Vol. 139, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 29, 1986 Pages 156-162

THE MAJOR EXCRETED PROTEIN (MEP) OF TRANSFORMED MOUSE CELLS AND CATHEPSIN L HAVE SIMILAR PROTEASE SPECIFICITY

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Received July 16, 1986

SUMMARY: The major excreted protein of transformed mouse cells is an acid activable cysteine protease (1). In this paper, oxidized insulin B chain is shown to be a substrate for this protease. By isolation and analysis of the insulin B peptides generated by the protease, the bond specificity of this protease was determined. The bonds preferentially cleaved are glu_{13}-ala_{14}, leu_{17}-val_{18}, and tyr_{26}-thr_{27}. No obvious preference for a specific amino acid was found in these studies. The bond specificity of this cysteine protease for oxidized insulin B chain has been compared with that of other proteases, and it is the same as that reported for cathepsin L, suggesting that the major excreted protein and cathepsin L may be the same protein.

INTRODUCTION: The proteins secreted by tumor cells are presumed to be involved in the many deleterious effects of a tumor on its host. We have been studying one such protein secreted by Kirsten virus transformed NIH 3T3 mouse fibroblasts termed MEP (2), which is a 39,000 dalton mannose 6-phosphate containing glycoprotein (3). Increased synthesis and secretion of this protein have been observed in mouse cells treated with tumor promoters (4, 5), growth factors (6, 7) or other transforming viruses (2). Cells sequester a portion of their MEP, and process it to two lower molecular weight proteins (29,000 and 21,000 daltons) which are found in lysosomes (8). Recent work with the purified secreted protein showed that it is the pro-form of an acid protease (1). The secreted protein can auto-digest at low pH to form an active cysteine protease which can rapidly degrade a number of different proteins including extracellular matrix proteins (1). In this paper, the amino acid bond specificity of the MEP protease with the oxidized B chain of insulin is determined. The specific cleavage sites of the MEP protease are the same as those reported for cathepsin L, suggesting that MEP and cathepsin L may be the same protease.

Abbreviations: MEP, major excreted protein of mouse transformed fibroblasts; OIB, oxidized insulin B chain; HPLC, high performance liquid chromatography.

0006-291X/86 $1.50

MATERIALS AND METHODS

Purification of MEP. MEP was purified to homogeneity by High Performance Liquid Chromatography (HPLC) described previously (1). All materials were obtained from Sigma unless otherwise specified.

Reaction with oxidized insulin B chain. MEP was incubated with oxidized insulin B chain (OIB) as described previously for the reaction of MEP with BSA (1). For analysis and separation of the OIB peptides, MEP (0.34 μg) was activated by preincubation at pH 3.0 in sodium formate buffer for 1-2 min, then OIB was added at concentrations between 8 and 80 μg in 14 μl. The samples were incubated for various lengths of time, and the proteolysis was terminated by neutralizing with 28 λl of 1M $NaHCO_3$, pH 9.8. The resulting peptide mixture was separated on a reverse phase C-18 column (Altex Ultrasphere ODS, 4.6 mm ID x 25 cm) on a Beckman HPLC system. The peptides were loaded onto the column with 0.05% trifluoroacetic acid (TFA) in water and eluted with various steps of 0.05% TFA in acetonitrile at a flow rate of 1.0 ml/min. The gradient steps were as follows: First the column was equilibrated with 5% TFA/acetonitrile, then a gradient lasting 5 min to achieve a final concentration of 15% TFA/acetonitrile was followed by a 40 min gradient to 45%, and finally a gradient lasting 5 min to 90% TFA/acetonitrile was used. The elution of peptides was monitored by measuring optical density at 210 nm on an Altex spectrophotometer and this was plotted and areas under the peaks were calculated using the Altex R-C1A Integrator. The optical densities at 280 nm and 260 nm were also monitored for some of the peaks to determine the presence of tyrosine and phenylalanine in the peptides. The eluted peaks were collected and dried in a Speed Vac Concentrator (Savant) and analyzed for amino(N)-terminal amino acid, for carboxy(C)-terminal amino acid and for total amino acid content (see below).

N-terminal amino acid analysis. Dansyl chloride treatment (9) was used to determine the N-terminal amino acid for the OIB peptides, and the dansylated amino acids were separated and identified by the method of Weiner et al. (10) using polyamide thin layer chromatography sheets backed with translucent plastic or opaque foil (Pierce).

C-terminal amino acid analysis. Carboxypeptidase Y (Pierce) (2.5 ng) was added to the peptide (about 4 nmoles) in 0.1M pyridine acetate, pH 5.5 and allowed to react for 1 hr. The buffer was removed by vacuum and the free amino acid dansylated and analyzed as above.

Determination of amino acid content. For total amino acid composition of the peptides, pre-column o-phthalaldehyde (OPA) derivatives were formed as previously described (11) and separated on a C-18 reverse phase HPLC system. The peptides were hydrolyzed as above and dissolved in 0.05% TFA in water. A volume containing approximately 70 pmoles of the peptide was combined with a similar volume of the OPA reaction mixture. The reaction mixture contained OPA (20 mM), β-mercaptoethanol (60 mM) and methanol (1.5%) in 1M borate, pH 10.4. This reaction was incubated at room temperature for exactly 1.5 min. The mixture was then immediately injected into a C-18 column (IBM Octadecyl 150 mm standard) equilibrated with 50 mM NaCl in water at 30°C. The flow rate was 2 ml/min, and the derivatized amino acids were eluted with a methanol gradient. The gradient steps were as follows: A 3 min gradient to 25% methanol, 25% methanol for 7 min, a 4 min gradient to 40% methanol, then a 3 min gradient to 50% methanol, a 4 min gradient to 55% methanol, and finally a 0.5 min gradient to 75% methanol. The OPA-amino acids were detected by fluorescence on a Gilson spectrofluorometer with excitation and emission wavelengths at 340 and 445 nm, respectively. The fluorescence yield of the various amino acids was determined by running a standard OPA-amino acid mixture through the column and determining the peak areas for each amino acid.

RESULTS: The cleavage of oxidized insulin B chain (OIB) by the MEP protease can be monitored by following the appearance of fluorescamine reactive groups (data not shown) or by the appearance of new peptides fractionated on a C-18 reverse phase column (Fig. 1). At initial time points, four peptides were

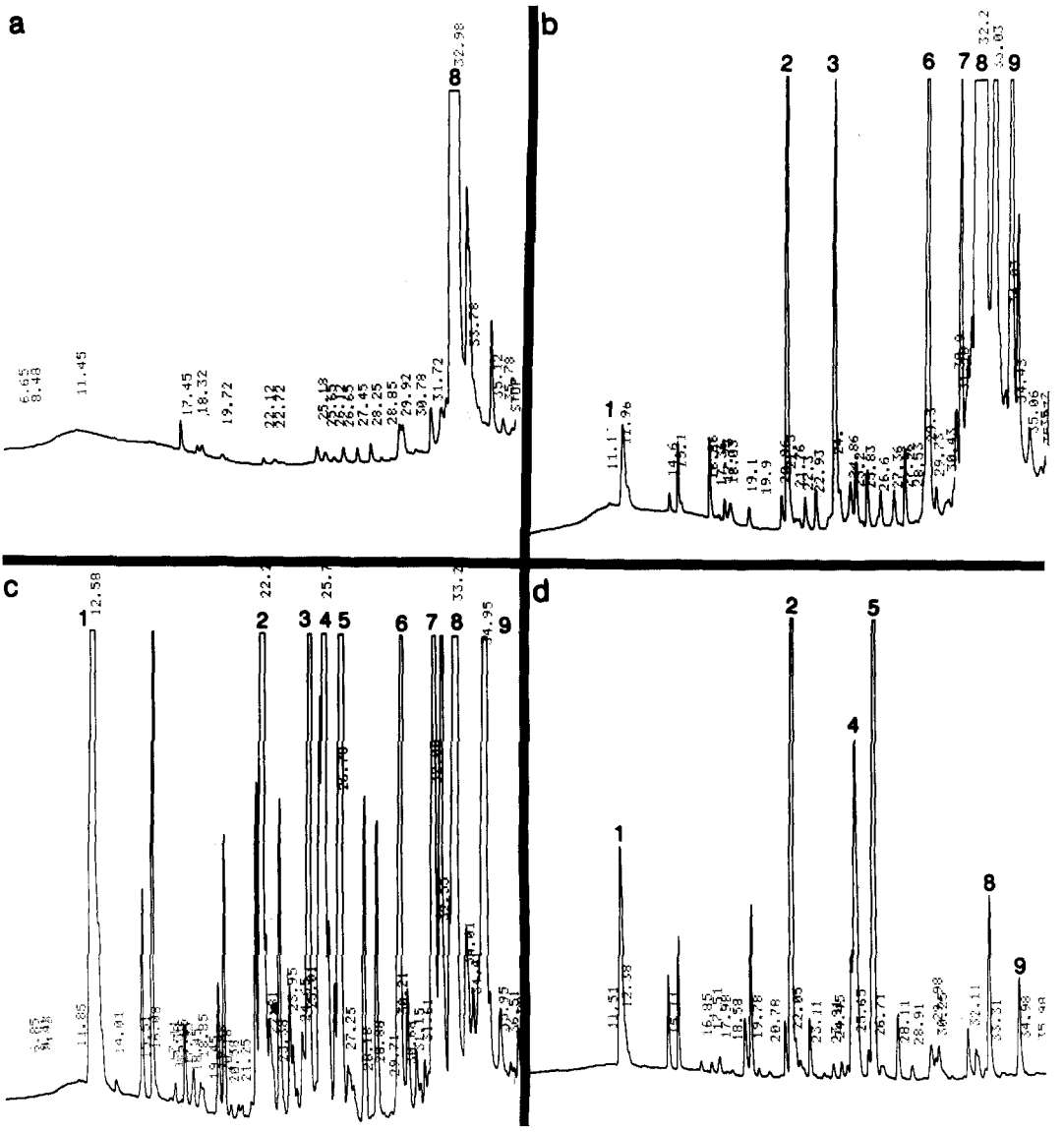

Fig. 1. HPLC separation of oxidized insulin B chain peptides digested with MEP: Activated MEP (0.3 µg) was added to OIB (80 µg) in 0.1M sodium formate, pH 3.0 and incubated for various times. The reaction was terminated and the peptides separated on a C-18 reverse phase column as described in the text by monitoring the optical density at 210 nm. The panels show the peptides isolated after 0 min (a), 8.5 min (b), 62 min (c), and 4 hr (d) reactions. The major peptides were numbered as described in the text. The range for optical density was 0.2 for panels a--c and 0.5 for panel d.

observed eluting from the C-18 column earlier than the starting material, OIB, and one eluted slightly later (Fig. 1, panel b). The stable cleavage products consisted of four major peptides seen after extensive incubation with MEP (Fig. 1, panel d).

a

ANALYSIS OF OXIDIZED INSULIN B CHAIN PEPTIDES

peak	N-term	C-term	amino acid content	peptide sequence
1	Thr	?	Thr,Ala,Lys	Thr(27)-Ala(30)
2	Phe	Glu,Gly	Asp,Cya,2Glx,2His,Ser,Gly, Val,Phe,2Leu	Phe(1)-Glu(13)
3	Val	ND	Cya,2Glx,2Gly,Thr,Arg,2Ala, 2Tyr,Val,2Phe,2Leu	Val(18)-Ala(30)
4	Ala	Leu	Ala,Tyr,2Leu	Ala(14)-Leu(17)
5	Val	Tyr	Cya,Glx,2Gly,Thr,Arg,Tyr, 2Phe,Val	Val(18)-Tyr(27)
6	Ala	ND	Cya,Glx,2Gly,Thr,Arg,2Ala,2Tyr, Val,2Phe,2Leu	Ala(14)-Ala(30)
7	Phe	Leu	Cya,Asp,2Glx,Ser,His,2Gly,Ala, Tyr,2Val,Phe,3Leu	Phe(1)-Leu(17)
8	Phe	Ala	2Cya,Asp,3Glx,Ser,2His,3Gly,Thr, Arg,2Ala,2Tyr,3Val,3Phe,4Leu	Phe(1)-Ala(30)
9	Phe	Tyr	2Cya,Asp,3Glx,Ser,2His,4Gly, Arg,Ala,2Tyr,3Val,3Phe,4Leu	Phe(1)-Tyr(27)

b

REPRESENTATION OF OXIDIZED INSULIN B PEPTIDES

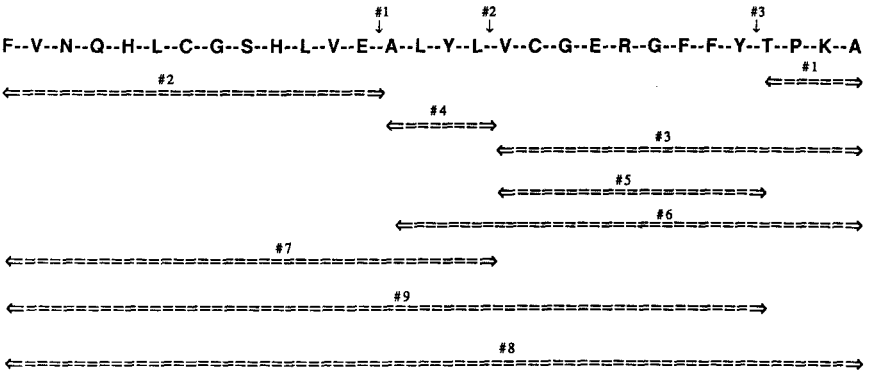

Fig. 2. The peptides generated by the MEP protease: panel a: Analysis of oxidized insulin B chain peptides. The peptides are numbered as in Fig. 1 and in the text. The peptides were analyzed for amino(N)-terminal, carboxy(C)-terminal and total amino acid content as described in MATERIALS AND METHODS. From these results, the sequence of the peptide within the OIB molecule was deduced. The three letter amino acid code is used with cya for cysteic acid and glx for acid hydrolyzed glutamine or glutamic acid. ND--not determined. panel b: Representation of oxidized insulin B peptides. The peptides are numbered as in Fig. 1 and in the text. The position of the peptides was deduced from the information in panel a. The three main cleavage sites of the MEP protease are numbered. The single letter code for the amino acids is shown.

The major peptides detected throughout the digestion were numbered based on their elution from the peptide column, number 1 being first and number 9 being last. Peptide number 8 was undigested OIB. All of the numbered peptides were isolated from the HPLC column and analyzed to determine their sequence.

MAJOR CLEAVAGE SITES IN OXIDIZED INSULIN B CHAIN BY SULFHYDRYL PROTEASES

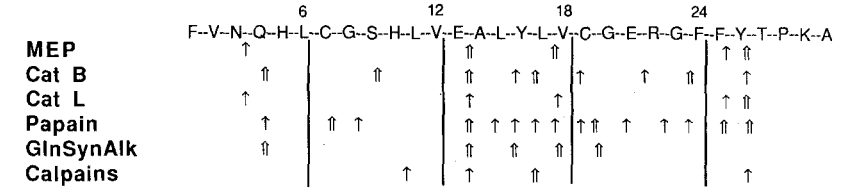

Fig. 3. Major cleavage sites in oxidized insulin B chain by cysteine proteases: The cleavage sites for several cysteine proteases on the OIB molecule are indicated by arrows: Major sites by double bar arrows and minor sites by single bar arrows. The proteases represented are MEP (from this work), cathepsin B (12), cathepsin L (13), papain (14), glutamine synthetase alkaline protease (15) and calpains (16).

The results of amino(N)-terminal, carboxy(C)-terminal and total amino acid analyses are shown in Fig. 2, panel a. The OPA reaction cannot detect proline (11) and without added detergent the OPA-lysine derivative has very low fluorescence yield due to quenching (11). Thus these two amino acids were not detected well in this system. The tyrosine and phenylalanine content of the final peptides was confirmed using absorption at 280 and 260nm (data not shown). The data show that peptides 1 and 2 do not contain tyrosine while all the other peptides do.

The structure of the major peptides correspond to three main cleavage sites in the OIB molecule (Fig. 2, panel b) numbered 1, 2 and 3. The sites include the glutamine-alanine bond at positions 13 and 14, the leucine-valine at 17 and 18, and the tyrosine-threonine bond at positions 26 and 27. These three sites seem to be cleaved at similar rates since the various resulting peptides appear at about the same rate.

A number of other minor peaks were evident eluting from the HPLC column (Fig. 1, panel d), for example between peaks 1 and 2. These peptides were analyzed for total amino acid content only. The amount of these peptides was less than 10% of the amount of the major final peptides (1, 2, 4 and 5) and thus probably represent minor cleavage sites for this enzyme. The minor peptides were glutamine$_4$-glutamine$_{13}$ and tyrosine$_{26}$-alanine$_{30}$ representing the minor cleavage sites between asparagine$_3$ and glutamine$_4$ and between phenylalanine$_{25}$ and tyrosine$_{26}$ (shown in Fig. 3). The presence of these minor cleavage sites was corroborated when the purified final four peptides were reacted again with four times more MEP than in the initial reaction. Only peptides 2 and 4 showed some cleavage as monitored by fluorescamine reactivity. The small amounts of new amino termini generated by this reaction were glutamic acid and glycine for peptide 2 and tyrosine and phenylalanine for peptide 4 suggesting the same minor cleavage sites.

DISCUSSION: It has been shown that the major excreted protein of transformed mouse cells (MEP) is an acid activable protease (1). MEP is a sulfhydryl protease inhibited by leupeptin and iodoacetic acid, but it is not activated by cysteine or β-mercaptoethanol in reactions at pH 3.0 (1). When comparing the enzymatic and molecular properties of MEP with the sulfhydryl acid proteases described in the literature (including cathepsins B, H, L, T, M, and S), none appeared exactly the same as the MEP protease (1,18). As discussed below, there are strong similarities between the peptide bond specificity of MEP and cathepsin L, suggesting that these two proteases may be the same.

Using oxidized insulin B chain (OIB) as a substrate, we have found that the MEP protease has a preference for the glu_{13}-ala_{14}, the leu_{17}-val_{18}, and the tyr_{26}-thr_{27} bonds. No bond specificity of MEP is seen. However, all three bonds do involve a tyrosine at or near the site of cleavage, suggesting that this is a key feature. The leu_{11}-val_{12} is not a major cleavage point for the MEP protease. It may be that the local environment of this site is unfavorable for MEP activity. There appear to be two other minor proteolytic sites, accounting for no more than 10% of the total peptide products. These minor peptides also appear when two of the four major peptides are digested with more MEP, indicating that they are the result of true sites of cleavage by MEP.

The comparison of the MEP protease bond specificity and that of other cysteine proteases is shown in Fig. 3. The MEP protease has a bond specificity which is the same as cathepsin L. However, cathepsin L cleaves some bonds within the insulin B chain molecule at different rates than MEP. This may reflect different assay conditions, different enzyme or substrate preparations and/or different quantitation methods. Although in the previous paper concerning the MEP protease activity (1), no activity was observed above pH 5 with carboxylate buffers, when other buffers are used such as arsenate or phosphate, activity for this enzyme can be observed with the OIB substrate above pH 5 (unpublished observations) as is seen for cathepsin L (17, 18). When the OIB peptides generated by the MEP protease in an arsenate buffered reaction at pH 6 are analyzed, the same peptides in the same approximate amounts are generated as at pH 3.0 (data not shown). This suggests that the pH of the reaction did not produce this apparent difference in bond preference between these proteases. Another important difference in these systems is that we use the auto-activated pro-form of MEP for our studies, whereas others use the active lysosomal form of cathepsin L.

Partial nucleotide sequence data for an MEP cDNA (19) has been obtained in this laboratory (Troen, B. T., Doherty, P. J., and Gottesman, M. M., manuscript in preparation). These data are consistent with sequences for MEP obtained by Denhardt et al. (Denhardt, D. T., Hamilton, R. T., Parfett, C. L. J., Edwards, D. R., St. Pierre, R., Waterhouse, P., and Nilsen-Hamilton, M., in press,

Cancer Res.) and for a mouse cysteine protease, which appears to be the same as MEP (Portnoy, D. A., Erickson, A. H., Kochan, J., Ravetch, J. V. and Unkeless, J. C., in press, J. Biol. Chem.). Comparisons of the amino acid sequence predicted by these data with amino-terminal peptide sequences for bovine cathepsin L (20) indicate 85% homology with the mouse MEP protein. This minor difference in amino acid sequence may reflect species differences and is consistent with the hypothesis, indicated by the enzymological data presented here, that MEP is mouse cathepsin L.

ACKNOWLEDGMENTS: We would like to thank David Denhardt and Daniel Portnoy for communication of their sequences results to us prior to publication, Michael Maurizi, James Riordan and Bruce Troen for helpful discussions, Joyce Sharrar for secretarial assistance and Ray Steinberg for help with photography.

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