Duck gp180 was previously identified by its ability to bind to the preS envelope protein of duck hepatitis B virus particles (Kuroki, K., Cheung, R., Marion, P. L., and Ganem, D. (1994) J. Virol. 68, 2091–2096). Cloning and sequencing of gp180 cDNA revealed that it is a polyprotein with three carboxypeptidase-like domains (Kuroki, K., Eng, F., Ishikawa, T., Turck, C., Harada, F., and Ganem, D. (1995) J. Biol. Chem. 270, 15022–15028). To evaluate enzymatic properties of this protein, a soluble 170-kDa form of the protein (gp170) lacking the C-terminal transmembrane domain and cytoplasmic tail was expressed in a baculovirus system. The purified 170-kDa protein cleaved 5-dimethylaminonaphthalene-1-sulfonyl (dansyl)-Phe- Ala-Arg with a pH optimum of 5.5–6.5. With this substrate at pH 5.5, the 170-kDa protein displayed a $K_m$ of 12 $\mu$M and a $K_{cat}$ of 57 s$^{-1}$. Dansyl-Pro-Ala-Arg and dansyl-Phe-Ala-Arg were cleaved with $K_m$ values of 17 and 21 $\mu$M, and $K_{cat}$ values of 57 and 17 s$^{-1}$, respectively. Constructs containing only the first or second carboxypeptidase domains also showed enzymatic activity. The effects of inhibitors and ions on enzyme activity of gp170 were generally similar to the effects of these compounds on purified bovine carboxypeptidase D. To evaluate the regions within gp180 necessary for binding preS, a series of deletion mutants were expressed in the 293T human kidney cell line. Deletions of the first and second domains, leaving the third domain intact, eliminated carboxypeptidase activity but retained preS binding. Deletion of the third domain eliminated preS binding but not carboxypeptidase activity. These results indicate that the third domain is responsible for preS binding, and this binding does not require carboxypeptidase activity.

Duck hepatitis B virus is a member of the hepadnavirus group, which includes human hepatitis B virus (1). These viruses are small enveloped DNA viruses that infect liver cells, causing acute and chronic hepatitis (1). The cellular receptor for human hepatitis B virus has not been identified. A candidate receptor for the duck hepatitis B virus (DHBV)$^1$ has recently been described (2, 3). This 180-kDa protein, named gp180, was identified based on its ability to co-precipitate with DHBV particles or recombinant DHBV envelope proteins (2). gp180 was found to bind with high affinity and specificity to the preS region of the DHBV large envelope protein (2). The binding domain within the preS region that is responsible for the gp180 interaction overlaps key areas that have been established as important for viral infectivity (4). The cDNA for gp180 has recently been cloned, revealing that gp180 is a member of the metallocarboxypeptidase gene family (3). Interestingly, gp180 contains an N-terminal signal peptide, three metallocarboxypeptidase-like domains, a putative C-terminal transmembrane domain, and a short cytoplasmic tail. Of the known carboxypeptidases, gp180 has the highest homology to carboxypeptidase E (CPE). Specifically, the first and second domains of gp180 have 39% and 43% amino acid identity with mammalian CPE, and the third domain has 29% amino acid identity.

CPE functions in the biosynthesis of numerous peptide hormones and transmitters (5, 6). CPE removes C-terminal Arg and Lys residues remaining after endoprotease cleavage of the prohormone. For many years, CPE was thought to be the only carboxypeptidase involved with neuropeptide biosynthesis. However, recent studies on the Cpe$^{fat}$/Cpe$^{fat}$ mouse suggest that additional CPE-like carboxypeptidases partially compensate for a defective CPE. These mice have a point mutation within the coding region of the CPE gene that renders the protein inactive (7). Despite the absence of active CPE in the Cpe$^{fat}$/Cpe$^{fat}$ mice, a reduced but detectable amount of peptide processing occurs, implying that additional carboxypeptidases may perform this step in vivo. A novel carboxypeptidase with CPE-like properties, named metallocarboxypeptidase D (CPD), was identified in bovine pituitary and in other tissues (CPD) (8). In bovine tissues, the major form of CPD is 180 kDa (8). In addition to the 180-kDa form, rat tissues also contain several smaller forms ranging from 100 to 180 kDa (9). In contrast, all other known carboxypeptidases are 30–60 kDa (10–19). The size of the major species of CPD is similar to that of gp180, raising the possibility that these proteins are homologs. In addition, the partial N-terminal amino acid sequence of bovine CPD is similar to that of the duck gp180, and both proteins are

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1 The abbreviations used are: DHBV, duck hepatitis B virus; kb, kilobase pair(s); dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; CPE, carboxypeptidase E; CPD, carboxypeptidase D; SV40, simian virus 40; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; GEMS, guanidinoethylmercaptosuccinic acid.
broadly distributed in many tissues (3, 9).

The purpose of the present study was to investigate the enzymatic properties of gp180 and to compare them to the properties of bovine CPD. To accomplish this, both gp180 and a 170-kDa form lacking the putative transmembrane domain and cytoplasmic tail (termed gp170) were expressed in a baculovirus system. The enzymatic properties of the purified gp170 protein are generally similar to those of the previously reported CPD, providing strong evidence that these proteins represent species homologs. In addition, the enzymatic activities of deletion mutants expressing only the first carboxypeptidase-like domain (termed gp75), only the second carboxypeptidase domain (termed gp60–120), or the combined first and second carboxypeptidase-like domains (termed gp120) were characterized to determine the regions responsible for carboxypeptidase activity. Furthermore, a series of deletion mutations were expressed in 293T cells to determine the regions responsible for binding to the DHV-B preS protein. Interestingly, this study showed that the third domain is responsible for binding preS but is not enzymatically active. In contrast, constructs deleting portions of the third domain did not bind preS but were enzymatically active. These results demonstrate that preS binding and carboxypeptidase activity are localized to different regions of the protein.

**MATERIALS AND METHODS**

**Plasmid Constructions**—For expression in the baculovirus system, the expression vector pVL1392L was created by inserting the double-stranded linker GCCGGCGCGCATGGCTTAAGTTAGGTTAATCTAG into the NotI and XhoI sites within the polylinker of the baculovirus expression vector pVL1392 (PharMingen). The linker contains an NcoI site (underline) that incorporates an ATG, an A/III site (bold), and stop codons in all three reading frames. For construction of pVL-170 expressing gp180, a 4.2-kb NcoI fragment containing the entire coding region of gp180 was inserted into the pVL-1392L. The initiating ATG of gp180 is encoded within one of the NcoI sites of the fragment. Incorporation of this fragment in the correct orientation generates a consensus Kozak sequence that includes the initiating ATG of gp180. For construction of pVL-170, a 4.2-kb NotI/BamHI fragment from pVL-180 was first subcloned into pcDNA3 (Invitrogen). The transmembrane and cytosolic domains of gp180 were then deleted by replacement with a unique XhoI/A/III fragment. The remaining vector was treated with Klenow and religated. From this subclone, a NotI/BamHI fragment encoding gp170 was then subsequently inserted into pVL1392 to generate the plasmid pVL-170; this plasmid encodes the lumenal portion of gp180 (residues 1–1307) with the additional residues LSEVI on the C terminus. For construction of pVL-120, a 1.4-kb StuIA/Ill fragment was deleted from pVL-180, and the remaining vector was then blunt-ended and religated; this pVL-120 plasmid encodes the residues 1–947 of gp180 (i.e., the first and second domains). For construction of pVL-75, a 2.6-kb Sacll/A/Ill fragment was deleted from pVL-180, and the remaining vector was blunt-ended and religated; this pVL-75 plasmid encodes residues 1–544 of gp180 (i.e., the first domain). For construction of pVL-60–120, a 1.3-kb SnaI/FspI fragment was deleted from pVL-120; the resulting plasmid encodes amino acid residues 1–66 fused to residues 492–948 (i.e., the second domain attached to the N-terminal signal sequence).

For expression in 293T cells, the transmembrane-spanning domain and the C-terminal tail of gp180 were replaced with a region of the human hepatitis B virus S protein so that the expressed protein could be readily isolated. For construction of pG180S4, an NcoI/XhoI fragment encoding the three carboxypeptidase domains of gp180 was blunt-ended with T4 DNA polymerase and subcloned into HindIII/BamHI-digested pSV45 that was also blunt-ended. The plasmid pSV45 contains the entire human hepatitis B virus surface protein (S protein) open reading frame cloned downstream of the simian virus 40 (SV40) early promoter (20). The HindIII site is downstream of the SV40 promoter, and the BamHI site is within the S protein coding region at amino acid residue 133. The resulting fusion protein contains the three carboxypeptidase-like domains of gp180 followed by residues 133–400 of the virus S protein. For construction of pG180DS16, the plasmid pG180S4 was digested with SaI and XhoI, treated with mung bean nuclease, and religated. For construction of pG180DS2, the plasmid pG180S4 was digested with BglII and religated. To construct pG180DS24, the plasmid pG180S4 was cleaved with HindIII, the ends were filled in with T4 DNA polymerase, the DNA was digested with BamHI and blunt-ended with mung bean nuclease, and the DNA was religated. To construct pG180DS33, the plasmid pG180S4 was cleaved with KpnI and Smll, treated with mung bean nuclease, and religated. All constructs were verified by sequencing.

**Expression and Purification of gp180, gp170, gp60–120 and gp1392 in SF9 Cells with Baculovirus**—The baculovirus expression vector (5 μg) containing gp180, gp170, gp120, gp75, or gp60–120 cDNA was combined with 0.25 μg of Bucagold viral DNA (PharMingen) and used to transiently infect 10⁷ SF9 cells using the standard calcium phosphate procedure recommended by PharMingen. The SF9 cells were grown in 4 ml of SF900 II serum-free medium (Life Technologies, Inc.) in a 25-cm² flask. Five days after the transfection, the medium was removed and a 0.1-ml aliquot was used to infect another 25-cm² flask containing 10⁶ SF9 cells in 4 ml of SF900-II serum-free medium. This second infection was harvested after 3 days, and 1 ml of the medium was used to infect 10⁸ cells in 50 ml of medium, growing in a 500-ml shaker flask. Medium from this third infection was subsequently used for large scale preparation of infected cells. Typically, 10 ml of the medium from the third infection was used to infect 2 x 10⁸ cells growing in 1 liter of SF900-II medium in shaker flasks.

For purification of gp170, gp120, gp75, and gp60–120, 1 liter of medium from cells infected for 2–3 days was collected after centrifugation at 30,000 x g for 30 min. The supernatant was adjusted to pH 5.5 with 2 M acetic acid and then applied to a 10-ml p–aminophenyl–Arg–Sepharose affinity resin, as described (8). This resin has previously been used for the purification of either CPE or CPD; whereas CPE can be eluted from the affinity column by increasing the pH to 8.0, mammalian CPD remains bound at pH 8 and can be eluted when Arg is included in the buffer (8). The affinity column was performed as described previously for the purification of CPD from bovine pituitary (8), using 5 mM Arg to elute the gp170. This eluate (50 ml) was concentrated to approximately 1 ml by filtration dialysis using a Centrifrap 30 (Amicon). Approximately 1 mg of purified protein (gp170, gp120, gp75, or gp60–120) was typically obtained from 1 liter of medium. For measurement of enzyme activity, the purified protein was diluted so that the Arg concentration was below 0.1 mM; previous studies have found that Arg concentrations of 1 mM or less have negligible effect on CPD enzyme activity. In addition to purification of gp170 and deletion mutants from medium, gp180 was purified from cells following extraction with 1 mM NaCl and 1% Triton X-100.

**Western Blot Analysis**—Protein was electrophoretically transferred to nitrocellulose from SDS-PAGE gels. Blots were blocked with 5% nonfat milk in 10 mM Tris 7.5, 150 mM NaCl, 0.1% Tween 20 and then incubated with 1:1000 dilution of polyclonal rabbit antiserum raised against portions of gp180. The anti-serum directed toward the C-terminal 57 amino acids of gp180 was raised against a glutathione S-transferase fusion protein that was expressed in _Escherichia coli_ and purified using a glutathione-agarose column. Another antiserum was raised against gp170 expressed in a baculovirus system and purified as described above. Following exposure of the blot to primary antiserum, the enhanced chemiluminescence method (Amersham Pharmacia Biotech) was used to detect bound antiserum.

**Carboxypeptidase Assays**—Assays using dansylated substrates were performed as described previously (21). Briefly, purified enzyme was combined with 100 μM substrate in a final volume of 250 μl in 100 mM NaAc, pH 5.5. After 30 min to 1 h at 37 °C, the reaction was terminated with the addition of 100 μl 0.5 M HCl and then 2 ml of chloroform was added. For kinetic analysis, purified enzyme was combined with substrate (12.5, 25, 50, 100, and 400 μM) and incubated at 37 °C for 30 min or 1 h. The amount of enzyme chosen was that which hydrolyzed a maximum of 20% of the peptide. The amount (nmol) of product was determined from standard curves determined for each peptide. The kinetic parameters were evaluated by fitting the data to _y_ = _m_1 / _x_ + _m_2 x + _x_ using the KaleidaGraph program ( _x_ = velocity, _x_ = substrate concentration, _m_1 = _V_m, max, _m_2 = _K_m). For inhibitor and ion analysis, the compounds were preincubated with enzyme on ice for 1 h prior to the enzyme assay. For analysis of the pH optimum, Tris acetate was used as buffer in place of the sodium acetate. Data represent the average of two to eight experiments, which were performed in duplicate.

Assays using the [3H]benzoyl–FAR substrate were performed as described previously (21) with minor differences. Briefly, purified enzyme immobilized on protein A-Sepharose (see above, re expression in 293T
cells) was combined with substrate (30 nM) in a final volume of 100 μl in 80 mM NaAc, pH 5.6, for 15 min at 37 °C. Reactions were terminated by the addition of 50 μl of 1 M HCl, and then 1 ml of chloroform was added. Samples were mixed on a vortex mixer and the aqueous phase discarded. Four hundred μl of the organic phase was transferred to scintillation vials and allowed to evaporate in a fume hood overnight. Scintillation fluid was added, and the samples were vortexed and counted for ³H. All constructs were tested twice by duplicate transfections. Determinations of enzyme activity from each of the transfections were performed in triplicate. Samples that scored negative had no detectable enzyme activity (<0.5% of total substrate detected in the organic phase). Those that scored positive cleared 35%, 8%, and 24% of the substrate for constructs pG180S4, pG180DS16, and pG180DS2, respectively.

Expression and Purification of Epitope-tagged Proteins in Kidney

293T Cells—Human embryonic kidney 293T cells (approximately 40% confluent in a 100-mm dish) were transfected with 7.5 μg of DNA using the calcium phosphate method (22). Approximately 16–18 h after transfection, cells were washed three times with cold phosphate-buffered saline (PBS) and then incubated for an additional 2 days in RPMI 1640 medium, supplemented with 10% fetal bovine serum. To prepare extracts, cells were first washed three times with cold phosphate-buffered saline and then lysed with 2 ml of cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100), which was supplemented with 1 mM phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, and 2 μg/ml pepstatin. Extracts were divided into two 1-ml aliquots and cleared by centrifugation in a microcentrifuge at maximum speed for 10 min at 4 °C. One-ml aliquots of the supernatant were incubated overnight at 4 °C on a rocking platform with 2 μl of rabbit anti-hepatitis B surface antigen (Biomedica Corp.) and 45 μl of protein A-Sepharose beads (90 μl of a 50% slurry preblocked with 1% bovine serum albumin). After adsorption, the beads were washed twice with 1 ml of lysis buffer and three times with 1 ml of lysis buffer containing 0.1% Triton X-100. Samples were aliquoted as a slurry into three microcentrifuge tubes and then centrifuged to pellet the beads bearing the immobilized fusion protein. Wash buffer was removed, and each aliquot was stored at −80 °C. Three aliquots were used for triplicate determinations in the enzyme assays. Each aliquot represents one-sixth of the purified protein recovered per dish of transfected cells.

To confirm the expression of the constructs and assess preS binding, duplicate transfections (performed on a separate day) were incubated in the absence of Met for 1 h and then labeled with [³S]Met/Cys (100 μCi/ml) in 4 ml of Met-free Dulbecco’s modified Eagle’s medium for 4 h at 37 °C. Extracts (2 ml) were prepared as described above and then diluted with an equal amount of lysis buffer. For confirmation of expression, a 1-ml aliquot (25% of the total extract) was subjected to immunoprecipitation with 5 μl of anti-S antibody and 50 μl of protein A-Sepharose for 4 h at 4 °C. For preS binding, a 1-ml aliquot (25% of the total extract) was incubated overnight at 4 °C with 50 μl of glutathione-agarose (Sigma) and 10 μg of a GST-preS fusion protein purified from the bacterial expression of plasmid pBES1 (described in Ref. 4). For preS binding competition between the ³S-labeled constructs and purified gp170, gp120, gp75, or guanidine-thiolcarboxypeptidase acid (GEMSA), 1-ml aliquots (12.5% of the total extract) were mixed with purified competitor and then incubated as described above with 50 μl of glutathione-agarose and 50 ng of the GST-preS fusion protein. Following the incubation, the protein A-Sepharose or glutathione-agarose beads were recovered by centrifugation and washed as described for the immunoprecipitation procedure above. Bound proteins were eluted from the beads by heating at 95 °C for 5 min in gel loading buffer containing 0.1% SDS and then subjected to denaturing SDS-PAGE. The gel was fixed in 30% methanol and 10% acetic acid, soaked in Fluorohance (Research Products International Corp.), dried, and then subjected to autoradiography at −80 °C.

RESULTS

Upon expression of gp180 in insect SF9 cells using the baculovirus expression system, an immunoreactive protein of 178 kDa is detected on Western blots (Fig. 1). Medium from these cells also shows some immunoreactive protein, although the apparent molecular weight is slightly smaller than the expressed cellular gp180 protein (Fig. 1). When a comparable Western blot was probed with an antiserum directed against the C-terminal 57 amino acids of gp180, a strong band of 178 kDa was detected in the gp180-expressing cells (not shown). Medium from these cells showed essentially no immunoreactive protein suggesting that the major form of gp180 in the medium is lacking this C-terminal region, presumably due to proteolytic processing. Expression of the form of the protein lacking the C-terminal 82 amino acids (gp170) with the baculovirus system shows both cellular and medium immunoreactive proteins of 170 kDa (Fig. 1). In addition, smaller forms are also detected in cells and to a lesser extent in the medium. Expression of only the first and second carboxypeptidase-like domains (gp120) or only the first domain (gp75) also shows both cellular and medium immunoreactive proteins of 123 and 76 kDa, respectively. The construct containing only the second carboxypeptidase domain (gp60–120) also showed a similar level of expression in medium upon Western blot analysis (data not shown).

Carboxypeptidase activity of the SF9 cells and medium infected with baculovirus for 3 days was measured with dansyl-Phe-Ala-Arg (dansyl-FAR) at pH 5.5. The background activity of cells infected with wild-type virus was 2.3 nmol of product/min of incubation with dansyl-FAR/10⁶ cells. The medium from these cells also contained 1.5 nmol/min/10⁶ cells of activity toward the substrate. Upon infection with the gp180-expressing baculovirus, the cells expressed 78 nmol/min/10⁶ cells of activity and the medium expressed 13 nmol/min/10⁶ cells of activity. Infection with gp170-expressing baculovirus produced 54 and 52 nmol/min/10⁶ cells of activity for the cell homogenates and medium, respectively. Infection with gp120-expressing baculovirus produced 156 and 167 nmol/min/10⁶ cells of activity for the cell homogenates and medium, respectively. Infection with gp60–120 expressing baculovirus produced 38 nmol/min/10⁶ cells of activity in the medium; activity for cell homogenates was not measured. These results indicate that gp180, gp170, gp120, gp75, and gp60–120 are able to cleave the dansyl-FAR substrate.

The baculovirus-expressed gp170, gp120, gp75, and gp60–120 were purified from medium using a substrate affinity column and conditions previously developed for the purification of bovine and rat CPD (Fig. 2). As found for mammalian CPD, duck gp170 remains bound to the affinity column when the pH is raised to 8.0 (not shown) and elutes when Arg is added (Fig. 2). After the affinity column purification, bands of 170, 123, 76, and 66 kDa are present for gp170, gp120, gp75, and gp60–120, respectively (Fig. 2). In contrast, attempts to purify the baculovirus-expressed gp180 from SF9 cells were less successful, with low recovery and purity (not shown). Thus, the purified gp170 was used for further characterization of the enzymatic properties. This enzyme was compared with CPD purified from bovine pituitary glands, and in some experiments also compared with gp120 and gp75.

FIG. 1. Western blot analysis of baculovirus-expressed gp180, gp170, gp120, and gp75. SF9 cells were infected with wild-type baculovirus (wt vir), or with baculovirus expressing the indicated protein. After 2 days of infection, cells were collected by centrifugation and lysed by sonication in 0.1 M sodium acetate, pH 5.5, buffer. An aliquot of the cells or medium was subjected to electrophoresis on a denaturing 8% polyacrylamide gel, and proteins were transferred to nitrocellulose at 100 mA overnight. The positions and molecular masses of prestained protein standards (Amersham Pharmacia Biotech) are indicated.

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Enzymatic Properties of gp180

**TABLE I**

| Compound                        | Concentration (mM) | Control* (%) |
|---------------------------------|-------------------|--------------|
| 1,10-Phenanthroline             | 1                 | 1.4          |
| Phenylmethylsulfonyl fluoride    | 1                 | 110          |
| Iodoacetamide                   | 1                 | 110          |
| p-Chloromercuriphenylsulfonate   | 0.1               | 51           |
| Leupeptin                       | 1                 | 110          |
| Benzamidine                     | 1                 | 110          |
| Tosylphenylalanylchloromethyl ketone | 0.1             | 110          |
| Tosyllysylchloromethyl ketone   | 0.1               | 110          |
| Guanidinomethylmercapto-  | 1                 | 12           |

* Variation in three separate experiments, each performed in duplicate, was less than 20%.

**TABLE II**

| Compound   | Concentration (mM) | Control* (%) |
|------------|-------------------|--------------|
| CoCl₂      | 1                 | 330          |
| ZnCl₂      | 1                 | 350          |
| CaCl₂      | 1                 | 200          |
| MnCl₂      | 1                 | 120          |
| CdCl₂      | 1                 | 61           |

* Variation in two separate experiments, each performed in duplicate, was less than 20%.

**TABLE III**

| Substrate | Enzyme | Km (mM) | Vₐₚ (µM/s) | Km/Kcat (µM⁻¹/s⁻¹) |
|-----------|--------|---------|------------|-------------------|
| Dansyl-FAR| CPD    | 63      | 51         | 0.81              |
|           | gp170  | 12 ± 2  | 57 ± 2     | 4.7 ± 0.9         |
|           | gp120  | 14 ± 2  | 22 ± 0.4   | 1.6 ± 0.3         |
|           | gp75   | 82 ± 8  | 9.2 ± 0.3  | 0.11 ± 0.04       |
|           | gp60–120| 26 ± 4  | 22 ± 0.2   | 0.83 ± 0.05       |
| Dansyl-FFR| CPD    | 54      | 22         | 0.41              |
|           | gp170  | 17 ± 2  | 57 ± 1     | 3.4 ± 0.6         |
|           | gp75   | 100     | 5.9        | 0.059             |
|           | gp60–120| 21 ± 4  | 17 ± 1     | 0.8 ± 0.2         |

* Data for CPD have been published previously (8).

The pH optimum of gp170 is 5.5–6.5 and is similar to that of purified bovine CPD (Fig. 3). This pH optimum distinguishes CPD from CPE and other metallocarboxypeptidases (8). The pH optima of the shorter constructs are generally similar to that of gp170, although the first domain (gp75) is less active at acidic pH values and the second domain (gp60–120) is more active at acidic pH values and the second domain (gp60–120) is more active than gp170, although the first domain (gp75) is less active at acidic pH values (Fig. 3). The chelating agent 1,10-phenanthroline inhibits both gp170 and bovine CPD (Table I), consistent with the proposal that these enzymes are metalloproteases. Inhibitors of serine proteases (phenylmethylsulfonyl fluoride) or cysteine proteases (iodoacetamide) do not substantially affect either gp170 or bovine CPD (Table I). However, the compound p-chloromercuriphenyl sulfonate partially inhibits both activities at 0.1 mM concentration, indicating a sensitivity to thiol-directed reagents. Other compounds, such as leupeptin, benzamidine, tosyl phenylalanyl chloromethyl ketone, and tosyldithiobenzyol chloride do not substantially affect either activity. CdCl₂ has a differential effect on the two activities, activating gp170 by 60% and inhibiting bovine CPD by approximately 40% (Table II). This activation of gp170 by CdCl₂ was observed in three separate experiments with comparable results (170%, 140%, and 160% of control).

The kinetic parameters of substrate hydrolysis were determined for dansyl-FAR and two related substrates (Table III). Of the three substrates examined, dansyl-FAR is cleaved with the highest Km and Kcat/Km by both gp170 and bovine CPD, and dansyl-FFR is cleaved with the lowest Kcat and Kcat/Km for both enzymes (Table III). The Km for all three substrates with gp170 are 3–5-fold lower than the Km for bovine CPD (Table III). In comparing dansyl-FAR hydrolysis with gp170, gp120, and gp75, the Km decreases by about 60% between gp170 and gp120, and by 50% between gp120 and gp75 (Fig. 3). The Kcat for gp75 is 6–7-fold higher than the Kcat for gp120 and gp170. Thus, the Kcat/Km for gp75 is only 2–3% of the value for gp170 (Fig. 3). In contrast, the construct that contains only the second carboxypeptidase domain (gp60–120) cleaves dansyl-FAR with a Kcat comparable to that with the construct containing both domains (gp120). The Km for gp60–120 is slightly higher than the Km for gp120, and so the Kcat/Km for gp60–120 is approximately 50% of that for gp120 (Table III).
Previously, gp180 was shown to bind to the preS region of DHBV. To determine the region of gp180 involved in this binding, a series of deletion mutations were transiently expressed in the human embryonic kidney (HEK) cell line 293T. This cell line differs from its progenitor, HEK293, in that the SV40 T antigen was introduced to allow for high levels of expression from vectors carrying an SV40 origin of replication. The recombinant proteins were C-terminally tagged with the human hepatitis B virus surface antigen (S protein) to facilitate purification of the expressed proteins by immunoprecipitation. In all constructs, the gp180 transmembrane domain and cytosolic tail was replaced with this tag (Fig. 4). The expression of each construct in the 293T cells was confirmed by immunoprecipitation using an antiserum to the S protein (Fig. 5). Although there was some variability in the expression levels and the recovery by immunoprecipitation, the fusion protein was detected for each construct (Fig. 5). Carboxypeptidase activity of the immunoprecipitate was assayed using the radioactive substrate $[3H]$benzoyl-Phe-Ala-Arg. The preS binding was assessed by the ability of the 293T expressed proteins to bind to the GST-preS fusion protein immobilized on glutathione-agarose beads (Fig. 5). Cells transfected with the construct containing all three carboxypeptidase-like domains (pG180S4) gave enzyme activity 56-fold above the background of the blank controls that lacked lysate. As expected, this construct bound the preS fusion protein (Fig. 5). A construct containing nearly all of the first two domains and lacking the third domain (pG180D16, Fig. 4), showed enzymatic activity (13-fold above background) but however did not bind preS (Fig. 5). Deletions within the first two domains (pGD180DS24 and pGD180DS33, Fig. 4) eliminated the carboxypeptidase activity, but did not interfere with preS binding (Fig. 5). A deletion of the second domain and the N-terminal half of the third domain (pG180DS24) eliminated preS binding but showed carboxypeptidase activity (Figs. 4 and 5).

To evaluate whether the three constructs that bound preS had comparable binding affinities, the ability of purified baculovirus-expressed gp170 to compete for binding was examined. Binding of S4 to preS was competed by increasing amounts of gp170 (Fig. 6A). Quantitation of this binding showed that approximately 40 ng of purified gp170 reduced the binding of S4 to preS by 50% (Fig. 6B). The binding of S24 and S33 to preS was also competed by purified gp170 (Fig. 6A). Quantitation of the results from two separate experiments showed that approximately 20–30 ng of purified gp170 reduced the preS binding by 50% (Fig. 6B). This result indicates that gp170 competes for binding of all three constructs with a generally similar potency. In contrast, 500 ng of purified gp75 (domain 1) or gp120 (domains 1 and 2) were unable to compete for preS binding to S33, which encodes for only the third domain (Fig. 7). Furthermore, 10 µM GEMSA (an active site inhibitor) does not compete for binding of S33 to preS (Fig. 7).

**DISCUSSION**

A major finding of the present study is that duck gp180 has carboxypeptidase activity. Furthermore, this activity is similar to that of bovine CPD. A key property that distinguishes CPD from other metallocarboxypeptidases, and which is shared by duck gp180 (or gp170), is the ability to bind to the p-aminoben-
Enzymatic Properties of gp180

With gp75 (domain 1), gp120 (domains 1 and 2), and the active-site inhibitor GEMSA. 293T cells transfected with the S33 construct were metabolically labeled with [35S]Met. Aliquots (1 ml) of the labeled extract were preincubated with either 10 μM GEMSA or 500 ng of baculovirus-expressed and purified gp75 or gp120. The preS binding was assessed as in Fig. 6.

The finding that Sf9 cells infected with gp180-expressing baculovirus secrete immunoreactive protein into the medium was observed in two separate experiments, including one in which the infection was performed for only 2 days to minimize cell death due to viral infection. Since the size of the secreted protein is not the same as the cellular form and the secreted protein does not react strongly with an antiserum raised against the C-terminal tail, it is likely that this secreted form results from proteolysis. In previous studies, both bovine and rat tissues were found to contain a variety of smaller forms of CPD, some of which were soluble (9). These smaller forms were not recognized by the C-terminally directed antiserum, suggesting that proteolysis of CPD cleaves the transmembrane domain near the C terminus and gives rise to the smaller soluble forms. An alternative explanation is that differential mRNA splicing gives rise to the various forms of CPD. In the present study, the finding that a form of gp180 is secreted from Sf9 cells is strong evidence in favor of proteolysis as a mechanism for generating the secreted form.

The results of the deletion analysis indicate that both the first and the second carboxypeptidase domains of gp180 are enzymatically active. This finding fits with the prediction based on the amino acid identity among these domains and other metallo-carboxypeptidases (3, 32). It is difficult to interpret the enzyme activity of deletion mutants as the removal of large portions of a protein can cause changes in structure and subsequently enzyme properties. For example, the $K_{cat}$ and the

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Fig. 6. A, preS binding to epitope-tagged gp170 and deletion mutants in the presence of gp170 competitor. 293T cells transfected with constructs that are able to bind preS (indicated with the last 2–3 characters of the plasmid name) were metabolically labeled with [35S]Met. Aliquots (1 ml) of the labeled extract were preincubated with the indicated amounts of purified gp170 (ng). GST-preS protein (50 ng) and glutathione-agarose beads were then subsequently incubated as described under “Materials and Methods.” Proteins bound to the beads were eluted with SDS-PAGE loading buffer and subjected to SDS-PAGE and autoradiography. B, autoradiograms shown in Fig. 6 were subjected to densitometry. Binding activity was normalized to the maxium binding activity detected in the absence of gp170 competitor for each construct. Error bars represent range of duplicate determinations for the S24 and S33 constructs. Points without error bars had range values smaller than the symbol size. Points for the S4 construct represent a single determination.

Fig. 7. Competition of preS binding to the third domain (S33) with gp75 (domain 1), gp120 (domains 1 and 2), and the active-site inhibitor GEMSA. 293T cells transfected with the S33 construct were metabolically labeled with [35S]Met. Aliquots (1 ml) of the labeled extract were preincubated with either 10 μM GEMSA or 500 ng of baculovirus-expressed and purified gp75 or gp120. The preS binding was assessed as in Fig. 6.
$K_{cat}/K_{m}$ for gp170 are 2–3-fold higher than for gp120, indicating that this third domain either has enzyme activity or influences the activity of the other domains. Even though the construct containing only this third domain (pG180DS33, Fig. 4) did not show measurable carboxypeptidase activity, it was able to bind preS protein. This result indicates that preS binding does not require carboxypeptidase activity. Further evidence in favor of this interpretation is the finding that 10 µM GEMSA does not influence preS binding to gp180 (Fig. 7).

Recently, the rat and human homologs of gp180 (i.e. CPD) were cloned and sequenced (32, 37). The deduced amino acid sequence shows 82% identity among the third domain of duck and rat proteins (32). This degree of sequence similarity is higher than the first domain of the rat and duck proteins (66% amino acid identity) and is nearly as high as the degree of sequence similarity among the second domain (83% amino acid identity). A comparison of human CPD and duck gp180 shows essentially the same degree of amino acid identity as between the rat and duck proteins (37). The high degree of sequence similarity within the third domain among duck, rat, and human proteins implies that this region has an important function. One possible function is to bind an endogenous preS-like factor. This factor could either be in the secretory pathway or act as a co-receptor or other factors may be necessary to confer a successful infection. Co-receptors are necessary for infectivity of other viruses such as human immunodeficiency virus, which requires both CD4 and a chemokine receptor (33). Another metalloprotease, aminopeptidase N, has been found to be a receptor for coronavirus (34, 35). Further studies are needed to examine whether the interaction of DHBV with gp180 is important for viral infection.

3 O. Varlamov and L. Fricker, submitted for publication.
gp180, a Protein That Binds Duck Hepatitis B Virus Particles, Has Metallocarboxypeptidase D-like Enzymatic Activity
Francis J. Eng, Elena G. Novikova, Kazuyuki Kuroki, Don Ganem and Lloyd D. Fricker

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