Cloning and Characterization of a Human Polyamine Oxidase That Is Inducible by Polyamine Analogue Exposure

Yanlin Wang, Wendy Devereux, Patrick M. Woster, Tracy Murray Stewart, Amy Hacker, and Robert A. Casero, Jr.

The Johns Hopkins Oncology Center, Bunting-Blaustein Cancer Research Building, Baltimore, Maryland 21231 [Y. W., W. D., T. M. S., A. H., R. A. C.], and Department of Pharmaceutical Sciences, Wayne State University, Detroit, Michigan 48202 [P. M. W.]

Abstract

Mammalian polyamine catabolism is under the control of two enzymes, spermidine/spermine N\textsuperscript{\textsubscript{\textalpha}}-acetyltransferase and the flavin adenine dinucleotide-dependent polyamine oxidase (PAO). In this study, the cloning and initial characterization of human PAO is reported. A 1894-bp cDNA with an open reading frame of 1668-bp codes for a protein of 555 amino acids. In vitro transcription/translation of this cDNA clone produces the expected Mr, 61,900 protein with PAO activity. The PAO activity of this clone is inhibited by MDL 72,527, a specific inhibitor of mammalian PAO. However, neither pargyline, a specific monoamine oxidase inhibitor, nor semicarbazide, a specific diamine oxidase inhibitor, inhibits the PAO activity of this clone. PAO has been referred to as being constitutively expressed. However, 24-h exposure of a non-small cell lung carcinoma cell line, NCI H157, to 10 \mu M of N\textsuperscript{2},N\textsuperscript{\textalpha},bis(ethyl)norspermine results in a 5-fold induction of PAO mRNA and a >3-fold induction of PAO activity. These results demonstrate that in at least one cell type, PAO is up-regulated in response to polyamine analogue exposure. The PAO clone described here should provide a useful tool, which will facilitate the dissection of the role of polyamine catabolism in normal growth and in response to the antitumor polyamine analogues.

Introduction

The polyamines putrescine, spermidine, and spermine are naturally occurring polycationic amines that have been demonstrated to be important in normal and neoplastic cell proliferation, differentiation, and in some cases, survival (1–3). Because of the absolute requirement of these compounds for cell growth, the polyamine metabolic pathway is a promising target for antiproliferative strategies (4). Although much work (1) on interfering with the polyamine metabolic pathway has focused on blocking synthesis by directly inhibiting the biosynthetic enzymes ornithine decarboxylase and S-adenosylmethionine decarboxylase, more recent work has centered on using the self-regulatory properties of polyamine metabolism to design active polyamine analogues. Along this line, several symmetrically and unsymmetrically substituted antitumor polyamine analogues have been synthesized (5, 6).

In addition to regulating the biosynthetic enzymes, some of the most effective antitumor polyamine analogues were found to profoundly increase the catabolism of polyamines (7). In some instances, it appears that the activity of polyamine catabolism is directly associated with the activity of the analogues (2, 8, 9).

Polyamine catabolism is mediated by the activity of two enzymes acting sequentially. The first and generally rate-limiting enzyme is SSAT (10). This enzyme catalyzes the addition of an acetyl group to the N\textsuperscript{\textalpha}-position of either spermidine or spermine. The acetylated polyamine then becomes the preferred substrate for the activity of PAO, a flavin adenine dinucleotide-dependent oxidase that results in production of 3-acetamido propanal, H\textsubscript{2}O\textsubscript{2}, and either spermidine or putrescine, depending on the starting polyamine (11, 12). It should be noted that unmodified spermine is also a substrate of PAO (11).

As stated above, the polyamine catabolic pathway has been implicated in the sensitivity of several tumor types to many antitumor polyamine analogues. This has been particularly true for SSAT. Several studies (2, 7, 13) have demonstrated that the activity of SSAT can increase several thousand-fold in response to exposure to polyamine analogues. However, it has been thought previously (12) that the activity of PAO was constitutive and primarily limited by the availability of the acetylated substrate. Although there are recent cloning reports of yeast and plant PAOs (14, 15), the direct study of the regulation of mammalian PAO has been hampered by the fact that no clone of the mammalian enzyme has been available. In this study, we report the cloning and characterization of human PAO and demonstrate that the expression of the enzyme can be significantly induced by exposure to the antitumor polyamine analogues. The results presented here suggest another mechanism by which various tumor types can respond differentially to exposure to the antitumor polyamine analogues.

Materials and Methods

Chemicals. The radionucleotides ([\textalpha]-\textsuperscript{32}P]dCTP and [\textalpha]-\textsuperscript{35}S]methionine) were supplied by Amersham Pharmacia Biotech (Piscataway, NJ). The TnT-coupled wheat germ extract system was purchased from Promega (Madison, WI). The TA cloning kit was purchased from Invitrogen (Carlsbad, CA). Trizol total RNA reagent was from Life Technologies, Inc. (Rockville, MD). Advantage cDNA Polymerase Mix system and a retroviral placenta cDNA library were from Clontech Laboratories, Inc. (Palo Alto, CA). Restriction and DNA-modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, MA), Life Technologies, Inc., and Sigma Chemical Co. (St. Louis, MO). Life Technologies, Inc. synthesized all of the oligomers used in the experiments. The DNA sequencing was performed with a Perkin-Elmer ABI Automated DNA Sequencer. N\textsuperscript{2},N\textsuperscript{\textalpha},bis(2,3-butadienyl)-1,4-butanediamine (MDL 72,527) was kindly supplied by Dr. Eugene Gerner (University of Arizona, Tucson, AZ). Other chemicals came from Sigma Chemical Co., Roche Molecular Biochemicals (Indianapolis, IN), Bio-Rad (Hercules, CA), Aldrich Chemical Company, Inc. (Milwaukee, WI), and J. T. Baker, Inc. (Phillipsburg, NJ).

Cloning of Human PAO. PCR (Advantage cDNA Polymerase Mix system) was used to clone human PAO cDNA. PCR was performed with a gene-specific primer pair (5'-CGGCGCTCGCCGACGACTTCTC-3' and 5'-AAAGCTACAGGCGCAGTGGAG-3') and cDNA from a human placenta library. The PCR products were then cloned into pcCR2.1 vector (pcCR2.1/PAOhx). To construct the pPAOh1 plasmid, the cDNA insert in pcCR2.1/PAOhx was removed by cutting with HindIII and EcoRV and then inserting the resultant fragment into pcDNA3.1 (+) vector in the same restriction sites.

In Vitro Transcription and Translation. In vitro transcription and translation reactions were performed with the TnT-coupled wheat germ extract system. Parallel reactions were prepared by adding an unlabeled amino acid mixture to one reaction and a [\textsuperscript{35}S]methionine containing amino acid mixture to the other, according to the supplied protocol. Vector pcDNA3.1 and....
pPAOh1 were linearized by SspI restriction and served as the templates. The labeled translation reactions products were separated by 10% SDS-PAGE, and radioactivity on the labeled PAO band was determined by Phosphor image analysis using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

RNA Purification and Northern Blot Assay. Total cellular RNA from the NCI H157 cell line was extracted using Trizol total RNA reagent according to the protocol from the manufacturer. Total RNA (20 µg) was separated on a denaturing 1.5% agarose gel containing 6% formaldehyde, transferred to Zetaprobe membrane (Bio-Rad), and hybridized with a random primer-labeled pPAOh1 cDNA as the probe. Blots were washed and probed with an 18S ribosomal cDNA probe as a loading control.

Determination of PAO Enzyme Activity. The cultured H157 cells with or without treatment of 10 µM N,N-bis(ethyl)norspermine (BENSpm) were homogenized with a Dounce tissue homogenizer in ice-cold 0.083 m sodium borate buffer (pH 9.0). The PAO activity in homogenates was assayed by the method of Suzuki et al. (16), which measures the H2O2 formed due to oxidation of spermine by converting homovanillic acid into a highly fluorescent compound in the presence of horseradish peroxidase. The samples were prepared in a 600-µl reaction containing 83 mM sodium borate buffer (pH 9.0), 0.04% mg of horseradish peroxidase, 100 µl of cell homogenate, 0.1 mg of homovanillic acid, and 250 µM spermine. Before the addition of homovanillic acid and spermine, the tubes were preincubated for 20 min with shaking at 37°C to remove endogenous substrates of H2O2-producing enzymes. After preincubation, homovanillic acid and spermine were added, and the reactions were incubated for 1 h at 37°C. The enzyme activity was stopped by the addition of 2.0 ml of 0.1 M NaOH solution. The fluorescence intensity was measured with excitation at 323 nm and emission at 426 nm. Background fluorescence was determined by addition of the spermine substrate into the reaction mixture only after inactivation of the enzyme by NaOH. Protein content of the cellular homogenate was determined using the Bio-Rad protein assay kit (Bio-Rad). One unit of PAO activity in cell homogenate was defined as that amount that transformed 1 pmol spermine/mg cell protein/60 min at 37°C. For determination of PAO activity in the product of the unlabeled TnT reaction, 10 µl of TnT reaction was used in the place of the homogenate. The PAO activity in the TnT reactions was represented by the fluorescent compound formed within the 1-h incubation. In some tests, the inhibitor for monoamine oxidase (pargyline), diamine oxidase (semicarbazide), or polyamine oxidase (MDL-72527) was used at the final concentration of 1.0 mM, 0.1 mM, and has the assigned accession no. AY033889.

Results

Molecular Cloning of Human PAO. The maize PAO was cloned recently by Tavladoraki et al. (14). Using the information provided by their work, PCR primers spanning the putative flavin adenine dinucleotide-binding site were made to be used in the PCR techniques. Using human placenta cDNA as a starting material, multiple homologues of the maize PAO were identified. After sequencing of each individual clone, BLAST homology searching of the National Center for Biotechnology Information human genome database revealed that the multiple clones were encoded by the same genomic sequence (accession no. AL1216785), which is located on chromosome 20p13. The longest clone, pPAOh1, is a total of 1894 bp and possesses an open reading frame of 1668 bp coding for a putative protein of 555 AA (Fig. 1A). This clone was chosen for further characterization. On the basis of the available GenBank data, pPAOh1 is the product of seven exons and six introns (Fig. 1B) spanning 38.9 kb of genomic DNA. The origin of transcription is not currently known.4

4 The nucleotide sequence reported in this paper has been submitted to the GenBank and has the assigned accession no. AY033889.
The labeling assay was performed in the presence of [35S]methionine with in vitro transcription and translation of human PAO h1 with wheat germ extract system. PAO, increasing concentrations of spermine were used in the calculation of initial velocity, and D protein.

Reaction products were then separated by 10% SDS-PAGE. The templates used in the assays were:

- BEMSpm (diamine oxidase inhibitor), MDL 72,527 (PAO inhibitor), and a no inhibitor control as indicated. The ordinate is measured in arbitrary fluorescent units/equal amounts of TnT reaction mix.
- The mean of at least two trials with a variation of <10%.

**Fig. 2.** Determination of PAO activity and \( K_m \) from TnT-produced protein. *In vitro* transcription and translation were performed with T7-coupled wheat germ extract system. TnT reaction (10 \( \mu \)l) was used for each assay with spermine as the substrate. A, PAO activity from TnT products using pPAOh1 or vector pcDNA3.1 as the template. B, effects of amine oxidase inhibitors on PAO activity in protein from TnT reaction (pPAOh1). The inhibitors used in the experiment: pargyline (monoamine oxidase inhibitor) + semicarbazide (diamine oxidase inhibitor), MDL 72,527 (PAO inhibitor), and a no inhibitor control as indicated. The ordinate is measured in arbitrary fluorescent units/equal amounts of TnT reaction mix. Bars, the mean of at least two trials with a variation of <10%.

C, in vitro transcription and translation of human PAOh1 with wheat germ extract system. The labeling assay was performed in the presence of [15S]methionine with 2 \( \mu \)g of linearized plasmid as the template in a 25-\( \mu \)l TnT reaction. The labeled transcription products were then separated by 10% SDS-PAGE. The templates used in the assays were:

- pPAOh1 or pcDNA3.1 vector as indicated. The arrow indicates the position of PAOh1 protein. D, increasing concentrations of spermine were used with equal amounts of TnT reaction products to determine initial velocity, and \( K_m \) was determined by a Lineweaver-Burke transformation.

**Fig. 3.** BENSpm-induced PAOh1 expression in the H157 cell line. A, PAO activity of NCI H157 cells after exposure to 10 \( \mu \)M BENSpm. The ordinate represents pmol H_2 O_2 produced/mg protein. Bars, the mean of two experiments with a variation of <10%.

**Discussion**

Polyamine metabolism has been a target of antineoplastic drug development since the recognition of the cellular requirements of polyamines for growth. The recent advance in the synthesis of analogues that alter polyamine metabolism by regulation rather than inhibition has lead to the discovery of how critical the control of polyamine metabolism is with respect to normal and neoplastic growth. Although it has been possible to extensively study the activity and regulation of the first step in polyamine catabolism because of the availability of mammalian clones (10), the extensive study of the mammalian PAO has been impaired. The cloning and characterization of human PAO presented here provides a tool and offers some unique insights into the regulation and importance of PAO. The 555 AA protein with an apparent \( M_r \) 62,000 agrees well with the size predicted for the mammalian protein purified previously (12, 18).

Previously (12), it was assumed that PAO was expressed in a constitutive manner or only slowly induced. In this study, it is clearly demonstrated that this is not always the case. The significant induction of PAO message and activity in the analogue-treated human nonsmall cell lung carcinoma cells clearly demonstrates that PAO can be up-regulated within 24 h in a manner similar to that observed for SSAT. The induction of PAO activity correlates well with the message level, suggesting that the major regulation of PAO activity is at the transcripational level. This appears to be in contrast to SSAT induction, where post-transcriptional regulation plays a large role in the regulation of SSAT expression (19, 20). It will be necessary to examine several other cell types to determine how common the induction of PAO is in response to analogues or other stimuli. If, as is the case for SSAT, PAO demonstrates a cell type-specific induction response to stimuli, it is possible that PAO may play a significant role in the sensitivity of cells to the antitumor polyamine analogues.

It is also important to note that alternative splicing may have a role

or diamine oxidases, specific inhibitors of each were included in the indicated reactions. Only the PAO inhibitor, MDL 72,527, was effective in inhibiting the human PAO h1 protein product. (Fig. 2B). To ensure equal additions of protein to the assays described above, parallel TnT reactions for each condition were prepared by adding an unlabeled amino acid mixture to one reaction and an [15S]methionine-containing amino acid mixture to the other. Protein produced in this manner yielded a major band of ~62 kDa after denaturing PAGE, consistent with the expected size of the open reading frame (Fig. 2C).

To determine the apparent \( K_m \) for the *in vitro* produced human PAO, increasing concentrations of spermine were used in the calculation of initial velocities of H_2 O_2 production as described above. The initial velocity of the reaction was determined for increasing concentrations of spermine ranging from 2.5 to 250 \( \mu \)M. The apparent \( K_m \) of the TnT-produced PAO using spermine as the substrate was determined by the Lineweaver/Burke transformation to be ~18 \( \mu \)M (Fig. 2D).

**Effects of BENSpm Treatment on PAO mRNA Expression and Enzyme Activity in NCI H157 Cells.** PAO has frequently been described as a constitutively expressed protein. To test this hypothesis, we exposed NCI H157 cells to 10 \( \mu \)M BENSpm for 24 h. This time and concentration were chosen because BENSpm has demonstrated the ability to highly induce SSAT in H157 cells and produce H_2 O_2-related apoptosis (3). BENSpm exposure resulted in ~5-fold increase in PAO message (Fig. 3A) and a >3-fold increase in PAO activity (Fig. 3B).
in how PAO is regulated. The significance of the multiple splice variants is not known and will take additional experimentation to address. Although pPAOh1 represents a longer clone than was predicted by Babbage, the submitting author of the genomic sequence AL121675, it is possible that even longer splice variants may exist that were not represented in our PCR clones. The multiple splice variants observed and those suggested by Babbage are consistent with the results of Libby and Porter (11) who identified two isoforms of PAO in the murine leukemia line, L1210. Further study will be required to determine the number and significance of the possible splice variants and resulting isoforms.

We have previously demonstrated that in NCI H157 cells, the induction of SSAT is associated with apoptosis. Additionally, we found that the onset of apoptosis could be delayed after treatment with the PAO inhibitor, MDL 72,527, suggesting that the \( \text{H}_2\text{O}_2 \) produced by PAO had a role in producing the toxic effects of BENSpm in NCI H157. However, in subsequent work by others (9) the addition of the PAO inhibitor had no effect on the cytotoxicity produced by BENSpm. This apparent contradiction in results may be the result of different levels of PAO activity expressed among the different cell types.

The current results suggest that a substantial increase in PAO mRNA after BENSpm treatment is a result of increased transcription of the PAO gene. However, because it is also possible that analogue treatment may alter message stability, nuclear run-on assays must be performed to confirm an increase in PAO transcription.

PAO is the terminal enzymatic step in polyamine catabolism. Although, previous studies have indicated that PAO is constitutively expressed, the data presented here with the newly cloned human PAO demonstrate that expression of PAO can be positively modulated by cellular exposure to a cytotoxic polyamine analogue. These results suggest that the sensitivity of tumor cells to the polyamine analogues may result from a combination of inducible SSAT and PAO activity. The availability of the human PAO clone will make further study of this important enzyme considerably easier.

References


Cloning and Characterization of a Human Polyamine Oxidase That Is Inducible by Polyamine Analogue Exposure

Yanlin Wang, Wendy Devereux, Patrick M. Woster, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/61/14/5370">http://cancerres.aacrjournals.org/content/61/14/5370</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 20 articles, 7 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/61/14/5370.full#ref-list-1">http://cancerres.aacrjournals.org/content/61/14/5370.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 42 HighWire-hosted articles. Access the articles at: <a href="http://cancerres.aacrjournals.org/content/61/14/5370.full#related-urls">http://cancerres.aacrjournals.org/content/61/14/5370.full#related-urls</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, use this link <a href="http://cancerres.aacrjournals.org/content/61/14/5370">http://cancerres.aacrjournals.org/content/61/14/5370</a>. Click on &quot;Request Permissions&quot; which will take you to the Copyright Clearance Center's (CCC) Rightslink site.</td>
</tr>
</tbody>
</table>