Metallothionein IIA Is Up-Regulated by Hypoxia in Human A431 Squamous Carcinoma Cells

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Abstract

The expression of metallothionein IIA (MT-IIA) was investigated in A431 human squamous carcinoma cells exposed to hypoxia (pO_2 < 0.01%) of atmospheric pO_2 and subsequent reoxygenation. Northern analysis showed that MT-IIA mRNA levels were significantly increased during 14 h of hypoxia and during reoxygenation. Western blotting confirmed that total MT protein levels were also increased in response to these stresses. Evidence of the transcriptional control of MT-IIA expression in hypoxic and in reoxygenated A431 cells was found using a 0.2-kilobase sequence of the proximal 5'-regulatory region of the MT-IIA gene in a chloramphenicol acetyltransferase reporter gene construct. Thus the proximal promoter of the human MT-IIA gene appears to contain a hypoxic response element(s). These observations indicate that MT-IIA may have an important role in the stress responses of cells in solid tumors.

Introduction

Metallothioneins are a family of ubiquitous low molecular weight proteins (M, 6000–7000) enriched in cysteine residues. The expression and regulation of 3 of the 6 or 7 functional members of the human MT family, MT-IA, MT-IB, and MT-IIA, have been extensively studied (1). Although MT-IA and MT-IB proteins have been found in all cultured human cells examined, MT-IIA is the predominant form and accounts for about 50% of the total cellular MT protein (1). MTs have well-established regulatory roles in metal ion homeostasis and in the detoxification of heavy metals (2). Because MTs are induced by a wide variety of environmental stresses and agents [e.g., X-radiation, heat shock, phorbol esters, interferon α, interleukins, and tumor necrosis factor] (3), it is possible that they function as protective cellular stress proteins. It has been suggested that MTs are major intracellular zinc-binding proteins and may determine the availability of this critical metal ion (4); e.g., MTs may regulate zinc-dependent transcription factors such as SP-1 and TFIIIA.

In this study, we found that MT-IIA mRNA and protein expression in A431 human SC cells were significantly increased during hypoxic stress and subsequent reoxygenation. We include this protein as a member of a group of ORPs (5). We also found evidence of a transcriptional component(s) in the regulation of MT-IIA induction by hypoxia and reoxygenation.

Materials and Methods

Cell Culture and Hypoxic Exposure. A431 cells were maintained as described previously (6). Cells were plated at 2.0 × 10⁶ cells/100-mm culture grade Petri dish (Corning, Corning, NY) in 10 ml of Dulbecco’s modified Eagle’s medium/10% fetal bovine serum, incubated in 5% CO_2/air at 37°C, and allowed to grow to approximately 80–90% confluence (4 days). The medium was changed 3 h before exposure to hypoxia. The dishes were placed inside specially designed aluminum chambers submerged in a 37°C water bath and attached to a 5% CO_2/N_2 manifold on a vacuum line (6). Oxygen was extracted at 37°C over 1.5 h by 7 cycles of pumping to a fixed pressure followed by filling with 5% CO_2/N_2. The final O_2 tension in the gas phase was approximately 0.01% of atmospheric O_2. After incubation at 37°C for up to 24 h, the chambers were opened in air for reoxygenation studies or in under 5% CO_2/N_2 in a humidified anaerobic chamber (Anaerobe Systems, Santa Clara, CA) for harvesting of protein. Aerobic controls were incubated for an equal time period in 5% CO_2/air at 37°C.

Northern Blotting. Total RNA was obtained by lysis with a 4 M guanidinium isothiocyanate solution followed by CsCl ultracentrifugation. The RNA was resolved in denaturing 1% agarose gels (15 pg/well) and blotted onto nylon membranes. The membranes were probed with a 32-P-labeled, 2.3-kilobase BamHI fragment (0.5–2.0 × 10⁶ cpmp/μg) of human MT-IA genomic DNA (7). Autoradiographs of the membranes were produced using Kodak XAR-5 film. Densitometry was performed by using a Lynx 4000 image analyzer (Applied Imaging, Santa Clara, CA).

Western Blotting. Cells were lysed with a Triton-based phosphate-buffered solution containing various protease inhibitors (6). Detergent-soluble proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 15% one-dimensional gels and transferred to Immobion P membranes (Millipore Corporation, Bedford, MA) according to the method of Aoki and Suzuki (8). MT protein was detected with murine monoclonal anti-MT (I and II) antibodies (Dako Corporation, Carpinteria, CA) and visualized by using a goat anti-mouse IgG polyclonal antibody conjugated with alkaline phosphatase exposed to a chemiluminescent substrate (Immum-Lite II detection system; Bio-Rad Laboratories, Hercules, CA).

Transfection Studies. An expression vector containing 0.2 kilobase of the human MT-IIA proximal promoter region placed immediately upstream of the bacterial CAT gene (gift of T. Mulcahy, University of Wisconsin-Madison) was transfected by electroporation (Gene Pulser; Bio-Rad, Richmond, CA) into 5 × 10⁶ A431 cells in a volume of 200 μl (250 V; 25 μF). After 48 h the electroporated cells were exposed to a selection medium (Dulbecco’s modified Eagle’s medium/10% fetal bovine serum/400 μg ml⁻¹ G418) to produce stable transfectants. Early passages (1–10) of pooled stable clones were used for hypoxia/reoxygenation studies. Total protein for CAT assays of hypoxic A431 cells was harvested in the humidified anaerobic chamber. We found no differences in CAT activities or in MT protein contents between duplicate hypoxic samples harvested in the anaerobic chamber and in the air. Cadmium chloride treatment (10 μM in serum-free medium for 3 h followed by 4 h of recovery) was used as a positive control for MT-IIA promoter activity. CAT activity assays and CdCl₂ treatments were performed according to established methods (9).

Results and Discussion

A431 human SC cells were exposed to hypoxia (pO_2 < 0.08 torr; 0.01% of atmospheric O_2) for up to 14 h followed by 24 h of reoxygenation. The A431 cells remained essentially 100% viable during these hypoxic treatments judging by trypan blue exclusion. Fig. 1 shows a Northern blot, from a single experiment, representative of human MT-IIA mRNA accumulated as a function of time during hypoxia and subsequent reoxygenation. The blot was reprobed for 28S rRNA which is presented as a control (Fig. 1). The MT-IIA message...
were reoxygenated after 14 h of hypoxia.

Time points include 8—14 h of hypoxia and 1, 2, 4, 6, and 24 h of reoxygenation (15, 16, 18, 20, 24, and 38 h from the start of hypoxia, respectively). Total aerobic control levels of MT protein. Triton-soluble protein (20 μg) was separated in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon P membrane, and probed with the anti-MT antibody as described in "Materials and Methods." Cells were reoxygenated after 14 h of hypoxia.

Fig. 1. Northern blot of MT-IIA mRNA and 28S rRNA. The lanes have been rearranged for clarity. Time points include 8—14 h of hypoxia and 1, 2, 4, 6, and 24 h of reoxygenation (15, 16, 18, 20, 24, and 38 h from the start of hypoxia, respectively). Lanes 1, 2, 3, 4, 6, and 9, aerobic control levels; n = 4, and this message enhancement was reported in the literature (7). Maximum levels of MT-IIA mRNA were attained by 14 h of hypoxia (Lane 1) followed by 12 h of reoxygenation. Additional reoxygenation (ReOx) time points are 4, 8, 10, and 12 h (Lanes 3, 4, 6, and 8, respectively). Lanes 2, 5, 7, and 9, aerobic control levels of MT protein. Triton-soluble protein (20 μg) was separated in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon P membrane, and probed with the anti-MT antibody as described in "Materials and Methods." Hx, hypoxia; A, air.

Fig. 2. Western blot analysis of total MT protein from A431 cells subjected to 14 h of hypoxia (Lane 1) followed by 12 h of reoxygenation. Additional reoxygenation (ReOx) time points are 4, 8, 10, and 12 h (Lanes 3, 4, 6, and 8, respectively). Lanes 2, 5, 7, and 9, aerobic control levels of MT protein. Triton-soluble protein (20 μg) was separated in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon P membrane, and probed with the anti-MT antibody as described in "Materials and Methods." Hx, hypoxia; A, air.

The identification of MT-IIA as an ORP offers another example of a hypoxic stress protein having a potential role in the development of drug resistance; e.g., overexpression of MT has been shown to confer resistance to the anticancer drugs cisplatin (17, 18), Adriamycin (19), and mitomycin C (20). Recent investigations indicate that MT pro-

Table 1 Transcriptional activation of the human MT-IIA 5'-regulatory region in hypoxic and reoxygenated A431 cell transfectants (experimental CAT activity/control CAT activity)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Transcriptional Activation</th>
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<tr>
<td>CAT&lt;sub&gt;HYPOXIA&lt;/sub&gt;/CAT&lt;sub&gt;AIR&lt;/sub&gt;</td>
<td>1.8 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>1.8 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>2.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT&lt;sub&gt;REOXYGENATION&lt;/sub&gt;/CAT&lt;sub&gt;AIR&lt;/sub&gt;</td>
<td>2.8 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>2.8 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>4.1 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>24</td>
<td>3.8 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT&lt;sub&gt;CONS&lt;/sub&gt;/CAT&lt;sub&gt;AIR&lt;/sub&gt;</td>
<td>3.9 ± 1.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>3.9 ± 1.3&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> Sample SD; n, 4.
<sup>b</sup> Sample SD; n, 7.
<sup>c</sup> Sample SD; n, 5.

with a CAT vector containing a 0.2-kilobase fragment of the human MT-IIA proximal promoter (see "Materials and Methods"). Cells were harvested for CAT assays at 8 and 14 h of hypoxia and at 4, 8, and 24 h of reoxygenation. The results of these studies (Table 1) indicate that hypoxic stress can increase transcription from the human MT-IIA proximal promoter; enhancements in CAT activity relative to the aerobic controls were observed at both 8 and 14 h of hypoxia (approximately 2- and 3-fold, respectively). CAT activities were also induced by reoxygenation and remained elevated for up to 24 h, presumably because of induction by oxidative stress and the relatively long half-life of the CAT protein (12—24 h). All inductions were within the same range as those found in the CdCl<sub>2</sub>-positive controls. Because we observed no differences in CAT activity in A431 cells transfected with a constitutive pSV2CAT reporter vector, these enhancements were probably caused by transcriptional activation rather than CAT message stabilization. Posttranscriptional regulation of MT-IIA mRNA could not be assessed because the apparent half-lives of hypoxic, reoxygenated, and aerobic MT-IIA mRNAs were longer than 8 h (data not shown). We are presently characterizing the putative hypoxic-responsive element(s) in this promoter region.

This study identifies MT-IIA as an ORP. ORPs and glucose-regulated proteins are subsets of cellular proteins induced in response to environmental stresses such as hypoxia and reoxygenation, hypoglycemia, heat, and exposure to chemicals and drugs. Many stress proteins probably have protective functions and may contribute to the ability of tumor cells to resist radiotherapy and some forms of chemotherapy. Using two-dimensional gel electrophoresis, we have detected the induction of at least 10 ORPs in a wide range of eukaryotic cells (10). Earlier work by our group demonstrated that ORP 80 is the same stress protein as GRP 78 (11) and that ORP 33 is HO-1 (10). We have also shown that the product of the c-jun gene (12) and the epidermal growth factor receptor (6) are ORPs. Other disparate proteins such as ornithine decarboxylase (13), xanthine oxidase (14), erythropoietin, platelet-derived growth factor β chain, endothelin 1, transforming growth factor β, tyrosine hydroxylase, and glycolytic enzymes (15) have been documented as hypoxic stress proteins in a variety of human cell types. More recently, the expression and activities of the bioreductase, DT-diaphorase, and y-glutamylcysteine synthetase have been reported to be up-regulated in response to hypoxia and reoxygenation (16).

The identification of MT-IIA as an ORP offers another example of a hypoxic stress protein having a potential role in the development of drug resistance; e.g., overexpression of MT has been shown to confer resistance to the anticancer drugs cisplatin (17, 18), Adriamycin (19), and mitomycin C (20). Recent investigations indicate that MT pro-

4 B. J. Murphy and K. R. Laderoute, unpublished data.

5 B. J. Murphy, K. R. Laderoute, and R. M. Sutherland, unpublished data.
teins have central roles in cellular stress responses. Many of the stresses that induce MT synthesis involve the production of reactive oxygen species which can be intercepted by MT thiols or thiolates (3). Interestingly, the possible involvement of oxidative stress in some mechanisms of apoptosis (or programmed cell death) (21) suggests that MT-IIA could be considered an inducible antiapoptotic gene. Another ORP, HO-i, also has antioxidant properties because its activity causes increases in the levels of bilirubin, which is considered to be a physiological antioxidant (22). Reoxygenation of hypoxic tumor cells may constitute an oxidative stress similar to the reperfusion of ischemic normal tissue. Because many solid tumors are known to contain regions of transient hypoxia, arising from inadequate or intermittent blood supply, we hypothesize that the regulation of MT-IIA by hypoxia/reoxygenation may have a survival advantage and contribute to malignant progression.

Acknowledgments

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References

1. Skroch, P., Buchanan, C., and Karin, M. Regulation of human and yeast metallothio-


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