Purification and Characterization of a Novel Stress Protein, the 150-kDa Oxygen-regulated Protein (ORP150), from Cultured Rat Astrocytes and Its Expression in Ischemic Mouse Brain*

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As the most abundant cell type in the central nervous system, astrocytes are positioned to nurture and sustain neurons, especially in response to cellular stresses, which occur in ischemic cerebrovascular disease. In a previous study (Hori, O., Matsumoto, M., Kuwabaraf, K., Maeda, M., Ueda, H., Ohtsuki, T., Kinoshita, T., Ogawa, S., Kamada, T., and Stern, D. (1996) J. Neurochem., in press), we identified five polypeptide bands on SDS-polyacrylamide gel electrophoresis, corresponding to molecular masses of about 28, 33, 78, 94, and 150 kDa, whose expression was induced/enhanced in astrocytes exposed to hypoxia or hypoxia followed by replacement into the ambient atmosphere (reoxygenation). In the current study, the ~150-kDa polypeptide has been characterized. Chromatography of lysates from cultured rat astrocytes on fast protein liquid chromatography Mono Q followed by preparative SDS-polyacrylamide gel electrophoresis led to isolation of a ~150-kDa band only observed in hypoxic cells and which had a unique N-terminal sequence of 15 amino acids. Antiserum raised to either the purified ~150-kDa band in polyacrylamide gels or to a synthetic peptide comprising the N-terminal sequence detected the same polypeptide in extracts of cultured rat astrocytes exposed to hypoxia; expression was not observed in normoxic but was induced by hypoxia within 24 h, augmented further during early reoxygenation, and thereafter decreased to the base line by 24 h in normoxia. ORP150 expression in hypoxic astrocytes resulted from de novo protein synthesis, as shown by inhibition in the presence of cycloheximide. In contrast to hypoxia-mediated induction of the ~150-kDa polypeptide, neither heat shock nor a range of other stimuli, including hydrogen peroxide, cobalt chloride, 2-deoxyglucose, or tunicamycin, led to its expression, suggesting selectivity for production of ORP150 in response to oxygen deprivation, i.e. it was an oxygen-regulated protein (ORP150). Northern and nuclear run-off analysis confirmed the apparent selectivity for ORP150 mRNA induction in hypoxia. Subcellular localization studies showed ORP150 to be present intracellularly within endoplasmic reticulum and only in hypoxic astrocytes, not cultured microglia, endothelial cells, or neurons subject to hypoxia. Consistent with these in vitro results, induction of cerebral ischemia in mice resulted in expression of ORP150 (the latter was not observed in normoxic brain). These data suggest that astroglia respond to oxygen deprivation by redirection of protein synthesis with the appearance of a novel stress protein, ORP150. This polypeptide, selectively expressed by astrocytes, may contribute to their adaptive response to ischemic stress, thereby ultimately contributing to enhanced survival of neurons.

Astrocytes are strategically located to exert neurotrophic functions, especially in response to environmentally stressful situations that threaten neuronal survival but are well tolerated by astrocytes (2, 3). Several examples of this beneficial impact of astrocytes on neuronal homeostasis include their ability to enhance the viability of neuronal cells subjected to glucose deprivation (4) and hypoxia (5). Mechanisms underlying these effects are likely to be complex, and they include such factors as regulation of extracellular potassium concentration (6) and glutamate uptake (7). Astrocytes can also express neurotrophic factors (8). In this context, we have reported that cultured rat astrocytes exposed to hypoxia followed by reoxygenation elaborate a neurotrophic cytokine, interleukin 6 (IL-6), suggesting that this is an inducible factor produced by astrocytes that could enhance neuronal survival (9).

Deprivation of environmental oxygen alters cellular biosynthetic and other properties, resulting in a rapid shift to anaerobic glycolysis (1, 10), depression of cellular proliferation (11), and the production of cytokines and growth factors (12–15). Another aspect of the cellular response to hypoxia is characterized by the expression of a set of stress proteins, termed oxygen-regulated proteins (ORP) (16), which are believed to have important roles in protecting cellular biosynthetic activities under stressful circumstances. In cultured astrocytes subject to hypoxia, expression of a group of stress proteins, with

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molecular mass values corresponding to ~28, 33, 78, 94, and 150 kDa, was observed. The 28-, 78-, and 94-kDa stress proteins had properties of glucose-regulated proteins (GRPs), based on parallel induction in normoxic astrocytes exposed to 2-deoxyglucose or calcium ionophore. Furthermore, the 78-kDa polypeptide proved to be identical to GRP78/Bip by the N-terminal amino acid sequence analysis (1).

In our previous study (1), we observed an ~150-kDa polypeptide expressed by hypoxic astrocytes that has been characterized in detail in the current experiments. The ~150-kDa polypeptide is selectively expressed by cultured rat astrocytes but not endothelial cells, microglia, or neurons subjected to oxygen deprivation. Identification of the first 15 N-terminal amino acid residues indicates that this polypeptide is unique. Based on its induction in hypoxic astrocytes but not in astrocyte cultures exposed to heat shock, hydrogen peroxide, 2-deoxyglucose, or other agents, it has been termed an oxygen-regulated protein-150 or ORP150. ORP150 is present intracellularly, localized to the endoplasmic reticulum, and induced in gerbil brain following experimentally induced ischemia. These data suggest that ORP150 may be a selective marker of astrocyte adaptation to oxygen deprivation, potentially enhancing the synthesis and/or export of proteins in the endoplasmic reticulum important for astrocyte and, possibly, neuronal survival in ischemia.

MATERIALS AND METHODS

Cell Culture and Achievement of Hypoxic Condition—Rat primary astrocytes and microglia were obtained from neonatal rats by a minor modification of previously described methods (9). Briefly, cerebral hemispheres were harvested from neonatal Sprague-Dawley rats within 24 h of birth, meninges were carefully removed, and brain tissue was digested at 37°C in minimal essential medium with 0.2%Trypsin (Life Technologies, Inc.) containing 0.02%EDTA (Boehringer Mannheim, Germany). Cells were harvested by centrifugation, and the cell pellet was resuspended and grown in minimal essential medium supplemented with fetal calf serum (10%, CellGrow, Boston, MA). After 10 days, cytosine arabinofuranoside (10 μg/ml; Wako Chemicals, Osaka, Japan) was added (48 h) to prevent fibroblast overgrowth, and cultures were shifted to 1%CO2 in humidified 95%air/5%CO2 at 37°C.

In certain experiments, cycloheximide (3 μM) and actinomycinD (5 mM) were added at the start of hypoxia, and the incubation was continued for 48 h (detection of glial fibrillary acidic protein) criteria. Cultures used for experiments were >98% astrocytes. Brain microvesSEL endothelial cells and neurons were separated from digested neonatal rat brain homogenates as described (17, 18). Cells within a density of about 5 × 10^5 cells/cm^2 in the above medium except neurons, which were cultured in astrocyte-conditioned medium supplemented with fetal calf serum (10%) and glucose (20 mM). When cultures achieved confluence, they were exposed to hypoxia using an incubator attached to a hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI), which maintained a humidified atmosphere with low oxygen tension, as described previously (1, 19). Where indicated, after exposure to hypoxia, cultures were returned to the ambient atmosphere (reoxygenation, R), at which time the conditioned medium was rapidly exchanged with fresh medium. Oxygen tension in the medium was measured using a blood gas analyzer (ABL-2, Radiometer, Sweden). Cell viability was assessed by several methods including morphological criteria, trypan blue exclusion, lactate dehydrogenase release, and evaluation of general protein synthesis based on incorporation of [3H]leucine into thricloroacetic acid-precipitable material (20).

Purification and Sequencing of the 150-kDa Polypeptide Induced by H2R—Astrocytes (about 5 × 10^6 cells) exposed to hypoxia for 48 h were harvested, cells were washed three times with phosphate-buffered saline (PBS), and proteins were extracted. The indicated amount of either ORP150 N-terminal or bovine serum albumin was blotted onto nitrocellulose paper and reacted with anti-ORP150 IgG (5 μg/ml) formed using an automated peptide sequencing system (Applied Biosystems Division, Perkin-Elmer Co., Ltd., Japan), and protein sequence data were compared with those available in the data banks using the Stretcher peptide sequence data base.

Preparation and Characterization of Antibodies to ORP150—The ~150-kDa gel band visualized by Coomassie Blue staining of fractions from preparative SDS-PAGE was destined and used to immunize New Zealand White rabbits by standard procedures (21). A peptide was also synthesized based on the N-terminal amino acid sequence of ORP150, aggregated by the multiple antigen peptide method (Sawadig Technology Inc., Tokyo) (22), and used to immunize rabbits by the same procedure. Animals received a second immunization at 4 weeks; 1 month later, immune serum was harvested, and IgG was prepared by affinity chromatography using immobilized protein A (Econopack, Bio-Rad). The titer of antisera raised against the N-terminal ORP150 synthetic polypeptide was assessed by the enzyme-linked immunosorbent assay. Protein concentration was measured by the Bio-Rad protein assay after overnight dialysis versus PBS (about 1 mg/ml in each case). To assess the specificity of anti-ORP150 IgG, Western blotting was performed by the method of Towbin et al. (23). In brief, either normoxic or hypoxic astrocytes were exposed to Nonidet P-40 (1%), and about 10 μg of extracted protein (in each case) was subjected to SDS-PAGE (7%), transferred to PVDF paper, and reacted with anti-ORP150 IgG (1:200 dilution, 5 μg/ml) raised against the purified native ORP150. Fractions (2 μl) from FPLC Mono Q of hypoxic astrocyte extracts were similarly subjected to Western blotting using the anti-ORP150 IgG.

To further test the specificity of anti-ORP150 IgG, studies were performed with synthetic peptide comprising the first 15 amino acid residues of ORP150. The indicated amount of either ORP150 N-terminal or bovine serum albumin was blotted onto nitrocellulose paper and reacted with anti-ORP150 IgG (5 μg/ml) raised to purified ORP150 as described above. In brief, protein extract prepared from about 5 × 10^8 astrocytes exposed to hypoxia for 48 h was incubated in Tris-buffered saline (about 12 ml) containing Nonidet P-40 (1%), EDTA (5 mM), and PMSF (1 mM) and was incubated for 12 h at 4°C with either anti-ORP150 IgG or preimmune IgG (1:50 dilution, 20 μg/ml in each case). Then, a suspension of Staphylococcus aureus protein A (0.4 ml/tube, 10% suspension of IgGSorb, The Enzyme Center, Malden, MA) was added to each tube and incubated for 1 h at 4°C. After centrifugation (4000 rpm for 10 min), the supernatant was collected, concentrated 50-fold by ultrafiltration, and an aliquot (20 μl) containing about 10 μg of protein was subjected to Western blotting, and blots were blocked with anti-ORP150 IgG raised to the N-terminal ORP150 synthetic peptide.

Induction of ORP150 in Cultured Astrocytes—Cultured astrocytes (about 5 × 10^5 cells) were exposed to hypoxia followed by reoxygenation, as described. In certain experiments, cycloheximide (3 μg/ml) was added at the start of hypoxia, and the incubation was continued for 48 h. At the indicated time points, cells were washed with three times with ice-cold PBS and lysed in the presence of PBS (200 μl) containing Nonidet P-40 (1%), EDTA (5 mM), and PMSF (1 mM). After centrifugation (5000 × g for 5 min at 4°C), the supernatant (about 1 μg of protein) was applied to SDS-PAGE (7.5%), transferred to PVDF paper, and stained with anti-ORP150 IgG raised to purified ORP150. To examine whether the exposure to hypoxia could induce ORP150 expression, cultured astrocytes maintained in normoxia were either subjected to heat shock (43°C for 3 h) or hydrogen peroxide (5 μM for 10 min) was added and subsequently incubated for 6 h prior to harvest. In other experiments, astrocytes were incubated under normoxic conditions and exposed to either cobalt chloride (20 μM) or tunicamycin (1 μM), and tunicamycin (1 μM) or tunicamycin (1 μM), and anti-HP372 monoclonal antibody (Amersham), or anti-heme oxygenase type 1 (HO-1) antiserum (Stressgen, USA) at 4°C for 3 h. Staining with anti-HP372 monoclonal antibody (Amersham), or anti-heme oxygenase type 1 (HO-1) antiserum (Stressgen, USA) at 4°C for 3 h. Staining with anti-HP372 monoclonal antibody (Amersham), or anti-heme oxygenase type 1 (HO-1) antiserum (Stressgen, USA) at 4°C for 3 h. Staining with anti-HP372 monoclonal antibody (Amersham), or anti-heme oxygenase type 1 (HO-1) antiserum (Stressgen, USA) at 4°C for 3 h. Staining with anti-HP372 monoclonal antibody (Amersham), or anti-heme oxygenase type 1 (HO-1) antiserum (Stressgen, USA) at 4°C for 3 h.
cell pellet was disrupted by Dounce homogenization. Fractionation of cell lysates was performed as described (24), and, following measurement of protein concentration, each fraction (about 1 \( \mu \)g of protein) was immunoblotted with anti-ORP150 IgG raised to purified ORP150.

Immunocytochemical studies were performed in hypoxic astrocytes as described (25). In brief, astrocytes plated on glass coverslips were exposed to hypoxia (1% \( O_2 \)) for 48 h, fixed in 4% paraformaldehyde for 30 min, and permeabilized using PBS containing Nonidet P-40 (1%), and incubated with either anti-ORP150 IgG (raised to purified ORP150) or preimmune rabbit IgG (in each case at 1:100 dilution, 10 \( \mu \)g/ml). Sites of primary antibody binding were visualized using rhodamine-conjugated anti-rabbit IgG (Sigma).

To further analyze the subcellular localization of ORP150 antigen in hypoxic astrocytes, about 5 \( \times \) 10^8 hypoxic astrocytes were pelleted and fractionated as described (26). In brief, astrocyte cell pellets frozen at \(-80^\circ\)C were thawed, resuspended in 10 ml of buffer A (0.25 M sucrose, 10 mM Hepes-NaOH, pH 7.5, 1 mM dithiothreitol, 1 mM PMSF, 1 \( \mu \)g/ml leupeptin, 0.1 mM 1-chloro-3-tosylamido-7-aminomethylcoumarin, and the cells were cavitated at 400 psi N\(_2\) pressure for 30 min by nitrogen cavitation bomb (Kontes Glass Co., Vineland, NJ). Following homogenization, the cell lysate was centrifuged at 10,000 \( \times \) g for 15 min at 4°C, and the pellet was resuspended in TNE buffer (10 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 \( \mu \)g/ml aprotinin, 1 mM PMSF). The clarified lysate was then centrifuged and each fraction (fractions 1–4) was collected. Material in the gel was transferred electrophoretically to PVDF paper, and the antigen band whose expression was induced in hypoxic astrocytes, was subjected to N-terminal sequence analysis. 15 cycles were performed on the antigen band by using a protein sequencer. The N-terminal sequence of ORP150 was enriched by this procedure and a peptide corresponding to ORP150 was identified as described above. Expression of ORP150 antigen in ischemic brain—To examine the expression of ORP150 antigen in the central nervous system, cultured rat microglia, brain microvessel endothelial cells, and cortical neurons were exposed to hypoxia for 24 h. Cells were lysed in PBS containing Nonidet P-40 (1%), and about 1 \( \mu \)g of protein extract was subjected to Western blotting by using the anti-ORP150 IgG raised to purified ORP150 as described above. Expression of ORP150 antigen in ischemic brain was studied using adult C57BL mouse (20–25 g) subjected to permanent occlusion of the middle cerebral artery (28). Animals were sacrificed 24 h after ischemia, and the frontal and parietal cortex was harvested and frozen in liquid nitrogen. The samples were thawed and homogenized in PBS containing Nonidet P-40 (1%), EDTA (5 mM), and PMSF (1 mM) and immunoblotted using anti-ORP150 IgG. Expression of ORP150 antigen in ischemic brain was also examined by immunohistochemical analysis (29). Mice were anesthetized with sodium pentobarbital, and 24 h after ischemia, they were perfused transcardially with 0.01 M PBS followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were immersed for 12 h in 4% paraformaldehyde in phosphate buffer (pH 7.4) and stored in 20% sucrose in 0.1 M phosphate buffer (pH 7.4). Brain sections were cut in a cryostat and stained with anti-ORP150 IgG (raised to purified ORP150) as described (9).

RESULTS

Viability of Astrocytes Exposed to Hypoxia—Oxygen tension in the medium fell to about 8 torr within 8 h after cultures were transferred to the hypoxia chamber. Cell viability was maintained throughout hypoxia (H) and following replacement of cultures back to normoxia (reoxygenation (R)), based on lack of lactose dehydrogenase release into the culture supernatant, continued trypsin blue exclusion, adhesion of cells to the culture substrate, and unchanged morphologic features. Total cellular protein synthesis in astrocytes exposed to hypoxia for 48 h, assessed by incorporation of [3H]leucine, decreased to 68 ± 1.8% of that observed in normoxic controls by 48 h. The reversibility of this approximately 30% suppression of protein synthesis was evident by the rebound to levels about 1.7 times that seen in normoxia within 3 h of restoring hypoxic cultures to the ambient atmosphere.

Purification of ORP150 and Northern Blotting and Nuclear Run-off Analysis—To confirm the induction of ORP150 mRNA in astrocytes by hypoxia, a partial ORP150 cDNA corresponding to 151–570-base pair deduced amino acid residues 51–190 were employed for Northern analysis (GenBank No. U41853). About 5 \( \mu \)g of total RNA was extracted from astrocytes exposed to hypoxia or hypoxia/reoxygenation for the indicated times by the AGPC method. RNA was separated by electrophoresis on 1.0% agarose/formamide gels and transferred overnight onto Biodyne B paper (Pall Bio-Support, NY). The membrane was prehybridized for 3 h at 42°C in hybridization buffer (0.9 M NaCl, 90 mM sodium citrate, pH 7.0) containing 5 \( \times \) Denhardt’s solution, SDS (0.5%), and heat-denatured salmon sperm DNA (100 \( \mu \)g/ml). ORP150 cDNA was radiolabeled with [\( ^{32}P \)]dCTP (NZ522, DuPont NEN) by the random hexamer procedure (27). After hybridization overnight at 42°C in hybridization buffer containing radiolabeled cDNA probe (5 ng/ml), filters were washed twice with 2 \( \times \) SSC, 0.5% SDS and 2 \( \times \) SSC, 0.5% SDS for 30 min at 50°C, exposed to x-ray film (Fuji Photo Film, Japan), and subjected to autoradiography. The level of ORP150 mRNA was evaluated by the comparison with \( \beta \)-actin mRNA.

Nuclear run-off analysis was performed as described (9) to determine the relative rates of ORP150 transcription. In brief, nuclear suspension (0.2 ml), obtained from about 2 \( \times \) 10^6 astrocytes prepared under the indicated conditions, was incubated with CTP, ATP, and GTP (0.5 mM each) in the presence of [\( ^{32}P \)]UTP (250 \( \mu \)Ci, 3000 Ci/mmole, DuPont NEN). Samples were subjected to phenol/chloroform extraction, and RNA was precipitated and resuspended in hybridization buffer. Hybridization to denatured rat ORP150 and rat \( \beta \)-actin (control) probes (20 \( \mu \)g each in each dot) blotted onto nylon membranes was performed at 42°C for 2 days. Filters were washed, dried, and exposed to Fuji x-ray film. Detection of ORP150 Antigen in Other Cell Types and Ischemic Brain—To examine the expression of ORP150 antigen in the central nervous system, cultured rat microglia, brain microvessel endothelial cells, and cortical neurons were exposed to hypoxia for 24 h. Cells were lysed in PBS containing Nonidet P-40 (1%), and about 1 \( \mu \)g of protein extract was subjected to Western blotting by using the anti-ORP150 IgG raised to purified ORP150 as described above. Expression of ORP150 antigen in ischemic brain was studied using adult C57BL mouse (20–25 g) subjected to permanent occlusion of the middle cerebral artery (28). Animals were sacrificed 24 h after ischemia, and the frontal and parietal cortex was harvested and frozen in liquid nitrogen. The samples were thawed and homogenized in PBS containing Nonidet P-40 (1%), EDTA (5 mM), and PMSF (1 mM) and immunoblotted using anti-ORP150 IgG. Expression of ORP150 antigen in ischemic brain was also examined by immunohistochemical analysis (29). Mice were anesthetized with sodium pentobarbital, and 24 h after ischemia, they were perfused transcardially with 0.01 M PBS followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were immersed for 12 h in 4% paraformaldehyde in phosphate buffer (pH 7.4) and stored in 20% sucrose in 0.1 M phosphate buffer (pH 7.4). Brain sections were cut in a cryostat and stained with anti-ORP150 IgG (raised to purified ORP150) as described (9).

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protein extracts from astrocytes (about 5 x 10^8 cells, 2-3 mg of protein) exposed to hypoxia for 48 h were filtered, diluted with 50 ml of PBS, and applied to FPLC Mono Q. The column was washed with 0.2 M NaCl and eluted with an ascending salt gradient; protein content in eluate was monitored by A(280nm). B, reduced SDS-PAGE (7.5%) was performed on protein extracts from either normoxic or hypoxic astrocytes (about 10 g of protein each, lanes N and H) and on aliquots of fractions (10 µl, fractions 1-20) from the Mono Q column (0.5 ml, lanes 1-20). Proteins in the gel were visualized by silver staining, and migration of simultaneously run molecular weight markers is indicated on the far left side of the gel (ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa), and myosin (200 kDa)). The migration of ORP150 is indicated by the asterisk. C, fractions eluted from FPLC Mono Q, which contained the ~150-kDa polypeptide (fractions 6 and 7) were pooled and concentrated by ultrafiltration. About 20 µg of protein was applied to reduced SDS-PAGE, and protein was detected by staining with Coomassie Blue. The asterisk indicates the migration of ORP150, and migration of molecular weight markers is shown on the far right side of the gel.

N, respectively). Similar results were observed with antibody to ORP150 generated against the synthetic peptide (data not shown). Immunoblotting, using anti-ORP150 IgG, of the eluate of the FPLC Mono Q column loaded with lysate of hypoxic astrocytes also visualized a band with a molecular mass ~150 kDa in fractions 6–10 (Fig. 2A, fractions 1–20). The latter band was most intense in fraction 7, consistent with our previous results (Fig. 1B). Further experiments demonstrated that anti-ORP150 IgG raised against purified ORP150 bound to N-terminal ORP150 peptide blotted onto nitrocellulose in a dose-dependent manner, diminishing as peptide concentration was lowered (Fig. 2B). Finally, although lysate of hypoxic astrocytes immunoblotted a band with a molecular mass ~150 kDa using anti-ORP150 IgG raised against the synthetic peptide (Fig. 2C, HYPOXIA), when astrocyte proteins were pre-adsorbed with anti-ORP150 IgG (antibody raised against purified ORP150), no band was observed (Fig. 2C, αORP150). In contrast, anti-ORP150 IgG raised against the synthetic peptide did not visualize a band in normoxic astrocyte lysates (Fig. 2C, NORMOXIA), whereas hypoxic astrocyte lysates pre-adsorbed with nonimmune IgG still showed the ~150-kDa band (Fig. 2C, preimmune). These data show that both antibodies, which raised to purified ORP150 and which raised to the N-terminal synthetic peptide (sites of primary antibody binding were detected as described in the text) in panels A and C, migration of simultaneously run molecular weight markers is shown on the left.

Expression of ORP150 in Cultured Astrocytes—Using anti-ORP150 IgG raised to purified ORP150, immunoblotting studies were performed on lysates of cultured astrocytes exposed to hypoxia (HYPOXIA) or normoxic (NORMOXIA) astrocytes (about 10^8 cells) was pre-adsorbed with either anti-ORP150 IgG raised against purified ORP150 (αORP150) or preimmune IgG (preimmune) as described in text. After the removal of IgG-ORP150 complexes using immobilized protein A, supernatant was concentrated (to about 20 µl in each case) and subjected to reduced SDS-PAGE (7.5%). Other samples applied to the gel were lysate of hypoxic (HYPOXIA) or normoxic (NORMOXIA) astrocytes (about 1 µg of protein). Following Western blotting, membranes were reacted with anti-ORP150 IgG (5 µg/ml) raised against the N-terminal synthetic peptide (sites of primary antibody binding were detected as described in the text). In panels A and C, migration of simultaneously run molecular weight markers is shown on the left.

Fig. 1. Isolation of ORP150 from rat cultured astrocytes by FPLC Mono Q (A and B) and preparative SDS-PAGE (C). A, protein extracts from astrocytes (about 5 x 10^8 cells, 2-3 mg of protein) exposed to hypoxia for 48 h were filtered, diluted with 50 ml of PBS, and applied to FPLC Mono Q. The column was washed with 0.2 M NaCl and eluted with an ascending salt gradient; protein content in eluate was monitored by A(280nm). B, reduced SDS-PAGE (7.5%) was performed on protein extracts from either normoxic or hypoxic astrocytes (about 10 g of protein each, lanes N and H) and on aliquots of fractions (10 µl, fractions 1-20) from the Mono Q column (0.5 ml, lanes 1-20). Proteins in the gel were visualized by silver staining, and migration of simultaneously run molecular weight markers is indicated on the far left side of the gel (ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa), and myosin (200 kDa)). The migration of ORP150 is indicated by the asterisk. C, fractions eluted from FPLC Mono Q, which contained the ~150-kDa polypeptide (fractions 6 and 7) were pooled and concentrated by ultrafiltration. About 20 µg of protein was applied to reduced SDS-PAGE, and protein was detected by staining with Coomassie Blue. The asterisk indicates the migration of ORP150, and migration of molecular weight markers is shown on the far right side of the gel.

Fig. 2. Immunoblotting of ORP150 using antibody raised against purified ORP150 or the N-terminal synthetic peptide. A, immunoblotting using anti-ORP150 antibody raised against purified ORP150. Extract from either normoxic (N) or hypoxic (H) astrocytes (about 10 µg in each case) or aliquots of fractions from FPLC Mono Q (1-20, from Fig. 1A) were subjected to reduced SDS-PAGE (7.5%) and transferred to PVDF paper. After blocking excess sites on the paper, PVDF was reacted with anti-ORP IgG (5 µg/ml), and sites of primary antibody binding were visualized as described in the text. B, the indicated amount of ORP150 synthetic peptide (ORP150) or bovine serum albumin (BSA) were dot-blotted on nitrocellulose paper and reacted with rabbit IgG obtained either before (Preimmune) or after (Postimmune) immunization of animals with purified ORP150. Sites of primary antibody binding were visualized as above. C, lysate of hypoxic astrocytes (from about 5 x 10^8 cells) was pre-adsorbed with either anti-ORP150 IgG raised against purified ORP150 (αORP150) or preimmune IgG (preimmune) as described in text. After the removal of IgG-ORP150 complexes using immobilized protein A, supernatant was concentrated (to about 20 µl in each case) and subjected to reduced SDS-PAGE (7.5%). Other samples applied to the gel were lysate of hypoxic (HYPOXIA) or normoxic (NORMOXIA) astrocytes (about 1 µg of protein). Following Western blotting, membranes were reacted with anti-ORP150 IgG (5 µg/ml) raised against the N-terminal synthetic peptide (sites of primary antibody binding were detected as described in the text). In panels A and C, migration of simultaneously run molecular weight markers is shown on the left.
hypoxia or hypoxia followed by reoxygenation (H/R). ORP150 antigen was detected within 24 h of subjecting cells to oxygen deprivation and continued at 48 h (Fig. 3, ORP150, HYPOXIA). Following replacement of hypoxic cultures into normoxia (Fig. 3, ORP150, REOX), intensity of the ORP150 band increased at 2 and 4 h, thereafter diminishing at 12 h and being undetectable by 24 h. This time course of ORP150 induction in response to hypoxia and H/R paralleled expression of GRP78 (Fig. 3, GRP78). In contrast, expression of HSP72 was only observed after H/R (Fig. 3, HSP72), and expression of heme oxygenase type 1 antigen (HO-1, molecular mass \( \sim 33 \) kDa) was detected at low levels in normoxic cultures and peaked by 24–48 h of hypoxia (Fig. 3, HO-1).

To further characterize astrocyte induction of ORP150, its expression in astrocyte lysates exposed to chemical agents was studied by immunoblotting. Treatment of normoxic astrocyte cultures with either 2-deoxyglucose or tunicamycin resulted in the expression of GRP78 (Fig. 4, GRP78). HO-1 antigen was induced in astrocytes by addition of hydrogen peroxide, cobalt chloride, or elevated temperature (Fig. 4, HO-1); whereas only elevated temperature induced HSP72 (Fig. 4, HSP72). None of these chemical stimuli elicited expression of ORP150 antigen in astrocytes (Fig. 4, ORP150). The role of de novo protein biosynthesis in ORP150 expression by hypoxic astrocytes was emphasized by disappearance of the ORP150 band from cultures subjected to hypoxia in the presence of cycloheximide (3 \( \mu \)g/ml, H + CX). Astrocytes maintained in normoxia were subjected to either heat shock (43°C for 3 h, HEAT); exposure to hydrogen peroxide (5 \( \mu \)M for 10 min, H, O2), followed by the incubation for 6 h; or exposure to cobalt chloride (1 \( \mu \)M, Co), 2-deoxyglucose (25 mM, 2DG), or tunicamycin (5 \( \mu \)g/ml, TM) for 24 h. Cells were then harvested as in Fig. 3, and about 1 \( \mu \)l from each sample was subjected to immunoblotting using the same antibody preparations.

Cellular Distribution of ORP150—Using ultracentrifugation to perform subcellular fractionation studies, immunoblotting of hypoxic astrocyte lysates indicated enrichment of ORP150 antigen in cytosolic and nuclear wash fractions. Consistent with these results, immunostaining of hypoxic astrocytes with anti-ORP150 IgG revealed a diffuse diffuse cytosolic distribution with diminished intensity in the nuclear region (Fig. 5B, panel B1), which was similar to the distribution of GRP78 under the same conditions (Fig. 5B, panel B2). In contrast, no staining was observed when anti-ORP150 IgG was replaced by preimmune IgG (Fig. 5B, panel B3).

Sucrose density gradient ultracentrifugation of hypoxic astrocyte lysates suggested a similar distribution of ORP150 and GRP78 antigens, suggesting localization of ORP150 in the endoplasmic reticulum (Fig. 5C; fractions 4 and 5, enriched for endoplasmic reticulum, contain most of the ORP150 and GRP78).
Induction of ORP150 Message in Hypoxic Astrocytes—Northern blot analysis using a partial cDNA for ORP150 demonstrated induction of ORP150 mRNA in hypoxia as well as during early reoxygenation, paralleling the time course for expression of ORP150 antigen (Fig. 6). Nuclear run-off analysis showed induction of the transcription of ORP150 mRNA during hypoxia, which appeared to increase further early in reoxygenation (Fig. 7).

Expression of ORP150 in the Other Cell Types and Ischemic Brain—Hypoxia-induced expression of ORP150 antigen was observed only in cultured astrocytes and not in other cell types, such as microglia, endothelial cells, or neurons harvested from neonatal rat brain (Fig. 8). ORP150 was also expressed in brain tissue subjected to ischemia using middle cerebral artery occlusion in mice. Immunoblotting with anti-ORP150 IgG of cortex extracts from ischemic side of the brain showed a ~150-kDa band, which was not observed in control sides (Fig. 9A). Immunohistochemical analysis using the same antibody also indicated expression of ORP150 antigen in areas of the brain subjected to ischemic injury by this procedure, especially in the penumbral area. In contrast, no staining was observed in brain tissue from nonischemic control sides (Fig. 9B).

**DISCUSSION**

Analysis of the astrocyte response to environmental challenge is particularly important as these cells withstand stress-
ful conditions that curtail neuronal viability (2, 3). In addition, astrocytes can sustain neuronal homeostasis through a variety of mechanisms, including the elaboration of neurotrophic factors. In a previous study, we found that cultured astrocytes exposed to hypoxia followed by replacement into normoxia (H/R) elaborated the neurotrophic cytokine IL-6, which enhanced survival of PC12 (9). To analyze mechanisms underlying the synthesis and release of IL-6 by astrocytes exposed to H/R, we characterized new products of protein synthesis under these conditions. Of the five polypeptides whose expression was dramatically enhanced/induced by hypoxia or H/R, based on SDS-PAGE analysis, we first characterized a 78-kDa polypeptide, which proved to be identical to GRP78 by N-terminal sequencing and reactivity with anti-GRP78 antisera (1). GRP78 functioned as a chaperon to enhance the elaboration of IL-6 by astrocytes exposed to H/R; in fact, IL-6 was present in conditioned media complexed with GRP78, and addition of antisense oligonucleotides for GRP78 suppressed release of IL-6. These data suggested the complexity of events activated by hypoxia and H/R, we characterized new products of protein synthesis under these conditions. Of the five polypeptides whose expression was dramatically enhanced/induced by hypoxia or H/R, based on SDS-PAGE analysis, we first characterized a 78-kDa polypeptide, which proved to be identical to GRP78 by N-terminal sequencing and reactivity with anti-GRP78 antisera (1). GRP78 functioned as a chaperon to enhance the elaboration of IL-6 by astrocytes exposed to H/R; in fact, IL-6 was present in conditioned media complexed with GRP78, and addition of antisense oligonucleotides for GRP78 suppressed release of IL-6. These data suggested the complexity of events activated by hypoxia and H/R for biosynthetic mechanisms likely to be affecting pathways from transcription to protein processing and release from the cell.

In this report, we have characterized a ~150-kDa polypeptide whose expression was induced by hypoxia or H/R in astrocytes. N-terminal sequence analysis defined this polypeptide as being unique, which is consistent with our preliminary analysis of the recently isolated partial cDNA (corresponding to deduced amino acid residues 1–884). Furthermore, the ~150-kDa polypeptide is produced selectively in response to oxygen deprivation, leading us to assign the name ORP150. This contrasts with HO-1 and GRP78, both of which display enhanced expression in response to hypoxia, as well as other stimuli. Our data suggest it is unlikely that heme-containing proteins, implicated in the induction of heme oxygenase or erythropoietin by heavy metals such as cobalt (30, 31), are involved in ORP150 expression. In addition, the lack of ORP150 induction following inhibition of glycolysis, following addition of 2-deoxyglucose, which induces GRP78 (1) and other GRPs (32), also points to the likelihood that distinct mechanisms underlying ORP150 expression, possibly mechanisms more directly related to oxygen deprivation.

Another facet of ORP150 induction deserving of comment concerns its selective expression in astrocytes, as opposed to other cell types, exposed to hypoxia or H/R. In cell culture, neurons, microglia, nor endothelial cells subjected to similar environmental conditions expressed ORP150. By contrast, polypeptides such as HO-1 and GRP78 are expressed in diverse cell types. Furthermore, in the mouse model of ischemia injury, immunocytochemical studies showed expression of ORP150 in ischemic area, suggesting that induction of ORP150 is part of the central nervous response to oxygen deprivation. These data suggest that ORP150 may be a marker of ischemic stress and lead us to speculate that it may have an important role in the successful adaptation of astrocytes to oxygen deprivation. In this context, the presence of ORP150 in endoplasmic reticulum places it at the critical locus for controlling de novo protein synthesis and processing.

These studies represent a first step in the characterization of a novel polypeptide induced in astrocytes by hypoxia or H/R. While the functional role of ORP150 to the adaptive astrocyte response to ischemic stress, including expression of neurotro-
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phic stimuli, remains to be defined, we have developed the reagents and tools that will permit this next level of analysis.

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Purification and Characterization of a Novel Stress Protein, the 150-kDa Oxygen-regulated Protein (ORP150), from Cultured Rat Astrocytes and Its Expression in Ischemic Mouse Brain

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