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G. 知的財産権の出願・登録状況

特になし

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

自然免疫センサーRIG-IによるHCV増殖阻害機構の解明とそれを基盤にした抗HCV製剤の開発

研究分担者　藤田　尚志　京都大学ウイルス研究所　教授

研究要旨：内在性のRIG-Iを細胞染色で検出できる抗RIG-I抗体を作製した。この抗体を用いて、HCVおよび各種RNAウイルスの感染細胞を染色、観察を行った。その結果、各種ウイルスは細胞質の特定の場で増殖を行い、その場にRIG-Iの局在が誘導されることが明らかとなった。また、RIG-Iだけではなく、一群のRNA結合蛋白質群が集合することが明らかとなった。このことはRIG-Iが単独でウイルスRNAを認識する、と云う従来の概念を書き換えるものと考えられる。

A．研究目的

HCV感染細胞の中でウイルスの増殖が起ったとき、宿主である肝細胞はそれを感知する機構をそなえている。本研究ではウイルスRNAが細胞のどこで感知されているのかを解明することを主目的としている。この「場」の解明は抗ウイルス薬の開発等に際して重要な情報となることが期待される。

B．研究方法

RIG-I を特異的に検出することの出来る、抗RIG-I抗体を作製し、それを免疫染色に用いてHCVやその他のウイルスの感染細胞のどこにRIG-Iが局在するか光学顕微鏡、電子顕微鏡を用いて検討を行なった。ヒト、動物に関する実験含まれていないため倫理面での問題は無い。

C．研究結果

我々の作製した抗RIG-I抗体はウイルス感染細胞でのRIG-Iの局在の変化を検出できるプローブとして使えることが判明した。HCVは油滴の周辺域で増殖をし、そこにウイルス抗原の集積が認められる。そこに一致してRIG-Iの局在が誘導されることが明らかとなった。また、RIG-I以外の関連蛋白質、MDA5、LGP2およびその他のRNA結合蛋白質も同様に局在が誘導されることが明らかとなった。

D．考察

HCVの複製場所にはウイルスセンサーであるRIG-Iの局在が誘導されることを明らかとした。このときRIG-Iのみではなく、その他のRNA結合蛋白質も集まってくる。このことはウイルスRNAの認識には多くのRNA結合蛋白質が直接、間接に関与することを示唆している。また、同時に認識からシグナル伝達の段階にもこれらの複数のRNA結合蛋白質が関与することを示唆している。

E．結論

HCVの増殖複合体へRIG-Iの局在が誘導される時にRIG-Iのみならず他の蛋白質の誘導も重要であることが強く示唆された。この局在を誘導するメカニズムの解明は抗HCV薬剤の開発に繋がると考えられる。

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G. 知的財産権の出願・登録状況

無し

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

HCVによる自然免疫システムの撹乱機構の解析

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研究要旨:昨年度までに、ヒト不死化肝PH5CH8細胞内でHCVのNS3-4AはCardifを切断することでそのシグナル伝達経路をほぼ完全に抑制するが、TRIFを介するシグナル伝達経路を抑制することができないことを明らかにした。また、NS5BによるIFN-βの産生は２本鎖RNAの合成によるTLR3経路およびRIG-I/MDA5経路の活性化、さらには両経路の下流の転写因子IRF-3の活性化を経て誘導されることを明らかにした。今年度は、(1)HCV遺伝子型2a型のJFH-1株、またHCVと近縁のGBV-BのNS3-4AでもCardifは切断できるが、TRIFは切断できないことを明らかにした。(2)JFH-1、GBV-B由来のNS5BによりPH5CH8細胞でIFN-βが産生されることを見出した。HCVおよびその近縁のGBV-BではNS5Bによる自己のRNA遺伝子合成の副産物である２本鎖RNAがIFN-βvを産生してしまう。このことはウイルスの複製にとっては負の効果をもたらす。HCVやGBV-BはNS3-4AがCardifを切断することで、この問題を克服し持続感染出来る仕組みを手に入れた可能性が示唆された。

A. 研究目的

C 型肝炎ウイルス(HCV)感染は高頻度に慢性肝炎を引き起こし、15-30 年を経て肝硬変、肝癌へと進行する。肝癌の原因の８割が HCV 感染によるが、肝癌に対する決定的な治療法がないのが現状である。HCV の持続感染状態である C 型慢性肝炎は肝細胞のがん化の重要な因子であるが、HCV による持続感染機構およびそれに起因する肝発がん機構については未だよく理解されていない。HCV の持続感染する機構を明らかにし、ウイルスの持続感染を断ち切ることができれば、肝発癌のリスクを軽減することが可能となる。本研究では、ウイルスに対する宿主の防御機構（自然免疫機構）としての IFN 産生システムを HCV がどのように撹乱抑制しているのかを解明することで、現状のインターフェロン／リバビリン併用療法の治療効果の改善を目的としている。これまでに、遺伝子型１b 型 HCV の NS5B が IFN-b の発現を誘導すること、NS3-4A が Cardif を切断し IFN-b の発現を抑制することを報告した。本年度は、遺伝子型 2a 型 HCV 株の JFH1 と HCV に近縁の GBV-B の NS5B、NS3-4A を用いて IFN-b の発現に及ぼす効果を検討した。

B. 研究方法

遺伝子型 2a 型 HCV 株の JFH1 あるいは HCV に近縁のウイルスである GBV-B 由来の NS5B 蛋白質を恒常的に発現するヒト不死化細胞 PH5CH8 細胞(PH5CH8/NS5B 細胞)を作成し、IFN-βの mRNA を定量比較した。

JFH1 が効率よく感染し、複製、粒子産生できる HuH-7 細胞由来の RSc 細胞に HCV cc を MOI 0.1 で感染させ、96 時間後に Trif、Cardif が切断されるかについて Western blot解析を行った。

GBV-B 由来の NS3-4A を Trif あるいは、Cardif とともに発現する PH5CH8 細胞を作成し、Trif、Cardif が切断されるかについて Western blot 解析を行った。

細胞内での二本鎖 RNA は免疫蛍光抗体法により調べた。カバーガラス上に播種した PH5CH8/NS5B 細胞に抗二本鎖 RNA 認識抗体を反応させた後、蛍光色素で標識した二次抗体を反応させた。その後、レーザー共焦点顕微鏡を用い、細胞内の二本鎖 RNA を観察した。

内在性の MDA5 の多量化については、回収した粗蛋白質画分を Native-PAGE にて分離して、抗 MDA5 抗体より検出した。

（倫理面への配慮）

本研究においては、実験及び解析に用いた材料は全てこれまでに確立されているものであり、本年度の

研究にはヒトの臨床材料を用いたものがない。そのために倫理面への特段の配慮はなかった。但し、実験に使用した細胞および核酸については蒸気滅菌を施した後に廃棄した。

C. 研究成果

ヒト不死化細胞 PH5CH8 細胞に JFH-1 由来の NS5B を恒常的に発現させた細胞では遺伝子型 1b 型 HCV 由来の NS5B 同様 IFN-β の mRNA が誘導された。一方、ベクタープラスミドを導入した PH5CH8 細胞では IFN-β の mRNA の誘導は認められなかった。

GBV-B 由来の NS5B を恒常的に発現させた PH5CH 細胞でも IFN-β の mRNA が誘導された。さらに、dsRNA を特異的に認識する抗体を用いた免疫蛍光抗体法で PH5CH8/NS5B 細胞で dsRNA を検出することが出来た。また、この細胞で、Native-PAGE による Western blot 解析により MDA5 の多量体の形成を確認できた。

これらの結果は 1b 型 HCV 由来の NS5B のみならず 2a 型 HCV 由来の NS5B、さらには HCV 近縁の GBV-B 由来の NS5B にも dsRNA 産生を介した IFN-β の誘導能があることを示唆しているものと思われる。

HuH-7 細胞由来の RSc 細胞に JFH-1 由来の HCVcc を感染させた時、Cardif は切断されたが、Trif は切断されなかった。また、PH5CH8 細胞に GBV-B 由来の NS3-4A を発現させた PH5CH8 細胞でも、Cardif は切断されたが、Trif は切断されなかった。

D. 考察

昨年度までに遺伝子型 1b 型 HCV 由来の NS5B による IFN-β の誘導、NS3-4A による Cardif の切断を報告した。本年度は遺伝子型 2a 型 HCV あるいは GBV-B 由来の NS5B により IFN-β の誘導、NS3-4A による Cardif の切断が起こるかについて検討した。

HCV2a 型、GBV-B のいずれの NS5B 蛋白質でも IFN-β の発現誘導が認められた。また、dsRNA の産生、MDA5 の多量化が認められたことより、IFN-β の産生は dsRNA 産生による RIG-I/MDA5 経路の活性化によるものであることが示唆された。

一方、HCV2a 型、GBV-B のいずれの NS3-4A 蛋白質でも Cardif は切断されたが、Trif は切断されなかった。

本年度は HCV の遺伝子型 1b 型で認められた NS5B による IFN-β の産生、NS3-4A による Cardif の切断が遺伝子型 2a、HCV 近縁のウイルスである GBV-B でも確認することができた。NS5B、NS3-4A による IFN-β 産生系への効果は HCV の遺伝子型全般あるいは、HCV 以外のフラビウイルスに共通した現象である可能性が示唆された。

NS5B は IFN-β 産生へ、NS3-4A は IFN-β 産生抑制という、一見相反する機能を有しているが、NS5B の機能に比べて NS3-4 の機能が優っているために、IFN-β 産生系のバランスは抑制に傾き HCV の持続感染を可能にしているのかもしれない。HCV は自己の遺伝子複製の副産物として産生される dsRNA による IFN-β の誘導を克服する手段として NS3-4A による Cardif を切断し IFN-β の誘導を断ち切る機能を進化の過程で獲得した可能性が考えられる。

NS5B (IFN-β 産生) を NS3-4A (IFN-β 抑制) に比べて優位にするような方法は、持続感染阻止の手段となるかもしれない。

E. 結論

2a 型 HCV あるいは GBV-B 由来の NS5B による IFN-β の誘導、NS3-4A による Cardif の切断を見出した。

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G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

IRF7を介した血清由来HCVの感染増殖抑制機構の解明とそれを応用したHCV増殖抑制戦略の構築

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研究要旨　ここれまでにC型肝炎ウイルス(HCV)の感染増殖が転写因子であるインターフェロン(IFN)調節因子(IRF)7の機能によって抑制されることを明らかにしてきたが、肝細胞における自然免疫のメカニズムは全く不明であった。そこで我々が開発した新たな不死化肝細胞を用いて肝細胞における自然免疫機構について解析をおこなった。この不死化肝細胞は我々が独自に樹立した細胞で有り長期にわたり初代培養肝細胞に類似した形質を維持している特徴を有している。他の肝癌由来細胞株と異なり、この不死化肝細胞では初代培養肝細胞同様にIRF7およびTLR8の恒常的な発現が認められた。この細胞ではセンダイウイルス感染後3時間で、IRF3ではなくIRF7がRIG-I非依存的に活性化されていた。この感染早期のIRF7活性化にはTLR8が機能していることが分かった。さらにこのTLR8からのIRF7依存的シグナルによって、もともと肝細胞中で発現の低いRIG-I遺伝子の発現が誘導されることが明らかとなった。RNA干渉法を用いてこの細胞のTLR8の発現を抑制することにより、HCV感染の初期においてTLR8はその増殖抑制に機能しているが、感染成立後にはほとんど影響を与えないことがわかった。このことから、肝細胞ではHCV感染初期にはTLR8によるIRF7依存的なRIG-Iの発現誘導機構が存在し、HCV感染検出の感度を増強する機能が存在していることが明らかとなった。このことからIRF7の機能を向上させる方法の開発により肝細胞を抗HCV状態に変化させることが可能であることがわかった。

A. 研究目的

血清由来C型肝炎ウイルスが効率良く感染増殖する不死化肝細胞を用いて、このウイルスの感染増殖を抑制する自然免疫機構、特にIRF7の肝細胞における機能の詳細を明らかにして、これを効果的に亢進させることによる抗HCV戦略構築を目指した。

B. 研究方法

1. 既に樹立している新規ヒト不死化肝細胞ならびに市販の初代培養肝細胞における各種自然免疫関連遺伝子の発現をRT—PCR法等で解析した。
2. この細胞センダイウイルスを感染させ、IRF3ならびにIRF7の経時的な活性化機構をその核内移行およびインターフェロン遺伝子プロモーターの活性化を指標に検討した。
3. TLR8からのシグナル経路の解析とHCV血清由来HCVの感染増殖の関連を解析した。

（倫理面への配慮）

この研究はあらかじめ京都大学医学部医の倫理委員会に申請し、審査の後に承認されたものである。不死化肝細胞作製に用いた肝臓や感染実験に用いた血液提供者へのインフォームドコンセントや個人情報の管理は上記委員会の規定通りにおこなわれており、倫理面に関する問題はない。

C. 研究結果

1. ヒト各組織からの総RNAを用いたRNAブロット法（市販のものを使用）の解析からIRF7が肝臓内で発現していることが確認されたが、不死化肝細胞と異なる２種類の初代培養肝細胞内ではIRF7遺伝子の恒常的な発現が認められた。また、TLR 8mRNAの発現も同様に観察された。このことから肝細胞においてIRF7とTLR8遺伝子が恒常的に発現していることがわかった。一方、これまでHCVレプリコンが効率良く複製し、また組換え体HCVの効率良い感染増殖が可能であ

ることがわかっている肝癌由来細胞株（HuH-7細胞）では両遺伝子の発現は認められなかった。またHCVなどのウイルス感染を検出するRIG-IのmRNAレベルはすべての肝由来細胞で極めて低レベルであることがわかった。

2. この不死化肝細胞にセンダイウイルスを感染させると3時間でIRF7タンパク質が核内に移行していることが観察できるがこの時IRF3はまだ細胞質に存在することが蛍光免疫組織染色で分かった。またこの時インターフェロンalpha遺伝子プロモーターの活性化がおこるがこれはRIG-I非依存的に活性化されることがわかった。この感染早期のIRF7活性化はTLR8アゴニストで生じることが分かった。
3. このTLR8からのIRF7依存的シグナルによって、もともと肝細胞中で発現の低いRIG-I遺伝子はセンダイウイルス感染によって6時間後に発現誘導されるが、これはTLR8に対するRNA干渉法によって抑制された。
4. 血清由来HCVの不死化肝細胞に対する感染は、この細胞を予めRNA干渉法を抑制した場合、効率良く観察することができたが、感染3日後に抑制した場合には感染後の増殖にはほとんど影響を与えないことがわかった。

D. 考察

1. 肝細胞ではTLR8によるIRF7依存的なRIG-Iの発現誘導機構が存在し、ウイルスの感染検出の感度を増強する機能が存在している可能性が示唆された。

E. 結論

1. 肝細胞ではIRF7がウイルス感染初期においてTLR8からのシグナルを伝え、RIG-I遺伝子を誘導してウイルスRNAの検出効率を上昇させ、またRIG-I活性化後はインターフェロン遺伝子の誘導に機能するというようにHCV感染初期と後期のそれぞれにおいて重要な役割を果たすことが分かった。
2. IRF7の機能を向上させる方法の開発により肝細胞を抗HCV状態に変化させることが可能であることが考えられた。

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第18回広島肝臓研究会　平成21年11月6日、広島　2009

G. 知的所有権取得状況

1. 特許取得　特になし。
2. 実用新案登録　特になし。
3. その他　特になし。

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

ウイルスの持続感染機序の解析及びその制御に関する研究

研究分担者　小原道法（財）東京都医学研究機構・東京都臨床医学総合研究所　副参事研究員

研究要旨：HCV感染病態モデルマウスを樹立することに成功し、このマウスでHCV遺伝子組換えワクチニアウイルス(rVV)による免疫活性化によるHCV排除を試みた。接種後１週目で、ｒVV-N25群の肝臓において壊死性細胞浸潤、肝細胞索の乱れ、肝細胞の膨化、グリコーゲン変性および脂肪変性といった慢性肝炎の病態の正常化が認められた。

A．研究目的

HCV感染病態モデルマウスを樹立することに成功し、このマウスでHCV-rVVによる免疫活性化によるHCV排除を試みた。

B．研究方法

HCVの持続感染成立機序、慢性肝炎・肝硬変・肝癌への推移機構解明の為に任意の時期にHCV遺伝子をスイッチング発現Tgマウスを樹立した。このマウスを用いて、HCV-rVVによる免疫活性化によるHCV排除を試みた。HCV-ｒVVはHCVの構造蛋白質を主に発現するｒVV-CN2、非構造蛋白質を発現する-N25、全蛋白質を発現する-CN5を用いた。

（倫理面への配慮）

動物実験は東京都臨床医学総合研究所の実験動物指針に基づいて行った。

C．研究結果

接種後１週目で、ｒVV-N25群の肝臓において壊死性細胞浸潤、肝細胞索の乱れ、肝細胞の膨化、グリコーゲン変性および脂肪変性といった慢性肝炎の病態の正常化が認められた。4週目のｒVV-N25接種群では形態異常の正常化、肝臓内のHCV蛋白の減少がみられた。

D．考察

ｒVV-N25のHCV蛋白の排除にはCD４およびCD8＋T細胞が重要であることが示唆された。しかし、肝臓の形態異常は抗CD8抗体および抗CD4抗体を投与したにも関わらず正常化していたことから、病態形成とHCV蛋白排除は別の機序であることが明らかとなった。

E．結論

ｒVV-N25接種によるHCV蛋白の制御には細胞死を伴わない何らかの蛋白排除機構が働いていることが示唆された。

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G．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

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Cochaperone Activity of Human Butyrate-Induced Transcript 1 Facilitates Hepatitis C Virus Replication through an Hsp90-Dependent Pathway▽

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Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a component of the replication complex consisting of several host and viral proteins. We have previously reported that human butyrate-induced transcript 1 (hB-ind1) recruits heat shock protein 90 (Hsp90) and FK506-binding protein 8 (FKBP8) to the replication complex through interaction with NS5A. To gain more insights into the biological functions of hB-ind1 in HCV replication, we assessed the potential cochaperone-like activity of hB-ind1, because it has significant homology with cochaperone p23, which regulates Hsp90 chaperone activity. The chimeric p23 in which the cochaperone domain was replaced with the p23-like domain of hB-ind1 exhibited cochaperone activity comparable to that of the authentic p23, inhibiting the glucocorticoid receptor signaling in an Hsp90-dependent manner. Conversely, the chimeric hB-ind1 in which the p23-like domain was replaced with the cochaperone domain of p23 resulted in the same level of recovery of HCV propagation as seen in the authentic hB-ind1 in cells with knockdown of the endogenous hB-ind1. Immunofluorescence analyses revealed that hB-ind1 was colocalized with NS5A, FKBP8, and double-stranded RNA in the HCV replicon cells. HCV replicon cells exhibited a more potent unfolded-protein response (UPR) than the parental and the cured cells upon treatment with an inhibitor for Hsp90. These results suggest that an Hsp90-dependent chaperone pathway incorporating hB-ind1 is involved in protein folding in the membranous web for the circumvention of the UPR and that it facilitates HCV replication.

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis in humans and infects approximately 170 million people worldwide (64). HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and is classified into six major genotypes (39). The virus forms small, round, enveloped particles and possesses a genome consisting of a single positive-stranded RNA with a nucleotide length of 9.6 kb. The viral genome encodes a single precursor polyprotein consisting of approximately 3,000 amino acids, which in turn is posttranslationally processed into 10 viral proteins by host and viral proteases. The structural proteins are cleaved from the N-terminal one-fourth of the polyprotein by the host signal peptidase and signal peptide peptidase (36, 43, 44), resulting in the maturation of capsid protein, two envelope proteins, and viroporin p7. The nonstructural protein 2 (NS2) protease cleaves its own carboxyl terminus, and then NS3 cleaves the appropriate downstream positions to produce NS3, NS4A, NS4B, NS5A, and NS5B (24, 60), which form the replication complex, together with several host proteins (14, 35).

NS5A is a membrane-anchored zinc-binding phosphoprotein that appears to possess diverse functions, including the suppression of host defense and the regulation of virus replication (1, 15, 58), but its biological function remains unclear. Several groups, including ours, have suggested that the molecular chaperone, heat shock protein 90 (Hsp90), and several cochaperones participate in the replication complex of HCV through interaction with NS5A or other NS proteins (45, 56, 65). Hsp90 is the highly conserved and ubiquitously expressed protein that acts as a key regulator for the turnover and the activities of more than 200 signaling proteins, including steroid receptors and cell-signaling kinases (66). The chaperone activity of Hsp90 contributes to the refolding of an unfolded protein in an ATP-dependent manner, and the execution of Hsp90-dependent protein folding requires the formation of a multichaperone complex containing other chaperones (e.g., Hsp70, Hsp104, and Hsp40) and cochaperones (e.g., p23, Hop, and immunophilins) (4, 18, 48). Geldanamycin or its derivatives, which are represented as specific inhibitors of Hsp90, can destabilize and then degrade client proteins (41, 55).

The host chaperone mechanism is involved in the folding of viral polymerase to support viral replication (6, 27). Moreover, host chaperones have been reported to play roles in the assembly of viral particles and the sorting of virus proteins (9, 32, 38). We have previously reported that Hsp90 chaperone activities and chaperone-associated proteins are required for the efficient propagation of HCV (45, 56) and that human butyrate-induced transcript 1 (hB-ind1) is involved in the propagation of HCV through interactions with NS5A and Hsp90 via the coiled-coil domain and the FXXW motif, respectively (56). hB-ind1 was first reported to be a multiple-membrane-spanning protein consisting of 362 amino acids that possesses a significant homology with a cochaperones, p23, that regulates

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Hsp90 function by its cochaperone activity (11). However, the roles of hB-ind1 in the life cycle of HCV have not been precisely clarified. In this study, we investigated the role of the Hsp90-related chaperone system, including hB-ind1, in the regulation of the RNA replication and particle production of HCV.

MATERIALS AND METHODS

Plasmids. The plasmids encoding hB-ind1, NS5A, Hsp90, and FK506-binding protein 8 (FKBP8) were prepared by methods described previously (45, 56). The DNA fragments encoding hB-ind1 mutants were prepared by PCR with the introduction of a silent mutation that is resistant to the short hairpin RNA in the hB-ind1 knockdown cells, as described previously (56). The human p23 gene and glucose-regulated protein 78 (GRP78) promoter region (−151 to +22) were amplified by PCR from the total cDNA and genomic DNA of Huh7 cells, respectively. The DNA fragments encoding mutants of hB-ind1 and p23 were prepared by the method of splicing by overlap extension (26) and introduced into pEF FLAGGs pGKpuro (28). The GRP78 promoter region was introduced between the KpnI and HindIII sites of pGL3-basic (Promega, Madison, WI) and designated pGRP78-luc. The reporter plasmid carrying a firefly luciferase gene under the control of the GR promoter (pGR-luc) was purchased from Panomics (Fremont, CA). The internal-control plasmid encoding a *Renilla* luciferase (pRL-TK) was purchased from Promega. The plasmid pFK-I_{389} neo/NS3-3′/NK5.1 (47) was kindly provided by R. Bartenschlager. The plasmids used in this study were confirmed by sequencing them with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cells and virus infection. All cell lines were cultured at 37°C under a humidified atmosphere and 5% CO_2. The human embryonic kidney 293T and hepatocellular carcinoma Huh7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS). The human hepatocellular carcinoma cell line Huh7.5.1 was kindly provided by F. Chisari (70) and was maintained in DMEM containing nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. The Huh9-13 cell line, which is a Huh7 cell line harboring a subgenomic HCV RNA replicon (35), was maintained in DMEM containing 10% FCS, nonessential amino acids, and 1 mg/ml G418 (Nakalai Tesque, Kyoto, Japan). The hB-ind1 knockdown cell line Huh-KD and control cell line Huh-ctrl were described previously (56). Huh-KD cells were transfected with each of the expression plasmids encoding wild-type or mutant hB-ind1 and cultured for 1 week in the presence of 10 μg/ml of puromycin. The remaining cells were used for the experiments described below. The viral RNA of JFH1 was introduced into Huh7.5.1 cells according to the method of Wakita et al. (62) for preparation of the infectious HCV particles in cell culture.

Antibodies. The rabbit anti-hB-ind1 antibody was prepared as described previously (56). Mouse monoclonal antibodies to HCV NS5A, influenza virus hemagglutinin (HA) and FLAG tags, and β-actin were purchased from Austral Biologicals (San Ramon, CA), Covance (Richmond, CA), and Sigma, respectively. Mouse anti-protein disulfide isomerase (PDI) immunoglobulin G2a (IgG2a) was from Affinity Bioreagents (Golden, CO). Mouse anti-double-stranded RNA (dsRNA) IgG2a (J1 and K2) antibodies were from Biocenter Ltd. (Szirak, Hungary). Alexa Fluor 488 (AF488)-conjugated anti-mouse IgG1, AF647-conjugated anti-rabbit IgG, and AF594-conjugated anti-mouse IgG2a and IgG2b antibodies were from Invitrogen (San Diego, CA).

Transfection, immunoblotting, and immunoprecipitation. Transfection and immunoprecipitation analyses were carried out as described previously (25, 45). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan). The protein bands of GRP78 and β-actin were quantified by Multi Gauge software (Fujifilm), and the values of GRP78 expression were normalized with those of β-actin.

Quantitative reverse transcriptase PCR. HCV RNA was estimated by the method described previously (56). Total RNA was prepared from cells by using an RNeasy minikit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using an RNA LA PCR in vitro cloning kit (Takara Bio Inc., Shiga, Japan) and random primers. Each cDNA was estimated with Platinum SYBR green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI Prism 7000 (Applied Biosystems). The internal ribosomal entry site regions of HCV and mRNAs of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), GRP78, and growth arrest- and DNA damage-inducible gene 153 (GADD153) were amplified using the primer pairs 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCACCCTATCA-3′, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGGTGAAGGTCGGAGTC-3′, 5′-CGCCAAGCGGCTCATC-3′ and 5′-AACCACCTTGAACGGCAAGA-3′, and 5′-AGCTGGAACCTGAGGAGAGA-3′ and 5′-TGGATCAGTCTGGAAAAGCA-3′, respectively. The values of the HCV genome or each mRNA were normalized with those of GAPDH mRNA. Each PCR product was detected as a single band of the correct size on agarose gel electrophoresis (data not shown).

In vitro transcription and RNA transfection. The plasmid pFK-I_{389} neo/NS3-3′/NK5.1 was linearized by treatment with ScaI and then transcribed in vitro using the MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The in vitro-transcribed RNA was electroporated into cells at 4 million cells/0.4 ml under conditions of 270 V and 960 μF using a Gene Pulser (Bio-Rad, Hercules, CA). The colony formation assay was carried out by a method described previously (45).

Indirect immunofluorescence assay. Cells cultured on glass slides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. After being washed twice with PBS, the cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with PBS containing 0.2% gelatin (gelatin-PBS) for 60 min at room temperature. The cells were incubated with gelatin-PBS containing rabbit anti-hB-ind1 antibody, mouse anti-NS5A IgG1, mouse anti-PDI IgG2a, mouse anti-FKBP8 IgG2b, or mouse anti-dsRNA IgG2a (J1 and K2) at 37°C for 60 min; washed three times with PBS containing 1% Tween 20; and incubated with gelatin-PBS containing AF488-conjugated anti-mouse IgG1 or AF647-conjugated anti-rabbit or AF594-conjugated anti-mouse IgG2a or IgG2b antibodies at 37°C for 60 min. Finally, the cells were washed three times with PBS containing 1% Tween 20 and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Correlative FM-EM. Correlative fluorescence microscopy-electron microscopy (FM-EM) allows individual cells to be examined both in an overview with FM and in a detailed subcellular-structure view with EM (51). The endogenous hB-ind1 and NS5A were stained and observed in the HCV replicon cells by the correlative FM-EM method as described previously (45).

Luciferase assay. Each plasmid was transfected into Huh7, Huh9-13, and interferon (IFN)-cured cells seeded in a 12-well plate, and the cells were treated with 1 μM dexamethasone (Sigma) for 12 h or with 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (DMAG) (Sigma) for 6 h at 36 h posttransfection and lysed in 200 μl of passive lysis buffer (Promega). Luciferase activity was measured in 20-μl aliquots of the cell lysates using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase cotransfected with the internal-control plasmid pRL-TK. The resulting values were expressed as the increase in relative light units (RLU).

Statistical analysis. Results were expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t* test.

RESULTS

The p23-like domain of hB-ind1 has cochaperone activity. Although we had previously reported that hB-ind1 regulates HCV RNA replication through interaction with NS5A and Hsp90, the molecular mechanisms underlying the regulation of HCV replication remained to be clarified. To gain more insights into the potential cochaperone activity of hB-ind1 in the Hsp90 chaperone system, we prepared expression plasmids encoding a wild-type p23 and three p23 mutants—one in which the FXXW motif was replaced with AXXA (p23AxxA), one in which the cochaperone domain of p23 was replaced with the p23-like domain of hB-ind1 (cp23), and one in which both substitutions were made (cp23AxxA) (Fig. 1A). HA-tagged Hsp90 was coexpressed with FLAG-tagged p23 or the FLAG-tagged p23 mutants in 293T cells (Fig. 1B). Hsp90 was coimmunoprecipitated with wild-type p23 and a cp23 mutant, but not with the p23AxxA or cp23AxxA mutants, indicating that the FXXW motif of hB-ind1, as is the case with that of p23

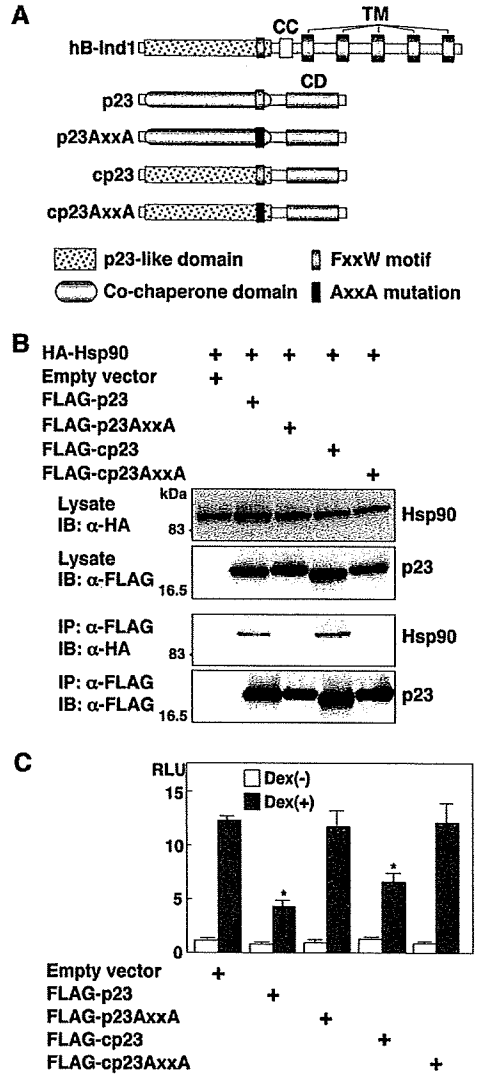

FIG. 1. Construction and characterization of p23 mutants. (A) Structures of hB-ind1, p23, and the three p23 mutants. hB-ind1 consists of a p23-like domain, an FXXW motif, a coiled-coil domain (CC), and a transmembrane domain (TM). p23 consists of a cochaperone domain, an FXXW motif, and a chaperone domain (CD). The three p23 mutants, p23AxxA, cp23, and cp23AxxA, were constructed by replacing the FXXW motif with AXXA, the cochaperone domain of p23 with the p23-like domain of hB-ind1, and both of the regions, respectively. (B) FLAG-tagged p23, p23AxxA, cp23, or cp23AxxA was coexpressed with HA-tagged Hsp90 in 293T cells and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting (IB). (C) The expression plasmid encoding FLAG-tagged p23, cp23, p23AxxA, or cp23AxxA was cotransfected with pGR-luc and pRL-TK plasmids into 293T cells and treated with 1 mM dexamethasone [Dex(+)] at 36 h posttransfection or untreated [Dex(−)], and the luciferase activities were determined at 12 h of incubation. The firefly luciferase activity was normalized with that of *Renilla* luciferase, and the GR-responsive promoter activity was indicated as the RLU. The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) versus the control value. The data shown are representative of three independent experiments.

(67), is also involved in binding to Hsp90. Hsp90 participates in the folding and stabilization of the ligand-binding domain of the glucocorticoid receptor (GR), together with p23 and other cofactors (49). p23 was shown to act not only in the activation (30), but also in the inhibition, of GR signaling (67). To examine whether hB-ind1 has the ability to work as a cochaperone in an Hsp90-dependent manner, each of the plasmids encoding p23 or the p23 mutants was cotransfected with a reporter plasmid carrying a firefly luciferase gene under the control of the GR promoter (pGR-luc), together with an internal-control plasmid (pRL-TK), and GR-mediated transcriptional activity was determined at 12 h after treatment with dexamethasone, a ligand of GR. Expression of the p23 or cp23 mutant, but not of the AXXA mutants, significantly inhibited GR-mediated transcription (Fig. 1C). These results indicate that the p23-like domain of hB-ind1 possesses cochaperone activity comparable to that of p23.

The p23-like domain of hB-ind1 is interchangeable with the p23 cochaperone domain during complex formation with NS5A, Hsp90, and FKBP8. Previous reports have suggested that HCV NS5A interacts with several host proteins, including FBL2 (63), vesicle-associated membrane protein-associated protein subtype A (VAP-A) (61), VAP-B (25), FKBP8 (45), and hB-ind1 (56), and that these interactions participate in the replication of HCV. We have shown that hB-ind1 interacts with NS5A and Hsp90 through the coiled-coil domain and the FXXW motif in the p23-like domain, respectively, and that coexpression of FKBP8 enhances the interaction of Hsp90 with hB-ind1 (56). To determine the effect of the mutation in the p23-like domain of hB-ind1 on interaction with Hsp90, NS5A, and FKBP8, we prepared an expression plasmid encoding wild-type hB-ind1 and three hB-ind1 mutants, one in which the p23-like domain was replaced with the cochaperone domain of p23 (chB-ind1), one in which the FXXW motif was replaced with AXXA (hB-ind1AxxA), and one in which both replacements were made (chB-ind1AxxA) (Fig. 2A). The FLAG-tagged wild-type or mutant hB-ind1 was coexpressed with HA-tagged Hsp90 (Fig. 2B, left) or HA-tagged NS5A (Fig. 2B, right) in 293T cells and immunoprecipitated with anti-FLAG antibody. Hsp90 was coprecipitated with wild-type hB-ind1 and the chB-ind1 mutant, but not with the hB-ind1AXXA and chB-ind1AXXA mutants (Fig. 2B, left), confirming that the FXXW motif is crucial for the interaction with Hsp90. In contrast, NS5A was coprecipitated with each of the hB-ind1 proteins, suggesting that mutation in the p23-like domain of hB-ind1 has no effect on the binding of hB-ind1 to NS5A through the coiled-coil domain (Fig. 2B, right). To determine the effect of FKBP8 expression on the interaction between hB-ind1 and Hsp90, FLAG-tagged wild-type hB-ind1 or the chB-ind1 mutant was coexpressed with HA-tagged FKBP8 and/or Hsp90 in 293T cells and immunoprecipitated with anti-FLAG antibody. The amounts of Hsp90 coprecipitated with hB-ind1 or chB-ind1 were increased by coexpression of FKBP8 (Fig. 2C). To further examine the interaction of hB-ind1 with Hsp90 and NS5A at an endogenous expression level in Huh9-13 cells harboring an HCV subgenomic RNA replicon, lysates of the replicon cells were subjected to immunoprecipitation analysis. Endogenous Hsp90 and NS5A were specifically coimmunoprecipitated with endogenous hB-ind1 (Fig. 2D). These results suggest that the p23-like domain of hB-ind1 is inter-

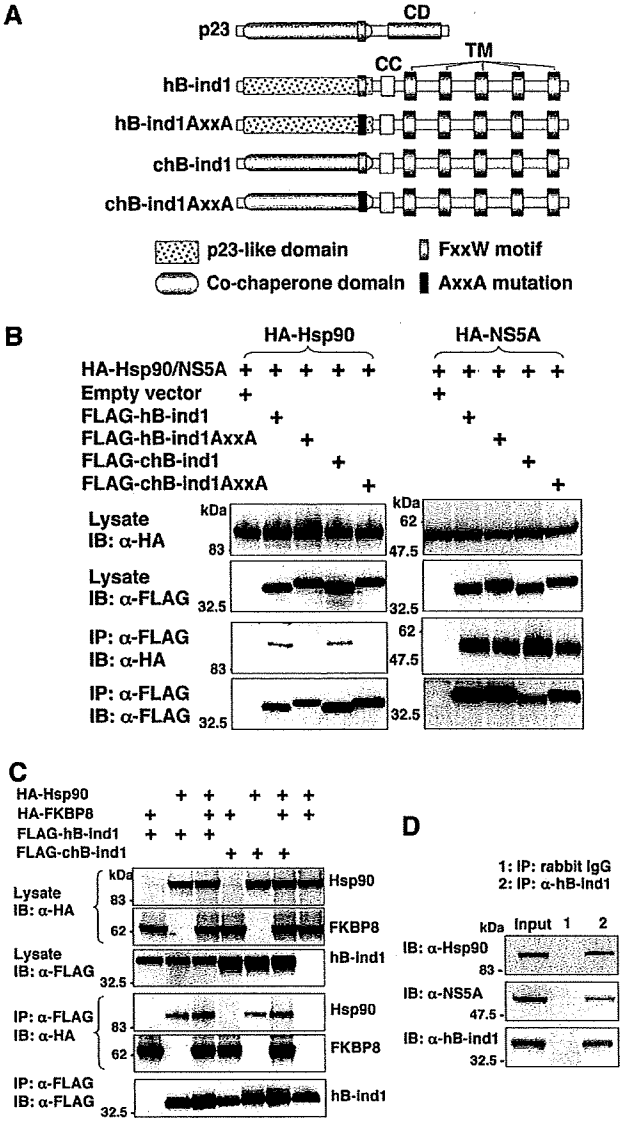

FIG. 2. Construction and characterization of hB-ind1 mutants. (A) Structures of p23, hB-ind1, and the three hB-ind1 mutants. The three hB-ind1 mutants, hB-ind1AxxA, chB-ind1, and chB-ind1AxxA, were constructed by replacing the FXXW motif with AXXA, the p23-like domain of hB-ind1 with the cochaperone domain of p23, and both of the regions, respectively. (B) FLAG-tagged hB-ind1, hB-ind1 AxxA, chB-ind1, or chB-ind1AxxA was coexpressed with either HA-tagged Hsp90 (left) or NS5A (right) in 293T cells and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting (IB). (C) HA-tagged Hsp90 and HA-FKBP8 were expressed with FLAG-tagged hB-ind1 and chB-ind1 in various combinations in 293T cells and immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were detected by immunoblotting. (D) Endogenous hB-ind1 in Huh9-13 cells harboring subgenomic HCV replicon RNA was immunoprecipitated with anti-hB-ind1 rabbit IgG (lane 2). The cell lysate was mixed with normal rabbit IgG as a negative control (lane 1). The immunoprecipitates were analyzed by immunoblotting with an antibody to Hsp90, NS5A, or hB-ind1. The data shown are representative of three independent experiments.

changeable with the cochaperone domain of p23 during complex formation with NS5A, Hsp90, and FKBP8.

Cochaperone activity in the p23-like domain of hB-ind1 is required for propagation of HCV. The p23-like domain of hB-ind1 has been suggested to be required for HCV propagation (56). However, the involvement of the cochaperone activity of hB-ind1 in HCV propagation has not been examined. To assess the effect of cochaperone activity in the p23-like domain of hB-ind1 on the RNA replication and particle production of HCV, each of the expression plasmids encoding the FLAG-tagged wild-type or mutant hB-ind1 carrying the silent mutations resistant to small interfering RNA was transfected into hB-ind1 knockdown (Huh-KD) cells and cultured for a week in the presence of puromycin. The expressions of FLAG-tagged hB-ind1 and the mutants in the Huh-KD cells were comparable to that of the endogenous hB-ind1 in the control (Huh-ctrl) cells transfected with an empty vector (Fig. 3A). Subgenomic HCV replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1 was transfected into these cells and cultured for 4 weeks in the presence of G418. Although the number of colonies was reduced in the Huh-KD cells compared with the Huh-ctrl cells after transfection with an empty vector, as described previously (56), the colony numbers were recovered by the expression of the hB-ind1 or chB-ind1 mutant, but not by that of the hB-ind1AXXA or chB-ind1AXXA mutants (Fig. 3B). Similarly, intracellular HCV RNA and infectious viral titers in the culture supernatants of Huh-KD cells infected with JFH1 virus were partially recovered by the expression of the hB-ind1 or chB-ind1 mutant, but not by that of the hB-ind1AXXA or chB-ind1AXXA mutant (Fig. 3C). These results suggest that cochaperone activity in the p23-like domain of hB-ind1 is required for HCV propagation and that the cochaperone domain of p23 can substitute for the p23-like domain of hB-ind1.

hB-ind1 colocalizes with NS5A, FKBP8, and dsRNA on the membranous web. Our previous report revealed the interplay among hB-ind1, Hsp90, FKBP8, and NS5A and showed that these interactions play an important role in HCV replication (56). However, the subcellular localization of the endogenous hB-ind1 in the replicon cells and JFH1 virus-infected cells has not been precisely assessed. To determine the subcellular localization of hB-ind1 in the context of HCV replication, the expression of hB-ind1 and NS5A in the replicon cells and JFH1 virus-infected cells was examined by immunofluorescence analyses (Fig. 4A). Endogenous hB-ind1 was colocalized with the endoplasmic reticulum (ER)-marker PDI and NS5A as dot-like structures in the Huh9-13 replicon cells (Fig. 4A, top) and in cells infected with JFH1 virus (Fig. 4A, bottom), and these dot-like structures disappeared in concert with the loss of NS5A expression by treatment with IFN-α in the replicon cells and was not observed in the mock-infected Huh7.5.1 cells. Furthermore, FKBP8 (Fig. 4B, top) and dsRNA (Fig. 4B, bottom) were colocalized with hB-ind1 and NS5A in the dot-like structures in Huh9-13 replicon cells. These results indicate that HCV replicating RNA is localized with hB-ind1, FKBP8, and NS5A in the dot-like compartments. HCV RNA replication or expression of viral proteins leads to formation of the convoluted membranous structures designated the membranous web (14, 23). The large structures of the replication complexes in the replicon cells indicate membranous webs with

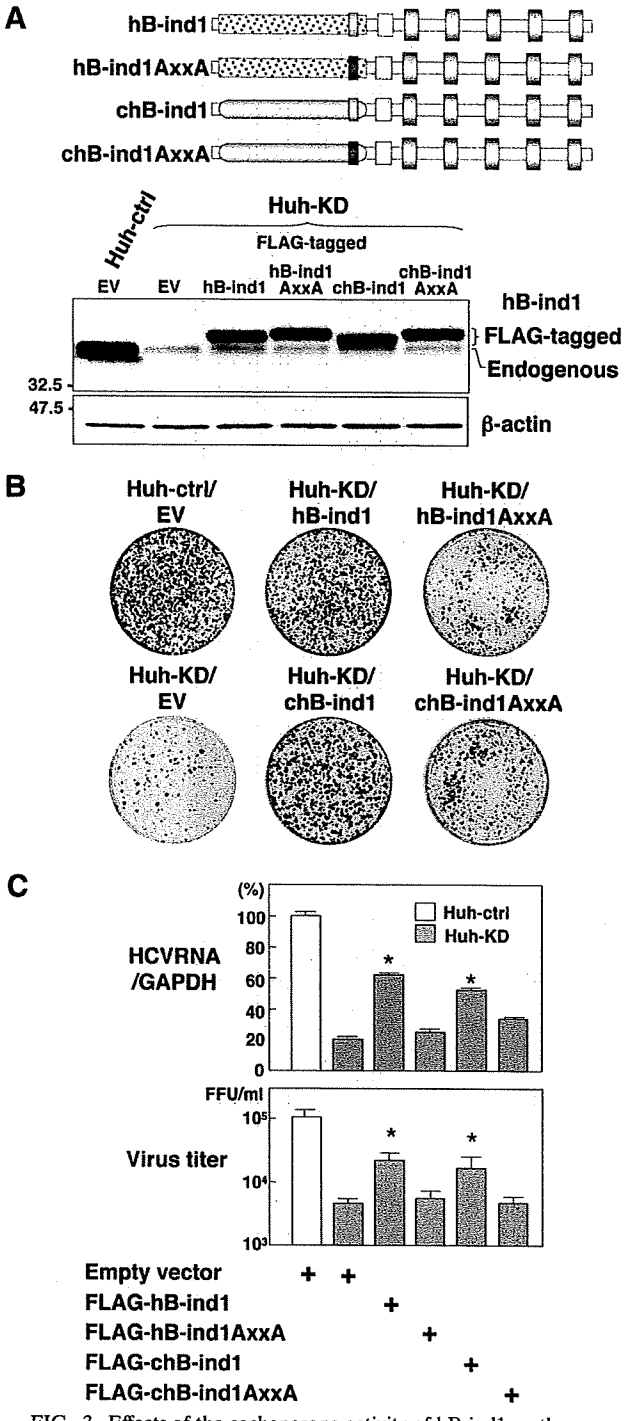

FIG. 3. Effects of the cochaperone activity of hB-ind1 on the propagation of HCV. (A) Huh-KD cells were transfected with either an empty vector or an expression plasmid encoding FLAG-tagged hB-ind1, hB-ind1AxxA, chB-ind1, or chB-ind1AxxA, which are resistant to small interfering RNA due to the introduction of silent mutations, and cultured for a week in the presence of 10 μg/ml of puromycin. The surviving cells were used in the subsequent experiments. The endogenous and exogenous expression of hB-ind1 and the mutants was detected by immunoblotting. The control cell line (Huh-ctrl) or the Huh-KD cell line transfected with an empty vector (EV) was used as a control. (B) Huh-KD cells were transfected with the plasmids and then selected with puromycin. The resulting cells were further transfected with a replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1, cultured for 4 weeks in the presence of 1 mg/ml of G418, and stained with crystal violet after fixation with 4% paraformaldehyde. The Huh-KD cell line transfected with an empty vector (EV) was used as a positive control. (C) The cells prepared as described above were infected with JFH1 virus and harvested at 3 days postinfection. The amount of intracellular HCV RNA was estimated by quantitative reverse transcriptase PCR and normalized with that of GAPDH mRNA. The values of HCV RNA are presented as percentages versus those of Huh-ctrl cells transfected with an empty vector. The culture supernatants were subjected to a focus-forming assay. Virus titers are presented as focus-forming units (FFU) per ml. The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) versus the value of the control. The data shown are representative of three independent experiments.

restricted motility (68). To further analyze the subcellular compartments, including hB-ind1 and NS5A, the same field of the Huh9-13 replicon cells was observed under FM and EM by using the correlative FM-EM technique (Fig. 5A, upper two rows). The large structures that included hB-ind1 and NS5A in the replicon cells were observed under FM and EM (white-boxed areas) and further magnified (black-boxed areas). Convoluted membranous structures that consisted of small vesicles and that were similar to the membranous web were observed. Another field of view yielded similar results (Fig. 5A, lower two rows). The membranous web resembling the convoluted structures was not observed in the Huh9-13 cells depleted of viral RNA by IFN treatment (Fig. 5B). Together, these results suggest that hB-ind1 interacts with NS5A on the membranous web in cells replicating HCV RNA.

Hsp90 is involved in the circumvention of the UPR during HCV replication. Hsp90 regulates the folding and stability of proteins in all eukaryotes (59), and inhibition of the chaperone pathway suppresses correct protein folding, which leads to induction of proteasome-mediated degradation of the unfolded proteins and the unfolded protein response (UPR). Our previous (46) and present studies (Fig. 4 and 5) showed that several cochaperone components are recruited in the membranous web, suggesting that the Hsp90 chaperone system participates in the replication complex to circumvent the induction of the UPR and to maintain the folding of the host and viral proteins in a replication-competent state. To determine the induction of the UPR by HCV replication, Huh9-13 replicon cells were transfected with a reporter plasmid carrying a firefly luciferase gene under the control of the GRP78 promoter, which is activated by the induction of the UPR, together with an internal-control plasmid. Although the GRP78 promoter activity was slightly enhanced in the Huh9-13 cells compared to that in the parental cells, a fourfold increase of GRP78 promoter activity in the replicon cells was observed after treatment with an Hsp90 inhibitor, DMAG, in contrast to the twofold increase in similarly treated parental Huh7 cells, and the activation of the GRP78 promoter was canceled by treatment with IFN-α despite DMAG treatment (Fig. 6A), suggesting that the Hsp90 chaperone system participates in the circumvention of the UPR induced by the replication of HCV RNA. In addition, activation of GRP78 at transcriptional and translational levels after treatment with DMAG was higher in the

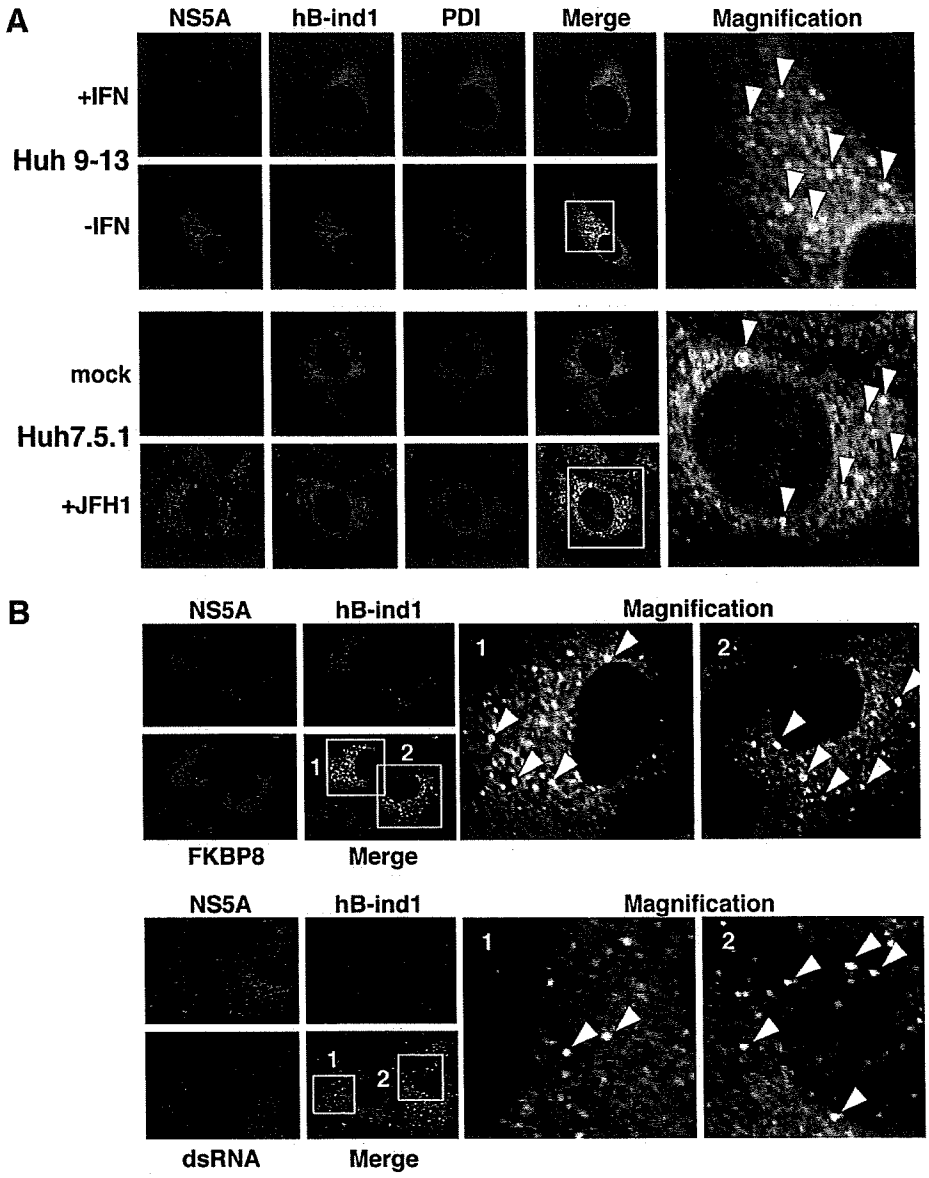

FIG. 4. Intracellular localization of hB-ind1 in replicon cells and infected cells. (A) Huh9-13 replicon cells with IFN-α or untreated and Huh7.5.1 cells infected with JFH1 virus or naïve cells were stained with antibodies against NS5A, hB-ind1, or PDI and examined by immunofluorescence assay. The boxed areas in the merged images are magnified and displayed on the right. The arrowheads indicate intracellular positions colocalized with NS5A, hB-ind1, and PDI. (B) Huh9-13 replicon cells were fixed, permeabilized, and stained with appropriate antibodies to NS5A, hB-ind1, and FKBP8 (top) or dsRNA (bottom). The boxed areas in the merged images are magnified and displayed on the right. The arrowheads indicate intracellular positions colocalized with NS5A, hB-ind, and FKBP8 or dsRNA. The images shown are representative of three independent experiments.

HCV replicon cells than in the parental cells or in cured cells, which were depleted of HCV RNA by treatment with IFN-α (Fig. 6B). Furthermore, DMAG treatment enhanced the transcription of the UPR marker protein GADD153 at a higher level in the replicon cells than in the parental Huh7 or the cured cells (Fig. 6C). These results suggest that the Hsp90-dependent chaperone system plays a crucial role in the folding of the host and viral proteins involved in HCV replication and in the regulation of UPR induction.

DISCUSSION

Studies of the relationship between Hsp90 and steroid receptors, such as GR, have revealed the activities of cochaperones (52, 67). Cochaperones, such as p23, appear to interact with and dissociate from Hsp90 and the client protein complex in a defined order. These cochaperones participate in the chaperone complex in a late step and promote the dissociation of the client proteins from Hsp90 to facilitate formation of the

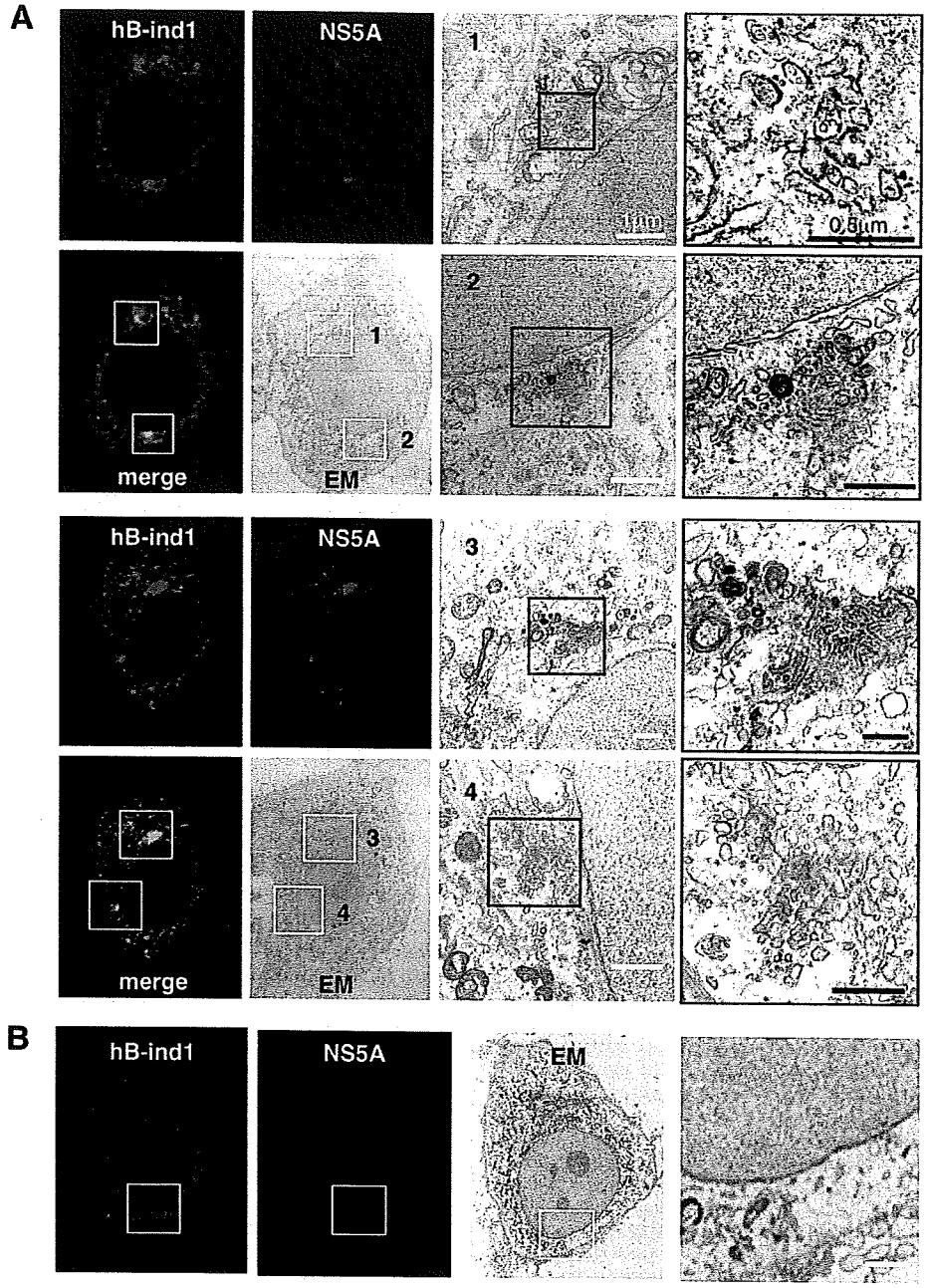

FIG. 5. hB-ind1 interacts with NS5A in the membranous web. Huh9-13 replicon cells were stained with specific antibodies to hB-ind1 and NS5A. Identical fields of Huh9-13 (A) or the cured cells (B) were observed under EM by using the correlative FM-EM technique. The white-boxed areas indicate the colocalized areas of hB-ind1 with NS5A. Magnified views of the white-boxed areas are displayed in the third column from the left. The right column contains further-magnified images of each of the black-boxed areas. Another field of view is presented in the lower two rows.

chaperone complex in the next chaperone cycle (16–18). In this study, we have shown that hB-ind1 participates in HCV replication and that the p23-like domain of hB-ind1 possesses cochaperone activity comparable to that of the cochaperone domain of p23, suggesting that hB-ind1 is involved in the recycling of the chaperone complex in the membranous web to maintain the function of the replication complex of HCV.

Previous studies have indicated that HCV proteins rearrange the ER membrane into the small convoluted membranous vesicles that are collectively known as the membranous web, and these vesicles have been suggested to be the intracellular compartments in which HCV replication takes place (14, 23, 68). In the living replicon cells, two forms of replication complexes, small and large vesicles, are detected, both of which include the viral replication complexes (68). Large vesicles, corresponding to membranous webs, exhibit restricted motil-