A Soluble Form of the Avian Hepatitis B Virus Receptor

BIOCHEMICAL CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE RECEPTOR LIGAND COMPLEX

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Avian hepatitis B virus infection is initiated by the specific interaction of the extracellular preS part of the large viral envelope protein with carboxypeptidase D (gp180), the primary cellular receptor. To functionally and biochemically characterize this interaction, we purified a soluble form of duck carboxypeptidase D from a baculovirus expression system, confirmed its receptor function, and investigated the contribution of different preS sequence elements to receptor binding by surface plasmon resonance analysis. We found that preS binds duck carboxypeptidase D with a 1:1 stoichiometry, thereby inducing conformational changes but not oligomerization. The association constant of the complex was determined to be $2.2 \times 10^7$ M$^{-1}$ at 37 °C, pH 7.4, with an association rate of $4.0 \times 10^{10}$ M$^{-1}$ s$^{-1}$ and a dissociation rate of $1.9 \times 10^{-3}$ s$^{-1}$, substantiating high affinity interaction of avianhepadnaviruses with their receptor carboxypeptidase D. The separately expressed receptor-binding domain, comprising about 50% of preS as defined by mutational analysis, exhibits similar constants. The domain consists of an essential element, probably responsible for the initial receptor contact and a part that contributes to complex stabilization in a conformation sensitive manner. Together with previous results from cell biological studies these data provide new insights into the initial step of hepadnaviral infection.

Hepatitis B viruses are a group of small enveloped hepatotropic partially double-stranded DNA viruses that cause acute and chronic infections in humans, mammals, and birds (1). In case of the human hepatitis B virus (HBV) chronic infections dramatically increases the risk for the development of primary hepatocellular carcinomas, and HBV therefore represents a major health problem to the world population (2). While many details of hepadnaviral genome replication are understood in considerable detail (reviewed in Refs. 3 and 4), our knowledge of the early events in HBV infection, namely the identity of the cellular receptor for HBV, is poor, and reflects the lack of a suitable infection system (5). However, promising results have been achieved in the duck hepatitis B virus (DHBV) model system where systematic infection studies using primary duck hepatocytes can reproducibly be performed (6). In this system infections can be suppressed by the simultaneous application of nucleocapsid-free, non-infectious subviral particles which are produced in an about 1000-fold excess over virus during infection (7). Both subviral particles and virions contain the same two envelope proteins, namely the large viral surface protein (L-protein) and the small viral surface protein (S-protein). Both proteins are transcribed from a common open reading frame and share the hydrophobic S moiety which is responsible for membrane anchoring. The L-protein additionally contains the 161-amino acid amino-terminal hydrophilic preS sequence which, on its own, inhibits the DHBV infection of primary duck hepatocytes. A subsequent deletion analysis showed that an 85-amino acid preS element constitutes the receptor-binding domain within preS (8).

In an attempt to identify receptor candidates for DHBV, Kuroki et al. (9) and independently Tong et al. (10) identified a glycoprotein of 170–180 kDa (gp180/p170) which binds DHBV particles and Escherichia coli-derived GST-preS polypeptides. Sequence comparisons of gp180/p170 cDNA with known sequences suggested that it represents the prototype of a new family of membrane bound carboxypeptidases (10, 11). Independently, several mammalian homologues of gp180/p170 were discovered in bovine, rat, mouse, and humans, and have been classified as metallocarboxypeptidase D (CPD) (12–15). The primary sequence analyses indicate that CPDs consist of three luminal/extracellular carboxypeptidase B-like domains (called A, B, and C), a hydrophilic transmembrane anchor and a highly conserved cytoplasmic tail. While in the A and B domains all essential amino acids for the enzymatic activity of CPDs investigated so far are conserved, the C domains lost most of them, despite their high overall sequence homology. It has therefore been hypothesized that this domain of CPD is catalytically inactive and serves a different, as yet unknown function (15). This assumption was recently confirmed for duck CPD, whose A and B domains displayed CPD activity, while the C-domain contains the DHBV preS-binding site (16).

CPDs from rat and duck have been shown to be resident trans-Golgi network membrane proteins which cycle between the trans-Golgi network and the plasma membrane (17, 18). Their localization, enzymatic activity, and the broad tissue distribution support the notion that CPDs are involved in processing of a variety of polypeptides that traverse the secretory pathway of various tissues (19). However, evolutionary conservation of an enzymatically inactive C-domain, recycling from the cell surface to the trans-Golgi network, and the uptake of DHBV particles strongly implies a yet unidentified function of

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1 The abbreviations used are: HBV, hepatitis B virus; DHBV, duck hepatitis B virus; CPD, carboxypeptidase D; dCPD, duck carboxypeptidase D; sCPD, soluble duck carboxypeptidase D; DpreS, duck hepatitis B virus preS polypeptide; SF, Spodoptera frugiperda; PARA, 4-aminobenzoyl-arginine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; AcNPV, Autographa californica nuclear polyhedrosis virus.

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CPDs, possibly involving binding of a natural ligand.

We have recently characterized the receptor-binding domain within the preS region of the DHBV L-protein and demonstrated that duck carboxypeptidase D is the primary receptor for avian hepatitis B viruses (8, 18). In the present study we have extended these results by the detailed biochemical analysis of the DHBV preS-duck carboxypeptidase D interaction. Using a set of preS deletion mutants, we investigated binding and dissociation rates of the preS receptor complex by real-time surface plasmon resonance spectroscopy. We defined a receptor-binding domain, comprising about one-half of preS, which consists of a short receptor attachment site and a conformation dependent stabilizing element. Binding of dCPD to this domain is strong and, considering the presence of multiple binding sites in viral particles, implies that DHBV binding to hepatocytes is probably irreversible. Together with findings from earlier studies our results allow a model to be proposed for the early steps in hepadnaviral infection with several implication regarding the mechanistics of hepadnaviral infection.

**EXPERIMENTAL PROCEDURES**

**Purification of Soluble Duck Hepatitis B Virus Receptor from S. frugiperda Cells**—The baculovirus transfer vector pVL-sdCPD was constructed by ligating the Nco I fragment of plasmid pBKSvp-gp180 (kindly provided by K. Kuroki, Kanasawa, Japan) into a modified version of plasmid pVL1393 (Fig. 1A). This vector contains an additional Nol site as a part of the start codon and a polylinker at the 3′-end which introduces an artificial stop codon. The NcoI/3′-XhoI fragment of pBKSvp-gp180 encodes the signal sequence of duck CPD and the three carboxypeptidase-like domains but lacks the carboxyl-terminal transmembrane anchor and the cytosolic tail.

Recombinant bacuvirus were obtained by co-infection of Spodoptera frugiperda (Sf9) cells with a mixture of 100 ng of linearized Baculo-Gold DNA (Pharmingen) and 5 μg of the baculovirus transfer vector pVL-sdCPD using the manufacturer’s protocol for lipofection with DOTAP (Boehringer-Mannheim). Two hours after lipofection, the medium was changed and recombinant viruses were collected after 5 days. The virus was purified according to the protocol of O’Reilly et al. (20), amplified in Sf9 cells by two additional rounds of infection and used for infection of High Five cells as described below.

**SDS-PAGE, Silver Staining, and Immunological Techniques**—Protein samples for SDS-PAGE were dissolved in sample buffer (200 mM Tris/Cl, pH 6.8, 6% SDS, 20% glycerol, 10% dithiothreitol, 0.1 mg/ml bromophenol blue, 0.1 mg/ml Orange G), boiled for 5 min, and subjected to electrophoresis in 7.5 or 13% polyacrylamide-SDS gels (21). After electrophoresis, proteins were either silver-stained according to the method described by Heukeshoven and Dernick (22) or transferred to a nitrocellulose filter for immunological analysis. As a primary antibody we used monoclonal antibodies of a polyclonal antibody against recombinant soluble dCPD. Binding assays at different pH values were performed in amounts that yielded 2,200–3,500 response units. Monomeric and dimeric proteins were removed and the plate was washed 3 times with binding buffer at 4 °C. Unbound proteins were removed and the plate was washed 3 times with binding buffer at 4 °C. Bound proteins were eluted with 50 mM SDS sample buffer at 80 °C and analyzed by PAGE and Western blotting. The samples were added in either standard binding buffer (1% Triton X-100, 50 mM Tris/Cl, 150 mM NaCl, pH 7.4) for solubilized membrane fractions or detergent-free binding buffer (50 mM Tris/Cl, 150 mM NaCl) for recombinant soluble dCPD. Binding assays at different pH values were performed in sodium acetate buffer, pH 3.5–5.5, sodium phosphate buffer, pH 6.0–8.0, and Tris buffer, pH 8.5–10.0. Binding was allowed to occur for 1–2 days at 4 °C. Unbound proteins were removed and the plate was washed 3 times with binding buffer at 4 °C. Bound proteins were eluted with 50 μl of SDS sample buffer at 80 °C and analyzed by PAGE and Western blotting.

**Surface Plasmon Resonance Spectroscopy**—Surface plasmon resonance analysis (BIAcore-Upgrade, BIAcore-System) of DHBV preS protein binding to dCPD was done at 37 °C in 1× HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Surfactant P-20 (Pharmacia)) at flow rates of 5–30 μl/min according to standard protocols provided by the manufacturer. DHBV preS proteins were coupled to a CM5 sensor chip via standard NHS/EDC activation chemistry (BIAcore amine coupling kit) in amounts that yielded 2,200–3,500 response units. Monomeric and oligomeric sdCPD at the indicated concentrations were injected for 5 min followed by 3 min elution with HBS buffer. The sensor chip was cleaned of sdCPD bound to immobilized DpReS by injection of 30 μl of regeneration solution (20 mM HCl). Binding constants of the sdCPD-DpReS interaction were calculated with the BIAevaluation program version 2.1 (Pharmacia). The baselines for the curves shown in Fig. 4 were adjusted to zero before calculation. Calculation of $K_{d}$ was done by fitting the data to the equation $R = R_{e} - R_{o} / K_{d}$.

**RESULTS**

**Purification of Soluble Duck Hepatitis B Virus Receptor from a Baculovirus Expression System**—To facilitate biochemical studies on the interaction of duck hepatitis B virus (DHBV) with duck carboxypeptidase D (dCPD), we constructed recom-
Recombinant baculoviruses were amplified in S. frugiperda 9 (Sf9) cells and used for infection of the High Five insect cell line (see “Experimental Procedures”). Omission of the transmembrane anchor and the cytoplasmic part resulted, as intended, in the secretion of a soluble dCPD variant of 170 kDa (sdCPD), as shown by Western blot analysis of culture supernatants using an antiserum against gp180 (Fig. 1 B). Treatment with endoglycosidases H and F led to a decrease of the molecular weight indicating that sdCPD had been modified by both complex and high mannose-type glycosylation (Fig. 1 B).

Purification of sdCPD from the culture supernatant of infected High Five insect cells was achieved by affinity chromatography on a PABA-Sepharose column as described previously (12). Silver staining of proteins from pooled fractions eluting from the PABA column revealed a single band at about 170 kDa with only minor contaminants. Western blot analysis using an anti-dCPD specific antibody verified the identity of the 170 kDa as dCPD (Fig. 1 D) as does analysis by Edman degradation. The NH₂-terminal sequence AHIKKAE A... indicated cleavage of the leader sequence at position 25 in insect

FIG. 1. Purification of sdCPD from a baculovirus expression system. A, the coding sequence of the three carboxypeptidase-like extracellular domains of dCPD, preceded by the secretory signal sequence, was inserted into the baculovirus transfer vector pVL1393. The vector was modified by introduction of a XhoI/XbaI/PstI stop linker (boxed sequence with underlined amber codon), and a NcoI site, downstream from the vector encoded BamHI site, containing the start codon. The NH₂-terminal methionine and the COOH-terminal valine of sdCPD are numbered. Numbers 25 and 29 indicate the cleavage sites of the signal peptidase as determined by NH₂-terminal sequencing. B, sdCPD is secreted as a glycoprotein into the culture supernatant of infected insect cells. Cell culture media of AcNPV (WT) and AcNPV-sdCPD (AcNPV-sdCPD) infected Sf9 cells were analyzed by Western blot for the presence of dCPD specific proteins in the absence (−) or presence of endoglycosidase H (Endo H) and endoglycosidase F (Endo F). The position of undigested sdCPD is indicated by an arrow. C, elution profile of sdCPD on a PABA affinity column. Culture supernatants of AcNPV-sdCPD infected High Five insect cells were applied to the column (start) and sdCPD (pool) was eluted with 50 mM arginine (elution). D, silver-stained SDS-gel electrophoresis and dCPD-specific Western blot analysis of the pooled fractions eluted from the affinity column, as shown in C.

FIG. 2. Separation of monomeric and oligomeric sdCPD by size exclusion chromatography. A, elution profile of affinity purified sdCPD on a calibrated Superdex 200 column. V₀ and the elution volumes of thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cobalamin (1.3 kDa) used for calibration are indicated at the top. Fraction numbers correspond to lane numbers in B. The apparent molecular mass of monomeric sdCPD (248 kDa) was calculated from standard curves as a mean value of three independent measurements. B, silver-stained SDS gel of fractions applied to (load = pool of the PABA affinity column) and eluted from the Superdex 200 column, as shown in A. sdCPD is indicated by an arrow. Lane numbers correspond to fraction numbers in A.
cells, and was found to be identical to previously published data for endogenous duck CPD (11) and bovine CPD (19). In addition, detection of a second sequence KAEAA . . . , lacking 4 amino acids, suggests that processing can also occur after lysine 28 in insect cells. The comparison of the yields of recombinant sdCPD obtained from SF9 cells with the yields obtained from High Five cells revealed an about 8-fold higher expression rate in the High Five cell line (25 μg of sdCPD per 10^7 SF9 cells in contrast to 200 μg/10^7 High Five cells).

Further analysis of affinity purified sdCPD on a calibrated Superdex 200 gel filtration column (Fig. 2A) resulted in two forms with different apparent molecular weights, which were, however, indistinguishable in SDS-PAGE (Fig. 2B) and Western blot analysis (data not shown). About 35% of sdCPD eluted in the void volume and represent oligomers with molecular masses of approximately 3,600 kDa as determined by electron microscopy (data not shown). 65% of sdCPD eluted at 248 kDa and represents monomeric sdCPD eluting at a slightly higher molecular mass than expected. Since proteins were initially purified by affinity chromatography on PABA-Sepharose it can be assumed that both forms have carboxypeptidase activity but differ considerably in their binding properties to DHBV preS, as shown below.

sdCPD Efficiently Inhibits the Infection of Primary Duck Hepatocytes with Duck Hepatitis B Virus—To investigate whether recombinant sdCPD exhibits receptor function comparable to the cell surface bound molecule on hepatocytes, we performed infection inhibition assays essentially as described previously for DHBV preS (DpreS) polypeptides (8). Primary duck hepatocytes were infected with DHBV in the presence of increasing amounts of monomeric or oligomeric sdCPD, and viral markers (intracellular viral DNA or secreted DHBV e-antigen) were quantified 6 days post-infection. As shown in Fig. 3A, both forms of sdCPD efficiently inhibit DHBV infection. About 7 molecules of monomeric sdCPD per viral particle were sufficient for 50% inhibition of infection (18). In comparison, oligomeric sdCPD, despite its inability to bind recombinant DHBV preS, as shown below, competed DHBV infection also remarkably well; with about 15 molecules needed for 50% inhibition. In these calculations we assumed that an approximately 1,000-fold excess of non-infective subviral particles is present in the infective serum. Taking into account that one particle consists of approximately 20 L-protein molecules with 50% having an inverse topology and therefore their receptor-binding domain is located inside the viral particle (27), we conclude that every single sdCPD molecule is able to bind viral particles. Ratios of 100 sdCPD molecules/particle almost completely block DHBV infection, indicating that viral particles bound to sdCPD are unable to infect hepatocytes. Insect cell-derived soluble duck carboxypeptidase D therefore constitutes a suitable tool for virus-receptor interaction studies at the molecular level.

Competitive inhibition of infection was also performed with the E. coli-derived preS polypeptides used in the binding studies described below. As shown in Fig. 3B, full-length DpreS and the DHBV preS fragment DpreS30–115, representing the receptor-binding domain, inhibit DHBV infection equally well in a concentration-dependent manner. Under the conditions used, the IC_{50} was determined to be 0.4–0.8 μM, corresponding to approximately 3000 DpreS molecules per viral particle.

Recombinant sdCPD Binds DHBV PreS Polypeptides Similarly to Authentic dCPD—To confirm that the biological activity of sdCPD in infection competition experiments correlates with its physical binding properties to DHBV preS polypeptides, we performed binding assays with the solid phase bound preS polypeptides depicted in Fig. 4A and compared the results with those obtained for authentic dCPD from duck liver lysates. As shown in Fig. 4B, authentic dCPD binds DpreS and the DpreS fragment consisting of amino acids 30–115 (DpreS30–115). The deletion mutant DpreS85–96, inactive in DHBV infection competition (8), was used as a control and showed no binding. Likewise, recombinant sdCPD eluted from the PABA column binds DpreS but not DpreS85–96 (Fig. 4C). However, even at a large excess of DpreS we did not observe complete binding of sdCPD (Fig. 4C, left frame, left lane) and therefore assumed that a fraction of sdCPD might be inactive in DpreS binding. This interpretation was confirmed in a quantitative BIAcore analysis showing that oligomeric sdCPD does...
not bind DpreS (see below).

In Fig. 5 the pH dependence of the DpreS-sdCPD interaction is shown. Binding was observed between pH 5.5 and 10.0 with an optimum at pH 6.5. Binding still occurred at pH 5.0. However, further protonation abolished binding completely.

**Determination of Binding Constants of the DHBV preS-sdCPD Complex by Real Time Surface Plasmon Resonance Spectroscopy**—To determine association and dissociation rates of the DHBV preS-sdCPD complex and accordingly deduce the affinity of DHBV to its cellular receptor we followed complex formation and dissociation by real time surface plasmon resonance analysis (BIAcore). DHBV preS polypeptides were covalently immobilized to CMS sensor chips (see “Experimental Procedures”). DHBV preS polypeptides were covalently immobilized to CMS sensor chips (see “Experimental Procedures”) and three different concentrations (0.06, 0.15, and 0.29 μM) of monomeric sdCPD were injected onto the surface at 37 °C (Fig. 6A). The kinetics of binding (100–400 s) and release (400–575 s) of sdCPD were calculated from the slopes of the curves. The association rate $k_a$ was determined to $4.0 \times 10^4$ M$^{-1}$ s$^{-1}$, the dissociation rate $k_d$ to $1.9 \times 10^{-3}$ s$^{-1}$, corresponding to a half-life for the complex of about 6.0 min at 37 °C. From these data we calculated the dissociation constant $K_d$ to $4.6 \times 10^{-8}$ M. Using the DHBV preS fragment from amino acid 30 to 115 (DpreS30–115), which has been identified by infection competition experiments as the receptor-binding domain (8), we observed similar constants: $k_a$ 7.1 $\times 10^4$ M$^{-1}$ s$^{-1}$, $k_d$ 2.7 $\times 10^{-3}$ s$^{-1}$; $t_{1/2}$, 4.3 min; $K_d$, $9.8 \times 10^{-8}$ M (Fig. 6B). Consequently full-length DpreS and DpreS30–115 are indistinguishable in both infection competition and dCPD interaction.

Consistent with our observation in the qualitative binding assay (Fig. 4B), the deletion mutant DpreSΔ85–96 showed no obvious interaction with sdCPD in the BIAcore experiment (Fig. 6D). This observation led us to conclude that amino acid residues 85–96 of DpreS contain absolutely essential elements for receptor interaction. Interestingly, anti-preS antibodies which are capable of blocking DHBV infection have been mapped to bind epitopes within this region$^{2,3}$ indicating their surface exposure.

Despite its ability to bind the substrate PABA, most likely via the two enzymatically active A and B domains, oligomeromic sdCPD was found to interact only weakly with DpreS (Fig. 6C). Thus oligomerization restricts DpreS binding.

**The Receptor-binding Domain of DHBV-preS Consists of an Essential Attachment Site and a Conformation Stabilizing Element**—We extended surface plasmon resonance analysis to investigate the contribution of particular sequence elements within DpreS to receptor binding, by testing the same set of deletion mutants (Fig. 7A) that have been characterized in infection competition experiments (8) for sdCPD interaction (Fig. 7, B-D). Deletions outside the receptor-binding domain (mutants DpreSΔ22–30 and DpreSΔ128–139) showed kinetics of association and dissociation similar to full-length DpreS (Fig. 7B). This indicates that the part of proS that binds the receptor folds independently of the deleted flanking amino

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$^2$ C. Kuhn, personal communication.

$^3$ L. Cova, personal communication.
injected onto immobilized DpreS for 300 s. At 400 s the injection was stopped and dissociation followed in running buffer for 150 s. The association rate $k_a$, the dissociation rate $k_d$ and the dissociation constant $K_d$ given in the inserted frame, were calculated from three independent measurements using the BIAbasys evaluation program version 2.1. Note that sudden changes in refractive indices for all 4 sensorgrams shown are due to differences in the composition between buffer and buffer. As neither significant binding nor release was observed, calculations of kinetic constants could not reasonably be performed.

Binding of DHBV preS to Monomeric sdCPD Induces Conformational Changes but Not Complex Oligomerization—To determine the stoichiometry of the DpreS:sdCPD complex in solution and to address the question whether binding of the ligand induces conformational changes in the receptor that lead to oligomerization, we performed a calibrated gel filtration analysis. To this aim, full-length DHBV preS or the fragment DpreS30–115 were incubated with sdCPD at 4 °C to allow complex formation and applied to a calibrated Superdex 200 column. At this temperature the complex stability is greatly enhanced and complex dissociation could be prevented. Eluted fractions were analyzed for the presence of DpreS polypeptides by Western blot analysis and apparent molecular weights of the corresponding peaks in the elution profiles were determined. In a control experiment, monomeric sdCPD and DpreS were analyzed separately (Fig. 8, A and B). Monomeric sdCPD did not form oligomers under the chosen conditions and eluted with an apparent molecular mass of 248 kDa (as described above). DpreS alone eluted at 46 kDa with an apparent molecular mass more than 2-fold higher than expected for the monomer (19,822 Da as determined by mass spectrometry (data not shown)). This behavior is probably not caused by dimerization (8), but reflects particular structural elements in the polypeptide that stabilize a non-globular conformation.

As shown in Fig. 8, C and D, both DpreS and DpreS30–115 co-elute in complex with sdCPD, with apparent molecular masses of 254 and 215 kDa, respectively. Consequently, binding of full-length DpreS or DpreS30–115 to sdCPD neither requires nor induces oligomerization of the sdCPD ectodomain. While the DpreS:sdCPD complex runs at an only slightly greater molecular mass compared with sdCPD alone (254 kDa), the DpreS30–115:sdCPD complex eluted at an even lower apparent molecular mass than free sdCPD (215 kDa) in gel permeation experiments. Hence, binding of DpreS to its receptor induces conformational changes that cause a significant reduction of the apparent molecular weight. The size of both of the complexes suggests that a single molecule DpreS binds to one molecule sdCPD in a 1:1 stoichiometry. This assumption was confirmed by a quantitative binding assay shown in Fig. 9. DpreS that has been applied in a molar ratio of 1:1 to covalently immobilized monomeric sdCPD is completely absorbed by sdCPD (right two lanes). An increase in the molar ratio of DpreS/sdCPD, from 1:1 to 1:2 or 1:4, did not lead to further preS-
binding, but resulted in additional amounts of DpreS remaining in the supernatant (Fig. 9, left four lanes).

**DISCUSSION**

We have investigated the interaction of the extracellular portion of the avian hepatitis B virus receptor, purified from a baculovirus expression system, with DHBV preS-polypeptides representing different parts of the ectodomain of the large viral envelope protein. We have found that the COOH-terminal truncated and thus soluble dCPD binds DHBV preS in a similar manner as to the full-length membrane-bound carboxypeptidase D, and therefore provides a valuable tool for studies of avian hepatitis B virus-receptor interactions. In particular, utilization of sdCPD had several advantages when compared with the authentic dCPD: (i) sdCPD could be purified in detergent-free buffers and consequently be used in cell culture assays. (ii) sdCPD becomes secreted into the culture medium and has by-passed all control elements of the secretory pathway. (iii) High level protein expression in serum-free culture medium is feasible in High Five insect cells and facilitates subsequent purification steps.

Recombinant sdCPD was produced in a monomeric and an oligomeric form. Monomeric sdCPD eluted at a slightly greater apparent molecular mass than expected (248 kDa instead of 170 kDa), which may be attributed to its three-domain structure rendering the protein an ellipsoidal overall shape. The monomeric fraction, comprising about two-thirds of total sdCPD, showed PABA as well as preS binding activity and was remarkably active in infection competition assays. In contrast, the remaining one-third of sdCPD had an apparent molecular mass greater than 1 MDa and was impaired in DpreS binding. Our initial assumption that this fraction consists of inactive aggregates, seems unlikely because (i) electron microscopic analysis revealed that the majority of molecules had a defined size of approximately 3,600 kDa as calculated from the Stokes radii (data not shown), (ii) oligomeric sdCPD was able to bind PABA and therefore probably possesses carboxypeptidase activity, and (iii) oligomeric sdCPD showed PABA binding, but resulted in additional amounts of DpreS remaining in the supernatant (Fig. 9, left four lanes).

**Fig. 7.** The receptor-binding domain of DpreS consists of an essential attachment site and elements that contribute to receptor complex stabilization. A, schematic drawing of terminal and internal DpreS deletion mutants used for the surface plasmon resonance analysis of DpreS-sdCPD interaction shown in B, C, and D. Numbers indicate positions of amino acids deleted (e.g., DpreS22–50 lacks amino acids 22–30 within DpreS1). Bars in light gray correspond to polypeptides that bind sdCPD with comparable binding constants as full-length DpreS (DpreS, DpreS22–30, DpreS22–139, and DpreS30–115). Bars in black represent polypeptides that did not show a detectable interaction with sdCPD (DpreS185–96 and DpreS101–109). Mutants with reduced binding constants are illustrated by the shading of the bars (DpreS38–115, DpreS43–115, DpreS52–130, DpreS63–70, and DpreS74–84). B, sensorgram overlays for the interaction of sdCPD with full-length DpreS (red). DpreS22–30 (blue), and DpreS101–139 (green). DpreS polypeptides were immobilized on a CM5 sensor chip as described under "Experimental Procedures." At 100 s the monomeric sdCPD was injected at a concentration of 0.15 μM for 300 s. After 300 s the sample was replaced with buffer and dissociation followed for 150 s. Changes in refractive indices (visible as jumps in the curves) represent differences in solution composition between the buffer and the protein solution. C, sensorgram overlays for the interaction of sdCPD with deletion mutants DpreS30–115 (blue), DpreS43–115 (red), DpreS67–70 (green), and DpreS52–130 (yellow). Sample application was performed as described in B. D, sensorgram overlays for the interaction of sdCPD with the internal deletion mutants DpreS67–70 (blue), DpreS74–84 (red), DpreS85–96 (yellow), and DpreS101–109 (green). Sample application was performed as described in B. Note that despite the changes in refractive indices caused by differences between buffer and protein solutions, DpreS67–70 (blue) and DpreS74–84 (red) in contrast to DpreS85–96 (yellow) and DpreS101–109 (green) clearly show time dependent binding and release of sdCPD. E, DpreS consists of an internal receptor-binding domain comprising amino acids 30–115 (bracket) and two flanking regions (1–29 and 116–161) which are non-essential with respect to receptor binding and serve other functions. The receptor-binding domain contains an essential (amino acids 85–109) and a stabilizing (amino acids 30–85) element.

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(iii) infection competition activity was, interestingly, only slightly reduced when compared with monomeric sdCPD, indicating that, at least in the presence of natural viral particles and living cells, oligomeric sdCPD can be converted to an active state (Fig. 3A). Nevertheless, it remains to be investigated if oligomerization of dCPD also occurs in vivo or is just an artifact of our expression system.

It has recently been shown that DpreS binds to the enzymatically inactive C-domain of dCPD (16). With respect to this observation, the impaired preS binding capability of oligomeric sdCPD suggests that it may also be the C-domain of dCPD that is involved in oligomerization. This could either mean that the preS-binding site is directly involved in homomeric contacts, or that the preS-binding site within the dCPD C-domain is inaccessible in the oligomer.

Despite the evolutionary loss of most essential amino acids required for carboxypeptidase activity, the C-domains of all CPDs investigated so far have conserved primary sequences, implying that they have preserved a still unknown function (15). One of these hypothetical functions could be binding to a yet unknown natural ligand. With respect to this idea and with respect to our observation of a ligand-independent formation of oligomers, binding of this natural component as well as of DHBV might also cause oligomerization and, as a consequence, might activate intracellular signal transduction pathways. The recently observed enhancement of DHBV infection by subviral particles at very low multiplicity of infections (28) is presumably achieved by utilizing this CPD-mediated signaling pathway, as the essential region responsible for enhancement of infection coincides with the primary receptor attachment site defined here.

Purified sdCPD from insect cells binds DHBV preS in a comparable manner to the authentic full-length dCPD from liver lysates. The dissociation constant for the interaction of a single preS moiety with a single receptor molecule at 37 °C was
found in the range of $10^{-8}$ M, indicating high affinity interaction at the molecular level. Taking into account that DHBV exhibits several receptor-binding sites on its surface and hence probably interacts with more than a single dCPD molecule, binding of virions to the hepatocyte is likely to be strong and irreversible. This conclusion, at first sight, appears to be incompatible with particular observations regarding in vitro DHBV infections of primary duck hepatocytes as well as in vivo infections of ducklings. For example, the question arises why in vitro infections of primary duck hepatocytes with serum-derived DHBV requires high multiplicity of infections and long incubation times (26). By contrast, in vivo infection has been described to be extremely effective (29), although dCPD is expressed in various other organs of the duck at much higher levels than in the liver (data not shown and Ref. 19). With regard to the low in vitro infection efficiency, one possible partial explanation is given by the observation that only a very limited number of receptor molecules (<100) are accessible on the surface of hepatocytes. Thus saturation of cell-surface exposed dCPD with viral particles could be achieved already at very low multiplicity of infections (assuming an at least 1000-fold excess of non-infectious subviral particles), and the rate of receptor cycling, as has been shown to occur for dCPD (18) and rat CPD (17), rather than kinetics of binding would determine infection efficiency.

Using a set of DHBV preS insertion mutants in an in vitro dCPD binding assay, Ishikawa et al. (30) mapped the viral determinants required for dCPD binding to amino acids 43–108 within preS. In a similar approach but using terminally deleted preS mutants, Tong et al. (10) mapped the dCPD-binding site to amino acids 87–102. These conflicting results are resolved by the kinetic analysis presented in this study. As the preS fragment from amino acids 87 to 102 overlaps with the essential receptor-binding site defined here, we assume that this polypeptide binds dCPD with a drastically reduced association constant when compared with full-length DpreS. This low affinity interaction was presumably measured by Tong et al. (10) and led them to conclude that amino acids 87–102 represent the complete dCPD-binding domain. In contrast, our data, however, indubitably demonstrate that amino acids preceding this primary attachment site also contribute substantially to dCPD interaction. This stabilization is conformation dependent explaining why insertions in this region of preS destabilize the preS-dCPD complex (30). In addition, insertions, in contrast to deletions, might alter the tertiary structure of preS in such a way that even low affinity interaction could be disrupted. Thus, the apparently conflicting data in fact support our conclusion that the receptor-binding domain within DHBV preS consists of an essential primary binding site which, on its own, interacts with low affinity and a more NH₂-terminal located region which enhances receptor affinity.

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