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 (pO₂ < 0.01 % of atmospheric pO₂) 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 (2). 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 × 10⁴ 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% CO₂/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% CO₂/N₂, 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% CO₂/N₂. The initial CO₂ tension in the chamber was 0.01% of atmospheric CO₂. After incubation at 37°C for up to 24 h, the chambers were opened in air for reoxygenation studies or in under 5% CO₂/N₂ 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% CO₂/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 and blotted onto nylon membranes. The membranes were probed with a 32P-labeled, 2.3-kilobase BamHI fragment (0.5-2.0 X 10⁶ cpm/g) of human MT-IIA 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 Immobilon P membranes (Millipore Corporation, Bedford, MA) according to the method of Aoki and Suzuki (8). MT protein was detected with murine monoclonal antibodies which recognize MT-LA257, 333 (9). The expression of metallothionein HA (MT-HA) was investigated in A431 human squamous carcinoma cells exposed to hypoxia (pO₂ < 0.08 torr; 0.01% of atmospheric O₂) 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. A431 human SC cells were exposed to hypoxia (pO₂ < 0.08 torr; 0.01% of atmospheric O₂) 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. MT-IIB protein was detected with murine monoclonal antibodies which recognize MT-LA257, 333 (9).
were reoxygenated after 14 h of hypoxia. Total RNA (15 μg) was isolated and resolved as described in "Materials and Methods."

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 2, 5, 7, and 8 showed no significant inductions during the initial 8 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 a 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.

![MT-IIA IS AN ORP](image1.png)

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>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>3.9 ± 1.3^b</td>
</tr>
<tr>
<td>14</td>
<td>2.8 ± 1.1^a</td>
</tr>
<tr>
<td>24</td>
<td>4.1 ± 1.4^c</td>
</tr>
<tr>
<td>38</td>
<td>3.8 ± 1.7^d</td>
</tr>
</tbody>
</table>

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 a 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. 2 shows an autoradiograph of a representative Western blot of MT protein from hypoxic and reoxygenated A431 cells resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by chemiluminescence. The blot shows significant message accumulations of MT-IIA mRNA levels within 4 h of the onset of hypoxia (data not shown), whereas A431 cells showed no significant inductions during the initial 8 h of hypoxia (Fig. 1).

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-i (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-

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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

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