Effect of mutation of two critical glutamic acid residues on the activity and stability of human carboxypeptidase M and characterization of its signal for glycosylphosphatidylinositol anchoring

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Human carboxypeptidase (CP) M was expressed in baculovirus-infected insect cells in a glycosylphosphatidylinositol-anchored form, whereas a truncated form, lacking the putative signal sequence for glycosylphosphatidylinositol anchoring, was secreted at high levels into the medium. Both forms had lower molecular masses (50 kDa) than native placental CPM (62 kDa), indicating minimal glycosylation. The predicted glycosylphosphatidylinositol-anchor attachment site was investigated by mutation of Ser^{406} to Ala, Thr or Pro and expression in HEK-293 and COS-7 cells. The wild-type and S406A and S406T mutants were expressed on the plasma membrane in glycosylphosphatidylinositol-anchored form, but the S406P mutant was not and was retained in a perinuclear location. The roles of Glu^{260} and Glu^{264} in CPM were investigated by site-directed mutagenesis. Mutation of Glu^{260} to Gln had minimal effects on kinetic parameters, but decreased heat stability, whereas mutation to Ala reduced the k_{cat}/K_m by 104-fold and further decreased stability. In contrast, mutation of Glu^{264} to Gln resulted in a 10000-fold decrease in activity, but the enzyme still bound to *p*-aminobenzoylarginine–Sepharose and was resistant to trypsin treatment, indicating that the protein was folded properly. These results show that Glu^{264} is the critical catalytic glutamic acid and that Glu^{260} probably stabilizes the conformation of the active site.

Key words: membrane anchoring, peptidase, peptide metabolism, recombinant protein expression, site-directed mutagenesis.

INTRODUCTION

Human carboxypeptidase (CP) M was discovered as a membrane-bound enzyme that cleaves C-terminal Arg and Lys residues from peptides, such as bradykinin, at neutral pH [1–3]. Purification and characterization of CPM showed it to be a 62 kDa glycoprotein that is anchored to plasma membranes via a glycosylphosphatidylinositol (GPI) anchor [2,4–6]. The cloning and sequencing of human CPM revealed the presence of a mildly hydrophobic stretch of 15 residues at the C-terminus that is a putative signal sequence for GPI attachment [7].

CPM is a member of the so-called 'regulatory' or CPN/E subfamily of zinc metallocarboxypeptidases [2,8] that now includes CPs M, E/H, N, D, Z, X1 and X2, and adipocyte enhancer-binding protein 1 (AEBP1) [7–13]. Whereas the overall identities of members of this family with the more distantly related 'pancreatic' CPs or CPA/B subfamily are low (15–20 %), sequence alignment has allowed the tentative assignment of several putative active-site residues [7,8,14].

A hallmark of zinc metalloenzymes is the presence of three amino acid residues (two His and one Glu in metallocarboxypeptidases) that co-ordinate the catalytic zinc atom [15]. Another important residue is the catalytic glutamic acid, corresponding to Glu^{270} in pancreatic CPA. This residue is generally accepted to play a critical role in catalysis by assisting the ionization of the metal-bound water [16], although some evidence has been provided to indicate the possible formation of a covalent acyl-enzyme intermediate with the substrate resulting from nucleophilic attack of the catalytic Glu [17,18]. Glu^{264} in human CPM aligns with the catalytic Glu^{270} of pancreatic CPA [7]. This residue is contained within a NCFEITLE^{264}LSC sequence that is highly conserved in other members of the CPN/E subfamily (Figure 1). However, there is relatively low overall identity with the corresponding CPA sequence of IKYSFTFE^{270}LRD, which is highly conserved in other members of the CPA/B subfamily [8,19] (Figure 1). AEBP1, a transcriptional repressor reported to have B-type CP activity [20,21], contains Tyr in the position of the putative catalytic Glu, but it does have a Glu four residues away that is conserved in all other members of the family (corresponding to Glu^{260} in CPM; Figure 1). Although this residue was identified as the putative catalytic Glu in AEBP1 in the original sequence alignment [20], other investigators have not been able to demonstrate that this protein has catalytic activity [11]. Nevertheless, the complete conservation of this residue indicates that it has an important function.

We undertook the present study to identify the membrane-anchor signal and the GPI attachment site of human CPM and to investigate the roles of its two conserved glutamic acid residues, Glu^{260} and Glu^{264}.

EXPERIMENTAL

Materials

Enzymes for molecular biological procedures were purchased from Promega and New England Biolabs. Radiochemicals were

Abbreviations used: AcNPV, *Autographica californica* nuclear polyhedrosis virus; AEBP1, adipocyte enhancer-binding protein 1; CP, carboxypeptidase; CPMwt, wild-type CPM; GPI, glycosylphosphatidylinositol; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; PI-PLC, phosphatidylinositol-specific phospholipase C.

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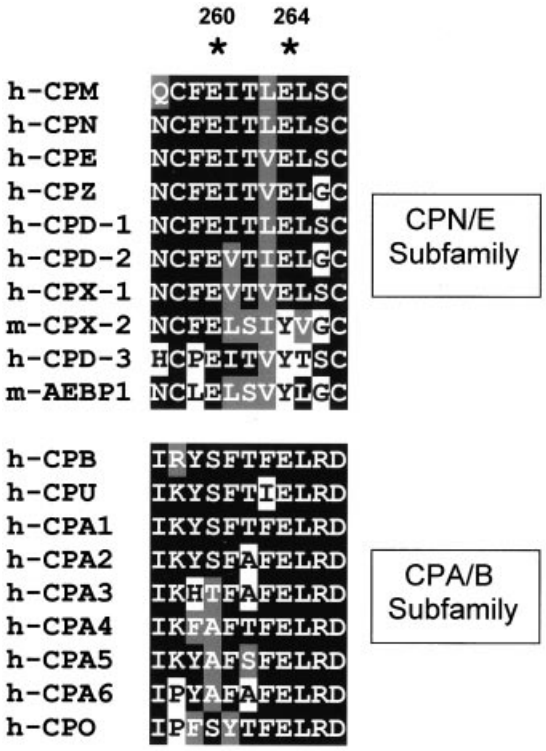

Figure 1 Comparison of sequences around the catalytic glutamic acid residue in mammalian metallocarboxypeptidases

Enzymes are categorized as either belonging to the CPN/E or CPA/B subfamilies based on overall sequence identity with CPs M, E and N or pancreatic CPs A and B, respectively [1,8,13,19]. Identical residues are denoted by black shading with reverse type; similar residues are shown with grey shading and reverse type; non-similar residues are not shaded. The positions of the two conserved glutamic acid residues are denoted by asterisks. h, human; CPD-1, CPD-2 and CPD-3 denote each of the three tandem CP repeat domains in the CPD sequence; m, mouse; AEBP1, a transcriptional repressor protein with sequence similarity to CPs that binds to the AE-1 site of the *aP2* gene [20].

from Amersham Biosciences. Sf900-II medium, Grace's insect medium and fetal bovine serum were from Gibco-BRL. The human kidney cDNA λgt10 library was a generous gift from Dr Graeme Bell (University of Chicago, Chicago, IL, U.S.A.). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was from ICN or Oxford Glycosystems. Circular or linear wild-type *Autographica californica* nuclear polyhedrosis virus (AcNPV) viral DNA, the pBluebac transfer vector and liposome transfection kit were from Invitrogen.

Insect cell culture

The *Spodoptera frugiperda* insect cell lines Sf9 and High Five (Invitrogen) were cultured in Sf900-II or Grace's insect medium with or without 10% heat-inactivated fetal bovine serum at 27 °C. The culturing, subculturing and transfection of the cells and the selection and purification of the recombinant virus were performed as described in the Invitrogen manual.

Construction of the transfer vector

The original CPM cDNA clone (HP-CPM) obtained from a human placental library [7] contained only part of the putative signal peptide and lacked an ATG initiation codon. To obtain the complete 5′ sequence, a CPM clone (HK-CPM) was isolated from a human kidney λgt 10 cDNA library as described in [22]. A 670 bp *Eco*RI–*Nco*I fragment from HK-CPM was ligated with the 1480 bp *Nco*I–*Eco*RI fragment from HP-CPM. The ligation mixture was separated by agarose-gel electrophoresis and the 2.2 kb DNA fragment isolated and subcloned into the *Eco*RI site of a pGEM-7Z cloning vector. The 2.2 kb *Eco*RI fragment containing the entire coding region of CPM was removed from the cloning vector, treated with T7 DNA polymerase to fill in the ends, and then ligated into the 14 kb pBlueBac transfer vector. The orientation of the inserted cDNA was determined by restriction endonuclease mapping. The cDNA lacking the C-terminal hydrophobic membrane anchor signal (CPMΔC) was constructed by cleavage of the CPM cDNA at a *Pvu*II site (upstream of the region coding for the C-terminal hydrophobic region), followed by addition of an *Xba*I linker to provide a stop codon and facilitate cloning. The 5′-end was also truncated to remove three upstream ATGs (present due to an artifact of the clone which contained an unrelated mitochondrial sequence at the extreme 5′-end) by cleaving at the *Sma*I and *Bss*HII sites, followed by blunt-end ligation and cloning into the *Bam*HI/*Xba*I site of a pGEM-7Z vector.

The E264Q mutant of CPM was generated by digesting the CPMΔC construct with *Hin*dIII to obtain a 240 bp fragment containing the coding sequence for Glu^{264}. The 240 bp *Hin*dIII fragment was cloned into a pGEM-7Z(−) vector, which had T7 and SP6 primer sites on each side of the polycloning region. Two mutation PCR primers (5′-TTGCAGCTGTCATGCTGTAA-3′ and 5′-CAGCTGCAACGTAATTTCAA-3′) were used with T7 and SP6 primers, respectively, to amplify two overlapping cDNA fragments, from bp 795 to 965 and from bp 957 to 998 (Glu^{264} is encoded by GAG at bp 960–962). Then, the 240 bp *Hin*dIII fragment encoding the Gln^{264} mutation was obtained by secondary PCR reaction with T7 and SP6 primers using a mixture of the first-round PCR products (in a 1:1 ratio) as the template, followed by digestion with *Hin*dIII. The mutated fragment was cloned back into the CPMΔC pGEM-7Z vector and sequenced to confirm that the desired mutation was present and that no other mutations were introduced by the PCR procedures used. Similar procedures were used to generate the E260A and E260Q mutants.

Production and purification of recombinant baculovirus

The pBlueBac plasmid containing the CPM cDNA was co-transfected into Sf9 cells along with wild-type AcNPV DNA using the calcium phosphate precipitation technique. Viruses resulting from homologous recombination between the transfer vector and AcNPV were detected by the formation of blue plaques on X-gal/agarose plates after 6–8 days of incubation at 27 °C. Blue and occlusion-negative (polyhedrin-free) plaques were picked up, resuspended in the medium and 5–6 cycles of plaque purification were performed, until only polyhedrin-free blue plaques were present on an individual plate. CPMΔC was cloned into the *Bam*HI/*Xba*I site of the baculovirus vector pVL1393, and recombinant virus was produced using the Pharmingen BaculoGold linearized baculovirus. Recombinant viruses containing the E260Q, E260A and E264Q mutants were generated using similar techniques.

Expression of recombinant human CPM

Cells were grown attached in flasks or culture dishes, or in suspension culture using spinner flasks. Cells were infected with a recombinant virus solution (ratio of virus/cells, 10:1) at room temperature for 60–90 min. The virus solution was replaced by

fresh serum-free medium and the cells were incubated at 27 °C for up to 5 days.

Immunogold labelling of CPM on Sf9 cells for scanning electron microscopy

Sf9 cells were harvested, collected by centrifugation (250 ***g***), washed in PBS and then resuspended in 0.25 % glutaraldehyde in PBS, pH 7.2. After 5 min of fixation at room temperature, cells were washed three times for 10 min each in PBS. All fluid changes were accomplished by centrifugation (1–2 s in a microfuge) and resuspension by pipette aspiration. Following the rinses in PBS, cells were treated three times for 10 min each with 0.1 % glycine in PBS to quench free aldehydes. To block non-specific binding of antibody, cells were incubated for 15 min in a solution of 5 % normal goat serum and 0.1 % BSA in PBS (BSA/PBS). Cells were exposed to primary antibody overnight at 4 °C. The primary polyclonal antiserum to purified human placental CPM was raised in rabbits and the IgG fraction purified as described [6]. Following incubation in the primary antibody, cells were rinsed five times for 5 min each in PBS and incubated with a secondary antibody conjugated to 15 nm colloidal gold particles (GARG15; Janssen Life Sciences, Piscataway, NJ, U.S.A.) for 1 h at room temperature. Both the primary and secondary antibodies were diluted in 1 % normal goat serum in BSA/PBS. Following this last incubation, cells were again rinsed five times in PBS for 5 min each. As controls, cells were incubated in the same manner either without primary antibody or with non-immune rabbit IgG.

For scanning electron microscopy, a drop of cell suspension was placed on a polylysine-treated, carbon-coated film that was applied to a conventional electron-microscope grid, and the cells were allowed to settle and attach for 30 min. The grid, with cells attached, was rinsed with water, dehydrated with an ascending ethanol series, and dried by the critical point method. Before scanning electron microscopy, the cells were coated with carbon by evaporation. Cell surfaces were imaged in correlative secondary electron imaging and backscattered imaging modes to reveal sites of immunogold labelling of CPM.

Enzyme assays

CP activity was measured with dansyl-Ala-Arg substrate as described in [23]. For kinetic studies, duplicate reactions were run to determine the initial rate of substrate hydrolysis (using four or five time points) at each of five or six substrate concentrations ranging from 0.4 to 3.5 times the K_m. Kinetic constants were determined by plotting [S] against [S]/v and fitted to the best straight line by linear regression. Correlation coefficients of $r = 0.995$ or better were obtained. Results are expressed as the mean ± S.E.M. of the results of three such experiments for each form of CPM tested.

Cell fractionation and immunoprecipitation

Infected Sf9 cells were harvested, collected by centrifugation (250 ***g***), washed with PBS and then resuspended in 50 mM Tris/HCl buffer, pH 8, containing 1 mM PMSF, 0.1 mM *p*-chloromercuriphenylsulphonate and 0.1 mM leupeptin. Cells were sonicated, and crude membrane and cytoplasmic fractions were obtained by centrifugation at 100000 ***g*** for 1 h. The membrane fraction was solubilized with 0.8 % (w/v) CHAPS for 1 h at 4 °C. Immunoprecipitation of CPM was performed essentially as described [24]. Samples (200 μl) were incubated with 50 μl of 1:10 diluted heat-inactivated polyclonal antiserum directed against CPM, or with normal rabbit serum as control.

Purification of recombinant CPM

CPMΔC and the E260Q, E260A and E264Q CPM mutants were expressed at high levels in the medium and were purified to homogeneity by two steps of ion-exchange chromatography. Briefly, the expression medium was concentrated by ultrafiltration, adjusted to pH 7.4, centrifuged and loaded on to a Q-Sepharose column equilibrated with 20 mM Tris/HCl, pH 7.4. Under these conditions, CPM does not bind and passes through the column, whereas many contaminant proteins are retained. The pass-through fractions were collected, concentrated and dialysed against 25 mM Mes, pH 6.3. The dialysed sample was applied to an S-Sepharose column (0.8 cm × 8.0 cm) pre-equilibrated with the dialysis buffer and the column was eluted with a linear salt gradient (250 ml × 250 ml) of 0–0.4 M NaCl in 25 mM Mes, pH 6.3. Active fractions were pooled and concentrated on an Amicon concentrator with a YM-10 membrane. In some cases, an additional FPLC purification step was performed using a Superdex-200 HR or Mono-S column to remove minor contaminants.

Synthesis of *p*-aminobenzoylarginine–Sepharose

p-Aminobenzoylarginine was synthesized similarly to the published procedure [25] with the following modifications. *p*-Nitrobenzoyl chloride (Aldrich; instead of isobutylchloroformate and *p*-nitrobenzoic acid) was reacted with an equimolar amount of L-arginine (Sigma) to generate *p*-nitrobenzoylarginine. The quantity of reactants was increased 10-fold, but the volume of solvent (tetrahydrofuran and *N*-methylmorpholine) was increased only 5-fold relative to the published procedure. After the reaction, the pH was lowered to 1.5 with HCl, the solution was filtered, and the solvent removed by rotary evaporation. The resulting syrup was extracted twice with ether to remove impurities and the *p*-nitrobenzoylarginine was purified on Sep-Pak C_{18} columns instead of Sephadex G-25 used in the original procedure. Briefly, 40 μl of trifluoroacetic acid was added to a 40 ml aliquot of the concentrate, which was applied to a 35 ml Sep-Pak C_{18} column. The column was washed with 34 ml of water/0.1 % trifluoroacetic acid and the *p*-nitrobenzoylarginine was eluted with 50 ml of 20 % acetonitrile/0.1 % trifluoroacetic acid in water. Solvent was removed by rotary evaporation and lyophilization. *p*-Aminobenzoylarginine was generated from *p*-nitrobenzoylarginine by standard reductive hydrogenation. The purity of both *p*-nitrobenzoylarginine and *p*-aminobenzoylarginine was checked by HPLC analysis on a reversed-phase column and the identity of both compounds was confirmed by MS.

p-Aminobenzoylarginine was coupled to epichlorohydrin-activated Sepharose as described for the preparation of arginine–Sepharose [26].

Binding of CPMΔC and the E264Q mutant to *p*-aminobenzoylarginine–Sepharose

Aliquots of purified CPMΔC or E264Q (23–49 μg) were diluted to 0.5 ml with buffer A (0.05 M Tris, pH 7.5, containing 0.1 M NaCl and 0.05 % CHAPS) and mixed in a 1.5 ml conical tube with 0.25 ml of *p*-aminobenzoylarginine–Sepharose for 1 h at 4 °C. Tubes were centrifuged briefly to settle the gel and the supernatant containing any unbound enzyme was removed. The gel was washed three times with 0.5 ml aliquots of buffer A and then eluted with four 0.25 ml aliquots of buffer A containing 0.1 mM DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA). All fractions were analysed by SDS/PAGE on a 9 % acrylamide gel and eluted protein was detected by silver staining.

Trypsin treatment of CPMΔC and E264Q

Aliquots of purified CPMΔC or E264Q were incubated in 0.05 M Hepes, pH 7.5, with 10 mM $CaCl_2$ (final volume, 22 μl) in the absence or presence of trypsin at trypsin/CPM molar ratios of 1:100, 1:30 and 1:10 for 4 h at 37 °C. PMSF (8 μl, 10 mM) was added to stop the reaction and prevent trypsin hydrolysis of CPM denatured during the heating in sample buffer prior to electrophoresis. Samples were analysed by SDS/PAGE on 10 % acrylamide gels and bands were detected by silver staining.

Construction of CPM GPI anchor signal mutants

WT-CPMpcDNA3 was constructed by ligating the *Bam*HI–*Nco*I fragment from CPMΔC pGEM-7Z (see above) and the 3′-end *Nco*I–*Eco*RI fragment from CPM pGEM-4Z [7] into the *Bam*HI/*Eco*RI sites of pcDNA3. S406A-CPMpcDNA3, S406P-CPMpcDNA3 and S406T-CPMpcDNA3 were all made using the QuikChangeXL Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, U.S.A.). In the PCR reaction, WT-CPMpcDNA3 was used as a template and the mutagenesis primers used were as follows: S406A-CPM, 5′-GCCAGACCAC-GCAGCTGCAACAAAG-3′ and 5′-GTTGCAGCTGCGTGG-TCTGGCAAAT-3′; S406P-CPM, 5′-GCCAGACCACCCAG-CTGCAACAAAG-3′ and 5′-GTTGCAGCTGGGTGGTCTG-GCAAAT-3′; S406T-CPM, 5′-GCCGACCACACAGCTGCA-ACAAAG-3′ and 5′-GTTGCAGCTGTGTGGTCTGGCAAA-T-3′. The sequence of the mutations was confirmed by DNA sequencing performed by the DNA Core Facility, University of Illinois at Chicago, IL, U.S.A.

Transfection and analysis of GPI anchoring

HEK-293 cells were plated in 100 mm dishes at about 50–70 % confluence the day before the transfection. For transfection, 10 μg of DNA from the wild-type or mutant CPM constructs was mixed together with 50 μl of SuperFect transfection reagent (Qiagen) in 300 μl of serum-free, antibiotic-free Dulbecco's modified Eagle's medium, kept at room temperature for 5–10 min, then diluted with 3 ml of Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and antibiotics and applied to PBS-washed HEK-293 cells. The cells were incubated with the transfection mixture in an incubator (5 % CO_2) at 37 °C for 3 h and then washed with PBS and cultured in Dulbecco's modified Eagle's medium with 10 % fetal bovine serum for 40–48 h. The cells were collected in ice-cold PBS (2 × 1.5 ml), centrifuged and then resuspended in 0.5 ml of cold PBS containing protease-inhibitor cocktail without metal chelators (Sigma). The cells were broken by freeze–thawing and sonication on ice. Soluble and membrane fractions were prepared by centrifugation at 100000 ***g*** for 30 min. The membrane fraction (pellet) was resuspended in 0.5 ml of PBS. A 100 μl aliquot of the suspended membrane fraction was incubated with an equal volume of either 1 % Triton X-100 detergent at 4 °C for total solubilization of activity or with water (control) or water containing 30 m-units of PI-PLC for 1 h at 37 °C. Samples were then centrifuged at 100000 ***g*** for 30 min and the supernatants were assayed to determine the amount of released CPM.

Immunostaining of cells expressing GPI anchor site mutants

COS-7 cells were plated on a circular microscope cover glass (18 mm) and grown to 50 % confluency. Cells were then co-transfected with 2 μg of the wild-type or mutant CPM pcDNA3 constructs and pEGFP-C1 (Clontech) encoding enhanced green fluorescent protein or with pEGFP-C1 alone (control) using SuperFect reagent. After a 48 h incubation, cells were fixed in 0.8 ml of PBS containing 4 % paraformaldehyde and 0.12 M sucrose for 20 min at room temperature. The fixed cells were washed three times for 5 min each with 100 mM glycine in PBS and then three more times with PBS with gentle shaking. After blocking non-specific binding with PBS containing 5 % normal goat serum (Sigma), 0.2 % BSA and 0.1 % Triton X-100 for 1 h, the cells were incubated with 0.5 ml of a 1:500 dilution of monoclonal antibody against CPM (Novocastra Laboratories) in 2 % normal goat serum, 1 % BSA and 0.1 % Triton X-100 in PBS at 4 °C overnight. After the incubation with the first antibody, the cells were washed three times with PBS with 0.1 % Triton X-100, blocked as above for 1 h. Cells were then incubated with 0.5 ml of a 1:750 dilution of AlexaFluor 546 goat anti-mouse IgG(H + L) conjugate (Molecular Probes, Eugene, OR, U.S.A.) in 2 % normal goat serum, 1 % BSA and 0.1 % Triton X-100 in PBS for 1 h. Finally, the cells were washed three times with PBS containing 0.1 % Triton X-100, rinsed very briefly with distilled water, mounted on a glass slide and analysed by confocal microscopy.

Protein assay

Protein concentrations were routinely determined by the dye-binding method of Bradford [27] with BSA as a standard. It is well known that inherent differences in dye binding by proteins can result in inaccurate protein values for highly purified samples when using this method [28,29]. To eliminate this source of error, the protein concentration of a purified sample of recombinant CPM was accurately determined by measuring the absorbance at 280 nm and using an absorption coefficient of 61290 $M^{-1} \cdot cm^{-1}$, calculated from the protein sequence as described in [30,30a]. Several dilutions of this same sample, assayed by the method of Bradford, gave protein concentrations that were an average of 2.29-times lower than that determined by the absorption coefficient. Thus for routine protein determinations of subsequent samples of purified CPM, the protein concentration was determined with the dye-binding assay and then multiplied by 2.29 to obtain the true value.

RESULTS

Production of recombinant CPM

When insect cells were infected with recombinant baculovirus containing the wild-type CPM (CPMwt) sequence, increasing amounts of B-type CP activity cleaving the dansyl-Ala-Arg substrate were found in cell lysates after 2–5 days of infection (Figure 2A). Cells were fractionated by differential centrifugation to obtain cytosolic and membrane preparations and the membrane fractions were solubilized with 0.8 % CHAPS. The highest levels of activity were in the membrane fraction, with less activity in the cytosolic fraction and no activity in the medium until after 4 days, when virus-induced cell lysis began (Figure 2A). To prove that the activity was CPM, the solubilized membrane fractions were immunoprecipitated with antiserum to human CPM, resulting in precipitation of 84, 90 and 91 % of the activity after 2, 3 or 4 days of infection, respectively. Furthermore, similar to CPM purified from human placenta [4], the membrane-bound CP activity had a pH optimum in the neutral range (between 6.5 and 7.5) and was inhibited almost completely by 1 mM guanidinoethylmercaptosuccinic acid (97 %) or 1 mM *o*-phenanthroline (96 %), but not by 1 mM PMSF or 0.1 mM leupeptin. Finally, cells infected with wild-type baculovirus produced no detectable CPM in the membrane fraction or cytosol at up to 4 days (results not shown).

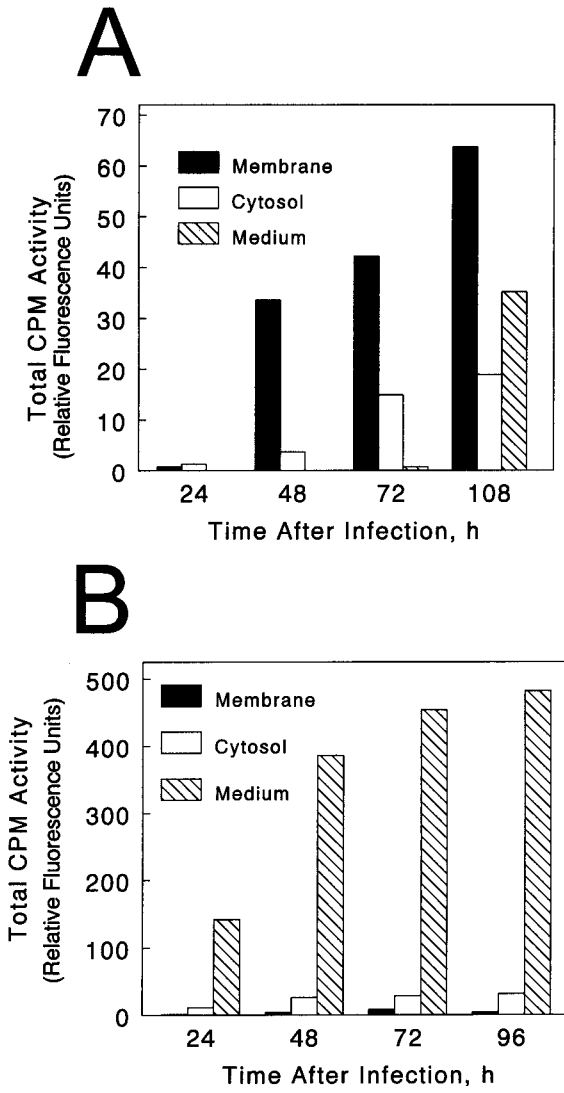

Figure 2 Expression of recombinant CPMwt and CPMΔC in baculovirus-infected insect cells

Cells were infected with recombinant baculovirus as described in the Experimental section and incubated at 27 °C for various times, after which the cells and medium were harvested and assayed for CPM activity with dansyl-Ala-Arg substrate. (**A**) Cells were infected with recombinant baculovirus containing the CPMwt construct; (**B**) cells were infected with recombinant baculovirus containing the CPMΔC construct.

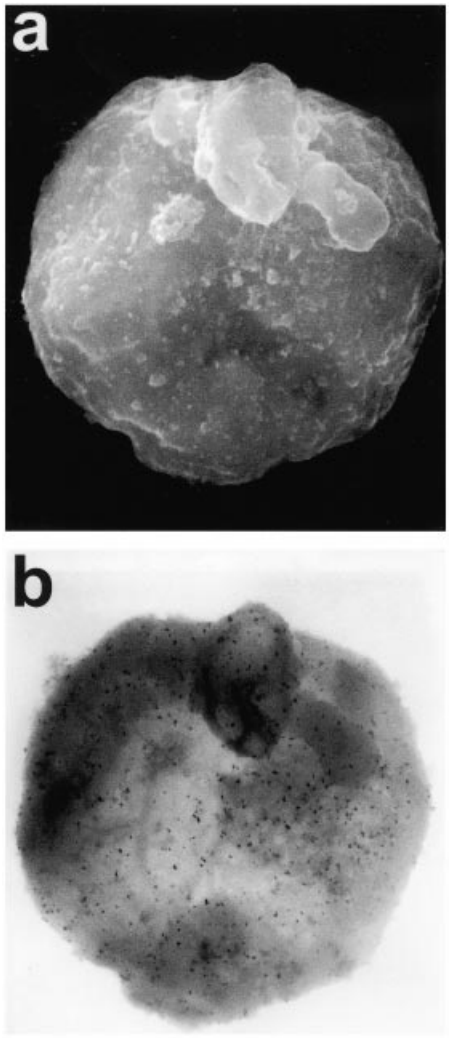

Figure 3 Scanning electron micrographs showing immunogold staining of an insect cell infected with CPMwt recombinant baculovirus

(**a**) Secondary electron (low-energy) image showing cell-surface features. (**b**) Backscattered electron (high-energy) image reveals areas of high density. Black particles show deposition of immunogold complexes indicating the presence of CPM on the cell surface. Magnification, × 7050.

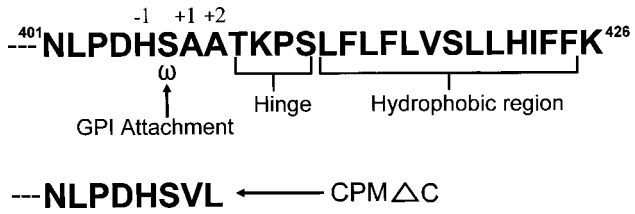

Figure 4 Schematic diagram of the C-terminal sequences of human CPMwt and CPMΔC

Important features are marked based on required sequence elements of functional GPI anchor signal sequences that have been identified in previous studies [33].

Further evidence for the plasma-membrane expression of CPMwt was obtained by immunogold electron microscopy (Figure 3). Incubation of infected cells with antiserum specific for human CPM followed by gold-labelled secondary antibody resulted in heavy labelling of CPM on the cell surface (Figure 3). Infected cells incubated either without primary antibody or with non-immune rabbit IgG were not labelled (results not shown). The recombinant, membrane-anchored CPM was readily solubilized with 0.8 % CHAPS detergent, as was found with naturally occurring CPM in placental microvilli [4] or Madin–Darby canine kidney cell membranes [6]. A unique characteristic of GPI-anchored proteins is their ability to be released from the membrane by bacterial PI-PLC [31]. We thus treated membrane fractions of insect cells infected with recombinant CPMwt with 20 m-units of bacterial PI-PLC for 1 h at 37 °C and determined the amount of CPM released. In the presence of buffer only, 9 % of the CPM activity was released during the 1 h incubation, whereas PI-PLC released 60 % of the activity. For comparison

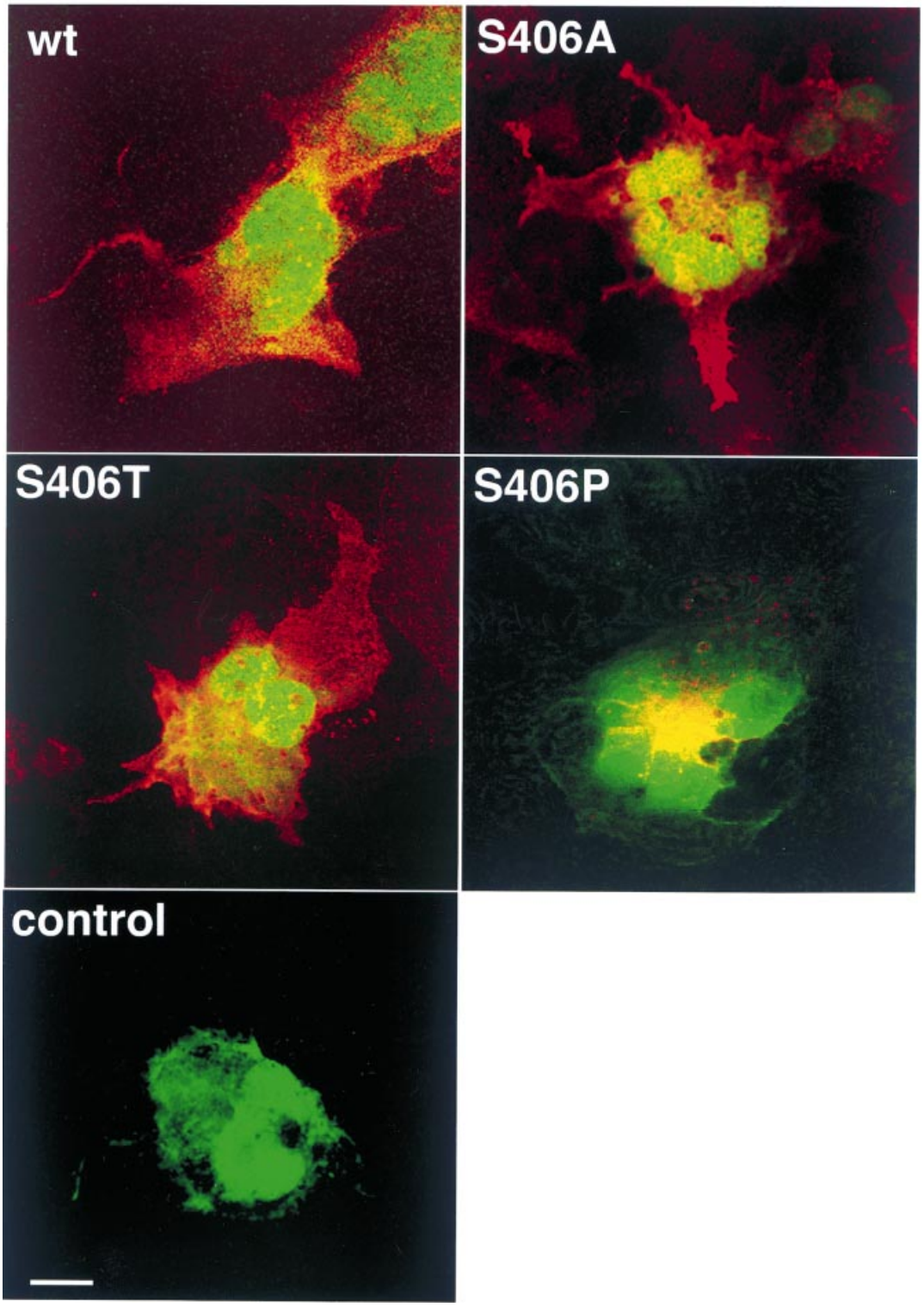

Figure 5 Localization of CPMwt and GPI-anchor site mutants expressed in COS-7 cells

COS-7 cells were co-transfected with wild-type (wt) or mutant CPM pcDNA3 constructs and pEGFP-C1 (encoding enhanced green fluorescent protein as a marker of transfection) or pEGFP-C1 alone (control), immunostained for CPM with monoclonal antibody and then analysed by confocal microscopy. Red staining indicates the localization on the cell membrane of CPMwt and the S406A and S406T mutants. The perinuclear yellow staining indicates retention of the S406P mutant in the endoplasmic reticulum/Golgi. No CPM staining was evident in cells transfected with pEGFP-C1 alone (control). Scale bar, 10 μm.

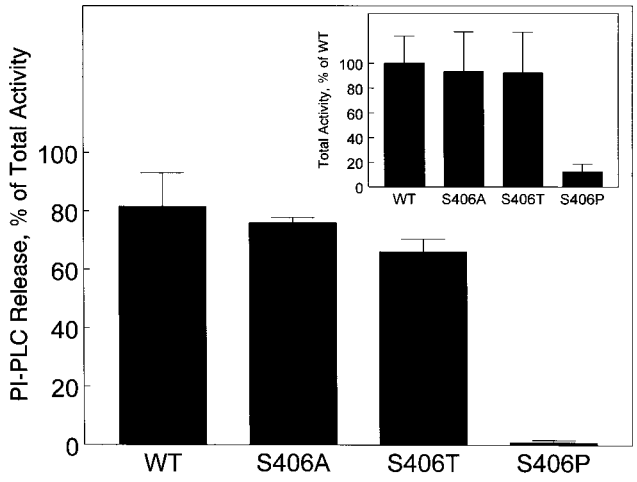

Figure 6 PI-PLC release of CPMwt and GPI-anchor site mutants from membranes of transfected HEK-293 cells

HEK-293 cells were transfected with CPMwt (WT) or mutant CPM pcDNA3 constructs and membrane fractions were prepared. The membrane fractions were incubated for 1 h with 1% Triton X-100 detergent at 4 °C (for total activity), water (as a control) or water containing 30 m-units of PI-PLC at 37 °C, followed by centrifugation at 100000 ***g*** for 30 min. The released CPM was measured in the supernatant with the dansyl-Ala-Arg substrate. For each set of experiments, mock-transfected cells were treated in the same way and background CP activity was subtracted from the results obtained with the transfected cells. For PI-PLC studies, CPM activity released by water treatment alone was subtracted from the activity released by PI-PLC. Results shown are the means ± S.E.M. from 3–7 separate experiments, each assayed in duplicate. Inset shows total CPM activity expressed in HEK-293 cell membranes after solubilization with 1% Triton X-100. PI-PLC release is expressed as the percentage of total membrane activity solubilized with 1% Triton X-100.

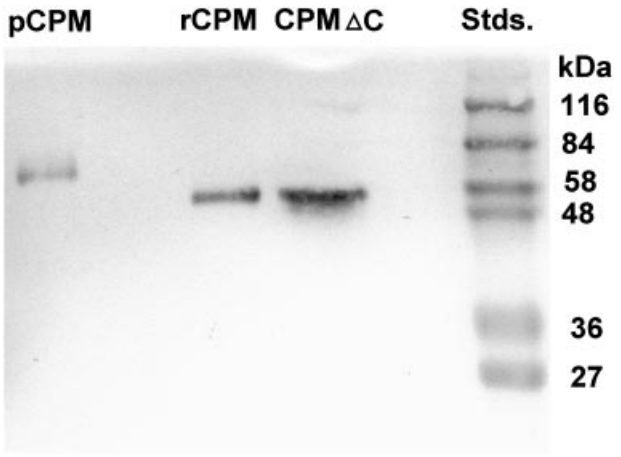

Figure 7 Western blot of purified human placental CPM and recombinant CPMwt and CPMΔC

Native CPM purified from human placenta or recombinant CPM from baculovirus-infected insect cells was electrophoresed, blotted and detected using antiserum to human placental CPM. Stds., molecular-mass markers; pCPM, placental CPM; rCPM, full-length CPM expressed in baculovirus-infected insect cells; CPMΔC, CPM lacking the C-terminal GPI-anchor signal sequence expressed in baculovirus-infected insect cells.

purposes, a placental microvillar fraction was used as a positive control; buffer alone resulted in release of 20% of the activity, while PI-PLC released a total of 80% of the CPM. These data indicate that recombinant CPMwt is correctly processed and anchored to the plasma membrane via a GPI anchor, as is the naturally occurring enzyme in mammalian tissues and cells [5,6,32].

Production of a soluble form of recombinant CPM

To prove that the C-terminal region of CPM functions as a GPI anchoring signal, a truncated cDNA lacking this region (CPMΔC) was constructed and incorporated into a recombinant baculovirus. The C-terminal sequence of the protein ends in -His^{405}-Ser-Val-Leu^{408}, with the last two residues being added because of the construction method used (Figure 4). Insect cells infected with the CPMΔC recombinant virus produced increasing amounts of CPM activity 1–4 days after infection (Figure 2B). In contrast with CPMwt, CPMΔC was almost completely secreted into the medium and at no time was any significant activity detected in the membrane fraction (Figure 2B). The expression level of the soluble CPMΔC was also much higher when compared with the cellular expression of CPMwt. Levels of up to 30–40 mg/l of culture medium could be attained with CPMΔC, whereas expression levels of CPMwt ranged from about 100–500 μg in the cells contained in a total of 1 l of culture. To prove that the signal peptide was processed correctly by the insect cells, the N-terminal sequence of the purified recombinant soluble CPMΔC was determined. A single sequence was obtained, Leu-Asp-Phe-Asn, which matches that of the mature N-terminus of CPM purified from human placenta [7], indicating correct processing in the insect cells.

Determination of the GPI-anchor attachment site

The above results show that the C-terminal sequence of CPM is necessary for GPI anchoring. The GPI-anchor attachment site is usually comprised of a three small amino acid residues (ω, $\omega+1$, $\omega+2$, where ω is the attachment site) followed by a 5–11 residue polar spacer or hinge region and a C-terminal hydrophobic domain [33,34]. Based on these considerations, the Ser^{406}-Ala-Ala-^{408} sequence of CPM would comprise the ideal $\omega/\omega+1/\omega+2$ combination (Figure 4). To determine if Ser^{406} is indeed the attachment site, we mutated it to Ala (S406A), Thr (S406T) or Pro (S406P) and expressed the wild-type or mutant proteins in either COS-7 cells (for immunohistochemical staining) or HEK-293 cells (for biochemical studies). As shown in Figure 5, CPMwt was well expressed on the cell membrane, exhibiting punctate staining typical of GPI-anchored proteins, and it was also efficiently released from membrane fractions by PI-PLC (Figure 6). The S406A mutant was very similar to the wild-type enzyme with regard to expression and release by PI-PLC (Figures 5 and 6), which is consistent with Ala being one of the preferred residues at the ω site [33]. The S406T mutant was expressed at a similar level and cell-surface staining was similar to that of the wild type or the S406A mutant, and PI-PLC release was similar (Figures 5 and 6). GPI anchoring of the S406T mutant was unexpected as Thr was reported to not be a viable ω attachment site in some studies [33,35–37]. However, it was recently reported that Thr can function as the attachment site in some contexts [38]. Because it is well established that proline is not allowed at the ω site [33,35,37,38], we mutated Ser^{406} to Pro. Indeed, the S406P mutant was expressed at much lower levels than the wild type and could not be released from the membrane by PI-PLC (Figure 6). In addition, there was no evidence of surface staining, but instead cells exhibited a perinuclear pattern of staining, consistent with either endoplasmic reticulum or Golgi localization (Figure 5). These results are consistent with previous findings showing that GPI-anchored proteins engineered to contain an uncleavable GPI anchor signal are retained and degraded in the endoplasmic reticulum/Golgi compartment [37,39–41].

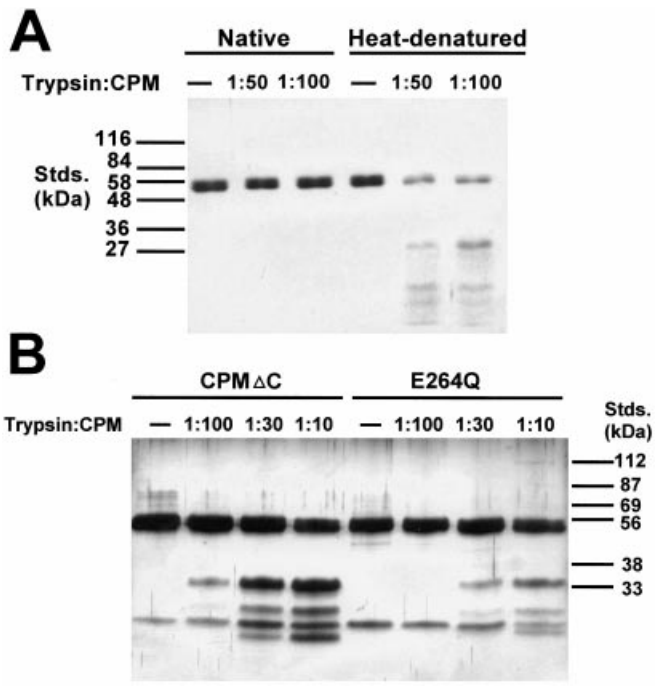

Figure 8 Trypsin hydrolysis of CPMΔC and the E264Q mutant

Purified CPMΔC or E264Q was incubated in 0.05 M Hepes, pH 7.5, with 10 mM $CaCl_2$ alone (—) or in the presence of trypsin at various molar ratios of trypsin/CPM as indicated for 4 h at 37 °C. Samples were analysed by SDS/PAGE on 10% acrylamide gels and detected by silver staining. Stds., positions of molecular-mass markers. (**A**) Trypsin treatment of CPMΔC (Native) or CPMΔC that had been heat-denatured for 15 min at 60 °C (Heat-denatured). (**B**) Trypsin treatment of CPMΔC or the E264Q mutant. A minor contaminant band at about 22 kDa is apparent in (**B**) in the CPM preparations used.

Molecular mass of recombinant CPM

The apparent molecular masses of CPMwt and CPMΔC were identical and ranged from 50 to 53 kDa on SDS/PAGE, depending on the molecular-mass standards used (Figure 7). The similar molecular masses were to be expected because, based on the predicted attachment site for the GPI anchor at Ser^{406}, CPMwt would contain 406 residues and CPMΔC 408 residues. However, the masses of both recombinant forms were much lower than that of native placental CPM (62 kDa). CPM is a glycoprotein with six potential Asn-linked glycosylation sites [7] and deglycosylation of the native protein reduces its molecular mass to 47.6 kDa [4]. To determine the mass more accurately, purified CPMΔC was analysed by matrix-assisted laser-desorption ionization–time-of-flight MS, which yielded a single peak of 49278 Da. Based on the calculated molecular mass of 46596 Da for CPMΔC, the enzyme would contain only 5.4% carbohydrate by mass, less than one-quarter of that found in the native placental CPM [4]. The smaller size of the recombinant protein is probably due to a lower level of glycosylation and less complex carbohydrate structures, as is common with proteins expressed in the baculovirus system [42]. Insect cells generally do not have the capacity for adding complex N-glycans and most commonly yield glycoproteins containing oligomannosidic N-glycans with five to nine mannose residues and two *N*-acetylglucosamine residues [42], which would add approx. 1300–2000 Da per site. The difference of 2682 Da between the calculated protein molecular mass and that determined for CPMΔC indicate glycosylation at two out of the possible six sites, which is consistent with the recently determined X-ray crystal structure of CPMΔC (D. Reverter, M. Braun, K. Maskos, F. Tan, J. K. Black, S. Balsitis, R. Huber, R. A. Skidgel and W. Bode, unpublished work).

Table 1 Kinetic constants of recombinant CPMΔC and Glu^{260} mutants with the substrate dansyl-Ala-Arg

Duplicate reactions measured at four or five time points were carried out at each of five or six substrate concentrations and kinetic constants were determined by plotting [S] against [S]/*v*. Results are expressed as the mean ± S.E.M. from the results of three separate experiments for each form of CPM tested. Mutated residues are shown in bold.

Enzyme	Sequence (258–267)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
CPMΔC	CF**E**ITLELSC	59 ± 0.5	5.12 ± 0.25	86780
E260Q	CF**Q**ITLELSC	102 ± 5	6.02 ± 0.38	59020
E260A	CF**A**ITLELSC	280 ± 20	0.233 ± 0.01	832
E260A + 0.5 M NaCl	CF**A**ITLELSC	42 ± 0.8	0.433 ± 0.003	10310

Purification and characterization of CPMΔC

The high level of expression of CPMΔC and its secretion into serum-free medium in a fully soluble form afforded a relatively straightforward purification of the enzyme to homogeneity, as revealed by SDS/PAGE (Figure 8) and a single N-terminal sequence (described above). To further prove that the soluble recombinant enzyme retained the characteristics of the solubilized membrane-bound form of human CPM purified from placenta, it was characterized with regard to substrate hydrolysis, pH optimum, inhibition by a specific B-type CP inhibitor and metal-ion activation. CPMΔC readily cleaved the synthetic dipeptide substrate dansyl-Ala-Arg with a K_m of 59 μM and a k_{cat} of 5.12 s^{-1} (Table 1). As a comparison, the kinetic constants were also determined for CPM purified from human placenta, which gave a K_m of 57 μM and a k_{cat} of 6.62 s^{-1}. These data indicate that the recombinant CPMΔC is fully active. The slightly higher turnover number with placental CPM could be due to either somewhat higher intrinsic activity or a slight underestimation of the protein concentration in the protein assay due to higher carbohydrate content of the placental enzyme.

Consistent with the properties of human placental CPM we determined previously [4], CPMΔC has a neutral pH optimum and is potently inhibited by the arginine analogue MGTA with a K_i of 2.0 nM (results not shown). Also characteristic of CPM, the ability of Co^{2+} to activate CPMΔC was pH-dependent; 1 mM $CoCl_2$ activated the enzyme by 2.0-fold at pH 7.5, but by 4.9-fold at pH 5.5, similar to previous results with CPM purified from human placenta [43].

Effect of mutation of Glu^{260} on CPM activity

The residue equivalent to Glu^{260} is strictly conserved among members of the CPN/E subfamily (Figure 1), indicating that it performs an important function. This was investigated by mutating Glu^{260} in CPMΔC to either Gln (E260Q) or Ala (E260A). The recombinant mutant enzymes were expressed in the baculovirus system and purified as described in the Experimental section. Both mutants were enzymically active, cleaving the CPM substrate dansyl-Ala-Arg, and exhibited properties similar to CPMΔC with regard to its pH optimum (7.0), inhibition by *o*-phenanthroline and MGTA and activation by cobalt (results not shown). To determine if the E260Q and E260A mutants also

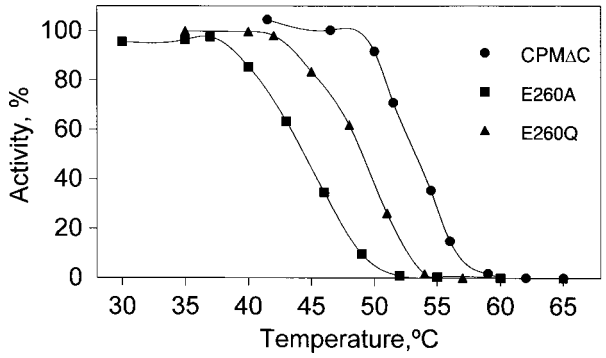

Figure 9 Heat stability of CPM and Glu^{260} mutants

CPMΔC or the E260Q or E260A mutant was incubated at various temperatures for 15 min, quenched on ice and then assayed for remaining CP activity with the dansyl-Ala-Arg substrate. Activity is expressed as a percentage of the control value determined for enzyme incubated for 15 min at room temperature. Results shown are the mean values for two (E260Q), three (CPMΔC) or four (E260A) determinations. Standard errors calculated for results with CPMΔC or E260A were ±2.9% or less and in most cases were smaller than the symbol and are thus not shown.

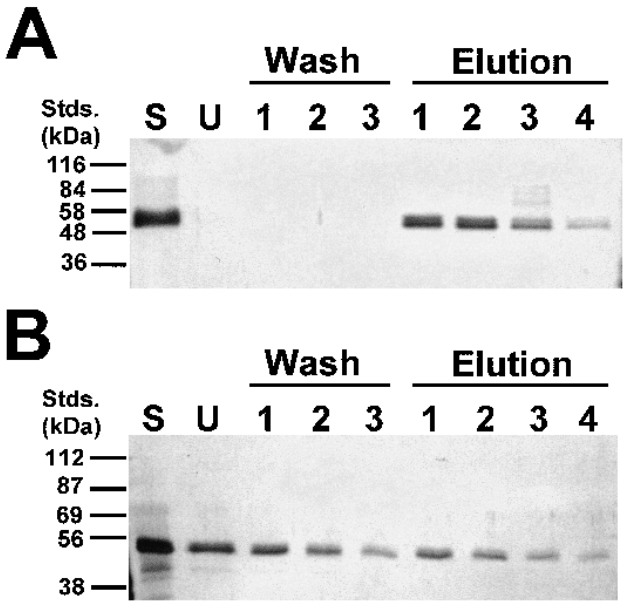

Figure 10 Binding and elution of CPMΔC and the E264Q mutant from *p*-aminobenzoylarginine–Sepharose

Purified CPMΔC or the E264Q mutant was bound to *p*-aminobenzoylarginine–Sepharose, and then washed and eluted in a batchwise fashion as described in the Experimental section. Fractions were analysed by SDS/PAGE and bands detected by silver staining. S, starting sample; U, unbound fraction after initial binding to the affinity resin; lane numbers refer to fraction numbers of elution with either wash buffer (Wash) or buffer containing 0.1 mM MGTA (Elution). (**A**) Binding and elution of CPMΔC. (**B**) Binding and elution of the E264Q mutant.

exhibited the same strict substrate specificity for C-terminal basic residues as the wild-type enzyme, they were incubated with a series of benzyloxycarbonyl-Ala-Xaa dipeptides (Xaa = Arg, Ile, Asn, Glu, Met, Ala, Gly or Leu). Whereas benzyloxycarbonyl-Ala-Arg was cleaved rapidly, none of the other peptides was hydrolysed, even after prolonged incubations (results not shown). To examine the activities of these mutants in more detail, kinetic constants with dansyl-Ala-Arg were determined (Table 1). The kinetic values with the E260Q mutant were only slightly altered compared with the CPMΔC control, with about a 2-fold increase in K_m and a small increase in k_{cat}, leading to only a small decrease in the specificity constant (Table 1). However, the activity of the E260A mutant was substantially decreased, with an almost 5-fold increase in the K_m value and a 22-fold decrease in the k_{cat}, leading to a 104-fold fall in the k_{cat}/K_m (Table 1). The addition of 0.5 M NaCl to the assay buffer did not significantly alter the activities of the wild-type enzyme with 200 μM dansyl-Ala-Arg (106±3% of control without added salt) or the E260Q mutant (116±3%), but did substantially increase the activity of the E260A mutant (219±5%; $n = 3$). The activity enhancement of E260A was not specific to NaCl, as similar increases were found with other salts such as $NaNO_3$ (249%), KNO_3 (256%), KCl (256%) and Na_2SO_4 (297%; mean values of two separate experiments each run in duplicate), indicating that the effect was probably due to an increase in ionic strength. Kinetic studies on the E260A mutant run in the presence of 0.5 M NaCl showed that the increase in activity was due to a large fall in K_m (over 6-fold) and a smaller increase in k_{cat} (almost 2-fold), leading to a 12-fold increase in k_{cat}/K_m, which was still 8-fold lower than that of the native CPMΔC (Table 1).

To determine the effect of the mutations on stability, enzymes were incubated for 15 min at various temperatures, cooled on ice and then assayed with dansyl-Ala-Arg for remaining activity. As shown in Figure 9, the three enzymes exhibited quite different heat-stability curves. The CPMwt was most stable, retaining 50% of its activity after 15 min at 53.3 °C, followed by E260Q, which was 50% inactivated at 49.0 °C. The E260A mutant was the least stable, being completely inactivated after incubation at temperatures above 50 °C and retaining 50% of its activity at only 44.5 °C (Figure 9). Nevertheless, these results show that the substantially lower activity of the E260A mutant is not due to its instability as the enzyme was stable at 37 °C (Figure 9), the temperature at which the kinetic constants were determined.

Identification of Glu^{264} as the catalytic glutamic acid in CPM

The CPMΔC construct was used to generate a mutant CPM in which the putative catalytic Glu^{264} was mutated to Gln (E264Q). When the recombinant virus containing this construct was used to infect insect cells, a protein of the correct size was detected in Western blots of the conditioned medium, but no CP activity was detected with dansyl-Ala-Arg. Despite the lack of activity, the mutant enzyme was eluted identically with CPMΔC when purified by ion-exchange chromatography and was purified to a similar extent (Figures 8 and 10). Western analysis with anti-CPM antibody showed a positive band at the same molecular mass as the major protein band (results not shown). Although undetectable in the crude medium, a very low level of activity could be detected in the purified E264Q mutant with the sensitive dansyl-Ala-Arg fluorimetric assay using high amounts of protein (≈ 5 μg per assay) and a prolonged incubation time (120 min). Although the activity was too low to obtain meaningful kinetic data, the specific activity of the E264Q mutant with 200 μM dansyl-Ala-Arg substrate was 0.52 nmol/min per mg (mean value from two separate preparations and purifications), which is over 10000-fold lower than the activity of the recombinant CPMΔC (5490 nmol/min per mg). At this level of activity, it cannot be ruled out that a slight contamination of the preparation with an insect cell CP could be responsible for the hydrolysis of dansyl-Ala-Arg measured. For example, it would require only a 0.01% contamination of the preparation with an enzyme that has an equivalent specific activity to CPM to account for the activity.

Thus, it is possible that the E264Q mutation completely eliminated the activity of CPM.

Although these results suggest that Glu^{264} is indeed the catalytic glutamic acid, the data could also be interpreted as evidence for an important structural role for Glu^{264} and that mutation to Gln results in a denatured, inactive protein. To address this question, we tested the susceptibility of the mutant protein to trypsin hydrolysis. Native CPM is relatively resistant to trypsin, as we reported before [6], but CPM denatured by heat treatment (15 min at 60 °C) was readily cleaved (Figure 8A). When treated with increasing amounts of trypsin (from a ratio of 1:100 to 1:10 trypsin/CPM), the E264Q mutant was actually somewhat more resistant to hydrolysis than CPMΔC (Figure 8B), indicating that it is not in a denatured state.

To explore the possibility that the E264Q mutant, although unable to cleave a C-terminal Arg might still bind to it, we applied the protein to the substrate affinity gel, *p*-aminobenzoylarginine–Sepharose. As shown in Figure 10, the E264Q mutant did bind to the affinity resin, although more weakly than CPMΔC as shown by the presence of the enzyme in the initial supernatant and the successive buffer washes. Under the same conditions, CPMΔC completely bound to the affinity gel and did not elute with the buffer washes (Figure 10A). Addition of the specific inhibitor, 0.1 mM MGTA, resulted in elution of both the E264Q mutant and CPMΔC (Figure 10) in a similar fashion. Taken together, the above data show that the lack of activity of the E264Q mutant is not due to production of an unfolded, denatured protein and support the conclusion that Glu^{264} is the catalytic glutamic acid. The increased resistance to trypsin treatment and weaker binding to the affinity resin indicate that the conformation of the E264Q mutant is slightly altered compared with CPMΔC containing Glu^{264}.

DISCUSSION

The C-terminal sequence of CPM is both necessary and sufficient for GPI anchoring, as shown by secretion of CPMΔC in soluble form in this study and our previous experiments in which angiotensin-converting enzyme (normally a type I transmembrane protein) was correctly GPI-anchored when engineered to contain the CPM GPI-anchor signal at the C-terminus [44]. A GPI-anchor signal typically consists of a three-residue cleavage/attachment motif (designated ω, $\omega+1$, $\omega+2$) made up of small amino acids, followed by a polar 'hinge' or spacer region, usually containing proline, and finally a mildly hydrophobic region at the C-terminus [33,35,37,38]. These sequence features are all present in the C-terminal domain of CPM (Figure 4), where the Ser^{406}-Ala-Ala-^{408} sequence, representing the ω, $\omega+1$, $\omega+2$ residues, contains the most highly preferred amino acid at each site [33]. Using the formula derived from experimentally determined probabilities for the ω and $\omega+2$ residues [33], the probability of attachment at Ser^{406} is calculated to be 1.0 (out of a maximum of 1.0). The only other possible attachment sites are Asp^{404} or Ala^{407}, but the probabilities are only 0.12 and 0.04, respectively. A recent study revealed a previously undiscovered preference for basic residues at the $\omega-1$ site [38]. In these experiments, the presence of a His at $\omega-1$ significantly increased GPI anchoring (about 2–5-fold), especially at sites representing weak signals [38]. The presence of His^{405} at $\omega-1$ in the CPM sequence indicates that this is a robust GPI-attachment site and may explain our finding that replacement of Ser^{406} with Thr, not usually found as an ω residue, still allowed significant membrane localization and PI-PLC release of the S406T mutant. Indeed, it was recently shown that Thr could function as a GPI-attachment site in rat acetylcholinesterase and that GPI anchoring at the Thr site was enhanced about 2-fold by the addition of His at $\omega-1$, yielding a protein that was GPI-anchored at about 70 % of the efficiency of the protein containing a preferred Asn at the ω site [38].

The higher level of expression of secreted CPMΔC than native GPI-anchored CPM in baculovirus-infected insect cells is similar to results found in transfected COS cells expressing soluble and GPI-anchored forms of alkaline phosphatase [45] or carbonic anhydrase IV [40]. The reason for the difference in expression of soluble versus GPI-anchored forms of the same protein is unknown, but could be due to either exceeding the capacity of the cell to process overexpressed GPI-anchored proteins or the presence of negative regulatory feedback that inhibits GPI-anchored protein synthesis. That the latter might occur is supported by the finding that co-transfection of COS cells with a GPI-specific phospholipase D and GPI-anchored alkaline phosphatase resulted in greatly increased release of alkaline phosphatase into the medium and an overall 3–4-fold greater expression than in cells transfected with alkaline phosphatase alone [46]. We found that release of CPM by treatment of cells with exogenous PI-PLC up-regulates enzyme synthesis in Madin–Darby canine kidney cells [32]. Increased GPI-anchored protein synthesis after release with PI-PLC was also reported for neural cell adhesion molecule of 120 kDa (N-CAM120) in a C6 rat astrocytoma cell line [47] and acetylcholinesterase in the parasite *Schistosoma mansoni* [48].

It has been reported that utilization of a native insect signal-peptide sequence (e.g. honeybee melittin) can greatly increase the expression level of heterologous proteins [49]. However, the native human CPM signal peptide correctly targeted CPMwt and CPMΔC to the secretory pathway in insect cells and resulted in an especially high level of expression of CPMΔC, showing that a native mammalian signal peptide can be efficiently processed in this system. The high-level expression of a soluble form of CPM that is only minimally glycosylated has facilitated the production of large amounts of the protein for determination of its structure by X-ray crystallography (D. Reverter, M. Braun, K. Maskos, F. Tan, J. K. Black, S. Balsitis, R. Huber, R. A. Skidgel and W. Bode, unpublished work).

The strict conservation of the glutamic acid residue corresponding to Glu^{260} in CPM in all members of the CPN/E subfamily (Figure 1) indicates that it plays an important structural or functional role. The lack of a large effect on kinetic constants when this residue was mutated to Gln indicates that it is not intimately involved in the catalytic process. The somewhat lower stability of the E260Q mutant indicates that it may play a structural role, e.g. via the formation of hydrogen bonds, to maintain the structure of the active-site pocket. Thus replacement of Glu with Gln would still allow the formation of hydrogen bonds, which would probably be weaker. This is supported by the properties of the E260A mutant, in which the substituted residue would be unable to form side-chain hydrogen bonds. Indeed, the E260A mutant had dramatically lower catalytic efficiency, due to both an increase in K_m and a decrease in k_{cat}, and was also much less stable to heat inactivation. The ability of increased ionic strength to partially reverse the loss in catalytic effectiveness is not directly explained by a loss of hydrogen bonds. However, it may be evidence for the possible formation of inappropriate interactions in the E260A mutant that are diminished by an increase in ionic strength. For example, inappropriate hydrogen bonds could be formed by the normal binding partners of Glu^{260}. In addition, the smaller side chain of Ala might allow interactions that would be sterically unfavourable in the native protein. These conclusions are supported by the recent determination of the crystal structure of CPM (D.

Reverter, M. Braun, K. Maskos, F. Tan, J. K. Black, S. Balsitis, R. Huber, R. A. Skidgel and W. Bode, unpublished work). In the structure, Glu^{260} is not directly in, but is very near, the active-site pocket, being some 7–11 Å from the side chain binding Asp or catalytic Glu. However, it does form critical hydrogen bonds with Lys^{60}, Ser^{169} and Asn^{171}. The importance of this hydrogen-bonding network is supported by the strict conservation of all four of these residues in all other active members of the CPN/E subfamily, which are located near other active-site residues in the primary sequence [8,9]. Thus, Lys^{60} is close to the zinc-binding His^{66}, and Ser^{169} and Asn^{171} are close to the third zinc-binding residue, His^{173}.

Whereas mutation of Glu^{260} to Gln had only minor effects on the catalytic efficiency of CPM, mutation of Glu^{264} to Gln resulted in at least a 10000-fold decrease in enzyme activity. It is possible that the mutation completely eliminated the activity as we cannot rule out the possibility of slight contamination with an insect cell CP that co-purified with the CPM mutant. This was not the result of production of a misfolded, denatured protein as the mutant still bound to an affinity resin and was resistant to trypsin treatment. In addition, a misfolded protein would probably be retained and degraded within the cell, not secreted at high levels as was the E264Q mutant. Thus, these data support the identification of Glu^{264} as the catalytic glutamic acid in human CPM.

The lack of activity of the E264Q mutant is consistent with either of the two proposed roles for the analogous catalytic Glu^{270} in pancreatic CPA: (i) as a nucleophile which forms a covalent acyl-enzyme intermediate with the substrate [17,18] or (ii) as a general base catalyst that promotes the ionization of a zinc-bound water molecule to attack the scissile peptide bond and function as a proton donor to the amino acid being released [16]. Gln is the same size as Glu and can form hydrogen bonds, but would be unable to act as a nucleophile or general base catalyst; thus its presence in the active site would be expected to result in a largely inactive enzyme, as we found. On the other hand, if Glu^{264} of CPM was not a general base catalyst or nucleophile, but participated in catalysis only by stabilizing the transition state and/or acting as a general acid catalyst to protonate the amine leaving group, mutation to Gln would probably result in a much higher level of residual activity. For example, in human matrilysin, where Glu^{198} is proposed to function in this way, mutation to Gln^{198} only reduced the k_{cat}/K_m by 590-fold [50].

Despite its lack of activity, the E264Q mutant was still retained on a *p*-aminobenzoylarginine–Sepharose resin, indicating that the active-site pocket maintained a functional conformation. However, the weaker binding of the E264Q mutant than native CPM to the affinity resin and its higher resistance to trypsin hydrolysis indicates that mutation to Gln resulted in subtle conformational changes. This is unlikely to be due to a steric effect as Gln and Glu are the same size, but could be due to formation of an inappropriate hydrogen bond with another residue in the active-site pocket.

Our results are consistent with the identification of Glu^{300} as the catalytic glutamic acid of rat CPE [51]. This residue aligns with Glu^{264} in the human CPM sequence (Figure 1), strongly suggesting that the homologous residue in other regulatory CPs also functions as the catalytic glutamic acid. In addition, in the X-ray crystal structure reported for domain 2 of duck CPD [52] and that recently determined for human CPM (D. Reverter, M. Braun, K. Maskos, F. Tan, J. K. Black, S. Balsitis, R. Huber, R. A. Skidgel and W. Bode, unpublished work), this Glu is appropriately positioned in the active-site pocket to perform its suggested catalytic role. Similar to our results, mutation of Glu^{300} in rat CPE to Gln resulted in an essentially inactive protein that still could bind to a substrate affinity resin, although more weakly than the native enzyme [51]. Recent studies have identified three unique members of the CPN/E subfamily, AEBP1 [20], domain 3 of CPD [9,10] and CPX2 [12], that have significant overall sequence identity with other members of the family, but lack several important catalytic residues, including the active-site Glu (Figure 1). All three members have the conserved glutamate corresponding to Glu^{260} in CPM (Figure 1) that was identified as the potential catalytic Glu in AEBP1 [20]. However, our results would argue against this possibility. The assignment of a catalytic function to this Glu is also inconsistent with the recent homology modelling of the structure of CPD-3 [53]. Interestingly, all three proteins have Tyr in the position of the catalytic Glu found in the other CPs, and the sequences preceding this residue are strikingly similar: Leu-Ser-Val-Tyr in AEBP1, Ile-Thr-Val-Tyr in domain 3 of CPD and Leu-Ser-Ile-Tyr in CPX2. Although AEBP1 has been reported to have B-type CP activity [20,21], two other groups have been unable to reproduce this finding [11,51,54] and domain 3 of CPD [55] as well as CPX2 [12] lack any detectable activity with typical B-type CP substrates. In addition, replacement of Glu^{300} in rat CPE with Tyr resulted in inactive protein that did not bind to a substrate affinity resin [51]. However, it is possible that these 'inactive' members of the CPN/E subfamily have evolved an unusual catalytic activity or more likely use the metallocarboxypeptidase α/β hydrolase fold as a structural scaffold for another function, such as binding to other proteins or peptides, as proposed for CPD-3 [53]. Indeed, domain 3 of duck CPD binds the duck hepatitis B-virus pre-S protein [55] and the precise binding site was recently identified as a 30-residue sequence (920–949) near the N-terminus of the domain [56].

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