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

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 cytoskeletal proteins. 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.

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
data demonstrate that RA410 is expressed intensely in astrocytes 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-cm² 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 μg/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 × 10⁶ cells/cm² 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 lactate dehydrogenase release.

Preparation of Total RNA and cDNA—Total RNA was extracted and purified from astrocytes (5 × 10⁶ 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 (12). For hypoxia samples, RNA was extracted 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 (5 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (300 units; Life Technologies, Inc.), oligo(dT) primer (2.5 μM T₁₁G), 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 (5 μg in each reaction) were used as PCR templates in 30 μl of reaction mixture containing arbitrary primer (1 μM), dNTP (200 μM each), and Toq 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 hypoxia and hypoxia/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 hypoxia/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 T7 primer Cycle Sequencing Core kit (Applediag, Systems 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 encodes the entire open reading frame of RA410. The latter was sequenced in both directions. To show homology to other vertebrate transport-related proteins, phylogenetic 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 purified from an isolate obtained by differential display (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 Immobion 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 32P-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. Inclusion 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 assess 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 CQDEVKRLKSIMGLEGEDE (amino acids 346–365; see Fig. 1B), 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 Pepptide Systems). Astrocytes (10⁶ 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 (10⁵) were exposed to hypoxia (24 h) or to hypoxia/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., Naper ville, 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 × 10⁵) 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-p-tosyl-L-lysine chloromethyl ketone), and the cells were centrifuged at 400 p.s.i. N₂ pressure for 30 min by nitrogen cavitation bomb (Kontes Glass Co., Vineland, NJ). Following homogenization, the cell lysate was desalted 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 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
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 phylogenetic 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 [32P]dCTP-labeled RA410 cDNA probe (upper panel) and β-actin probe (lower panel). In B, the amino acid
The consensus sequences of other vesicle transporters. The consensus sequences of these vesicle transport-related proteins are indicated in the tree. The phylogenic analysis of the multiple alignment is shown. The tree is rooted assuming a clock, which might not be justified.

**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 lactate 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 32P-labeled RA410 cDNA probe confirmed the presence of the transcripts in RNA pools obtained from reoxygenated astrocytes (Fig. 1A). 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. 1B) 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 peptide. Identical physiological variables remained within normal limits. The normal limits for these parameters were set as follows: mean arterial blood pressure, 90–130 mm Hg; PCO2, 30–50 mm Hg; PO2, 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 μg RA410-derived peptide) for 12 h at 4 °C prior to its incubation with tissue sections.

**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, phylogenetic 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.**
Expression of RA410 antigen occurred within 2 h of reoxygenation, RA410-derived synthetic peptide (data not shown). Expression disappearance when antibody was preincubated with excess antibody. The results of Western blotting of subcellular fractions (Fig. 4, A) revealed the distribution of RA410 transcripts to be highest in testis, with lower levels in heart, intestines, and brain (Fig. 3).

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

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. 6A) and in the processes of astrocytes (Fig. 6B), 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-
component of cytochrome c dependent manner by DPI (Fig. 7). Superoxide dismutase. Superoxide was formed almost immediately by astrocytes with DPI (50 μM) in reoxygenated astrocytes was blocked by pretreatment of astrocytes subjected to hypoxia/reoxygenation as described above is shown. Superoxide generation by astrocytes exposed to hypoxia (1 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.

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. 7A). In contrast, astrocytes exposed to either hypoxia or normoxia alone demonstrated no superoxide generation (Fig. 7A). 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. 7B). The addition of antagonists of nitric-oxide synthases, 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 [3H]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. 8A), whereas contralateral nonischemic cortex (control) was negative (Fig. 8B). Higher power views of adjacent sections of ischemic cortex display colocalization of RA410 antigen (Fig. 8D), with cells also staining for glial fibrillary acidic protein (Fig. 8E). In contrast, samples of ischemic cortex stained with anti-RA410 IgG in the presence of excess peptide used as immunogen showed no staining (Fig. 8C), indicating that staining of RA410 (Fig. 8D) 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-spooling 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.

Acknowledgments—We thank Dr. T. Fukui for synthesizing peptides and Drs. Y. Furutani and K. Hosoki (Dainippon Pharmaceutical Co. Ltd.) for suggestions and discussions.

REFERENCES
1. Siesjo, B. K. (1988) Crit. Care Med. 16, 954–963
2. Pelham, R. B. H. (1986) Cell 46, 959–961
3. Swanson, A. R., and Choi, D. W. (1993) J. Cereb. Blood Flow Metab. 13, 162–169
4. Hori, O., Matsumoto, M., Maeda, M., Kuwahara, K., Ueda, H., Ohbutsu, T., Kinoshita, T., Ogawa, S., Stern, D., and Kamada, T. (1996) J. Neurochem. 66, 973–979
5. Kuwahara, K., Matsumoto, M., Ikeda, J., Hori, O., Ogawa, S., Maeda, Y., Kitagawa, K., Inuta, N., Kinoshita, T., Stern, D. M., Yanagi, H., and Kamada T. (1996) J. Biol. Chem. 271, 5025–5032
6. McCord, J. M. (1985) N. Engl. J. Med. 312, 159–161
7. Maeda, Y., Matsumoto, M., Ohbutsu, T., Kuwahara, K., Ogawa, S., Hori, O., Shui, D. Y., Kinoshita, T., Kamada, T., and Stern, D. (1994) J. Exp. Med. 180, 2297–2308
8. Hori, O., Matsumoto, M., Maeda, Y., Ueda, H., Kinoshita, T., Stern, D., Ogawa S., and Kamada, T. (1994) J. Neurochem. 62, 1489–1495
9. Matsu, N., Ogawa, S., Imai, Y., Takagi, T., Tohyama, M., Stern, D., and Wanaka, A. (1995) J. Biol. Chem. 270, 28216–28222
10. Ogawa, S., Gerlach, H., Esposito, C., Macaulay, A. P., Brett, J., and Stern, D. (1996) J. Clin. Invest. 85, 1090–1098
11. Chomczynski, P., and Sacchi, N. (1986) Anal. Biochem. 162, 156–159
12. Liang, P., and Pardee, A. B. (1992) Science 257, 967–971
13. Hein J. J. (1990) Methods Enzymol. 183, 626–645
14. Feinberg, A., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
15. Takeichi, M. (1991) Science 251, 1451–1455
16. Towbin, H., Stracheln, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
17. Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994) Cell 77, 53–62
18. Horii, S., Breit, J., Slattery, T., Cao, R., Zhang, J., Chen, J. X., Nagashima, M., Lundh, E. R., Vijay, S., Nitecki, D., Morser, J., Stern, D., and Schmidt, A. M. (1985) J. Biol. Chem. 270, 25752–25761
19. Vidugiriene, J., and Mensa, A. K. (1993) J. Cell Biol. 121, 987–996
20. Kubota, Y., Inagaki, S., Shimada, S., Kito, S., Edkstein, F., and Tohyama, M. (1987) Brain Res. 413, 179–184
21. Curnutte, J. T., Kuver, R., and Scott, P. J. (1987) J. Biol. Chem. 262, 5563–5569
22. Nagasawa, H., and Kogure, K. (1989) Stroke 20, 1037–1043
23. Kitagawa, K., Matsumoto, M., Tagaya, M., Hata, R., Ueda, H., Niinobe, M., Handa, N., Fukunaga, K., Kimura, K., Mikolsiba, K., and Kamada, T. (1991) Brain Res. 561, 203–211
24. Dascher, C., Ossing, R., Gallwitz, D., and Schmitt, H. D. (1991) Mol. Cell. Biol. 11, 872–885
25. Alate, M. K., Rushon, L., Hosono, K., and Keraenen, S. (1991) Yeast 7, 643–650
26. Cowles, C. R., Emr, S. D., and Horazdovsky, B. F. (1994) J. Cell Sci. 107, 3449–3459
27. Banta, L. M., Vida, T. A., Herman, P. K., and Emr, S. D. (1990) Mol. Cell. Biol. 10, 4638–4649
28. Hata, Y., Slaughter, C. A., and Sudhof, T. C. (1993) Nature 366, 347–351
29. Salzberg, A., Cohen, N., Halachmi, N., Kinchuz, Z., and Lev, Z. (1993) Development (Camb.) 117, 1309–1319
30. Hosono, R., Hekimi, S., Kamiya, Y., Sassu, T., Murakami, S., Nishiwaki, K., Mira, J., Taketo, A., and Kodaira, K.I. (1992) J. Neurochem. 58, 1517–1525
31. Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T. E. (1991) Cell 64, 649–665
32. Sorger, P. K., and Pelham, H. R. (1987) EMBO J. 6, 991–998
33. Henn, F. A., Halajian, H., and Hamburger, A. (1972) Brain Res. 43, 435–442
34. Schousboe, A. L., Hertz, L., and Svenneby, G. (1977) Neurochem. Res. 2, 217–223
35. Arthur, F. E., Slivers, R., and Bowman, P. (1987) Dev. Brain Res. 36, 155–161
36. Frei, K., Mailpiereu, U., Leist, T., Zinkernagel, R., and Schwab, M. (1989) Eur. J. Immunol. 19, 689–695
37. Pechan, P. A., Chowdhury, K., and Seifert, W. (1992) Neuroreport 3, 469–472
38. Vacca, K., and Wendt, E. (1992) Exp. Neurol. 118, 62–68
39. Janecek, K. (1991) Brain Res. 564, 86–90
40. Benveniste, E. T. (1992) Am. J. Physiol. 263, C1–C16
41. Sogaard, M., Tani, K., Ye, R., Geromans, S., Tempst, P., Kirchhausen, T., Rothman, J., and Sollner, T. (1994) Cell 78, 937–948
42. Shreck, R., Reiber, P., and Baerlea, P. A. (1991) EMBO J. 10, 2247–2254