Reconstitution of hepatitis C virus protease activities in yeast

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Abstract The hepatitis C virus (HCV) protease genes (NS2/3 and NS3) were expressed in yeast with their natural substrates fused to a ligand-dependent transcriptional activator, the retinoic acid receptor (RARβ). RARβ can activate transcription in yeast cells in response to retinoic acids. We hypothesized that *cis*-cleavage at the NS2–3 or NS3–4A junctions by the appropriate HCV proteases would release RARβ, thereby activating transcription of a reporter gene. Our results from Western blot analyses and reporter gene activation indicate that the wild-type NS2/3 and NS3 enzymes are catalytically active in yeast cells, whereas mutations in the catalytic domain of NS2(C993V) and NS3(S1165A) lead to inactive enzymes. We conclude that HCV NS2/3 and NS3 protease activities can be reconstituted in yeast.

Key words: HCV protease; Retinoic acid receptor; *Saccharomyces cerevisiae*

1. Introduction

Hepatitis C virus (HCV), which belongs to the *Flaviviridae* family, is a positive-sense single-stranded RNA virus with a genome of 9.6 kb in length. The viral proteins are produced from a single long open reading frame encoding a polyprotein of 3010 amino acid residues in the order of NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH [1,2]. Polyprotein processing is mediated by host signal peptidases as well as by HCV encoded proteases, NS2/3 and NS3 [3,4]. The enzymatic activity of the NS2/3 HCV protease requires amino acid sequences located in the C-terminal half of the NS2 gene linked to the N-terminal one third of the NS3 gene (aa 907–1207) (unpublished observation) and is responsible for the cleavage at the NS2/NS3 junction. Cleavage at the NS2/NS3 junction is a rapid zinc-dependent intramolecular event. Previously, His-952 and Cys-993 have been described as essential residues for enzymatic activity since mutation of either one of these amino acids inactivates the enzyme [5,6]. The amino-terminal domain of NS3 also encodes a serine protease, which cleaves at the NS3/4A junction in *cis* followed by the cleavage at the NS4A/B, NS4B/5A and NS5A/B sites in *trans* [7,8]. Efficient proteolytic activity of NS3 requires the NS4A protein as a cofactor [9,10]. Since viral proteases are indispensable for viral replication, they are attractive targets for developing antiviral therapies. Indeed, many academic and industrial laboratories have developed in vitro assays for the identification of specific protease inhibitors against NS3 and to a lesser extent, NS2/3 protease [11,12]. *Saccharomyces cerevisiae* has been exploited as a novel host to study mammalian transcription factors [13–15] as well as to determine substrate specificity of hepatitis C virus NS3 protease [16]. In this report, we further employed this yeast expression system to examine the feasibility of studying HCV protease activity in yeast cells. Our results indicate that this genetic system is a useful tool to study viral protease activity in vivo, thus allowing the development of a screening method to identify specific viral protease inhibitors.

2. Materials and methods

2.1. Cells

The *S. cerevisiae* strain (BJ2168) containing the YEp$_c$RARβ and YEpA plasmids has been described previously [14,15]. Growth and transformation of yeast cells were performed according to standard procedures [17]. Double transformant yeast strains were grown in synthetic dropout medium to maintain expression plasmids.

2.2. Plasmids

YEp$_c$RARβ is a copper inducible expression plasmid for the human retinoic acid receptor-β fused at its N-terminus to the ubiquitin gene under control of the CUP1 promoter. DNA sequences encompassing the HCV NS2/3 protease domain, composed of full-length NS2 protein linked to the N-terminal 181 amino acids of NS3 (aa 810–1207 of the HCV polyprotein), were constructed by standard PCR amplification, using cDNA of HCV H strain as a template [3]. Sequences composed of full-length NS3 protease linked to NS4A and the first 19 amino acids of NS4B (aa 1027–1730) were constructed similarly. The resulting PCR fragments were cloned in frame by blunt-end ligation between the ubiquitin and the RARβ gene at the *Eag*I site of YEp$_c$RARβ [15]. The NS2/3(C993V) and the NS3(S1165A) mutants were obtained using the QuickChange™ site-directed mutagenesis kit (Stratagene). The resulting constructs, YEp$_c$NS2/3-RARβ, YEp$_c$NS2/3-RARβ(mut), YEp$_c$NS3/4AB$_{19}$-RARβ, and YEp$_c$NS3/4AB$_{19}$-RARβ-(mut) were sequenced to confirm the integrity of the genes. When expressed in yeast, the 76 amino acid ubiquitin fragment will be cleaved off the fused protein by the endogenous ubiquitinase in yeast producing the NS2/3-RARβ and NS3/4AB$_{19}$-RARβ proteins. The yeast reporter plasmid YEpA containing the lac-Z gene under the control of the CYC1 promoter and the *Apo*AI site A enhancer (GACTGAACCCTTGACCCCTGCCC) has been described elsewhere [14,15].

2.3. Western blot analysis

Yeast cell lysates were prepared and subjected to Western blot analyses as described previously [14,15]. Rabbit polyclonal antibody C-19 raised against human RARβ (Santa Cruz Biotechnology) was used to probe the blot. HRP-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech) was used as secondary antibody and the protein was detected using the ECL™ detection reagents (Amersham Pharmacia Biotech).

2.4. Transcription assay

Double transformant yeast strains carrying the reporter plasmid YEpA and one of the following expression constructs: YEp$_c$RARβ,

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$YEp_cNS2/3$-RARβ, $YEp_cNS2/3$-RARβ(mut), $YEp_cNS3/4AB_{19}$-RARβ or $YEp_cNS3/4AB_{19}$-RARβ(mut), were grown in synthetic dropout medium in the absence or presence of 100 μM cupric sulfate and 100 nM all-*trans* retinoic acid (Sigma). When cell density reached late log phase, yeast cells were assayed for β-galactosidase induction as described previously [14,15].

2.5. Indirect immunofluorescence

An overnight yeast culture (OD = 1.0 at 600 nm) was fixed for 1 h with 4% formaldehyde. The cells were then pelleted, washed once with potassium phosphate (0.1 M, pH 7.5) and resuspended in the same buffer containing zymolyase (50 μg/ml) (Invitrogen) for 20 min at 30°C. The cells were washed twice with phosphate buffer before and after a 30 s treatment with 1% Triton X-100. The permeabilized cell suspension (50 μl) was spotted onto polylysine coated slides for 5 min and subsequently washed twice with PBS and overlayed with 4% BSA for 30 min at room temperature (RT) in a humid chamber. A rabbit polyclonal IgG anti-RARβ (Santa Cruz Biotechnology) was used as a primary antibody. After 1 h incubation at RT, the slides were washed three times, 5 min each with PBS and incubated with a donkey anti-rabbit $(Fab')_2$ FITC-conjugated antibody (Jackson Laboratories) for 1 h at RT. After three washes with PBS, the slides were overlayed with DAPI solution (1 μg/ml) (Sigma) for 1 min and mounted using the Prolong antifade (Molecular Probes).

3. Results and discussion

3.1. NS3 protease activity in yeast

The concept of reconstituting HCV NS3 protease activity is illustrated schematically in Fig. 1A. *Cis*-cleavage between NS3 and NS4A at aa 1657 will generate two cleaved products, i.e. NS3 and $NS4AB_{19}$-RARβ. The interaction of NS4A with the N-terminal amino acids of NS3 will promote cleavage of NS4A from NS4B, resulting in the final chimeric receptor, $NS4B_{19}$-RARβ. In contrast, the mutant chimera will be expressed as an uncleaved product (NS3–$4AB_{19}$-RARβ). Upon expression of the wild-type $NS3/4AB_{19}$-RARβ polyprotein in yeast, the NS3 protease would produce the following intermediates: $NS4AB_{19}$-RARβ (63 kDa), $NS4B_{19}$-RARβ (57 kDa) as well as the uncleaved form, $NS3/4AB_{19}$-RARβ (133 kDa). Indeed, all these polyproteins were detected by Western blot at the expected size using a polyclonal antibody against RARβ as shown in Fig. 1B. In contrast, when the protease activity of NS3 was abolished by substitution of the serine at position 1165 by an alanine (NS3(S1165A)), only the uncleaved form, $NS3/4AB_{19}$-RARβ (133 kDa) was observed by Western blot. This observation further supports the notion that NS3 is a serine protease [5,12]. However, a specific serine protease inhibitor for HCV NS3 protease is not available commercially to validate this yeast genetic system. To demonstrate the size of $NS4AB_{19}$-RARβ intermediate, a construct YEp_cNS4AB_{19}-RARβ expressing this protein was used to transform yeast cells. As expected, the polyprotein has the predicted size of 63 kDa (Fig. 1B). In some cases, low level of protein expression was detected under non-induced condition (in the absence of cupric sulfate), this is due to the leaky metallothionein (CUP1) promoter. The yeast strain carrying the expression construct, $YEp_cNS3/4AB_{19}$-RARβ, and the reporter plasmid, YEpA, exhibited a ligand-dependent transactivation, which was similar to that observed for RARβ (Fig. 1C). However, yeast strains harboring the mutant construct $YEp_cNS3/4AB_{19}$-RARβ(mut) or the YEp_cNS4AB_{19}-RARβ construct exhibited little or no reporter gene activation (Fig. 1C). These data clearly indicate that $NS4B_{19}$-RARβ is the only intermediate that can function efficiently as a transcriptional activator in yeast and support the notion that the NS3/$4AB_{19}$-RARβ polyprotein must undergo two cleavage reactions by the NS3 to produce $NS4B_{19}$-RARβ. The fact that $NS4AB_{19}$-RARβ fusion is not transcriptionally active suggests that either the 54 residues of NS4A affect the conformation of the fusion protein, which interferes with the ability of RARβ to bind to DNA or ligand, or the highly hydrophobic moiety of NS4A directs the fusion protein to yeast membranes, thereby preventing translocation of the protein to the nucleus.

The indirect immunofluorescence study indicated that RARβ from both active and inactive NS3 protease constructs were mostly cytoplasmic in yeast cells in the absence of retinoic acid (Fig. 2C,E). However, in the presence of ligand, RARβ from the inactive NS3 protease construct still remained cytoplasmic (Fig. 2F). Possible reasons include: (1) the size of the uncleaved $NS3/4AB_{19}$-RARβ polyprotein was too big to translocate into the nucleus, (2) the ligand binding domain at the C-terminus of RARβ was masked by the large N-terminal domain of NS3, or (3) the hydrophobic NS4A sequences prevented translocation. In contrast, cleavage of the polyprotein by the active form of NS3 led to a RARβ exhibiting both cytoplasmic and nuclear localization in the presence of ligand (Fig. 2D). The cytoplasmic form of the RARβ is most likely the uncleaved $NS3/4AB_{19}$-RARβ and/or the partially cleaved $NS4AB_{19}$-RARβ, whereas the nuclear form is the $NS4B_{19}$-RARβ. Nuclear localization of the $NS4B_{19}$-RARβ protein would correlate directly with the transcriptional activation of the lac-Z gene (Fig. 1C).

3.2. NS2/3 protease activity in yeast

The concept of reconstituting HCV NS2/3 protease in yeast is based on the assumption that the N-terminal hydrophobic domain of NS2 is cotranslationally anchored in the endoplasmic reticulum (ER) membranes (Fig. 3A, bottom). Thus, the chimeric enzyme/substrate will be anchored to the cytoplasmic surface of the ER in the absence of autocatalytic protease activity (Fig. 3A). Autocleavage between aa 1026 and 1027 will release the chimeric receptor (NS3-RARβ), which functions as a ligand-activated transcription factor leading to reporter gene induction. In contrast, the mutant chimera, which has an active site point mutation (C993V), will not exhibit autocleavage. The uncleaved chimeric protein should be retained on cytoplasmic surface of the ER membrane and therefore cannot function as a ligand-activated transcription factor.

In order to demonstrate the specific cleavage between NS2 and NS3 by the NS2/3 protease in this system, a construct expressing the wild-type NS2/3-RARβ fusion protein was used to transform yeast cells. Yeast cells carrying the expression plasmid, $YEp_cNS2/3$-RARβ, revealed two distinct protein bands that correspond to the predicted size of the uncleaved chimera (97 kDa) (top arrow) and the cleaved product, NS3-RARβ (74 kDa) (middle arrow). In contrast, yeast cells harboring the mutant construct, $YEp_cNS2/3$-RARβ(mut), resulted only in an uncleaved product (97 kDa protein) (Fig. 3B). Under non-induced condition, low levels of protein were detected for RARβ (bottom arrow), uncleaved wild-type NS2/3-RARβ and cleaved NS3-RARβ due to the leaky metallothionein promoter (CUP1). These observations clearly indicate that NS2–NS3 *cis*-cleavage can occur in yeast cells with an efficiency of cleavage of about 40–50% under the conditions used in the present study, whereas the mutant chimera cannot undergo self cleavage. We have consistently observed that the expression level of the NS2/3-RARβ mutant

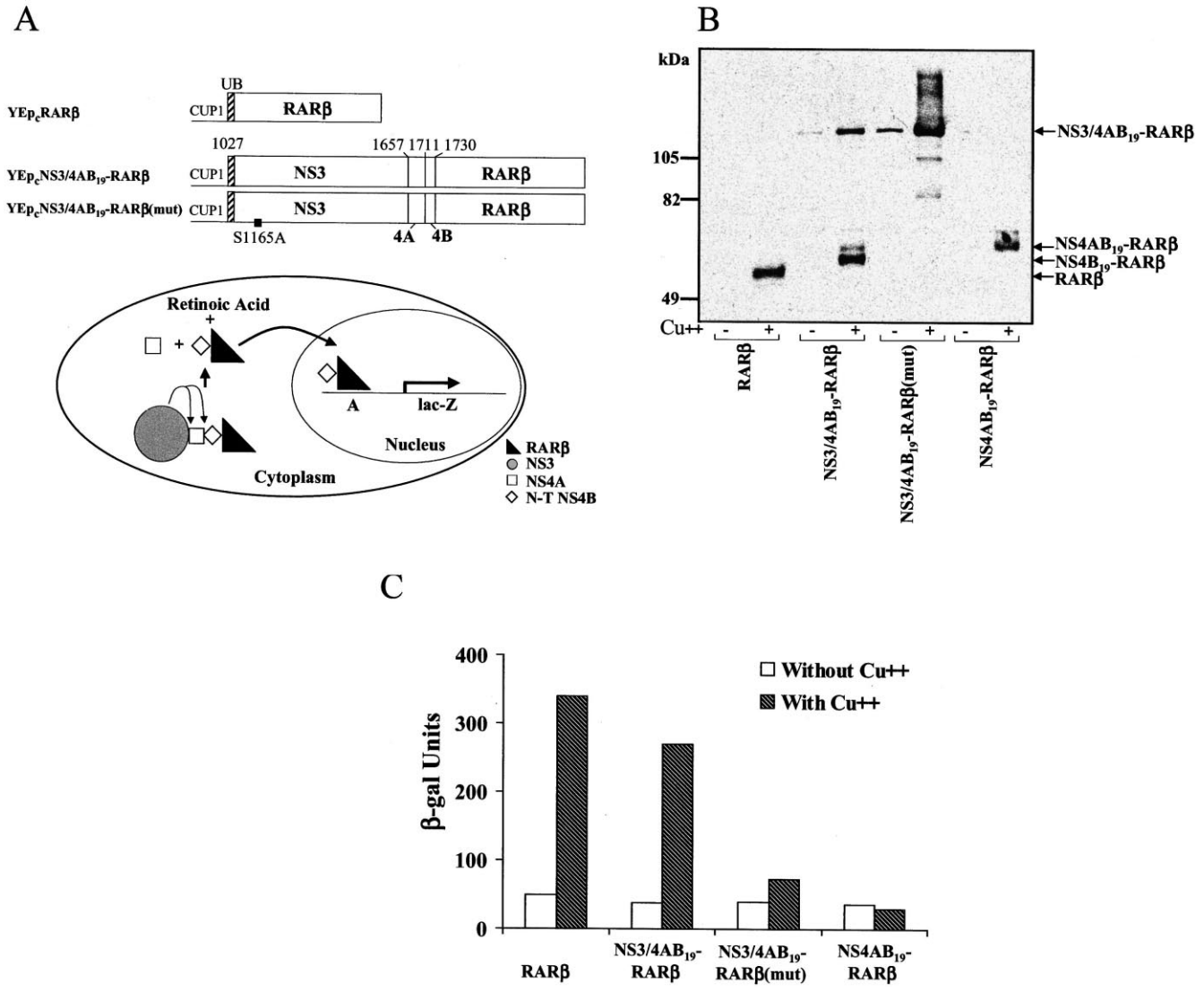

Fig. 1. A: Reconstitution of HCV NS3 protease activity in yeast. For the wild-type chimeric construct (YEp$_c$NS3/4AB$_{19}$-RARβ), the entire NS3 (aa 1027–1657), NS4A (aa 1658–1711) and the N-terminal 19 aa of NS4B (aa 1712–1730) were inserted in frame between the ubiquitin gene (UB) and the RARβ. The mutated version (YEp$_c$NS3/4AB$_{19}$-RARβ(mut)) of this chimeric construct has a point mutation (S1165A) (top), which will disrupt the *cis*- and *trans*-cleavage activity of NS3. The construct YEp$_c$RARβ was used as a control. Each of these expression constructs was used to transform yeast cells carrying the reporter plasmid, which contains two copies of a specific enhancer, site A (GACTGAACCCTTGACCCCTGCCC) upstream of the CYC1 promoter (bottom). B: Western blot analysis of NS3/4AB$_{19}$-RARβ. Yeast cell lysates were prepared from yeast strains expressing RARβ, NS3/4AB$_{19}$-RARβ, NS3/4AB$_{19}$-RARβ(mut), or NS4AB$_{19}$-RARβ under non-induced (−) or induced (+) conditions as described in Section 2. Equal volume of cell lysate was electrophoresed on 10% SDS–PAGE. Molecular weight standards are indicated in kDa. C: Ligand-dependent transcriptional activation of NS3/4AB$_{19}$-RARβ in yeast. Double transformant yeast strains carrying the reporter plasmid and the expression constructs for RARβ, NS3/4AB$_{19}$-RARβ, NS3/4AB$_{19}$-RARβ(mut), or NS4AB$_{19}$-RARβ were grown in synthetic dropout medium containing 100 nM all-*trans* retinoic acid in the absence (empty bar) or presence (hatched bar) of 100 μm cupric sulfate. After overnight incubation at 30°C, cells were harvested and analyzed for β-gal activity as described in Section 2. The results are the average of two separate experiments.

protein is much lower than that of the wild-type. NS2 is a highly hydrophobic protein with four putative transmembrane domains. It has been shown that NS2 protein is anchored to the cytoplasmic surface of the endoplasmic reticulum (ER) when expressed in mammalian cells [18]. Thus, it is most likely that the uncleaved NS2/3-RARβ mutant protein is cotranslationally anchored to the ER membrane through the NS2 protein. It is possible that the association of the NS2/3-RARβ mutant polyprotein with the ER membrane has an 'inhibitory effect' on the synthesis and/or the accumulation of these chimeras in yeast cells. In fact, we were not able to detect the mutant chimera by indirect immunofluorescence (data not shown). In contrast, the expression level of the wild-type NS2/3-RARβ polyprotein was comparable to that of RARβ based on Western blot analyses. Yeast transformants harboring the plasmids YEp$_c$RARβ or YEp$_c$NS2/3-RARβ exhibited ligand-dependent transcriptional activation (Fig. 3C). We have observed that the yeast strain carrying the plasmid, YEp$_c$NS2/3-RARβ(mut), was unable to activate transcription when yeast cells were incubated with both retinoic acid and cupric sulfate (unpublished observations). A direct comparison in transcriptional activation between the wild-type and the

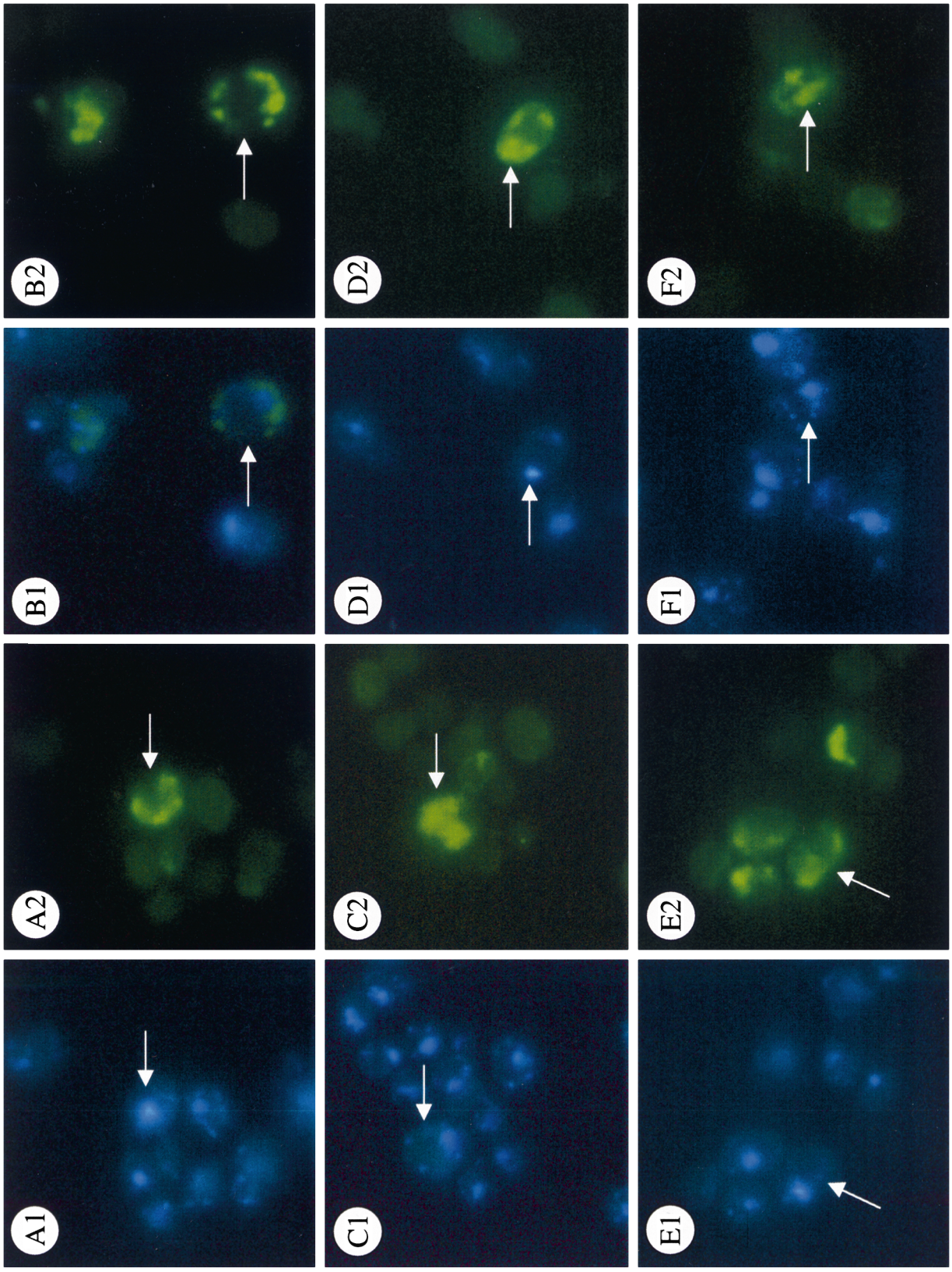

Fig. 2. Localization of the RARβ protein in yeast cells by indirect immunofluorescence. Yeast transformed with $YEp_cNS2/3$-RARβ (A, B), $YEp_cNS3/4AB_{19}$-RARβ (C, D) and $YEp_cNS3/4AB_{19}$-RARβ(mut) (E, F) were probed with a rabbit polyclonal antibody raised against RARβ. DNA staining using DAPI reagent is shown in panels A1, B1, C1, D1, E1 and F1. Specific RARβ expressing cells are shown in panels A2, B2, C2, D2, E2 and F2. Cells of interest are indicated by an arrow. A, C and E: Without retinoic acid. B, D and F; With retinoic acid.

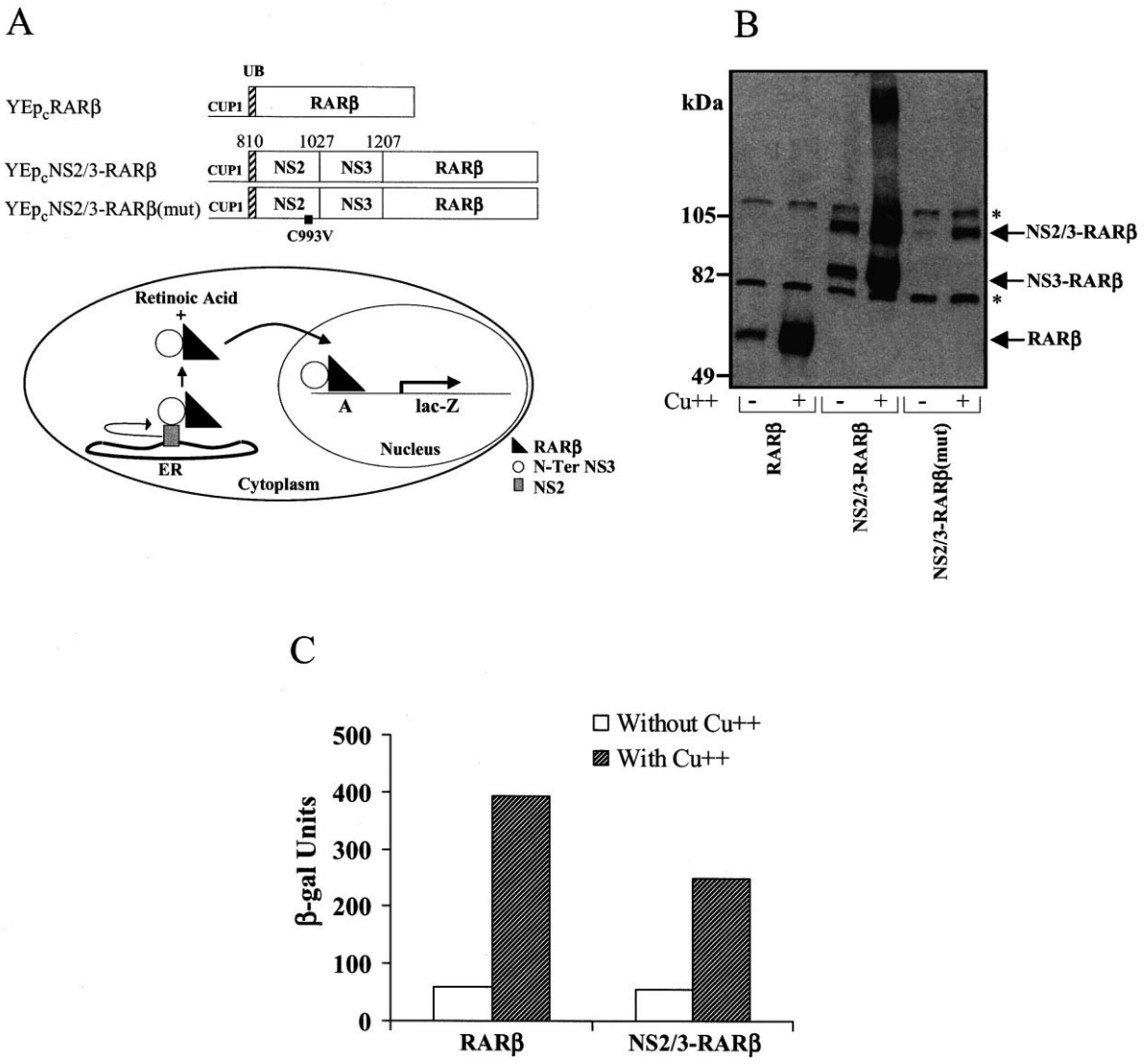

Fig. 3. A: Reconstitution of HCV NS2/3 protease activity in yeast cells. The three ubiquitin (UB) fusion yeast expression plasmids are shown in top. Each of these expression constructs was used to transform yeast cells carrying the reporter plasmid, which contains two copies of a specific enhancer, site A (GACTGAACCCTTGACCCCTGCCC) upstream of the CYC1 promoter (bottom). The resulting double transformant yeast strain was analyzed for reporter gene (lac-Z) induction as well as protein expression. B: Western blot analysis of NS2/3-RARβ. Yeast cell lysates were prepared from yeast strains expressing RARβ, wild-type NS2/3-RARβ or mutant NS2/3-RARβ(mut) under non-induced (−) or induced (+) conditions as described in Section 2. Equal volume of cell lysate was electrophoresed on 10% SDS–PAGE. The arrows (from top to bottom) indicate the size of uncleaved NS2/3-RARβ, NS3-RARβ and RARβ respectively. Non-specific immunoreactive bands were indicated by asterisk. Molecular weight standards are indicated in kDa. C: Ligand-dependent transcriptional activation of NS2/3-RARβ in yeast. Double transformant yeast strains carrying the reporter plasmid and the expression constructs for RARβ or wild-type NS2/3-RARβ were grown in synthetic dropout medium containing 100 nM all-*trans* retinoic acid in the absence (empty bar) or presence (hatched bar) of 100 μm cupric sulfate. After overnight incubation at 30°C, cells were harvested and analyzed for β-gal activity as described in Section 2. The results are the average of two separate experiments.

mutant chimera is not feasible due to the dramatic difference in the level of protein expression. The lack of activity by the mutant NS2/3-RARβ may simply be due to either the low level of protein synthesis or retention of the mutant protein at the ER membrane. If the latter is the case, then this would predict that the conversion of uncleaved NS2/3-RARβ (97 kDa) to the cleaved products NS2+NS3-RARβ is required for reporter gene induction in yeast cells.

Indirect immunofluorescence indicated that RARβ from both uncleaved and cleaved NS2/3-RARβ polyprotein were localized in the cytoplasm of the cells in the absence of retinoic acid (Fig. 2A). In contrast, RARβ were detected mainly in the nucleus when the cells were incubated with retinoic acid (Fig. 2B). It is not clear in the present study whether nuclear localization of RARβ represents both cleaved (NS3-RARβ) and uncleaved (NS2/3-RARβ) chimeras. Nevertheless, these observations are in agreement with the mechanism of steroid hormone action [19,20].

3.3. NS2/3 and NS3 proteases are attractive antiviral targets for small molecule inhibitors

HCV non-structural protein processing is achieved by NS2/3 and NS3 proteases, which are essential for viral replication. Therefore, these proteases are good targets for antiviral ther-

apy. In the present study, we were able to reconstitute NS2/3 protease activity in yeast cells, which can be exploited as an efficient screening tool to identify novel small molecule inhibitors. This can be achieved by screening compounds that can inhibit the reporter gene induction (lac-Z) or the conversion of uncleaved NS2/3-RARβ polyprotein to a cleaved NS3-RARβ product as detected by Western blots. Likewise, the same screening strategy can be applied to NS3 protease. In summary, we have exploited the yeast genetic system to reconstitute HCV protease activities, which can be used to develop a screening assay to identify small molecule inhibitors for both HCV proteases. Furthermore, this system will also be useful for structure–function analyses of these proteases in cells.

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