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Characterization and Localization of Mitochondrial Oligopeptidase (MOP) (EC 3.4.24.16) Activity in the Human Cervical Adenocarcinoma Cell Line HeLa

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Abstract In this study we describe the partial purification and characterization of the HeLa cell oligopeptidase M or endopeptidase 3.4.24.16. The HeLa enzyme was isolated initially by its ability to hydrolyse a nonapeptide substrate (P9) which was cognate to the N-terminal cleavage site of preproTGFα. The enzyme was shown to be a metalloprotease as it was inhibited by Zn^{2+}-chelating agents and DTT, and had an approximate molecular weight of 55–63 kD determined by gel filtration. Neurotensin, dynorphin $A^{1–17}$ and $GnRH^{1–9}$ were rapidly degraded by the enzyme while $GnRH^{1–10}$ and somatostatin were not. Neurotensin was cleaved at the Pro^{10}-Tyr^{11} bond, leading to the formation of neurotensin (1–10) and neurotensin (11–13). The K_m for neurotensin cleavage was 7 μM and the K_i for the specific 24.16 dipeptide inhibitor (Pro-Ile) was 140 μM which were similar to those observed from the human brain enzyme [Vincent et al. (1996): Brain Res 709:51–58].

Through the use of specific antibodies, the purified HeLa enzyme was shown to be oligopeptidase M. This enzyme and its closely related family member thimet oligopeptidase were shown to co-elute during the isolation procedure but were finally separated using a MonoQ column. Oligopeptidase M is located mainly in mitochondria though it was detected on the plasma membrane in an inactive form. The results obtained demonstrate the first recorded instance of this enzyme in human tissue cultured cells, and raise the issue of its function therein. J. Cell. Biochem. 66:297–308, 1997.

Key words: oligopeptidase M; neurolysin; thimet oligopeptidase; peptide hydrolysis; TGFα

It has been shown that peptidases can regulate the physiological action of peptides by inactivating them or by changing their structure and, thus, their action. Examples of such enzymes include angiotensin converting enzyme (EC 3.4.15.1) [Ondetti et al., 1977], neprilysin (EC 3.4.24.11) [Casey et al., 1991] and endopeptidase 24.16 (EC 3.4.24.16) [Checler et al., 1983; Barrett et al., 1995]. Endopeptidase 24.16 was initially shown to cleave neurotensin in rat neural tissue [Checler et al., 1983], but has subsequently been shown to process angiotensin I, bradykinin and dynorphin-8 [Barelli et al., 1993]. This enzyme is now listed as neurolysin by the IUBMB Enzyme Nomenclature committee [Webb, 1989].

In a recent study it was shown that the oligopeptidase M (MOP), isolated from rat hepatocytes, shared all the biochemical properties of neurolysin (endopeptidase 24.16) found in neural tissue [Serizawa et al., 1995]. Subsequent sequence analysis confirms that these two enzymes are the same (for further discussion see [Barrett et al., 1995]). In order to avoid confusion we will refer to both MOP and 24.16 as the same enzyme in this manuscript. MOP (24.16) has also been shown to be the same as pig angiotensin-II binding protein [Kato et al., 1994]

Abbreviations: MOP, oligopeptidase M (EC 3.4.24.16); TOP, thimet oligopeptidase (EC 3.4.24.15); GnRH, gonadotropin releasing hormone; TGFα, transforming growth factor-α; DTT, dithiothreitol.

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and the rabbit microsomal endopeptidase [Kawabata et al., 1993], but different to the 24.16 variant found in rat testes [Rodd and Hersh, 1995]. MOP and its closely related homologue thimet oligopeptidase (TOP) (EC 3.4.24.15) are members of the M3 family of metallopeptidases (for review see [Barrett et al., 1995]). This single, diverse family contains homologous exopeptidases and endopeptidases which possess a zinc-binding motif (HEXXH) [Rawlings and Barrett, 1993]. MOP is distinguished from TOP through their different products of cleavage of neurotensin and the differential sensitivity of MOP to the dipeptide inhibitor Pro-Ile [Dauch et al., 1991]. TOP is primarily a cytosolic metallopeptidase which is thiol activated and acts on peptide substrates of up to 17 amino acid residues [Barrett et al., 1995] and has about 60% amino acid homology with MOP (EC 24.16) [Barrett et al., 1995; Dauch et al., 1995]. MOP (EC 24.16) has been found in the cytoplasm and plasma membrane of a wide range of tissues [Barrett et al., 1995; Checler et al., 1986; Barelli et al., 1994; Vincent et al., 1996a]. Serizawa et al. [1995] found that MOP was present in rat liver mitochondria though this enzyme was not detected in the mitochondrial fraction of astrocytes and neurons [Vincent et al., 1996a]. MOP and TOP can be distinguished immunologically by the use of polyclonal antibodies produced by Serizawa et al. [1995], who had previously used the monoclonal antibody raised against the rabbit soluble angiotensin-II protein by Soffer et al. [1991] to characterize MOP.

In this paper, we describe the partial purification and characterization of MOP from tissue cultured HeLa cells, originally derived from an adenocarcinoma of the cervix uteri [Jones et al., 1971]. We have previously shown that there is significant TGFαase activity on the HeLa cell membrane and that this increased following exposure to low doses of UVC [Piva et al., 1997]. As there was no detectable induction of mRNA or protein synthesis of the TGFαase following UV exposure [Brown et al., 1993] it was suggested that there may be intracellular reservoir of the enzyme which migrates to the cell surface to explain the increased levels of activity. We therefore attempted to purify this putative TGFαase from HeLa cell extracts using a synthetic nine amino acid peptide (P9) which is cognate with the N-terminal cleavage site of preproTGFα [Brown et al., 1992]. An enzyme activity was found in HeLa cell lysates which cleaved P9, and the nature of the enzyme responsible was sought. The TGFαase found on the HeLa cell membrane differs from the mitochondrial MOP with regard to substrate specificity and inhibition profiles. The use of the ^{125}I-labelled P9 substrate proved to be a quick and cheap method for assaying both MOP and TOP activity which had similar product profiles. This is the first reported incidence of this enzyme in human tissue which is not of neural origin and it was found to be similar to the endopeptidase 24.16 recently isolated from the human brain [Vincent et al., 1996b]. Evaluation of the physiological role of this intramitochondrial and plasma membrane endopeptidase represents an exciting challenge.

MATERIALS AND METHODS

All chemicals and biochemicals were obtained from Sigma (St Louis, MO), except for the following: dynorphin A$^{1-17}$, neurotensin, somatostatin, N-acetyl neurotensin, gonadotropin releasing hormone (GnRH), Pro-Ile and pepstatin A were obtained from Auspep (Melbourne, Australia), trypsin, penicillin and streptomycin were obtained from Commonwealth Serum Laboratories (Melbourne, Australia), RPMI tissue culture medium was obtained from Gibco (Grand Island, NY), foetal bovine serum (FBS) was obtained and processed in-house, Whatman channelled TLC plates (LK6D) were obtained from FSE Scientific (Brisbane, Australia), Na^{125}I (100 mCi/ml) was obtained from NEN/Du Pont (Boston, MA) and chloramine-T was obtained from BDH Chemicals Ltd. (Poole, England). GnRH$^{1-9}$ and z-pro-prolinol were provided by Dr. A.I. Smith, Baker Medical Research Institute, Melbourne. K118 and R646/P polyclonal antibodies were a gift from Dr. A.J. Barrett, Strangeways Research Laboratory, Cambridge, England. All other reagents were of AR grade. All aqueous solvents were prepared with Milli-Q purified water.

Cell Culture

HeLa S_3 (ATCC CCL2.2) cells were grown in RPMI 1640 media containing penicillin (100 mg/ml), streptomycin (100 mg/ml) and 10% (v/v) FBS (pH 7.2 at 37°C) in 1 litre suspension culture flasks (Bellco, Vineland, NJ). Every day approximately 20% of the suspension was decanted from the flask and replaced by an equal volume of fresh medium. The cells were har-

vested twice weekly when 700–800 ml of the cell suspension was removed from the flask and replaced by an equal volume of fresh medium. Cell viability was routinely monitored to ensure maximal viability was being maintained in culture. Cells were harvested when the cell density reached 5×10^5 cells/ml. All organelle isolation and enzyme purification steps were performed at 4°C unless otherwise stated.

Preparation of Intact Mitochondrial and Plasma Membranes

Approximately 10^9 HeLa cells were removed from spinner flasks, centrifuged (200*g* for 5 min) and washed twice with phosphate-buffered saline before being suspended in 5 ml homogenisation buffer (H-buffer) (5 mM HEPES, 210 mM mannitol, 70 mM sucrose, 1 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM leupeptin, 10 mM pepstatin and 10 mM zinc acetate, pH 7.4). HeLa cell mitochondria were isolated as previously described [Moreadith and Fiskum, 1984].

The plasma membranes were isolated from the supernatant obtained from the medium speed mitochondrial centrifugation step (10 min at 11,000*g*). This supernatant fraction was then centrifuged (100,000*g* for 60 min) and the pellet (cell plasma membrane) and supernatant (cell cytosol) collected and used in immunoblots.

The washed mitochondrial pellet was suspended in 10 ml TEZMB buffer (20 mM triethanolamine, 10 mM zinc acetate 1 mM 2-mercaptoethanol and 0.05% Brij-35, pH 7.4) containing 0.5% digitonin and after 10 min was sonicated (2×10 s bursts, 30% output on a Branson Sonifier 250). The sonicate was centrifuged (100,000*g* for 60 min) and the supernatant (mitochondrial extract supernatant) used in the enzyme purification studies.

The levels of the mitochondrial cytochrome C oxidase [Storrie and Madden, 1990] and plasma membrane 5′-nucleotidase activity [Gentry and Olsson, 1975] were measured after each step of the isolation procedures in order to monitor the isolation of the mitochondrial and plasma membrane fractions.

Whole Cell Supernatant Preparation

Digitonin was added to the original cell homogenate, already containing 0.1% digitonin (as described above), until it reached a final concentration of 0.5%. This cell homogenate would contain free mitochondrial enzymes as the mitochondrial membranes are degraded in the presence of 0.5% digitonin [Loewenstein et al., 1970]. After 10 min the homogenate was sonicated (2×10 s bursts, 30% output on a Branson Sonifier 250) before being centrifuged (10 min at 200*g*). The supernatant was recentrifuged (100,000*g* for 60 min) and the resultant supernatant applied to a DEAE sephadex column and partially purified using hydroxyapatite, blue dextran and MonoQ matrices as described below. Gel filtration was not used for the cell cytosol preparation.

HeLa Mitochondrial Oligopeptidase Partial Purification

The mitochondrial extract supernatant was applied to a column (1.6×10 cm) of DEAE-sephadex equilibrated with TEZMB. The sample was loaded at a flow rate of 0.5 ml/min. Proteins were eluted with a 300 ml linear gradient of increasing NaCl (0 M–0.5 M NaCl in TEZMB). Active fractions (4 ml), as assessed by P9 and N-acetyl neurotensin hydrolysis, were then applied to a column (1×8 cm) of hydroxyapatite equilibrated with PPB buffer (1 mM potassium phosphate, 10 mM zinc acetate, 1 mM 2-mercaptoethanol, 0.05% Brij-35, pH 7.4). Proteins were eluted with a 150 ml linear gradient of 1–250 mM potassium phosphate in PPB at a flow rate of 0.5 ml/min and 2 ml fractions were collected. The active fractions were pooled and concentrated to 400 µl using Amicon centriprep-50 and centricon-30 concentrator spin columns. The concentrated fractions were incubated with 400 µl blue dextran (equilibrated in PPB) for 5 min. Blue dextran has been shown to adsorb irrelevant proteins [Serizawa et al., 1995]. This mixture was centrifuged (3,000*g* for 5 min) and 200 µl of the supernatant was applied to a Superose 12 gel column (at room temperature) equilibrated in TEZMB containing 110 mM NaCl. Proteins were eluted at a flow rate of 0.15 ml/min and 0.3 ml fractions were collected. Active fractions were diluted with an equal volume of TB buffer (20 mM Tris-Cl, 50 mM NaCl, 0.05% Brij-35, 10 mM zinc acetate, pH 7.6) and applied to a MonoQ column (at room temperature) equilibrated in TB. Proteins were eluted with a linear gradient of 50–250 mM NaCl in TB at a flow rate of 1.5 ml/min and 1.5 ml fractions were collected. Active fractions were utilised in the characterization assays and aliquots analysed on SDS-PAGE gels.

SDS-Polyacrylamide Gel Electrophoresis

Proteins from different purification steps were separated by SDS-PAGE under denaturing conditions using 10% acrylamide gels. Gels were stained using silver nitrate [Merril et al., 1984].

Peptidase Assays and Inhibition Studies

The nonapeptide H_2N-YVAAAVVSH-NH_2 (denoted P9 herein) was prepared and purified within this institute prior to being iodinated using $Na^{125}I$ as previously described [Brown et al., 1992].

Peptidase fractions were assayed in a final volume of 40 µl containing 20 ng ^{125}I-P9 and 10 mM bestatin. Bestatin was added to the assay mixture to inhibit any contaminating aminopeptidase activity [Suda et al., 1976]. Extracts (35 µl) from the assay medium were applied to channelled TLC plates and separated by ascending chromatography [butanol:H_2O:acetic acid, 100:30:10 (BAW), pH 2.6] [Brown et al., 1992]. Briefly, after development, the TLC plate was subjected to phosphorimaging (Storage phosphorscreen, Kodak). The image was realised by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA) and quantitated using ImageQuant software (Version 3.15). Standards (P9-derived peptides) were added to each plate prior to analysis.

Enzyme specific activity was expressed as ng P9 degraded/min per mg of protein. Protein levels of the samples were determined using the bicinchoninic acid (BCA) method (Sigma) using BSA as the standard.

Selective inhibition of the enzyme was achieved by pre-incubating the sample for 10 min with an equal volume of either 1) thimet oligopeptidase (TOP) antibody (R646/P) (240 µg/ml) or 2) oligopeptidase M (MOP) antibody (K118) (10 mg/ml), prior to the addition of ^{125}I-P9 or neurotensin in a manner analogous to that previously described [Serizawa et al., 1995].

Activity of the HeLa Cell Peptidase Toward Other Synthetic Peptides

The specificity of the HeLa cell peptidase present in fraction 14 of the MonoQ step was determined by HPLC analysis. In each experiment, 30 µl peptide (2–4 nmol) in TB buffer (pH 7.4) was added to 20 µl enzyme (final concentration of peptide 20–40 mM). After 60 min incubation at 37°C, the reaction was terminated by boiling for 3 min followed by the addition of 500 µl 0.09% (v/v) trifluoroacetic acid (TFA). The tubes were centrifuged (20,000*g* for 5 min) and the supernatant applied to a C18 column pre-equilibrated in solvent A (0.09% TFA) and peptide fragments eluted by a linear gradient of 0–50% solvent B (80% CH_3CN/0.09% TFA) over 35 min. The collected fractions were lyophilised, resuspended in 20 µl TFA and the molecular weight of the peptide products determined by mass spectrometry using a Finnigan Mat Lasermat Mass Spectrometer.

Kinetic Analysis of Neurotensin Degradation

The K_m for neurotensin hydrolysis was determined over the concentration range 0–200 mM using the procedure described above. The level of the peptides recovered in the reaction mixture by HPLC were determined using the Bio-Rad integration software and the rate of hydrolysis expressed as nmol neurotensin hydrolyzed/min per 20 µl peptidase. The inhibitory effect of the specific 24.16 dipeptide inhibitor Pro-Ile [Dauch et al., 1991] on neurotensin degradation was measured over the concentration range 0–1 mM. The kinetic parameters (K_m, V_{max}) of neurotensin hydrolysis by the HeLa peptidase and the K_i of Pro-Ile inhibition were determined using Enzpack 3 software (Biosoft, Cambridge, England).

RESULTS

Purification and Identification of Peptidase Activity

A peptidase was partially purified from HeLa cells grown in suspension as described in Materials and Methods using P9 as a substrate. N-acetyl neurotensin was also used to monitor 24.16-like activity during the early steps of purification (data not shown). The results obtained from a typical enzyme purification from the mitochondrial fraction of 10^9 cells are shown in Table I. Very much less than 10 µg of enzyme was obtained from 10^9 cells giving a yield of 1% of cell enzyme activity on P9 but with a much greater than 94-fold purification. SDS-PAGE analyses of the fractions after each chromatographic separation are shown in Figure 1. The amount of protein recovered in the final run was so small that only faint bands were observed in a silver-stained gel. Since we could not detect any protein using either the BCA or fluorescamine protein estimation assays, an accurate determination of the degree of purity for

TABLE I. Partial Purification of Oligopeptidase M From HeLa Cell Mitochondria

Fraction	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Mitochondrial extract	125.6	30	4.2	100	1
DEAE	81.9	8.4	9.8	65	2.3
Hydroxyapatite	36.0	1.5	24.7	29	5.9
Blue dextran	5.0	0.08	64	4	15.2
Gel filtration	3.9	<0.01	>394	3	>94
MonoQ	1.3	$\ll 0.01$	$\gg 394$	1	$\gg 94$

The mitochondrial fraction was isolated from 1 g of HeLa cells. Activity of the enzyme was determined by using the substrate ^{125}I-P9 as described in Materials and Methods. One unit is defined as the amount of enzyme that cleaves 1 ng P9/min.

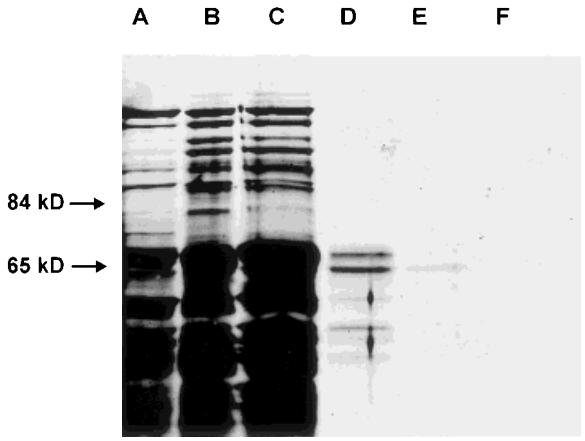

Fig. 1. Purification of oligopeptidase M from HeLa cells. SDS-PAGE gel of samples obtained during the partial purification of oligopeptidase M from the mitochondrial fraction of HeLa cells. Protein was stained with silver stain. **A:** DEAE-sephadex fraction. **B:** Hydroxyapatite fraction. **C:** Blue dextran fraction. **D:** Gel filtration fraction 12. **E:** Gel filtration fraction 13. **F:** MonoQ fraction 13 (final product).

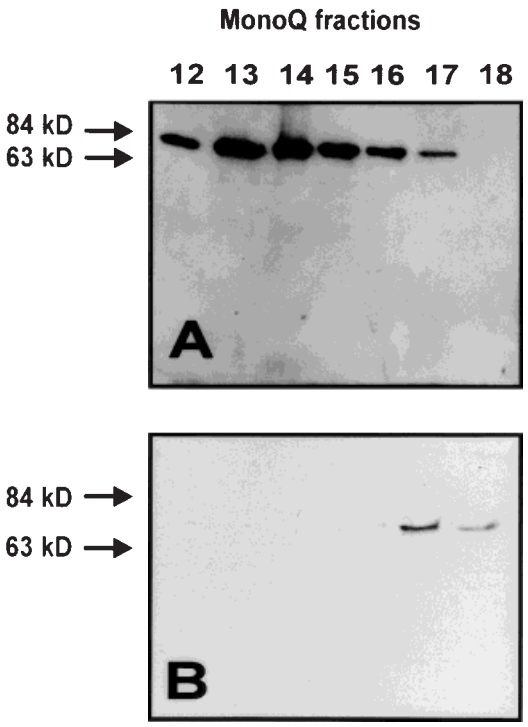

Fig. 2. Distribution of oligopeptidase M and thimet oligopeptidase in MonoQ fractions of a whole cell supernatant preparation. Immunoblots of samples from an SDS-polyacrylamide gel of fractions obtained from the MonoQ run of the whole cell supernatant were developed with either **(A)** K118 (MOP antibody) or **(B)** R646/P (TOP antibody).

the HeLa enzyme was not feasible. We were able to show an approximate molecular weight of 55–63 kD for the HeLa enzyme using gel filtration.

Through the use of two antibodies, K118 (specific for oligopeptidase M) and R646/P (specific for thimet oligopeptidase), both kindly supplied by Dr. A.J. Barrett, we were able to discriminate these peptidases during the purification procedure. In a recent study it was shown that after 10 min incubation of the purified rat MOP or TOP with their respective antibody, greater than 90% of the enzyme activity was inhibited [Serizawa et al., 1995]. We also used these antibodies in Western blots and immunoinhibition studies.

In the whole cell extracts TOP co-purified with MOP through the DEAE, hydroxyapatite and blue dextran separation steps. TOP was shown by immunoblotting to elute later than MOP from the MonoQ column (Fig. 2) which was detected in fractions 12–17 of the MonoQ column eluate with maximal staining observed in fractions 13–15. On the other hand TOP was detected mainly in fractions 16–18. The effect of these antibodies on the hydrolysis of P9 by the MonoQ fractions confirmed that MOP predominates in fractions 12–14 while TOP predominates in fractions 16–18 (data not shown).

MOP has been shown to be a mitochondrial enzyme [Serizawa et al., 1995]. Mitochondria isolated from HeLa cells [Moreadith and Fis-

kum, 1984] were solubilised and the extracts separated using the procedures described for the cell extracts. TOP and MOP were both detected in the initial HeLa cell mitochondrial fraction, but after blue dextran adsorption, gel filtration and MonoQ chromatography MOP alone was detected (Fig. 3). The active MOP fractions of the mitochondrial MonoQ run (Fig. 4) were shown to cleave P9 between the hydrophobic amino acids Ala-Ala (producing P3 and P4) and Ala-Val (producing P5). The activity of fraction 14 of the MonoQ step containing the mitochondrial enzyme was inhibited 83% and 13% by K118 and R646/P, respectively. Fraction 14 of the MonoQ step was used for further characterization studies of the mitochondrial MOP enzyme as described below.

Neurolysin-Like Activity of the HeLa MOP

Serizawa et al. [1995] suggest that MOP is the same enzyme as endopeptidase 24.16 neurolysin. This enzyme has been shown to cleave neurotensin to produce two fragments: neurotensin (1–10) and neurotensin (11–13) [Serizawa et al., 1995; Checler et al., 1986, 1995; Vincent et al., 1996b]. The HeLa mitochondrial enzyme was also shown to hydrolyse neurotensin producing neurotensin (1–10) and neurotensin (11–13) fragments as seen in Figure 5. A K_m of 7 µM and a V_{max} of 64 pmol/min per 20 µl enzyme were obtained. The activity of the HeLa mitochondrial enzyme was inhibited by 1) K118 (Fig. 5) and 2) Pro-Ile, a specific 24.16 dipeptide inhibitor [Dauch et al., 1991], but not by the

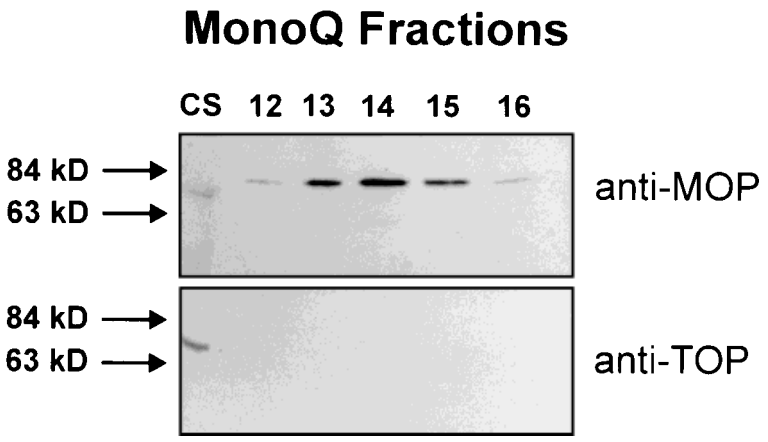

Fig. 3. Distribution of oligopeptidase M and thimet oligopeptidase in MonoQ fractions of a mitochondrial extract. Immunoblots of the cytosolic supernatant (CS) and the MonoQ fractions of a mitochondrial extract after separation by SDS-PAGE were developed with either **(upper)** K118 (MOP antibody) or **(lower)** R646/P (TOP antibody) as described in Materials and Methods.

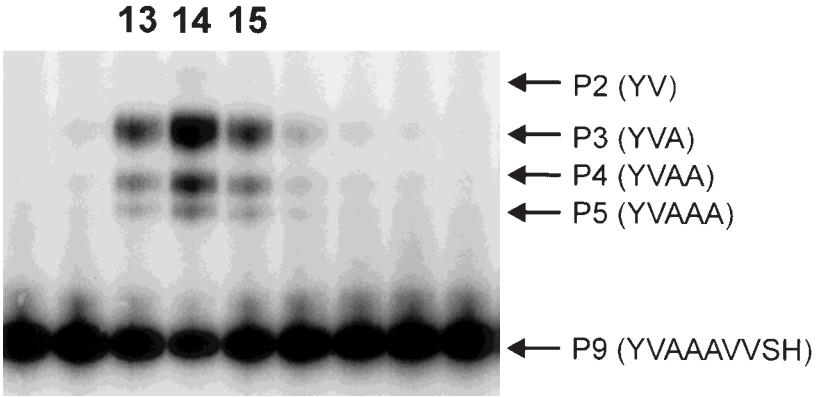

Fig. 4. Hydrolysis of P9 by MonoQ fractions of the mitochondrial preparation. Fractions from the final MonoQ run were incubated with ^{125}I-P9 and the hydrolytic products separated by TLC, developed onto a phosphor screen to give the above phosphorimage as described in Materials and Methods. Structures of the peptide fragments are P2 YV; P3 YVA; P4 YVAA; P5 YVAAA; and P9 YVAAAVVSH.

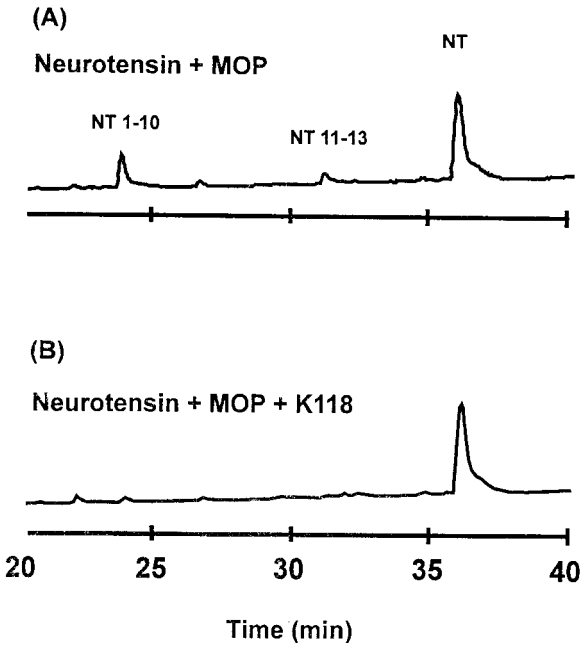

Fig. 5. Immunoinhibitory effect of K118 (MOP antibody) on the hydrolysis of neurotensin (NT) by the HeLa MOP. Neurotensin (2 nmol) was incubated for 1 h at 37°C with 20 µl of HeLa MOP from fraction 14 of the MonoQ run in the absence **(A)** or presence **(B)** of K118. HPLC analysis was performed as described in Materials and Methods. Peaks derived from NT are labelled as follows: peak (A) NT; (B) NT(1–10) and (C) NT(11–13).

TOP antibody R646/P. The K_i for Pro-Ile was calculated to be 140 µM, which was similar to that reported for the rat (90 µM) [Dauch et al., 1991] and human brain enzyme (270 µM) [Vincent et al., 1996b].

N-acetyl neurotensin (8–13), $GnRH^{1-9}$ and dynorphin A^{1-17} were also degraded by the HeLa enzyme, while somatostatin (LHRH) and GnRH were not. $GnRH^{1-9}$ (88 µM) was degraded at twice the rate (110 pmol/min per 20 µl enzyme) of neurotensin (57.6 pmol/min/20 µl of enzyme) at the same concentration.

Inhibition Profile of the HeLa Cell MOP

The HeLa enzyme was shown to be a metalloenzyme as EDTA and 1,10-phenanthroline inhibited activity as can be seen in Table II. Other inhibitors had no effect on enzyme activity except for 1) 1 mM DTT, which elicited a 50% inhibition of activity, agreeing with that seen previously in rat liver MOP [Serizawa et al., 1995] and 2) 1 mM $ZnCl_2$, which totally inhibited activity which was similar to that seen in other studies on zinc proteases [Barrett and Brown, 1990; Larsen and Auld, 1991].

TABLE II. Effect of Protease Inhibitors on MOP Activity Observed in HeLa Cells Compared to That Observed in Other Tissues

Inhibitor (concentration)	HeLa cells	Human brain[a]	Rat brain[a]	Rat liver[b]	Pig brain[c]
Control	100	100	100	100	100
EDTA (1 mM)	20	3[d]	1[d]	0[d]	20
1,10 phenanthroline (1 mM)	0	6	3	0	6
PMSF (1 mM)	100	106	96		
E 64 (100 µM)	100	107[e]	94[e]		
Leupeptin (10 µM)	100	89[e]	78[e]		
$ZnCl_2$ (1 mM)	0				
DTT (1 mM)	50				

Inhibitors (at the final concentrations listed) were added to 20 µl of fraction 14 from the cell supernatant MonoQ run prior to the addition of 20 ng ^{125}I-P9 in a final volume of 50 µl. Analysis of the hydrolysis of P9 was as described in Materials and Methods. Activity in the absence of inhibitors (control) was expressed as 100% and the effect of the inhibitors on activity normalised to this value. Results are expressed as the means of three separate experiments.

[a] Vincent et al. [1996b].
[b] Serizawa et al. [1995].
[c] Dando et al. [1993].
[d] 10 mM inhibitor used.
[e] 1 mM inhibitor used.

Plasma Membrane Isoform of MOP

The main portion of MOP is found in the mitochondria though a significant fraction was detected in the plasma membrane with a trace detected in the cytosol as seen in Figure 6. In order to confirm that these fractions were not contaminated with mitochondrial fragments we used the marker enzymes cytochrome C oxidase [Storrie and Madden, 1990] and 5′-nucleotidase [Gentry and Olsson, 1975] to detect mitochondrial and plasma membrane fragments, respectively, during the isolation procedure. Table III shows that cytochrome C oxidase activity was undetected in the plasma membrane fraction, indicating that this fraction was not contaminated with mitochondrial membranes. The level of 5′-nucleotidase activity in the mitochondrial pellet was less than that detected in the plasma membrane, but still represents significant contamination of the mitochondrial fraction with cell membranes. The activity of the hydrolysis of P9 in the plasma membrane fraction was not inhibited by 1 mM Pro-Ile indicating that no significant peptidase activity attributable to MOP was present in the plasma membrane fraction. This result suggests that

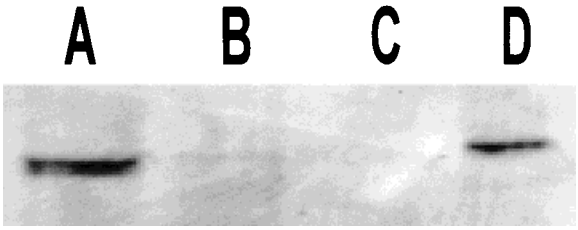

Fig. 6. Subcellular localization of oligopeptidase M in HeLa cells. Immunoblot of samples subcellular fractions after separation by SDS-PAGE were developed with K118 (MOP antibody) as described in Materials and Methods. **Lane A:** Mitochondrial pellet. **Lane B:** Supernatant obtained after the 11,000*g* spin of cell homogenate. **Lane C:** Supernatant obtained following the 100,000*g* spin. **Lane D:** Plasma membrane pellet following the 100,000*g* spin of the cell supernatant.

TABLE III. Monitoring the Subcellular Fractionation of HeLa Cells

Cell fraction	Relative cytochrome C oxidase activity	Relative 5′-nucleotidase activity
Intact cell	1	1
Cell homogenate	17	2
Cytosol	5	1
Plasma membrane	—[a]	139
Mitochondrial supernatant	138	—
Mitochondrial pellet	355	89

Samples were assayed for cytochrome C oxidase and 5′-nucleotidase activity after each step of the cell fractionation procedure as described in Materials and Methods. The level of cytochrome C oxidase and 5′-nucleotidase activity/mg protein in the fractions was normalised with intact cells representing 1.0 and that of the various fractions were compared, therewith. Results expressed are the means of three separate experiments.

[a] Not detected.

MOP was present in the plasma membrane as an inactive apoenzyme.

DISCUSSION

This study describes the first reported purification and characterization of oligopeptidase M (MOP) (EC 3.4.24.16) from human tissue cultured cells. This enzyme has also been called neurolysin and endopeptidase 24.16 in the literature. It has been previously characterised in rat brain [Checler et al., 1986], rat liver [Serizawa et al., 1995] and most recently in human brain [Vincent et al., 1996b]. MOP has been shown to be a species variant of both the pig-soluble angiotensin II-binding protein and rabbit microsomal endopeptidase and is a close homologue of thimet oligopeptidase (TOP) (EC 3.4.24.15) [Serizawa et al., 1995].

The first steps in the purification of HeLa MOP were performed using DEAE and hydroxyapatite resins in a manner analogous to that used in purifying the rat and human endopeptidase 24.15/24.16 enzymes [Checler et al., 1986; Serizawa et al., 1995; Vincent et al., 1996b]. The HeLa MOP was shown to elute at similar positions in the gradients applied to these columns compared to that seen for rat endopeptidase 24.16 [Checler et al., 1986]. Despite the similarity in elution conditions the hydroxyapatite column did not effect as good a separation of the human TOP and MOP as that seen for the pig [Millican et al., 1991] and rat enzymes [Checler et al., 1986]. Hydroxyapatite fractions 24–30 contained both MOP and TOP which were separated after the MonoQ run (see Figure 2 for the whole cell preparation and Figure 3 for the mitochondrial preparation). In other studies using porcine and murine tissue [Millican et al., 1991] these enzymes were separated using hydroxyapatite and it may be that the human enzymes share a greater degree of homology which makes purification more difficult.

In the whole cell supernatant MOP elutes from the MonoQ column before TOP, with overlap occurring in some fractions (Fig. 2). The MOP antibody was shown to inhibit activity in fractions 12–18, even though it did not detect the protein in fraction 18 on the Western blot. Since the TOP antibody did not inhibit activity in fractions 12–14 of the MonoQ run, only MOP is present in these fractions, but in the other fractions (15–18) both enzymes were shown to be present. The MOP antibody did not detect any protein in fraction 18 but showed significant inhibition of P9 hydrolysis. This may be due to the fact that antibodies have different sensitivities when their immunoblotting characteristics are compared with their ability to inhibit some function of their antigen (such as receptor binding, enzymatic activity and infection suppression; for further discussion see [Roitt, 1991]). The difference may also be related to the affinity of the antibodies for the enzyme and the degree by which they inhibit activity: in this case the R646/P antibody may have a lower affinity for TOP than does the K118 antibody for MOP. Another explanation for the observed difference elicited by the K118 antibody in fraction 18 is that the MOP present is an isoform of that seen in the other fractions.

The R646/P and K118 antibodies were effective in Western blots and there appears to be no cross reactivity between the two, as seen in the immunoblot of the MonoQ run of the mitochondrial preparation (Fig. 3). The TOP antibody inhibited 13% of the P9 hydrolyzing activity of fraction 14 from the mitochondrial MonoQ run compared to 81% inhibition by the MOP antibody on this fraction, which agrees with that seen on the Western blots.

Very small quantities of MOP were obtained from the HeLa mitochondrion, despite using 1 g cells (Table I). In concentrating the sample (16 → 1 ml) following the hydroxyapatite run there was a significant loss of activity (sevenfold reduction in protease activity). The loss in activity could have been due to protein adsorption to the membrane of the centricon filters. The silver-stained SDS-PAGE (Fig. 1) shows some very faint bands which indicate that several proteins are present after the final MonoQ run. MOP has been isolated and characterised from hepatic and neural tissue [Checler et al., 1986; Serizawa et al., 1995; Vincent et al., 1996b]. In those studies where MOP has been isolated and characterized only very small amounts have been isolated from large amounts of starting material e.g. 1.7 mg MOP isolated from 61 g human neural tissue [Vincent et al., 1996b]. Immunologically, MOP has been detected at high concentrations in the brain, liver and testes but at low levels in other tissues of the rat [Checler et al., 1989, 1995].

In this study through the use of the ^{125}I-labelled P9 substrate we were able to detect enzyme activity throughout the purification process (Fig. 4), even though the level of protein in the fractions was below measurable limits. The high sensitivity and rapid analysis of the P9 assay [Brown et al., 1992] made it a valuable tool in this study. MOP was shown to cleave between the small hydrophobic amino acids in P9 (YVA†A†A†VVSH) as well as aromatic and charged residues in neurotensin (ELYENKPRRP†YIL) and GnRH$^{1-9}$ (EHWSY†GLRP). Of particular interest is the novel ability of MOP to cleave post-proline. Knight et al. [1995] concluded that TOP could be regarded as a peptidylpeptidase as it would hydrolyse toward the C-terminal of oligopeptides, releasing C-terminal fragments of up to six residues. Dando et al. [1993] showed that all the scissile bonds for the human TOP were located at least three residues into the oligopeptide substrate from a free N- or C-terminus. A similar conclusion can be reached concerning the action of MOP on peptides as seen from the substrates processed by this enzyme [Serizawa et al., 1995; Checler et al., 1995], and the P3, P4 and P5 products from P9 in this study.

MOP is similar to TOP as they are both metallopeptidases and sensitive to EDTA and 1,10-phenanthroline but not to serine or thiol protease inhibitors [Serizawa et al., 1995; Tisljar, 1993]. They are both inhibited by mercurial and thiol blocking compounds such as N-ethylmaleimide [Serizawa et al., 1995]. TOP is activated by 1 mM DTT while MOP is inhibited [Barrett et al., 1995; Serizawa et al., 1995]; this difference might be explained by the five conserved cysteine residues which are present in TOP but not in the rat MOP [Barrett et al., 1995]. The HeLa enzyme has the same properties as the human brain and rat hepatocyte MOP enzyme with regard to its inhibition profiles and sensitivity to DTT (Table II), indicating its identity with them. Activity of the HeLa enzyme was inhibited by 1 mM $ZnCl_2$ which was similar to that observed with other zinc metalloproteases which have been exposed to high concentrations of Zn^{2+} [Barrett and Brown, 1990; Larsen and Auld, 1991].

HeLa MOP also possesses endopeptidase 24.16-like activity [Checler et al., 1986; Vincent et al., 1996b] as it was shown to degrade neurotensin between the Pro^{10}-Tyr^{11} bond (Fig. 5). The enzyme was inhibited by Pro-Ile (K_i = 140 μM), a value which was similar to that seen in human neural tissue (270 μM) [Vincent et al., 1996b] but was less than that observed in rat liver (500 μM) [Serizawa et al., 1995]. Recently a new variant, endopeptidase 24.16B, has been discovered which is activated by 1 mM DTT and has a high K_i value for Pro-Ile of 6 mM [Rodd and Hersh, 1995]. The 24.16B variant degrades neurotensin at the Arg^8-Arg^9 bond and the Pro^{10}-Tyr^{11} bond which differs from the HeLa MOP described herein.

Millican et al. [1991] isolated a neurotensin degrading enzyme from porcine brain which also processed GnRH. This enzyme cleaved the Tyr^5-Gly^6 and Gly^6-Leu^7 bonds of GnRH at a much slower rate than that observed for neurotensin. Lew et al. [1994] showed that endopeptidase 24.15 has a higher affinity for GnRH$^{1-9}$ than GnRH and suggested that GnRH metabolism in the rat hypothalamus occurs via a two step process where Gly^{10}-NH_2 is removed from

GnRH by prolyl endopeptidase prior to cleavage of the Tyr^5-Gly^6 bond by endopeptidase 24.15. Interestingly, the HeLa MOP also degrades $GnRH^{1-9}$ with the same specificity as endopeptidase 24.15 but is unable to process GnRH. This suggests that MOP may be involved in the hypothalamic degradation of GnRH, which may explain why it is found in this tissue [Dauch et al., 1992].

Endopeptidase 24.16 has been shown to process somatostatin but at rates 1/500th that of neurotensin [Dando et al., 1993]. We were unable to confirm somatostatin cleavage for the HeLa MOP, but this could be due to the low levels of enzyme used in the assay mixture.

HeLa MOP degraded dynorphin A^{1-17}, which suggests that this enzyme may be involved in regulating those peptides involved in steroid biogenesis. Pro-dynorphin-derived peptides and luteinising hormone are released by gonadotrope cells in the rat adenohypophysis which influence steroid production [Spampinato et al., 1995]. MOP is located mainly in the intermembrane space of the mitochondria, where the initial, rate-limiting step of steroid synthesis occurs. Cholesterol influx into the inner mitochondrial membrane from the outer membrane is the rate limiting step for the synthesis of pregnenolone, which is the precursor for all steroid molecules [Guarneri et al., 1992]. The influx of cholesterol from the outer mitochondrial membrane is stimulated by the regulatory protein, steroidogenic acute regulatory protein (StAR), which is also located in the inter membrane space [King et al., 1995]. Little is known about the role of StAR in steroid production, but it is possible that MOP may regulate its bioactivity by processing it further when it reaches the inter membrane space.

MOP has been detected in the mitochondria of rat hepatocytes [Serizawa et al., 1995], though not in astrocytes or neurons [Vincent et al., 1996a]. We have also shown that in the HeLa cell that MOP is primarily a mitochondrial enzyme though was detected in immunological blots of plasma membrane extracts (Fig. 6). The plasma membrane ectopeptidase activity was not sensitive to Pro-Ile inhibition, which raises the possibility that it may reside on the cell surface in an inactive form, along with several other ecto-endopeptidases previously described [Brown et al., 1993]. Many other proteins have been shown to be transported into the mitochondria, where upon cleavage they become activated [Klaus et al., 1996]. Most imported precursor proteins are cleaved either by the mitochondrial intermediate peptidase or mitochondrial processing peptidase [Branda and Isaya, 1995]. The function of the plasma membrane MOP is not known and may represent an intermediate in the pathway between the cytoplasmic and mitochondrial forms of the enzyme. This pathway may be analogous to the transport of lysosomal acid phosphatase from the cytoplasm to lysosomes via the cell membrane [Braun et al., 1989].

Endopeptidase 24.16 was the only enzyme shown to degrade neurotensin in a wide variety of cell lines [Checler et al., 1993]. It has also been shown to inactivate neurotensin in vivo in the gastrointestinal tract [Barelli et al., 1994; Checler et al., 1995]. Endopeptidase 24.16 has been identified in a wide range of tissues and occurs in many subcellular compartments, which suggests that his enzyme probably performs a number of different functions [Checler et al., 1993, 1995; Barrett et al., 1995]. We have shown that in the HeLa cell that this enzyme is found in two different sites and processes a wide range of substrates.

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