Pro-205 of large hepatitis delta antigen and Pro-62 of major hepatitis B surface antigen influence the assembly of different genotypes of hepatitis D virus

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Hepatitis B surface antigen (HBsAg) is essential for the assembly and infection of hepatitis D virus (HDV). The assembly efficiency of genotype 1 HDV is higher than that of genotype 2, whilst the P62L substitution of major HBsAg further compromises the assembly of genotype 2 and 4 HDV. This study investigated the influence of proline residues in the carboxyl end of the large hepatitis delta antigen (HDAg-L) on the assembly of HDV of different genotypes. Expression vectors containing the HDAg-L gene or full-length HDV genome of genotype 1, 2 or 4 were co-transfected with plasmids expressing HBsAg proteins that bore either proline or leucine residues at position 62. Of the eight HDV genotypes, only genotype 1 has Pro-205 in HDAg-L, whereas genotypes 2 and 4 have Arg-205. The Arg-205 to Pro-205 substitution in HDV-2 and -4 markedly increased the assembly efficiencies of HDAg-L and whole HDV genomes, even in the presence of HBsAg with Leu-62. In contrast, secretion of genotype 1 HDV or HDAg-L was reduced significantly when arginine or alanine replaced Pro-205. When HBsAg contained Pro-62, the influence of Pro-205 on assembly decreased. In conclusion, both Pro-205 of the HDAg-L and Pro-62 of the major HBsAg play critical roles in the assembly of HDV of different genotypes. The presence of Pro-205 in genotype 1 HDV may account for its higher assembly efficiencies and wider distribution.

INTRODUCTION

Hepatitis B surface antigen (HBsAg), provided by helper hepatitis B virus (HBV), is essential for the envelopment, secretion and infectivity of defective hepatitis delta virus (HDV). Inside the HBsAg-enveloped HDV virion, there is a ribonucleoprotein (RNP) complex composed of a 1.7 kb single-stranded circular HDV RNA genome, and the hepatitis delta antigen (HDAg) of two molecular forms (Wang et al., 1986; Modahl & Lai, 2000; Taylor, 2003). The small HDAg (HDAg-S) is essential for trans-activating HDV RNA replication, whilst the large HDAg (HDAg-L) is needed for virus assembly (Kuo et al., 1989; Chao et al., 1990; Modahl & Lai, 2000; Taylor, 2003). HDV infection is an important factor in the aetiology of fulminant hepatitis (Govindarajan et al., 1984; Hadler et al., 1984; Wu et al., 1994). It can also worsen underlying chronic hepatitis B and lead to cirrhosis, liver failure or hepatocellular carcinoma (HCC). Alternatively, it may run a subclinical, slowly progressive course (Rizzetto et al., 1983; Govindarajan et al., 1986; Wu et al., 1995a, b).

HDV is classified into at least eight major clades or genotypes (HDV-1 to -8) according to phylogenetic analysis (Imazeki et al., 1990; Casey et al., 1993; Wu et al., 1998; Sakugawa et al., 1999; Ivanishina et al., 2001; Le Gal et al., 2006). The global distribution of HDV genotypes is shown in Fig. 1 (Casey et al., 1993; Pujol & Devesa, 2005). HDV-1 is distributed worldwide, whereas HDV-2 and HDV-4 are restricted to areas in the Far East, including Taiwan, Japan and Yakutia (Russia). HDV-3 is localized in South and Central America and HDV-4 is found only in Taiwan and Okinawa, whilst HDV-5 to -8 are found in Africa (Imazeki et al., 1990; Casey et al., 1993; Wu et al.,
The reason for the disparate global distribution of HDV genotypes remains unclear. Historical and geographical reasons may only partly account for the variations. Infections caused by different HDV genotypes vary in clinical course and prognosis (Casey et al., 1993; Wu et al., 1995b). Both HDV-1 and HDV-2 are prevalent in Taiwan. HDV-2 infection is associated less frequently with fulminant hepatitis in the acute stage and unfavourable long-term outcomes in the chronic stage (Wu et al., 1995b). The efficiency of HDV-1 assembly is generally higher than that of HDV-2 (Hsu et al., 2002).

The HDV packaging signal (PAS) at the C-terminal 19 aa of HDAg-L is markedly divergent (73.7 %) between HDV-1 and HDV-2, whilst genotype 4 is has higher similarity in this domain to HDV-2 (78.9 %) (Wu et al., 1998). HDV-2 assembly is reduced by substitution of proline residues in the nuclear-export signal (NES) at the C-terminal region of HDAg-L, indicating that prolines are critical for HDV assembly (Lee et al., 2001; O’Malley & Lazinski, 2005). Alanine, instead of naturally occurring amino acid variants, has been used to replace proline in two studies (Lee et al., 2001; O’Malley & Lazinski, 2005).

Based on inter-group divergence of 8 % or more in the complete nucleotide sequence, HBV can also be classified into eight different genotypes (A–H). Genotypes B and C are most prevalent in Asia (Kao et al., 2000; Chu & Lok, 2002). In Taiwan, genotype B is most prevalent, followed by genotype C (Kao et al., 2000). Based on phylogenetic analysis, HDV was recently classified into at least eight major clades (types 1–8) (Le Gal et al., 2006). Genotype 1 HDV has a worldwide distribution, whereas the remaining seven genotypes are restricted to certain areas. Genotype 2 is predominant in Taiwan, followed by genotypes 1 and 4 (Wu et al., 1995b, 1998).

Fig. 1. Worldwide genotype distributions of HBV and HDV. Based on 8 % or more divergence in the whole genomic nucleotide sequence, HBV was classified into eight genotypes (A–H). Genotypes B and C are most prevalent in Asia (Kao et al., 2000; Chu & Lok, 2002). In Taiwan, genotype B is most prevalent, followed by genotype C (Kao et al., 2000). Based on phylogenetic analysis, HDV was recently classified into at least eight major clades (types 1–8) (Le Gal et al., 2006). Genotype 1 HDV has a worldwide distribution, whereas the remaining seven genotypes are restricted to certain areas. Genotype 2 is predominant in Taiwan, followed by genotypes 1 and 4 (Wu et al., 1995b, 1998).

Effects of substitutions at position 205 of HDAg-L on its packaging with HBsAg

The predicted amino acid sequences of the 19 aa extension at the C-terminal domain of HDAg-L for the eight HDV genotypes are shown in Table 1. The predicted amino acid sequences of the 19 aa extension at the C-terminal domain of HDAg-L for the eight HDV genotypes are shown in Table 1. The predicted amino acid sequences of the 19 aa extension at the C-terminal domain of HDAg-L for the eight HDV genotypes are shown in Table 1. The predicted amino acid sequences of the 19 aa extension at the C-terminal domain of HDAg-L for the eight HDV genotypes are shown in Table 1.
genotypes are shown in Fig. 2. A simple virus-like particle (VLP) model was used to compare the assembly and secretion efficiencies of VLPs in the presence of HDAG-L and HBsAg with naturally occurring or engineered amino acid substitutions.

Interestingly, only HDAG-L of HDV-1 showed proline at aa 205. Proline was substituted to arginine (R) or glycine (G) in the remaining genotypes (Fig. 2a). To further evaluate the role of Pro-205 of HDAG-L in assembly and secretion of HDV-2 and HDV-4, especially when interacting with major HBsAg with leucine at aa 62, the original amino acid was replaced with alanine (A), glutamine (Q), arginine (R) or proline (P) of the naturally occurring variants at positions 204 and 205 in the C-terminal domain of HDAG-L of HDV-1, -2 and -4 (Fig. 2b).

HDAG-L mutants were produced at levels similar to that of the wild type (Fig. 3a, c). The assembly and secretion efficiencies of HDV-2 and HDV-4 HDAG-L with an R205A substitution in the presence of HBsAg containing Leu-62 were only modestly decreased or nearly unchanged compared with the already low secretion levels of HDAG-L produced by the wild-type parental plasmids (Fig. 3b). In contrast, substitution of Pro-205 with alanine or arginine in HDV-1 markedly decreased the assembly and secretion efficiencies of VLPs containing HDAG-L to 2–9 % of those of wild-type parental plasmids. The assembly and secretion efficiencies of HDV-2 or HDV-4 HDAG-L with the R205P substitution were enhanced markedly (by 214–321 %) compared with those of wild-type or mutant R205A-containing HDAG-L.

The effects of Pro-62-containing HBsAg on HDV-1, -2 and -4 assembly and secretion are shown in Fig. 3 (c, d). Pro-62-containing HBsAg did not modulate HDAG-L expression levels, regardless of HDV genotype, mutant or wild type (Fig. 3c). Levels of secreted R205A-substituted HDAG-L were decreased only slightly compared with those expressed by HDV-1, HDV-2 and HDV-4 wild-type parental plasmids (Fig. 3d). These results indicated that the P205N mutation of genotype 1 HDAG-L did not change the assembly and secretion efficiencies of VLPs significantly (Fig. 3d).

In contrast, levels of secreted R205P-substituted HDAG-L expressed by HDV-2 or HDV-4 plasmids were increased slightly compared with those of R205A-substituted HDAG-L, but comparable to those of the parental wild type (Fig. 3d). Notably, alanine substitution at position 204 of HDAG-L did not affect the assembly and secretion efficiencies of VLPs, regardless of Leu-62- or Pro-62-containing HBsAg.

Effects of natural variations in the C-terminal domain of HDAG-L on its packaging with HBsAg

To investigate whether proline residues or amino acid variations at sites other than residue 205 of the HDAG-L C terminus also affected the assembly of HDV-1, -2, and -4, Huh-7 cells were co-transfected with pHBVenv6 plasmids.

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Fig. 2. Amino acid sequences of the C-terminal domains spanning residues 196–214 of HDAG-L. (a) The eight genotypes of HDV. Dots indicate amino acids identical to those of the genotype 1 HDV HDAG-L (25-L). (b) Wild-type and mutant HDAG-L expression plasmids used in this study. Dots indicate amino acids identical to those of the reference wild-type genotypes 1, 2 and 4 HDV HDAG-L, respectively (25-L, 24-L and 1025-L). WT, Wild type.
expressing HBsAg with Leu-62 or Pro-62, and HDAg-L plasmids with single amino acid substitutions to variants of genotypes 1, 2, and 4 (Fig. 4). Huh-7 cells co-transfected with pHBVenv6 (Leu-62 or Pro-62) and a wild-type or residue 205-mutated HDAg-L expression plasmid produced similar HDAg-L and Hsc70 protein levels in cell lysates (Fig. 4a, c). Consistent with results described in the previous section, marked differences in HDAg-L levels were detected in the culture media from the wild-type or residue 205-mutated HDAg-L expression plasmids co-transfected with pHBVenv6 expressing HBsAg with Leu-62 (Fig. 4b). In the presence of HBsAg with Pro-62, VLP assembly and secretion efficiencies were similar, irrespective of the context of wild-type or residue 205-mutated HDAg-L (Fig. 4d). Whilst D or P substitutions at aa 197, 204 and 208, based on naturally occurring variants of different genotypes, did not alter the assembly and secretion efficiencies of VLPs significantly, the results support a critical role for Pro-205, but not prolines at other residues, of HDAg-L in VLP assembly and secretion.

**Effects of natural amino acid substitutions at position 205 of HDAg-L on HDV assembly**

In order to confirm that different HDV assembly efficiencies were not due to varying HDV replication efficiencies between wild type and mutants, similar experiments were repeated using expression plasmids of the entire HDV-1 genome with or without P205R substitution. HDV-4 genomes with or without R205P substitution were co-transfected with two kinds of pHBVenv6 plasmid expressing HBsAg with Leu-62 or Pro-62 into Huh-7 cells.

**Fig. 3.** Effects of substitution with alanine or naturally occurring variant amino acids at position 205 of HDAg-L on the assembly of VLPs. (a, c) Huh-7 cells grown in 10 cm diameter dishes (6 × 10⁶ cells per dish) were co-transfected with 5 μg expression plasmids of HDAg-L (genotype 1, 25-L; genotype 2, 24-L; genotype 4, 1025-L) and 5 μg of two kinds of pHBVenv6 plasmid expressing HBsAg with Leu-62 or Pro-62 per dish. Both cells and culture media were collected on day 6 post-transfection. Cellular lysates were analysed for intracellular HDAg-L expression and equal loading of protein sample by Western blotting using human anti-HDV antiserum and a monoclonal antibody specific for the heat-shock protein Hsc70, respectively. (b, d) Analysis of HDAg-L in secreted VLPs in culture medium. VLPs in 9 ml culture medium were pelleted by high-speed centrifugation (40,000 r.p.m. for 5 h at 4 °C in a Beckman SW41 rotor) through a 20% sucrose cushion. The pellets were dissolved in SDS sample buffer and analysed for extracellular HDAg-L by Western blotting. Values under each lane are relative assembly efficiencies of HDAg-L of residue-substituted mutants compared with those of wild type.
The same amounts of HDV RNA and two forms of HDAs were detected in lysates of Huh-7 cells transfected by genotype 1 and 4 HDV expression plasmids (Fig. 5a). Equal amounts of loaded samples were demonstrated by similar amounts of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA and Hsc70 protein in each lane. However, HDV assembly and secretion decreased markedly when the HDV-1 plasmid with P205R substitution was co-transfected with pHBVenv6 plasmids expressing HBsAg with Leu-62. In contrast, HDV assembly and secretion increased markedly when the HDV-4 plasmid with R205P substitution was co-transfected with the same pHBVenv6 plasmids (Fig. 5b). The assembly and secretion of genotype 1 (with or without P205 mutation) and 4 (with or without R205 mutation) HDV were not affected significantly by co-transfection with pHBVenv6 plasmids expressing HBsAg with Pro-62 (Fig. 5b). Results for the assembly of virus particles containing whole HDV genomes were consistent with those obtained from the VLP model mentioned above.

**DISCUSSION**

The number and position of proline residues in HDAg-L PAS vary according to genotype (Fig. 2a). This study indicates that proline 205, located in the PAS, is a more important determinant of HDAg-L assembly and secretion than the proline content of an HDV genotype. For example, genotypes 2 and 4 PAS also contain five proline residues, but they have arginine rather than proline at position 205 of genotype 1. Both HDV-2 and HDV-4 exhibited lower assembly and secretion efficiencies than HDV-1. Pro-204 is less influential than Pro-205 for the packaging and secretion of genotypes 2 and 4 HDAg-L in this study. Interestingly, only HDV-1 of the eight genotypes has a proline at residue 205 in the PAS region of HDAg-L (Fig. 2a). The results here
suggest that this unique characteristic of HDV-1 may facilitate its assembly and secretion, and contribute to its worldwide distribution. Nonetheless, further studies on the assembly and secretion efficiencies of HDV-5, -6, -7 and -8 are needed to strengthen this hypothesis.

In previous reports, the reduction of HDV assembly in HDAg-L mutants with P205A was assumed to be related to impaired nuclear export of HDAg-associated RNP (Lee et al., 2001). However, a later report by O’Malley & Lazinski (2005) indicated that assembly defects persisted under conditions where the mutants had access to the site of VLP assembly, cytosol and the endoplasmic reticulum. It is assumed that, aside from a proposed role in nuclear export, proline at residues 201 and 205 may play a more direct role in their interaction with HBsAg (O’Malley & Lazinski, 2005).

In this study, the assembly of genotype 2 and 4 HDAg-L with arginine at residue 205 appears unimpaired, particularly in the presence of HBsAg with Pro-62. The assembly of HDV requires interaction between HDAg-L and HBsAg, and Hwang & Lai (1993) demonstrated that HBsAg interacts specifically with HDAg-L. This interaction requires the isoprenylation of HDAg-L. However, prenylated small HDAg without the C-terminal extension of HDAg-L is insufficient to mediate the interaction between HDAg and HBsAg (Hwang & Lai, 1993). O’Malley & Lazinski (2005) also demonstrated no difference in the extent of farnesylation between HDV-1 and HDV-2. Therefore, the difference in assembly efficiency between genotypes 1 and 2 of HDV may be due to differences in the interactions between HDAg-L of different genotypes and HBsAg.

As arginine or glycine, instead of proline, is present at position 205 in non-genotype 1 HDAg-L, the changes of the charges of the amino acids at this position may affect interactions between HDAg-L and HBsAg and subsequent HDV assembly. The hydrophobic character of prenylated HDAg-L facilitates its targeting to the endoplasmic reticulum. Higher assembly and secretion efficiencies of HDV may reflect stronger interaction between a more hydrophobic HBsAg containing Pro-62 (or Leu-62) and a more hydrophobic HDAg-L containing Pro-205. The efficiencies of HDV assembly and secretion may be lower in interactions between HBsAg with hydrophobic Leu-62, and genotype 2 and 4 HDAg-L with basic Arg-205 or genotype 3 HDAg-L with hydrophilic Gly-205 (O’Malley & Lazinski, 2005).

In addition to the important role of Pro-205 of HDAg-L in the assembly of HDV, the findings here indicate that the assembly and secretion of HDAg-L require the collaboration of Pro-62 of the major HBsAg. Leu-62 of HBsAg is a minor HBV variant of genotypes B and C, based on 1532 HBsAg sequence samples retrieved from GenBank (data not shown). Leu-62 is found in the HBsAg sequences of 1.5% (three of 212) and 5% (12 of 239) of genotype B and C HBV isolates, respectively. Conversely, the remaining 1081 HBV isolates, comprising six HBV genotypes (265 genotype A, 473 genotype D, 196 genotype E, 93 genotype F, 22 genotype G and 32 genotype H HBV sequences) exhibit Pro-62 in the CYL-I region. As the presence of HBsAg with Leu-62 will further compromise the assembly and secretion of genotype 2 and 4 HDV, this may contribute to the more restricted distribution of HDV-2 and HDV-4.

Fig. 5. Effects of substitution with naturally occurring variant amino acids at position 205 of HDAg-L on HDV virion assembly. Equal amounts of two different genotypes of HDV and two kinds of pHVenv6 plasmid expressing HBsAg with Leu-62 or Pro-62 were co-transfected into Huh-7 cells. Both cells and culture media were collected on day 9 post-transfection. (a) Cellular lysates were analysed for intracellular HDV RNA and HDAg expression by Northern blotting using digoxigenin (DIG)-labelled cDNA probes and by Western blotting using human anti-HDV, respectively. Equal loading of RNA and protein samples was assessed by hybridization with a DIG-labelled RNA probe for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and a monoclonal antibody specific for heat-shock protein (Hsc70), respectively. (b) Analysis of HDV RNA, HDAgs and HBsAgs in culture media. Analyses of intracellular and extracellular HDV RNA, HDAgs and HBsAgs were performed as described in the legend to Fig. 3.
Jenna & Sureau (1998) analysed the effects of deletion of various amino acid segments within cytosolic loop I (between residues 28 and 80) of the small HBsAg, exposed on the cytosolic side of the endoplasmic reticulum, on the interaction with HDV RNP and subsequent virion assembly. They found that an insertion of Gly-Ala to the deleted segment of aa 61–64 resulted in poor expression of HBsAg and deficient secretion of HBsAg particles and HDV virions (Jenna & Sureau, 1998). Another study (Shih et al., 2008) revealed that P62L in cytosolic loop I impaired the assembly efficiency of genotype 2 and 4 HDV in the absence of obvious effects on HBsAg expression and secretion. The critical roles of Pro-205 of the HDAG-L and Pro-62 of the major HBsAg in the assembly of HDV of different genotypes may be due to the stronger HDAG-L and HBsAg interactions, as explained above. Taken together, the current study provides a mechanistic explanation for the higher assembly efficiency of HDV genotype 1 than genotype 2 (Hsu et al., 2002).

In a previous study (Wu et al., 1995b), genotype 1 HDV infections were associated more frequently with unfavourable outcomes than genotype 2. The higher assembly efficiency of genotype 1 HDV than that of genotype 2 may result in the spread of HDV into more hepatocytes and lead to more severe hepatitis, due to host immune attack (Hsu et al., 2002). By swapping the C-terminal 19 aa of HDAG-L between genotype 1 and 2 HDV, it is demonstrated that this region determines the HDV assembly efficiency (Hsu et al., 2002). This study also discloses that Pro-205 is critical in determining the higher assembly efficiency of genotype 1 HDV.

Transforming growth factor beta (TGF-β) plays an important role in liver fibrosis and cirrhosis (Castilla et al., 1991). Choi et al. (2007) reported that HDAG-L may induce liver fibrosis through TGF-β-induced signal transduction. Further studies are warranted to clarify whether Pro-205 of HDAG-L will also influence higher expression of TGF-β and more severe liver fibrosis compared with HDAG-L of other genotypes. However, other confounding factors, such as the genotype of HBV, mutations or variants of HBsAg, viral loads of HBV and/or HDV or co-existing hepatitis C virus or human immunodeficiency virus infection, may also influence disease outcomes (Smedile et al., 1991; Sù et al., 2006; Shih et al., 2008).

In summary, Pro-205 of the PAS of HDAG-L and Pro-62 of HBsAg play significant roles in the assembly and secretion efficiencies of HDV of different genotypes. These may contribute to disease manifestations and global HDV genotype distribution.

**METHODS**

**Construction of HDAG-L expression plasmids of different genotypes for single-residue substitution mutants.** The original three plasmids expressing HDAG-L of different HDV genotypes were cloned into pCMV-EBNA (Clontech) and constructed as described previously (Hsu et al., 2002). TW2577 (25-L), TW2476 (24-L) and TW1025 (1025L) belonged to HDV-1, -2 and -4 isolates, respectively. HDAG-L expression plasmids with single-residue substitutions at aa 197, 204, 205 and 208, which differed among genotypes, were constructed by using the PCR overlap-extension method. The aspartate at residue 197 and prolines at residues 204, 205 and 208 of HDAG-L of genotype 1 HDV were substituted with valine, arginine and leucine, which occur naturally in HDV-2 and HDV-4 isolates.

**Construction of plasmids expressing wild-type or mutated HBsAg.** Plasmids expressing the entire large, middle and major HBsAg of pHBVenv6 were constructed and described previously (Shih et al., 2008). The cytomegalovirus promoter in these plasmids controlled HBsAg expression. The expression vector for the L62P-mutated HBsAg was constructed by using the PCR overlap-extension method. Upon transfection of these plasmids into Huh-7 cells, genomic-sense HDV RNA was produced.

**Culture and transfection of Huh-7 cells.** The well-differentiated HCC cell line Huh-7 (Nakabayashi et al., 1982) was used in this study. Huh-7 cells (10⁶ cells) were transfected with FuGENE HD transfection reagent (Roche), according to the supplier’s instructions. Briefly, 5 μg HDV- and 5 μg pHBVenv6-expressing plasmid DNA were co-transfected into Huh-7 cells with 15 μl FuGENE HD transfection reagent. The medium was changed 6–8 h post-transfection. Subsequently, the medium was replaced and collected on days 3, 6 and 9 as described previously (Wu et al., 1991; Shih et al., 2008).

**Western blot analysis.** Extraction of intracellular and extracellular HDAGs and Western blot analysis were performed as described previously (Shih et al., 2008) using day 3 cell lysates and day 6 supernatants. The isolated proteins were separated by SDS-PAGE (12% gel), blotted onto nitrocellulose membranes and stained for HDAGs with anti-HDV-positive human serum (1:5000) (Shih et al., 2008) or for the reference protein, heat-shock cognate protein (Hsc70), with monoclonal antibody HSC70 (B-6) (1:5000; Santa Cruz Biotechnology). The relative efficiency of assembly was estimated by comparing HDAG-L levels in the supernatant, secreted by residue-substituted mutants, with those of the wild type by using an Alphalmager 2000 Documentation and Analysis System and Alphaljmage 2000 software package (Alpha Innotech).

**Analysis of HDV RNA.** Total cellular RNAs from harvested Huh-7 cells or virus particles from concentrated supernatant after high-speed centrifugation (40 000 r.p.m. for 5 h at 4 °C in a Beckman SW41 rotor) were extracted by TRIzol reagent (Life Technologies). RNA purification was performed according to the manufacturer’s instructions. In total, 20 μg RNA was analysed by Northern blotting as described previously (Shih et al., 2008). After fixation by UV illumination, RNA was hybridized with digoxigenin (DIG)-labelled cDNA probes derived from different genotypes of HDV templates as described previously (Shih et al., 2008). Hybridization was performed by incubation with DIG-Hyb solution (DIG labelling and detection kit; Roche Diagnostics System) at 55 °C overnight. For controls, hybridization with DIG-labelled G3PDH gene was performed simultaneously.
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