Close Relationship of the Major Excreted Protein of Transformed Murine Fibroblasts to Thiol-dependent Cathepsins


INTRODUCTION

In 1978 Gottesman (1) described a low molecular weight protein that was secreted from transformed murine fibroblasts in larger amounts than from the untransformed parent cells. One reason for the increased abundance of the secreted protein is the elevated amount of mRNA in the transformed cells; exposure of the cells to the tumor promoter TPA also increases the mRNA levels (2, 3). MEP is secreted by epidermal cells, both in culture and in vivo (in mouse skin), and in both cases TPA enhances its production (4). It is a glycosylated protein and carries the mannose 6-phosphate group believed to target proteins to lysosomes (5). The M, 39,000 protein is encoded in a 1.8-kilobase mRNA the abundance of which is increased 6–12 h after stimulation of density-arrested BALB/c-3T3 cells with PDGF (6). The PDGF-induced cellular protein called pII has been shown to be MEP (7).

Nilsen-Hamilton et al. (8) showed that various growth factors and lectins (fibroblast growth factor, nerve growth factor, congoavlin A) stimulated the release of MEP from both Swiss and BALB/c-3T3 cells, and further that the amount secreted was increased by various agents (nigericin, monensin, NH4Cl, chloroquine) that are known to inhibit proteolysis in lysosomes. The amount of MEP secreted by the cell is also modulated by degradation of newly synthesized protein in the lysosomes.

We are interested in understanding the changes in gene expression that occur in cells stimulated to proliferate by interactions with growth factors. As one aspect of this study, we have cloned cDNA copies of two mRNAs [mitogen-regulated protein (proliferin), Parfett et al. (9); MEP, this paper] encoding secreted proteins the abundance of which in the cell culture medium is increased when mouse fibroblasts are exposed to any of several growth factors. We anticipate that studies of this sort will reveal what we presume to be an interlocking set of cascades that lead from the initial stimuli to the final events, DNA replication and cell division. In this report we describe the isolation and partial characterization of a cDNA clone of the MEP mRNA. From the sequence of the encoded protein we infer that MEP is likely to be a murine thiol-dependent cathepsin. While this work was in progress, Doherty et al. (3) also reported cloning the mRNA for MEP.

MATERIALS AND METHODS

Cells and RNA. The mouse BNL and Swiss 3T3 fibroblast cells are described in Refs. 9 and 10, respectively; NIH-3T3 cells were obtained from Dr. D. Fujita. All were grown in Dulbecco’s minimal essential medium with 10% calf serum. Polyadenylated mRNA was isolated as described by Edwards et al. (10).

Isolation of the cDNA Clone. Sucrose-formamide density gradient fractions enriched in MEP poly(A)mRNA isolated from BNL cells were identified by in vitro translation in a reticulocyte system (DuPont-NEN), followed by immunoprecipitation of the [35S]methionine-labeled proteins with MEP antibody and protein A-Sepharose and electrophoresis in 12% polyacrylamide gels. The antisera was raised in rabbits that had been immunized with MEP purified as described by Nilsen-Hamilton et al. (4). Complementary DNA was prepared using the procedure of Land et al. (11) from sucrose-formamide fractions containing the most MEP mRNA. Cloning into λgt10 and λgt11 and immunological screening of the λgt11 phage plaques was performed as described by Parfett et al. (9) using materials and information made available by Drs. T. Huynh and R. Davis.

RNA Analysis and DNA Sequencing. Nick translation of plasmid DNAs, Northern blotting of cellular RNA, and hybridizations were all performed using standard protocols (12). Formaldehyde-treated RNA was electrophoresed through a 1.1% agarose gel containing formaldehyde. Autoradiography of blots labeled with 32P and fluorography of protein gels impregnated with PPO were performed at -70°C using intensifying screens in the former case and preflashed film in the latter case (9, 10). Sequencing of the inserts in M13mp8 and M13mpl8 was performed using standard protocols (12). Formaldehyde-treated RNA was electrophoresed through a 1.1% agarose gel containing formaldehyde. Autoradiography of blots labeled with 32P and fluorography of protein gels impregnated with PPO were performed at -70°C using intensifying screens in the former case and preflashed film in the latter case (9, 10). Sequencing of the inserts in M13mp8 and M13mpl8 was performed using standard protocols (12). Formaldehyde-treated RNA was electrophoresed through a 1.1% agarose gel containing formaldehyde. Autoradiography of blots labeled with 32P and fluorography of protein gels impregnated with PPO were performed at -70°C using intensifying screens in the former case and preflashed film in the latter case (9, 10). Sequencing of the inserts in M13mp8 and M13mpl8 was performed using standard protocols (12). Formaldehyde-treated RNA was electrophoresed through a 1.1% agarose gel containing formaldehyde. Autoradiography of blots labeled with 32P and fluorography of protein gels impregnated with PPO were performed at -70°C using intensifying screens in the former case and preflashed film in the latter case (9, 10). Sequencing of the inserts in M13mp8 and M13mpl8 was performed using standard protocols (12).

RESULTS

Isolation of a MEP cDNA Clone. A λgt11 library prepared from poly(A) mRNA enriched for mRNA encoding MEP was screened with an antiserum raised against MEP; positive plaques were picked and rescreened until homogeneous plaque preparations were generated. The insert (present as an EcoRI fragment) from a selected plaque stock was recloned into...
M13mp8. Both the replicative form DNA and single-stranded DNA produced by two clones with the inserts in opposite orientations (designated 1 and 8) were used to hybrid select mRNA from poly(A)mRNA isolated from TPA-treated NIH3T3 cells. The mRNA was translated in a reticulocyte system and the resulting [35S]methionine-labeled proteins were immunoprecipitated with anti-MEP antibody.

Fig. 1 shows a fluorogram of selected lanes from two different sodium dodecyl sulfate-polyacrylamide gels of the [35S]methionine-labeled proteins translated from the hybrid-selected mRNA and of immunoprecipitated products. Lanes 1–4 contain, respectively, the translation products of the mRNA annealed to single-stranded DNA generated by clone 1, by clone 8, to the (denatured) double-stranded replicative form from clone 8, and from a control unrelated clone (16C8). Lanes 7–9 show the proteins immunoprecipitated with MEP antibody from those reactions analyzed in Lanes 1–3, respectively. The major species in Lanes 7 and 9 migrates roughly as expected for the M, 39,000 MEP protein, providing strong evidence that the cDNA clone does indeed correspond to the mRNA-encoding MEP.

The material synthesized in vitro migrates slightly faster than the species found in the cell culture medium (Lane 6), presumably because it is not glycosylated (it may also still retain its leader sequence). Lower molecular weight forms are presumably the result of proteolytic degradation of the full-size MEP protein or are the products of partially degraded mRNA. Lane 0 shows the in vitro translation products of the total poly(A) mRNA, and Lane 5 shows the proteins immunoprecipitated from this reaction with anti-MEP. The subclone (clone 1, Lanes 1 and 7) that produces single-stranded DNA able to hybrid select MEP mRNA is the one that yielded the coding sequence on the sequencing gels.

Confirmation that the isolated clone encoded MEP was obtained from Northern blot analyses of the mRNA species in various cells under different conditions. Cells known to be secreting MEP contained a 1.8-kilobase mRNA that was recognized by the clone and migrated just ahead of the 18S rRNA (Fig. 2). This is precisely the size previously determined for MEP mRNA (6). When quiescent, serum-deprived 3T3 cells were stimulated to proliferate, the abundance of the polyadenylated cytoplasmic MEP mRNA increased, reaching a maximum between 6 and 12 h, depending upon the synchronization, and then declined slowly. We infer that increased transcription is responsible for the increase since both actinomycin and 5,6-dichloro-l-β-d-ribofuranosylbenzimidazole blocked it. Fibroblast growth factor was also a good inducer of MEP mRNA (data not shown). The (more delayed) increase in the cytoplasmic abundance of mitogen-regulated protein mRNA, which runs at about 1 kilobase and has been shown to be the result of new transcription (10), was even more impressive.

To ascertain whether MEP expression varied during a normal cell cycle, we used centrifugal elutriation to fractionate an exponentially growing culture of Ehrlich ascites cells into cells of different sizes. Flow cyt fluorimetry verified that cells in the various fractions had the expected amount of DNA. Northern blot analysis of the MEP mRNA content in the different fractions did not reveal significant changes in the abundance of the MEP mRNA (data not shown).

Sequence of the MEP Clone and Its Encoded Polypeptide. The strategy used to sequence the 638-base pair cDNA is indicated in the upper part of Fig. 3; the lower portion of the figure gives...
The sequence of nucleotides in the coding strand of the insert is given above the nucleotide sequence. The SmaI, HinfI, and EcoRV sites were used to generate subclones for sequencing in the indicated directions; arrows, regions sequenced.

**DISCUSSION**

Cysteine proteases (e.g., cathepsins B, H, and L) are a widely distributed class of lysosomal endopeptidases that require a sulfhydryl group for activity (19, 20). Their role in metabolism is not well defined, although various studies have implicated them both in intracellular protein catabolism and in the breakdown of extracellular proteins, e.g., during tissue remodeling. Sloane and Honn (21) have reviewed the experimental data suggesting their involvement in metastasis; perhaps most compelling is the correlation between cathepsin B activity in homogenates of murine solid tumors and the lung colonization potential of the B16 melanoma variants that gave rise to the tumors. The hypothesis that such proteases facilitate the invasion of surrounding tissues by the malignant cell is an attractive one. Alternatively, increased uptake of secreted cathepsins (MEP?) by cells expressing mannose 6-phosphate receptors may have physiological importance to limited proteolysis, with important consequences for subsequent cellular behavior. For example, protein kinase C is irreversibly activated in vitro by a Ca²⁺-dependent neutral thiol protease (22).

The amount of MEP secreted by mouse fibroblast cells in vitro by a Ca²⁺-dependent neutral thiol protease (22).

**MEP PROTEIN** (partial) 212 amino acids vs ABPA,MIB library

The best scores are: 93%

**Alignment with:**

<table>
<thead>
<tr>
<th>MEP</th>
<th>Cathepsin H</th>
<th>Rat</th>
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<tr>
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**RESEMBLANCE OF MEP TO CYSTEINE PROTEASES**

The sequence of nucleotides in the coding strand of the insert is given above the nucleotide sequence. The SmaI, HinfI, and EcoRV sites were used to generate subclones for sequencing in the indicated directions; arrows, regions sequenced.

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The alignment with cathepsin H is shown in detail with : signifying identity and — signifying a conservative replacement. The FASTP program used to search the library and the method of calculating similarity scores are discussed by Lipman and Pearson (15). Number in parentheses after the mean score of 21.4, SD of the almost entirely random matches, is near the initial and optimal (after introducing gaps and detecting homologies outside of the region first uncovered) similarity scores.

Fig. 3. Unique restriction sites in the 638-base pair MEP clone and its nucleotide sequence. The SmaI, HinfI, and EcoRV sites were used to generate subclones for sequencing in the indicated directions; arrows, regions sequenced.

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When the amino acid sequence of the polypeptide was compared to the sequences present in the 1985 National Biomedical Research Foundation protein data bank for sequences homologous to the partial sequence deduced for MEP, the alignment with cathepsin H is shown in detail with : signifying identity and — signifying a conservative replacement. The FASTP program used to search the library and the method of calculating similarity scores are discussed by Lipman and Pearson (15). Number in parentheses after the mean score of 21.4, SD of the almost entirely random matches, is near the initial and optimal (after introducing gaps and detecting homologies outside of the region first uncovered) similarity scores.

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not blocked but was indeed enhanced by inhibition of protein synthesis (23). We have found, as have Rabin et al. (24), that the growth factor-induced increase in MEP mRNA abundance is dependent upon a new gene transcription. Under our conditions both platelet-derived growth factor and fibroblast growth factor enhanced MEP mRNA abundance; however, the relative abundance of MEP mRNA in various stages of the cell cycle of normally cycling Ehrlich ascites cells (separated by centrifugal elutriation into populations at different points in the cell cycle) did not vary significantly (data not shown).

Although we have not yet obtained a sequence for the entire MEP mRNA, it is clear that the region we have sequenced constitutes an internal segment of the protein and possesses very significant homology to a region of the cysteine proteases including the active site. For example, the glutamine at position 20, the cysteines at positions 23, 26, and 66, and the histidine at position 166 (cathepsin H numbers) are conserved in all the sequences (25). The proposed site for glycosylation of MEP (Fig. 3) is only 6 residues away from the site in cathepsin H that is believed to be glycosylated (26).

There appears to be little doubt that MEP is related to the cysteine proteases. Consistent with this conclusion is the recent report that MEP is an activable acid protease, apparently autoactivated at low pH; the appearance of lower molecular mass polypeptides (M, 29,000 and 20,000) derived from MEP correlated with the acquisition by the protein preparation of the ability to digest various proteins, including extracellular matrix proteins (27). The activity was inhibited by iodoacetate and leupeptin and had a pH optimum around 3.5; however, thiol compounds did not enhance activity.

It will be fascinating to compare the detailed structure of the active site for proteolysis contained in MEP with that in the thiol proteases. Like many proteases, the proteolytic activity of MEP is not revealed until after the structure/conformation of the “mature” protein is altered, but unlike the cathepsins (B, H, L) or the cysteine protease described by Reckliese and Mort (28), there is no requirement for a thiol for activation. The limited information available is consistent with the idea that the active site of MEP is only partly related to the active site in the thiol proteases and that its activity is masked in the purified mature protein.

ACKNOWLEDGMENTS

We thank David Lipman, William Pearson, John Donelson, and David Mount for providing us with their computer programs; Linda Bonis and Beth Orphan for preparing the manuscript; Martha Holman and Marilyn McLeod for superb technical assistance; Dan Portnoy for bringing a sequencing error to our attention; and Anneliese Reckliese for a careful critique of the manuscript. D. T. D. also thanks Martin Hollenberg for making some of this work possible.

Note Added in Proof

D. A. Portnoy, A. H. Erickson, J. Kochan, J. V. Ravetch, and J. C. Unkeless have sequenced a full-length cDNA clone of a mouse cysteine proteaseinase that contains the exact sequence reported here for MEP in J. Biol. Chem., in press, 1986.

REFERENCES


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