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A vitellogenic-like carboxypeptidase expressed by human macrophages is localized in endoplasmic reticulum and membrane ruffles

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Abstract

Carboxypeptidase, vitellogenic-like (CPVL) is a serine carboxypeptidase of unknown function that was first characterized in human macrophages. Initial studies suggested that CPVL is largely restricted to the monocytic lineage, although it may also be expressed by cells outside the immune system. Here, we use a new monoclonal antibody to characterize the properties and localization of CPVL in human macrophages to elucidate a possible function for the protease. CPVL is up-regulated during the maturation of monocytes (MO) to macrophages, although the protein can be seen in both. In primary macrophages, CPVL is glycosylated with high mannose residues and colocalizes with markers for endoplasmic reticulum, while in MO it is more disperse and less clearly associated with endoplasmic reticulum. CPVL is highly expressed in lamellipodia and membrane ruffles, which also concentrate markers of the secretory pathway (MIP-1α and tumour necrosis factor-α) and major histocompatibility complex (MHC) class I and II molecules. CPVL can be seen on early latex bead and *Candida albicans* phagosomes, but it is not retained in the maturing phagosome, unlike MHC class I/II. CPVL has a mixed cytosolic and membrane-associated localization but is not detectable on the outer plasma membrane. We propose that CPVL may be involved in antigen processing, the secretory pathway and/or in actin remodelling and lamellipodium formation.

Keywords
antigen processing/presentation, lamellipodia, membrane ruffles, secretory pathway, serine carboxypeptidase

Introduction

Carboxypeptidases are a large group of proteases that cleave one or two amino acids from the carboxy terminus of proteins or peptides (Skidgel & Erdos 1998) and are classified

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according to their active site mechanism into three main groups, cysteine, metallo- and serine carboxypeptidases. The latter have been extensively studied, with numerous examples in plants and yeast, but only two classical serine carboxypeptidases have been cloned and characterized from mammalian sources, carboxypeptidase, vitellogenic-like (CPVL) and protective protein/cathepsin A (PPCA, also known as lysosomal protective protein, cathepsin A and carboxypeptidase A). Extra-lysosomal PPCA has been shown to have deamidase, esterase and carboxypeptidase activities, hydrolysing a variety of substrates *in vitro*, including tachykinins, but within the lysosome, it forms a multienzyme complex with neuraminidase and β-galactosidase, protecting them from degradation (Galjart *et al*. 1988; Hiraiwa 1999). The human lysosomal storage disease galactosialidosis is the result of a genetic defect of PPCA (D'Azzo *et al*. 1982). A more distantly related serine carboxypeptidase, prolylcarboxypeptidase (PRCP, angiotensinase C), is concentrated in lysosomes and catalyses the conversion of angiotensin II to angiotensin 1–7 (Odya *et al*. 1978; Skidgel & Erdos 1998).

CPVL was first cloned and characterized in this laboratory during a search for human macrophage-restricted genes using differential display polymerase chain reaction (PCR) (Mahoney *et al*. 2001). CPVL expression is notable in a number of different tissues, including spleen, placenta, heart and kidneys, but within the immune system, CPVL appears to be largely, if not wholly, restricted to myeloid cells, and its expression is up-regulated during maturation of peripheral blood monocytes (MO) into macrophages *in vitro*. Expression of CPVL can be modulated by inflammatory stimuli; the protein is down-regulated in macrophages cultured in medium with IFN-γ and *Staphylococcus aureus* (Mahoney, 2003), and *in vivo*, CPVL is strongly expressed in human alveolar macrophages (Stanton *et al*. 2003). The designation of CPVL as a true serine carboxypeptidase awaits demonstration of enzymatic activity, although the primary sequence displays the expected serine carboxypeptidase active site. The function of this enzyme is not known and attempts to detect protease activity in the supernatants of CPVL-transfected 293T cell cultures were unsuccessful, although this may be because these cells do not process CPVL in the appropriate manner (Mahoney *et al*. 2001). The primary sequence of CPVL contains a putative signal sequence, four potential N-linked glycosylation sites and four myristylation sites, but no transmembrane domain, suggesting that it may be luminal in an organelle and/or involved in the secretory pathway.

In the present study, we report on the biochemistry and subcellular localization of CPVL in human macrophages using a newly produced monoclonal antibody specific to the protein, as well as an enhanced green fluorescent protein (EGFP)-CPVL construct. The results suggest a number of possible roles for this novel carboxypeptidase, including processing/transport of peptides for loading onto major histocompatibility complex (MHC) class I molecules, processing/sorting of secretory molecules and actin remodelling in macrophage lamellipodia/membrane ruffles.

Materials and methods

Cell lines and reagents

Unless otherwise stated, reagents were obtained from Sigma (Poole, Dorset, UK). Chinese hamster ovary (CHO) cells were grown in tissue culture flasks in F-12 nutrient mixture (Gibco, Paisley, UK) with 10% heat-inactivated foetal calf serum (FCS) and 50 U/ml penicillin and 50 μg/ml streptomycin. THP-1 human monocytic cells were cultured in RPMI-1640 medium (Gibco) with L-glutamine, penicillin/streptomycin and 10% FCS and stimulated with phorbol myristate acetate (PMA) to produce adherent macrophage-like cells.

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were from R&D systems, Minneapolis, MN, USA. Rabbit polyclonal antibody (Ab) against tumour necrosis factor (TNF)-α was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse mAb against HLA-DR and rabbit polyclonal against human cathepsin D were from Biomeda (Foster City, CA, USA), and mAbs against HLA-ABC and macrophage-inhibitory protein (MIP)-1α were obtained from Serotec (Oxford, UK). Mouse mAb against endosome autoantigen 1 (EEA1) was from BD Transduction laboratories (Oxford, UK). Rabbit polyclonal Ab against calreticulin was from Affinity Bioreagents (Golden, CO, USA). Mouse monoclonal against golgin 97 was from Molecular Probes (Paisley, UK). Mouse mAbs against ERGIC-53 and PDI were provided by H.P. Hauri (University of Basel, Switzerland) and David Vaux (Sir William Dunn School of Pathology, UK), respectively. Rabbit polyclonal Ab against coronin 1 was a gift from Anthony Segal (University College London, London, UK). Rabbit polyclonal Ab against a synthetic CPVL peptide sequence was previously provided by this lab (Mahoney *et. al*., 2001). Antibody binding was detected with the following fluorophore-Ab conjugates: cy3- or cy5-conjugated $F(ab')_2$ donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Alexa-Fluor 488- and 647-conjugated goat anti-rabbit IgG (Molecular Probes). For costaining with other mouse mAbs, anti-CPVL mAb CC338 was directly conjugated with Alexa-Fluor 488 (Molecular Probes), following the manufacturers' instructions. Polymerized actin was stained with TRITC-phalloidin.

Production of a monoclonal antibody against CPVL

Recombinant CPVL was produced in bacteria as follows. CPVL without its 21 amino acid signal sequence was PCR amplified using the forward primer GAGAGCTAGCCTGT TTCGCTCCCTATACAGAAGT (Nhe I site underlined) and reverse primer GAGAAAGCTTTTATCCAACATAAGGAT CCCATCC (Hind III site underlined). The resulting PCR product was digested and ligated into the Nhe I and Hind III sites of the bacterial expression vector pET-28a(+) (Novagen, San Diego, CA, USA) using standard methods. The accuracy of the entire coding sequence of the resulting plasmid was confirmed by automated sequencing. The CPVL-pET-28a(+) construct was used to transform competent BL21-DE3 cells, followed by IPTG induction. Recombinant CPVL protein was produced as insoluble inclusion bodies, which were isolated by sonication and washed with 0.5% Triton X-100 and 10 mM EDTA. The protein obtained was estimated to be >80% pure by SDS–PAGE.

Young adult BALB/c mice were inoculated subcutaneously with 15 μg recombinant CPVL in Titermax gold Adjuvant twice over a 5-week period and mice with a high antibody response to CPVL selected using enzyme-linked immunosorbent assay (ELISA) analysis of sera. Eleven weeks after the second inoculation, mice were inoculated intraperitoneally with 15 μg CPVL 3 and 4 days before they were killed and spleens removed for fusion with SP2/0 cells (Jones & Howard 1995). All cultures were maintained in RPMI 1640-Gmax (Gibco) containing 10% heated-inactivated FCS, penicillin, streptomycin, HAT (hypoxanthine, aminopterin, thymidine supplement) and B-cell-cloning factor (Origen^{TM}, IGEN, Gaithersburg, MD, USA). Supernatants from colonies were tested using ELISA and then using Western blot for specificity. Colonies were cloned using limiting dilution. A single clone was selected, based on its specificity by Western blot, grown in the above media and antibody purified from the supernatant on GammaBind Plus sepharose (Amersham Biosciences, Buckinghamshire, UK). The resulting protein was characterized as IgG2b and named CC338.

Isolation and culture of human monocyte-derived macrophages and monocyte-derived dendritic cells

Human MO were isolated from buffy coats (provided by the National Blood Service, Southmead Hospital, Bristol) or fresh blood following the methods described previously (Davies & Gordon 2004). Briefly, PBMCs isolated on a Ficoll density gradient (Amersham Biosciences) were plated on gelatin-coated dishes for 60–90 min, after which time nonadherent cells were washed off and the remaining cells (>90% MO) were cultured in *X-vivo* 10 medium (Cambrex, Walkersville, MD, USA) supplemented with 1% heat-inactivated autologous serum. After 24 h, the MO were washed off the dishes, counted and cultured further in the same medium at a density of $2–5 \times 10^5$ cells/ml, for 7–12 days to produce mature monocyte-derived macrophages (MDM). For TNF-α staining, MDM were stimulated with lipopolysaccharide (LPS) (50 ng/ml) for 30 min. For monocyte-derived dendritic cells (MoDC), MO were cultured in medium with the addition of GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) for 7 days or with GM-CSF (50 ng/ml) only to produce nonadherent 'pre-DCs' (Santambrogio L. and Stern L.J., personal communication). MoDC were further matured by treatment with LPS (50 ng/ml) for 2–48 h. For some experiments, MO were isolated from PBMC either by positive selection with CD14-coated magnetic beads or by negative selection using monocyte isolation kits (Miltenyi Biotech, Surrey, UK) (>98% MO), following density-gradient centrifugation of diluted peripheral blood on Ficoll. These cells were cultured in *X-vivo* 10 with 1% heat-inactivated autologous serum.

Western blot

Cells were lysed in 2% NP-40 in Tris/saline/EDTA (10 mM/150 mM/2 mM) with protease inhibitors, PMSF and 0.5% deoxycholate on ice for 60 min. Lysates were centrifuged to remove nuclei and protein concentration determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). After boiling for 5 min, proteins were separated on 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. After blocking with 5% milk overnight, the membranes were probed with CC338 or CPVL pAb, then incubated with HRP-conjugated secondary antibodies and detected using enhanced chemiluminesence (ECL). For deglycosylation assays, lysates were incubated with enzymes overnight according to the manufacturer's protocol.

Phagocytosis assays

To study phagocytosis, we incubated MDM with 2-μm latex beads (Polysciences Inc., Warrington, PA, USA) or live *Candida albicans*. Particles were preincubated with heat-inactivated autologous human serum for 30 min at 37 °C and fed to MDM in the same serum-containing medium. In all experiments, particles were allowed to bind for 5–30 min at 37 °C, depending on the particle and conditions. Non-bound particles were washed off and the cells incubated for the remainder of the experiment in media with autologous serum.

Construction of a CPVL-EGFP expression vector

The full-length CPVL-coding sequence was amplified using PCR from pooled human leucocyte RNA using gene-specific primers, 5′- ATACTCGAGGCCACCATGGTTGGTGCCAT GTGGAAGGTGATT and 3′-ATAGACCGGTGTTCCAAC ATAAGGATCCCATCCTTT. The amplified cDNA fragment was sequenced to confirm its identity and digested with *Xho*I and *Age*I, the restriction sites incorporated in the gene-specific primers. The digested cDNA fragment was purified and subcloned into pEGFP-N1 (BD Clontech, Basingstoke, UK) to generate pCPVL-EGFP, which encodes a fusion protein containing the full-length CPVL and a C-terminal EGFP.

Confocal microscopy

Cells were grown on nitric acid-treated, methanol-washed 13-mm diameter glass coverslips in 24-well tissue culture plates or cytospun onto coverslips. After fixation with 4% paraformaldehyde in PBS (20 min at room temperature), cells were blocked and permeabilized in PBS with 0.5% BSA, 0.1% Triton X-100 and 1% goat serum for 20 min at room temperature. Primary antibodies were diluted (5–10 μg/ml) in this blocking/permeabilization buffer and added to the cells for 60 min at room temperature, after which time the cells were washed five times in PBS, then incubated with secondary antibody (5–10 μg/ml) for 60 min at room temperature, washed five times in PBS, dipped in distilled water to remove PBS crystals and mounted on glass slides with fluorescent mounting medium (Dako, Carpinteria, CA, USA). Where two mouse mAbs were used for double labelling, cells were first stained with the nonconjugated antibody, incubated with fluorescently labelled anti-mouse conjugate, blocked in 10% mouse serum and then finally incubated with the Alexa-conjugated CC338. Immunofluorescence was analysed on a Bio-Rad Radiance 2000 laser-scanning confocal microscope with a ×63 oil immersion lens and the resulting images processed in Adobe Photoshop. All images displayed are representative of the cell population studied.

CPVL ELISA

Ninety-six-well plates were coated with recombinant CPVL in bicarbonate buffer (10 μg/ml, 50 μl per well) overnight at room temperature. After washing with PBS, the plates were blocked with 3% BSA in PBS for 60 min and then washed with PBS/Tween 20 (0.1%). Primary antibody (CC338 or polyclonal anti-CPVL) was diluted in PBS and incubated on the plates for 60 min at room temperature. The plates were washed with PBS/Tween and then incubated with alkaline phosphatase-conjugated secondary antibody for 30 min. Colorimetric reactions following addition of *p*-nitrophenyl phosphate were measured at a wavelength of 405 nm.

Flow cytometry

Human MDM were detached from tissue culture plastic by gentle scraping following incubation with lidocaine/EDTA (4 mg/ml/2 mM) on ice. All subsequent steps were carried out on ice. Cells were transferred to a 96-well plate and washed three times with a solution of 2 mM EDTA and 1% BSA in PBS. For analysis of intracellular protein, cells were fixed with 2% paraformaldehyde (in PBS) for 20 min and then permeabilized with 0.1% Triton X-100 (in PBS with 1% BSA) for 15 min. The cells were blocked with 10% human serum, incubated with primary Ab for 60 min and then with Alexa 488-conjugated secondary Ab for 60 min. For surface staining, unfixed cells were blocked with 10% human serum and incubated with primary and secondary Abs as described above and then fixed with 2% paraformaldehyde. The cells were analysed on a FACScan (Becton Dickinson, Oxford, UK).

Electron microscopy

For immuno-electron microscopy (EM), human MDM cells grown on tissue culture plastic were fixed in 4% (approximately 10 min) and then 8% paraformaldehyde in 250 mM HEPES buffer (pH 7.4) for 2 h. Fixed cells were washed in 250 mM HEPES buffer containing 50 mM glycine for 1 h and released from the plastic by scraping, embedded in 3% gelatin, infused with 2.3 M sucrose and snap-frozen in liquid nitrogen. Ultrathin cryosections (approximately 65-nm thick) were cut and collected using a 1:1 mixture of 2.3 M sucrose and 2% methylcellulose and labelled with rabbit anti-CPVL polyclonal antibody (1:25 dilution) followed by goat anti-rabbit IgG conjugated to 10 nm gold (British Biocell, UK). Sections were examined in a Zeiss (LEO) Omega 912 electron microscope (Zeiss/LEO Electron Microscope Ltd, Oberkochen, Germany) equipped with a Proscan-cooled slow-scan charge-coupled device camera (2048 × 2048 pixels). All digital images were captured with the integrated Soft Imaging Software (SIS) image analysis package (Soft Imaging Software, GmbH, Münster, Germany) and absolute measurements were recorded directly from the images.

Results

Characterization of CC338, a new mAb against CPVL

We previously generated a rabbit polyclonal Ab specific for CPVL, raised against a synthetic peptide corresponding to the

C terminal 14 amino acids plus an additional cysteine at the amino terminus [6]. Whilst this antibody has proved useful for biochemical and histological identification of CPVL (Mahoney *et al.* 2001; Stanton *et al.* 2003), it does not stain macrophage monolayers for confocal analysis. To address this issue, we raised a new mAb against CPVL in mice using recombinant CPVL and selected to detect CPVL in human macrophage lysates using Western blot analysis (using the polyclonal Ab as reference). The resulting mAb, named CC338, recognizes native CPVL in human macrophages using Western blot, CPVL-EGFP in transiently transfected CHO cells, recombinant CPVL using ELISA and native CPVL in human alveolar macrophages (Figure 1). The antibody also detects CPVL in cells expressing the monocyte/macrophage marker CD68 in other human tissues, including spleen and kidney (not shown). Orthologues of the CPVL gene have been found in a number of other species (see the *MEROPS* database: http//merops.sanger.ac.uk/; Rawlings *et al.* 2004), including *Danio rerio, Mus musculus* and *Rattus norvegicus*. We have found that CC338 also recognizes protein in primary murine macrophages and the RAW264.7 macrophage cell line, as well as in bovine alveolar macrophages, MDM and dendritic cells (not shown).

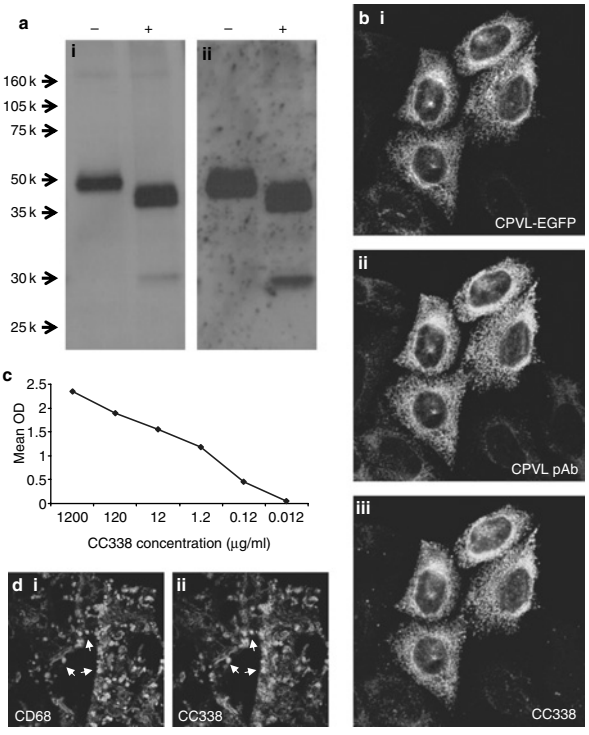

Figure 1 Confirmation of the specificity of CC338 for carboxypeptidase, vitellogenic-like (CPVL). (ai) CC338 detects CPVL in human macrophage cell lysates using Western blot before (–) and after (+) deglycosylation with endoglycosidase H. (aii) The same blot stripped and re-probed with anti-CPVL polyclonal antibody to confirm the specificity of CC338. (b) Both CPVL antibodies recognize CPVL-EGFP in transiently transfected Chinese hamster ovary cells. (c) enzyme-linked immunosorbent assay showing titration of CC338 with 50 ng rCPVL. (d) CC338 stains $CD68^+$ alveolar macrophages in human lung tissue (arrows).

CPVL is glycosylated in the endoplasmic reticulum of macrophages

As has previously been shown (Mahoney *et al.* 2001; Stanton *et al.* 2003), within the human immune system, CPVL is largely restricted to the monocytic lineage. Western blot analysis of cell lysates demonstrated that CPVL is present in MO, macrophages and PMA-stimulated THP-1 cells but not in monocyte-depleted PBMC and that protein levels are higher in macrophages than in MO (Figure 2a). CPVL is also expressed in MoDC and its expression is unaffected by treatment with LPS over 48 h (Figure 2b). The primary protein sequence of CPVL has four putative N-glycosylation sites, and CPVL in macrophage lysates is insensitive to treatment with O-glycosidase (Figure 2c) but sensitive to *N*-glycosidase

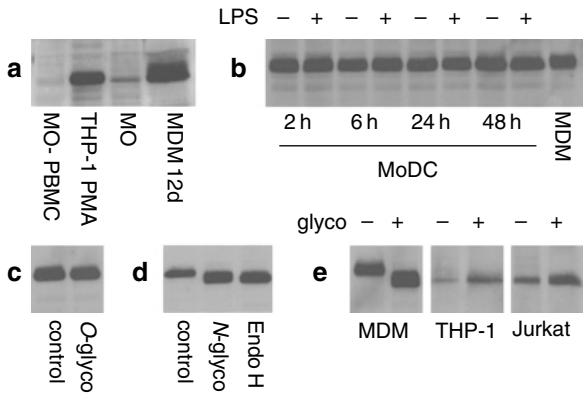

Figure 2 Western blot analysis of carboxypeptidase, vitellogenic-like (CPVL) expression in human immune cells. (a) CPVL is expressed by monocytes (MO), monocyte-derived macrophages (MDM) and PMA-stimulated THP-1 cells, but not by monocyte-depleted peripheral blood mononuclear cells (MO^-PBMC). Protein levels are higher in MDM than in MO. (b) CPVL expression in monocyte-derived dendritic cells (MoDC) is unaffected by treatment with LPS for up to 48 h and is comparable with that in MDM. (c) CPVL in MDM is unaffected by treatment with O-glycosidase. (d) Treatment with *N*-glycosidase or endoglycosidase H (endo H) lowers the CPVL band in MDM. (e) CPVL in THP-1 and Jurkat cell lines are insensitive to treatment with a cocktail of *O*-glycosidase, *N*-glycosidase and endo H (glyco). In all experiments, protein loading for all samples was equal.

treatment (Figure 2d). Moreover, this glycosylation is entirely endoglycosidase H (endo H)-sensitive, which would suggest that CPVL is glycosylated with high-mannose residues in the endoplasmic reticulum (ER) (Figure 2d). In contrast, CPVL in PMA-stimulated THP-1 cells is not sensitive to treatment with a cocktail of *N*-glycosidase, *O*-glycosidase and neuraminidase (Figure 2e). CPVL is also detectable in the Jurkat T-cell line and is insensitive to deglycosylation (Figure 2e). After deglycosylation, a second, faster migrating band (approximately 29 kDa) can be seen in human MDM lysates (Figure 1a). The nature of this species is not known. Immunofluorescent analysis of cells using confocal microscopy confirms that CPVL colocalizes strongly with the ER markers PDI (not shown) and calreticulin in macrophages but less so in PMA-stimulated THP-1 cells, MO and nonstimulated THP-1 cells (Figure 3a–d). Similarly, in transfected CHO cells, CPVL-EGFP colocalizes with PDI (Figure 4a) and calreticulin (not shown). CPVL is not evident in the Golgi apparatus or in the ER-Golgi intermediate compartment of macrophages (Figure 4b,c).

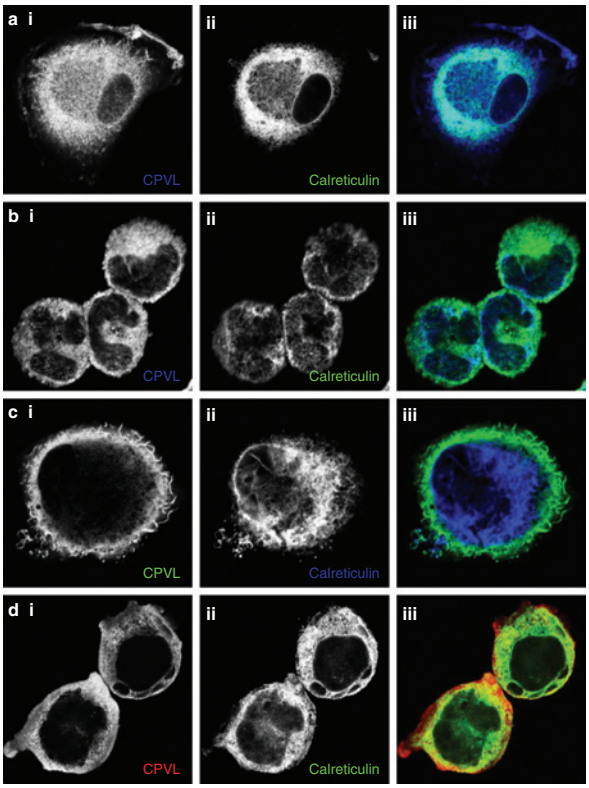

Figure 3 Confocal analysis of carboxypeptidase, vitellogenic-like (CPVL) localization in monocyte-derived macrophages (MDM) and THP-1 cells. (a) CPVL colocalizes strongly with the endoplasmic reticulum marker calreticulin in MDM but not in (b) PMA-stimulated THP-1 cells. (c) Colocalization of CPVL with calreticulin is similarly low or absent in (c) peripheral blood monocytes and (d) nonstimulated THP-1 cells.

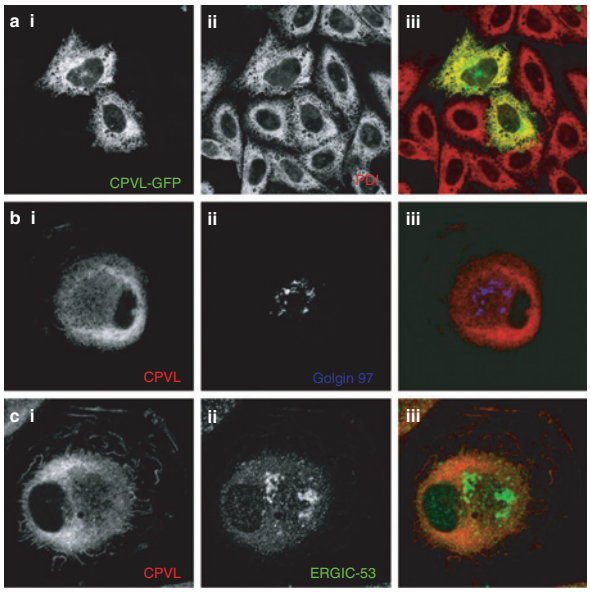

Figure 4 (a) In transfected Chinese hamster ovary cells, carboxypeptidase, vitellogenic-like (CPVL)-EGFP colocalizes strongly with PDI. In monocyte-derived macrophages, CPVL does not colocalize with (b) the Golgi marker golgin 97 or with (c) ERGIC-53, a marker of the Golgi-endoplasmic reticulum intermediate compartment.

CPVL is concentrated in lamellipodia and membrane ruffles

As well as its association with the ER, CPVL is also concentrated within actin-rich lamellipodia and membrane ruffles (the latter defined in this case as smaller structures, not necessarily at the leading edge) in apparently punctate structures (Figure 5a,b). Within these membrane structures, CPVL colocalizes with MHC class I and II molecules (Figure 5c,d). In contrast, CPVL does not colocalize with actin in lamellipodia/membrane ruffles in immature and LPS-stimulated moDC (not shown), although it is surrounded by actin in membrane ruffles (or 'blebs') in GM-CSF-derived 'pre-DCs' (Figure 5e). We have found that the lamellipodia and ruffles of macrophages stain strongly for numerous other molecules important for macrophage biology, including FcR (CD64), CD14, coronin 1 (TACO) and the epidermal growth factor-like domain containing TM7 (EGF-TM7) receptors, CD97 and EMR2 (unpublished results). We hypothesize that these structures may facilitate active (macrophage-initiated) phagocytosis: membrane regions that can protrude in a specific direction

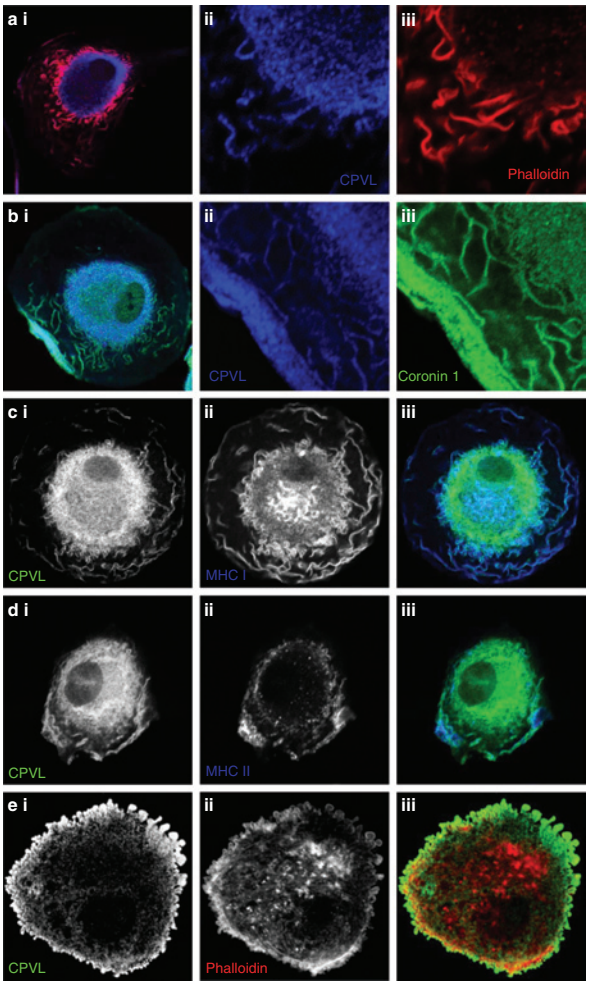

Figure 5 Carboxypeptidase, vitellogenic-like (CPVL) is expressed in membrane ruffles/lamellipodia in monocyte-derived macrophages (MDM). (a) CPVL can be seen as punctuate staining in TRITC-phalloidin-stained actin-rich membrane ruffles/lamellipodia (ii and iii, enlarged from i). Within these membrane structures, CPVL colocalizes with (b) the actin-binding protein, coronin-1 (ii and iii enlarged from i) (c) MHC class I and (d) MHC class II. (e) CPVL is present in actin-rich ruffles, or 'blebs' in pre-DC (arrows).

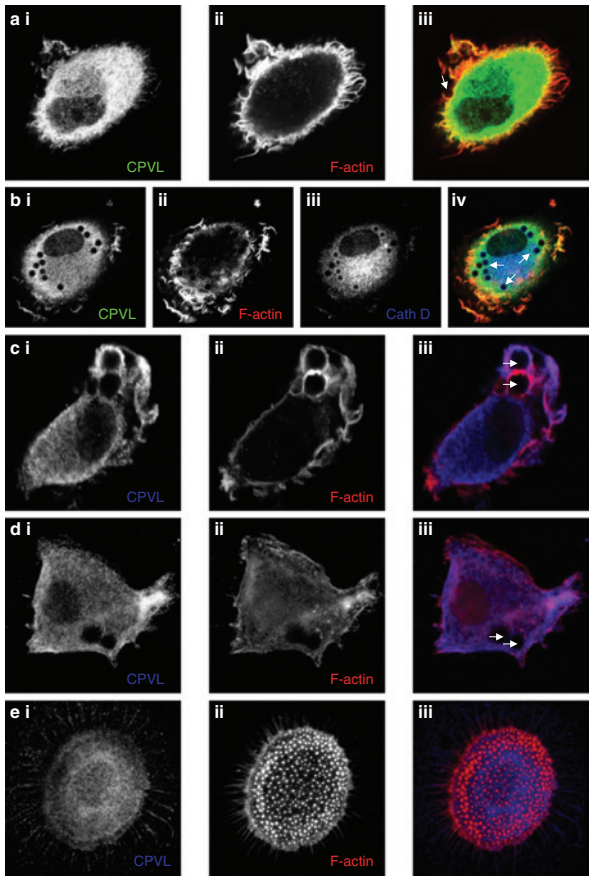

Figure 6 Carboxypeptidase, vitellogenic-like (CPVL) and phagocytosis in monocyte-derived macrophages (MDM). (a) CPVL colocalizes with actin on the early (5-min incubation) latex bead phagosome (phagocytic cup, arrow) but (b) is absent from the cathepsin D-positive latex bead phagolysosome (60-min incubation, arrows). Similarly, CPVL is present on the live *Candida albicans* (c) early phagosome (15-min incubation) but not the (d) late phagosome (60-min incubation). (e) CPVL does not colocalize with TRITC-phalloidin-stained actin in MDM podosomes.

with the necessary phagocytic machinery intact to allow rapid formation of a phagocytic cup and subsequent ingestion. As such, CPVL may itself be involved in this process. To test this, we studied phagocytosis of latex beads and *C. albicans* by confocal microscopy. Whilst CPVL can be seen in early (5-min incubation) latex bead phagosomes (Figure 6a), it is not retained in the mature, cathepsin D-positive phagolysosome (60-min incubation, Figure 6b), unlike MHC class I and class II, which are present on the latex bead phagosome for up to 5 h (not shown). Similarly, CPVL can be seen in early (15 min) *C. albicans* phagosomes but is not retained after 60 min (Figure 6c,d), so may be involved in the early transition from lamellipodia to phagosome. Although the primary sequence of CPVL has no actin-binding domain, it may still have a role in actin cytoskeleton remodelling/lamellipodia formation. The association of CPVL with actin is specific for lamellipodia/membrane ruffles; the protease is not present in actin-rich podosomes (Figure 6e).

CPVL and the secretory and endosomal pathways

As CPVL has no transmembrane domain, we were intrigued by its presence in lamellipodia near the plasma membrane (PM). High-speed centrifugation of macrophage lysates was

employed to produce membrane and nonmembrane fractions. Western blot analysis of these fractions showed CPVL to be equally abundant in both (Figure 7a). To further elucidate the localization of CPVL within macrophages, we analysed cells by electron microscopy following immunogold labelling (Figure 7b). CPVL can be seen throughout the cytoplasm, as well as associated with vesicles. Where it is on or near vesicles, it is not clear whether it is on the inner or outer surface. The latter would indicate that CPVL might be recruited to these vesicles from the cytoplasm, perhaps playing a role in vesicle transport or fusion. However, localization in the ER and the presence of a signal peptide would favour the hypothesis that CPVL travels from the ER via a secretory pathway. Some CPVL can also be seen on or near the PM; however, analysis of nonpermeabilized macrophages using FACS revealed that CPVL is not present on the outer PM (Figure 7b). To test whether CPVL might be involved in the secretory pathway, we looked at immunofluorescent staining of secretory molecules and found that CPVL colocalizes with both MIP-1α and TNF-α (Figure 7d,e). We have so far been unable to detect CPVL in macrophage culture supernatants by immunoprecipitation and Western blot (not shown), which would suggest that it is not itself secreted. CPVL shows little colocalization with some markers of the endocytic pathway, including EEA1, lysosomal membrane glycoprotein 1 (LAMP1), but more with cathepsin D (Figure 8a–c). These markers, along with mannose-6-phosphate receptor, mannose receptor and scavenger receptor A, are largely absent from membrane ruffles (not shown). Similarly, CPVL shows no association with fluorescently labelled dextran (MW 10,000) at any stage of its uptake by macrophages (not shown).

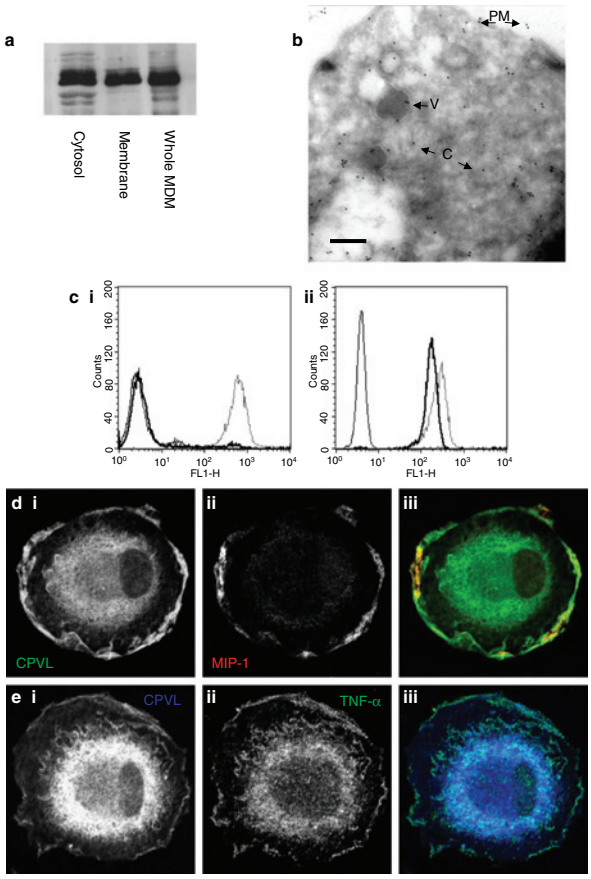

Figure 7 (a) Western blot shows that carboxypeptidase, vitellogenic-like (CPVL) is present in both cytosol and membrane fractions of monocyte-derived macrophages (MDM). (b) Immunogold labelling of CPVL in MDM cryosections. CPVL can be seen both in the cytosol (C), near the plasma membrane, and associated with vesicles (V, arrows). Scalebar = 250 nm (c) Flow cytometry of (i) live and (ii) fixed and permeabilized MDM. CPVL (bold line) is not expressed on the surface of cells, unlike MHC class II (dotted line) but is expressed at similar levels to MHC class II in permeabilized cells. The plain line represents an Ig-matched antibody control for CC338. CPVL colocalizes with the secretory molecules (d) MIP-1α and (e) TNF-α (stimulated with LPS, 50 ng/ml, for 30min).

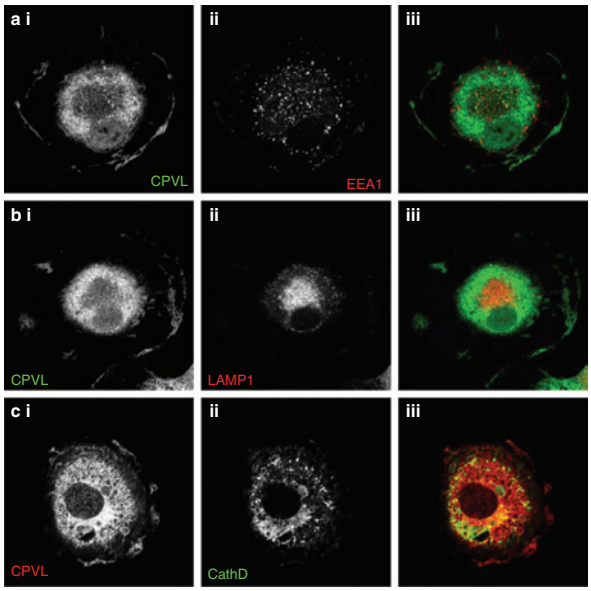

Figure 8 Carboxypeptidase, vitellogenic-like (CPVL) and the endocytic pathway in monocyte-derived macrophages (MDM). CPVL shows little colocalization with (a) early endosome autoantigen 1 (EEA1) or with (b) lysosomal membrane glycoprotein 1, a marker of lysosomes/late endosomes but more with (c) cathepsin D.

Discussion

Although relatively widespread in plants and yeast, only three serine carboxypeptidases have been characterized in mammals, PPCA, PRCP and CPVL. As such, little is known of possible roles for these proteases in mammalian cells. The protective functions of PPCA are well documented, but the enzyme clearly has numerous other potential functions, including hydrolase activity against a number of substrates (Hiraiwa 1999). The role of CPVL in human macrophages is unknown, and it has not yet been demonstrated to have enzymatic activity. However, by characterizing its intracellular localization and cell biology, we aimed to identify possible roles for CPVL and narrow the list of potential substrates. Although high levels of CPVL mRNA in the heart and kidney (Mahoney *et al.* 2001) suggest it may be present in some nonimmune cells, the fact that it is restricted to monocytic cells, particularly macrophages, within the immune system makes it an intriguing molecule. Macrophages play a number of important and specific roles in the immune system, from phagocytosis and organization of the innate immune response to antigen processing and modulation of subsequent acquired immune and inflammatory responses.

We have shown that, specifically in macrophages, CPVL is glycosylated and retained in the ER, whilst in MO, it appears to be more dispersed in the cell and less closely associated with the ER. The basis for these differences between MO and macrophages is not clear, although the abundance of CPVL in the ER of macrophages may correlate with the increase in protein observed using Western blot. In MO, CPVL may pass quickly through the ER, while in macrophages, which produce larger amounts of the protein, it may be stored in the ER for transport to its site of action. In THP-1 cells, both stimulated and nonstimulated, CPVL is absent from the ER, as shown using immunofluorescent staining, and this corresponds with the lack of glycosylation seen on CPVL in these cells. We have also transfected a modified CPVL, with EGFP tagged onto the C terminus, into CHO cells, and this too is retained in the ER. However, CPVL-EGFP is not seen in lamellipodia or membrane ruffles, suggesting that it may be incorrectly processed by CHO cells, or that the GFP is removed during processing. We have so far been unsuccessful in attempts to transfect CPVL into a monocytoid cell line.

Although CPVL colocalizes with MHC class II in lamellipodia/membrane ruffles, it does not remain associated with these molecules on phagosomes. CPVL shows no significant association with EEA1 or LAMP1 nor does it colocalize with endocytosed fluorescently labelled dextran in macrophages or with caveolin 1-positive vesicles (unpublished results). However, CPVL does colocalize with cathepsin D in some intracellular vesicles, although the nature of these vesicles is not clear, and it does not colocalize with cathepsin D on mature latex bead phagosomes. The biosynthesis of class II molecules and processing of antigens are known to occur largely in endocytic and lysosomal vesicles and the latter is likely to involve cathepsin D (see Watts 2001). The concentration of MHC class I molecules in lamellipodia/membrane ruffles may be relevant to the presence of CPVL in these structures. Antigens for loading on MHC class I molecules are largely generated via the proteasome system in the cytosol, independent of normal endocytic pathways, although there is evidence for the involvement of other proteases (see Kloetzel & Ossendorp 2004). Processing of some peptides may also occur in the ER (Bonifacino & Lippincott-Schwartz 1991). The transport of processed peptides from the cytosol to the ER for loading onto class I molecules is mediated using TAP (transporter associated with transport) genes (Androlewicz *et al.* 1993; Neefjes *et al.* 1993; Shepherd *et al.* 1993), while in DC soluble antigens that have escaped proteolysis **have** been shown to enter the lumen of the ER, from where they may be translocated to the cytosol for degradation Ackermann *et al.* 2004). The localization of CPVL both in the cytosol and associated with vesicles, along with its expression in the ER, could suggest a role in processing/transport of peptides for loading onto class I molecules and could explain why CPVL was not detected in the Golgi or endocytic pathways, despite its presence in the ER. However, CPVL is not retained on the phagosome with class I and does not colocalize with intracellular pools of MHC class I. CPVL, along with MHC class I and MIP-1α, is evident in newly formed lamellipodia/membrane ruffles of MO cultured on glass coverslips for 10 min, whereas MHC class II does not associate with these structures within the first 90 min of culture (unpublished results). This early appearance on lamellipodia in newly spreading MO, along with it's presence on the early, but not maturing phagosome, could suggest a role for CPVL in the formation of lamellipodia/membrane ruffles, more specifically in actin remodelling. The absence of CPVL in the actin-rich ruffles of DC may indicate that the specific functions of these structures may differ between macrophages and DC, although localization of CPVL within actin-positive ruffles in pre-DCs may suggest that these differences are subtle.

Little is known about the secretory system of macrophages, but carboxypeptidases are known to be involved in the regulated secretory pathway of cells of the central nervous system (CNS). In particular, carboxypeptidase E (CPE) is instrumental in the sorting and processing of prohormones, including pro-opiomelanocortin and proenkephalin (Loh *et al.* 2002). CPE exists both as a membrane-bound prohormone-sorting receptor, probably in the *trans*-Golgi network (Cool *et al.* 1997) and as a

soluble processing enzyme within secretory granules (Song & Fricker 1995). CPD, a B-type metalloprotease with CPE-like activity in the CNS, has also been identified in membrane fractions of murine macrophage cell lines (McGwire *et al.* 1997) and has been implicated in arginine-dependent nitric oxide release (Hadkar & Skidgel 2001), although nothing is known of its intracellular localization in these cells. We have shown that CPVL colocalizes with MIP-1α and TNF-α in lamellipodia, which could suggest that its site of action is along the secretory pathway. Also, CPVL appears to exist in both membrane-bound and soluble forms. Thus, it may be involved in the sorting/processing/activation of secretory molecules, such as cytokines and/or chemokines. The lack of CPVL bound to the cell surface or secreted in the media of macrophage cultures would suggest that CPVL is not itself secreted nor acts as a PM-bound exoenzyme but concentrates just below the cell surface in lamellipodia/membrane ruffles. However, whilst we have not been able to detect secreted CPVL, we can not rule out the possibility that a secreted form of the protein might not be recognized by our antibodies. Development of a fully functional recombinant CPVL would allow better characterization of its processing, as well as enabling study of its enzymatic activity.

In summary, we have shown, using a newly characterized mAb, that CPVL has a distinctive localization in human macrophages, concentrating in the ER, where it is glycosylated, and in lamellipodia/membrane ruffles. On the basis of its localization, CPVL may play a role in the biosynthesis of secretory molecules, or could be involved in the processing and/or transport of peptides for loading onto MHC class I molecules, or in MHC class II-dependent APC functions, but it is unclear whether it is involved in endocytosis. CPVL exists both in a membrane-bound form and as a soluble, cytosolic molecule. Although enzymatic activity has not yet been demonstrated for CPVL, we have suggested possible functions of this novel serine carboxypeptidase, which may prove to be a useful marker for human macrophages.

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