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CLONING AND PRODUCTION OF FUNCTIONAL ACTIVE RECOMBINANT HEPATITIS B VIRUS SURFACE ANTIGEN BINDING PROTEIN

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Endonexin II present on the surface of human hepatocytes has recently been identified as a hepatitis B virus surface antigen (HBsAg) binding protein. A full-length cDNA clone encoding human endonexin II was isolated from a human liver cDNA library and was placed under the control of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV). Infection of *Spodoptera frugiperda* cells with recombinant virus resulted in the production of high amounts of recombinant protein. This protein has the same molecular weight and iso-electric point as native human endonexin II. It can be easily purified by methods analogous to those described for the native protein. Moreover, the recombinant product binds very efficiently to hepatitis B surface proteins (HBsAg) in a similar fashion as native human endonexin II.

Hepatitis B is still a major world health problem. Its manifestations are numerous and chronic hepatitis B infection is unequivocally related with liver cirrhosis and hepatocellular carcinoma, resulting in significant mortality (15). Hepatitis B virus (HBV) displays a strict host- and tissue specificity, which has been the subject of extensive investigation. An obvious cause for host and tissue tropism might be the existence of a specific cellular receptor for the virus, enabling it to enter the liver cell. A receptor can even be the sole determinant for viral tropism (10). Therefore, one possible means of vaccination or treatment of viral hepatitis B could be the prevention of the virus from entering the liver cell by blocking the interaction between virus and cellular receptor.
HBsAg exists in three forms, the small, middle and large HBsAg with identical carboxyl termini. Middle and large HBsAg are composed of small HBsAg with N-terminal extensions of 55 and 181 amino acids respectively (the preS2 and preS1 regions)(15). Recently we identified a specific small HBsAg binding protein on human liver cells as EII (4). Human EII belongs to the family of annexins, a group of closely related and highly conserved Ca^{2+}-dependent phospholipid-binding proteins.
To investigate the usefulness of EII as a therapeutic agent for HBV infections, and to be able to conduct research in order to localize the exact binding epitopes on the viral surface proteins and on the receptor itself, large amounts of purified protein are generally required. To achieve this, we isolated EII cDNA from a human liver cDNA library. This cDNA was cloned in the well characterized baculovirus expression system (11). The

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recombinant protein is produced in high amounts, is easy to purify and binds efficiently to HBsAg. We consider this recombinant protein to be a very important tool in future hepatitis B research.

METHODS

Screening of cDNA library for EII - Using amino acid sequence information from the HBsAg binding protein (4), the 36-mer 5'-TTTTTCATTTGTTCCAGCTCCCTTCAAG-GCATGTTT-3' was synthesized on a Perkin Elmer DNA synthesizer. This oligo-nucleotide was labelled with τ-^{32}P-ATP using T4-polynucleotide kinase (Life Technologies) according to the manufacturers instructions. A human liver cDNA library in phage lambda-ZAP (a kind gift from Dr. Lamers, Acad. Med. Centre, Amsterdam) was screened with this probe using standard plaque lifting and hybridization techniques. cDNA inserts were rescued in plasmid pBluescript by co-infection with helper phage R408.
DNA manipulations - Restriction enzymes and other DNA-modifying enzymes were purchased from Life Technologies. High temperature DNA sequencing was performed using a Taq-polymerase based kit (Promega) according to the manufacturers instructions.
Cells and viruses - Sf9 cells were grown as monolayer cultures at 27°C in TC100 medium (Life Technologies), supplemented with 10% FBS and 40 μg/ml gentamycin. Seeding of the cells and virus infections were performed as described by Summers and Smith (14).
Construction of baculovirus transfer vectors pVLendo-1.4AE and pVLendo-1.4EE - cDNA inserts from rescued phage lambda-ZAP were purified by digestion with EcoR1, electrophoresis in 0.6% agarose gels and electro-elution using a biotrap apparatus (Schleicher & Schüll). 116 of the 150 non-coding nucleotides at the 5'-terminus were removed by digestion at a unique AvaII site in the EII cDNA, 34 nucleotides upstream from the start codon. The AvaII-EcoR1 fragment and the original EcoR1 EcoR1 fragment were supplied with BamH1 and KpnI termini by cloning in the SmaI site of pGEM3Zf[-] (Promega). This was followed by digestion with BamH1 and KpnI and cloning in the BamHI-KpnI sites of baculovirus transfer vector pVL941. Resulting transfer plasmids were named pVLendo-1.5EE (1.5 Kb EII EcoR1-EcoR1 fragment), pVLendo-1.4AE (1.4 Kb AvaII-EcoR1 fragment) and pVLendo-1.4EA (1.4Kb EcoR1-AvaII fragment, non-coding orientation)(Figure 1).
Cotransfection and purification of recombinant baculovirus - The recombinant pVLendo transfervectors were cotransfected with AcNPV DNA into Sf9 cells using lipofectin reagent (Life technologies). After 5 days cell cultures were harvested and screened for presence of recombinant virus by a standard dot-blot procedure (8, 12) using agarose gel purified, ^{32}P-dCTP labelled EII cDNA. Presence of wild-type baculovirus was excluded by stripping and reprobing the blots with purified polyhedrin cDNA. Nomenclature for the recombinant viruses was Acendo-1.5EE, Acendo-1.4AE and Acendo-1.4EA, according to the previous used nomenclature for the various plasmids.
Iso-electric focusing, SDS-PAGE and western blotting -
For analyis of recombinant EII production, cells were homogenized in buffer H (20 mM HEPES pH 7.4, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM phenyl-methyl-sulfonyl fluoride) using a glass-glass Potter Elvehjem. 20 μg protein were subjected to SDS-PAGE. Semi-dry western blotting was performed on a Novablot apparatus (Pharmacia). Immunoblots were incubated for 1 hr at room temperature with PBS, 0.05% Tween-20 (PBST), 1% gelatine, followed by an overnight incubation with 1:5000 diluted rabbit anti-human EII antiserum (5). Bound antibodies were detected by a 2 hrs incubation with peroxidase-conjugated Swine anti-rabbit immunoglobulin (1:800, Dakopatts) and colour development in 50 mM Tris, pH7.6, 0.06% diaminobenzidine, 0.03% Cobalt chloride. Iso-electric focusing was performed on pre-cast ampholine pH gradient gels in a Phast-System apparatus (Pharmacia, Uppsala, Sweden), followed by staining with Coomassie Brilliant Blue. pH markers in the range 3-10 were purchased from the same manufacturer.

Immunocytochemical studies on Sf9 cells, infected with recombinant virus - 100,000 Sf9 cells were seeded in 4 wells plates (Costar) with glass cover slips and infected with wild type or recombinant virus with a multiplicity of infection (m.o.i.) of 1. Cells were washed with PBS and fixed in ice-cold methanol for 10 minutes at 1, 2, 3 and 4 days post infection. After drying, cells were stored at -20°C until analysis. Immediately before use, cells were thawed in ice-cold methanol and washed in PBS. Endogenous peroxidase activity was blocked by a 10 min incubation with methanol/3%H_2O_2. Aspecific binding sites were blocked in PTTB (PBS , 0.05% Tween-20, 0.1% Triton-X100, 4% BSA). The cells were incubated overnight at 4°C with a 1:1500 dilution of rabbit anti-EII antiserum in PTTB. Incubation of Sf9 cells infected with recombinant baculoviruses with pre-immune serum or AcNPV infected cells with anti-EII, served as negative controls. After extensive washing with PBS-0.05% Tween-20 (PBST), peroxidase conjugated swine anti rabbit IgG (1:40 in PTTB) was added. After 1 hr, cells were washed in PBST. Peroxidase was visualized by reaction with 0.1% diaminobenzidine in PBS with 0.3% H_2O_2 for 10 minutes. Cells were counterstained with haematoxylin, dehydrated, mounted permanently in eukitt and examined by light microscopy.
Isolation and purification of recombinant EII - Sf9 cells were seeded in 160 cm^2 tissue culture flasks and infected with baculovirus Acendo-1.4AE with a m.o.i. of 5. Four days post infection cells were harvested and recombinant EII was isolated according to the procedure for isolating native EII (3).
Radio-labelling of proteins - 5 μg of recombinant EII was labelled with $Na^{125}I$ (Amersham, Buckinghamshire, UK) using Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril, Pierce, Rockford, IL USA) according to manufacturers prescription.
Binding of recombinant EII to HBsAg - Equimolar amounts of small or large HBsAg or BSA as a control protein were coated on high binding capacity 96 wells micro-titre dishes (Greiner, Germany). After blocking aspecific binding sites with PBS with 3% gelatin for 2 hrs at room temperature), the wells were washed twice with PBST and once with PBS. 100,000 cpm of iodinated recombinant EII in PBS, supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was added to the wells. After a two hour incubation at room temperature, the dishes were washed extensively with PBS/Ca^{2+}/Mg^{2+} until no more radioactivity was released. Bound protein was released by adding PAGE sample buffer to the wells. Released proteins were denatured at 100°C for 5 min and subjected to SDS-PAGE and autoradiography. In parallel experiments, after the two hrs incubation period, the nonreversible cross-linking agent disuccimidylsuberate (DSS, Pierce) in dimethylsulfoxide (DMSO) was added in a final concentration of 1 mM. After the crosslinking reaction (30 min at 4°C), 100 mM glycine was added to stop the reaction. Formed complexes were released in PAGE sample buffer and subjected to SDS-PAGE and autoradiography.

RESULTS AND DISCUSSION

In previous work we have demonstrated that human EII is involved in the Ca^{2+}-dependent binding of HBsAg to the human liver cell (4, 9). EII is a member of the family of annexins, a group of highly conserved proteins with Ca^{2+}-dependent phospholipid binding properties. The function of these proteins is not clear. It has been suggested that they may be involved in endocytosis and exocytosis by promoting membrane fusion (2, 6, 7).
To conduct research to the mechanism of virus entry, large amounts of functionally active HBsAg binding protein are required. To achieve this goal, we chose to clone the EII cDNA in the well defined baculovirus expression system. A full lenght cDNA fragment

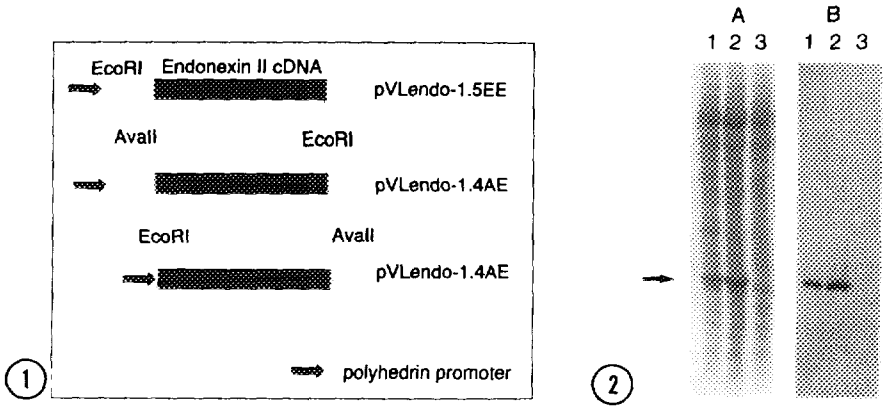

FIG. 1. EII cDNA, picked from a human liver cDNA library in phage lambda-ZAP, was cloned in the SmaI site of plasmid pGEM3Zf[-]. Using the flanking BamH1 and KpnI restriction sites, this cDNA was cloned unidirectional in the BamH1/KpnI-digested baculovirus transfervector pVL941. Besides the complete cDNA fragment, an AvaII-EcoR1 fragment lacking 135 5'-terminal non-coding nucleotides was cloned in pVL941 in order to minimalize the distance between promoter and initiation codon. For nomenclature for the resulting transfervectors see text.

FIG. 2. 3 days after infection with Acendo-1.5EE (lanes 1), Acendo-1.4AE (lanes 2) or cendo-1.4EA (lanes 3), Sf9 cells were lysed in SDS-PAGE sample buffer. Panel A represents a Coommassie-Brilliant-Blue-stained 10% polyacrylamide gel, panel B the concomitant Western blot, stained with rabbit-anti human EII. The arrow indicates the position of recombinant EII.

of 1470 base pair, as confirmed by DNA sequencing, was isolated from an human cDNA library and cloned in the multiple cloning site of pBluescript. The plasmid was digested with EcoRI and AvaII. Resulting AvaII-EcoR1 and EcoR1-EcoR1 fragments were cloned in the baculovirus transfer vector pVL941 to yield pVLendo-1.4AE, pVLendo-1.5EE and pVLendo-1.4EA (non-coding orientation of AvaII-EcoR1 fragment, functioning as a control)(Figure 1). After purification of recombinant virus infected cells were screened for recombinant protein production. Figure 2 shows a Coommassie Brilliant Blue stained SDS PAGE gel and the corresponding Western blot of cell homogenates, infected with Acendo-1.5EE, Acendo-1.4AE and Acendo-1.4EA. As expected, removal of the 135 bp at the 5'-noncoding region led to a significant increase of EII expression (compare lanes 1 and 2). Iso-electric focusing and SDS-PAGE of recombinant EII, revealed that the iso-electric point and molecular weight are identical to that of native EII (figure 3A and 3B, compare lanes 1 and 2).

From amino acid sequence data it is known that EII does not contain a consensus signal sequence. Therefore this protein has been regarded as a non-transmembrane protein. However, annexin I has shown to be efficiently secreted from cells, despite the absence of a signal peptide (3, 16) and secretion of recombinant rat endonexin II from insect cells is

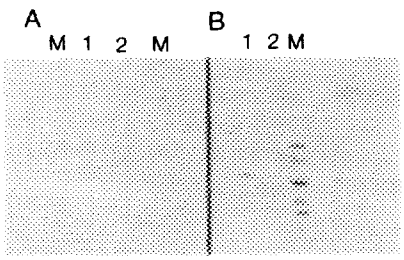

FIG. 3. Comparison of recombinant and native EII. Panel A shows a Phast System IEF gel, pH 4-6.8. Lane M: pH markers pI 9.3, 8.65, 8.45, 8.15, 7.35, 6.85, 6.55, 5.85, 5.20, 4.55, 3.5, lane 1: recombinant EII; lane 2: native EII. Both gels were stained with Coommassie Brilliant Blue following the manufacturers instructions. Panel B: Phast System SDS-PAGE gradient gel (8-25% polyacrylamide). Lane M: Mw markers 92, 68, 45, 29, 21 and 14 kD; lane 1: purified recombinant EII; lane 2: purified native EII.

recently reported (14). Using immunocytochemical analysis of Sf9 cells (Figure 4), expressing recombinant EII, we demonstrate that this protein is at least membrane associated, being a prerequisite for a receptor protein. Whether EII is also a transmembrane protein has to be established yet, but seems to be expected from binding studies in which the binding between intact human hepatocytes and HBsAg can be inhibited by incubation with anti-EII antibodies (Submitted elsewhere).

For purification of recombinant EII from Sf9 cells, a modified version of the purification procedure of EII from human placenta was used (6)(Figure 5). Lane 1 contains 2 μg protein of total homogenate of Sf9 cells, infected with Acendo-1.4AE. However, lane 3 shows that considerable amounts of recombinant EII can not be precipitated with Ca^{2+} (considering that the volume of the supernatant was approximately 20 times the volume of the resuspended pellet). This is probably due to the large excess of recombinant protein as compared to the amount of phospholipids present. To purify this non-precipitable protein, the Ca^{2+} precipitation step was repeated twice using the phospholipid pellet from the first precipitation step after it had been cleared from EII with EGTA (lane 5 and 6 respectively). The pooled EGTA supernatants contained only one protein band on SDS PAGE and IEF gels (Figure 3, lanes 2). Purified recombinant EII was labelled with ^{125}I using the Iodogen method and used in binding assays with HBsAg as described in Methods. It is clear from the autoradiograph in figure 6 that binding of recombinant EII to HBsAg is strictly Ca^{2+}- and Mg^{2+}- dependent.

Figure 6B shows the results of a binding experiment in which the binding capacity of small HBsAg, large HBsAg and synthetic preS1 was investigated. As is the case for native human EII, large HBsAg also is capable of efficient binding to recombinant EII (lanes 2, duplicate experiments). There is a negligible binding to synthetic preS1 peptide

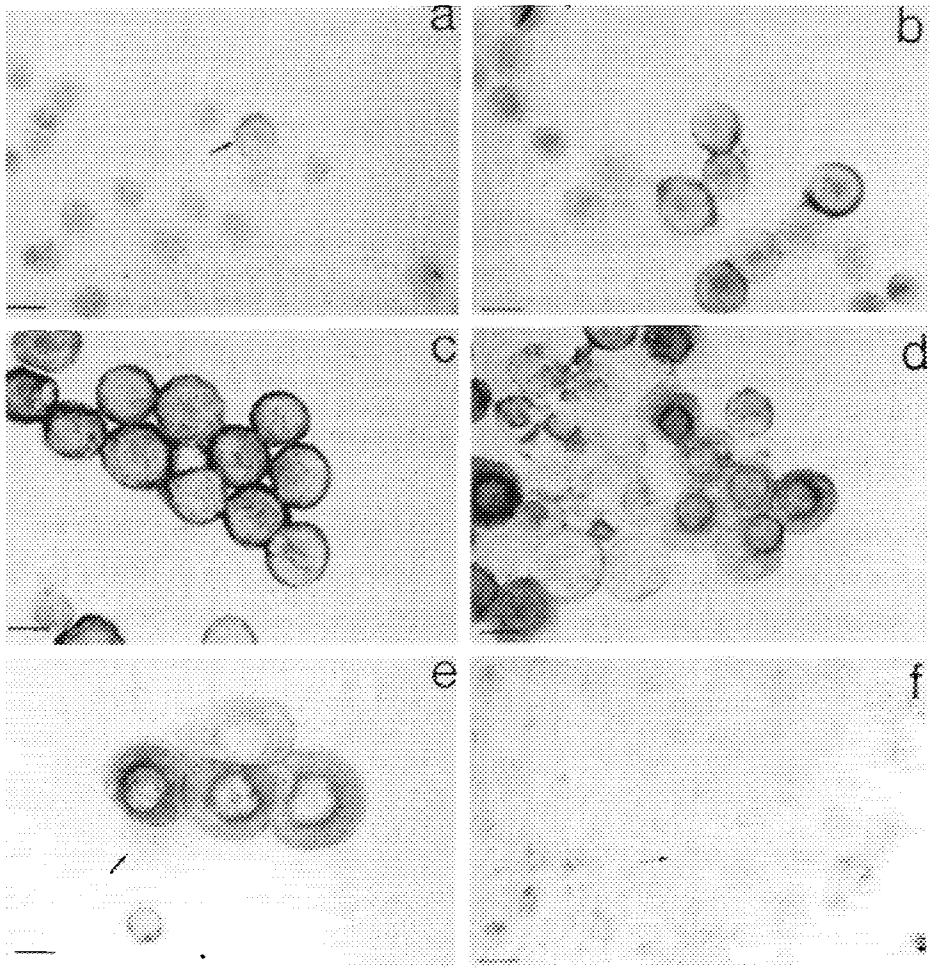

FIG. 4. Immunoperoxidase staining of recombinant virus-infected Sf9 cells. Sf9 cells were infected with Acendo-1.4AE, fixed and stained with anti-EII as stated in Materials and Methods. panels a-e : 1-5 days post infection, panel f : wild-type AcNPV infected Sf9 cells. The arrow in panel e shows leakage of recombinant protein, the arrow in panel f points at polyhedrin accumulations.

(lanes 3) and no binding at all to BSA (lanes 4). Figure 7 shows the complexes that are formed when radiolabelled EII is cross-linked to small HBsAg (lane 2). A protein complex with an apparent molecular weight of 85 kilodaltons is formed. This protein complex might be the result of one EII molecule (34 kD) and two HBsAg molecules (2x26 kD) or two molecules of EII with one molecule of HBsAg.

In summary, the recombinant EII produced in this system has the same molecular weight and iso-electric point as the native protein. Moreover, it behaves identical as the native protein in regard to HBsAg and phospholipid binding, as it can be isolated using a protocol for the isolation of native EII. When cross-linking studies are performed with

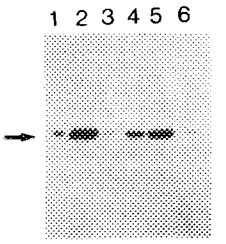

FIG. 5. Purification of recombinant EII. Recombinant EII was isolated from Ac-endo1.4AE infected Sf9 cells using its property as a Ca^{2+}-dependent phospholipid binding protein. Each lane contains 2 μg of protein. Lane 1 shows a total homogenate, lane 2 the pellet after Ca^{2+}-precipitation as described in Materials and Methods. Lane 3 contains proteins, present in the supernatant after Ca^{2+} precipitation. After washing the phospholipid pellet with buffer H containing EGTA, EII is released in the supernatant (lane 4). The washed pellet is however capable of renewed binding to EII, as is shown in lane 5 (after second round of binding and EGTA treatment) and lane 6 (after third round of binding and EGTA treatment).

major HBsAg and EII, complexes are found with molecular weights that indicate the presence of two binding sites on EII for HBsAg, or two binding sites on HBsAg for EII. The availability of large amounts of recombinant EII, functionally active in regard to

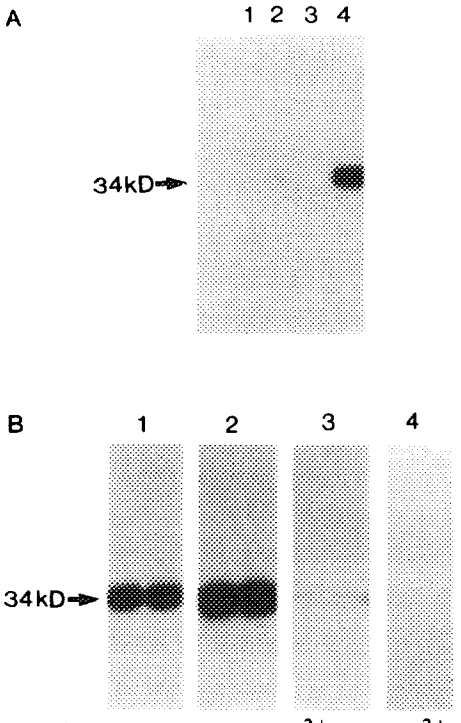

FIG. 6. Functional activity of recombinant EII. A) Ca^{2+}- and Mg^{2+}-dependent binding of recombinant EII to HBsAg. Radio-labelled recombinant EII was added to solid-phase-coated HBsAg as described in Methods. After incubation, bound EII was released, subjected to SDS-PAGE and autoradiography. Binding assays of EII on major HBsAg were performed in PBS (lane 1), PBS with Ca^{2+} (lane 2), PBS with Mg^{2+} (lane 3) and PBS with both Ca^{2+} and Mg^{2+} (lane 4). B) Binding of recombinant EII to solid phase coated major HBsAg (lanes 1), large HBsAg (lanes 2), preS1 (lanes 3) and BSA (lanes 4) (all experiments in duplicate).

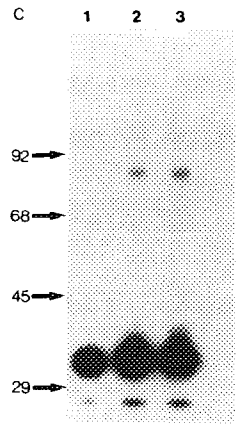

FIG. 7. DSS cross-linking of EII bound to major HBsAg. Lane 1: no crosslinker added; lanes 2 and 3: DSS added (duplicate experiment). Arrows represent molecular weight markers: Downward 92 kD, 68 kD, 45 kD, 29 kD and 21 kD.

HBsAg binding, may therefore be of considerable importance in future hepatitis B research.

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