Squamous Cell Carcinoma Antigen 1-mediated Binding of Hepatitis B Virus to Hepatocytes Does Not Involve the Hepatic Serpin Clearance System

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The cellular receptor for hepatitis B virus (HBV) has not yet been identified. A recent candidate is a homologue of squamous cell carcinoma antigen 1 (SCCA1), a serpin. This study confirms that transfection of SCCA1 into mammalian cells (both hepatocyte-derived and of non-hepatocyte origin) results in increased HBV binding. Furthermore, virus bound to transfected cells is protected significantly from degradation by trypsin (75% compared with 30% in untransfected cells). The possibility that HBV enters cells via the hepatic clearance system for serpin-enzyme complexes was investigated by analysis of the reactive site loop of SCCA1. Functional and deletion mutants of SCCA1 were constructed by site-directed mutagenesis and compared with the wild type construct. In no case was virus binding reduced by functional alterations or deletions within the reactive site loop. A possible role for the low density lipoprotein receptor-related protein (LRP) in binding virus was investigated. SCCA1 transfection of Huh7 cells was shown to result in up-regulation of LRP expression, reaching levels observed in total liver. However, the use of receptor-associated protein (RAP), a competitive ligand for LRP, suggests that LRP up-regulation is not responsible for enhanced virus binding to SCCA1-transfected cells.

Hepatitis B virus (HBV) is a member of the hepadnavirus family and exhibits extreme host and tissue specificity, being able to infect only humans and higher primates and its replication being limited almost exclusively to hepatocytes. The molecular basis of this hepatotropism remains poorly understood but may be attributable to a combination of factors, including liver-specific transcription factors required by the virus for replication, and liver-specific expression of the host cell receptor utilized during the initial stages of infection (1).

The early stages of the HBV life cycle, virus binding and internalization, remain obscure, largely because of the lack of a permissive cell culture system. Many studies have made use of primary hepatocytes, but difficulties in obtaining these cells, and variability between and within batches, hamper the use of primary tissue. Moreover, primary hepatocytes lose susceptibility to HBV infection within days (2).

The cellular receptor for HBV has proved elusive. Despite the proposal of numerous candidates, reviewed by De Meyer et al. (1), no single molecule thus far has proved convincing. A candidate proposed recently by De Falco et al. (3) and referred to by them as HBV-binding protein (HBV-BP), bears significant sequence homology to a serine proteinase inhibitor (serpin), squamous cell carcinoma antigen 1 (SCCA1). HBV-BP was shown to interact with the pre-S1 domain of the HBV surface protein, which is now widely accepted to be the viral ligand involved in the initial interaction with the cellular receptor (4, 5). Furthermore, transfection of HBV-BP into HepG2 cells resulted in a 2-fold increase in virus binding capacity, whereas Chinese hamster ovary cells transfected with HBV-BP showed a newly acquired susceptibility to virus binding, albeit at low levels. It was reported also that virus particles were internalized to a greater degree in cells expressing HBV-BP. These results have not yet been confirmed.

The possibility that SCCA1, or a close homologue, functions as the receptor for HBV is exciting, particularly when considering the biology of serpins, a well conserved superfamily of over 100 proteins present in viruses, plants, vertebrates, and invertebrates (6). Serpins are involved in the regulation of a variety of proteinases, including those of the coagulation, complement, fibrinolytic, and inflammatory pathways (6, 7). Inhibitory serpin functions via an exposed reactive site loop (RLS) of about 20 amino acids, and irreversibly inhibit proteinases through a suicide substrate inhibition mechanism. The enzyme recognizes and binds to the RSL, forming a serpin-enzyme complex (SEC) (8). This leads to cleavage of the RSL between the reactive P1 and P1' residues, causing a significant and irreversible conformational change with complete insertion of the RSL loop into a β-sheet. The enzyme is inactivated reversibly by the formation of an acyl ester linkage between the active site serine and a serpin side chain (7–9). Although the SEC is kinetically stable, it is thermodynamically unstable, and would eventually break down releasing inactivated (cleaved) serpin, and active proteinase. Therefore, the irreversible inhibition of the proteinase requires removal of the SEC from the circulation (7).

Although SCCA1 is classed as a serpin by sequence identity, functionally it is a cysteine protease inhibitor. This raises ques-

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tions regarding the relevance of studies of serpins, especially those involving the RSL, to investigations of SCCA1. Serpins generally are limited to inhibiting serine proteinases, but there are at least two other serpins that inhibit cysteine proteinases (although both also inhibit serine proteinases) (10, 11). Furthermore, SQN-5, a mouse serpin similar to SCCA1 and SCCA2, also has been shown to inhibit both chymotrypsin-like serine proteinases and papain-like cysteine proteinases, using an RSL-dependent inhibitory mechanism (12, 13). Replacement of the RSL of SCCA2 (which inhibits serine proteinases) with that of SCCA1 confers cysteine proteinase inhibitory properties on the modified SCCA2, confirming that the RSL is the only region required for cysteine proteinase inhibition (9). Replacement of the RSL of the archetypal serpin, α1-antitrypsin, with that of SCCA1 resulted in a modified α1-antitrypsin that inhibited papain in the same way as SCCA1 and enabled characterization of the serpin-proteinase complex (14). Formation of a serpin-cysteine proteinase complex and the inhibition of that protease relied critically on RSL insertion into the body of the molecule (14). Therefore, the body of a serpin molecule is capable intrinsically of supporting cysteine protease inhibition and the complex formed between the serpin and the cysteine proteinase shares architecture analogous with that observed in the inhibition of serine proteinases by serpins (14).

Although little is known about the mechanism of action of SCCA1, there is evidence for the removal of other SECs (e.g. thrombin-antithrombin, α-protease inhibitor-trypsin, and α1-antichymotrypsin-cathepsin G) from circulation via a receptor-mediated clearance mechanism. SECs are removed from circulation largely by liver-enriched, or even liver-specific, pathways; clearance studies of radiolabeled SECs in animal models show localization to the liver and a specific association with hepatocytes (7). The low density lipoprotein receptor-related protein (LRP), which is highly expressed in hepatocytes, has been implicated in the hepatic clearance of SECs from the circulation (15). The type I transmembrane proteins are an evolutionarily conserved group of cell-surface receptors that bind several unrelated ligands (16). Bound ligands are delivered via clathrin-coated pits to endosomes for degradation through receptor-mediated endocytosis (15), a strategy that is often utilized by viruses to enter host cells (reviewed in Ref. 17). Furthermore, LRP has been implicated in virus binding and entry, serving as the receptor for the minor group of human rhinoviruses (18). There have also been reports that other members of the low density lipoprotein receptor family (LRF) act as receptors for subgroup A Rous sarcoma virus (19) and for members of the Flaviviridae (20).

The RSL of a serpin consists of a loop projecting from the body of the protein, comprising a hinge region and a variable reactive center loop. Site-directed mutagenesis has been used to examine the role of individual residues within the RSL (9, 13, 21). Mutations within the hinge region affect RSL mobility and the rate at which the RSL inserts into the body of the serpin. Mutation of the P14 residue from alanine to arginine blocks RSL insertion and abrogates inhibitory activity, although the protein still functions as a substrate, i.e. the protease is capable of binding to the serpin but cleavage of the RSL does not result in an SEC (13). Mutation of the variable region has shown that the P3 residue also is critical in the interaction between cathepsin S and SCCA1. Mutation of the P3 phenylalanine to alanine results in a loss of cathepsin S inhibition (13).

The SCCA1-mediated binding of HBV to mammalian cells reported by De Falco et al. (3), and confirmed below by ourselves, raises questions regarding the mechanism of virus binding. It has been reported that SCCA1 is expressed in a wide range of tissues, including the skin, lungs, prostate, testis, thymus, and tonsils (detectable by RT-PCR and/or immunohistochemistry), but not in the liver (22). Although this report is not supported by the fact that we and others (5) have been able to amplify SCCA1 cDNA from HepG2 cells, a hepatocyte-derived cell line, by nested RT-PCR, the discrepancy most likely is due either to the sensitivity of nested RT-PCR in comparison with immunohistochemistry and single round RT-PCR, or to differences between the tumor-derived HepG2 cells and primary hepatocytes.

The reported absence of expression of SCCA1 by hepatocytes (22), along with the fact that serpins are secreted, rather than expressed on the plasma membrane, suggests that the role of SCCA1 in virus entry may not be as a receptor expressed on the cell surface. An alternative hypothesis is that HBV hijacks the mechanism for hepatic clearance of SECs to enter hepatocytes. We have investigated this hypothesis using two strategies. First, we have determined whether the function of SCCA1, as a proteinase inhibitor, is required for enhanced virus-cell binding. These studies included site-directed mutagenesis of the reactive site loop, followed by virus-cell binding assays. Second, we investigated a potential role for the LRP in SCCA1-mediated virus binding and uptake utilizing competitive binding assays.

**EXPERIMENTAL PROCEDURES**

**Cloning and Sequencing of SCCA1 cDNA**

Total RNA was purified from HepG2 cells using the SV Total RNA isolation system (Promega). Reverse transcription (Superscript II reverse transcriptase, Invitrogen) was primed using oligonucleotide SCCA1outer1 (5'-ctggagagaaaaagctattatgtggg-3') and SCCA1outer2. First round primers were SCCA1outer1 (5'-caagctgattcagactcagtag-3') and SCCA1outer2. Second round PCR utilized primers including restriction sites to facilitate cloning, SCCA1-XhoI (5'-gcgctagcagggagagatgcc-3') and SCCA2-His (5'-ggattgaaggggatttgacgta-3'). PCR was performed using Pfu Turbo (Stratagene). Cycling for both reactions was performed in the PerkinElmer Geneamp PCR system 2400 as follows: 94 °C for 1 min, 25 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, followed by 1 cycle of 72 °C for 10 min. Sequencing was performed by Qiagen Sequencing Services, Germany. The sequence of this clone has been submitted to the GenBank™/EMBL database (accession number AJ515706).

The SCCA1 cDNA was cloned as a XhoI-BstBI fragment into the multiple cloning site pCDNA 3.1 V5 His (Invitrogen). The ligated DNA was transformed into One-Shot™ chemically competent Escherichia coli (Invitrogen) and purified using the Endo-free Plasmid Maxi kit (Qiagen). Sequencing (Qiagen) was performed to confirm that the sequence of the SCCA1 insert in pCDNA SCCA1 corresponded to the published SCCA1 sequence (accession number U19556). All restriction and modifying enzymes were obtained from New England Biolabs unless otherwise stated.

**Construction of pGEX 4T-3 SCCA Poly-His**

The SCCA1 cDNA was subcloned from pCDNA SCCA1 into pGEX 4T-3 (Amersham Biosciences) as follows: the SCCA1 cDNA was excised from pCDNA SCCA1 by XhoI and PmeI digestion followed by purification from low melting point agarose. The vector was digested with NotI, blunt-ended, and then further digested with SalI (to utilize the compatible cohesive ends resulting from SalI and XhoI digestions). The XhoI-PmeI SCCA1 insert was ligated into the vector in-frame to produce a construct encoding a GST-SCCA1 fusion construct. Restriction digests and DNA sequencing confirmed that the insertion had been successful. A single colony of E. coli BL21 cells was inoculated into L broth and incubated overnight at 37 °C, 225 rpm. This culture (500 μl) was used to inoculate 100 ml of L broth, and incubated at 37 °C, 225 rpm until the culture reached an A600 of 0.6. Cells were induced to express the fusion protein by the addition of 100 μl of 100 μM isopropyl-1-thio-β-D-galactopyranoside, followed by incubation at 30 °C for 3 h. Bacteria were pelleted at 3000 rpm for 10 min, and the pellet stored overnight at −20 °C. Cells were resuspended in 10 ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8) and lysed using sonication. The crude bacterial extract was centrifuged to pellet insoluble debris.
(3000 rpm, 15 min). Purification was performed using ProBond resin (Invitrogen) equilibrated in binding buffer and packed into 1-ml spin columns. Columns were washed with washing buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) until the absorbance at 280 nm was less than 0.06, then proteins were eluted stepwise using 50, 200, 350, and 500 mM imidazole (Sigma) in washing buffer. The fusion protein was concentrated to 1 mg/ml using Vivaspin 20-ml centrifugal concentrators (Vivascience). In certain experiments the GST tag was removed from the fusion protein using thrombin protease (Promega) as per the manufacturer’s instructions. The wild type SCCA1 construct, pCDNA SCCA1, was used as a template for mutagenic PCR using the Pro-Pro mutant cassette within the wild type RSL with the Pro-Pro mutant cassette.

**Construction of Reactive Site Loop Mutants**

Reactive site mutants (Table I) were constructed using three different approaches.

**P3/P14 Mutants**—Site-directed mutagenesis was utilized to create modified SCCA1 constructs in pCDNA3.1 V5 His. Mutagenesis was performed using the GeneEditor in vitro site-directed mutagenesis system (Promega) as per the manufacturer’s instructions. The mutant oligonucleotides utilized were AlaP14Arg (5′-aggagggcgaagagctgccgacgc-3′) and PheP3Ala (5′-ggaggttcgggcagctggagcagc-3′). Coding changes are indicated in bold and underlined. To facilitate screening, a PstI restriction site was removed in each oligonucleotide (bold) without altering the predicted amino acid sequence. Transferrants were screened for successful mutagenesis by restriction enzyme digestion and by DNA sequencing.

**Pro-Pro Mutant**—Mutation of the putative cleavage site from P1 serine to proline was performed using the mutagenic oligonucleotide Pro-Pro (5′-gtgctagctgtagctgtagctgtagcaccacct-3′). This oligonucleotide encodes the putative cleavage site P1 proline and also encompasses a BamHI restriction site to facilitate replacement of a cassette within the wild type RSL with the Pro-Pro mutant cassette. The wild type SCCA1 construct, pCDNA SCCA1, was used as a template for mutagenic PCR using the RSL-reverse primer (Table II) and the Pro-Pro primer. Cycling was performed in the PerkinElmer Geneamp PCR system 2400 as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 1 cycle of 72 °C for 5 min. Both the ampiclon and the parental plasmid were digested with BstBI and BamHI, and the mutated cassette was ligated into the parental vector. Ligated DNA was transformed into One-Shot™ Chemically Competent E. coli (Invitrogen) and purified using the Endo-free Plasmid Maxi kit (Qiagen). Sequencing was performed to confirm that the desired changes had been made.

**Deletion Mutants**—Deletion mutants were constructed using a PCR ligation method. Essentially two primers, RSL-forward and RSL-reverse, were designed flanking the entire RSL cassette, and encompassing the Kap1 and BstBI restriction sites. Each deletion mutant was constructed by amplifying the area on either side of the deletion (A and B) using primers designed to incorporate regions of reverse complementarities (Table II). The two amplicons were then gel purified, diluted and mixed together, and denatured and annealed to allow the complementary regions of amplicons A and B to anneal to one another. PCR ligation was performed using the two outer primers (RSL-forward and RSL-reverse). Cycling for both reactions was performed in the PerkinElmer Geneamp PCR system 2400 as follows: 94 °C for 5 min, 35

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**Table I**

| Amino acid sequences (wild type (WT) residues 329–375) of the RSL mutants created from pCDNA SCCA1 (WT) | WT | E | G | A | E | A | A | A | A | T | A | V | A | F | G | S | S | P | P | P | T | S | T | N | P3 | E | G | A | E | A | A | A | A | T | A | V | A | F | G | S | S | P | P | T | S | T | N | P14 | E | G | R | E | A | A | A | A | T | A | V | V | F | G | S | S | P | T | S | T | N | Pro | E | G | A | E | A | A | A | A | T | A | V | V | F | G | S | S | P | T | S | T | N | Δ H | E | G | A | E | A | A | A | A | T | A | V | V | F | G | S | S | P | T | S | T | N | Δ V | E | G | A | E | A | A | A | A | T | A | V | V | F | G | S | S | P | T | S | T | N | Δ S | E | G | A | E | A | A | A | A | T | A | V | V | F | G | S | S | P | T | S | T | N | Δ 20 | E | G | A | E | A | A | A | A | T | A | V | V | F | G | S | S | P | T | S | T | N |

**Deletion Mutants**—Deletion mutants were constructed using a PCR ligation method. Essentially two primers, RSL-forward and RSL-reverse, were designed flanking the entire RSL cassette, and encompassing the Kap1 and BstBI restriction sites. Each deletion mutant was constructed by amplifying the area on either side of the deletion (A and B) using primers designed to incorporate regions of reverse complementarities (Table II). The two amplicons were then gel purified, diluted and mixed together, and denatured and annealed to allow the complementary regions of amplicons A and B to anneal to one another. PCR ligation was performed using the two outer primers (RSL-forward and RSL-reverse). Cycling for both reactions was performed in the PerkinElmer Geneamp PCR system 2400 as follows: 94 °C for 5 min, 35
cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by 1 cycle of 72 °C for 5 min.

The ligated product of the second round PCR was then digested with KpI and BstBI and ligated into the parental wild type vector pCDNA SCCA1, which also had been digested with KpI and BstBI. Ligated DNA was transformed into One-Shot™ Chemically Competent E. coli (Invitrogen) and purified using the Endo-free plasmid maxi kit (Qiagen). Sequencing was performed to confirm that the desired deletions had been made.

Quantitative Real-time RT-PCR—Total cellular RNA was purified using the RNAs™ Total RNA isolation system (Promega) and quantified by spectrophotometry at 260 nm. Reverse transcription (SuperScript II reverse transcriptase, Invitrogen) was primed using random hexamers (Promega). Quantitative real-time PCR was performed using the Rotor-Gene amplification system (Biogene, Kimbolton, UK). Each 25-μl PCR reaction contained 1 μl of DNTPs (2 mM), 0.5 μl of each primer (10 pmol/μl), 10 units of SYBR Green (Biogene), 2.5 μl of 10× reaction buffer, 1 unit of Taq polymerase (Qiagen), and 1 μl of cDNA. Primers used for the amplification of GAPDH, which served as a housekeeping gene control, were as follows: GAPDH-forward, 5′-tgatgcatacgagggggtgga-3′; and GAPDH-reverse, 5′-tccttgagccagctggcagc-3′. These primer sequences correspond to bases 840–900 of GAPDH (GenBank accessionnumber M20012). The primers used for the amplification of LRP, which was sequenced, were: LRP-forward, 5′-catctcaggtgccgggaatacagc-3′; and LRP-reverse, 5′-accgactgggaaacaaagtcc-3′. These primer sequences correspond to bases 1079–1103 of LRP (GenBank accession number M20014). The relative expression levels of GAPDH and LRP were calculated according the threshold cycle (Ct) for each degree change. Relative levels of GAPDH and LRP expression were calculated as the ratio of the sample with the lowest level of each product, and normalized using the value of the sample with the lowest level of each product, and the data were expressed as the ratio of LRP to GAPDH. Specificity of the desired PCR products was confirmed by melting curve analysis and confirmed by agarose gel electrophoresis and ethidium bromide staining.

Bacterial Expression and Functional Analysis of Recombinant RAP—A plasmid encoding a GST-RAP fusion protein, pGEX-KG-RAP, was kindly provided by Prof. J. Herz (University of Texas Southwestern Medical School) (25). This plasmid, and a control plasmid, pGEX 4T 3 (Amershamsciences), which expresses GST, were transformed into E. coli BL21 and induced to express recombinant protein as described above. Harvested cells were resuspended in 1 ml of lysis buffer (15% trichloroacetic acid, 0.5% sodium dodecyl sulfate, 100 mM EDTA, 50 mM sodium acetate, 200 mM NaCl, and 50 mM mercaptooctylisothiouronium). Samples were incubated overnight at 4 °C on a rotator. The resin was washed six times with ice-cold PBS, using centrifugation at 3,500 × g for 5 min. Recombinant proteins were eluted by the addition of 100 μl of glutathione elution buffer (50 μM Tris-HCl, 1 mM reduced glutathione, 8.0) and incubated at room temperature for 5 min before centrifugation at 3,500 × g for 5 min. The protein-containing supernatant was removed and stored on ice. Reduced glutathione was removed by washing the protein three times with PBS using Nanosep 10K filters. Recombinant protein was analyzed for homogeneity using SDS-PAGE and Coomassie Blue staining, diluted to 1 mg/ml, and stored at −20 °C.

Competitive Binding Assays Using rGST-RAP—Competitive binding assays were performed to confirm that recombinant GST-RAP competed with methylamine activated and radiolabeled A2M (33), a known ligand of LRP, prepared as described previously (26). Huh7 cells were seeded into 24-well plates at a density of 4 × 103 cells/well. Cultures were incubated overnight at 37 °C in 5% CO2. Cell culture medium was removed and replaced with 1 ml/well of Dulbecco's minimal essential medium (no serum) containing varying amounts of either rGST-RAP or rGST (control). Cells were incubated for 20 min before the addition of 10 μg of rA2M. Cells were incubated for 4 h at 37 °C in 5% CO2, then washed three times with PBS, and solubilized in 200 μl/well of 0.1% n-DOC. Scintillation counting was performed by adding 150-μl aliquots of fractions to 2 ml of scintillate (Omniscint “HiSafe,” Wallac). Counting was performed for 5 min per sample using a Beckman LS 6500 multipurpose scintillation counter.

The effect of rGST-RAP on SCCA1-mediated virus-cell binding was assayed using two approaches. First, competitive binding assays were performed where transiently transfected cells were maintained in the presence of rGST-RAP (or rGST) for 15 min prior to the addition of the virus suspension. Cells were maintained continuously in the presence of the competitor and virus-cell binding assays were performed as above. An alternative strategy made use of the observation that cells exposed to low levels of recombinant RAP (200 nM) showed reduced expression of LRP (27, 28). Therefore, experiments were performed in which cells were transfected as previously, and then maintained in the presence of 200 nM rGST-RAP (or 200 nM rGST) for 48 h prior to performing cell-virus binding assays, as above. Reduced expression of LRP in SCCA1-transfected HuH7 cells maintained in the presence of rGST-RAP was confirmed using real-time RT-PCR as described above.

RESULTS

Cloning, Sequencing, and Expression of SCCA1 cDNA—A full-length cDNA for SCCA1 was amplified from total RNA (isolated from 105 HepG2 cells) using a nested RT-PCR, and cloned as a fusion construct into the mammalian expression vector pCDNA 3.2 V5 His, to produce pCDNA SCCA1. Western blotting of lysates from cells transfected with pCDNA SCCA1 and with a control plasmid, pCDNA 3.1 V5 His LacZ) confirmed expression of a protein of ~44 kDa, which corresponds to the expected size of SCCA1 with the addition of a carboxy-terminal V5 poly-His tag (Fig. 1).

Sequence analysis of the cloned cDNA for SCCA1 was performed for comparison with GenBank™ sequences for SCCA1 (accession number U19556) and the sequence reported for HBV-BP (33). A number of differences were observed in the predicted amino acid sequence. These changes are shown in Table III. Comparison of HBV-BP and pCDNA SCCA1 with the Draft Human Genome (using Blast+) was performed, and in both cases a unique match was found, namely SCCA1.

To express recombinant SCCA1 in a bacterial system, we subcloned the cDNA for SCCA1 V5 pol-His into a bacterial expression vector, pGEX 4T-3, in-frame with GST to produce a GST-SCCA1 fusion protein. Recombinant SCCA1 (rSCCA1)
was batch purified, and examined for purity using SDS-PAGE, Coomassie Blue staining, and Western blotting using an anti-V5 antibody (Fig. 2A). We confirmed that a protein of 71 kDa (corresponding to SCCA1 with the addition of a GST tag) was overexpressed in transformed E. coli BL21, and purified to 90% purity. Confirmation of function of the rSCCA1 was obtained by assaying inhibition of papain-mediated proteolysis of a fluorescent substrate (Fig. 2B). Kinetic studies comparing rSCCA1 with a mock purified cell lysate from untransformed E. coli BL21 confirmed that rSCCA1 competitively inhibited papain, a proteinase weakly targeted by SCCA1 in vitro (Fig. 2C).

Transfection and Virus Binding Assays—We investigated the effect of transfection of pCDNA SCCA1 on binding of HBV to Huh7 cells (a human hepatocyte derived line) and COS7 cells (monkey kidney cells). Cells were transiently transfected, and expression was allowed to proceed for 48 h before virus-cell binding assays were performed. Cell-associated virus DNA was analyzed using PCR, Southern blotting, and scanning densitometry.

In both Huh7 and COS7 cells, we observed an increase in cell-associated virus DNA in transfected cells compared with control (mock transfected) cells (Fig. 3A). These results mirror those reported for HBV-BP, although in that report HepG2 cells and Chinese hamster ovary cells were utilized for transfection studies (3). The enhanced binding of HBV to transfected cells was considerably more marked for Huh7 than COS7 cells. This observation is not attributable to transfection or expression efficiency, indeed, SCCA1 expression (as monitored using Western blotting) was considerably higher in COS7 cells, where it had less effect on virus binding, than in Huh7 cells.

![Table III](image-url)
Trypsinization, which is reported to remove virus particles associated with, but not internalized by, cells (29), was used to assess the possibility that the cell-associated virus DNA had been internalized. We observed that virus DNA bound to SCCA1-transfected Huh7 cells is protected from trypsin degradation significantly (with ~75% of bound virus DNA remaining cell-associated, compared with only 30% of total virus DNA bound to mock transfected controls) (Fig. 3B). This suggests that SCCA1 transfection mediates at least partial internalization of viral particles. Despite this observation, repeated attempts to demonstrate convincingly the presence of replicative intermediates of HBV DNA in SCCA1-transfected cells were unsuccessful.

Soluble recombinant SCCA1, expressed as a GST fusion protein and subsequently cleaved from the GST tag using thrombin, was shown to inhibit the binding of HBV to transfected cells (data not shown). This supports the data reported by De Falco et al. (3), who interpreted this as further evidence of a role for SCCA1 as a cellular receptor for HBV. However, if our hypothesis that an SCCA1-HBV complex enters hepatocytes via the SEC hepatic clearance system is correct, this inhibition may be explained by excess rSCCA1 bound to proteinases present in the serum, saturating the receptors used for clearance of SECs.

RSL Mutagenesis—Our results confirm that SCCA1 mediates enhanced binding of HBV to cells. The biology of serpins, and in particular the hepatic clearance mechanisms for SECs, stimulated our interest in whether enhanced virus-cell binding to SCCA1-transfected cells was mediated by the reactive site loop.

We utilized a site-directed mutagenesis approach to determine whether subtle mutations that alter the specificity of SCCA1 would abrogate the enhanced binding of HBV to transfected cells. The construct pCDNA SCCA1 was used as the template for construction of two functional mutants, the P14 RSL mutant, which contained a single coding change (AlaP14Arg) and the P3 RSL mutant (PheP3Ala). Both these changes result in lack of inhibition of target proteinases (9). We confirmed that both RSL mutants were no longer inhibitory for papain using the proteolysis inhibition assay described above (data not shown). Transfection and virus binding studies were performed to compare cells transfected with the P3 and P14 RSL mutants with those transfected with wild type SCCA1 and mock transfected cells. In neither case did functional mutation of the RSL, resulting in loss of proteinase inhibitory activity, also result in abrogation of enhanced virus-cell binding. There was no significant difference in binding between the wild type SCCA1 and either RSL mutant (Fig. 4A).

A third construct (Pro-Pro) targeted the putative cleavage site at residues P1 and P1’ (serine-serine) specifically. Mutation of the cleavage site to proline-proline, which results in loss of inhibitory activity for papain in proteolysis assays, was used to investigate the significance of the cleavage site in enhanced virus binding. Transfection and binding studies showed that the Pro-Pro mutant enhanced virus binding to the same degree as the wild type SCCA1 (Fig. 4B).

These results suggest that functional activity of SCCA1 as a proteinase inhibitor is not required for enhanced virus-cell binding. To determine whether more drastic changes in the RSL affected SCCA1-mediated virus-cell binding, we constructed four deletion mutants in which part of, or the entire, RSL was deleted. All mutants were shown not to inhibit papain (data not shown). The constructs were transfected into Huh7 cells and virus-cell binding was measured. As with the P3 and P14 mutants, in no case was there a decrease in virus-cell binding compared with cells transfected with wild type SCCA1 (Fig. 4C). Surprisingly, in the cases of the ΔΔ SCCA1 and the ΔΔΔ SCCA1 mutants, virus-cell binding was enhanced more than for wild type SCCA1-transfected cells. These data show that the reactive site loop is not required for SCCA1-mediated virus-cell binding.

Up-regulation of LRP Expression in SCCA1-transfected Cells—Total RNA was purified from resected liver tissue, HepG2 cells, mock transfected Huh7 cells, and Huh7 cells transfected with pCDNA SCCA1. Real-time RT-PCR was performed using primers specific for GAPDH, and the relative amount of GAPDH in each sample was determined using comparative means. Samples were normalized using GAPDH val-
ues for subsequent experiments. Expression of LRP in each sample was then analyzed using primers specific for the LRP cDNA.

LRP expression was shown to be reduced in the hepatocyte-derived cell lines, HepG2 and Huh7 cells, compared with primary liver tissue (Fig. 5). Huh7 cells transfected with pCDNA SCCA1 (which results in increased binding of HBV to cells) showed substantially higher levels of LRP expression, with an increase of ~60% in relative copy numbers from 1.14 ± 0.11 in untransfected Huh7 cells to 1.83 ± 0.09 in pCDNA SCCA1 transfected Huh7 cells (Fig. 5). The level of LRP expression in transfected Huh7 cells slightly exceeds that seen in total liver tissue.

**Competitive Binding Assays Using RAP**—RAP is a 39-kDa protein that was identified by the fact that it consistently co-purified with LRP (30). Herz et al. (25) showed that recombinant RAP (as a GST fusion protein) binds reversibly to the large subunit of LRP, and also inhibits the binding and uptake of LRP ligands including very low density lipoproteins and activated A2M. RAP has been used as a competitive inhibitor in ligand binding studies involving the expression of LRP (31, 32) or as a means of reducing the expression of LRP in cells exposed to low levels of recombinant RAP (27, 28). Both approaches were used to investigate the possibility that enhanced virus-cell binding in SCCA1-transfected cells resulted directly from the up-regulation of LRP.

Recombinant GST-RAP was expressed in a bacterial system, purified (Fig. 6) and assayed for protein function by competitive binding assays with methylamine-activated A2M (Fig. 7A). To investigate whether RAP-mediated reduction in available LRP molecules resulted in reduced virus cell binding, Huh7 cells transiently transfected with pCDNA SCCA1 were exposed to rGST or rGST-RAP (3 μg/well) in transfected cells. Compared with mock transfected cells (unt) and transfected cells in the absence of competitor (trans). Mean ± S.D., n = 6. In the presence of higher levels of recombinant protein (10 μg/well) there was nonspecific reduction of virus binding in wells containing either GST or GST-RAP.

**Fig. 6.** Coomassie Blue staining of purified rGST-RAP and rGST following SDS-PAGE. For both rGST and rGST-RAP, induced (Un) bacterial lysates show high levels of expression of desired proteins compared with uninduced (Un) cells. Purified (Pu) protein is shown to be a homogenous single protein band of 31 (rGST) or 68 kDa (rGST-RAP).

**Fig. 7.** A, competitive binding assay measuring the cell-associated 14C-A2M* (cpm). Binding assays were performed in the presence of increasing amounts of rGST-RAP (black) or rGST (gray, negative control) ranging from 0.3 to 10 μg. Mean ± S.D., n = 3. B, competitive virus binding assays in the presence of rGST-RAP (3 μg/well) or rGST (negative control, 3 μg/well) in transfected cells. Compared with mock transfected cells (unt) and transfected cells in the absence of competitor (trans). Mean ± S.D., n = 6. In the presence of higher levels of recombinant protein (10 μg/well) there was nonspecific reduction of virus binding in wells containing either GST or GST-RAP.
DISCUSSION

These results confirm that expression of SCCA1 enhances the binding of HBV to hepatocyte-derived cell lines and cells of non-hepatocyte origin. Transfection of SCCA1 into cells results in an increase in cell-associated virus DNA. Furthermore, virus binding is associated with at least partial internalization because bound virus particles are protected from removal from cells by trypsin. Although there is evidence for internalization, we have been unable to detect replicative intermediates of HBV DNA in these cells. Therefore, it appears that there remains, even in hepatocyte-derived SCCA1-transfected cells, a “block” downstream of binding, which prevents transfected Huh7 cells from supporting a complete round of virus replication.

The observation that enhancement of HBV binding to SCCA1-transfected cells was considerably more marked for hepatocyte-derived cells than for COS7 cells supports the hypothesis that a dual or even more complex receptor system is required for virus binding, an (as yet unknown) additional component being expressed endogenously in hepatocyte-derived cells. If this is the case, i.e. SCCA1 acts as a co-receptor, then non-hepatocyte-derived cells expressing SCCA1 may be a valuable tool for identifying the second (unknown) component of a putative receptor complex.

The SCCA1-mediated hepatic clearance of serpins in vivo is an attractive model for enhanced virus binding to SCCA1-transfected cells, especially considering the reported absence of SCCA1 expression in the liver. This hypothesis was tested by investigating the serpin RSL and LRP, the receptor that mediates the removal of SECs, using SCCA1-transfected cells.

Site-directed mutagenesis was utilized to create SCCA1 RSL functional and deletion mutants, which were compared with wild type SCCA1. In no case did mutation of the RSL result in abrogation of enhanced virus binding to transfected cells. These results discount convincingly the possibility that SCCA1-mediated virus-cell binding is dependent on the function of SCCA1 as a proteinase inhibitor, and therefore dependent on the reactive site loop. The reasons for the further enhancement of virus binding to cells transfected with the Δ8 and Δ20 RSL deletion mutants are not yet known, but may be because of the altered conformation of the SCCA1 molecule resulting from the large deletions that have been introduced, and may suggest that another region of the SCCA1 protein interacts with HBV.

We have also investigated the effect of SCCA1 expression in Huh7 cells on the levels of LRP expression. The levels of expression of LRP in two hepatocyte-derived cell lines, Huh7 and HepG2, were shown to be reduced compared with total liver. This suggested that the limited capacity of both cell lines for HBV binding and entry may be related to down-regulation of LRP in those cell lines. SCCA1 expression in Huh7 cells resulted in up-regulation of LRP, slightly exceeding the levels in total liver and suggesting, therefore, that SCCA1-mediated virus-cell binding may be because of increased expression of LRP in the transfected cells. However, the use of a competitive ligand for LRP, RAP, did not reduce the levels of cell-associated HBV DNA. Maintenance of transfected cells in the presence of RAP, previously reported and shown above to result in decreased expression of LRP, also did not lead to the concomitant reduction in levels of bound virus.

This study supports the assertion by De Falco et al. (3) that SCCA1 expression results in enhanced virus binding and internalization. However, this enhancement is not attributable either to the function of SCCA1 as a proteinase inhibitor, or to attachment of HBV (putatively bound to the serpin) to hepatocytes via the hepatic clearance system for serpin-enzyme complexes.

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