Perturbation of epidermal growth factor receptor complex formation and Ras signalling in cells harbouring the hepatitis C virus subgenomic replicon

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Hepatitis C virus non-structural NS5A protein inhibits epidermal growth factor (EGF)-stimulated activation of the Ras–ERK mitogen-activated protein kinase pathway at a point upstream of Ras activation. To determine the mechanism of this inhibition, the events occurring between the EGF receptor and Ras in Huh-7 cells harbouring the HCV subgenomic replicon were investigated. It was shown that, following EGF stimulation, these cells exhibited decreased EGF receptor tyrosine phosphorylation, aberrant recruitment of the adaptor proteins ShcA and Grb2 to the EGF receptor, reduced phosphorylation of ShcA and reduced Ras activation in comparison with control cells. These data are consistent with effects of NS5A and/or other components of the replicon on multiple events occurring upstream of Ras.

INTRODUCTION

Hepatitis C virus (HCV) is the leading cause of liver disease and transplantation in the developing world and is estimated to infect 3% of the global population (WHO, 1999). More than 80% of those infected will develop a chronic disease culminating in liver fibrosis, cirrhosis and hepatocellular carcinoma. The HCV genome is a 9.5 kb positive-sense RNA molecule that is translated in a cap-independent fashion via an internal ribosome entry site to generate a 3000 residue polypeptide, cleaved by host and viral proteases to produce 10 mature viral proteins.

The NS5A protein is one of six non-structural proteins that are believed to form a membrane-bound RNA-replication complex (Macdonald & Harris, 2004). Further to this role, NS5A has been shown to modulate a range of cellular signalling pathways, including the mitogen-activated protein kinase (MAPK) pathways. In particular, we and others have shown that NS5A expression is able to inhibit the Ras–ERK MAPK pathway (Tan et al., 1999; Georgopoulou et al., 2003; Macdonald et al., 2003). This pathway is activated by growth factors binding to their cognate receptors; this induces autophosphorylation of the receptors on tyrosine residues, which then act as ligands for the Src homology 2 (SH2) domains of adaptor proteins, such as ShcA and Grb2. Grb2 associates with a guanine nucleotide-exchange factor, Sos, which induces GDP/GTP exchange on the GTP-binding protein Ras (Tari & Lopez-Berestein, 2001). Ras then binds to and activates a serine kinase, Raf, which triggers a kinase cascade ultimately leading to c-Fos expression and concomitant activation of the AP-1 transcription factor (of which c-Fos is a component). We previously demonstrated that transfection of a dominant-active mutant of Ras was able to override the NS5A-mediated block to this pathway and we therefore concluded that NS5A was acting between the epidermal growth factor receptor (EGFR) and the activation of Ras (Macdonald et al., 2003). The mechanism of this inhibition is unknown, although it has been shown that NS5A is able to bind Grb2 (Tan et al., 1999; Macdonald et al., 2004). By doing so, it is possible that NS5A might inhibit the formation of the Grb2–Sos complex, thereby breaking the link between the activated EGFR and Ras. However, it has previously been stated that, in HeLa cells expressing NS5A, there was no effect on the levels of the Grb2–Sos complex (Tan et al., 1999), although these data have not been presented. We therefore sought to investigate further the mechanisms by which NS5A is able to disable EGF-mediated Ras–ERK signalling by analysing EGFR-proximal signalling events in Huh-7 cells harbouring an HCV subgenomic replicon (expressing the non-structural proteins NS3–NS5B) at very early time points following EGF stimulation. By using a combination of immunoprecipitation and immunoblotting, together with an assay for Ras activation, we have demonstrated that multiple EGFR-proximal events are perturbed in replicon cells, compared with naive Huh-7 cells.

METHODS

Cell culture. Parental Huh-7 cells and Huh-7 cells harbouring the culture-adapted subgenomic HCV replicon (FK5.1) (Krieger et al.,...
were cultured in modified Eagle’s medium supplemented with 1 % (v/v) non-essential amino acids, 10 % (v/v) fetal calf serum, 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Cell lines were incubated at 37 °C in a humidified 5 % CO₂ incubator. To generate the FK5.1 replicon cell line, Huh-7 cells were electroporated with T7 transcripts produced from the Scal linearized plasmid pFK-I<sub>399</sub>neo/NS3-3’/S.I (provided by Professor Ralf Bartenschlager, University of Heidelberg, Germany). Transfected cells were allowed to recover for 24 h before the addition of 1 mg G418 ml⁻¹ to the culture medium. Selection with G418 was maintained for 16 days, during which time the medium was changed every 3–4 days. G418-resistant colonies were maintained as a polyclonal cell line in medium supplemented with 250 μg G418 ml⁻¹.

**Immunoprecipitation and Western blot analysis.** To analyse the EGFR complex, cells were lysed in EGFR lysis buffer (50 mM Tris/ HCl (pH 8.0), 50 mM NaCl, 1 % NP-40, 1 mM Na<sub>2</sub>VO₄, 100 mM NaF and protease inhibitors (Boehringer Mannheim)) at 4 °C for 30 min. Lysates (500 μg total protein) were precipitated with antibodies directed against EGFR (Upstate), ShcA (Upstate), Grb2 (Santa Cruz) or Sos (Santa Cruz) for 1 h at 4 °C, followed by incubation with either protein G- or protein A-conjugated agarose beads (Sigma) for a further 2 h at 4 °C under rotation. Immune complexes were washed in lysis buffer and bound proteins were analysed by SDS-PAGE and Western blot. PVDF membranes were probed by using an in-house polyclonal antiserum to NS5A or antibodies to EGFR (Upstate), Grb2 (Santa Cruz), Sos (Santa Cruz), phosphotyrosine (4G10; Upstate) or ShcA (Upstate). Cell lysates (50 μg total protein) were also probed with antibodies for the phosphorylated forms of ERK, ShcA (Tyr-317), pan-ERK and Ras (all from Cell Signalling Technology) following the manufacturer’s recommendations. Western blots were visualized by using the ECL system (Amersham Biosciences).

**Protein expression and purification.** The bacterial-expression vector pGST-RBD encoding aa 51–131 of Raf-1 fused to glutathione S-transferase (GST) (de Rooy & Bos, 1997) was obtained from Dr Walter Kolch (Beatson Institute for Cancer Research, Glasgow, UK). The GST–RBD fusion protein was purified by using standard techniques (Smith & Johnson, 1988; Macdonald et al., 2004).

**Affinity precipitation of Ras–GTP.** Cells were seeded into 90 mm dishes and allowed to reach 70–80 % confluence, then serum-starved overnight and stimulated with 100 ng EGF ml⁻¹. After treatment, cells were washed in ice-cold PBS, and 200 μl magnesium-containing lysis buffer [MLB: 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 % (v/v) NP-40, 0.25 % sodium deoxycholate, 10 % (v/v) glycerol, 10 mM MgCl₂, 1 mM EDTA and protease inhibitors (Boehringer Mannheim)] was added. Samples were lysed on ice for 20 min and centrifuged (13 000 × g) for 10 min to pellet the debris. The protein concentration of each sample was determined by using the BCA assay (Pierce). Active Ras was precipitated from the cleared lysate (200 μg) by adding 20 μl glutathione–agarose beads pre-bound with GST–RBD and the samples were incubated under rotation at 4 °C for 30 min. The beads were washed three times in 300 μl MLB and resuspended in 30 μl 4 × SDS loading buffer prior to analysis by SDS-PAGE. Levels of Ras–GTP were detected by Western blot analysis with a monoclonal anti-Ras antibody (Upstate) and compared with a blot of total cellular Ras, taken from the lysate.

**RESULTS**

**EGF-mediated stimulation of ERK phosphorylation is blocked in replicon cells**

We have shown previously that ERK1/2 phosphorylation is reduced at late time points after treatment of replicon cells with EGF (from 0.5 to 4 h) (Macdonald et al., 2003). As the Ras–ERK pathway is normally activated rapidly (within 2 min) after EGF treatment, it was conceivable that our previous study had missed an early transient peak of ERK1/2 phosphorylation in replicon cells. To test this hypothesis, parental Huh-7 and replicon cells were treated with EGF (100 ng ml⁻¹) and harvested at time points up to 20 min post-treatment. Fig. 1(a) shows that, in parental Huh-7 cells, the increase in phosphorylation of ERK1/2 was essentially complete by 2 min and levels of phosphorylated ERK1/2 remained constant up to 20 min. In contrast, there was no increase in the level of phosphorylated ERK1/2 in the replicon cells, even at 20 min after EGF treatment. Overall levels of ERK1/2 were the same in both cell lines (Fig. 1b). This result confirmed that the block in ERK1/2 phosphorylation in replicon cells occurred at very early times after EGF treatment. It is important to point out at this stage that the replicon cells used in this study were a polyclonal population, derived by pooling all of the G418-resistant cells obtained following transfection of Huh-7 cells with FK5.1 RNA. It was therefore unlikely that the differences observed between replicon and naive cells were the result of clonal variations in Huh-7 cells.

**Reduced EGFR phosphorylation and adaptor-protein recruitment in replicon cells following EGF treatment**

Following EGF binding, the EGFR undergoes autophosphorylation on several tyrosine residues, thus providing binding sites for SH2 domains of adaptor proteins, in particular ShcA and Grb2. To examine these early events in EGF signalling, we immunoprecipitated EGFR from parental Huh-7 and replicon cells and probed the immunoprecipitates with antibodies to phosphotyrosine, ShcA and Grb2 by Western blotting. Fig. 2(a) shows that, as expected, in parental cells, there was a large increase in...
tyrosine phosphorylation of EGFR at 2 min after EGF treatment, and that this was sustained until 10 min after treatment and then declined [Fig. 2a(ii)]. In replicon cells, EGFR-bound ShcA increased very slightly, but not to the levels seen in the parental cells. Interestingly, significant levels of ShcA were associated with EGFR in the absence of EGF stimulation or EGFR tyrosine phosphorylation [Fig. 2a(ii), lanes 1 and 6]. This was unexpected, as it is generally assumed that ShcA can only bind to the phosphorylated receptor via its SH2 or phosphotyrosine-binding domains; however, there is evidence in the literature for ShcA association with non-ligand-bound EGFR (Ravichandran, 2001). This could be via other EGFR-interacting factors; for example, Shc contains a number of PxxP motifs that have been reported to interact with the SH3 domains of Src family tyrosine kinases.

Following phosphorylation of EGFR and ShcA recruitment, the SH2 domain of the adaptor protein, Grb2, can associate with phosphotyrosines present on either EGFR or ShcA. Consistent with this, Western blotting of EGFR immunoprecipitates with an antibody to Grb2 revealed a transient association of Grb2 with EGFR in parental Huh-7 cells: Grb2 was present in the EGFR immunoprecipitate at 2 min after EGF treatment but, by 5 min after treatment, no Grb2 could be detected in association with EGFR [Fig. 2a(iii)]. In contrast, no EGFR-associated Grb2 was observed in replicon cells at any time. We concluded that the amount of Grb2 associated with EGFR following EGF stimulation was reduced to below detectable levels in replicon cells. When ShcA was immunoprecipitated from these lysates, Western blotting with an antibody to phosphorylated EGFR confirmed that there were reduced levels of the latter in replicon cells [Fig. 2b(i)]. Following recruitment of ShcA to the phosphorylated EGFR, ShcA is tyrosine-phosphorylated by EGFR on residue 317 (Fan et al., 2004), providing a binding site for the Grb2 SH2 domain and resulting in subsequent signalling through the Ras–ERK pathway. To analyse phosphorylation of Tyr-317, we probed cell lysates with a phospho-specific antibody [Fig. 2c(i)]. This revealed that, in parental cells, ShcA was tyrosine-phosphorylated rapidly (within 2 min); however, in replicon cells, this event was both delayed (until 5 min) and significantly impaired. Taken together, these data are consistent with perturbation of receptor-proximal events in the EGF-signalling cascade.

**EGF signalling promotes an association of NS5A with the Grb2–Sos complex**

A C-terminal PxxP motif in NS5A has been shown to interact with the SH3 domains of Grb2; however, these domains also interact with PxxP motifs within Sos. Although the Grb2–Sos interaction has been reported to be constitutive and unaffected by EGF signalling (Okada & Pessin, 1996), it is conceivable that the interaction between NS5A and Grb2 would disrupt the Grb2–Sos interaction and thereby block Ras–ERK signalling. To test this, we immunoprecipitated either Grb2 or Sos from EGF-stimulated

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**Fig. 2.** EGF-stimulated EGFR phosphorylation and adaptor-protein recruitment are reduced in replicon cells. Parental Huh-7 cells (lanes 1–5) or Huh-7 cells harbouring the FK5.1 culture-adapted HCV subgenomic replicon (lanes 6–10) were treated with 100 ng EGF ml$^{-1}$ for the indicated time periods before harvesting. (a) Lysates were immunoprecipitated with an antibody specific for EGFR and immunoprecipitates were analysed by Western blotting with antibodies specific for phosphotyrosine (i), ShcA (ii), Grb2 (iii) and EGFR (iv). The three bands present in (ii) represent the three isoforms of ShcA (p46, p52 and p66). (b) Lysates were immunoprecipitated with an antibody specific for ShcA and immunoprecipitates were analysed by Western blotting with antibodies specific for phosphotyrosine (i) or ShcA (ii). (c) Total cell lysates were analysed by Western blotting with antibodies specific for ShcA phosphorylated on Tyr-317 (i) or total ShcA (ii).
parental Huh-7 or replicon cells and analysed the immunoprecipitates by Western blotting. Fig. 3[a(i)] shows that, prior to EGF treatment, no NS5A was associated with Grb2; however, at 2 min post-treatment, there was a dramatic increase in the level of NS5A associated with Grb2. This interaction was sustained until 20 min after treatment, with only a slight decline. Likewise, when lysates were immunoprecipitated with an anti-Sos antibody, NS5A could be detected in the precipitates only after 2 min EGF treatment [Fig. 3b(i)]. In both parental and replicon cells, the level of Grb2 associated with Sos showed a marginal decline over the 20 min time course [Fig. 3b(ii)]. Although the decline appeared to be more pronounced in replicon cells, this was unlikely to be due to displacement by NS5A, as the interaction of NS5A with Grb2–Sos preceded the decline in Sos-associated Grb2 by several minutes. Judging by the intensity of the NS5A signal in the Grb2 and Sos immunoprecipitates, it seemed likely that NS5A was binding directly to Grb2, rather than to Sos [compare Fig. 3a(i) and b(i)].

Ras activation is impaired in cells expressing the HCV subgenomic replicon

Recruitment of Grb2–Sos to the cell membrane brings Sos into the proximity of Ras, where it is able to activate the latter by promoting GDP/GTP exchange. Therefore, it was important to determine whether Ras activation was altered in cells expressing the HCV subgenomic replicon. To do this, we precipitated active (GTP-bound) Ras from lysates of EGF-stimulated cells by using a GST-fusion protein containing the Ras-binding domain of Raf-1 (GST–RBD). Fig. 4(a) shows that, in parental cells, EGF-mediated activation of Ras peaked at 2 min and was maintained until at least 10 min, after which it declined gradually. In replicon cells, the kinetics of activation of Ras were similar, in that levels peaked at 2 min; however, overall levels of Ras–GTP were reduced and, furthermore, the intensity of the signal declined earlier than in the parental cells. Overall levels of Ras were similar in both cell populations (Fig. 4b). A further control for the assay is presented in Fig. 4(d): Ras–GTP was recognized specifically by the RBD portion of the fusion protein, as GST expressed alone was unable to precipitate Ras from cell lysates. Thus, these data demonstrated that

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**Fig. 3.** EGF stimulation-dependent association of NS5A with the Grb2–Sos complex. Parental Huh-7 cells (lanes 1–5) or Huh-7 cells harbouring the FK5.1 culture-adapted HCV subgenomic replicon (lanes 6–10) were treated with 100 ng EGF ml$^{-1}$ for the indicated time periods before harvesting. (a) Lysates were immunoprecipitated with an antibody specific for Grb2 and immunoprecipitates were analysed by Western blotting with antibodies specific for NS5A (i) or Grb2 (ii). The non-specific band seen in all lanes in (i) is the Ig heavy chain (~50 kDa). (b) Lysates were immunoprecipitated with an antibody specific for Sos and immunoprecipitates were analysed by Western blotting with antibodies specific for NS5A (i), Grb2 (ii) or Sos (iii).

**Fig. 4.** Ras activation is reduced in replicon cells. Parental Huh-7 cells (lanes 1–5) or Huh-7 cells harbouring the FK5.1 culture-adapted HCV subgenomic replicon (lanes 6–10) were treated with 100 ng EGF ml$^{-1}$ for the indicated time periods before harvesting. Total cell lysates were incubated with purified GST–RBD and washed, and bound protein was analysed by Western blotting with a Ras-specific antibody (a). Total cell lysates were also analysed directly for total Ras levels (b) and the integrity and purity of the GST–RBD fusion protein was confirmed by Coomassie blue staining (c). As a control, total cell lysates were also incubated with purified GST and washed, and bound protein was analysed by Western blotting with a Ras-specific antibody (d), with the integrity and purity of GST in this assay being shown in (e). Total cell lysates were analysed by Western blotting with an NS5A-specific antiserum (f).
the activation of Ras in response to EGF stimulation is impaired in replicon cells, providing an explanation for the decreased activation of the ERK–MAPK pathway in these cells.

**DISCUSSION**

The data presented in this paper demonstrate that the EGFR–Ras–ERK signalling cascade is perturbed in replicon cells. A schematic diagram of the Ras–ERK pathway and the individual events that are shown to be inhibited within replicon cells is illustrated in Fig. 5. Taken together with previous observations (Macdonald et al., 2003), our findings are consistent with the hypothesis that this disruption is mediated by NS5A and results in the inhibition of growth factor-induced activation of the transcription factor AP-1. Although we cannot at this stage rule out the involvement of other non-structural proteins, this seems unlikely for the following reasons. Firstly, we have previously shown that AP-1 activation via the Ras–ERK pathway is blocked both by NS5A alone and also by the full-length HCV polyprotein. Secondly, we have recently demonstrated (Macdonald et al., 2005) that AP-1 activation is also inhibited in cells harbouring the FK5.1 replicon and that this block is abrogated by mutation of an SH3 domain-binding proline motif in NS5A (a mutation that also abrogates Grb2 binding). One discrepancy that needs to be clarified is the reported ability of the Core protein to stimulate multiple MAPK pathways (Fukuda et al., 2001; Erhardt et al., 2002). However, it should be pointed out that these studies were performed in cells expressing Core protein alone, and our data, using the full-length HCV polyprotein, would suggest that the inhibitory effect of NS5A on the Ras–ERK pathway is dominant.

The observation that EGF autophosphorylation is inhibited in replicon cells was unexpected, as it has not been reported that any of the HCV non-structural proteins interact directly with this receptor. Indeed, we were unable to detect NS5A complexed to EGFR (data not shown), so this is unlikely to be due to a direct interaction with NS5A. However, EGFR is known to be dephosphorylated by a number of protein tyrosine phosphatases (Tiganis, 2002), including cdc25A and Shp1/2, the latter containing SH2 domains that mediate recruitment to phosphorylated EGFR and thereby switch off the signal. One possible explanation, therefore, is that the activity or expression of protein tyrosine phosphatases might be increased in replicon cells. Interestingly, two recent reports provide an indirect precedent for such a hypothesis: firstly, upregulation of protein phosphatase 2A was reported in HCV-positive liver biopsies and HCV-transgenic mice (Duong et al., 2004) and, secondly, treatment of cells with recombinant HCV glycoproteins was reported to activate Shp2 (Balasubramanian et al., 2003).

Our results are consistent with the hypothesis that NS5A, by interacting with the Grb2–Sos complex, is able to perturb Sos-mediated Ras activation without disrupting the Grb2–Sos interaction. The EGF-dependent association of NS5A with the Grb2–Sos complex is highly reminiscent of the Sprouty (Spry1/2) protein family (Hanafusa et al., 2002). Spry1/2 has been shown to bind to Grb2 in a growth factor-dependent fashion, preventing recruitment of Grb2 to the receptor, but not disrupting the Grb2–Sos complex. The mechanism underpinning this has been shown to involve growth factor-stimulated translocation of Spry to the membrane and subsequent tyrosine phosphorylation of Spry, facilitating association with the SH2 domain of Grb2. In contrast, NS5A interacts with the SH3 domains of Grb2; however, it is conceivable that this interaction might block the association of the Grb2 SH2 domain with either Shc or the EGFR, possibly by steric hindrance. This might explain why no Grb2 was observed complexed to EGFR in replicon cells, in comparison with naive cells [Fig. 2a(iii)]. Interestingly, the activation of Ras is only partially abrogated in replicon cells although, downstream of Ras, the block in ERK phosphorylation is more pronounced. This suggests that there may be further, as yet unidentified, ways in which NS5A and/or other non-structural proteins are able to modulate the Ras–ERK pathway. In this regard, the hepatitis E virus ORF3 protein was recently shown to activate ERK by inactivating an ERK-specific phosphatase (Kar-Roy et al., 2004). It is intriguing to speculate that, as in the case of the EGFR

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**Fig. 5.** Schematic diagram of the interactions between NS5A and receptor-proximal events in the EGFR signalling pathway. After EGF binding, EGFR homodimerizes and undergoes autophosphorylation, thus providing a binding site for the SH2 domain of ShcA. EGFR then phosphorylates ShcA on Tyr-317, providing a binding site for the SH2 domain of Grb2. Once recruited to the membrane, Sos promotes GDP→GTP exchange on Ras, resulting in activation of the ERK pathway. A proline motif within NS5A (Macdonald et al., 2004) interacts with one of the SH3 domains of Grb2. This does not disrupt the Grb2–Sos interaction, but perturbs the ability of Sos to activate Ras. In addition, we propose that NS5A, either alone or in conjunction with other non-structural proteins, inhibits both EGFR autophosphorylation and ShcA phosphorylation as indicated.
phosphatases discussed above, HCV might activate an ERK-specific phosphatase.

The physiological significance of ERK inhibition for virus replication remains to be determined. However, it is pertinent to note that ERK signalling is involved in many processes, such as control of the cell cycle: growth-factor signalling through ERK is required for hepatocytes to transit a block in late G₁ (Talamin et al., 1999) and many viruses, such as murine coronavirus (Chen et al., 2004), human parvovirus B19 (Morita et al., 2003) and Kaposi’s sarcoma-associated herpesvirus (Izumiya et al., 2003), have been shown to effect a G₁ block to favour virus replication. Interestingly, a recent study observed elevated levels of G₁-phase hepatocytes in biopsy material taken from patients with HCV compared with alcoholic liver disease, providing indirect evidence for a G₁ block in HCV infection (Marshall et al., 2005). We propose, therefore, that the inhibition of Ras–ERK signalling by NS5A may play a role in cell-cycle perturbation during HCV infection. We have also shown that NS5A, either alone or in the context of the subgenomic replicon, can activate phosphoinositide 3-kinase, resulting in increased resistance to the induction of apoptosis (Street et al., 2003). Given that a G₁ block can result in the induction of apoptosis (Chang et al., 2004), the differential effects of NS5A on these two signalling cascades may represent a coordinated strategy to modulate the cellular environment to favour virus replication and persistence. This is an attractive hypothesis that is currently under investigation in our laboratory. It also suggests that the interactions between NS5A and cellular SH3 domain proteins, such as Grb2, are valid targets for antiviral-drug development.

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