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Fine Mapping of Virus-Neutralizing Epitopes on Hepatitis B Virus PreS1

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We identified the epitopes on the preS1 which induce antibodies that neutralize both *ad* and *ay* subtypes of hepatitis B virus (HBV). Previously we generated murine monoclonal antibodies KR359 and KR127 that bind specifically to the preS1 of HBV. In this study we have performed fine mappings of the epitopes of the antibodies by examining their reactivity with GST fusion proteins, which contain a series of deletion mutants of the preS1. KR359 and KR127 specifically recognize aa 19–26 and 37–45 of the preS1, respectively. The antibodies neutralized both *adr* and *ayw* subtypes of the virus in an *in vitro* neutralization assay using *in vitro* infection of adult human hepatocyte primary culture by HBV. The epitopes showed little sequence divergence and the antibodies bound to the preS1 of all the HBV subtypes and variants tested. © 2000 Academic Press

INTRODUCTION

Human hepatitis B virus (HBV) is a small enveloped DNA virus which causes acute and chronic hepatitis in humans (Ganem and Varmus, 1987). The HBV envelope contains three related surface glycoproteins called the large (L), middle (M), and small (S) proteins. All these proteins are the product of a single open reading frame, which is divided into the preS1, preS2, and S regions (Heermann *et al.,* 1984). They arise from separate translation initiations at each of the three inframe AUG codons and share a common C-terminal S open reading frame. The S protein (P24) and its glycosylated form (GP27) are encoded by the S region. The M protein (GP33/GP36) contains preS2 (55 amino acids [aa]) and S regions, and the L protein (P39/GP42) contains preS1 (108 or 119 aa, depending on the antigenic subtype), preS2, and S regions. The S protein carries the common group-specific determinant *a* and two sets of mutually exclusive subtype determinants *d/y* and *w/r* and thus the four major subtypes (*adw, adr, ayw,* and *ayr*) of the HBsAg denote the antigenic types of HBV (Courouce *et al.,* 1982).

In addition to the infectious virus particles, cells infected by HBV produce large quantities of noninfectious subviral particles of two other morphological forms, 22-nm spherical particles and filaments of 22-nm diameter. The S protein is found in large quantities on all three particle types, whereas the L protein is preferentially

localized on infectious virus particles (Stibbe and Gerlich, 1983; Heermann *et al.,* 1984). Thus, the L protein was speculated to have a pivotal function in viral infectivity and assembly (Neurath *et al.,* 1986; Persing *et al.,* 1986; Ou and Rutter, 1987). Also, the aa 21–47 of the preS1 was proposed to contain a specific binding site for human hepatocyte receptor (Neurath *et al.,* 1986; Pontisso *et al.,* 1989).

The envelope proteins carry epitopes involved in immune defense mechanisms (Dreesman *et al.,* 1982; Heermann *et al.,* 1987; Klinkert *et al.,* 1986; Milich, 1987). During infection, the antibodies are detectable at various stages of the disease and convalescence: anti-preS antibodies develop early, whereas anti-S antibodies appear later and signal virus elimination (Klinkert *et al.,* 1986; Heermann *et al.,* 1987; Milich, 1987; Neurath *et al.,* 1989). In the case of S protein, the common *a* determinant was shown to elicit the protective and neutralizing antibodies (Bhatnager *et al.,* 1982; Dreesman *et al.,* 1982); moreover, the monoclonal antibody specific to the common *a* determinant was demonstrated to have neutralizing activity (Iwarson *et al.,* 1985; Ogata *et al.,* 1993). On the contrary, the role of anti-preS antibodies in virus neutralization is less well studied. In the case of preS1, antiserum to peptide preS1(aa 21–47) was shown to neutralize HBV infection in chimpanzee (Neurath *et al.,* 1989). However, no anti-preS1 monoclonal antibody with virus-neutralizing activity has been reported. This may be a result of the lack of reproducible *in vitro* HBV infection system using a cell line and experimental animal model except chimpanzees. Recently, we found that *in vitro* neutralization assay using *in vitro* infection of adult human hepatocyte culture by HBV is specific and reproducible enough to evaluate the neutralizing activities of anti-HBsAg monoclonal antibodies (Ryu *et al.,* 1997).

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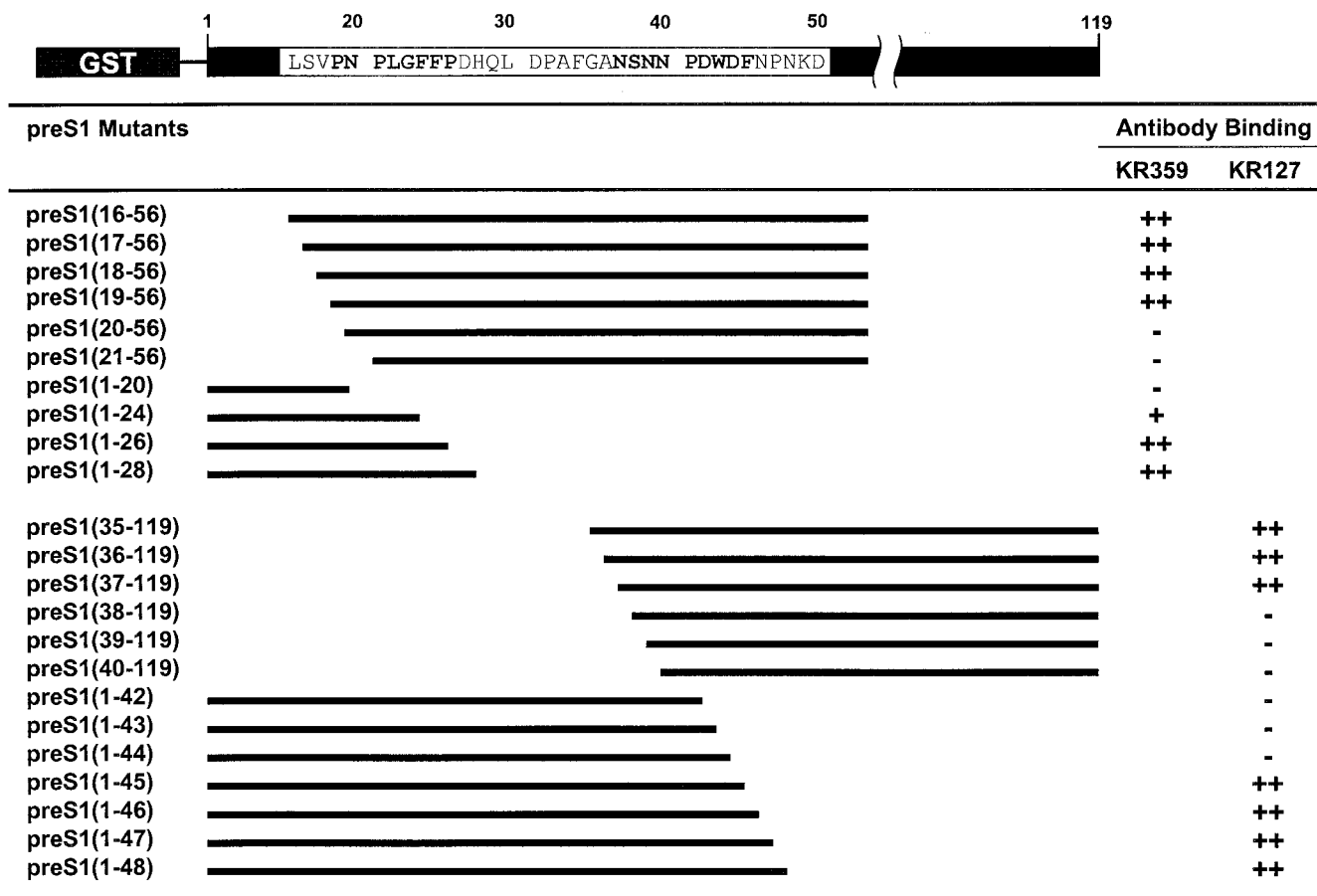
HBV PreS1(1-119)

FIG. 1. Schematic representation of the GST fusion proteins containing the preS1 or the deletion mutants used for epitope mapping. The letters in bold style indicate the epitope of KR359 or KR127 antibody.

In this study, we have mapped the epitopes of the anti-preS1 monoclonal antibodies KR359 and KR127, and showed that each of the antibodies neutralizes both the *adr* and *ayw* subtypes of the virus in the *in vitro* neutralization assay. The neutralizing epitopes (aa 19–26 and 37–45) showed little sequence divergence among HBV subtypes and the antibodies bound to the preS1 of all the HBV variants tested.

RESULTS AND DISCUSSION

Epitope mapping of anti-preS1 monoclonal antibodies KR127 and KR359

Previously, we generated anti-preS1 monoclonal antibodies KR359 (IgG1, κ) and KR127 (IgG2a, κ) by immunizing mice with the preS1 peptide (aa 1–56) conjugated to keyhole limpet hemocyanin, and confirmed that the antibodies bind to native HBV particles, but not to preS2 or S protein (Ryu *et al.,* 2000). In addition, the epitopes of KR359 and KR127 on the preS1 were roughly mapped as aa 21–35 and aa 35–47, respectively. In this study, fine mappings of their epitopes were performed.

To determine the epitope of KR359, a series of amino- or carboxyl-terminal deletion mutants of the preS1 region gene were synthesized by polymerase chain reaction (PCR) and fused to the 3' end of the glutathione S-transferase (GST) gene to construct a series of expression plasmids (Fig. 1). Each plasmid was introduced into *Escherichia coli*, and the protein extracts of the induced recombinant cells were analyzed by 12.5% SDS–polyacrylamide gel electrophoresis (PAGE). The series of GST-preS1 fusion proteins were efficiently expressed in *E. coli* and could be visualized by Coomassie blue staining (Figs. 2A and 2B). Western blot analysis of the amino-terminal deletion mutants using KR359 showed that the preS1(aa 19–56, 19–56) retained the full binding activity to the antibody, while the preS1(20–56) almost lost the binding activity (Fig. 2C), indicating that the amino acid at position 19 is necessary for the antibody binding. In the case of the carboxyl-terminal deletion mutants, the preS1(1–20) did not bind to the antibody and the preS1(1–24) bound weakly, while the preS1(1–26) retained the full binding activity (Fig. 2D). The results indi-
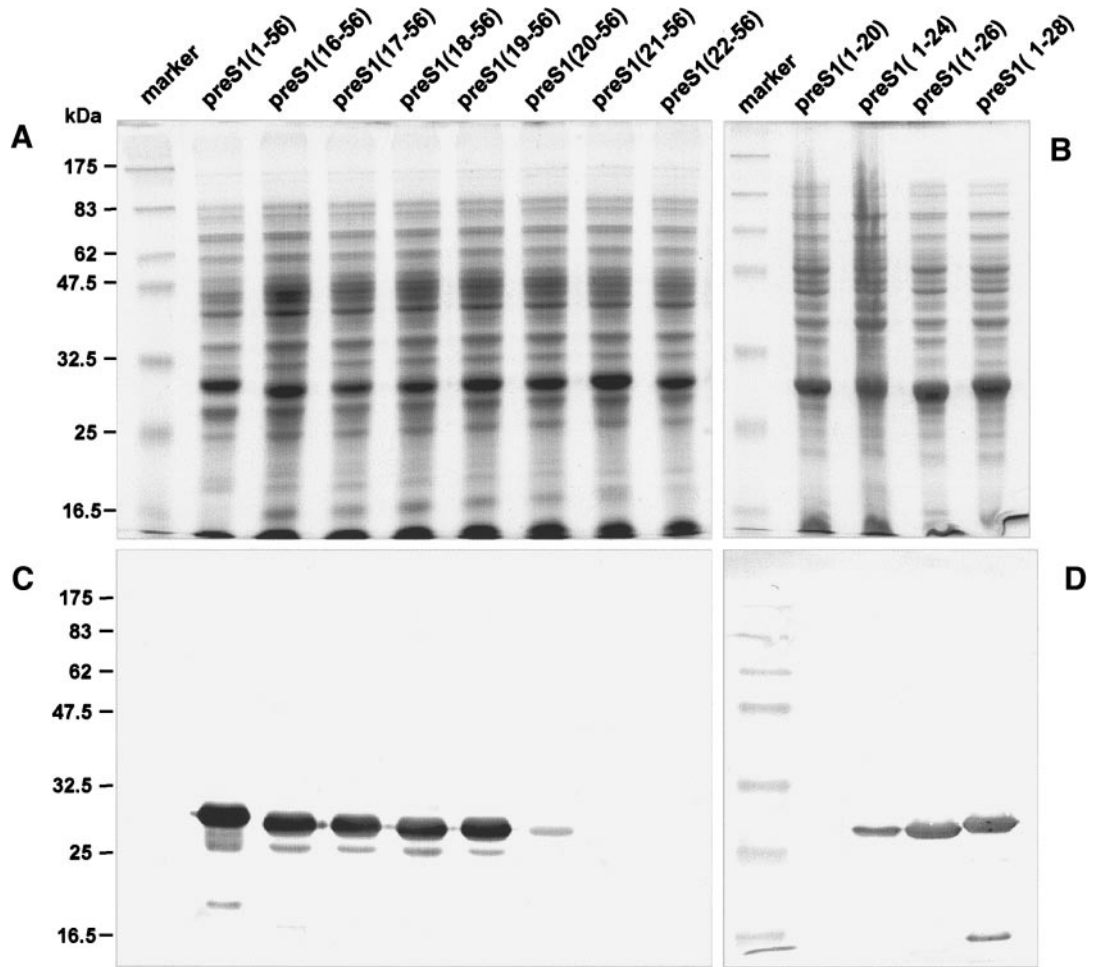
FIG. 2. Mapping of the epitope recognized by anti-preS1 monoclonal antibody KR359. The cell extracts of recombinant *E. coli* harboring GST fusion protein containing a series of deletion mutants as well as molecular weight markers were subjected to 12.5% SDS–PAGE (A, B) and Western analysis using KR359 (C, D). The smaller fragments below the main GST-preS1 fusion proteins are thought to be partially degraded forms of the preS1 fusion proteins.

cate that the epitope of KR359 is located at aa 19–26 of the preS1.

To map the fine epitope of KR127, a series of amino- or carboxyl-terminal deletion mutants of the preS1(1–119) were constructed (Fig. 1) and expressed in *E. coli* as GST fusion proteins (Fig. 3A). Western blot analysis using KR127 showed that the preS1(37–119) and preS1(1–45) retained the binding activity to KR127, whereas the preS1(38–119) and preS1(1–44) almost completely lost the binding activity (Fig. 3B). These data indicate that the epitope of KR127 is located at aa 37–45 of the preS1. The results of the epitope mapping are summarized in Fig. 1.

Virus-neutralizing activities of KR127 and KR359 antibodies

In vitro neutralization assay (Ryu *et al.,* 1997) was employed to assess the virus-neutralizing activities of KR127 and KR359 antibodies. Adult human hepatocyte primary culture was infected with either the *adr* (Ryu *et al.,* 1997) or the *ayw* (Sells *et al.,* 1987) subtype of HBV particles, with or without preincubation with various concentrations of each antibody, and incubated for 17 days while the growth medium was changed every 2 days. On Day 17 postinfection, the HBsAg secretion by the infected cells was measured by radioimmunoassay. As shown in Table 1, the HBsAg secretion rate decreased with the increasing concentration of antibody, and the antibodies almost completely blocked the infection by both *adr* and *ayw* subtypes of HBV at a concentration of 10 μg/ml. The same results were observed from Southern blot analysis of the infected cells on Day 17 using the ^{32}P-labeled HBV DNA as a probe (Fig. 4). No covalently closed circular (CCC) or single-stranded (SS) form of HBV DNA, which are abundant in active viral replication, was detected from the cells infected with the mixture of *adr* (Fig. 4A) or *ayw* (Fig. 4B) subtype of HBV with 10 μg/ml of KR127 or KR359. Unlike KR127 or KR359, *ayw*
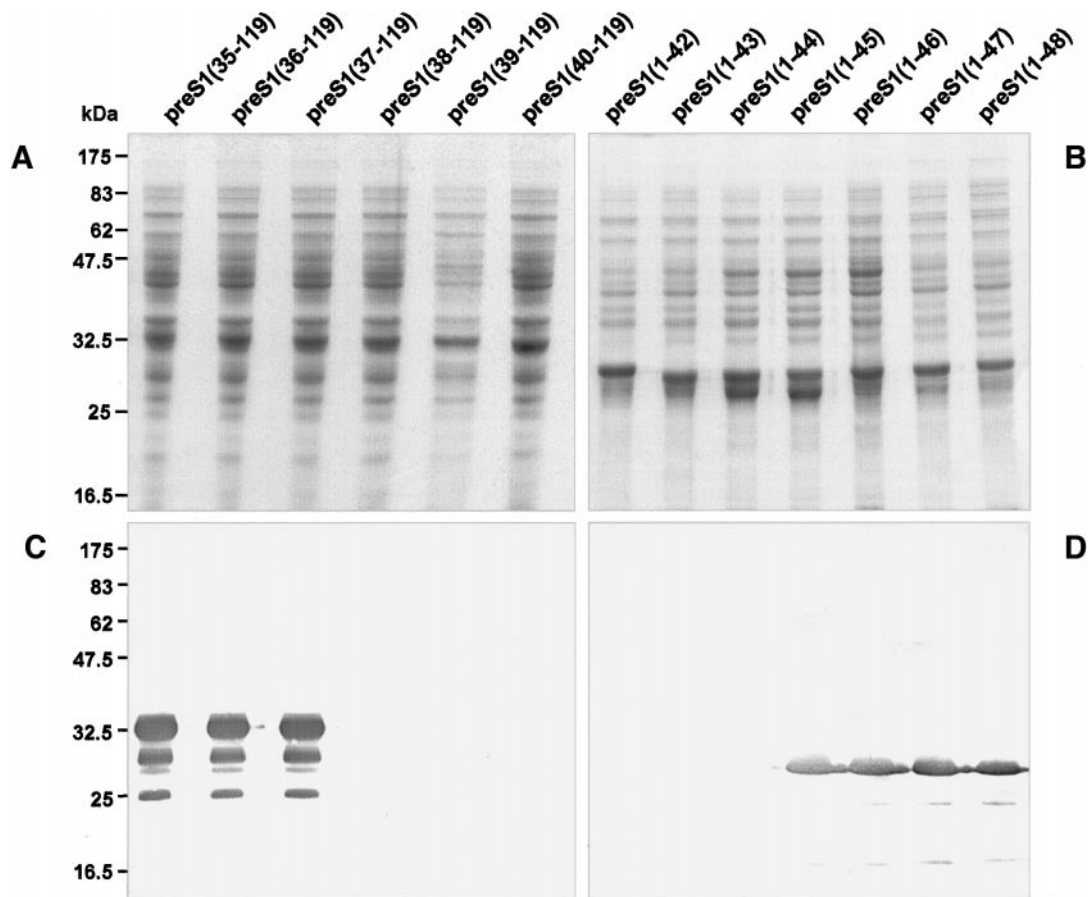
FIG. 3. Mapping of the epitope recognized by anti-preS1 monoclonal antibody KR127. The cell extracts of recombinant *E. coli* harboring GST fusion protein containing a series of deletion mutants as well as molecular weight markers were subjected to 12.5% SDS–PAGE (A, B) and Western analysis using KR127 (C, D). The smaller fragments below the main GST-preS1 fusion proteins are thought to be partially degraded forms of the preS1 fusion proteins.

subtype-specific anti-preS1 monoclonal antibody 5a-19-1 (Budkowska *et al.,* 1995), included as a control, neutralized only the *ayw* subtype of the virus. However, it showed more potent neutralizing activities than either KR127 or KR359. The neutralizing activity of KR127 or KR359 at 10 μg/ml concentration was almost the same as that of 5a-19-1 at 0.1 μg/ml concentration (Table 1, Fig. 4). The lower neutralizing activities of KR127 and KR359 may be ascribed to their lower affinities for the virus particle compared with that of 5a-19-1.

TABLE 1

Radioimmunoassay of *in Vitro* HBV Neutralization

Ab conc[a] (μg/ml)	KR359		KR127		5a-19-1	
	adr P/N[b] (%)[c]	*ayw* P/N (%)	*adr* P/N (%)	*ayw* P/N (%)	*adr* P/N (%)	*ayw* P/N (%)
Control[d]	1.0 (3)	1.0 (8)	1.0 (3)	1.0 (7)	1.0 (2)	1.0 (5)
10	1.2 (4)	2.0 (16)	2.5 (7)	1.3 (10)	34.8 (77)	1.1 (6)
1	5.7 (19)	9.2 (72)	16.4 (46)	6.6 (48)	33.9 (75)	1.1 (6)
0.1	21.6 (71)	9.9 (78)	27.9 (78)	10.6 (78)	43.7 (96)	1.4 (8)
0.01	29.0 (95)	12.6 (99)	34.8 (97)	11.5 (84)	46.5 (102)	10.4 (56)
0	30.5 (100)	12.8 (100)	35.7 (100)	13.7 (100)	45.4 (100)	18.8 (100)

[a] Antibody concentration.

[b] The P/N ratio of each antibody-treated sample was divided by that of the control sample.

[c] Relative P/N ratio.

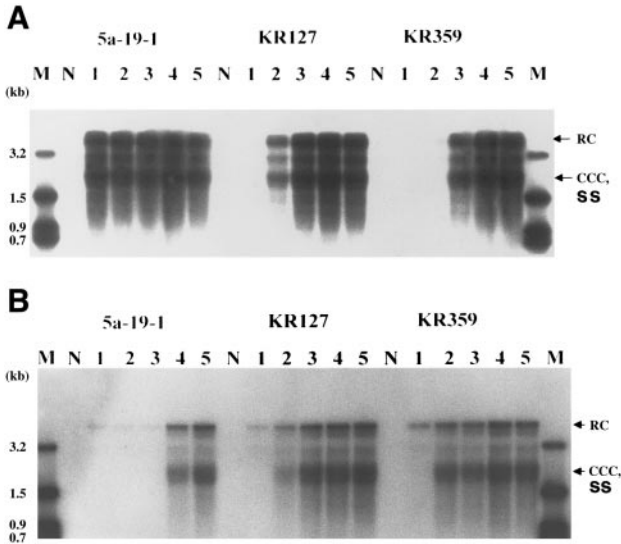[d] Culture supernatant from uninfected hepatocytes.

FIG. 4. Southern blot analysis of *in vitro* HBV-neutralization assay. The intracellular HBV DNA was isolated from uninfected (lane N) or the infected human hepatocytes by *adr* (A) or *ayw* (B) subtype of HBV preincubated with 10 μg/ml (lane 1), 1 μg/ml (lane 2), 0.1 μg/ml (lane 3), 0.01 μg/ml (lane 4), mock (lane 5) of each of the antibodies (5a-19-1, KR359, KR127), and subjected to Southern blot analysis using ^{32}P-labeled 3.2-kb HBV DNA as a hybridization probe. Molecular weight markers are indicated in kilobases on the left. RC and CCC represent relaxed circular and covalently closed circular forms of HBV genome, respectively. SS represents single-stranded HBV DNA. In (B), the RC form of HBV DNA detected in lane 1 seems to be derived from the HBV particles that tightly bound to the hepatocytes but did not infect the cells (Gripon *et al.,* 1993).

Binding abilities of KR127 and KR359 to HBV subtypes and variants

HBV variants carrying mutations on the preS region have been identified from chronic HBV carriers (Gerken *et al.,* 1991; Takayanagi *et al.,* 1993; Tran *et al.,* 1991;

Pollicino *et al.,* 1995) and some immune escape mutants of HBV with various mutations in the S region have been reported (Carman *et al.,* 1990; Yamamoto *et al.,* 1994; Kohno *et al.,* 1996). Therefore, sequence variations of the two neutralizing epitopes among different subtypes and variants of HBV were investigated. The amino acid sequences of aa 17–53 of all the preS1 listed in GenBank databases were aligned and compared with those of this study (Fig. 5). The sequence comparison revealed that two amino acid substitutions at position 19 (from proline to serine) and position 25 (from phenylalanine to leucine, named F25L) occurred in the epitope (aa 19–26) of KR359, while two amino acid substitutions at position 38 (from serine to threonine) and position 39 (from asparagine to histidine, threonine, or alanine) occurred in the epitope (aa 37–45) of KR127. No correlation, however, was found between the type of amino acid changes and HBV subtypes. In the case of changes outside of the epitopes, glycine to arginine substitution at position 35 was frequently found in the *ayw* subtype. The most divergent sequence was identified to carry six substitutions at positions 18, 19, 35, 38, 39, and 51 (named AYW). The amino acid substitutions at position 48 from asparagine to isoleucine (named N48I) or valine was also identified.

To test the binding abilities of KR359 and KR127 to the preS1 variants, the sequences of preS1(1–56) carrying F25L (S81946), N48I (M74498), or AYW (A32622), as presented in Fig. 5, were synthesized by recombinant PCR and expressed in *E. coli* as GST fusion proteins (Fig. 6A). Western blot analysis of the cell lysates showed that KR127 (Fig. 6B) and KR359 (Fig. 6C) bound to all the preS1 variants tested, indicating that the amino acid substitutions in the epitope of KR359 or KR127 did not affect the reactivity of the preS1 with the antibody. The result suggests that the antibodies could bind to all the

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ACC No   Subtype        Amino Acid Sequence
                         _____          _____
Query    (adr)     17SVPNPLGFFPDHQLDPAFGANSNNPDWDFNPNKDHWP53
D23680   (adr)     17-------------------------------------53  (9)
X14193   (adr)     17-----------------------------------R--53  (1)
A32621   (adw)     17----------------------------------Q--53  (5)
M74498   (adw/ayw) 17-----------------------------I-----53  (9)
A32626             17---------------V-------------I-----53  (3)
I01492             17---------------------H-------------53  (3)
S81946   (adr)     17--------L--------------------------53  (1)
P03142   (ayw/ad)   6--------L-------------T--------I-----42  (3)
A32624   (ayw)     17---------------------------V--D--53  (5)
A34129             17---------------V-------------V--D--53  (1)
U55223   (ayw)      6-TS--------------R--------------T--42  (1)
P03139   (ayw/ad)   6-TS--------------R--T-----------T--42  (2)
A32622   (ayw/ad)   6-TS--------------R--TA----------T--42  (5)
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FIG. 5. Sequence alignment of aa 17–53 of the various subtypes and variants of HBV preS1. Query is the preS1 sequence of *adr* subtype (Kim and Hong, 1995). A hyphen indicates an amino acid residue identical to that of query sequence. The epitopes of KR359 and KR127 are indicated by the lines on the top of the sequences. The sequences whose subtypes are identified in the GenBank databases are noted. Among the sequences whose subtypes are not indicated, those starting with number 20 may be *ad* subtype, while those with number 9 may be *ay* subtype because the first 11 amino acid of *ad* subtype are missing in *ay* subtype. The number of identical sequences among total 48 preS1 sequences listed in the GenBank databases are written in parentheses after amino acid sequences.
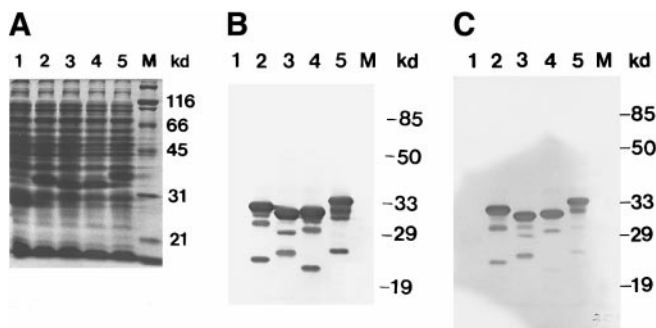
FIG. 6. Binding abilities of KR127 and KR359 to preS1 variants. Cell lysates containing GST (lane 1), the GST fusion proteins with preS1(1–56) of the query sequence (lane 2), N48I (lane 3), AYW (lane 4), F25L (lane 5), or molecular weight standards (lane M) were subjected to 12.5% SDS–PAGE (A) and Western blot analysis using KR127 (B) or KR359 (C).

subtypes of HBV. This is in contrast with *ad* or *ayw* subtype-specific monoclonal antibodies (Petit *et al.,* 1989; Budkowska *et al.,* 1995).

Since the epitope of KR127 shows the sequence variation at positions 38 and 39, we examined the importance of the residues in antibody binding. The preS1(1–56) mutants carrying alanine substitution at Ser[38] or Asn[39] were produced and performed Western blot analysis. The result showed that the alanine substitutions did not affect the reactivity of the preS1 with KR127 (data not shown), indicating that the two residues are not critical in antibody binding. This agrees with the result that KR127 reacted with the preS1 of the *ayw* subtype as well as the *adr* subtype (Fig. 6). It is likely that the residues 38 and 39 may not interact directly with the antibody-combining site of KR127. The structural study on the interaction of the antibody-combining sites with the epitope remains to be done for precise explanation.

Several anti-preS1 murine monoclonal antibodies that recognize epitopes on the aa 20–50 region are known (Petit *et al.,* 1989; Kuroki *et al.,* 1990; Budkowska *et al.,* 1995; Kuttner *et al.,* 1999). Of these, F35.25 is *ad* subtype-specific antibody and recognizes the amino-terminal part of aa 32–53 (Petit *et al.,* 1989), while 5a-19-1 is *ayw* subtype-specific and recognizes aa 36–43 (corresponding to *ad* subtype) (Budkowska *et al.,* 1995). On the other hand, 116-9 antibody binds to both *ad* and *ay* subtypes and recognizes aa 27–35 (Mimms *et al.,* 1990; Kuroki *et al.,* 1990), and MA 18/7 antibody recognizes aa 30–35 of preS1 (Kuttner *et al.,* 1999). The numbering systems in describing the epitopes are used for the *ad* subtype. None of these antibodies was demonstrated for the virus-neutralizing activity. Comparison of the epitopes of the antibodies shows that the epitopes (aa 19–26, 37–45) of our monoclonal antibodies are different from those of the other antibodies mentioned earlier, suggesting that KR127 and KR359 are new anti-preS1 monoclonal antibodies.

In conclusion, we identified the fine epitopes of two

anti-preS1 monoclonal antibodies, KR359 and KR127, to be aa 19–26 and aa 37–45, which are different from those of previously known monoclonal antibodies, and confirmed that the antibodies neutralize both *adr* and *ayw* subtypes of the virus. The antibodies bound to the preS1 of all the HBV variants tested, suggesting that they may bind to and neutralize all the subtypes of HBV. The information on the neutralizing epitopes will be useful in vaccine development. In addition, these neutralizing monoclonal antibodies will be valuable in investigating the mechanism of virus neutralization and the development of therapeutics for the prevention and therapy of HBV infection and hepatitis.

MATERIALS AND METHODS

Expression of GST-preS1 fusion proteins

Serially truncated preS1 or preS1(aa 1–56) of *adr* or *ayw* subtype were individually expressed as a fusion protein with GST. The coding sequences of preS1 of *adr* subtype were synthesized by PCR from pGSTpreS1–56 or pGSTpreS1–119 (*adr* subtype) (Kim and Hong, 1995) using 5′- and 3′-primers and subcloned into the *Bam*HI-*Eco*RI sites of pGEX-2T (Pharmacia) to yield expression plasmids. The preS1(aa 1–45) of *ayw* subtype was synthesized by PCR from pAYW (kindly provided by Dr. Y. D. Yun at Mogam Institute of Biotechnology, Korea) and subcloned into pGEX-2T to yield expression plasmid. The 5′- or 3′-primer contains a *Bam*HI or *Eco*RI sequence, respectively. To construct the preS1 variant, the 5′- and 3′-DNA fragments containing the mutagenic sequence were synthesized by PCR from the pGSTpreS1–56 and recombined by subsequent recombinant PCR (Lewis and Crowe, 1991). To express the GST-preS1 fusion proteins, the expression plasmid was introduced into *E. coli* DH5α cells and the expression of the fusion protein was induced by 0.4 mM isoprophyl-β-D-thiogalactopyranoside at 37°C for 4 h. The induced cells were harvested and the cell lysates were subjected to 12.5% SDS–PAGE and Western analysis.

Western analysis

After electrophoresis, protein bands were transferred to nitrocellulose membrane. Each blot was probed with anti-preS1 monoclonal antibody, developed with goat anti-mouse IgG alkaline phosphotase conjugate, and stained with nitroblue tetrazolium (NBT, GibcoBRL) and 5-bromo-2-chloro-3-indolyl phosphate (BCIP, GibcoBRL) as previously reported (Kim and Hong, 1995).

In vitro neutralization assay

Adult human hepatocytes were prepared by enzymatic dissociation of noncancerous liver fragments and cultured as described previously (Gripon *et al.,* 1993). All experimental procedures were in compliance with

French laws and regulations, and were approved by the National Ethics Committee.

In vitro HBV infection and neutralization assays were carried out as described previously (Gripon *et al.,* 1993; Ryu *et al.,* 1997). Briefly, human hepatocytes were seeded at a density of 10^6 cells per well containing 2 ml of normal growth medium and were infected 3 days later with the *adr* (Ryu *et al.,* 1997) or *ayw* (Sells *et al.,* 1987) subtype of HBV particles (about 3×10^7 viral genomic equivalent). For a neutralization assay, the virus particles were preincubated with various concentrations of antibody at room temperature for 1 h and inoculated onto the cultured hepatocytes. The hepatocytes were covered with 1 ml of the serum-free culture medium containing 4% PEG. The infected cells were washed with the growth medium and further incubated for 17 days, with the medium renewed every 2 days. On Day 17 postinfection, the culture medium was diluted by 10-fold and the HBsAg concentration of the diluted culture medium was assayed using a radioimmunoassay kit (Abbott Laboratories, Chicago, IL).

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