JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 106, No. 1, 99–102. 2008
DOI: 10.1263/jbb.106.99

In Vivo Protein Delivery to Human Liver-Derived Cells Using Hepatitis B Virus Envelope Pre-S Region

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Received 4 December 2007/Accepted 16 April 2008

Human hepatocyte-specific delivery of green fluorescent protein was succeeded in the mouse *xenograft* model by fusion with hepatitis B virus surface antigen pre-S regions (pre-S(1+2)), not with each pre-S region. The entire pre-S region would be useful for human liver-specific delivery of therapeutic proteins and bio-imaging fluoroproteins in biomedical field.

[**Key words:** hepatitis B virus, pre-S region, human liver, *in vivo* targeting, drug delivery system]

Several small peptides (up to 20 amino acids), when injected intravenously in animal models, have been shown to accumulate in specific tissues or organs by recognizing their blood vessels. These peptides are called homing peptides, and they are considered to be useful for tissue- or cancer-specific delivery of therapeutic compounds (1). However, a few homing peptides have succeeded in delivering large molecules *in vivo*, *e.g.*, tumor necrosis factor (TNF)α (2) and quantum dots (diameter 3–5 nm) (3). The delivery might have been possible because of a weak interaction between the homing peptides and the target organs and tissues, since the homing peptides were selected under artificial conditions (*i.e.*, using an *in vivo* panning method). Under natural conditions, various viruses have acquired their cell- and tissue-specificity (*i.e.*, tropism) during their evolution. The specificity of these viruses is usually determined by their surface proteins. The part of the surface proteins responsible for the cell- and tissue-specific delivery of a whole virus *in vivo* is considered to be useful as a targeting molecule for large molecules.

Hepatitis B virus (HBV) infects liver cells specifically in human and chimpanzee, not those in other animals. The hepatophilicity is determined by the pre-S regions (pre-S(1+2); pre-S1, 119 amino acid residues (aa) in HBV subtype *adr*; pre-S2, 55 aa) at the N-terminal half of HBV surface antigen (HBsAg) L protein (4). Further studies showed that pre-S1 [21–47] is indispensable for the specific binding of HBV to hepatocytes (5) and pre-S2 [41–52] is necessary for both poly-albumin-mediated cell attachment of HBV (6) and cell permeable ability of HBV (7, 8). In this study, we investigated whether the pre-S region can transport green fluorescent protein (GFP) to transplanted tumors in a mouse *xenograft* model. The results shown here will facilitate the use of viral surface proteins as an efficient *in vivo* targeting molecule in biomedical field (*e.g.*, drug delivery system, bio-imaging).

DNA fragments encoding pre-S proteins (pre-S1, pre-S2, pre-S(1+2)) were inserted into the pGEX-6p-GFP plasmid (GE, Piscataway, NJ, USA) to express the N-terminally GST (glutathione *S*-transferase)-GFP-fused pre-S proteins in *E. coli* BL21 (Fig. 1A). The transformant harboring each plasmid was cultured, and the crude lysate was subjected to the glutathione-coupled Sepharose column. The GFP-pre-S proteins (GFP-pre-S1, GFP-pre-S2, GFP-pre-S(1+2)) were eluted by digestion with PreScission proteinase (GE). Approximately 1.5 mg of GFP-pre-S protein was obtained from each transformant grown in 1 *l* broth. These GFP-pre-S proteins were confirmed more than 95% purity by SDS–PAGE analysis followed by CBB staining (Fig. 1B). The molecular mass of each GFP-pre-S protein coincided well with the mo-

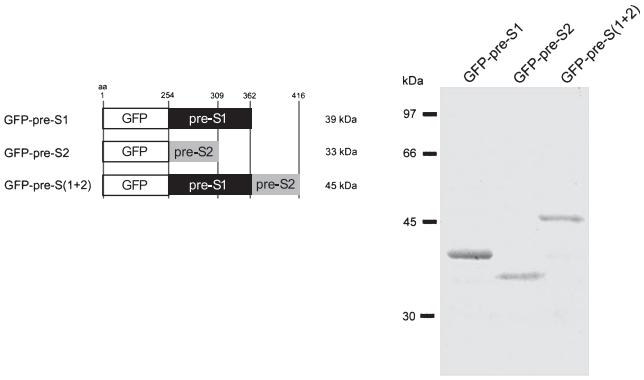

FIG. 1. Production of N-terminally GFP-fused pre-S proteins. (A) GFP-pre-S1, GFP-pre-S2, and GFP-pre-S(1+2) proteins. Amino acid residues (aa) are indicated in the upper margin. (B) The purified GFP-pre-S protein was analyzed with SDS–PAGE followed by CBB R-250 staining.

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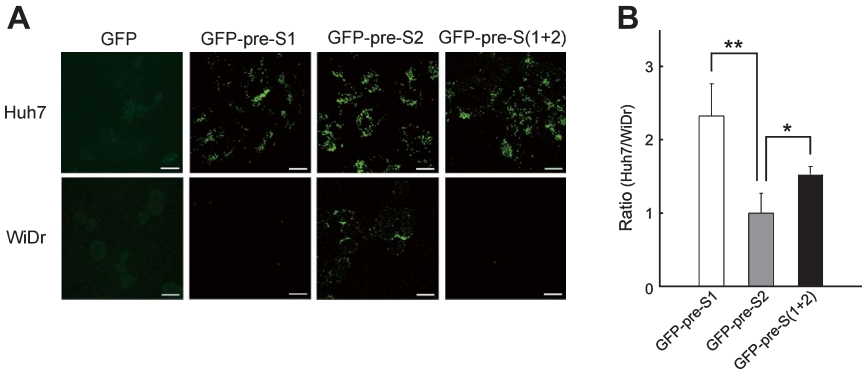

FIG. 2. Accumulation of GFP-pre-S proteins in cultured cells (*in vitro*). (A) About 2.5×10^5 cells of Huh7 and WiDr grown on a 3.5-cm glass-bottom dish were mixed with 30 μg of GFP-pre-S proteins, and then incubated for 6 h, fixed with 2% (w/v) paraformaldehyde, washed with PBS twice, and then observed under a LSM5 Pascal confocal laser microscope (Carl Zeiss, Oberkochen, Germany). Scale bars: 10 μm. (B) Ratio of the relative fluorescent unit (RFU) derived from GFP-pre-S proteins in Huh7 to that in WiDr cells. RFU was calculated from the TIFF files taken under the same conditions. Ten cells in the same visual field were analyzed with NIH image J software (Bethesda, MA, USA). n=7; mean±s.d.; * <0.05 and ** <0.001, *t*-test.

lecular weight calculated from each amino acid sequence.

We added 30 μg GFP-pre-S proteins to human hepatocellular carcinoma Huh7 cells and human colon adenocarcinoma WiDr cells. The fluorescent intensity of each cell was determined from the images with NIH image software. When either GFP-pre-S1 or GFP-pre-S(1+2) was applied, the GFP-derived fluorescence was observed only in Huh7 cells, while GFP alone produced no fluorescence in any cell (Fig. 2A). Comparing the fluorescence derived from GFP-pre-S1, GFP-pre-S2, or GFP-pre-S(1+2) in Huh7 cells with that in WiDr cells (Fig. 2B), both pre-S1 and pre-S(1+2) regions were revealed to possess enough activity for the *in vitro* targeting of human liver-derived cells. Intriguingly, GFP-pre-S2 produced fluorescence in both cells. Since the pre-S2 [41–52] region is a translocation motif (TLM) showing an energy-independent cell-permeable ability (7, 8), the GFP-pre-S2 might have the ability to penetrate into every type of cell.

When the intracellular localization of GFP-pre-S(1+2) in Huh7 cells was investigated by an immunocytochemical method using DAPI (4′,6-diamidino-2-phenylindole), the protein was found to accumulate as dots around the nucleus (Fig. 3, upper panels). Next, since Lamp-2 (late endosome-associated protein-2) protein localizes in the lysosomes and late endosomes in the perinuclear region, the cells were stained with anti-Lamp-2 antibody (Fig. 3, lower panels). A part of GFP-pre-S(1+2) was found to co-localize with Lamp-2 as dots, suggesting that GFP-pre-S(1+2) is incorporated not by membrane fusion but by endocytosis and is finally sorted to either the late endosome or lysosome.

We injected 300 μg of GFP-pre-S proteins intravenously into the tail vein of *xenograft* model mice harboring human hepatocellular carcinoma NuE- and WiDr-derived tumors. Sixteen hours after the injection, both tumors and tissues were isolated and embedded in synthetic resin. Slices of 5-μm width were analyzed under a laser scanning confocal microscope. As shown in Fig. 4A, green fluorescence was detected in the NuE-derived tumors of the mice administrated with GFP-pre-S(1+2). Neither fluorescence was observed in WiDr-derived tumors, brains, hearts, lungs, livers, spleens nor kidneys (Fig. 4B), nor was fluorescence observed in the same tissues of the mice administrated with

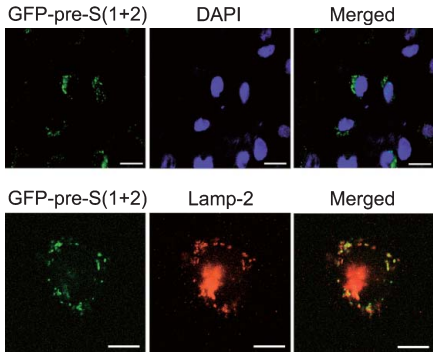

FIG. 3. Intracellular localization of GFP-pre-S(1+2) in Huh7 cells (*in vitro*). After the cells were treated with GFP-pre-S(1+2) for 6 h, the nuclei were stained with DAPI and observed under a fluorescent microscope IX-70 (Olympus, Tokyo) (upper panels). For immunocytochemical observation, the permeabilized cells were reacted with the anti-Lamp-2 monoclonal antibody (dilution factor 1000, clone H4B4; DSHB, Iowa City, IA, USA) overnight at 4°C, and then with the Cy3-conjugated anti-mouse IgG (dilution factor 1000; Jackson ImmunoResearch Laboratories, West Baltimore Pike, PA, USA) at room temperature for 30 min. The fluorescence was visualized under a laser scanning confocal microscope (lower panels). Scale bars: 10 μm.

GFP-pre-S1, GFP-pre-S2, or GFP (data not shown). These data agreed well with the hepatophilic property of HBV (only to human and chimpanzee, not to other animals) (4).

After infection, various viruses are circulated in the body and then sorted to target tissues *in vivo* by the action of their surface proteins, implying that the affinity of surface proteins to target tissues is sufficient to allow the pinpoint delivery of large molecules in the body, even nano-sized virions. Considering the homing peptide as an *in vivo* transporter of large molecules, only a few homing peptides have succeeded in making the delivery to specific tissues *in vivo*, such as TNFα and quantum dots (2, 3). The action of homing peptides has often been restrained by the conjugation of large molecules, which has restricted us to using homing peptides for the delivery of small molecules (*e.g.*, peptides, chemical compounds) (9, 10). In this study, the systemic injection of GFP-pre-S(1+2) allowed the specific accumulation of GFP-derived fluorescence in the human hepatocyte-derived tumors