Characterization of the interaction between hepatitis C virus NS5B and the human oestrogen receptor alpha

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The RNA-dependent RNA polymerase (NS5B) of hepatitis C virus (HCV) is part of the viral replicative complex and plays a crucial role in HCV replication. It has been described that NS5B interacts with cellular proteins, and that interactions between NS5B and host proteins are crucial for viral replication. Some of the host factors involved in the HCV replication cycle include the oestrogen receptor alpha (ESR1), protein kinases (c-Src) and chaperones (Hsp70). In this report, we determine the requirements for the interplay between NS5B and the domain C of ESR1 (ESR1C) by using Förster Resonance Energy Transfer. NS5B–ESR1C and ESR1C–ESR1C interactions are dependent on ionic strength, indicating that contacts are mainly electrostatic. Additionally, NS5B residues involved in NS5B oligomerization were also essential for NS5B–ESR1C interaction. The study of the interactions among viral and host factors will provide data to establish innovative therapeutic strategies and the development of new antiviral drugs.

Viruses are obligate parasites that need cellular machineries to replicate and propagate; therefore, two different antiviral strategies might coexist either directed against virally or cellular-encoded activities (Suzuki, 2010; Winkler, 2008). Furthermore, generation and selection of resistant-to-treatment mutants is much more difficult in the case of host targets than for compounds targeting virally encoded proteins. Because HCV variants with one or even two nucleotide substitutions are generated every day, almost all resistant-to-treatment mutants could be generated daily (Guedj et al., 2010). For this reason, search, identification and characterization of host proteins involved in virus life cycles have become a hot spot in virology during the past years (Georgel et al., 2010; Vidalain & Tangy, 2010). Some of them have been identified and, actually, they have been proposed to be used as targets for antiviral development (Flisiak et al., 2009; Georgel et al., 2010; Gerold & Rice, 2011; Khattab, 2009; Nagy et al., 2011; Paeshuyse et al., 2006; Watashi et al., 2005).

The viral protein NS5B replicates the genetic material through its RNA-dependent RNA polymerase (RdRp) activity (Behrens et al., 1996; Lohmann et al., 1997). It has been described that NS5B interacts with cellular proteins and these interactions are crucial for viral replication. One of the host proteins that interact with NS5B is the oestrogen receptor alpha (ESR1). ESR1 belongs to the nuclear receptor superfamily, a group of ligand-dependent transcription factors that include steroid receptors and some orphan receptors (Mangelsdorf et al., 1995). HCV NS5B is targeted to the endoplasmic reticulum membrane through the interaction with ESR1 (Watashi et al., 2007). Actually, tamoxifen, 17β-oestradiol and tomerifene have been identified as potent suppressors of HCV replication (Gastaminza et al., 2010; Hayashida et al., 2010; Watashi et al., 2007). ESR1 is a 565 aa protein with six different functional domains, named A–F. Domain C is responsible of ESR1 dimerization and DNA binding, whereas E–F are the ligand-binding domains. Watashi and colleagues described the ESR1 C domain (ESR1C) as the region involved in the interaction NS5B–ESR1 (Watashi et al., 2007). However, the region of the polymerase participating in the interaction is not mapped.

With these antecedents we have cloned ESR1C fused to fluorescent proteins (FP) cyan and citrine. Both FP variants carry the A206K mutation that prevents FP dimerization (Zacharias et al., 2002). The ORFs encoding ESR1C and mutants ESR1C255M and ESR1C258M were amplified from plasmids pCMV-FLAG-ERαC, pCMV-FLAG-ERα(255M) and pCMV-FLAG-ERα(258M), respectively (Watashi et al., 2007). FP cyan was fused by PCR either at ESR1C N (cyan-ESR1C) or C terminus (ESR1C-cyan). Citrine was also fused by PCR at the N-end (citrine-ESR1C) (Fig. 1a). All PCRs were carried out using the Pfu-Turbo-Polymerase (Stratagene) and the final constructs were verified by DNA sequencing. All constructs were tagged with a 6 × His at the C-end for protein purification purposes.

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Proteins described in this study were overexpressed and purified to apparent homogeneity using previously described protocols (Bellón-Echeverría et al., 2010). ESR1C fused to FP migrate at a position corresponding to their predicted molecular mass of 39 kDa (Fig. 1a), whereas NS5B fused to FP migrate at approximately 90 kDa (Bellón-Echeverría et al., 2010; Clemente-Casares et al., 2011 and data not shown). The spectroscopic properties of ESR1C-cyan, cyan-ESR1C and citrine-ESR1C (Fig. 1a) were analysed in an F-7000 spectrofluorometer (Hitachi). Proteins fused to cyan were excited at the excitation wavelength of 432 nm (\(\lambda_{\text{ex}}\) for cyan) to obtain fluorescence emission spectra from 460 to 600 nm. Proteins fused to citrine were excited at 460 nm (\(\lambda_{\text{ex}}\) for citrine) to obtain fluorescence emission spectra from 500 to 600 nm. The emission spectra for ESR1C-cyan and cyan-ESR1C (Fig. 1b) were indistinguishable from each other and both showed a maximum peak at 478 nm and a minor peak at 500 nm (Fig. 1b, continuous and dashed lines). The emission spectra for ESR1C-citrine showed a maximum peak at 530 nm when excited at 460 nm (Fig. 1b, punctuated line). In all cases, the emission spectra correspond unequivocally either to cyan or citrine.

Next, we analysed the ESR1C–ESR1C interactions by calculating the ratio of Förster Resonance Energy Transfer (FRET). For FRET analyses, donor and acceptor proteins were mixed at 50 nM each one, and \(\lambda_{\text{ex}}\) was set at 432 nm to obtain fluorescence emission spectra from 460 to 600 nm. At optimal FRET, fluorescence increases at 530 nm (FRET signal), while fluorescence at 478 nm (cyan emission) decreases with an isosbestic wavelength at ~512 nm. As negative controls we subtracted the spectra obtained in the...
presence of the corresponding protein fused to citrine because this FP cross-excites slightly at 432 nm. Finally, the data were used to calculate a simple ratio of FRET (emission at 530 nm/emission at 478 nm) (Bellón-Echeverría et al., 2010). To evaluate the specificity of the ESR1C–ESR1C interaction and discard the interaction between FP, we obtained the emission spectra of ESR1C-cyan in the presence of purified recombinant citrine. The obtained spectrum almost totally overlapped with the spectrum obtained for ESR1C-cyan (Fig. 1c). ESR1C–ESR1C interaction was analysed by mixing equimolar concentrations of ESR1C-cyan and citrine-ESR1C. The FRET signal for this preparation was even greater than the FRET signal obtained for the positive control (NS5B–NS5B interaction, compare dashed and punctuated lines in Fig. 1d). Therefore, we were able to detect the ESR1C–ESR1C interaction by measuring FRET emission (Fig. 1d, dashed line). The interaction between ESR1C and NS5B was also measured by FRET. We obtained the emission spectrum of a mixture containing ESR1C-cyan and NS5B-citrine at equimolar concentration. In this case, and as for ESR1C self-interaction, we detected FRET emission indicating that interaction between ESR1C and HCV polymerase can also be identified and quantified by FRET (Fig. 1d, dot-bar-dot line). Therefore, we have

**Fig. 2.** Interaction ESR1C–NS5B. (a) FRET ratio calculated for NS5B-citrine mixed with cyan-ESR1C or ESR1C-cyan. Data are shown in percentage and they were normalized to the ratio of FRET for the interaction NS5B-citrine ESR1C-cyan that was considered 100%. (b) Relative ratio of FRET for the interaction ESR1C–NS5B (solid line), NS5B–NS5B (dashed line) and ESR1C–ESR1C (dotted line) as a function of NaCl concentration. (c) Effect of the presence of magnesium or manganese. Results are the mean and SEM of at least 10 independent experiments. Data are shown in percentage and they were normalized to the ratio of FRET for the interaction NS5B–ESR1C at 10 mM in the presence of manganese that was considered 100%. (d) Effect of the initiating ribonucleotide (GTP, named G), other ribonucleotides (ATP, CTP and UTP, named ACU) and RNA in the presence of magnesium or manganese. Experiments were performed at 10 mM NaCl. Data are shown in percentage and they were normalized to the ratio of FRET for the interaction NS5B–ESR1C in the presence of manganese that was considered 100%. *P<0.005 (Student’s t-test) when comparing the points representing primer-extension and de novo initiation conditions. Results are the mean and SEM of at least six independent experiments.
developed a new tool to analyse ESR1C–ESR1C and ESR1C–NS5B interactions.

We generated two different ESR1C constructs fused to cyan, with the fluorescent tag located at the N or C terminus (Fig. 1a). This strategy was performed in an attempt to detect changes in the ratio of FRET caused by the relative orientation of the interacting proteins with their respective FP. We calculated the ratio of FRET for the interaction of ESR1C-cyan and cyan-ESR1C with NS5B and we did not observe statistically significant differences (Fig. 2a).

Next, we analysed the relationship among ESR1C–ESR1C and ESR1C–NS5B interactions and the ionic strength. FRET ratios for NS5B–NS5B, ESR1C–ESR1C and ESR1C–NS5B were calculated at different NaCl concentrations (Fig. 2b). In all cases, FRET ratio decreased as ionic strength increased, indicating that the interaction is mainly electrostatic. Furthermore, the effect was stronger for the ESR1C–NS5B interaction than for the others (Fig. 2b, compare solid line against dashed and punctuated lines).

It has been described that different divalent cations are related to different polymerase activities. Thus, magnesium is the preferred divalent cation for elongation activity, whereas manganese is the preferred divalent cation for de novo initiation (Ranjith-Kumar et al., 2002). The presence of these cations could be affecting the overall conformation of the polymerase and therefore, could also be regulating the NS5B–ESR1C interaction. For this reason, we performed FRET experiments in the presence of magnesium or manganese. The results did not show differences between these divalent cations (Fig. 2c). We also performed experiments in the presence and in the absence of nucleotides using magnesium or manganese as divalent cations. The results (Fig. 2d) did not show significant differences in the ratios of FRET when comparing absence/presence of GTP (the initiating nucleotide) and absence/presence of the other ribonucleotides (ATP, CTP and UTP), independently of the divalent cation used in the experiment. Therefore, ESR1C–NS5B interaction occurs preferentially at low NaCl concentration and this is independent of the presence of magnesium, manganese or ribonucleotides. Finally, we performed FRET experiments in

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**Fig. 3.** ESR1C–NS5B interaction is affected by mutations on both proteins. (a) Effects produced by ESR1C mutants 255M (grey bars) and 258M (white bars) on the NS5B–ESR1C interaction. Data are shown in percentage and were normalized to the ratio of FRET for the interaction of NS5B with the ESR1C wild-type (black bars) at 10 mM NaCl concentration that was considered 100%. NaCl concentration used in each experiment is shown in the lowest part of the graph. Results are the mean and SEM of five independent experiments. *P*-values (Student’s *t*-test) are shown. (b) Effects produced by NS5B mutants G1F5, E18A, W397A, H428A and H502A on the NS5B–ESR1C interaction. Data are shown in percentage and were normalized to the ratio of FRET for the interaction of NS5B wild-type with ESR1C that was considered 100%. Results are the mean and SEM of five independent experiments. *P*-values are shown at the bottom. (c) Model proposed to explain the interaction NS5B–ESR1. Two NS5B monomers are depicted with dashed lines indicating NS5B–NS5B interactions.
the presence of the four nucleotides (ATP, CTP, GTP and UTP) and the RNA LE19 that can be replicated by de novo initiation or by primer-extension mechanisms (Ranjith-Kumar et al., 2002). The divalent cations were Mn$^{2+}$, which has been described as favouring de novo initiation and inhibits the primer-extension activities, or Mg$^{2+}$ that is essential for primer extension activity (Ranjith-Kumar et al., 2002). Both cations were added at a 5 mM final concentration. Under de novo initiation conditions the ratio of FRET was indistinguishable from the control. However, when primer extension reaction conditions were tested, the ratio of FRET underwent almost a 25% decrease (Fig. 2d). Therefore, de novo initiation conditions, previously related to NS5B–NS5B interactions (Chinnaswamy et al., 2010), instead of primer extension reaction conditions are important for NS5B–ESR1C interaction (Fig. 2d).

In previous work, Watashi and co-workers reported that the ESR1–NS5B interaction was lead by the C domain of ESR1 (ESR1C) and that some ESR1C mutants were unable to interact with NS5B (Watashi et al., 2007). For this reason, mutants ESR1C255M and ESR1C258M were fused to cyan, overexpressed in bacteria and purified as described above. We obtained the ratio of FRET for these mutants and compared them to wild-type protein. Both mutants showed significant diminished FRET ratio values at 50 mM NaCl concentration (Fig. 3a). At the lowest tested NaCl concentration (10 mM) at which the interaction would be more favoured, only ESR1C255M mutant showed a diminished interaction with NS5B (Fig. 3a). These data could be indicating that (i) mutant phenotype is dependent on ionic strength or, (ii) FP fused in the proximity of the mutation could alter the interaction phenotype. Because both mutants are very close (Watashi et al., 2007), the latter would be in contradiction to the data obtained for the 255M mutant. In any case, the previously reported deleterious binding phenotype was obtained at 50 mM NaCl for both mutants.

Finally, we wanted to know if NS5B–NS5B oligomerization is required for the ESR1C–NS5B interaction. For this reason, we analysed the FRET ratios for the interactions between ESR1C and NS5B point mutants E18A, W397A, H428A and H502A. All of these mutants, but W397A, were defective for the ESR1C–NS5B interaction (Fig. 3b). Mutants E18 and H502 have been previously related to defects in NS5B self-interaction (Bellón-Echeverría et al., 2010; Qin et al., 2002). Mutants W397A and H428A showed an open conformation and the ability to bind dsRNA, although this phenotype was more evident for H428A than for W397A (Chinnaswamy et al., 2008). Interestingly, H428A mutant did not oligomerize, whereas W397A mutant showed oligomerization values similar to wild-type NS5B (I. Bellón-Echeverría, unpublished results). Therefore, all mutants defective for NS5B oligomerization were also defective for ESR1C–NS5B interaction (Fig. 3b). To test the robustness of this hypothesis we used a NS5B mutant with increased NS5B–NS5B interactions. This mutant is a chimera of genotype 1b with the alpha F helix from genotype 5 (G15F) and it showed almost a 40% increment in NS5B–NS5B FRET ratio when compared with the NS5B genotype 1b wild-type (P. Clemente-Casares, unpublished results). The ratio of FRET for the NS5B(G1F5)–ESR1C interaction showed a significant increase when compared with NS5B wild-type (Fig. 3b).

All of these results allow us to infer that the oligomerization state of NS5B is important for ESR1–NS5B interaction. Because ESR1 in the absence of ligand is largely located in the cytosol as a monomer, we suggest a model in which two interacting NS5B molecules form the unit of interaction with ESR1 (Fig. 3c). In conclusion, we have developed a FRET-based method that allows us to study the molecular determinants of the ESR1–NS5B interaction to test the effect of currently approved drugs and also to support the development of new virus-directed antiviral strategies.

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References


