (7). In brief, cerebral hemispheres were harvested from neonatal Harlan Sprague Dawley rats within 24 h of birth, and brain tissue was digested at 37 °C using Dispase II (3 mg/ml; Boehringer, Mannheim, Germany). The mixture was plated in 175-cm^2 culture flasks (two brains/flask), and cells were grown in minimal essential medium supplemented with fetal calf serum (10%; CellGrow, Life Technologies Inc.). After 10 days, culture flasks were incubated for 48 h with cytosine arabinofuranoside (10 mg/ml; Wako Chemicals, Osaka, Japan) to prevent fibroblast overgrowth and agitated on a shaking platform (Bioshaker BR-30L, Taitek, Tokyo, Japan) to separate astrocytes from remaining microglia and oligodendroglia, and the adherent cell population was then identified by morphologic and immunohistochemical criteria (detection of glial fibrillary acidic protein). Cells were then replated at a density of 5×10^4 cells/cm^2 in the above medium. When cultures achieved confluence, they were exposed to hypoxia using an incubator attached to a hypoxia chamber that maintained a humidified atmosphere with low oxygen tension (Coy Laboratory Products, Grass Lake, MI), as described previously (10). Where indicated, after exposure to hypoxia, cultures were returned to the ambient atmosphere (reoxygenation), at which time the conditioned medium was rapidly exchanged with fresh medium. Oxygen tension in the medium was monitored using a blood gas analyzer (ABL-2, Radiometer, Stockholm, Sweden). Cell viability was assessed by several methods, including morphologic criteria, trypan blue exclusion, and lactose dehydrogenase release.

Preparation of Total RNA and cDNA—Total RNA was extracted and purified from astrocytes (≈5×10^8 cells) exposed to hypoxia for 24 h or to hypoxia/reoxygenation (24 h of hypoxia followed by 1 h of reoxygenation) using the acid guanidinium/phenol/chloroform method (11). For hypoxic samples, RNA extraction was performed inside the hypoxia chamber after all reagents were equilibrated in the hypoxic atmosphere. Then, purified RNA (≈3 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (300 units; Life Technologies, Inc.), oligo(dT) primer (2.5 μM $T_{11}GT$), and dNTP mixture (20 μM each) for 60 min at 37 °C. cDNAs synthesized from RNAs were subjected to differential display (see below).

Differential Display—The polymerase chain reaction (PCR),[1] recovery, and reamplification of cDNAs obtained from astrocytes exposed to hypoxia and hypoxia/reoxygenation were performed as described (12) with minor modifications. In brief, reverse transcriptase products obtained from total RNA (≈25 ng in each reaction) were used as PCR templates in 30 μl of reaction mixture containing arbitrary primer (1 μM), dNTP (200 μM each), and *Taq* polymerase (2 units of AmpliTaq; Takara Shuzo, Tokyo, Japan). Thermocycling was performed for 39 cycles with the indicated parameters (95 °C for 30 s, 40 °C for 1 min, and 72 °C for 1 min (5 min for last cycle)) using the same arbitrary 12-mer oligonucleotide as both the upstream and downstream primers. In one set of experiments, PCR products obtained from hypoxic and hypoxic/reoxygenated astrocytes were screened using 60 different primers. After separation by 5% polyacrylamide gel electrophoresis, PCR products were visualized by staining with ethidium bromide. cDNA bands specifically amplified in hypoxic/reoxygenated astrocytes were excised and eluted from the gels. Then, the eluted material was reamplified by PCR using the same primers and conditions employed in differential display except for an increased concentration of dNTP mixture (40 μM).

Cloning and Sequencing of cDNA Fragments—Reamplified cDNA fragments were cloned into the pT7Blue T-vector (Novagen, Madison, WI). Plasmid DNA sequencing of cloned fragments was carried out using the *Taq* Dye Primer Cycle Sequencing Core kit (Applied Biosystems Inc., Tokyo, Japan) with either the M13 forward sequencing primer or reverse primer. The cDNA sequences were analyzed and compared for homology with those available in the EMBL and GenBank™ DNA data bases and the SWISSPROT protein data base. One of the cDNA fragments, tentatively named *RA410*, was subjected to further analysis. A rat cDNA library was screened allowing isolation of a cDNA (2 kilobase pairs) that encompassed the entire open reading frame of *RA410*. The latter was sequenced in both directions. To show homology to other vesicle transport-related proteins, phylogenic tree analysis was performed as described (13).

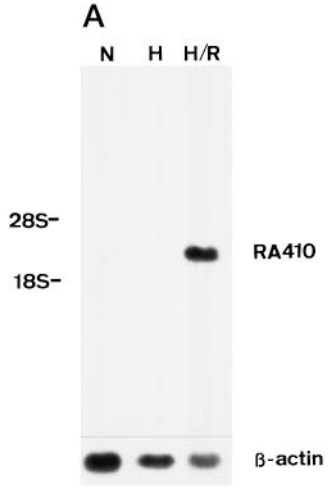
Northern Analysis—To study induction of *RA410* transcripts during reoxygenation, Northern blot analysis was performed using a cDNA probe synthesized from an RA410 fragment as described previously (9). In brief, total RNA extracted from hypoxic or hypoxic/reoxygenated astrocytes (≈5 μg) by the acid guanidinium/phenol/chloroform method was subjected to formaldehyde-agarose (1%) gel electrophoresis and transferred overnight to Immobilon N membranes (Millipore Corp., Bedford, MA). RNA was fixed to the membrane by exposure to UV irradiation prior to hybridization with cDNA probes. The membrane was prehybridized for 3 h at 65 °C in hybridization buffer (6 × SSC (0.9 M NaCl and 90 mM sodium citrate, final pH 7.0), 5 × Denhardt's solution (0.5% Ficoll, 0.5% polyvinylpyrrolidone, and 0.5% bovine serum albumin), 0.5% SDS, and 100 μg/ml heat-denatured salmon sperm DNA). The membrane was probed with ^{32}P-labeled cDNA fragments of RA410 by the random hexamer procedure (14). After hybridization, filters were washed twice with 2 × SSC and 0.5% SDS and with 0.1 × SSC and 0.5% SDS for 30 min at 65 °C, exposed to x-ray film (Kodak X-Omat, Eastman Kodak Co.), and subjected to autoradiography. Induction of *RA410* mRNA was evaluated by comparison with β-actin mRNA. In some experiments, either cycloheximide (10 μg/ml) or diphenyl iodonium (DPI) (50 μM; both reagents from Sigma) was added to the culture 15 min prior to reoxygenation, and total RNA was extracted 30 min after reoxygenation. To access the distribution of *RA410* transcripts under normal conditions, RNA was prepared and purified from adult Harlan Sprague Dawley rats (200–300 g) by the method described previously (15), and Northern blot analysis was performed as described above.

Production of Anti-RA410 Peptide Antibody and Western Blotting—To obtain antibody reactive with RA410 antigen, a peptide with the sequence CQEDEVKRLKSIMGLEGEDE (amino acids 346–365; see Fig. 1*B*), which contains an extra cysteine residue at the N terminus to facilitate conjugation, was synthesized and conjugated to bovine serum albumin using a kit from Sigma. Rabbits were immunized by conventional methods, and antisera were obtained from two rabbits, each immunized with 1 mg of synthetic peptide. Antibody titers of these antisera were studied by enzyme-linked immunosorbent assay, and the IgG fraction was purified by affinity chromatography using a column with immobilized RA410-derived synthetic peptide (ProtOn Kit1, Multiple Peptide Systems). Astrocytes (≈10^6 cells) exposed to hypoxia/reoxygenation for the indicated times were washed three times with phosphate-buffered saline, pelleted, and lysed in buffer containing 1% Nonidet P-40. Samples were prepared for SDS-polyacrylamide gel electrophoresis (10%; 5 μg of protein/lane), and RA410 antigen was visualized by immunoblotting (16) using anti-RA410 IgG. Protein content was determined using the Bio-Rad microprotein assay kit.

Subcellular Localization of RA410—To localize RA410 antigen in reoxygenated astrocytes, cells (≈10^8) were exposed to hypoxia (24 h)/reoxygenation (3 h) and harvested by centrifugation, and the cell pellet was disrupted by Dounce homogenization. Fractionation of cell lysates was performed as described (17), and following measurement of protein concentration, each fraction (≈5 μg of protein) was immunoblotted with anti-RA410 IgG. Immunocytochemical studies were performed on either hypoxic or reoxygenated astrocytes as described (18). In brief, astrocytes plated on glass coverslips (ChamberSlide, Nunc Inc., Naperville, IL) were exposed either to hypoxia (24 h)/reoxygenation (3 h) or to hypoxia only (24 h), fixed in Zamboni buffer for 2 h, washed twice with 0.2 M phosphate-buffered saline, and incubated with either anti-RA410 IgG or preimmune rabbit IgG (10 μg/ml in each case). Sites of primary antibody binding were visualized using fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma).

To further analyze the subcellular localization of RA410 antigen in hypoxic astrocytes, cells (≈5×10^8) were pelleted and fractionated as described (19). In brief, astrocyte cell pellets frozen at −80 °C were thawed and resuspended in 10 ml of buffer A (0.25 M sucrose, 10 mM HEPES/NaOH, pH 7.5, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone), and the cells were cavitated at 400 p.s.i. N_2 pressure for 30 min by nitrogen cavitation bomb (Kontes Glass Co., Vineland, NJ). Following homogenization, the cell lysate was clarified by centrifugation at 10,000 × *g* for 15 min at 4 °C, and the pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The clarified lysate was then centrifuged and fractionated by a series of sucrose steps: 38, 30, and 20% sucrose (all prepared in 10 mM HEPES/NaOH, pH 7.5, and 1 mM dithiothreitol) at 100,000 × *g* for 3 h at 4 °C. Layered

[1] The abbreviations used are: PCR, polymerase chain reaction; DPI, diphenyl iodonium; IL, interleukin.



B

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RA410          MVGSKMAASIRERQTVALKRMLNFNVPHVKNSPGEPVWKVLIYDRFGQDIISPLLSVKELRDMGITLHLLLHSDRDPIRDVPAVYFVMPTEENIDRLCQD
                                                 * .***** *  . ..**..*.*..* . ***.* *.. **.*..****.*** **.**** . .*
MAVEEIASRKDISLRDMQISAILKMLFLNKDLNNNDNITTITDDIFNQQEIIWKVLILDIKSTATISSVLRVNDLLKAGITVHSLIKQDRSPLPDVPAIYFVSPTKENIDIIVND

LRNQLYESYYLNFISAISRSKLEDIANAALAANAVTQVAKVFDQYLNFITLEEDMFVLCNQNKELVSYRAINRPDITDTEMETVMDTIVDSLFCFFVTLGAVPIIRCSRGTAAEM
*... *...*.**.*...*. ***.*...  ..  ... .*.****.**. * ..* *    .   .* ..* *..*..*.... ..*.*.**   .*....**** ..*..**.
LKSDKYSEFYINFTSSLPRNLLEDLAQQVSITGKSDKIKQVYDQYLDFIVTEPELFSL----EISNAYLTLNDPKTTEEEITGLCANIADGLFNTVLTINSIPIIRAAKGGPAEI

VAVKLDKKLRENLRDARNSLFTGDPLGTGQFSFQRPLLVLVDRNIDLATPLHHTWTYQALVHDVLDFHLNRVNLEESTGVENSPTGARPKRKNKKSYDLTPVDKFWQKHKGSPFP
.* **..***. . ....*  ....* ... *..* .*...*****.*. . *.*.** .* *....  * *..   .. **..... .*. ..*.**..* * ** ...  ***
IAEKLGTKLRDFVINTNSS--STSTLQGND-SLERGVLIILDRNIDFASMFSHSWIYQCMVFDIFKLSRNTVTIP-LESKENGTDNTTAKPLATKKYDIEPNDFFWMENSHLPFP

EVAESVQQELESYRAQEDEVKRLKSIMGLEGEDEGAISMLSDNTAKLTSAVSSLPELLEKKRLIDLHTNVATAVLEHIKARKLDVYFEYEEKIMSKTTLDKSLLDVISDPDAGTP
*.**.*...*..*.....*..* ... .... * ..    ...*... ..*..**** .**. ** * *. .*.*.......**..** *..  *..* .. .**...* ....
EAAENVEAALNTYKEEAAEITRKTGVTNISDLDPNS----NNDTVQIQEVVKKLPELTAKKNTIDTHMNIFAALLSQLESKSLDTFFEVEQDPGSTKTRSR-FLDILKDGKTNNL

EDKMRLFLIYYISAQQAPSEVDLEQYKKALTDAGCNLSPLQYIKQWKAFAKMASTP----------ASY--GNTTTKPM-GLLSRVMNTGSQFVMEGVKNLVLKQQNLPVTRIL
***.* *.. *...  . ..  ... .. ..........*.*. . ..* .... .          ...  .* * . . ** .  ...*   ...*.*.*. .....*.*...
EDKLRSFIVLYLTSTTGLPKDFVQNVENYFKENDYDINALKYVYKLREFMQLSNMSLQNKSLEDGSDSAFKPSNLTLSGIYGLTKGKLQGGVGSLISGIKKLLPEKKTIPITNVV

DNLME-----MKSNPETDDYRYFDPKMLRSNDSSVPRNKSPFQEAIVFVVGGGNYIEYQNLVDYIKGK--QGKHILYGCSEIFNATQFIKQLSQLGQK
*..*.      *.  .**.* *.***. *..... *. . ......*********.***** .. ...  . *...**...* ....*....*.**
DAIMDPLNSSQKNLETTDSYLYIDPKITRGSHTRKPK-RQSYNKSLVFVVGGGNYLEYQNLQEWAHSQLHNPKKVMYGSTAITTPAEFLNEISRLGASNSSNNDA     SLY-1
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C

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RA410 (rat)              253  LVLVDRNIDLATPLHHTWTYQALVHDV  279
Sly1p (yeast)            261  LIILDRNIDFASMFSHSWIYQCMVFDI  287
Sec1p (yeast)            247  LIITDRTLDPFAPILHDFSYQAMAYDL  273
Vps45p (yeast)           227  LLILDRNTDPITPLLQPWTYQSMINEY  253
Vps33p (yeast)           274  LIILERNTDPITPLLTQLTYAGILDDL  300
Munc18 (rat)             230  LLILDRGFDPSSPVLHELTFQAMSYDL  256
Rop (D. melanogaster)    241  LLILDRGFDCVSPLLHELTLQAMAYDL  267
Unc-18 (C. elegans)      310  LIIIDRGYDAITPLLHELTLQAMCYDL  336
β-COP (human)            300  LIVLDRGFDCVSPLLHELTLQAMAYDL  326

Consensus                     LLILDRNIDQATPLLHELTYQAMAYDL
                               ILV  GF  IS     W F  LVF V
                               VVI   T          L  I   I
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D

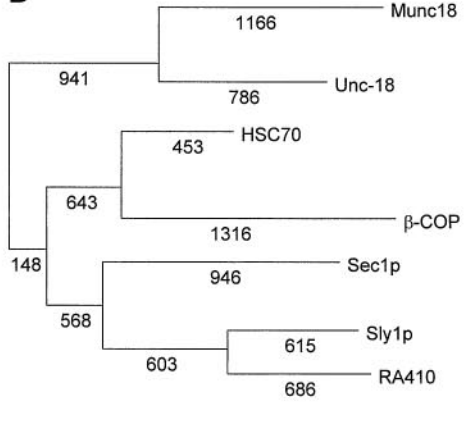

FIG. 1. **RA410: expression of mRNA in astrocytes subjected to hypoxia/reoxygenation (*A*), predicted amino acid sequence (*B*), homology to other vesicle transport polypeptides (*C*), and phylogenic tree analysis (*D*).** In *A*, RNA was extracted and pooled from three different astrocyte cultures. About 5 μg of RNA from normoxic (*lane N*), hypoxic (*lane H*), or reoxygenated (*lane H/R*) cultures was subjected to Northern blot hybridization using [^{32}P]dCTP-labeled *RA410* cDNA probe (*upper panel*) and β-actin probe (*lower panel*). In *B*, the amino acid

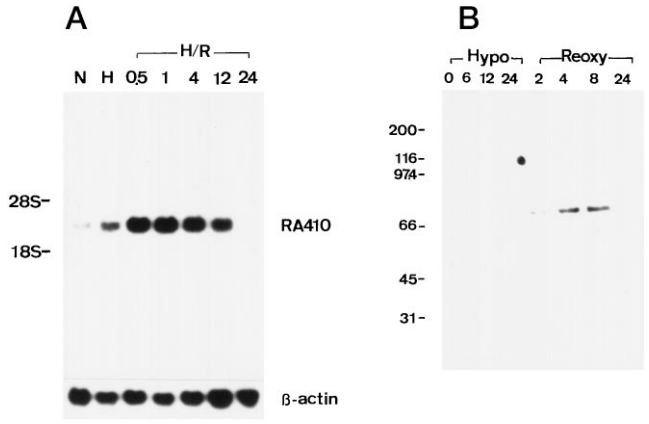

FIG. 2. **Expression of *RA410* mRNA (*A*) and RA410 antigen (*B*) in cultured astrocytes subjected to hypoxia/reoxygenation.** In *A*, total RNA was extracted from astrocytes at the end of hypoxia (0 h, the time at which reoxygenation was begun) and at the indicated time points after the start of reoxygenation (0.5–24 h). Then, ≈5 μg of total RNA was subjected to Northern analysis as described in the legend to Fig. 1*A*. In *B*, astrocytes exposed to hypoxia (*Hypo*; 0–24 h) followed by reoxygenation (*Reoxy*; 2–24 h) were harvested, and protein extracts (≈5 μg) were subjected to reducing SDS-polyacrylamide gel electrophoresis (10%) followed by immunoblotting with anti-RA410 polyclonal antibody (10 μg/ml). The migration of standard proteins is indicated on the left: carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase (116 kDa), and myosin (200 kDa). *N*, normoxia; *H*, hypoxia; *H/R*, hypoxia/reoxygenation.

fractions (fractions 1–4) were collected by puncturing the tube at the desired depth and gently withdrawing the fluid. The pellet at the bottom of the tube was resuspended in 3 ml of buffer A (precipitate fraction 5). Following measurement of protein concentration, each fraction (≈5 μg of protein) was subjected to Western blotting using anti-RA410 IgG. Enrichment of cellular structures/organelles in subcellular fractions was identified by the presence of marker enzymes: alkaline phophodiesterase I (plasma membrane), α-mannosidase II (Golgi apparatus), and dolichol-P-mannose synthetase (endoplasmic reticulum) (19).

Immunoelectron microscopic analysis to identify RA410 antigen within reoxygenated astrocytes was performed as described (20). In brief, cultured astrocytes exposed to hypoxia (24 h)/reoxygenation (3 h) were fixed in Zamboni buffer for 2 h, washed twice with 0.2 M phosphate-buffered saline, and incubated with either anti-RA410 IgG or preimmune rabbit IgG (10 μg/ml in each case). Sites of primary antibody binding were identified with biotinylated goat anti-rabbit IgG following reaction with avidin-biotin-peroxidase complex (Vector Labs, Inc.) and staining with diaminobenzidine and uranyl acetate. Tissues were then flat-embedded in Epon 812, and ultrathin sections were stained with lead citrate, followed by observation at 75 kV on an H-600 electron microscope (Hitachi, Tokyo, Japan).

Assay of NADPH Oxidase Activity—Cell-surface NADPH activity was measured as described (21) by incubating astrocytes (5×10^7 cells) for 3 h at 37 °C in 2 ml of phosphate-buffered saline, 5 mM $CaCl_2$, and 5 μg/ml cytochrome *c*. Where indicated, DPI (50 μM), an inhibitor of neutrophil-type NADPH oxidase, was added to the incubation mixture. Following the incubation period, cell suspensions were centrifuged ($1000 \times g$ for 5 min), and absorbance at 540 nm in the supernatant was measured using a Model UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). Superoxide production was determined based on the difference in absorbance at 540 nm in the absence and presence of superoxide dismutase (1 μg/ml; Sigma).

Expression of RA410 in Ischemic Brain—Expression of RA410 antigen was studied following transient unilateral (2 h) occlusion of the middle cerebral artery in male Harlan Sprague Dawley rats (250 g) (22). Blood gases, pH, and mean arterial blood pressure were monitored throughout the operation. Experiments were completed only if these

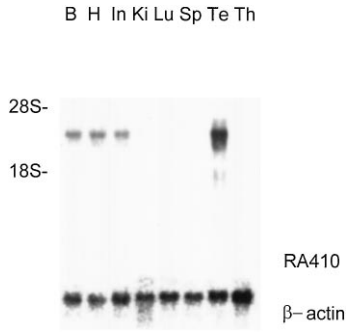

FIG. 3. **Distribution of *RA410* mRNA in normal tissue.** Total RNA was prepared from various tissues (brain (*B*), heart (*H*), small intestine (*In*), kidney (*Ki*), lung (*Lu*), spleen (*Sp*), testis (*Te*), and thymus (*Th*)), and ~5 μg of total RNA was subjected to Northern analysis as described in the legend to Fig. 1*A*.

physiological variables remained within normal limits. The normal limits for these parameters were set as follows: mean arterial blood pressure, 90–130 mm Hg; PCO_2, 30–50 mm Hg; PO_2, 100–130 mm Hg; and arterial blood pH, 7.25–7.45. Six hours after termination of arterial occlusion, rats were perfusion-fixed with 4% paraformaldehyde. Serial coronal sections (20-μm thickness) were obtained using a vibratome and were appropriately prepared and stained with anti-RA410 IgG. The same sections was further stained with anti-glial fibrillary acidic protein antibody (23), followed by visualization of the primary antibody using fluorescein isothiocyanate-labeled secondary antibody. Where indicated, anti-RA410 IgG was preincubated with excess antigen (10 μM RA410-derived peptide) for 12 h at 4 °C prior to its incubation with tissue sections.

RESULTS

Viability of Astrocytes Exposed to Hypoxia/Reoxygenation—Oxygen tension in the medium fell to 8 torr within 3–5 h after cultures were transferred to the hypoxia chamber. Cell viability was maintained throughout hypoxia and following return of cultures to normoxia (reoxygenation). This conclusion is based on lack of lactose dehydrogenase release into culture supernatants, continued trypan blue exclusion, adherence to the culture substrate, and unchanged morphologic features (data not shown).

Isolation of the cDNA for RA410 by Differential Display—Using the primer 5′-TGG TAC GGT ATA-3′ for differential display, a portion of a cDNA, termed *RA410*, was amplified from the cDNAs prepared from pooled RNA of hypoxia/reoxygenated astrocytes. A full-length cDNA was then isolated. Northern blotting using ^{32}P-labeled *RA410* cDNA probe confirmed the presence of the transcripts in RNA pools obtained from reoxygenated astrocytes (Fig. 1*A*). The apparent migration of the band corresponded to 2.0 kilobase pairs, suggesting that the cDNA (also 2 kilobase pairs) was likely to be full-length. The deduced amino acid sequence, derived from nucleotide sequencing of the *RA410* cDNA, included 637 amino acids (Fig. 1*B*) and resulted in a predicted polypeptide molecular mass of ≈70 kDa. Comparison with data bases indicated the presence of a consensus sequence associated with vesicle transport-related factors (Fig. 1, *C* and *D*). Of known vesicle transport proteins, RA410 had greatest homology (38%) to Sly1p (24), a yeast vesicle transport-related factor (Fig. 1, *C* and *D*). In addition, similarities were observed to Sec1p (yeast) (25), Vps45p (yeast) (26), Vps33p (yeast) (27), Munc18 (rat) (28), Rop (*Drosophila melanogaster*) (29), Unc18 (*Caenorhabditis elegans*) (30), β-COP (human) (31), and HSC70 (human) (32).

sequence predicted from the cDNA of RA410 (*upper line*) is compared with that of Sly1p (*lower line*), a yeast vesicle transport peptide. Identical amino acid residues in each peptide are indicated by *asterisks*. In *C*, the predicted amino acid sequence of RA410 (residues 253–279) is compared with the consensus sequences of other vesicle transporters. The consensus sequences of these vesicle transport-related proteins are indicated in the *lower panel*. In *D*, phylogenic analysis of the multiple alignment is shown. The tree is rooted assuming a clock, which might not be justified. The *number* in the tree represents the exact length of each branch, which is roughly proportional to the *horizontal lines*.

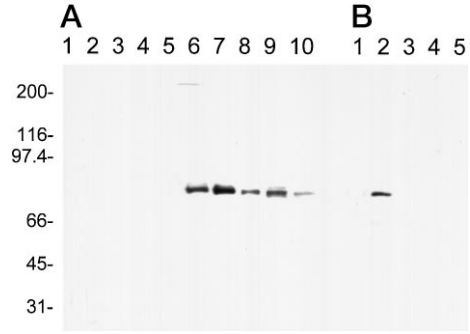

FIG. 4. **Localization of RA410 peptide in cultured rat astrocytes after reoxygenation.** *A*, subcellular fractionation of hypoxic astrocyte lysates. Astrocytes (≈10^8 cells) were exposed to hypoxia/reoxygenation, harvested by centrifugation, and fractionated as described under "Materials and Methods." Fractions (≈5 μg of protein) were subjected to immunoblotting using anti-RA410 antibody. The lanes show fractions containing crude nuclear pellet (*lane 1*), purified nuclei (*lane 2*), nuclear debris (*lane 3*), purified nuclear extract (*lane 4*), nuclear wash (*lane 5*), cytosolic membranes (*lane 6*) and protein (*lane 7*), membranes (*lane 8*), membrane wash (*lane 9*), or washed membrane pellet (*lane 10*). *B*, sucrose density gradient centrifugation of hypoxic/reoxygenated astrocyte lysates. Cavitates of reoxygenated astrocytes (≈5 × 10^8 cells) were separated by sucrose gradient centrifugation as described under "Materials and Methods." Each fraction (≈5 μg of protein) was immunoblotted using anti-RA410 IgG. The lanes represent fractions containing cytosol (*lane 1*), plasma membrane (*lane 2*), Golgi (*lane 3*), and endoplasmic reticulum (*lanes 4* and *5*). The migration of simultaneously run molecular mass markers (in kilodaltons) is shown on the left.

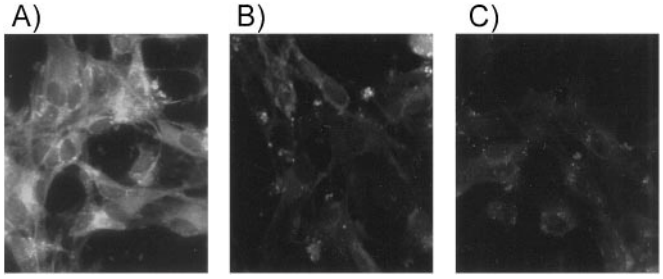

FIG. 5. **Immunocytochemical analysis of hypoxic astrocytes.** Astrocytes plated on coverslips were exposed either to hypoxia (24 h)/reoxygenation (3 h) (*A* and *C*) or to hypoxia alone (24 h) (*B*), permeabilized, and incubated either with anti-RA410 IgG (*A* and *B*) or with preimmune IgG (*C*). Sites of primary antibody binding were detected using fluorescein isothiocyanate-conjugated anti-rabbit IgG. Magnification × 400.

Induction of RA410 in Cultured Astrocytes by Hypoxia/Reoxygenation—Northern blot analysis of RNA from cultured astrocytes using ^{32}P-labeled *RA410* cDNA probe showed a striking increase in transcripts within 0.5 h of reoxygenation; levels of *RA410* mRNA reached a plateau within 0.5–1 h and subsequently declined to the base line by 24 h (Fig. 2*A*). Polyclonal antibody raised against a synthetic peptide derived from the predicted RA410 protein sequence recognized a band in lysates of reoxygenated astrocytes of ≈70 kDa (Fig. 2*B*). This is close to the expected molecular mass (≈70 kDa) based on the sequence deduced from full-length *RA410* cDNA. The specificity of this immunoreactive band for RA410 was confirmed by its disappearance when antibody was preincubated with excess RA410-derived synthetic peptide (data not shown). Expression of RA410 antigen occurred within 2 h of reoxygenation, achieved a maximum by 4–8 h, and returned to base line within 24 h, consistent with the pattern of *RA410* message expression (Fig. 2*B*).

Distribution of RA410 Message in Normal Tissue—Northern blot analysis using RNA obtained from normal rat tissue revealed the distribution of *RA410* transcripts to be highest in testis, with lower levels in heart, intestines, and brain (Fig. 3).

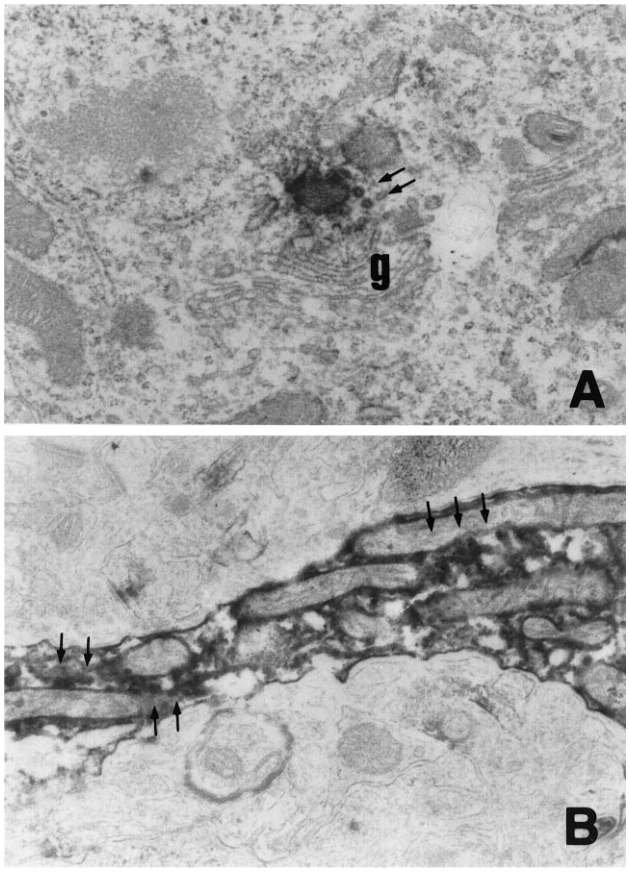

FIG. 6. **Immunoelectron microscopic analysis of RA410.** Cultured astrocytes were exposed to hypoxia (24 h)/reoxygenation (3 h), followed by staining with anti-RA410 antibody. Immunodeposits were observed corresponding to sites of RA410 antigen in large vesicles associated with the Golgi apparatus (*g*) (*A*) and in the processes of astrocytes (indicated by *arrows*) (*B*). Magnification x 10,000.

Localization of RA410 in Reoxygenated Astrocytes—To localize RA410 antigen in reoxygenated astrocytes, fractionated cell lysates prepared from reoxygenated cultures were subjected to immunoblotting using anti-RA410 IgG. RA410 antigen was detected mainly in the membrane fraction (Fig. 4*A*). Sucrose gradient separation of cytosolic fractions also showed RA410 antigen mainly in the plasma membrane and partially in the Golgi subfraction (Fig. 4*B*). These data were consistent with the results of immunocytochemical analysis of cultured astrocytes using anti-RA410 IgG (Fig. 5*A*); hypoxic/reoxygenated astrocytes demonstrated RA410 antigen, whereas no significant staining was detected in astrocytes subjected to hypoxia alone (Fig. 5*B*). Controls in which hypoxic/reoxygenated astrocytes were stained with preimmune IgG displayed no staining (Fig. 5*C*).

Immunoelectron microscopic analysis using anti-RA410 IgG in astrocytes exposed to hypoxia/reoxygenation revealed labeling of the antigen in large vesicles associated with the Golgi apparatus (Fig. 6*A*) and in the processes of astrocytes (Fig. 6*B*), but not in the Golgi apparatus itself. This is consistent with the results of Western blotting of subcellular fractions (Fig. 4, *A* and *B*) and suggests that RA410 may participate in post-Golgi vesicle transport.

Generation of Superoxide in Reoxygenated Astrocytes and Expression of RA410—To assess mechanisms underlying expression of RA410 in astrocytes exposed to hypoxia/reoxygenation, elaboration of superoxide was measured using the cyto-

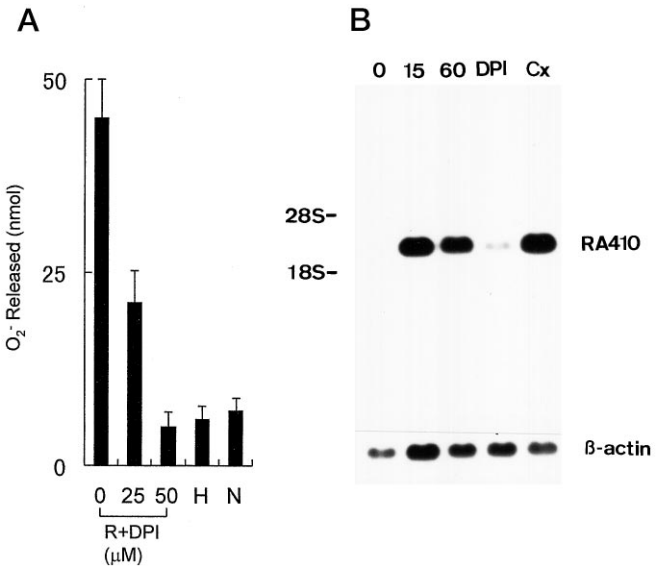

FIG. 7. **Generation of superoxide in cultured astrocytes after reoxygenation (*A*) and effect of DPI on induction of *RA410* message (*B*).** *A*, superoxide generation by astrocytes (5×10^7 cells) during the first 3 h of reoxygenation (*R*) following a 24-h period of hypoxia was measured using the cytochrome *c* reduction assay by determining the difference in absorbance at 540 nm in the absence and presence of superoxide dismutase (1 μg/ml; this difference is shown by the *first bar*). Suppression of superoxide generation on addition of DPI (25–50 μM) to astrocytes subjected to hypoxia/reoxygenation as described above is shown. Superoxide generation by astrocytes exposed to hypoxia (*H*) (24 h) or normoxia (*N*) alone is also shown. For these experiments, $n = 6$, and the mean ± S.D. is shown. *B*, cycloheximide (*Cx*; 10 μg/ml) or DPI (50 μM) was added to the culture 15 min before reoxygenation, and at the indicated times, total RNA was harvested and subjected to Northern blot analysis. Astrocyte RNA was obtained at the end of hypoxia (*lane 0*) or 15 and 60 min after reoxygenation.

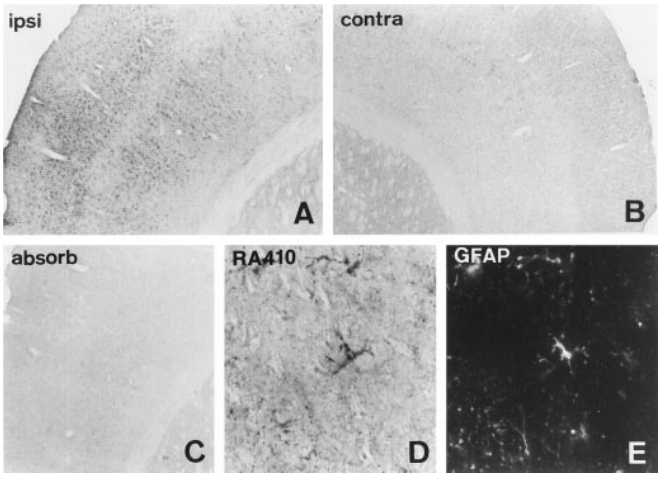

FIG. 8. **Expression of RA410 antigen in ischemic brain.** Immunohistochemical analysis was performed on rat brain 1 h after unilateral occlusion of the middle cerebral artery for 2 h. Ipsilateral cerebral cortex (*ipsi*) was reacted with anti-RA410 antibody (*A*, magnification × 4; and *D*, magnification × 40). Adjacent sections were also analyzed with anti-RA410 IgG in the presence of RA410 synthetic peptide (*C*, magnification × 40). The same section used in *D* was further stained with anti-glial fibrillary acidic protein (*GFAP*) antibody, followed by detection with fluorescein isothiocyanate-labeled secondary antibody (*E*, magnification × 40). Contralateral nonischemic cortex (*contra*) was also stained with anti-RA410 antibody (*B*, magnification × 4).

chrome *c* assay; superoxide generation was measured as the component of cytochrome *c* reduction suppressed on addition of superoxide dismutase. Superoxide was formed almost immediately after reoxygenation and was suppressed in a dose-dependent manner by DPI (Fig. 7*A*). In contrast, astrocytes exposed to either hypoxia or normoxia alone demonstrated no superoxide generation (Fig. 7*A*). Expression of *RA410* mRNA in reoxygenated astrocytes was blocked by pretreatment of astrocytes with DPI (50 μM) in parallel with suppression of reactive oxygen intermediate formation (Fig. 7*B*). The addition of antagonists of nitric-oxide syntheses, such as methyl-nitro N-nitro L-arginine, or placing cultures in arginine-free medium had no affect on cytochrome *c* reduction or expression of *RA410* mRNA, indicating that DPI exerted its effect most likely by inhibiting an NADPH oxidase-like activity, rather than by interfering with formation of nitric oxide (data not shown). The addition of cycloheximide to astrocytes, sufficient to diminish incorporation of [^{3}H]leucine into a fraction precipitable in trichloroacetic acid by >90%, did not diminish the level of *RA410* message, suggesting that RA410 expression might reflect an immediate early-type mechanism in reoxygenated astrocytes triggered by superoxide produced as a consequence of activation of an NADPH-like activity.

Expression of RA410 Immunoreactivity in Ischemic Rat Brain—To evaluate expression of RA410 *in vivo*, immunohistochemical analysis was performed on mouse brain following transient *unilateral* occlusion of the middle cerebral artery. At lower power, staining of ipsilateral ischemic cortex was observed (Fig. 8*A*), whereas contralateral nonischemic cortex (control) was negative (Fig. 8*B*). Higher power views of adjacent sections of ischemic cortex display colocalization of RA410 antigen (Fig. 8*D*), with cells also staining for glial fibrillary acidic protein (Fig. 8*E*). In contrast, samples of ischemic cortex stained with anti-RA410 IgG in the presence of excess peptide used as immunogen showed no staining (Fig. 8*C*), indicating that staining of RA410 (Fig. 8*D*) was specific.

DISCUSSION

Astrocytes have a central role in the maintenance of central nervous system homeostasis through modulation of the ionic milieu, removal of excitatory neurotransmitters, and maintenance of the blood-brain barrier (33–35). In addition to these housekeeping activities, neurotrophic properties of astrocytes support neuronal differentiation and survival through production of growth factors, such as nerve growth factor, basic fibroblast growth factor, and ciliary neurotrophic factor (36–38). Another facet of astrocyte biology concerns their ability to adapt to environmental stress, such as that represented by ischemia/reperfusion, modeled in our studies by hypoxia/reoxygenation. In contrast to the vulnerability of neurons to changes in ambient conditions, external challenges allow astrocytes to undergo activation, resulting in a change in cellular phenotype, as in tissue remodeling in the central nervous system (39). Consistent with this view, astrocytes have produced cytokines, including interleukin (IL)-1, IL-6, interferons, and transforming growth factor-β, in response to changes in the cellular environment, allowing them to orchestrate a cytokine cascade in brain (40).

We have previously demonstrated that expression of IL-6, a potent neurotrophic cytokine in the central nervous system, occurs in cultured astrocytes exposed to hypoxia/reoxygenation (7). Although induction of IL-6 mRNA is initiated late during hypoxia, maximal transcription, translation, and elaboration of this cytokine does not begin until reoxygenation, leading us to hypothesize that reoxygenation sets in motion a series of events culminating in astrocyte expression of a neurotrophic phenotype. We have delineated several components of this potential pathway. RA301, a *Drosophila* Tra-2-like RNA-splicing factor, also first identified using differential display applied to hypoxic/reoxygenated astrocytes, is likely to contribute to maturation of nascent RNAs, as its expression was necessary for maximal IL-6 production by the reoxygenated astrocyte (9).

The 78-kDa glucose-regulated protein (GRP78/BiP), a major stress protein in astrocytes expressed in response to oxygen deprivation, functions as a molecular chaperone by retaining immature IL-6 in the endoplasmic reticulum until the cellular metabolic environment is restored (8). RA410, likely to be a vesicle transport-related polypeptide, is also a potential contributor to protein trafficking in response to cellular stress. In this context, RA410 has the greatest homology to Sly1p, a yeast polypeptide that has a major role in vesicle transport and protein secretion (41). Our pilot studies are consistent with this concept and demonstrate that suppression of RA410 expression causes intracellular (Golgi) retention of IL-6, suggesting that this polypeptide is required for optimal trafficking of IL-6 through hypoxic/reoxygenated astrocytes.

Expression of *RA410* mRNA is enhanced by inhibition of protein synthesis using cycloheximide, suggesting an immediate early-type mechanism. In this case, the stimulus for expression of this vesicle transport-related factor appears to reside in reoxygenation-mediated activation of an NADPH-like activity with subsequent generation of superoxide. Similarly, reoxygenation-associated induction of RA301 is also regulated by reactive oxygen intermediates; the addition of the NADPH oxidase inhibitor diphenyl iodonium resulted in a marked decline in *RA301* message/RA301 antigen in cultured astrocytes (9). Our pilot studies demonstrate that the level of IL-6 transcripts is not suppressed by NADPH oxidase inhibitors, suggesting that diphenyl iodonium exerts its effect(s) at steps distal to transcription/mRNA stability. Such regulation appears to involve, at least in part, expression of RA301 and RA410. These data provide insight into the complex events activated in astrocytes by hypoxia/reoxygenation. In addition, a role for reactive oxygen intermediates as signal transducers of the stress signal is emphasized, analogous to their role in NF-κB activation (42).

In summary, we have cloned and characterized a novel vesicle transport-related factor, RA410, from hypoxic/reoxygenated astrocytes. Such expression of RA410 is regulated by the generation of reactive oxygen intermediates by an NADPH oxidase-like activity. Expression of RA410 in cultured astrocytes subject to environmental stress suggests that the markedly enhanced protein synthesis that occurs at this time represents a redirection of the biosynthetic apparatus to facilitate expression and release of new gene products.

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Cloning of a Putative Vesicle Transport-related Protein, RA410, from Cultured Rat Astrocytes and Its Expression in Ischemic Rat Brain

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