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Characterization of Thiol-, Aspartyl-, and Thiol-metallo-peptidase Activities in Madin-Darby Canine Kidney Cells

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Abstract We combined fluorogenic substrates or internally quenched fluorescent peptides with specific inhibitors in the pH profile of proteolytic activity experiments in order to detect proteolytic activities in lysates of MDCK cells. Hydrolytic activities related to cathepsin B, L, and D were observed. Serine-proteinase was not detected; however, we clearly demonstrated the presence of a thiol-metallo-endo-oligopeptidase, also called thimet-oligopeptidase (TOP). This peptidase from MDCK cells has substrate and inhibitor specificities as well as an activation profile with mercaptoethanol that are indistinguishable from the recombinant rat testis TOP (EC 3.4.24.15). In addition, polyclonal purified antibodies to this enzyme depleted the TOP activity of MDCK cells in whole homogenate. Although we present only preliminary data, TOP is secreted by MDCK cells. The presence of TOP in a phenotype polarized MDCK cells can have special significance in the cytoplasmic selection, transport, or clearance of short peptides due to restriction of the enzyme to sequences from 6 to 17 amino acids. Therefore, the MDCK cell could be a very useful cellular model with which to study some of the suggested TOP biological functions as processing of biological active peptides and antigen presentation. J. Cell. Biochem. 76:478–488, 2000.

Key words: MDCK cells; THIMET; endooligopeptidase; cathepsin B; cathepsin L; cathepsin D

The Madin-Darby canine kidney (MDCK) cell line is one of the best-characterized cultured monolayer cells that display many features of in vivo epithelia [Gstraunthaler, 1988; Madin and Darby, 1958; Cho et al., 1989]. As MDCK is derived from renal cortical cells and has many structural and functional similarities to the cortical collecting duct epithelial cells, these cells have been used to study water and ion transport regulation [Oberleitner et al. 1990, 1991; Vallés, 1997]. In addition, MDCK cell epithelial preparation as well as in isolated form responds to hormones as cortisol [Baker and Fanestil, 1977], vasopressin [Rindler et al., 1979], glucagon [Beckner et al., 1985], bradykinin [Jan et al., 1998], angiotensin [Morrison et al., 1989], as well as to protease as tissue kallikrein [Vallés et al., 1997] and drugs as amiloride [see reviews by Handler et al., 1980; Morrison et al., 1989]. MDCK cells, as other epithelial cells, are able to perform specialized vectorial functions such as absorption and exocrine secretion because they have a polarized phenotype; that is, their plasma membranes are divided into apical and basolateral domains, separated by tight junctions. As a result of these specific functions, MDCK cells have been useful for studies of polarized secretion [Fritz and Lowe, 1996; Lösch and Koch-Brandt, 1995; Roush et al., 1998]. MDCK cells were demonstrated to secrete vectorially over expressed proteolytic enzymes involved in renal physiology, namely, neutral endopeptidase (NEP) [Jalal et al., 1991] and tissue kallikrein [Abe et al., 1995]. Some specific proteases have been described in MDCK cells as carboxypeptidase M [Deddish et al.,

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1990], a basic carboxypeptidase found in urine purported to participate on the kinin metabolism, and membrane type 1 matrix metalloproteinase [Kadono et al., 1998] that was described to be involved in formation of branching tubules in MDCK cells. In addition, it was demonstrated that human influenza virus depends on endogenous cellular proteases for replication in MDCK cell lines [Noma et al., 1998].

This article reports the presence of a thiol-metallopeptidase in MDCK cells, besides cathepsin B, L, and D activity, in whole homogenate, using fluorogenic substrates and internally quenched fluorescent peptides [Juliano et al., 1990; Chagas et al., 1990]. For the metallopeptidase hydrolytic activity, the substrate and inhibitor specificities, as well as the activation profile with mercaptoethanol, are indistinguishable from rat testis thiol-activated metalloendopeptidase (EC 3.4.24.15). Unlike typical proteinases, this enzyme presents strict selectivity for oligopeptides, which characteristically hydrolyze peptides containing less than 17 amino acids, without a clearly define specificity for any amino acid. The generic name of this enzyme is thimet oligopeptidase (TOP) [Barrett et al., 1995; Camargo et al., 1997; Lew et al., 1995; Shrimpton et al., 1997; Tsljar, 1993]. TOP is proposed to be involved in the metabolism of neuropeptides including gonadotropin-releasing hormone, bradykinin, neurotensin, and opioid peptides [Dando et al., 1993; Lew et al., 1995; Camargo et al., 1994; 1997; Shrimpton et al., 1997]. Owing to the distribution of TOP in the cytosol of several mammalian tissues and the similarity between the length of TOP-susceptible peptide substrates and class I peptide epitopes, it has been proposed that TOP participates in the proteolytic processes of antigen presentation [Heemels et al., 1995; Engelhard, 1994]. According to this view, TOP was demonstrated to be inhibited with high affinity by major histocompatibility class (MHC) class I epitopes [Portaro et al., 1999]; more direct evidence was described relating TOP to antigen presentation [Silva et al., 1999]. A further indication that TOP plays a possible role in the cytoplasmic selection, transport, or clearance of short peptides was the demonstration that the soluble angiotensin II-binding proteins described in pig liver [Sugiura et al., 1992] and TOP were very similar, but not identical, proteins [McKie et al., 1993]. However, soluble angiotensin II-binding protein and neurolysin (3.4.24.16) are identical and this enzyme has high sequence homology and hydrolytic properties very similar to those exhibited by TOP [Serizawa et al., 1995; Dauch et al., 1995; Kato et al., 1997].

MATERIALS AND METHODS

Cell Culture

MDCK cells were purchased from American Type Culture Collection and stored in liquid nitrogen freezer until used. Cells were thawed and grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin, in bottles of 25 cm^2 of surface. Cells were grown at 37°C in a humid atmosphere of 95% air/5% CO_2. When cultures became confluent, subcultures were prepared after detaching cells with 0.05% trypsin and 0.5 mM EDTA. Cells from confluent monolayers were used for enzyme studies. Monolayers were washed five times with ice-cold phosphate-buffered saline (PBS), and the cells harvested by scraping with rubber spatula. Trypan blue dye exclusion tests were performed to indicate cell viability.

Cell Disruption

Whole cell homogenates were obtained suspending the washed cells in PBS, cooled on ice, and disrupted by sonication (3 × 30-s pulses at a power setting of 60%), using a Sonic Dismembranator with a microprobe (Heat Systems, Farmingdale, NY). The resulting suspensions were centrifuged at 1,000*g* for 5 min, at 4°C. The supernatants were removed, aliquoted, and stored at −20°C. The total protein concentrations were determined according to Bradford [1976].

Secretion Experiments

Confluent MDCK cells were washed and incubated in DMEM without calf serum addictive (pH 7.4) under the same conditions described for growing cells. Aliquots of media were collected after 5 min, 1 h, 6 h, and 16 h, for proteolytic activity measurements. A solution constituted of 1 ml of these media, 1 µM E-64, 5 µM pepstatin, 1 mM PMSF, and 5 mM mercaptoethanol in a thermostated cuvette at 37°C was stirred for 10 min. Then, 10 µl of substrate stock solution (Abz-RPPGFSPFRQ-EDDnp)

prepared in 30% DMSO was added, and the initial velocities of the substrate hydrolysis were obtained by continuously recording the fluorescence for 5–10 min. Similar experiments were performed in the presence of Cpp-AAF-pAb (N-(1-carboxy-3-phenylpropyl)-Ala-Ala-Phe-para-aminobenzoic) a specific inhibitor of TOP.

Preparation of the Recombinant Rat Testes TOP and Its Immobilization on Sepharose for an Affinity Column Preparation

The purified recombinant rat testes TOP (rTOP) was obtained as previously described [Glucksman and Roberts, 1995]. Briefly, *Escherichia coli* containing the TOP cDNA inserted in the plasmid pGEX-4T-1 (Pharmacia-LKB Uppsala, Sweden) was grown with antibiotic selection to an absorbance reading of 0.6 at 600 nm, when the expression of the TOP-fusion gene was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside. Bacteria were disrupted by two cycles of freezing and thawing, followed by sonication. After removal of bacterial debris, the supernatant was incubated with gluthathione-Sepharose beads. Part of these beads were treated with thrombin to cleave out the rTOP in its soluble form (rTOP). Thrombin was removed by exhaustive filtration in a Centricon 50 (Amicon, Beverly, MA). A general yield of 2.5 mg of pure protein per liter of culture was obtained, assayed by native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amino acid composition of the purified enzyme [Asp, 57; Glu, 97; Ser, 32; Gly, 46; His, 16; Arg, 38; Thr, 35; Ala, 58; Pro, 34; Tyr, 18, Val, 43; Met, 18; Ile, 18; Leu, 71; Phe, 27; Trp (not determined); Lys, 41] reproduced the theoretical composition values of the rat testes TOP [Barrett et al., 1995]. The homogeneity of the enzyme preparation was confirmed by MALDI-TOF mass spectrometry (TofSpec-E, Micromass, Manchester, UK). Calculated MW, MH^+ = 78315,51; obtained MW, MH^+ = 79748,33. A difference of approximately 2% is a reasonable error for the low accuracy condition for the external calibration employed. The purified enzyme was aliquoted in vials containing about 1 μg of enzyme in 2% serum albumin and 30% glycerol. After an initial reduction in 15% the enzyme activity remained constant for 2 months at −70°C.

Part of the beads containing rTOP were covalently immobilized for antibody purification, employing the following procedure. The beads were washed with 0.1 M borate, pH 8.0, and equilibrated with 0.2 M triethanolamine, pH 8.2, followed by the addition of 40 mM dimethylpimelimidate (DMP) (Pierce) in the same triethanolamine buffer, and kept at 25 C for 1 h. The beads were washed sequentially with 40 mM ethanolamine, pH 8.2, 0.1 M borate pH 8.0, and PBS containing reduced 20mM glutathione. The resin was stored in PBS containing azide 0.5%.

Antiserum Preparation

Polyclonal antiserum against purified rTOP was raised in rabbits as previously described [Silva et al., 1999]. The antiserum was purified in a column of Sepharose-glutatione, followed by affinity chromatography, in a column with the beads containing immobilized rTOP, described above; 5 M NaI eluted the antibodies. They were dialyzed extensively against 0.1 M sodium phosphate, pH 7.4. The obtained antibodies (27 mg) was immobilized in 3 ml Sepharose-protein A (Pierce) resin employing DMP, as described above for rTOP immobilization.

Peptides

Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were purchased from Novabiochem (San Diego, CA). All the internally quenched fluorescent peptides of general structure Abz-peptidyl-EDDnp (qf peptides) containing Gln at the C-terminus were synthesized by the solid-phase method [Hirata et al., 1994], whereas the peptide without Gln was synthesized by the classic solution method [Juliano et al., 1990]. All peptides were subjected to purification by preparative reverse-phase high-performance liquid chromatography (HPLC). The HPLC column elutes were monitored by absorbency at 220 nm and by fluorescence emission at 420 nm after excitation at 320 nm. MALDI-TOF mass spectrometry and amino acid composition were used to check the molecular weight and purity of synthesized peptides. The concentrations were determined by pico-Tag amino acid analysis after acid hydrolysis [Heinrikson and Meredith, 1984], as well as by colorimetric determination of 2,4-dinitrophenyl group (extinction coefficient at 365 nm was 17,300 $M^{-1}cm^{-1}$).

Fluorimetric Assays

Fluorimetric assays using qf peptides [Chagas et al., 1990, Juliano et al., 1990] were per-

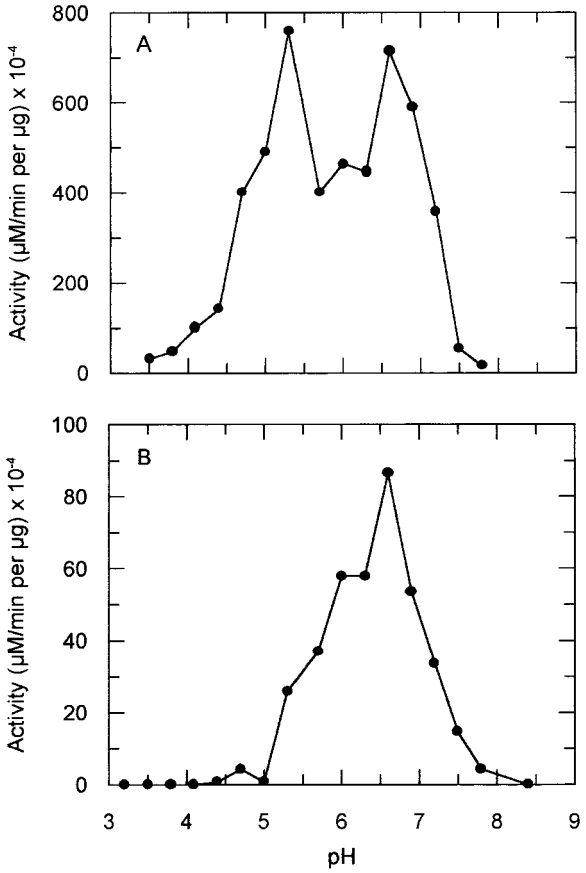

Fig.1. pH profile of hydrolytic activity of lysate of Madin-Darby canine kidney (MDCK) cells assayed with Z-Phe-Arg-MCA (**A**) and Z-Arg-Arg-MCA (**B**) in presence of 1 mM EDTA, 1 mM PMSF, 5 μM pepstatin, and 5 mM DTT. Preincubation time: 10 min. Buffer: 0.1 M potassium biphthalate (pH 3.0–3.5), 0.1 M sodium acetate (pH 3.5–5.5), 0.1 M sodium phosphate (pH 5.5– 8.0), and 0.1 M Tris-HCl (8.0–9.0), 37°C. Specific activity units = [(μM/min)/ μg of total protein] × 10^{-4}.

formed on a Hitachi model F-2000 spectrofluorimeter set at λ_{ex} = 320 nm and λ_{em} = 420 nm. Before starting the reaction by the addition of 10 μl of substrate stock solution, prepared in 30% DMSO, the lysate (10–100μl) was preincubated for 10 min in a thermostated cuvette at 37°C, with 2.0 ml of buffer containing the inhibitors of hydrolytic activity we wanted to suppress (1μM E-64, 5 μM pepstatin, 1 mM PMSF, 1 mM EDTA) and thiol activator when necessary (5 mM DTT or 5 mM mercaptoethanol). Initial velocities were obtained by continuously recording the fluorescence for 5–10 min. The following buffers were used: 0.1 M potassium biphthalate (pH 3.0–3.5), 0.1 M sodium acetate (pH 3.5–5.5), 0.1 M sodium phosphate (pH 5.5–8.0), and 0.1 M Tris-HCl (8.0–9.0).

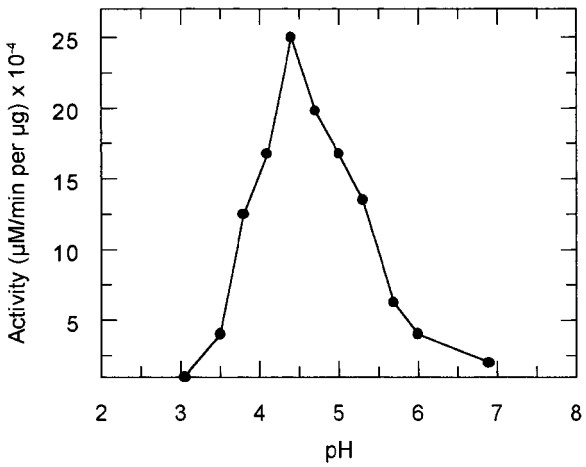

Fig. 2. pH profile of hydrolytic activity of lysate of Madin-Darby canine kidney (MDCK) cells assayed with Abz-KPILFFRQ-EDDnp in the presence of 1 μM E-64, 1 mM PMSF, and 1 mM EDTA. Preincubation time: 10 min. Buffer: 0.1 M potassium biphthalate (pH 3.0–3.5), 0.1 M sodium acetate (pH 3.5–5.5), and 0.1 M sodium phosphate (pH 5.5–8.0) 37°C. Specific activity units = [(μM/min)/ μg of total protein] × 10^{-4}.

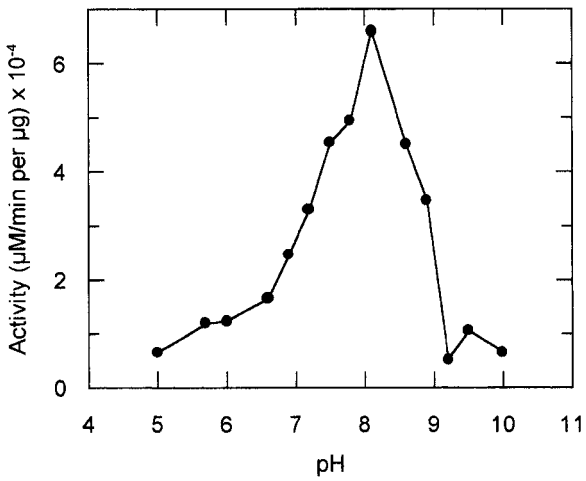

Fig. 3. pH profile of hydrolytic activity lysate of Madin-Darby canine kidney (MDCK) cells assayed with Abz-RPPGFSPFRQ-EDDnp in the presence of 1 μM E-64, 1 mM PMSF, 5 μM pepstatin, and 5 mM mercaptoethanol. Preincubation time: 10 min. Buffer: 0.1 M sodium acetate (pH 3.5–5.5), 0.1 M sodium phosphate (pH 5.5–8.0), and 0.1 M Tris-HCl (8.0–9.0), 37°C. Specific activity units = [(μM/min)/ μg of total protein] × 10^{-4}.

TOP-like Activity Assays for HPLC Analysis

The TOP-like activity assays for HPLC analysis were performed in a final volume of 500μl of 0.1 M sodium phosphate buffer pH 8.0, containing 2 μg of purified rTOP or 10–50 μl of MDCK whole cell lysate with 5 mM mercaptoethanol, and approximately 30 μM of the substrates. Samples were incubated at 37°C, and the reac-

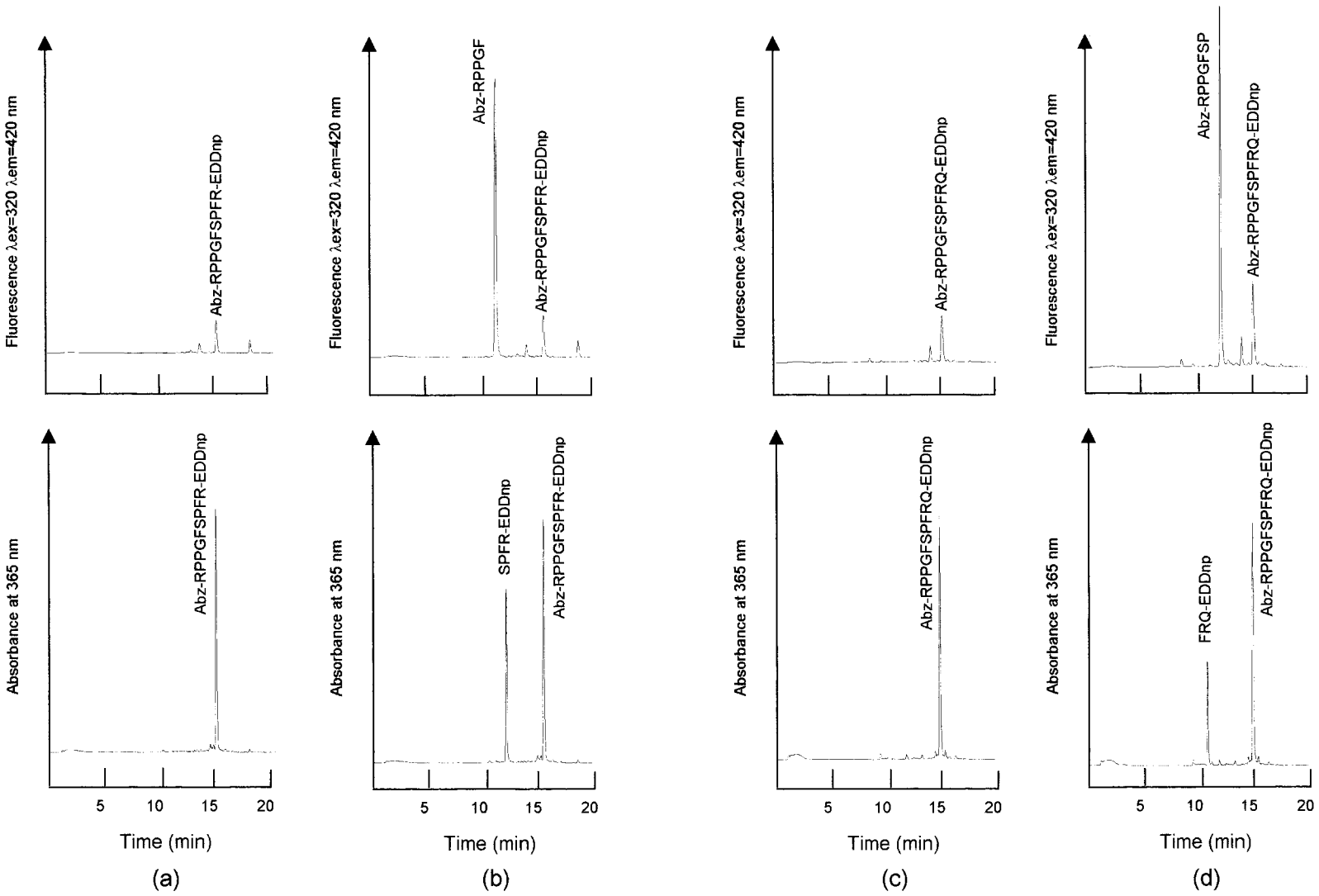

Fig. 4. HPLC profiles of the reaction mixtures of lysate of Madin-Darby canine kidney (MDCK) cells with qf substrates analogues of bradykinin: Abz-RPPGFSPFR-EDDnp in the absence (**a**) and presence (**b**) of lysate. Abz-RPPGFSPFRQ-EDDnp in the absence (**c**) and presence (**d**) of lysate. Assay conditions: 0.1 M sodium phosphate buffer pH 8.0, added to 1μM E-64, 1 mM PMSF, and 5 μM pepstatin. Preincubation with 5 mM mercaptoethanol during 10 min.

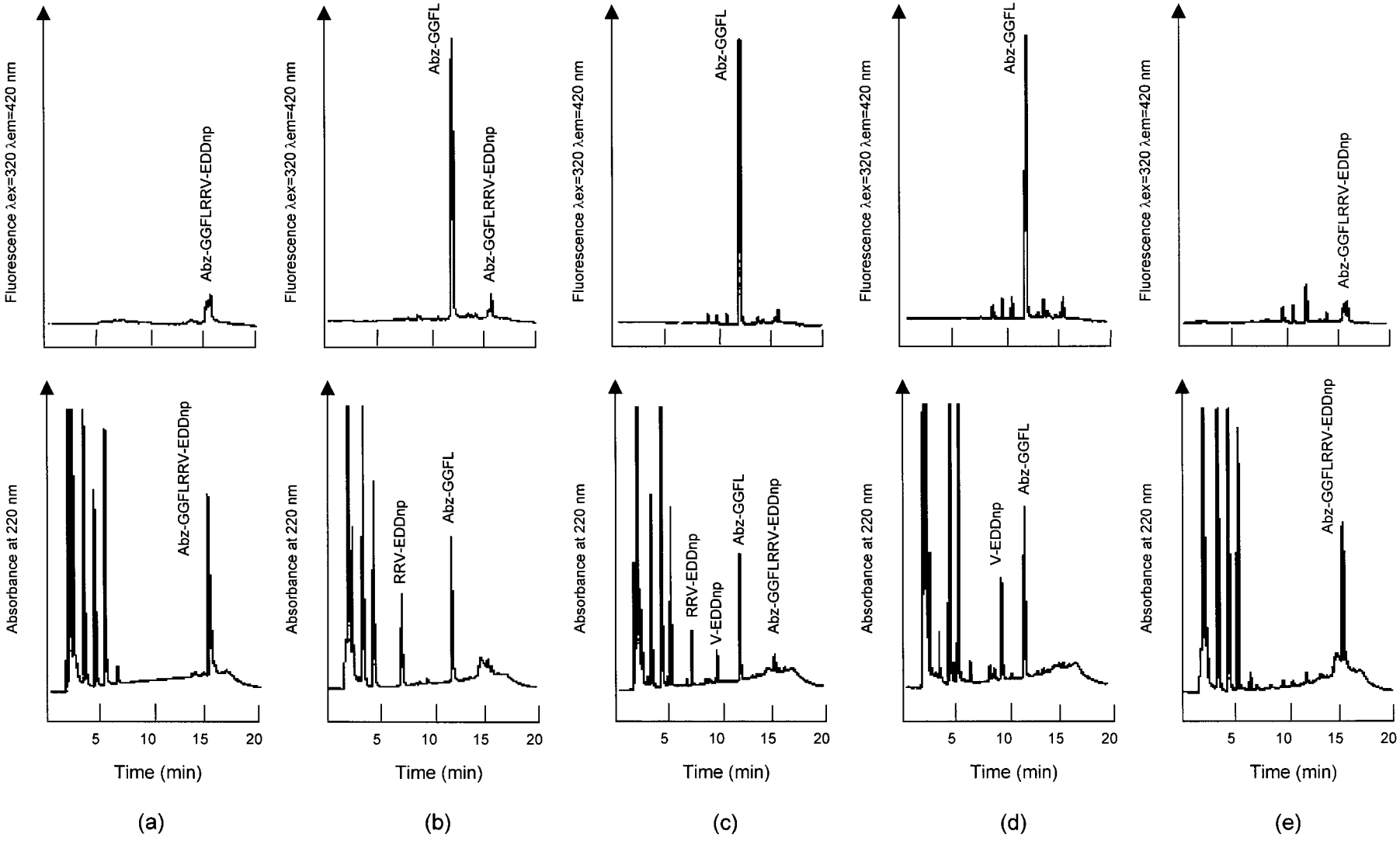

Fig. 5. High-performance liquid chromatography (HPLC) profiles of the reaction mixtures of lysate of MDCK cells with qf substrate analogue of (2–8)-dynorphin, Abz-GGFLRRV-EDDnp. (**a**) Peptide in the absence of lysate; (**b**) peptide in the presence of 5 nM rat recombinant TOP (rTOP); (**c**) peptide in the presence of lysate of Madin-Darby canine kidney (MDCK) cells with 1 μM E-64, 5 μ pepstatin and 1 mM PMSF; (**d**) peptide in the presence of lysate of MDCK cells without inhibitors; (**e**) peptide in the presence of lysate of MDCK cells depleted of TOP by affinity chromatography. Assays conditions: 0.1 M sodium phosphate buffer pH 8.0. Preincubation with 5 mM mercaptoethanol for 10 min.

tion was terminated by the addition of 10 µl of 10% (v/v) trifluoroacetic acid.

HPLC Analyses and Cleavage Site Determination

Each cleaved peptide bond was ascertained by reverse-phase chromatography of the hydrolysates. The peptide fragments were separated using a C_{18} µBondapak column (4.6 × 250 mm; Millipore, Bedford, MA) with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid in 15 min at a flow rate of 2.0 ml/min. Effluent peptides were monitored by an ultraviolet (UV) detector Shimadzu SPD-10-A (Tokyo, Japan) at $\lambda = 220$ nm and a fluorescent detector Shimadzu RF 535, at $\lambda_{ex} = 320$ nm, $\lambda_{em}=420$ nm with both detectors arranged in tandem. Cleavage sites of peptides were identified by mass spectroscopy as indicated above, as well as by peptide sequencing, using a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan).

Amino Acid Analysis

Amino acid composition, concentration of the peptides, and the purified rTOP were determined as follows: the samples were digested for 22 h at 110°C in 6 N HCl containing 1% phenol in vacuum-sealed tubes and then subjected to amino acid analysis using a pico-Tag station [Heinrikson and Meredith, 1984].

RESULTS

The activities related to thiol-, aspartyl-, and serine-protease in the soluble fraction of whole MDCK cell lysates were examined using susceptible substrates and specific inhibitors for each class of proteases through pH profile of hydrolytic activities. Figure 1 shows the pH profile of hydrolytic activity of MDCK cell lysate on Z-Phe-Arg-MCA and Z-Arg-Arg-MCA in the presence of pepstatin, PMSF, and EDTA. These hydrolyses were fully activated by 5 mM DTT and disappeared in the presence of 1 µM E-64. Two peaks of activity were detected with Z-Phe-Arg-MCA. By contrast, the pH profile of Z-Arg-Arg-MCA hydrolysis presented only one peak that overlaps to the same pH interval of the more alkaline peak detected with Z-Phe-Arg-MCA. This substrate is hydrolyzed by both cathepsin L and B; however, Z-Arg-Arg-MCA is hydrolyzed only by cathepsin B [Barrett, 1980; Barrett and Kirschke, 1981]. Therefore, MDCK cells seem to have at least two thiol-proteases, with a substrate, inhibitor specificity, and pH range of activity very similar to those of cathepsin B and L.

Aspartyl-protease activity was detected by the pH profile of hydrolysis of the internally quenched fluorescent peptide Abz-KPILFFRQ-EDDnp in the presence of 1 µM E-64, 1 mM PMSF, and 1 mM EDTA (Fig. 2). This peptide was synthesized with the same sequence previously described as a good substrate for cathepsin D [Scarborough et al., 1991, 1993], in which Abz (ortho-aminobenzoic acid) and Q-EDDnp [glutaminyl-(2,4-dinitrophenyl)ethylenedianine] were introduced as fluorescent and quencher groups, respectively. Cathepsin D as well as MDCK lysate hydrolyzed Abz-KPILFFRQ-EDDnp at the F-F bond, with similar pH profile activity and were inhibited by 5 µM pepstatin.

Serine-protease activity was assayed in MDCK lysate with Abz-KPILFFRQ-EDDnp and Z-Phe-Arg-MCA, which are substrates for chymotrypsin and trypsin, respectively. The lysate did not hydrolyze these substrates in the presence of 1 µM E-64, 5 µM pepstatin, and 1 mM EDTA at a pH range of 5–9. However, in the same condition, the peptide Abz-RPPGFSPFRQ-EDDnp, which has the added bradykinin sequence of Abz and Q-EDDnp, was hydrolyzed at the P-F peptide bond by MDCK lysate. This hydrolysis was not inhibited by 1 mM PMSF and was increased by the removal of EDTA and by the addition of 5 mM mercaptoethanol; these data led us to exclude the presence of serine-protease activity in our MDCK cell homogenates.

The pH profile of hydrolysis of Abz-RPPGFSP-FRQ-EDDnp (Fig. 3) presented only one peak of activity centered at pH 7.5–8.0 in the presence of 1 µM E-64, 5 µM pepstatin, 1 mM PMSF,

TABLE I. Inhibitory Profile of TOP-like Activity in Whole Soluble MDCK Cells Homogenate

Inhibitor	Concn/ pre-incubation time	Inhibition (%)
para-Hydroxymercuribenzoate	1 µM/1 min	40
Captopril	50 µM/15 min	10
EDTA	2.6 mM/35 min	85
Cpp-AAF-pAB[a]	5 µM/5 min	90

[a]N-(1-carboxy-3-phenylpropyl)-Ala-Ala-Phe-para-aminobenzoic (specific inhibitor of TOP) [Orlowski et al., 1988].

after preactivation with 5 mM mercaptoethanol. We examined this hydrolytic activity of MDCK lysate in greater detail, investigating the susceptibly of Abz-RPPGFSPFR-EDDnp under the same conditions. This peptide, a homologue of Abz-RPPGFSPFRQ-EDDnp in which Gln was absent, was hydrolyzed at F-S instead of P-F bond. Figure 4 shows the HPLC profile of both peptides, before and after the reaction with MDCK lysate. This hydrolysis at two different peptide bonds in the bradykinin internally quenched fluorescent analogue that depends on the presence of Gln at C-terminal end of peptide is a TOP peculiar activity [Camargo et al., 1997]. Further characterization of this activity was performed investigating this TOP-like activity of MDCK lysate on the peptide Abz-GGFLRRV-EDDnp, a typical substrate for TOP [Camargo et al., 1997; Juliano et al., 1990]. This peptide was cleaved at the L-R bond by rTOP (Fig. 5b), the same site hydrolyzed by MDCK cell lysate both in the presence and in the absence of inhibitors (Fig. 5c,d). In addition, this hydrolytic activity of MDCK lysate was depleted by filtering the lysate through a 3-ml Sepharose-protein A column, in which the purified polyclonal antibodies to rTOP were immobilized (Fig. 5–e). The peptide related to the N-terminal fragment (RRV-EDDnp) gradually disappeared and was converted in V-EDDnp. This conversion was faster in the absence of inhibitors (Fig. 5c,d). Therefore, this result indicates that MDCK cell lysates also have one or more aminopeptidases.

The presence of a TOP-like enzyme in MDCK cells was further demonstrated by the inhibitor assays, as shown in Table I. Similar to TOP, the endopeptidase activity was partially inhibited by para-hydroxymercuribenzoate and EDTA, inhibited by Cpp-AAF-pAB [N-(1-carboxy-3-phenylpropyl)-Ala-Ala-Phe-para-aminobenzoic], a specific inhibitor of TOP [Orlowski et al., 1988], and resistant to inhibition by captopril, an inhibitor of angiotensin-converting enzyme (ACE). Finally, the endopeptidase activity of MDCK cell lysate exhibited behavior very similar to that of TOP in the presence of an increasing concentration of mercaptoethanol, as shown in Figure 6, the activation was observed up to 10 mM mercaptoethanol, followed by gradual inhibition in higher concentrations.

TOP activity was detected in the medium of confluent MDCK cells 5 minutes, and 1 h, 6 h, and 16 h after being washed and incubated in

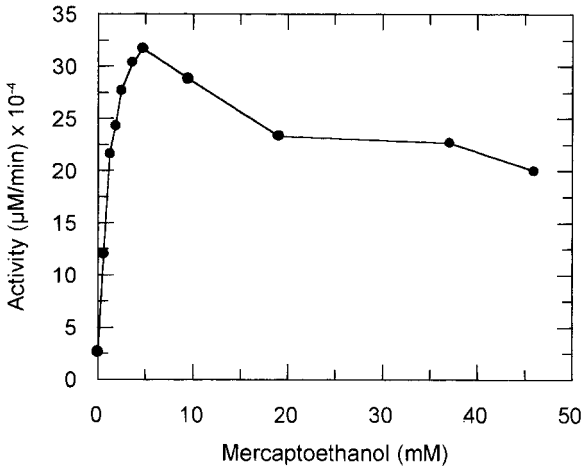

Fig. 6. TOP-like activity of lysate of Madin-Darby canine kidney (MDCK) cells on Abz-RPPGFSPFRQ-EDDnp preincubated with different β-mercaptoethanol concentration for 10 min. Assayed in 0.1 M of sodium phosphate buffer pH 8.0, added to1μM E-64, 1 mM PMSF, and 5 μM pepstatin.

DMEM without calf serum addictive (Fig. 7). These hydrolytic activities were completely inhibited by Cpp-AAF-pAB. Trypan blue dye exclusion tests indicated that the cells were viable. In addition, we assayed thiol- and aspartyl-protease activities, which were not detected in the medium in which these experiments were performed, further indicating that most of the cells were integral. The secretion was not cumulative; however, we do not know whether the enzyme is inactivated at a rate similar to that secreted or the secretion process in inhibited by the enzyme itself or by any other factor.

DISCUSSION

The combination of fluorogenic substrates or internally quenched fluorescent peptides that are very sensitive, some of which are specific substrates for classes of proteases, with specific inhibitors in the pH profile of proteolytic activity experiments allowed us to detect thiol- and aspartyl-protease activities in MDCK cell lysate. The results obtained indicated that these enzymes are cathepsin B, L, and D. By contrast, serine-proteinase was not detected, although we clearly demonstrated the presence of TOP-like peptidase and obtained an indication of the existence of aminopeptidase.

It is interesting to note that both TOP-like peptidase from MDCK cells and rat rTOP hydrolyzed the same substrates at the same sites. It is particularly noteworthy that, in both cases, the cleavage site at the P-F bond in peptide

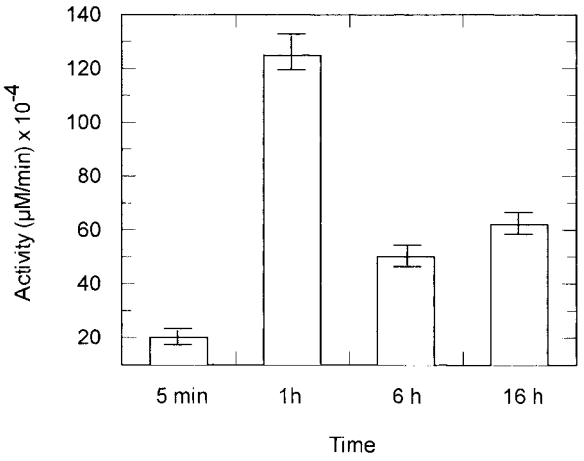

Fig. 7. TOP-like activity in the culture medium of confluent Madin-Darby canine kidney (MDCK) cell cultures at different times as specified. Each measurement was made at least in triplicate. Standard deviations are indicated at the top of each bar. Assay condition: substrate (30μM Abz-RPPGFSPFRQ-EDDnp) was added to 2.0 ml of the collected aliquots of culture media without calf serum. Preincubation with 5 mM mercaptoethanol for 10 min.

Abz-RPPGFSPFRQ-EDDnp changed to F-S in Abz-RPPGFSPFR-EDDnp. A similar change in the cleavage site was observed in the hydrolysis by rat rTOP [Camargo et al., 1997] and by MDCK cell TOP, in shorter analogues of these peptides, namely, Abz-GFSPFRQ-EDDnp and Abz-GFSPFR-EDDnp (data not shown). Abz-GGFLRRV-EDDnp is cleaved by recombinant TOP at L-R bond; however, adding the amino acid Gln as in Abz-GGFLRRVQ-EDDnp the hydrolysis also occurs at R-R bond [Camargo et al., 1997]. These simultaneous cleavages of two peptide bonds were also detected with TOP-like peptidase from MDCK cells. The absence of specificity to any particular amino acid for cleavage site, the changes in hydrolysis sites, and the selectivity to the size of the substrate, 7–17 residues suggest that conformational determinants are involved in this particular behavior of TOP [Jacchieri et al., 1998].

Another interesting characteristic of TOP is its activation by mercaptans, recently demonstrated to be dependent on the dissociation of two or three molecules of the enzyme-breaking intermolecular disulfide bonds [Shrimpton et al., 1997]. Although speculative, this has been proposed as a mechanism that controls the TOP activity inside the cell. In addition, the response to mercaptans is one of the criteria for differentiating TOP from neurolysin, as the latter enzyme is not activated by DTT, and TOP is inhibited at concentrations higher than 0.5 mM of mercaptan [Serizawa et al., 1995]. On the basis of these results and on the depletion of hydrolytic activity by purified antibodies to rTOP, we conclude that MDCK cells express TOP.

Proteolysis in cytosol is a subject that has been receiving particular attention in processes that control apoptosis (cell cycle) [Kutsyi et al., 1999] and antigen-presentation, mainly that related to proteasome activities [Kisslev et al., 1999; Portaro et al., 1999; Silva et al., 1999]. Oligopeptidases are emerging as specialized peptidyl hydrolases involved in the modification, destruction, or even transport of peptides inside the cell [McKie et al., 1993]. In addition to TOP, several distinct oligopeptidases have been characterized inside the cells: (1) prolyl oligopeptidase, which cleaves after prolyl residues of biological active peptides (Polgár, 1995], and a β-propeller domain structure in this enzyme appears to control this oligopeptidase activity [Folop et al., 1998]; (2) neurolysin, which has high homology to TOP [Serizawa et al., 1995; Dauch et al., 1995; Kato, 1997]; and (3) cytosolic tripeptidyl peptidase, a subtilisin- like peptidase of unknown function, a particle larger than the 26S proteasome [Geier et al., 1999]. Therefore, the presence of TOP in a phenotype polarized MDCK cells could have special significance in the cytoplasmic selection, transport, or clearance of short peptides. Although we have only preliminary data, MDCK cells seem to secrete TOP, and it is possible that this enzyme is also secreted by tubular cells, playing a role in the control of biological active peptides on kidney.

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