

Peer Review Information

Journal: Nature Structural & Molecular Biology

Manuscript Title: Structural basis of hepatitis B virus receptor binding

Corresponding author name(s): Umeharu Ohto, Sam-Yong Park, Koichi Watashi, Norimichi Nomura

Reviewer Comments & Decisions:

Decision Letter, initial version:
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Message: 24th Apr 2023

Dear Dr. Ohto,

Thank you again for submitting your manuscript "Structural basis of hepatitis B virus receptor binding". I sincerely apologize for the unusual delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 2 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that while both reviewers appreciate the quality of the work, Reviewer #1 suggests improvements to the data presentation, and Reviewer #2 emphasizes the need to introduce the origin and rationale of the PreS1 sequence used and to place the findings more appropriately in the context of previous literature. Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that

should be reported, please submit an updated version of the Reporting Summary along with your revision.

Please follow the links below to download these files:

Reporting Summary:

<https://www.nature.com/documents/nr-reporting-summary.pdf>

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our [Digital Image Integrity Guidelines](https://www.nature.com/nature-portfolio/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (<http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html>). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as

Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below: <https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data>

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDb and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at <http://www.nature.com/nsmb/authors/submit/index.html#costs>

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit <http://www.springernature.com/orcid>.

Please use the link below to submit your revised manuscript and related files:

[redacted]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,
Sara

Sara Osman, Ph.D.
Associate Editor
Nature Structural & Molecular Biology

Referee expertise:

Referee #1: cryo-EM, host-pathogen interaction

Referee #2: structural biology, HBV

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In this paper, Asami et al. describe a single-particle cryo-EM structure of the myristoylated preS1 domain of HBV large surface protein bound to human NTCP. The structure reveals that the N-terminal half of the peptide inserts deeply into the outward-facing tunnel of NTCP and the C-terminal half contacts extensively on the extracellular surface. Knowledge of the structure and mechanism of how HBV large surface protein interacts with human NTCP to facilitate HBV viral entrance and initiate infection will enhance the development of inhibitors for the treatment of HBV infections. Therefore, the topic of this paper is significant. The work is of high quality, the paper is concise and well-written, and the structures obtained are interesting. One point lowering significance slightly is that a peptide of 47 amino acids representing only the preS1 domain of HBV large surface protein was used for structural determination. The HBV surface protein is a membrane protein consisting of four TMs. It is not known if this full-length membrane protein binds human NTCP in the same manner as observed from the preS1 peptide. Nevertheless, the cryo-EM structure provides new insight in the molecular mechanism of viral entry. Overall, this is an excellent paper that makes significant contributions to understanding the interaction between HBV large surface protein and human NTCP, an essential step for the initiation of an infection. This reviewer recommend to accept this manuscript for publication in NSMB with minor revision.

Minor comments:

On p.4 (1st page of main text), it stated "... 6-TM core domain ... and the 3-TM panel domain ... (Fig. 1b)". Fig. 1b only shows the density map and does not indicate the 6-TM core and 3-TM panel domains. This figure is needed to modify and indicate the core and panel domains. Labeling the ribbon diagram will help.

In Fig. 1, a, b, c, ... has to be in order. Fig. 1a has to be shown first and then b, c, etc.

It would be better to show the hydrophobic tunnel in the side view of the ribbon diagram in Fig. 1c.

On p.5 (2nd page of main text), it was indicated that preS1 binds NTCP with high affinity. What is the binding affinity of preS1 with NTCP? Has it been reported in the literature before?

Where is the myristoylated moiety in Fig. 1a?

On p.5, It was indicated "NTCP (residues 1-320)". Is this a full-length protein? If not, how long is the full-length protein?

What are the sizes of the Fab fragments? It is good to mention it.

It is a good idea to include a top view ribbon diagram of NTCP (with the top view density side by side) in Fig. 1d. Fig. 1c does need to include the tunnel.

On p.7, it stated "... the tunnel of NTCP was increased by approximately 7 Å ...". Does it mean that the diameter of the tunnel was increased by 7 Å? Please clarify it.

Fig. 2 is so confusing and complicated. The authors should do a little better job on it. The labels of TM 1, 2, 3, etc. is very difficult to see. In Fig. 2b and the inserts, the dotted lines cannot be crossover each other. It makes it very confusing.

On p.7, in the last two lines, the statement "N terminal residues ...". It should be "The N terminal residues ...". In the same sentence, it stated "... enter the tunnel along TM5". What is the meaning of "along TM5"? Did the authors observe the process of entering the tunnel from the cryo-EM data? If not, the statement may need to be modified (such as "The N terminal residues ... occupy the entrance of the tunnel ...").

On p.8, it was indicated that preS1 interacts with NTCP via several hydrogen bonds. How long are these hydrogen bonded distances? The authors may need to indicate these distances in Fig. 2.

In Fig. 2c, are the backbone oxygen of D152 and backbone N of T3 electronegative enough form a hydrogen bond, given that the backbone oxygen of D152 has already made a hydrogen bond with the side chain oxygen of T3?

In Fig. 2g, f and i, please indicate the hydrogen bonded distances.

On p.10, the statement "... suggesting that HBV achieves high-affinity binding ...". It would be better to modify this statement to "... suggesting that HBV may achieve high-affinity binding ...".

On p.10, please specify in the text what the V7 and P8 mutate to in the double mutants.

On p.10, the authors claim that the L11W mutation showed little effect because this mutant maintains similar hydrophobic interactions. A point mutation does not affect the binding could mean that this residue is not a critical residue for binding. The authors may need to rephrase this sentence.

On p.10, fluorescence experiment indicated that preS1 (residues 36-48) does not bind NTCP and myr-preS1 (residues 2-33) only binds NTCP very weakly (or could even be non-specific). It could suggest that the full-length protein may bind NTCP in a very different manner (different from that was shown from the cryo-EM structure). The authors may need to address and discuss about what may happen when the full-length protein interacts with NTCP.

On p.11, the statement "... T268 and V272 are located more extracellularly form hydrophobic contacts with ...". Does it mean "... T268 and V272 are located more extracellularly and form hydrophobic contacts with ...".

On p.11, it was indicated that Q264, T268 and T272 showed reduced binding. Where is Q264 in Fig. 2h?

On p.12, it was indicated that T268 and V272 have minimal effects on bile acid transport. Why is that the V272W mutant has a much higher bile acid uptake in comparison with the wild-type transporter (Fig. 3f).

The authors may need to address in the "Discussion" section that it is not known if the full-length protein would bind NTCP in the same manner.

Reviewer #2:

Remarks to the Author:

Asami et al present the structures of NTCP and HBV preS1 in complex with one of two different Fab fragments. NTCP is the sodium-taurocholate co-transporting polypeptide; it is a hepatocyte specific bile acid receptor that has been co-opted as the receptor for Hepatitis B virus (HBV) and Hepatitis D virus (HDV). Pre-S1 is the N-terminal segment of the large form of the HBV surface antigen (L-HBs); the HBs open reading frame has three in-frame start codons resulting in large, medium and small forms of the surface protein. In Asami's structure the authors find that the N-terminal ~33 residues of pre-S1 form two compact loops that are jammed into the transmembrane tunnel of NTCP and the C-terminal 15 residues bind to the exterior of the NTCP. The bound peptide forms extensive contacts with NTCP, blocks the NTCP tunnel, and modestly deforms the NTCP structure. The work is a novel example of a virus recognizing a target by folding an intrinsically disordered peptide. It is of importance to understanding infection by HBV and HDV. It is of great importance to understanding the mechanism of entry inhibitors a class of drugs recently approved for treatment of HDV but still not fully understood.

Most of my comments on the structure are minor but there are substantial difficulties with the lack of context for the experimental system.

Major issues

1. The introduction needs to explain that HBs is the surface protein for HBV and HDV
2. Pre-S1 was identified as necessary for binding to cells for infection. An appropriate reference may be Gripon et al J Virol 2005 79:1613. Pre-S1 is also necessary for identifying NTCP as the HBV and HDV receptor (ref 3 and 4 but never stated).
3. Pre-S1 is the basis Bulervitide (also known as myrcludex B) an antiviral entry inhibitor. This is
4. There are nine HBV genotypes. Which genotype is your pre-S1 derived from? There are six amino acid differences between your pre-S1 and bulervitide. Are these predicted to affect binding? This would be an important application of this structure. Extended figure 2 should be more relevant to human HBV.
5. The size exclusion data implies that binding the peptide makes the NTCP more compact. Is this the case? If so it should be discussed.
6. An RMSD of 0.95Å is meaningless if a subset of helices move by 7Å. Fig 1e implies that the panel domain moves independently of the core. Describe independent RMSD values and how the panel moves.
7. In the discussion, the "unprecedented" induced fit is atypical for viruses but there is an extensive literature on binding-induced folding of intrinsically disordered peptides. This should a topic of discussion. As starting points, consider:
Mechanism of coupled folding and binding of an intrinsically disordered protein. K Sugase, HJ Dyson, PE Wright - Nature, 2007 - nature.com

Intrinsically disordered protein AK Dunker, JD Lawson, CJ Brown, RM Williams... - Journal of molecular graphics and modeling

8. The description of Figure 3 is too limited. These data relate the structure to function. However descriptions of experiments in text are limited or absent. "HBV susceptibility" presumably is a test for HBV infection. Myrcludex appears to be used as a control but is never defined. The data are not explained. The use of 12,000 genome equivalents/cell for infection is extraordinarily high.

Minor issues

1. Line 3 of the abstract, "to" appears to be extra
2. Line 7 of abstract, compact-folded should be replaced by either compact or folded.
3. Reference 3 and 14 are identical
4. The recent structure of the HBs protein would make an improvement on the generic and wrong parallel bundle of helices in Fig 4 and extended figure 1 as it provides an understanding of where preS1 and PreS2 will be located. doi: 10.1126/sciadv.abo4184
5. The call to Fig. 1b at the bottom of page 4 should be 1c.
6. The term high affinity for binding of peptide to NTCP should be quantified rather than described as high affinity. An IC50 value of about 8nM is reported in Gripon et al
7. What is the point of the sequence data in extended figure 2.
8. Figure 1 is too crowded and not well -organized.
9. Page 8, the phrase "PreS1 then folds back at the height of the crossover region defined between TM3 and TM8 ... " is hard to understand.
10. Addition of more labels to extended figure 6 would be helpful
11. On page 11 the phrase "T268 and V272 are located more extracellularly form hydrophobic contacts" is confusing
12. Page 12 delete "long-awaited" from the opening of discussion

Author Rebuttal to Initial comments

Response to reviewers

The comments of all reviewers were very useful in helping us to improve the quality of our manuscript.

Our responses to the comments of each reviewer can be found below.

The Figures and Table have been reorganized as follows:

Original	Revised
Fig. 1a, b, d, e	modified
Fig. 2a	Fig. 1f
Fig. 2b	Fig. 2a
Fig. 2c	Fig. 2b (modified)
Fig. 2d	Fig. 2c (modified)

Fig. 2e	Fig. 2d (modified)
Fig. 2f	Fig. 2e (modified)
Fig. 2g	Fig. 2f (modified)
Fig. 2h	Fig. 2g (modified)
Fig. 2i	Fig. 2h (modified)
Fig. 2j	Fig. 2i (modified)
Fig. 2k	Ext. Data Fig. 4b
Fig. 3a, d, e, f	modified
Fig. 4	modified
Ext. Data Fig. 1	Ext. Data Fig. 1a (modified)
	Ext. Data Fig. 1b (newly created)
	Ext. Data Fig. 1c (newly created)
	Ext. Data Fig. 5e (newly created)
	Ext. Data Fig. 6b (newly created)

The figure legends have been modified accordingly in the revised manuscript.

Referees' comments:

Referee #1 (Remarks to the Author):

In this paper, Asami et al. describe a single-particle cryo-EM structure of the myristoylated preS1 domain of HBV large surface protein bound to human NTCP. The structure reveals that the N-terminal half of the peptide inserts deeply into the outward-facing tunnel of NTCP and the C-terminal half contacts extensively on the extracellular surface. Knowledge of the structure and mechanism of how HBV large surface protein interacts with human NTCP to facilitate HBV viral entrance and initiate infection will enhance the development of inhibitors for the treatment of HBV infections. Therefore, the topic of this paper is significant. The work is of high quality, the paper is concise and well-written, and the structures obtained are interesting. One point lowering significance slightly is that a peptide of 47 amino acids representing only the preS1 domain of HBV large surface protein was used for structural determination. The HBV surface protein is a membrane protein consisting of four TMs. It is not known if this full-length membrane protein binds human NTCP in the same manner as observed from the preS1 peptide. Nevertheless, the cryo-

EM structure provides new insight in the molecular mechanism of viral entry. Overall, this is an excellent paper that makes significant contributions to understanding the interaction between HBV large surface protein and human NTCP, an essential step for the initiation of an infection. This reviewer recommend to accept this manuscript for publication in NSMB with minor revision.

We appreciate the reviewer for the careful consideration of our manuscript. We believe that all the comments and suggestions helped to improve the manuscript. Please find our point-by-point responses below.

Minor comments:

On p.4 (1st page of main text), it stated "... 6-TM core domain ... and the 3-TM panel domain ... (Fig. 1b)". Fig. 1b only shows the density map and does not indicate the 6-TM core and 3-TM panel domains. This figure is needed to modify and indicate the core and panel domains. Labeling the ribbon diagram will help.

Indicating the core and panel domains in Fig. 1b makes the figure difficult to read, and this information is redundant with Fig. 1c. In the revision, we have referred to Extended Data Fig. 1a here, which schematically shows domain structure.

In Fig. 1, a, b, c, ... has to be in order. Fig. 1a has to be shown first and then b, c, etc.

We have changed the figure order (revised Fig. 1).

It would be better to show the hydrophobic tunnel in the side view of the ribbon diagram in Fig. 1c.

We have shown the tunnel in the revised Fig. 1c.

On p.5 (2nd page of main text), it was indicated that preS1 binds NTCP with high affinity. What is the binding affinity of preS1 with NTCP? Has it been reported in the literature before?

Although direct binding affinity between myristoylated preS1 peptide and NTCP has not been reported, many studies have demonstrated that myristoylated preS1 peptide efficiently inhibits HBV infection, strongly suggesting a high-affinity interaction between preS1 and NTCP.

We have included this point in the revised manuscript as follows (page 4, line 14~17),

“A myristoylated preS1 peptide (2-48 aa) effectively inhibits HBV/HDV infection, with a 50% inhibitory concentration of nM order or even lower^{3,7,10}, and has been recently approved in clinical use [Bulevirtide, also known as Myrcludex B (Myr-B)] for treating HDV infection in the European Union and Russia¹¹ (Fig. 1a).”

1. Where is the myristoylated moiety in Fig. 1a?

We indicated the possible myristoyl moiety density in the revised Fig. 1b.

2. On p.5, It was indicated “NTCP (residues 1-320)”. Is this a full-length protein? If not, how long is the full-length protein?

The full-length NTCP consists of 349 residues. We used human NTCP lacking 29 residues at the C-terminus (residues 1–320).

We have clearly stated this point in the revised manuscript. (page 6, line 3)

3. What are the sizes of the Fab fragments? It is good to mention it.

We have described the size of Fab. (page 4, line 9)

4. It is a good idea to include a top view ribbon diagram of NTCP (with the top view density side by side) in Fig. 1d. Fig. 1c does need to include the tunnel.

We added a ribbon diagram in the revised Fig. 1d according to the reviewer’s suggestion.

Also, we indicated the tunnel in the revised Fig. 1c.

5. On p.7, it stated “... the tunnel of NTCP was increased by approximately 7 Å ...”. Does it mean that the diameter of the tunnel was increased by 7 Å? Please clarify it.

The width of the tunnel’s opening in this sentence refers to the distance between the Cα atoms of TM1 P22 and TM5 K157.

We have clearly stated this point in the revised manuscript. (page 7, line 15)

6. Fig. 2 is so confusing and complicated. The authors should do a little better job on it. The labels of TM 1, 2, 3, etc. is very difficult to see. In Fig. 2b and the inserts, the dotted lines cannot be crossover each other. It makes it very confusing.

We appreciate the reviewer's critical comment. We have modified Fig. 2 for better viewing.

7. On p.7, in the last two lines, the statement "N terminal residues ...". It should be "The N terminal residues ...". In the same sentence, it stated "... enter the tunnel along TM5". What is the meaning of "along TM5"? Did the authors observe the process of entering the tunnel from the cryo-EM data? If not, the statement may need to be modified (such as "The N terminal residues ... occupy the entrance of the tunnel ...").

Following the reviewer's suggestion, we have modified the sentence as follows (page 8, line 8), "The N terminal residues 2–10^{preS1} (superscript preS1 refers to preS1 residues) occupy the entrance of the tunnel along TM5;"

8. On p.8, it was indicated that preS1 interacts with NTCP via several hydrogen bonds. How long are these hydrogen bonded distances? The authors may need to indicate these distances in Fig. 2.

We acknowledge the reviewer's comment but indicating the hydrogen bond distances in Fig. 2 is rather busy. As an alternative, we have created an additional figure, Extended Data Fig. 6b, which lists the hydrogen bond distances between preS1 and NTCP.

9. In Fig. 2c, are the backbone oxygen of D152 and backbone N of T3 electronegative enough form a hydrogen bond, given that the backbone oxygen of D152 has already made a hydrogen bond with the side chain oxygen of T3?

We are very sorry for confusing the reviewer. The original assignment of a hydrogen bond between the side chain of T3preS1 and the backbone oxygen of D152 was incorrect. The distance between these atoms (3.44 Å) and their arrangement was unsuitable for hydrogen bond formation.

Accordingly, we have updated Fig. 2b (original Fig. 2c).

10. In Fig. 2g, f and i, please indicate the hydrogen bonded distances.

This comment is related to the comment #8. We have created an additional figure, Extended Data Fig. 6b, which lists the hydrogen bond distances between preS1 and NTCP.

11. On p.10, the statement “... suggesting that HBV achieves high-affinity binding ...”. It would be better to modify this statement to “... suggesting that HBV may achieve high-affinity binding ...”.

Following the reviewer’s suggestion, we have modified the sentence. (page 10, line 16)

12. On p.10, please specify in the text what the V7 and P8 mutate to in the double mutants.

We have specified the mutation (V7D/P8D). (page 11, line 6)

13. On p.10, the authors claim that the L11W mutation showed little effect because this mutant maintains similar hydrophobic interactions. A point mutation does not affect the binding could mean that this residue is not a critical residue for binding. The authors may need to rephrase this sentence.

We appreciate the reviewer’s concern that L11 might not be a critical residue for NTCP binding. However, we would like to point out that L11 of preS1 makes extensive contact with NTCP residues (Fig. 2d, i), and previous mutational analysis has also suggested the importance of this residue for NTCP binding (Schulze et al., J. Virol. 2010). Therefore, our original interpretation of the L11W mutation is reasonable.

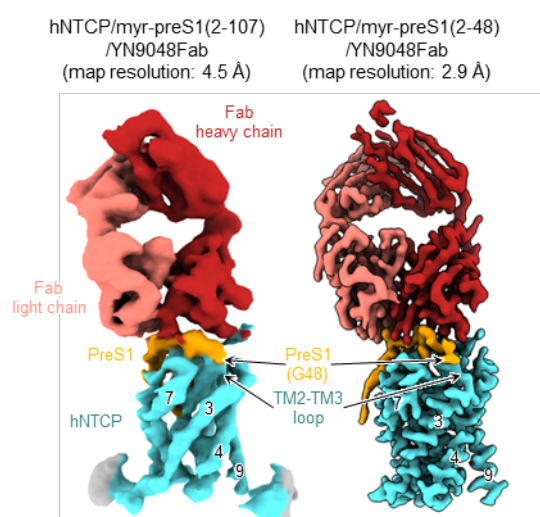
In the revised manuscript, we have elaborated more on this point as follows (page 11, line 8~12),

“Mutation at L11, the amino acid residing at the deepest position in the tunnel, to Trp in myr-preS1 (2-48) showed little effect on preS1 binding. Given that L11 makes extensive contact with NTCP (Fig. 2d, i) and has been suggested to be essential for the binding based on the previous mutational analysis¹⁰, our result suggests that Trp could maintain similar hydrophobic interactions with TM1 and TM3b as Leu”

14. On p.10, fluorescence experiment indicated that preS1 (residues 36-48) does not bind NTCP and myr-preS1 (residues 2-33) only binds NTCP very weakly (or could even be non-specific). It could suggest that the full-length protein may bind NTCP in a very different manner (different from that was shown from the cryo-EM structure). The authors may need to address and discuss about what may happen when the full-length protein interacts with NTCP.

Although not included in the manuscript due to the poor data quality (4.5 Å resolution), we have conducted cryo-EM analysis of NTCP in complex with the full-length myr-preS1 (2-107) (please see figure below) and found no other interactions between NTCP and preS1 in addition to those found for preS1 (2-48), suggesting that the rest of the preS1 region (49-107) is not involved in the interaction with NTCP or only loosely interacts with NTCP, which is consistent with the previous observations that the N-terminal region of preS1 is the most crucial region for HBV attachment to the cells.

Therefore, we believe that the structural features of the interaction between NTCP and preS1 observed in



the cryo-EM structure represent the essential part of the interaction between NTCP and preS1. However, there may be additional interactions between NTCP and full-length LHB protein. Thus, we have briefly discussed this point in the revised manuscript as follows (page 14, line 11),

“In addition to these interactions, other interactions may occur when full-length LHB interacts with NTCP.”

15. On p.11, the statement “... T268 and V272 are located more extracellularly form hydrophobic contacts with ...”. Does it mean “... T268 and V272 are located more extracellularly and form hydrophobic contacts with ...”.

Following the reviewer’s suggestion, we have modified the sentence. (page 12, line 10)

16. On p.11, it was indicated that Q264, T268 and T272 showed reduced binding. Where is Q264 in Fig. 2h?

We have added Q264 in the revised Fig. 2g (original Fig. 2h).

17. On p.12, it was indicated that T268 and V272 have minimal effects on bile acid transport. Why is that the V272W mutant has a much higher bile acid uptake in comparison with the wild-type transporter (Fig. 3f).

V272 is located at the tunnel entrance, and in this position, the larger side chain of Trp can be accommodated without any steric clashes with surrounding residues in either the outward- or inward-open conformation of NTCP. It is difficult to precisely explain the reason for the higher transporter activity of the V272W mutant. Still, the residues with larger side chains at this position may be more favorable for transport activity.

18. The authors may need to address in the “Discussion” section that it is not known if the full-length protein would bind NTCP in the same manner.

This comment is related to the comment #14. We have briefly discussed this point in the revised manuscript as follows (page 14, line 11),

“In addition to these interactions, other interactions may occur when full-length LHB interacts with NTCP.”

Referee #2 (Remarks to the Author):

Asami et al present the structures of NTCP and HBV preS1 in complex with one of two different Fab fragments. NTCP is the sodium-taurocholate co-transporting polypeptide; it is a hepatocyte specific bile acid receptor that has been co-opted as the receptor for Hepatitis B virus (HBV) and Hepatitis D virus (HDV). Pre-S1 is the N-terminal segment of the large form of the HBV surface antigen (L-HBs); the HBs open reading frame has three in-frame start codons resulting in large, medium and small forms of the surface protein. In Asami’s structure the authors find that the N-terminal ~33 residues of pre-S1 form two compact loops that are jammed into the transmembrane tunnel of NTCP and the C-terminal 15 residues bind to the exterior of the NTCP. The bound peptide forms extensive contacts with NTCP, blocks the NTCP tunnel, and modestly deforms the NTCP structure. The work is a novel example of a virus recognizing a target by folding an intrinsically disordered peptide. It is of importance to understanding infection by HBV and HDV. It is of great importance to understanding the mechanism of entry inhibitors a class of drugs recently approved for treatment of HDV but still not fully understood.

Most of my comments on the structure are minor but there are substantial difficulties with the lack of context for the experimental system.

We appreciate the reviewer for the careful consideration of our manuscript. We believe that all the comments and suggestions helped to improve the manuscript. Please find our point-by-point responses below.

Major issues

1. The introduction needs to explain that HBs is the surface protein for HBV and HDV

2. Pre-S1 was identified as necessary for binding to cells for infection. An appropriate reference may be Gripon et al J Virol 2005 79:1613. Pre-S1 is also necessary for identifying NTCP as the HBV and HDV receptor (ref 3 and 4 but never stated).

3. Pre-S1 is the basis Buleviritide (also known as myrcludex B) an antiviral entry inhibitor. This is

We have rewritten the introduction according to the reviewer's comments #1, #2, and #3 as follows (page 4, line 6~17),

“HBV large surface protein (LHBs) is a four-helices transmembrane (TM) protein composed of the preS1, preS2, and S domains, of which the N-terminal myristoylated preS1 domain is essential for HBV attachment to host cells for establishing productive infection³⁻⁶ (Extended Data Fig. 1a). A satellite virus of HBV, hepatitis D virus (HDV), which co-infects with HBV in cells, also carries the identical surface proteins to HBV. By using the preS1 domain as a bait, sodium-taurocholate co-transporting polypeptide (NTCP)/solute carrier family 10 member 1 (SLC10A1) has been identified as an indispensable host entry receptor for HBV and HDV that physically interacts with the myristoylated preS1 domain of LHBs with high affinity⁷⁻⁹. A myristoylated preS1 peptide (2-48 aa) effectively inhibits HBV/HDV infection, with a 50% inhibitory concentration of nM order or even lower^{3 10 7}, and has been recently approved in clinical use [Buleviritide, also known as Myrcludex B (Myr-B)] for treating HDV infection in the European Union and Russia¹¹ (Fig. 1a).”

4. There are nine HBV genotypes. Which genotype is your pre-S1 derived from? There are six amino acid differences between your pre-S1 and bulervitide. Are these predicted to affect binding? This would be an important application of this structure. Extended figure 2 should be more relevant to human HBV.

We appreciate the reviewer for raising an important issue regarding HBV genotypes lacking in our original manuscript.

In the cryo-EM analysis, we used preS1 peptide from the HBV genotype B strain according to the Elife paper (Yan et al., 2012). The revised manuscript includes this information (result section, page 6, line 1) and (method section, page 23, line 11). We have also shown the sequence of preS1 used in this study and that of Myrcludex B in the revised Fig. 1a. We expect the interactions between preS1 and NTCP observed in this study should be preserved in other preS1 sequences from different genotypes.

We have discussed this point in the discussion section as follows (page 16, line 9~),

“In the cryo-EM analysis, we used a preS1 peptide sequence from the HBV genotype B strain, which differs by six residues from Myr-B (positions 24, 28, 34, 37, 40, and 43) (Fig. 1a, Extended Data Fig. 1b, c). The first half of the preS1 peptides stuck into the NTCP tunnel are perfectly conserved between the two sequences. The nonconserved residues are located in the surface regions of the preS1-NTCP interface and are not directly involved in the interaction with NTCP (Extended Data Fig. 1c). Furthermore, loop A of preS1 (residues 7 to 19) is highly conserved among different HBV genotypes except for minor differences at positions 7, 8, and 14 (Fig. 1f, 2a, i, Extended Data Fig. 1b). Although the C-terminal half of preS1 exhibits more sequence variations among different genotypes, the residues N26^{preS1}, W32^{preS1}, N35^{preS1}, K38^{preS1}, D39^{preS1}, W41^{preS1}, and A44^{preS1} that interact with NTCP are well conserved (Fig. 2h and Extended Data Fig. 1b). These suggest that the preS1-NTCP binding mode observed in this study is essentially conserved in other preS1 from different HBV genotypes or Myr-B.”

5. The size exclusion data implies that binding the peptide makes the NTCP more compact. Is this the case? If so it should be discussed.

We would like to thank the reviewer for raising an interesting point, but that is not true. NTCP alone elutes with a slightly larger elution volume than the NTCP/preS1 complex. The NTCP eluted around 13-14 ml is probably a misfolded or denatured NTCP with no preS1 binding activity.

6. An RMSD of 0.95Å is meaningless if a subset of helices move by 7Å. Fig 1e implies that the panel domain moves independently of the core. Describe independent RMSD values and how the panel moves.

We appreciate the reviewer's suggestion. The revised manuscript has included the independent RMSD values for the overall, core, and panel regions as follows (page 7, line 9),

“Overall, the NTCP in complex with myr-preS1 adopts the same conformation as the apo-NTCP structure previously determined in the outward-facing conformation (PDB: 7VAD) (Asami et al., 2022) with RMSD values of 1.33 Å (overall), 1.13 Å (core), and 1.39 Å (panel). (Fig. 1e, Extended Data Fig. 5e)”

The relative positions of the core and panel domains mostly stay the same in the two structures, with only the regions near the preS1 binding site showing a slightly larger structural displacement to accommodate the preS1 peptide. The extracellular sides of TM1 and TM5 show the most significant displacement, as exemplified by the distance between TM1 P22 and TM5 K157, and are thus described in the manuscript.

7. In the discussion, the “unprecedented” induced fit is atypical for viruses but there is an extensive literature on binding-induced folding of intrinsically disordered peptides. This should a topic of discussion.

As starting points, consider:

Mechanism of coupled folding and binding of an intrinsically disordered protein. K Sugase, HJ Dyson, PE Wright - Nature, 2007 - nature.com

Intrinsically disordered protein AK Dunker, JD Lawson, CJ Brown, RM Williams... - Journal of molecular graphics and modeling

We appreciate the reviewer's suggestion. The folding-induced binding mechanism of intrinsically disordered protein is well known in the literature but has not been observed in virus-host interactions as we have described.

We have included this point in the discussion section with the suggested references. (page 14, line 2~7)

“Although there are extensive examples of such induced-folding of unstructured proteins coupled with binding^{31,32}, this is in sharp contrast to the other virus surface protein-receptor interactions where the pre-structured domain of a viral protein is involved, including those between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein and angiotensin-converting enzyme 2 (ACE2)^{33,34}, and between Ebola virus spike protein and Niemann-Pick C1³⁵”

8. The description of Figure 3 is too limited. These data relate the structure to function. However descriptions of experiments in text are limited or absent. “HBV susceptibility” presumably is a test for HBV

infection. Myrcludex appears to be used as a control but is never defined. The data are not explained. The use of 12,000 genome equivalents/cell for infection is extraordinarily high.

The original manuscript omitted detailed explanations due to the page limitation. The revised version has now included detailed descriptions. (page 10, line 18~page 11, line 12; page 12, line 11~page 13, line 6)

Although the reviewer pointed out that 12,000 GEq/cell is too high for HBV infection, multiple groups used the similar or even much higher amount of HBV inoculum [more than 100,000 GEq/cell in Ni Y et al. Gastroenterology 146(4): 1070-1083 (2014); 50,000 GEq/cell in Zakrzewicz D et al. Viruses 14(6): 1259 (2022); 8,000 GEq/cell in Liu Y et al. 4(9): 100535 (2022)] for HBV infection assay. The amount of HBV inoculum used in our study is not abnormally too high.

Minor issues

1. Line 3 of the abstract, “to” appears to be extra

Following the reviewer’s suggestion, we have deleted “to”. (page 2, line 3)

2. Line 7 of abstract, compact-folded should be replaced by either compact or folded.

Following the reviewer’s suggestion, we have deleted “compact-”. (page 3, line 7)

3. Reference 3 and 14 are identical

We appreciate the reviewer for pointing out our mistake. We have removed the original ref. 19.

4. The recent structure of the HBs protein would make an improvement on the generic and wrong parallel bundle of helices in Fig 4 and extended figure 1 as it provides an understanding of where preS1 and PreS2 will be located. doi: 10.1126/sciadv.abo4184

We appreciate the reviewer for a good suggestion. We modified the drawing of LHBs in Fig. 4 and Extended Data Fig. 1a based on the current structural information of LHBs.

5. The call to Fig. 1b at the bottom of page 4 should be 1c.

We have referred to Extended Data Fig. 1a here. (page 4, line 4)

6. *The term high affinity for binding of peptide to NTCP should be quantified rather than described as high affinity. An IC50 value of about 8nM is reported in Gripon et al*

We appreciate the reviewer's comment. This comment is also related to the comment #1, #2, and #3. Accordingly, we have rewritten the introduction as follows (page 3, line 14~17),

“A myristoylated preS1 peptide (2-48 aa) effectively inhibits HBV/HDV infection, with a 50% inhibitory concentration of nM order or even lower^{3,7,10}, and has been recently approved in clinical use [Bulevirtide, also known as Myrcludex B (Myr-B)] for treating HDV infection in the European Union and Russia¹¹ (Fig. 1a).”

7. *What is the point of the sequence data in extended figure 2.*

The sequence alignment of NTCP (Extended Data Fig. 2) is necessary to discuss the species tropism of HBV infection. (page 17, line 4~)

8. *Figure 1 is too crowded and not well -organized.*

We appreciate the reviewer's critical comment, which might be a comment on Fig. 2. We have modified Fig. 2 for better viewing.

9. *Page 8, the phrase “PreS1 then folds back at the height of the crossover region defined between TM3 and TM8 ... “is hard to understand.*

We have rephrased the sentence. (page 8, line 13)

“PreS1 then folds back near the crossover region between TM3 and TM8 at the tunnel's midpoint and forms a loop structure between V7^{preS1} and L19^{preS1} (loop A) (Fig. 1f, 2i).”

10. *Addition of more labels to extended figure 6 would be helpful*

We have added more labels on Extended Data Fig. 6a.

11. *On page 11 the phrase “T268 and V272 are located more extracellularly form hydrophobic contacts” is confusing*

We appreciate the reviewer for pointing out our mistake. The “and” is accidentally omitted in the original manuscript. (page 11, line 10)

“whereas T268 and V272 are located more extracellularly and form hydrophobic contacts with W32^{preS1}”

12. Page 12 delete “long-awaited” from the opening of discussion

We have deleted “long-awaited”. (page 13, line 15)

Decision Letter, first revision:

Message: Our ref: NSMB-A47255A

16th Jul 2023

Dear Dr. Ohto,

Thank you for submitting your revised manuscript "Structural basis of hepatitis B virus receptor binding" (NSMB-A47255A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in a couple of weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, it is important that we have a copy of the main text as a word file. If you could please send along a word version of this file as soon as possible, we would greatly appreciate it; please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,
Sara

Sara Osman, Ph.D.
Associate Editor
Nature Structural & Molecular Biology

Reviewer #1 (Remarks to the Author):

The authors have addressed all comments and substantially improved the content and figures of the manuscript. I support publication in NSMB.

Reviewer #2 (Remarks to the Author):

Asami et al present the structures of NTCP and HBV preS1 in complex with one of two different Fab fragments. NTCP is the sodium-taurocholate co-transporting polypeptide; it is a hepatocyte specific bile acid receptor that has been co-opted as the receptor for Hepatitis B virus (HBV) and Hepatitis D virus (HDV). Pre-S1 is the N-terminal segment of the large form of the HBV surface antigen (L-HBs); the HBs open reading frame has three in-frame start codons resulting in large, medium and small forms of the surface protein. In Asami's structure the authors find that the N-terminal ~33 residues of pre-S1 form two compact loops that are jammed into the transmembrane tunnel of NTCP and the C-terminal 15 residues bind to the exterior of the NTCP. The bound peptide forms extensive contacts with NTCP, blocks the NTCP tunnel, and modestly deforms the NTCP structure. The work is a novel example of a virus recognizing a target by folding an intrinsically disordered peptide. It is of importance to understanding infection by HBV and HDV. It is of great importance to understanding the mechanism of entry inhibitors a class of drugs recently approved for treatment of HDV but still not fully understood.

I served as reviewer 2 on the original manuscript. My review emphasized the need to provide context. The authors have responded to most of my suggestions. I have a few additional comments that they should address.

Regarding my query 5 and the authors' response, I wrote "size exclusion data implies that binding the peptide makes the NTCP more compact". To which the authors replied "not true" and then said the earlier time is due to misfolding. Are the authors' saying

1. The NTCP eluting early has no pre-S1-binding activity and appears to be persistently inactive and is removed from subsequent experiments

Or,

2. In the absence of pre-S1, the NTCP is misfolded or denatured but addition of pre-S1 appears to induce correct folding and a more compact state

Or,

3. They have insufficient data to decide.

The authors' should include an appropriate comment about interpreting the figure. If addition of pre-S1 leads to recovery of fold, the point I made in the original review, it indicates that binding a ligand restricts NTCP freedom of motion, useful for understanding NTCP mechanism of action.

Regarding query 8:

Though there are a few other examples of the use of high amounts of HBV to induce infection, most of these are older studies. 12,000 genomes is 1-2 orders of magnitude higher than most in the field would use. This is for your future information.

Regarding Figure 4, the authors should provide a brief description of the illustration and include a citation explain the unusual outline for the membrane bound HBs

On line 107, you note "The width of the opening (distance between the Ca atoms of TM1 P22 and TM5 K157) of the tunnel of NTCP was increased by approximately 7 Å due to the presence of preS1 peptide". Yet in the same paragraph (line 101) you note an RMSD of only 1.3Å. This paradox should be explained in text.

Author Rebuttal, first revision:**Response to reviewers**

The comments of all reviewers were very useful in helping us to improve the quality of our manuscript. Our responses to the comments of each reviewer can be found below.

Referees' comments:**Referee #2 (Remarks to the Author):**

Asami et al present the structures of NTCP and HBV preS1 in complex with one of two different Fab fragments. NTCP is the sodium-taurocholate co-transporting polypeptide; it is a hepatocyte specific bile acid receptor that has been co-opted as the receptor for Hepatitis B virus (HBV) and Hepatitis D virus (HDV). Pre-S1 is the N-terminal segment of the large form of the HBV surface antigen (L-HBs); the HBs open reading frame has three in-frame start codons resulting in large, medium and small forms of the surface protein. In Asami's structure the authors find that the N-terminal ~33 residues of pre-S1 form two compact loops that are jammed into the transmembrane tunnel of NTCP and the C-terminal 15 residues bind to the exterior of the NTCP. The bound peptide forms extensive contacts with NTCP, blocks the NTCP tunnel, and modestly deforms the NTCP structure. The work is a novel example of a virus recognizing a target by folding an intrinsically disordered peptide. It is of importance to understanding infection by HBV and HDV. It is of great importance to understanding the mechanism of entry inhibitors a class of drugs recently approved for treatment of HDV but still not fully understood.

[We appreciate the reviewer for his/her positive rating on our manuscript.](#)

I served as reviewer 2 on the original manuscript. My review emphasized the need to provide context. The authors have responded to most of my suggestions. I have a few additional comments that they should address.

Regarding my query 5 and the authors' response, I wrote "size exclusion data implies that binding the peptide makes the NTCP more compact". To which the authors replied "not true" and then said the earlier time is due to misfolding. Are the authors' saying

1. The NTCP eluting early has no pre-S1-binding activity and appears to be persistently inactive and is removed from subsequent experiments

Or,

2. In the absence of pre-S1, the NTCP is misfolded or denatured but addition of pre-S1 appears to induce correct folding and a more compact state

Or,

3. They have insufficient data to decide.

The authors' should include an appropriate comment about interpreting the figure. If addition of pre-S1 leads to recovery of fold, the point I made in the original review, it indicates that binding a ligand restricts NTCP freedom of motion, useful for understanding NTCP mechanism of action.

The NTCP eluted earlier than the 1:1 complex was inactive possibly due to the misfolding or denaturing. The misfolded or denatured proteins sometimes elute earlier than correctly folded protein and give broad peak in SEC purification. This is a common observation when we purify detergent solubilized membrane protein in heterologous expression systems.

To clarify this point, we added the following description in the method section.

“The NTCP/myr-preS1 complex (1:1) fractions (14.5-17.5 ml) were collected and concentrated using an Amicon Ultra centrifugal filter (100-kDa MW cut-off). The NTCP eluting early around 13-14 ml appeared to be misfolded or denatured and was not used in the subsequent experiments.”

Regarding query 8:

Though there are a few other examples of the use of high amounts of HBV to induce infection, most of these are older studies. 12,000 genomes is 1-2 orders of magnitude higher than most in the field would use. This is for your future information.

We thank the reviewer for the suggestion.

Regarding Figure 4, the authors should provide a brief description of the illustration and include a citation explain the unusual outline for the membrane bound HBs

According to the suggestion from the reviewer, we added a brief description of Fig. 4.

“NTCP adopts two structures, inward-facing and outward-facing, during bile acids transport. Myr-preS1 only targets and stabilizes the outward-facing conformation of NTCP by anchoring its myristoyl group to the host membrane and occupying the extracellular side of membrane-penetrating tunnel.”

On line 107, you note “The width of the opening (distance between the C α atoms of TM1 P22 and TM5 K157) of the tunnel of NTCP was increased by approximately 7 Å due to the presence of preS1 peptide”. Yet in the same paragraph (line 101) you note an RMSD of only 1.3Å. This paradox should be explained in text.

The 7 Å is a distance between specific pair of atoms (the C α atoms of TM1 P22 and TM5 K157) and the RMSD is root mean square deviation for all atoms in NTCP. Therefore, there is no discrepancy between these two figures.

Final Decision Letter:

Message 24th Nov 2023

:

Dear Dr. Ohto,

We are now happy to accept your revised paper "Structural basis of hepatitis B virus receptor binding" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Sincerely,
Sara

Sara Osman, Ph.D.
Associate Editor
Nature Structural & Molecular Biology