Vol. 18, pp. 67–77 (2021)
doi: 10.2142/biophysico.bppb-v18.008

Biophysics and Physicobiology
https://www.jstage.jst.go.jp/browse/biophysico/

Regular Article

Application of fluorescent-based technology detecting protein-protein interactions to monitor the binding of hepatitis B virus X protein to DNA-damage-binding protein 1

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Received August 7, 2020; accepted March 12, 2021; Released online in J-STAGE as advance publication March 17, 2021

The hepatitis B virus X protein (HBx) and the V protein of paramyxovirus simian virus 5 (SV5-V) interact with DNA damage-binding protein 1 (DDB1), a cellular enzyme involved in DNA repair and cell cycle regulation, to stimulate viral activity. DDB1 has several cellular substrates, and the amino acid sequences of the binding sites in the viral proteins and their substrates are notably dissimilar. To determine whether HBx binds preferentially to DDB1, despite differences in the amino acid sequences, we developed a system to monitor DDB1 binding in living cells through a protein-protein visualization system, designated fluorescent-based technology detecting protein-protein interactions (Fluoppi). HBx in association with DDB1 formed clear fluorescent puncta. The number of these fluorescent puncta increased with an increase in the amount of HBx. The binding of HBx to DDB1 inhibited the cellular substrate DDB1-CUL4A-associated factor 9 (DCAF9) from binding to DDB1. The inhibitor nitazoxanide prevented the viral proteins HBx and SV5-V from binding to DDB1 but did not inhibit the binding of DCAF9 or HBx(ΔNC), which constitutes the binding site of HBx. Our results demonstrate that the Fluoppi system is useful for monitoring the binding of HBx to DDB1 as well as for examining the effect of drugs on DDB1-Hbx binding.

Key words: ubiquitination, drug screening, hepatitis B virus X protein, DDB1-CLU4A-associated factor 9, Fluoppi

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Introduction

Protein ubiquitination is necessary for the selected degradation of abnormal proteins through a three-enzyme (E1-E2-E3) cascade. E3 ubiquitin ligase, which is represented by the superfamily of cullin-Really Interesting New Gene (RING) domain complexes, has a versatile recognition mechanism and acts upon proteins with a variety of amino acid sequences and structures [1]. Cullin-RING complexes are composed of cullin proteins and a RING, and incorporate an adapter protein to link to DNA

◀ ***Significance*** ▶

The hepatitis B virus X protein (HBx) binds DDB1 to activate viral amplification and is expected to be the target of new viral inhibitors. Although DDB1 binds to several cellular substrates and some viral proteins, with diverse amino acid sequences, it remains to be determined how it recognizes DDB1. The Fluoppi system shows that binding of HBx to DDB1 interferes with the binding of DCAF9 to DDB1. The Fluoppi system is also useful for monitoring the effects of drugs inhibiting HBx-binding. Our results indicate that the Fluoppi system provides the insights into the mechanism through which substrates and drugs interact with DDB1 in cells.

damage-binding protein 1 (DDB1) [2]. The DDB1-containing E3 ligase has 30 substrates that were isolated by tandem-affinity purification and identified by mass spectrometry [3], including well-known substrates such as DDB2 [4,5] and previously unknown substrates such as the DDB1-CUL4A-associated factor (DCAF) proteins, which recruit proteins for the E3 machinery [6,7].

Cullin-RING E3 complexes are often subverted by pathogenic viruses [8,9]. Some viral proteins associate with the DDB1-containing E3 ubiquitin ligase to degrade antiviral regulatory factors in host cells [10], with hepatitis B virus X protein (HBx) as one of the most well-known examples. HBx associates with DDB1 to degrade the structural maintenance of chromosomes 5/6 (Smc5/6) complex, which binds to hepatitis B virus (HBV) genomic covalently closed circular DNA (cccDNA) to inhibit the transcription of several HBV mRNAs [11–18]. Thus, HBx subverts DDB1 to promote transcription of the HBV genome. The paramyxovirus simian virus 5 V protein (SV5-V) also binds DDB1 through the α-helical structure to promote the degradation of STAT proteins [17,19,20]. Several structural analyses revealed that HBx and SV5-V associate with DDB1 through the α-helical structure in a similar way to the docking of the DCAF family [6,21]. However, the amino acid sequences of binding sites in the HBx, SV5-V, and DCAF families are notably dissimilar (Fig. 1). Despite the versatility of the DDB1 binding mechanism, it remains unclear whether viral proteins bind to DDB1 more efficiently than intracellular substrates. Therefore, it is not known how DDB1 recognizes viral proteins and other substrates with a variety of amino acid sequences. To understand the binding mechanism, we developed a system to monitor the interaction of DDB1 with viral proteins or host substrates by imaging in living cells.

Various luminescent protein-based imaging methods have been developed to monitor protein-protein interactions in living cells, such as fluorescence resonance energy transfer (FRET) [22], split luciferase (NanoBit, Promega) [23], and fluorescence-based technology detecting protein-protein interactions (Fluoppi, MBL) [24–26]. Because of its simplicity and ease of application in the study of protein-protein interactions, we decided to use the Fluoppi system to investigate how DDB1 associates with various substrates with dissimilar amino acid sequences. The Fluoppi system uses fluorescent Azami Green (AG) tags that form a tetrameric complex and assembly helper (Ash) tags that form homo-oligomers. When a protein with the AG tag binds to another protein with the Ash tag, AG is concentrated locally in the cell and becomes visible as fluorescent puncta. Therefore, the Fluoppi system can monitor protein-protein interactions in living cells in real time.

In this study, the binding of the viral proteins HBx and SV5-V, and the cellular substrate DCAF9, a well-known member of the DCAF family [27,28], was monitored through the visualization system of protein-protein interactions, Fluoppi. HBx, SV5-V, and DCAF9 could bind to DDB1, and HBx competes with DCAF9 for DDB1 binding. In addition, we investigated whether the Fluoppi system is useful for monitoring the effects of drugs on the binding of HBx to DDB1, using the inhibitor nitazoxanide (NTZ) [29]. Although NTZ was effective against the binding activity of DDB1 to the viral proteins HBx and SV5-V, it was not effective against the binding activity of DDB1 to DCAF9 and a deletion mutant of HBx, both of which have binding sites for DDB1. Our results suggest that the Fluoppi system is applicable for monitoring protein-protein interactions between DDB1 and viral proteins such as HBx and SV5-V, or other cellular proteins.

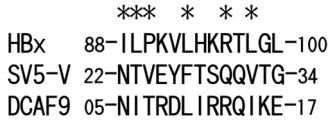

Figure 1 Sequence alignment of the DDB1-binding motif, which represents the binding sites of the viral proteins, and DCAF9, found in crystal structures of DDB1 complexes. The sequence alignment of DDB1-binding motifs [21] is shown. Asterisks indicate residues in DDB1 in the crystal structures. Each number indicates the position of the amino acid of each substrate.

Materials and Methods

Expression plasmids

The HBV nucleotide sequence of the X open reading frame (ORF), the region encoding HBx (GenBank accession: AB246345), was amplified by polymerase chain reaction. The amplified gene was then introduced into the BamHI and EcoRV cloning sites of the expression vector pAsh-MCL [Medical Biological Laboratories (MBL)] by standard molecular cloning techniques. The deletion mutants of HBx were prepared by site-directed mutagenesis, including HBx(ΔNC) (amino acid residues 67–139), retaining the α-helix domain essential for binding to DDB1, and HBx(ΔH) (amino acid residues 1–84 and 101–155) without the α-helix domain. The ORFs of full-length viral SV5-V (GenBank accession: NC_006430.1, amino acid residues 1–222) and human DCAF9 (GenBank accession: AB527722, amino acid residues 1–626) were inserted into the multicloning site of pAsh-MCL. The ORF of full-length human DDB1 (GenBank accession: NM_001923) was inserted into the multicloning site of phAG-MCL (MBL). Expression plasmids without the Ash tag were constructed by inserting the sequences encoding HBx or DCAF9 into the multicloning sites of pCAG-Neo PA tag-N (FUJIFILM).

Cell culture and transfection

HEK293T cells were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle medium (NISSUI) supplemented with 10% (vol/vol) fetal bovine serum (Gibco), 2 mM L-glutamine, and 1 mM kanamycin (Gibco). Cells were seeded in 24-well plates at a density of approximately 0.3×10^5 cells per well. The next day, the cells were transfected with 100 ng of hAG-tagged DDB1, and with 0–250 ng of Ash-tagged HBx, SV5-V, and DCAF9 using PEI MAX (Polysciences, Inc.) following the manufacturer's instructions. The cells were examined for fluorescent puncta 16 h after transfection.

Confocal microscopy imaging

Cells were cultured on ϕ6 mm coverglasses (Matsunami) at 37°C. At 16 h after co-transfection, the coverglasses were washed with phosphate-buffered saline (PBS(–)). The cells were fixed with 50% acetone/50% ethanol for 15 min at 4°C. The coverglasses were mounted with fluorescence mounting medium (ProLong; Life Technologies) on glass slides and cured for 24 h. The cells were imaged using an inverted microscope (FV3000, Olympus) with a 60× objective lens (NA 1.35) and FV31S-SW FLUOVIEW software.

Quantification of protein-protein interactions

The Fluoppi system was used to monitor protein-protein interactions in cells. HEK293T cells were transfected as described above. At 16 h after co-transfection, the cells were stained with Hoechst33342 (Dojindo) for 30 min. Image acquisition was performed using IN Cell Analyzer 6000 (GE Healthcare) with a 20× objective lens. Azami-Green (AG) and blue (Hoechst33342) fluorescence on the images was used to count the fluorescent puncta of complexes and nuclei, respectively. Five images were obtained per well. The numbers of fluorescent puncta and nuclei in the five images were counted using ImageJ software [25,30]. Each image contained approximately 500 cells. The number of fluorescent foci was calculated as the average value for one of the five images, which was normalized to 500 nuclei. The standard errors of the number of fluorescent puncta were calculated from the normalized number of fluorescent puncta.

Competitive assays with HBx and DCAF9

To determine whether HBx competes with the intracellular substrate DCAF9, the number of fluorescent puncta was counted after co-transfection of AG-DDB1, Ash-tagged substrates, and substrates without the Ash tag. Using a fixed amount of Ash-HBx or Ash-DCAF9 plasmid (200 ng) and AG-DDB1 plasmid (100 ng), HEK293T cells were co-transfected with various amounts of HBx or DCAF9 plasmids without the Ash tag, as described above (0–200 ng). At 16 h after co-transfection, the number of fluorescent puncta was counted using IN Cell Analyzer 6000 and ImageJ.

Inhibition assays with the Fluoppi system

Cells treated with or without NTZ (Funakoshi) were prepared in the same way as described above for transfection. The cells were co-transfected with AG-DDB1 and the substrates Ash-HBx, Ash-HBx(ΔNC), Ash-SV5-V, or Ash-DCAF9, and incubated for 14 h. After addition of NTZ or dimethyl sulfoxide (DMSO; Wako) as a control, the cells were incubated for 2 h at 37°C. The number of fluorescent puncta was quantified as described above.

Results

Binding of HBx to DDB1 formed clear fluorescent puncta in living cells

To determine whether the Fluoppi system is useful for measuring the interaction of HBx with DDB1, we observed the interaction by co-transfection of AG-tagged DDB1 (AG-DDB1) and Ash-tagged HBx plasmids. To this end, three types of expression plasmids (Fig. 2) were constructed to determine whether the Fluoppi system could monitor the interaction between HBx and DDB1. The constructs express the full-length HBx fused with the Ash tag (Ash-HBx), the Ash-HBx(ΔH) deletion mutant of the α-helix domain, which is essential for the binding of DDB1, and the Ash-HBx(ΔNC) control mutant containing the α-helix domain.

AG-DDB1 plasmids were co-transfected with Ash plasmids into HEK293T cells, and fluorescence was observed 16 h after co-transfection. The green fluorescence of AG-DDB1 was uniformly diffuse in the cytoplasm (Fig. 3, Supplementary Fig. S1), and no fluorescent puncta could be seen. Thus, Ash did not affect the formation of fluorescent puncta.

To determine whether HBx forms a complex with DDB1 in the cells, HEK293T cells were co-transfected with

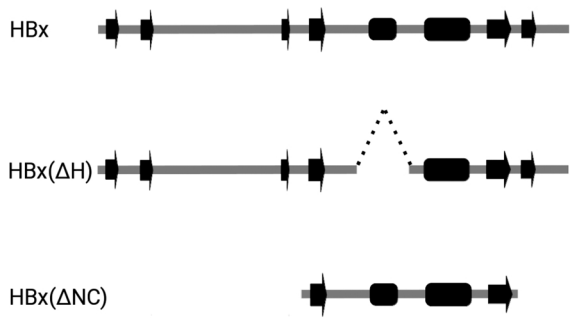

Figure 2 Domain structures of wild-type HBx and its truncated mutants used for DDB1-binding analysis. HBx carries the full-length HBx protein (wild type). HBx(ΔH) has a truncated H-box domain. HBx(ΔNC) retains the H-box domain. Squares and arrows show the α-helix and β-sheet, respectively.

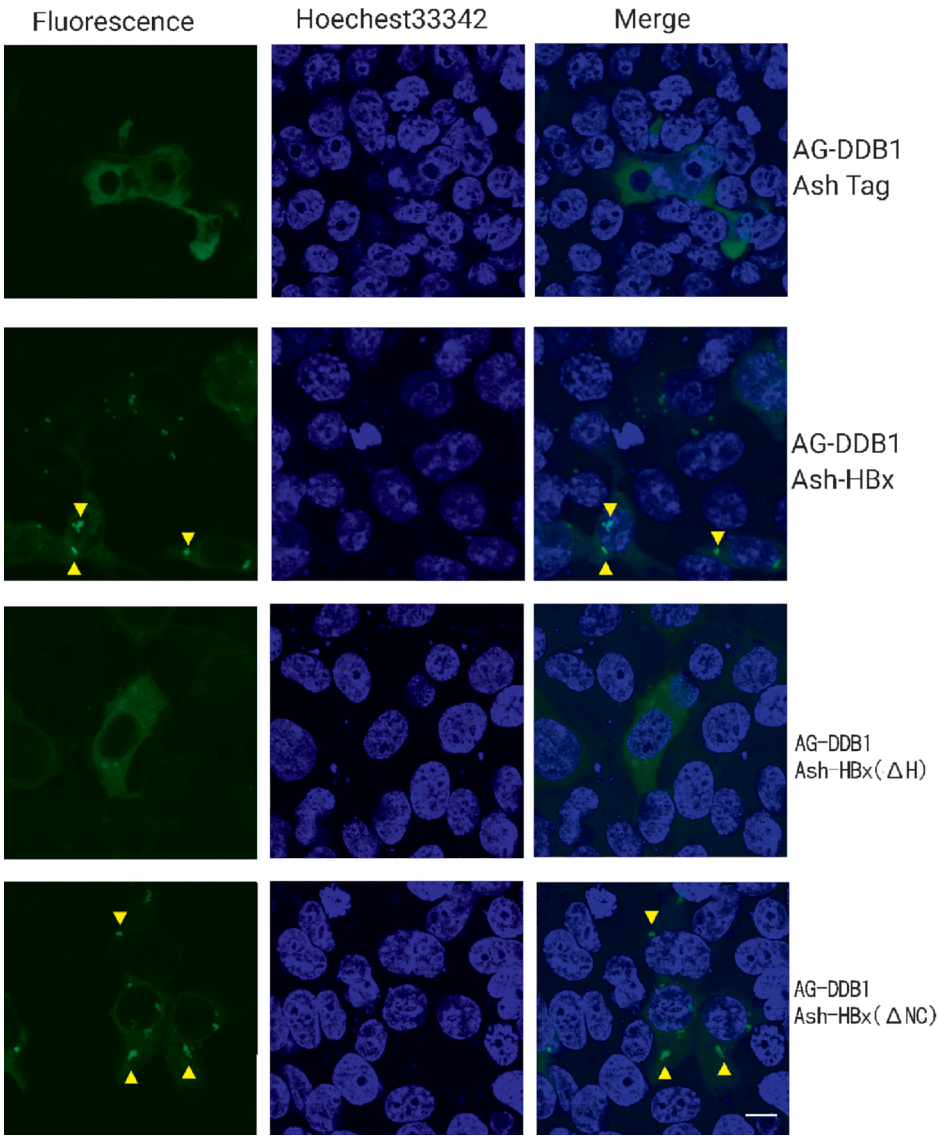

Figure 3 The interaction between HBx and DDB1 generates fluorescent puncta in living cells. Fluorescence images of cultured HEK293T cells expressing AG-tagged DDB1 and Ash-tagged HBx, HBx deficient in the DDB1-binding domain (HBx(ΔH)), or the DDB1-binding domain (HBx(ΔNC)) are shown. HEK293T cells were visualized with a confocal microscope 16 h after co-transfection. Cell nuclei were stained with Hoecht33342. Fluorescent puncta are indicated by yellow triangles. Scale bars indicate 10 μm.

the Ash-HBx and AG-DDB1 plasmids, and incubated for 16 h. Several clear fluorescent puncta appeared in the cytoplasm (Fig. 3) and uniformly diffused fluorescence was not observed. Co-transfection of AG-DDB1 and Ash-HBx(ΔNC) resulted in clear fluorescent puncta in the cytoplasm, as observed for binding of Ash-HBx to AG-DDB1 (Fig. 3). By contrast, the co-transfection of AG-DDB1 and Ash-HBx(ΔH) showed no or only obscure fluorescent puncta (Fig. 3). The image obtained from IN Cell Analyzer 6000 showed no fluorescent puncta of AG-tagged DDB1 to the Ash-tagged HBx(ΔH) complex (Supplementary Fig. S1).

The number of fluorescent puncta increased with the amount of HBx

The complex of Ash-HBx and AG-DDB1 formed clear fluorescent puncta in cells that were co-transfected with these plasmids, and analyses of mutants indicated that this interaction was specific for the domain of HBx. To verify the binding kinetics of HBx to DDB1, we next investigated whether the number of fluorescent puncta depends on the amount of substrate. Sixteen hours after co-transfection, the number of fluorescent puncta of the DDB1-HBx complex increased gradually as the amount of HBx plasmids increased, with a constant amount of the DDB1 plasmid

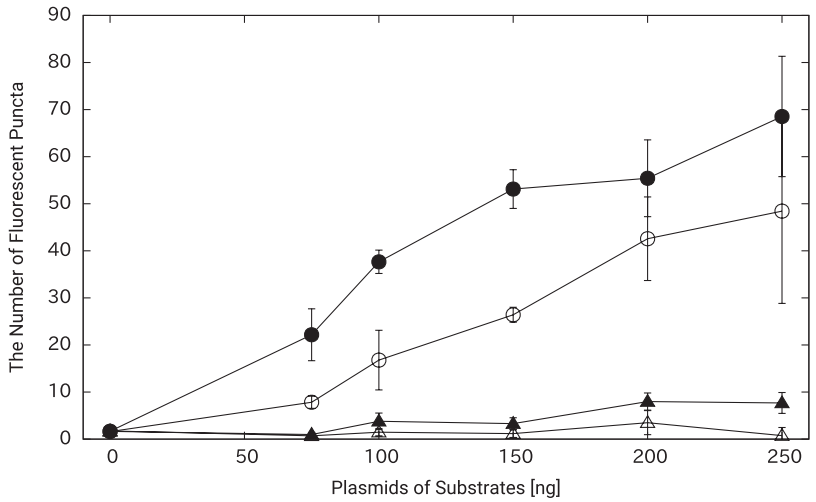

Figure 4 Association curves of wild-type HBx and mutant HBx to DDB1. The number of fluorescent puncta increased depending on the amount of plasmid. Open circles, filled circles, filled triangles, and open triangles show the number of fluorescent puncta of HBx-DDB1, HBx(ΔNC)-DDB1, HBx(ΔH)-DDB1 complexes, and Ash-tag-DDB1 complex, respectively. Error bars represent standard errors derived from normalized fluorescent puncta for five measurements.

(Fig. 4). The number of fluorescent puncta increased with an increasing concentration of HBx plasmid up to 200 ng. Therefore, the change in the number of fluorescent puncta in terms of the concentration of HBx plasmids followed an apparent association curve of DDB1 to HBx.

Similarly, the number of fluorescent puncta of the DDB1-HBx(ΔNC) complex increased as the concentration of HBx plasmid increased. The number of fluorescent puncta did not saturate at higher concentrations of the plasmid. In contrast to HBx and HBx(ΔNC), the DDB1-HBx(ΔH) complex did not form, even at high concentrations of the plasmids. The same result was observed for the DDB1-Ash complex. These results are consistent with those displayed in Figure 3, demonstrating the formation of fluorescent puncta in living cells after co-transfection with DDB1 and HBx or HBx(ΔNC).

Binding of DDB1 to DCAF9 and SV5-V formed clear fluorescent puncta in living cells

To test whether the Fluoppi system can detect the complexes of other substrates with DDB1, we used the intracellular substrate DCAF9 and the viral protein SV5-V. The intracellular substrate DCAF9, which is a member of the DCAF family and has anti-obesity activity, has already been shown to associate with DDB1 through tandem affinity purification and mass spectrometric analysis, and based on the crystal structure [6,21,28,31]. To confirm the binding of DCAF9 to DDB1 using the Fluoppi system, HEK293T cells were co-transfected with Ash-tagged DCAF9 (Ash-DCAF9) and AG-tagged DDB1 (AG-DDB1). After co-transfection of Ash-DCAF9 and AG-DDB1 plasmids, fluorescent puncta appeared in the cytoplasm (Fig. 5).

The viral protein SV5-V binds to DDB1 to degrade STAT1 [19]. Therefore, we further determined whether SV5-V could bind to DDB1 in living cells. After co-transfection with Ash-tagged SV5-V (Ash-SV5-V) and AG-DDB1, fluorescent puncta appeared in the cytoplasm (Fig. 5).

The number of fluorescent puncta of DDB1 complexes increases with the increase in the amount of SV5-V and DCAF9 plasmids

To determine how the number of fluorescent puncta of DDB1-SV5-V or DDB1-DCAF9 complexes increased depending on the amount of SV5-V or DCAF9 plasmids, the puncta were counted 16 h after co-transfection with AG-DDB1 and the plasmids encoding the substrates. The number of fluorescent puncta of these DDB1 complexes is plotted against the concentrations of transfected plasmids in Figure 6. The number of fluorescent puncta increased with increasing amounts of the substrate plasmid up to 250 ng, similar to the results for the DDB1-HBx complex. In contrast, when the cells were transfected with Ash and DDB1 plasmids, no fluorescent puncta appeared up to 250 ng.

HBx competes with DCAF9 for binding to DDB1

The results summarized above demonstrated that the Fluoppi system can monitor the interactions of DDB1 with HBx, SV5-V, and DCAF9. Although Li *et al.* [19] showed that the α-helical DDB1-binding peptide of the woodchuck hepatitis virus X (WHx) protein competes with DCAF9, it remains unknown whether interacting HBx and DCAF9 proteins can compete with each other to bind to DDB1. To determine whether HBx competes with DCAF9 to bind

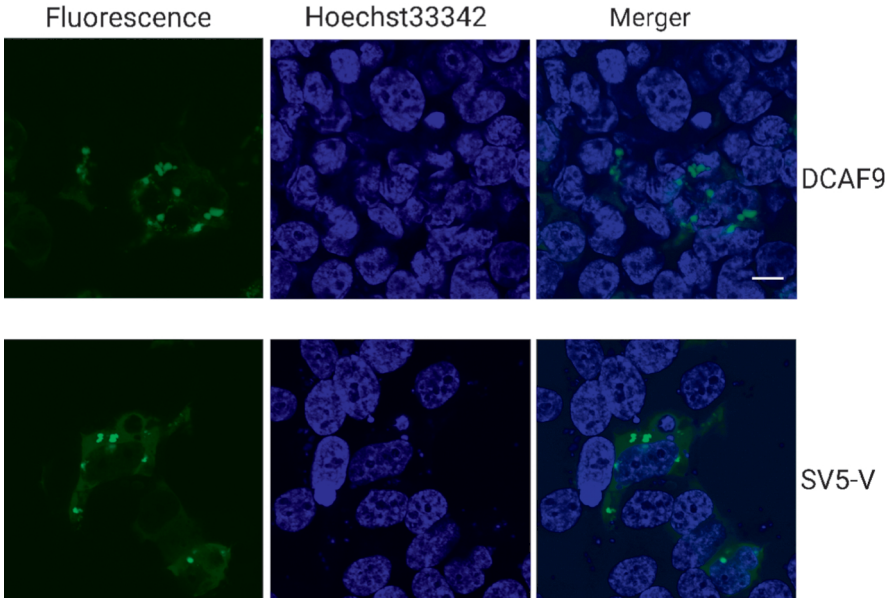

Figure 5 Interactions of DDB1 with DCAF9 and SV5-V generate fluorescent puncta in living cells. Fluorescence images of cultured cells coexpressing AG-DDB1 and Ash-DCAF9 or Ash-SV5-V are shown. AG-DDB1 and the substrates Ash-DCAF9 or Ash-SV5-V were transfected into HEK293T cells. After transfection, the cells were incubated for 16 h at 37°C. Cell nuclei were stained with Hoechst33342 and fixed on a cover glass. Scale bars indicate 10 μm.

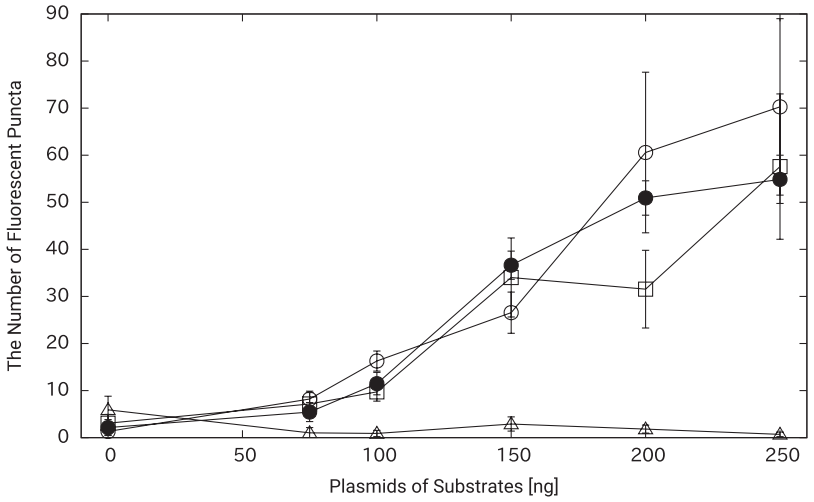

Figure 6 Association curves of the substrates DCAF9 and SV5-V with DDB1. The number of fluorescent puncta increased depending on the amount of the plasmids encoding the substrates. HEK293T cells were transfected with DDB1-binding proteins, HBx, SV5-V, and DCAF9. The number of fluorescent puncta was counted 16 h after transfection. Filled circles, open squares, open circles, and open triangles show the number of fluorescent puncta of HBx-DDB1, SV5-V-DDB1, DCAF9-DDB1, and Ash-tag-DDB1 complex, respectively. Error bars represent standard errors derived from normalized fluorescent puncta for five measurements.

DDB1, we investigated how the number of fluorescent puncta in DDB1-HBx or DDB1-DCAF9 complexes decreased with increasing amounts of DCAF9 or HBx plasmids without the Ash tag. Although the Ash tags produce clear fluorescent puncta with AG tags through homo-oligomerization, a pair of proteins interacting without an Ash tag cannot produce fluorescent puncta [24]. Therefore, when a substrate without an Ash tag binds to an AG-tagged molecule, fluorescence is absent; hence, the number of fluorescent puncta decreases in proportion to the increasing amount of substrate without an Ash tag.

The number of fluorescent puncta in the DDB1-HBx complex changed with an increasing amount of the expression plasmid of HBx or DCAF9 without an Ash tag.

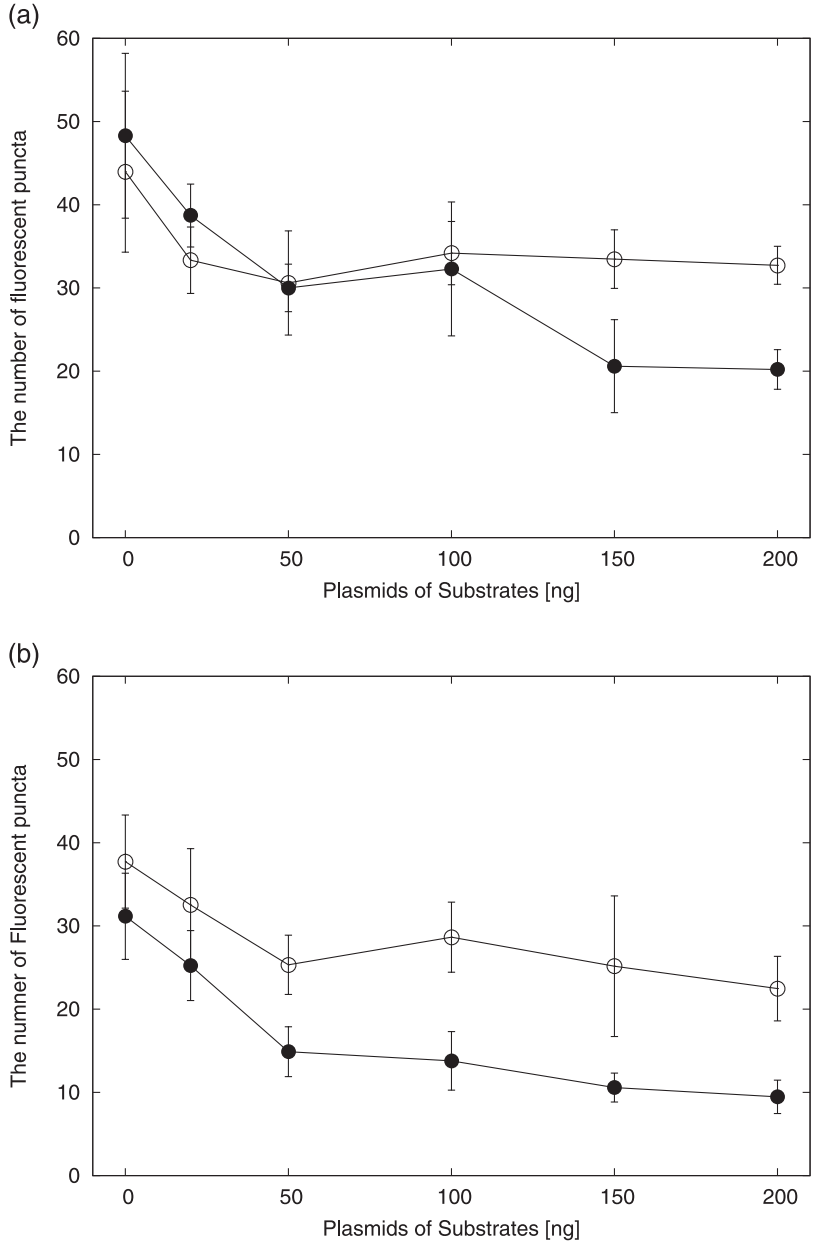

Figure 7 HBx markedly decreases the number of fluorescent puncta of DDB1-DCAF9 although DCAF9 only slightly decreased the puncta of DDB1-HBx. (a) HEK293T cells were co-transfected with AG-DDB1, Ash-HBx, and the substrates without an Ash tag (HBx and DCAF9). The number of fluorescent puncta of the HBx-DDB1 complex decreased with increasing amounts of HBx plasmids without an Ash tag (filled circles). Upon addition of DCAF9 without an Ash tag, the reduction in the number of complexes of HBx to DDB1 (open circles) is smaller when competing with HBx plasmids without an Ash tag. (b) HEK293T cells were transfected with AG-DDB1, Ash-DCAF9, and the substrates without an Ash tag (HBx and DCAF9). The number of fluorescent puncta for the DCAF9-DDB1 complex markedly decreased as the amount of competitor plasmid, HBx without an Ash tag (filled circles), increased. By contrast, the reduction in the number of complexes of DCADF9 to DDB1 (open circles) is smaller when HBx without an Ash tag was added.

The number of fluorescent puncta of the DDB1-HBx complex decreased to approximately 50% when the same amount of HBx plasmid without the Ash tag (200 ng) was added (Fig. 7). In contrast, the number of fluorescent puncta of the DDB1-HBx complex decreased to approximately 80% when up to 200 ng of the DCAF9 plasmid without the Ash tag was added.

Next, the changes in the number of fluorescent puncta of the DDB1-DCAF9 complex were examined through the co-expression of plasmids of HBx or DCAF9 without the Ash tag. Addition of DCAF9 plasmids without the Ash tag reduced the number of fluorescent puncta of the

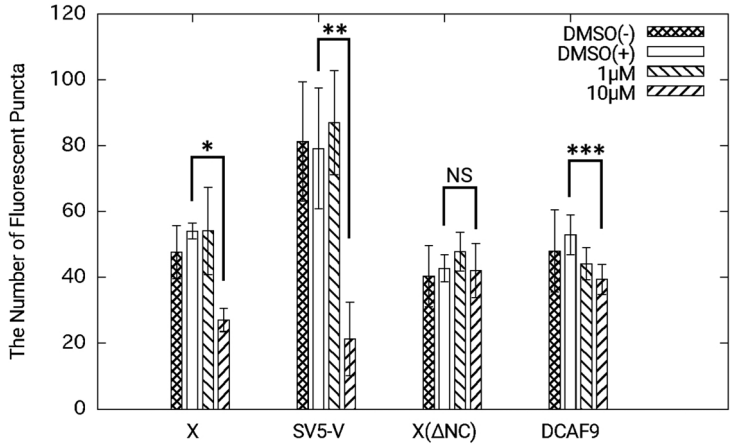

Figure 8 NTZ inhibits the interactions of intact viral proteins with DDB1. HEK293T cells were co-transfected with expression plasmids, AG-tagged DDB1 and the substrates, Ash-tagged HBx, Ash-tagged SV5-V, Ash-tagged HBx(ΔNC), and Ash-tagged DCAF9. At 14 h after transfection, NTZ (1, 10 μM) or DMSO was added and incubated for 2 h. The number of fluorescent puncta is shown as the average number of five measurements per well. Error bars represent standard errors derived from the normalized fluorescent puncta from five measurements. NS, not significant: * $P=6.1\times10^{-7}$, ** $P=3.1\times10^{-4}$, *** $P=4.0\times10^{-3}$ (t test).

DDB1-DCAF9 complex to approximately 50% with an equal amount of Ash-DCAF9 plasmid (Fig. 7). In contrast, the fluorescent puncta of the DDB1-DCAF9 complex were reduced to 30% with addition of the HBx plasmid without the Ash tag.

Inhibition assay of the binding of HBx to DDB1

To determine whether the Fluoppi system can be used to monitor the inhibitory effect of HBx binding to DDB1, we used the inhibitor NZT, which was previously reported to decrease the binding of HBx to DDB1 in HEK293T cells by approximately 40% within 2 h [29]. The half-maximal inhibitory concentration of NTZ for the HBx-DDB1 interaction was 10 μM. Cell viability decreased with increasing concentrations of NTZ above 50 μM.

To confirm whether NTZ inhibits the binding of HBx to DDB1 using the Fluoppi system, HEK293T cells were transfected with HBx and DDB1. After 14 h of incubation, the transfected cells were treated with 1 and 10 μM NTZ for 2 h at 37°C and the number of fluorescent puncta was quantified. Consistent with the previous analysis of NTZ [29], the number of fluorescent puncta declined to approximately 50% (Fig. 8).

We further determined whether NTZ could inhibit the binding of DCAF9, SV5-V, or HBx(ΔNC) to DDB1 (Fig. 8). For DDB1-HBx, HEK293T cells were transfected with DDB1 and DCAF9, SV5-V, and HBx(ΔNC) plasmids. The number of fluorescent puncta of the DDB1-SV5-V complex decreased to 50% at 10 μM NTZ, as for HBx-DDB1. Remarkably, at 10 μM, NTZ had no effect on the fluorescent puncta of DCAF9 and HBx(ΔNC) complexed with DDB1.

Discussion

The interactions between DDB1, the viral proteins HBx and SV5-V, and the cellular substrate DCAF9 were monitored using the Fluoppi system. Our results indicate that HBx competes with DCAF9 for binding to DDB1.

After co-transfection of HBx or HBx(ΔNC) with DDB1, fluorescent puncta appeared in the cytoplasm (Fig. 3). The number of fluorescent puncta increased depending on the concentration of the plasmids encoding the substrates (Fig. 4). In contrast, the mutant of HBx with deletion of the binding domain [HBx(ΔH)] formed blurry fluorescent puncta in the cytoplasm. The number of fluorescent puncta of the DDB1-HBx(ΔH) complex was the same as that of the DDB1-Ash complex. Although the H-box motif is essential for the association of DDB1, HBx could bind weakly through a bipartite binding mechanism, in which DDB1 associates with HBx through residues outside the H-box sequence [13,21,32]. The crystal structure shows that the α-helical domain of HBx docks into the pocket of DDB1 [21]. Our mutational studies of HBx showed that HBx(ΔNC) and HBx bound specifically to DDB1, whereas HBx(ΔH) might not bind to DDB1 or might have extremely weak binding affinity. Because the number of fluorescent puncta of DDB1-HBx increased with an increasing concentration of HBx plasmids, the change in the number of fluorescent puncta might be related to the apparent binding affinity. Consequently, this Fluoppi system might be able to monitor the protein-protein interaction between HBx and DDB1 in living cells. The Fluoppi system does not require specific skills for monitoring protein-protein interactions compared to other systems such as FRET and NanoBit [22,23]. In addition, the Fluoppi system can detect and quantify protein-protein

interactions in living cells as well as binding.

DDB1 has several intracellular substrates whose binding sites have a variety of amino acid sequences, and viral proteins such as SV5-V have no recognizable target sequences [21,33]. The results of the present study show that HBx, SV5-V, and DCAF9 are associated with DDB1 in living cells despite the wide variety of amino acid sequences (Fig. 5), and the number of fluorescent puncta of HBx, SV5-V, and DCAF9 increased with increasing concentrations of the plasmids encoding the substrates; accordingly, their association curves were highly similar (Fig. 6). These results indicate that the Fluoppi system could be useful to study another viral protein, SV5-V, and the cellular substrate, DCAF9. However, this study could not determine the reason for the similar association curves of these substrates despite the variability of their amino acid sequences. DCAF9 has been reported to be a substrate of the DDB1-containing E3 ubiquitin ligase [28]. Although the crystal structure of the complex between DDB1 and DCAF9 reveals an association with the H-box of DCAF9 [21], the crystal structure of intact DCAF9 has not been solved. Other unsolved binding sites of DCAF9 based on the crystal structure might be involved in the binding affinities to DDB1.

Although the DDB1-containing E3 ubiquitin ligase has several cellular substrates, the degradation of Smc5/6 requires the interaction of HBx with DDB1. The viral proteins SV5-V, WHx, and HBx compete with DCAF family members such as DCAF9 and DDB2 for binding with DDB1 [21,34]. Using the Fluoppi system, we found that HBx interferes with the binding of DCAF9 to DDB1. Our competition assays further showed that HBx prevented DCAF9 from binding to DDB1 and that HBx expression markedly reduced the number of fluorescent puncta of the DDB1-DCAF9 complex. Therefore, HBx might interfere with binding of the substrates to DDB1, such that the DDB1-containing E3 ligase might be directed to degrade the Smc5/6 complexes. Consequently, transcription of HBV cccDNA is activated to promote viral replication [13,35].

Although the amino acid sequences of these binding sites are dissimilar, viral proteins and the DCAF family dock into the pocket of DDB1 through the α-helix in a similar way [21]. Based on the present results, it is difficult to determine which of the substrates has the highest binding affinity. The amino acids in the binding pocket of DDB1 and those in other binding domains might coordinate the binding affinities of the substrates for DDB1. However, stoichiometric studies are needed to determine how each amino acid of the viral proteins and the DCAF family contribute to DDB1 binding. Such studies might be useful for understanding, biophysically and biochemically, how DDB1 recognizes the DCAF family and the viral proteins despite the diversity of amino acid sequences at the binding sites.

NTZ holds promise as a novel inhibitor of the HBx-DDB1 interaction [29], although it remains unknown whether NTZ binds to the pocket of DDB1. Our Fluoppi system showed that the number of fluorescent puncta of HBx and SV5-V declined by 50–60% after addition of 10 μM NTZ. This inhibitory effect was the same as reported previously [29]. Interestingly, NTZ did not inhibit the binding of DCAF9 and HBx(ΔNC) to DDB1, as was the case for SV5-V and HBx. If NTZ associates with the binding pocket of DDB1, the binding of DCAF9 and HBx(ΔNC) would be expected to be inhibited. The crystal structure of the complex between DDB1-containing E3 ligase and intact DCAF9 remains to be determined. The DCAF family might use WD40 domains and sequence regions outside WD40 to bind to DDB1 [6]. The results of the present study suggest that NTZ might bind to other sites outside the pocket of DDB1 to inhibit the binding of HBx and SV5-V to DDB1. It is possible that NTZ targets DDB1 without interfering with the docking of its substrates [8]. If NTZ binds to the pockets of DDB1 to which the α-helix domain of HBx associates, NTZ might inhibit the binding of HBx(ΔNC). However, NTZ did not inhibit binding of HBx(ΔNC). This result indicates that NTZ does not bind to the pockets of DDB1. Determining the mechanism by which NTZ inhibits the binding of the viral proteins HBx and SV5-V might provide insight into the design of inhibitors. Mutational studies with biochemical and structural analyses of the DDB1-NTZ complex could help to explain the binding mechanism, which could in turn inform the development of antiviral drugs.

Conclusion

We developed a monitoring system for the binding of DDB1 to viral proteins and cellular substrates using the Fluoppi system. Our results indicate that HBx interferes with binding of the cellular substrates to DDB1. Thus, viral proteins might prevent DCAF9 from binding to DDB1; consequently, the virus might propagate more efficiently. In addition, our results confirm that NTZ inhibits the binding of viral proteins to DDB1, although NTZ might not bind to the pocket of DDB1.

Acknowledgement

This study was supported in part by the Program on the Innovative Development and Application of New Drugs for Hepatitis B from the Japan Agency for Medical Research and Development under grants JP16fk0310503, JP17fk0310119, JP18fk0310119, JP16fk0310512, and JP20fk0310101.

Conflicts of Interest

All authors declare that they have no conflict of interest.

Author Contributions

K.O. wrote the manuscript. K. A. and Y. T. reviewed the manuscript and approved its final form.

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(*Edited by Haruki Nakamura*)