Cap-dependent and hepatitis C virus internal ribosome entry site-mediated translation are modulated by phosphorylation of eIF2α under oxidative stress

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Chronic hepatitis C is often associated with oxidative stress. *Hepatitis C virus* (HCV) utilizes an internal ribosome entry site (IRES) element for translation, in contrast to cap-dependent translation of the majority of cellular proteins. To understand how virus translation is modulated under oxidative stress, HCV IRES-mediated translation was compared with cap-dependent translation using a bicistronic reporter construct and hydrogen peroxide (H_2O_2) as a stress inducer. In H_2O_2-sensitive HeLa cells, H_2O_2 repressed translation in a time- and dose-dependent manner, concomitant with the kinetics of eIF2α phosphorylation. A phosphomimetic of eIF2α, which mimics the structure of the phosphorylated eIF2α, was sufficient to repress translation in the absence of H_2O_2. In H_2O_2-resistant HepG2 cells, H_2O_2 activated both HCV IRES-mediated and cap-dependent translation, associated with an increased level of phospho-eIF2α. It was postulated that H_2O_2 might stimulate translation in HepG2 cells via an eIF2α-independent mechanism, whereas the simultaneous phosphorylation of eIF2α repressed part of the translational activities. Indeed, the translational repression was released in the presence of a non-phosphorylatable mutant, eIF2α-SA, resulting in further enhancement of both translational activities after exposure to H_2O_2. In HuH7 cells, which exhibited an intermediate level of sensitivity towards H_2O_2, both HCV IRES-mediated and cap-dependent translational activities were upregulated after treatment with various doses of H_2O_2, but the highest level of induction was achieved with a low level of H_2O_2, which may represent the physiological level of H_2O_2. At this level, the HCV IRES-mediated translation was preferentially upregulated compared with cap-dependent translation.

INTRODUCTION

Hepatitis C is a severe medical problem affecting around 3 % of the world's population (Lauer & Walker, 2001). *Hepatitis C virus* (HCV) is a single-stranded, positive-sense RNA virus (Kato *et al.*, 1990). Its genome encodes a single polyprotein of ~3010 aa, which is processed into structural (core, envelope glycoproteins E1 and E2, p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (Moradpour *et al.*, 2002).

In contrast to cap-dependent translation of the majority of cellular mRNAs, the initiation of HCV translation is cap-independent and mediated by a highly conserved internal ribosome entry site (IRES) element located within the 5′ non-coding terminal region (NTR) and extends into the core

protein open reading frame (Reynolds *et al.*, 1995; Tsukiyama-Kohara *et al.*, 1992). The involvement of the X region in the 3′ NTR and viral proteins in the modulation of HCV IRES-mediated translation has also been implicated (Boni *et al.*, 2005; Ito *et al.*, 1998; Kalliampakou *et al.*, 2005). Both canonical and non-canonical eukaryotic initiation factors (eIFs) have been shown to play essential roles in IRES-dependent translation. The HCV IRES can directly recruit the 40S ribosomal subunit before forming an initiation complex with eIF2 and eIF3 (Pestova *et al.*, 1998). eIF2Bγ and eIF2γ have also been identified as co-factors in HCV IRES-mediated translation (Krüger *et al.*, 2000). Several cellular RNA-binding proteins, including La autoantigen and polypyrimidine tract-binding protein, have been implicated in efficient HCV IRES-mediated translation (Ali & Siddiqui, 1995, 1997).

Accumulation of reactive oxygen species (ROS) and the generation of oxidative stress have been implicated in the development of a number of inflammatory diseases, including viral hepatitis (Schwarz, 1996). ROS can cause oxidative damage to intracellular macromolecules and modulate cellular signal transduction pathways. Immune recognition of infected hepatocytes triggers the release of ROS (e.g. superoxide anion, hydrogen peroxide) from sequestered phagocytes and activated macrophages. It is evident that oxidative stress is associated with HCV infection. Chronic hepatitis C patients present elevated blood and hepatic levels of ROS, with increased lipid peroxidation and decreased hepatic glutathione (De Maria et al., 1996; Paradis et al., 1997). Intrahepatic gene expression profiling using microarray analysis has demonstrated upregulation of the oxidative stress-inducible genes in hepatitis C samples (Yamashita et al., 2001). Data from studies in vitro and in vivo suggest the direct involvement of HCV replication and gene expression in the generation of ROS. Replication of the subgenomic replicon of HCV in neomycin-selected cultured cells results in increased oxidative stress (Qadri et al., 2004). HCV core, NS3 and NS5A proteins are capable of inducing oxidative stress in cultured hepatocytes, monocytes and isolated mitochondria, and transgenic mice carrying the structural proteins exhibit elevated levels of ROS and are more susceptible to oxidant injury (Bureau et al., 2001; Gong et al., 2001; Korenaga et al., 2005; Moriya et al., 2001; Okuda et al., 2002). In the case of NS5A, it has been proposed that oxidative stress is triggered by the increased efflux of Ca^{2+} from the endoplasmic reticulum as a result of endoplasmic reticulum stress (Gong et al., 2001).

It is unclear how the virus itself can circumvent oxidative stress in terms of replication, translation and survival. To understand how virus translation is modulated under oxidative stress, we compared HCV IRES-mediated and cap-dependent translational activities using a bicistronic reporter construct (Collier et al., 1998).

METHODS

Cell culture. HeLa and HuH7 cells were maintained in Dulbecco's modified Eagle's medium. The medium of HuH7 cells was supplemented with $1\times$ non-essential amino acids. HepG2 cells were maintained in Eagle's minimal essential medium and $1\times$ non-essential amino acids. Wild-type A549 cells and A549 cells stably expressing eIF2α-SA were obtained from Costas Koumenis (Koumenis et al., 2002) and maintained in Ham's F12 medium. All media were supplemented with 10 % fetal calf serum, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 2 mM glutamate.

XTT viability assay. An XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay was performed according to the manufacturer's instructions (Cell Proliferation kit II; Roche). Cells seeded in 96-well plates were treated with H_2O_2 for 24 h before the addition of XTT. Readings were taken at 450 nm using a 650 nm reference filter and were corrected for background absorbance. The values shown represent means ± SEM of three independent experiments performed in triplicate and were expressed relative to the untreated control, which was set as 1 and represented 100 % viability.

ROS measurement. The generation of intracellular ROS was measured using the probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma) (Wang & Joseph, 1999). Cells seeded in 96-well plates were washed with PBS and pre-loaded with freshly prepared 100 μM DCFH-DA for 30 min at 37 °C. Following several PBS washes, cells were exposed to 100 μl medium containing serial dilutions of H_2O_2. The fluorescence from each well was measured immediately using a microplate fluorimeter (Twinkle; Berthold Technologies) with the excitation filter set at 485 nm and the emission filter at 535 nm with the temperature maintained at 37 °C. Data were collected every 5 min for 90 min. Each data point represents the mean ± SEM of three independent experiments performed in triplicate.

Plasmids. The bicistronic construct pRL encoding the Renilla luciferase (RLuc) and firefly luciferase (FLuc) genes under the control of a T7 promoter and a genotype 1b HCV IRES has been described previously (Collier et al., 1998). In this study, the bicistronic construct was subcloned to generate the plasmid pRFHCV1b, which contained the RLuc gene under the control of the cytomegalovirus (CMV) promoter and the FLuc gene under the control of the HCV IRES (see Fig. 1c). Briefly, a SacI–HindIII fragment containing the bicistronic construct was excised from pRL and subcloned into the vector pEGFP-N1 (Clontech). From this, a XhoI–ApaI fragment was excised and subcloned into the vector pcDNA3.1 (Invitrogen) to generate pRFHCV1b. Translation from the RLuc gene was cap-dependent, whereas translation from the FLuc gene was IRES-mediated. A promoterless (ΔCMV) pRFHCV1b plasmid was constructed by excising an MluI–XhoI fragment containing the CMV promoter from pRFHCV1b. Plasmids encoding eIF2α, eIF2α-SA, eIF2α-SD and the empty vector control hCD2 were obtained from David Ron (Novoa et al., 2001).

Transfection. DNA transfection was performed in 24-well plates according to the manufacturer's instructions using PolyFect (Qiagen) (HeLa cells), SuperFect (Qiagen) (HepG2 cells) or TransPEI (Eurogentec) (HepG2, HuH7 and A549 cells). Cells were then treated with H_2O_2 for the indicated time and harvested for a dual luciferase assay and for RT-PCR. Morphological changes in cells were captured by phase-contrast microscopy (Zeiss) at the time of harvest and again at 24 h post-treatment. RNA transfection was performed according to the manufacturer's instructions using a TransIT-mRNA Transfection kit (Mirus).

Dual luciferase assay. The activities of RLuc and FLuc were measured in relative light units over 10 s with a luminometer (Lumat LB9507; Berthold Technologies) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Protein concentrations were determined using the Bradford assay or an RC-DC protein assay kit (Bio-Rad). Luciferase activities, normalized with respect to total protein, were expressed relative to the untreated or empty vector controls. The IRES/cap ratio represented the ratio of FLuc to RLuc activity and was expressed relative to the control, which was set as 1. The values obtained represented means ± SEM of three independent experiments performed in triplicate.

RT-PCR. Total RNA from each well was extracted into 200 μl TRIzol (Invitrogen) or RNA-Bee (Tel-Test) according to the manufacturers' instructions. We confirmed that RNA extracted by this method was free from contaminating DNA by the absence of PCR amplification from DNase-free RNase I-digested RNA samples. Nevertheless, to ensure that the fragments detected were not amplified from residual vector DNA, the RNA sample was pre-treated with 1 U RNase-free DNase I (Roche). Total RNA (1 μg) was reverse
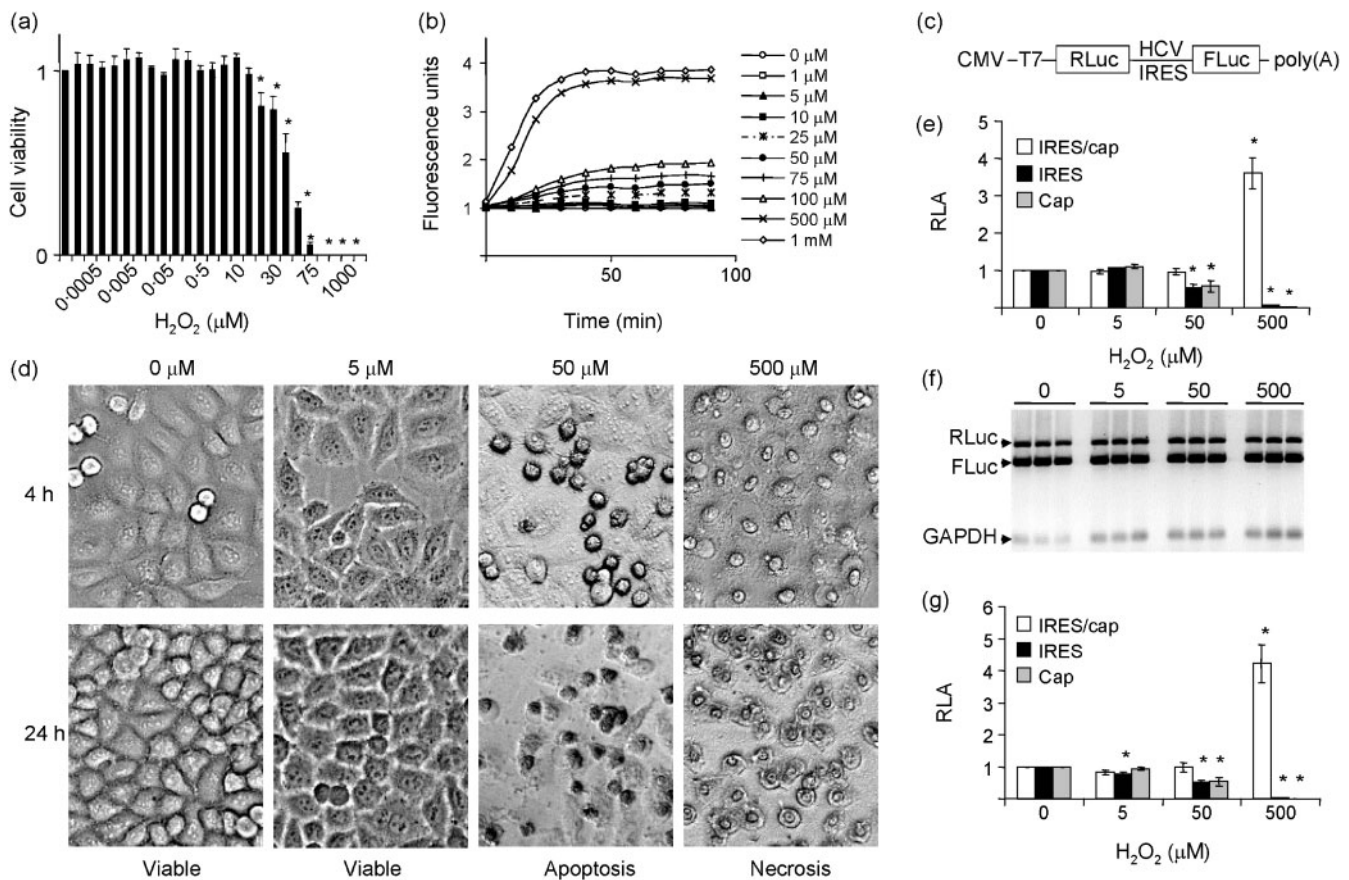
Fig. 1. H_2O_2 represses HCV IRES-mediated and cap-dependent translation in HeLa cells. (a) XTT assay showing the viability of HeLa cells (10 000 cells per well in 96-well plates) after treatment with 0–1000 μM H_2O_2 for 24 h. (b) DCFH-DA fluorometric assay showing the time course of ROS generation in HeLa cells (10 000 cells per well in 96-well plates) after treatment with 0–1000 μM H_2O_2. (c) Diagrammatic representation of the bicistronic construct pRFHCV1b. (d–f) HeLa cells were transiently transfected with the bicistronic construct pRFHCV1b for 16 h and then treated with H_2O_2 for 4 and 24 h. Phase-contrast microscopy was used to detect morphological changes at 4 and 24 h post-treatment (d). At 4 h post-treatment, relative HCV IRES- and cap-dependent translational activities were determined (e) and RT-PCR fragments from transfected samples were analysed on ethidium bromide-stained gels (f). (g) Relative IRES- and cap-dependent translational activities in HeLa cells transfected with the bicistronic RNA transcript from pRL for 4 h followed by treatment with H_2O_2 for 4 h. *$P<0.05$; RLA, relative luciferase activity.

transcribed and amplified by multiplexed or separate RT-PCR using the Titan One Tube RT-PCR System (Roche) with the following primer pairs: FLuc: 5′-CTGAAGGGATCGTAAAAACAGC-3′ and 5′-GATTACCAGGGATTTCAGTCG-3′; RLuc: 5′-CCACATATTGA-GCCAGTAGC-3′ and 5′-CCATGATAATGTTGGACGAC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-CCTGTT-CGACAGTCAGCCG-3′ and 5′-CGACCAAATCCGTTGACTCC-3′. The reverse transcription step was performed at 50 °C for 30 min, followed by 2 min of denaturation at 94 °C and 35 cycles of PCR using the following conditions: 94 °C for 10 s, 60 or 50 °C for 30 s and 68 °C for 45 s or 2 min, with a final extension at 68 °C for 5 min. The intensity of the luciferase bands was measured using IMAGEQUANT 5.0, normalized against an internal control (GAPDH) and expressed relative to the 0 μM H_2O_2-treated controls, which were set as 1.

Western blotting. Cells treated with H_2O_2 were harvested into 1× SDS-PAGE sample buffer. Protein concentrations were measured using the Bradford assay or an RC-DC protein assay kit. Western blotting was performed as described previously (Chan & Egan, 2005) using antibody specific for phospho-eIF2α (Cell Signalling), diluted 1:1000. Blots were stripped and reprobed with antibody recognizing total eIF2α (BioSource or Cell Signaling), diluted 1:1000. The intensity of the bands was measured with IMAGEQUANT 5.0, normalized against total eIF2α and expressed relative to the 0 h control, which was set as 1.

***In vitro* transcription.** *In vitro* transcripts were generated from the T7 promoter of pRL (Collier *et al.*, 1998) or pRFHCV1b (this study) using mMESSAGE mMACHINE and MEGAscript transcription kits (Ambion) according to the manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using analysis of variance. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

H_2O_2 represses HCV IRES-mediated and cap-dependent translation in HeLa cells

Cells respond to different levels of H_2O_2 by undergoing proliferation, apoptosis or necrosis (Davies, 1999). A physiological level of H_2O_2 is important for signalling, whereas a cytotoxic level of H_2O_2 can be considered as oxidative stress. HCV infection is frequently associated with oxidative stress, and apoptosis and necrosis are common features of HCV-infected liver (De Maria *et al.*, 1996; Dhillon & Dusheiko, 1995; Paradis *et al.*, 1997). Therefore, we sought to study the translational responses of HCV under conditions of oxidative stress that induced apoptosis or necrosis. The range of H_2O_2 concentrations that induced different cellular responses in HeLa cells were established using XTT assays and phase-contrast microscopy (Fig. 1a, d). HeLa cells remained viable until the concentration of H_2O_2 reached 10 µM. Concentrations greater than 10 µM were cytotoxic. The concentrations of H_2O_2 that induced cell death correlated with intracellular elevation of ROS, as measured by a fluorometric assay employing DCFH-DA (Fig. 1b). Concentrations of H_2O_2 that did not affect cell viability (< 10 µM) did not increase intracellular ROS levels over that of the untreated control. To test the effect of oxidative stress on HCV IRES-mediated translation, HeLa cells were transiently transfected with the bicistronic construct pRFHCV1b (Fig. 1c) and then treated with concentrations of H_2O_2 that have been shown to generate intracellular ROS and to elicit different growth responses in HeLa cells (Fig. 1e). Bicistronic reporter vectors are now commonly used to study IRES-mediated translation relative to cap-dependent translation (Hellen & Sarnow, 2001). After 4 h of treatment, a low level of H_2O_2 (5 µM) that had little or no effect on cell viability also failed to affect translational activities significantly. In contrast, repression of both HCV IRES-mediated and cap-dependent translation was observed at apoptotic (50 µM) and necrotic (500 µM) levels of H_2O_2. At 50 µM H_2O_2, both HCV IRES-mediated and cap-dependent translation were reduced to a similar extent, resulting in an unchanged IRES/cap ratio. However, treatment with 500 µM H_2O_2 caused considerably greater reduction in cap-dependent translation than HCV IRES-mediated translation, resulting in an overall fourfold increase in the IRES/cap ratio. These results suggested that initiation of translation from the HCV IRES was less sensitive or more resistant than cap-dependent translation to severe stress conditions. Using semi-quantitative RT-PCR, equal amounts of transcripts were amplified from each transfection reaction, confirming that changes in the IRES activity were translational and not transcriptional (Fig. 1f). For further confirmation of the effects of H_2O_2 on translational activities and to exclude the possibility that the effects obtained were derived from a cryptic promoter within the IRES sequence (Dumas *et al.*, 2003), we repeated the experiments using RNA transfection with the pRL transcript (Collier *et al.*, 1998) and obtained similar responses in translational activities after treatment with H_2O_2 (Fig. 1g).

FLuc activity does not derive from a cryptic promoter or spliced transcripts

The existence of cryptic promoters within IRES sequences has been reported in several cellular IRESs and the HCV IRES (Dumas *et al.*, 2003; Han & Zhang, 2002). As we used DNA transfection in some of our experiments, it could be argued that the effects obtained were derived from a cryptic promoter. For example, the difference in band intensity of the RLuc and FLuc fragments in Fig. 1(f) could be due to the production of additional FLuc transcripts from a cryptic promoter sequence within the HCV IRES. However, it was equally possible that it merely reflected the difference in PCR efficiency of the two sets of primers. Supporting the latter possibility was the similar degree of differential amplification of the RLuc and FLuc fragments from an *in vitro* transcript generated from the T7 promoter of the bicistronic vector pRFHCV1b and from transfected cells (Fig. 2a). To exclude the possibility of the presence of a cryptic promoter within the HCV IRES sequence, we compared the RLuc and FLuc activities in cells transfected with the bicistronic construct with and without the CMV promoter (Fig. 2b). Removal of the CMV promoter abolished the CMV-driven RLuc activity and, at the same time, FLuc activity, suggesting the absence of a cryptic promoter in our bicistronic construct pRFHCV1b, in at least three of the cell lines (HeLa, HepG2 and A549) used in this study. Also, in agreement with results from other studies (Sherrill *et al.*, 2004; Van Eden *et al.*, 2004a, b), we did not detect any aberrantly spliced transcripts in HeLa and HepG2 cells following transfection with the bicistronic DNA, as only an RT-PCR fragment of the expected size of 1·93 kb was amplified from the outer primers (Fig. 2c).

H_2O_2-induced translational repression correlates with eIF2α phosphorylation in a time- and dose-dependent manner

Evidence suggests that H_2O_2 can induce phosphorylation of eIF2α (O'Loghlen *et al.*, 2003). When we examined H_2O_2-treated HeLa cells, we also revealed a time- and dose-dependent phosphorylation of eIF2α in these cells (data not shown). Therefore, we investigated the role of phospho-eIF2α in modulating both HCV IRES-mediated and cap-dependent translation under oxidative stress. Indeed, the kinetics of eIF2α phosphorylation correlated with the kinetics of translational repression observed in HeLa cells after treatment with H_2O_2 (Fig. 3a, b). The degree of eIF2α phosphorylation remained unaffected following treatment with sub-apoptotic levels of H_2O_2 (5 and 10 µM). This was reflected in the absence of change in translational activities observed at these concentrations. However, increased phosphorylation of eIF2α was detected at apoptotic and necrotic levels of H_2O_2 (50 and 500 µM, respectively) and these concentrations also repressed translational activities.
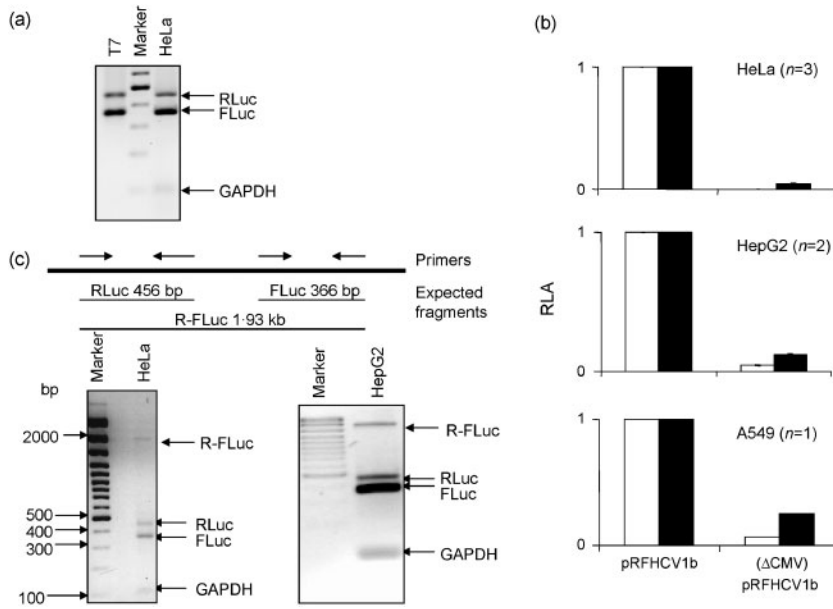
Fig. 2. Analysis of the bicistronic DNA construct. (a) Ethidium bromide-stained gel comparing the RT-PCR fragments amplified directly from transcripts synthesized *in vitro* from the T7 promoter of the bicistronic plasmid pRFHCV1b with those amplified from HeLa cells following transfection with pRFHCV1b. (b) RLuc (empty bars) and FLuc (filled bars) activities of the promoterless (ΔCMV) pRFHCV1b relative to those of the CMV-driven bicistronic pRFHCV1b. (c) Expected fragment sizes and ethidium bromide-stained gels showing the RT-PCR fragments amplified from HeLa and HepG2 cells following transfection with pRFHCV1b. RLA, Relative luciferase activity.

A phosphomimetic of eIF2α represses HCV IRES-mediated and cap-dependent translation in the absence of oxidative stress

To confirm the role of eIF2α phosphorylation in modulating translational activities, we transfected HeLa cells with the eIF2α mutants eIF2α-SA and eIF2α-SD (Fig. 3c). As a control, cells were transfected with wild-type eIF2α, which resulted in a slight elevation in translational activities. Phosphorylation of eIF2α occurs at serine 51 (Choi *et al.*, 1992). Replacement of serine with alanine results in a non-phosphorylatable eIF2α, eIF2α-SA, which acts as a dominant-negative mutant by exchanging with endogenous

eIF2α in the ternary complexes (Choi *et al.*, 1992). Transfection of cells with the non-phosphorylatable eIF2α-SA resulted in further translational activation compared with transfection with wild-type eIF2α. Conversely, in the phosphomimetic eIF2α-SD, serine 51 has been replaced with aspartate to mimic the structure of phosphorylated eIF2α (Choi *et al.*, 1992). In this case, even without stimulation with H_2O_2, transfection of HeLa cells with eIF2α-SD alone was sufficient to repress both HCV IRES-mediated and cap-dependent translation, suggesting that phosphorylation of eIF2α plays a modulating role in both HCV IRES-mediated and cap-dependent translation.
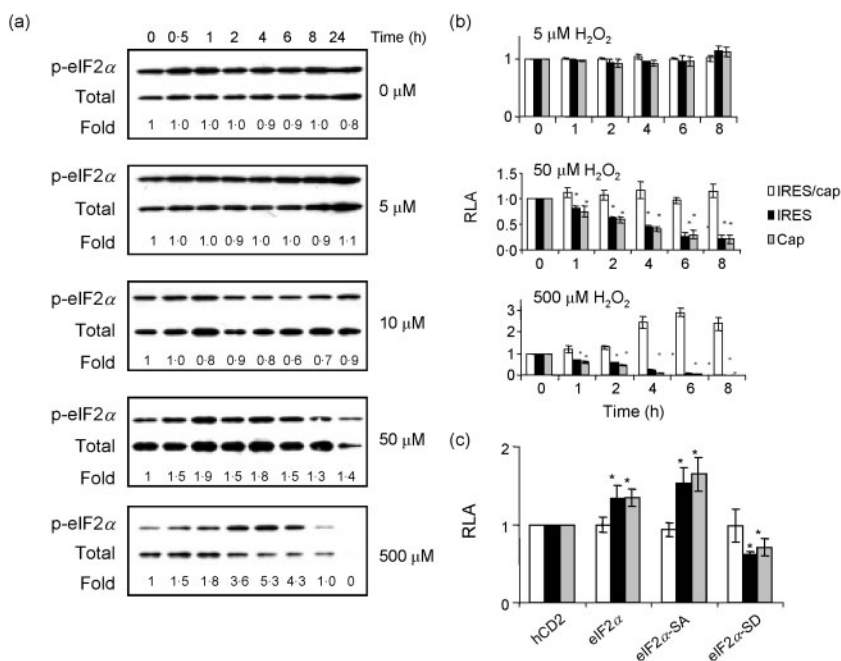

Fig. 3. (a, b) H_2O_2-induced eIF2α phosphorylation correlates with translational repression. Western blots (a) were used to determine the levels of eIF2α phosphorylation in HeLa cells after treatment with 0–500 μM H_2O_2 over a time course of 24 h. Dual luciferase assays (b) showed the corresponding IRES- and cap-dependent activities and the IRES/cap ratio under similar treatment conditions. (c) A phosphomimetic of eIF2α represses translation in the absence of oxidative stress. Relative IRES- and cap-dependent translational activities were determined in HeLa cells co-transfected for 48 h with the bicistronic construct pRFHCV1b and one of the following: the empty vector hCD2 or plasmid expressing eIF2α, eIF2α-SA or eIF2α-SD. *$P < 0.05$; RLA, relative luciferase activity.

H₂O₂ activates HCV IRES-mediated and cap-dependent translation in HepG2 cells

HCV is a hepatotropic virus and therefore we examined the translational responses in a hepatocyte cell line, HepG2. We chose HepG2 cells because they are highly differentiated hepatocytes and physiologically resemble primary hepatocytes more closely; thus, HepG2 is a good cell model in studies of HCV, which infects differentiated hepatocytes. HepG2 cells were more resistant to H_2O_2 and required 500 μM exogenously added H_2O_2 and a correspondingly greater increase in the intracellular level of ROS to induce mild apoptosis (Fig. 4a, b). Translational activities were not affected by low concentrations of H_2O_2, such as 20 μM, that did not affect intracellular ROS levels or cell viability (Fig. 4c). Translation was activated by higher concentrations of H_2O_2, such as 200 μM, which also caused a sharp rise in intracellular ROS levels but which did not affect cell viability or transcriptional activity (Fig. 4c, d). We confirmed that the changes in IRES activity were translational and not transcriptional by the amplification of equal amounts of transcripts from each transfection reaction using semi-quantitative RT-PCR (Fig. 4d) and also by using RNA transfection (data not shown). At 2 mM H_2O_2, both

luciferase and transcriptional activities decreased, probably as a result of cell toxicity.

Under *in vivo* conditions, cells could be exposed to sustained or transient levels of H_2O_2. We therefore examined the effect of transient exposure to H_2O_2 on HepG2 cells (Fig. 5a–c). A 30 min transient exposure of HepG2 cells to levels of H_2O_2 up to 2 mM did not affect viability, but was sufficient to induce transient elevation in intracellular ROS levels and, in turn, activated IRES- and cap-dependent luciferase activities in a dose-dependent manner. We confirmed that changes in the IRES activity were translational and not transcriptional by the amplification of equal amounts of transcript from each transfection reaction by using semi-quantitative RT-PCR (Fig. 5d).

Non-phosphorylatable eIF2α enhances translation after H₂O₂ treatment

When we examined the pattern of eIF2α phosphorylation in HepG2 cells exposed to 200 μM H_2O_2, there was also an increase in the level of phospho-eIF2α, which peaked at 2 h before returning to baseline level at 24 h post-treatment (Fig. 6a). As phospho-eIF2α repressed translation in HeLa
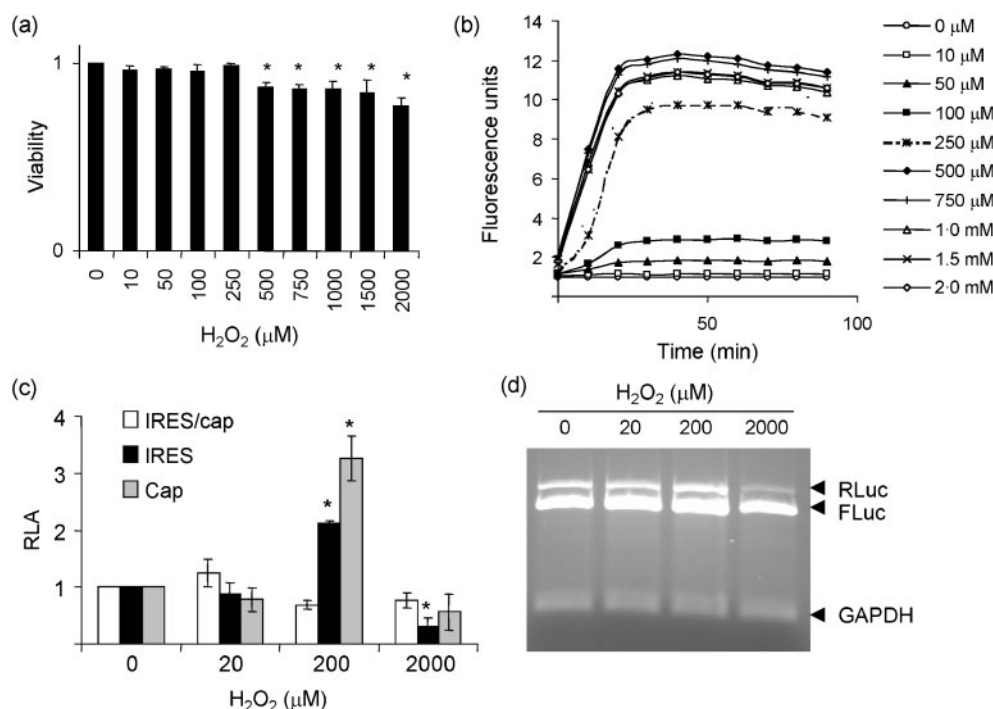

Fig. 4. Sustained exposure to H_2O_2 activates HCV IRES-mediated and cap-dependent translation in HepG2 cells. (a) XTT assay showing the viability of HepG2 cells (10 000 cells per well in 96-well plates) after treatment with 0–2 mM H_2O_2 for 24 h. (b) DCFH-DA fluorometric assay showing the kinetics of ROS generation in HepG2 cells (10 000 cells per well in 96-well plates) after treatment with 0–2 mM H_2O_2. (c, d) HepG2 cells were transiently transfected with the bicistronic construct pRFHCV1b for 16 h and then treated with H_2O_2 for 5 h. Relative IRES- and cap-dependent translational activities were determined at 5 h post-treatment (c) and RT-PCR fragments from transfected samples were analysed on ethidium bromide-stained gels (d). *$P<0.05$; RLA, relative luciferase activity.
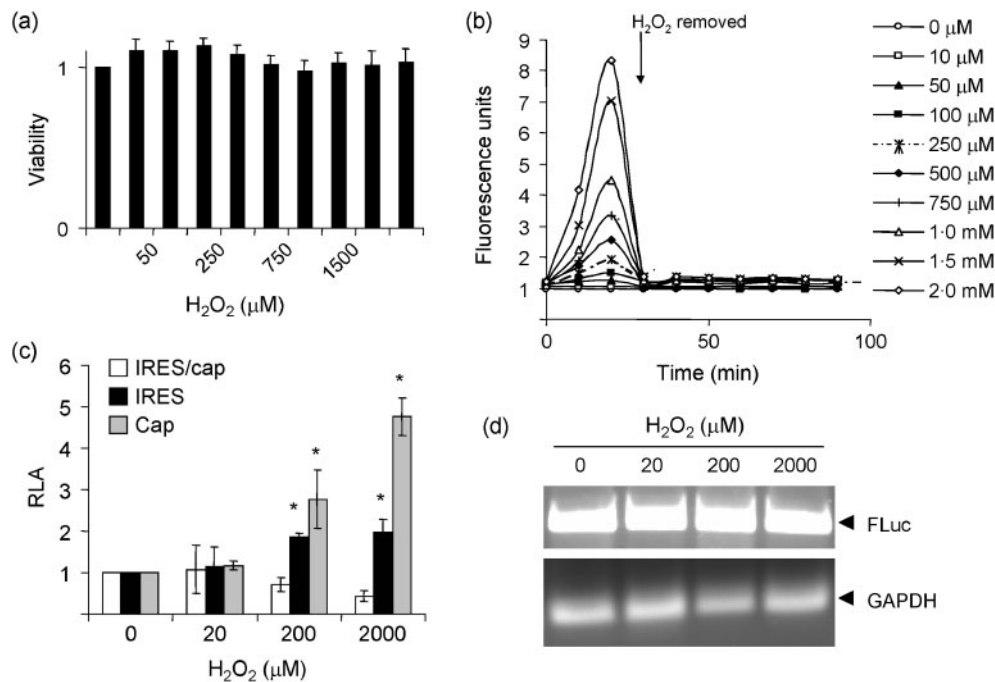
Fig. 5. Transient exposure to H_2O_2 activates HCV IRES-mediated and cap-dependent translation in HepG2 cells. (a) XTT assay showing the viability of HepG2 cells (10 000 per well in 96-well plate) after treatment with 0–2 mM H_2O_2 for 30 min. The conditioned medium was then replaced with fresh medium and readings were taken at 24 h post-treatment. (b) DCFH-DA fluorometric assay showing the kinetics of ROS generation in HepG2 cells (10 000 cells per well in 96-well plates) after treatment with 0–2 mM H_2O_2 for 30 min. The conditioned medium was then replaced with fresh medium before readings were taken. (c, d) HepG2 cells were transiently transfected with the bicistronic construct pRFHCV1b for 16 h and then treated with H_2O_2 for 30 min. The conditioned medium was then replaced with fresh medium. Relative IRES- and cap-dependent translational activities were determined at 5 h post-treatment (c) and RT-PCR fragments from transfected samples were analysed on ethidium bromide-stained gels (d). *$P<0.05$; RLA, relative luciferase activity.

cells in contrast to HepG2 cells where translational activities were increased after treatment with 200 μM H_2O_2, we reasoned that H_2O_2 might have stimulated translation in HepG2 cells via an eIF2α-independent mechanism, whereas the simultaneous phosphorylation of eIF2α repressed part of the translational activities. Indeed, in HepG2 cells transfected with the non-phosphorylatable eIF2α-SA mutant, further enhancement of both translational activities was observed after exposure to 200 μM H_2O_2, suggesting a role for the non-phosphorylatable eIF2α-SA in releasing the translational repression (Fig. 6b). In contrast, HepG2 cells transfected with the phosphomimetic eIF2α-SD failed to exhibit any translational upregulation in response to 200 μM H_2O_2.

In our transient co-transfection experiment, we achieved a low but consistent and significant level of translational rescue using the non-phosphorylatable eIF2α-SA mutant. The low level obtained could be due to the low efficiency of transient transfection. This is especially true with HepG2 cells, which have been known to be rather difficult to transfect. To address the role of phospho-eIF2α in translational repression further, we sought the use of a homogeneous population of cells stably expressing the non-phosphorylatable mutant eIF2α-SA. As stable eIF2α-SA hepatocyte cell lines are not available, we used a lung carcinoma cell line, A549, stably expressing eIF2α-SA instead (Koumenis et al., 2002). This cell line has also been well characterized in that the eIF2α-SA mutant cells fail to exhibit eIF2α phosphorylation under oxidative stress (Koumenis et al., 2002). First, we confirmed that wild-type A549 was similar to HepG2 in its sensitivity and translational responses to H_2O_2 treatment (Fig. 7). Wild-type A549 also exhibited a H_2O_2-resistant phenotype, although sensitivity was observed at concentrations >400 μM H_2O_2 (Fig. 7a). Similar to HepG2 cells, both HCV IRES-mediated and cap-dependent translation in wild-type A549 were upregulated following exposure to 200 μM H_2O_2 (Fig. 7b). We then examined the effect of stable expression of eIF2α-SA on translation. The eIF2α-SA mutant A549 was as resistant as wild-type A549 to H_2O_2 (Fig. 7a). In the eIF2α-SA mutant cells, HCV IRES-mediated and cap-dependent translation was further enhanced upon H_2O_2 stimulation compared with wild-type A549 (Fig. 7b). These results also indicated that phospho-eIF2α plays a modulating role on translation in H_2O_2-resistant cells.
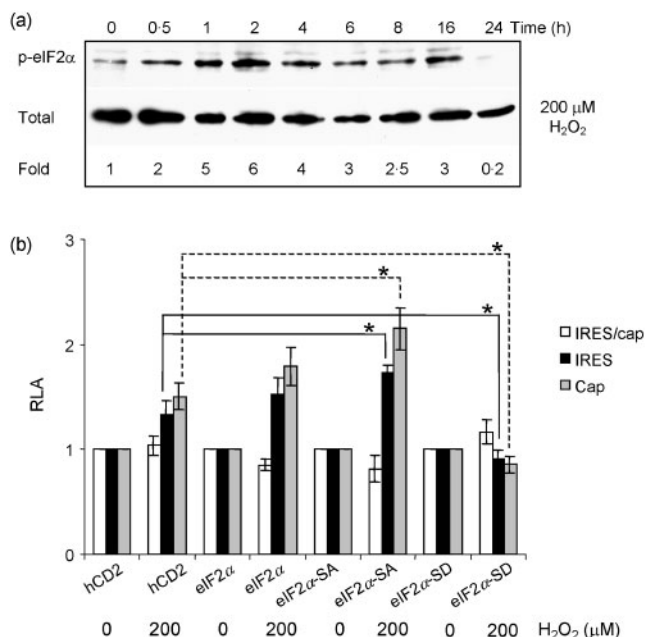
Fig. 6. Non-phosphorylatable eIF2α releases translational repression in HepG2 cells. (a) Western blots showing the levels of eIF2α phosphorylation in HepG2 cells after treatment with 200 μM H_2O_2 over a time course of 24 h. (b) Relative IRES- and cap-dependent translational activities in HepG2 cells co-transfected for 48 h with the bicistronic construct pRFHCV1b and one of the following: the empty vector hCD2 or plasmid expressing eIF2α, eIF2α-SA or eIF2α-SD. Cells were untreated or treated with 200 μM H_2O_2 for 5 h before harvest. Translational activities at 200 μM H_2O_2 were expressed relative to those of untreated cells transfected with the same plasmid construct. *$P < 0.05$; RLA, relative luciferase activity.

H_2O_2 activates HCV IRES-mediated and cap-dependent translation in HuH7 cells at physiological and cytotoxic levels

Since the introduction of a replicon system (Lohmann *et al.*, 1999), the hepatocyte cell line HuH7 has been employed widely in the study of HCV because it represents the cell line most supportive of HCV replication and even of a complete infectious cycle with the recently established cell-culture system (Wakita *et al.*, 2005). Therefore, we sought to characterize the translational responses to H_2O_2 in HuH7 cells. The sensitivity of HuH7 cells to H_2O_2 was in between that of HepG2 and HeLa cells (Fig. 8a, b). H_2O_2 at 20 μM induced a slight elevation of intracellular levels of ROS, but cells remained as viable as the untreated control; thus, this concentration may represent a physiological level of H_2O_2. At 100 μM H_2O_2, a high level of cell death was observed with a modest elevation of intracellular levels of ROS. Thus, this concentration may represent a cytotoxic level of H_2O_2 and can be considered as oxidative stress. At 50 μM H_2O_2, a low (although insignificant) level of cell death was observed with a modest elevation of intracellular levels of ROS. Thus, this

concentration may represent a critical dose of H_2O_2 functioning as a physiological/cytotoxic switch. These all have implications in HCV studies, because, for the duration of a chronic infection, the virus will have frequent exposure to different levels of H_2O_2, ranging from physiological to cytotoxic to pathological. Transfection of HuH7 cells with the bicistronic pRL IRES RNA transcript showed an inverse dose-dependent upregulation in both cap-dependent and HCV IRES-mediated translational activities (Fig. 8c). A physiological dose of 20 μM H_2O_2 induced the highest levels of translational activities, with less marked increases in translational activities at 50 μM H_2O_2, followed by 100 μM H_2O_2. Moreover, only at 20 μM H_2O_2 was the HCV IRES preferentially upregulated by twofold compared with cap-dependent translation. At 50 and 100 μM H_2O_2, both cap-dependent and HCV IRES-mediated translation responded similarly. Interestingly, elevation of the level of eIF2α phosphorylation was only obvious at 100 μM H_2O_2 where the level peaked at 30 min (Fig. 8d).

DISCUSSION

In this study, we investigated the response of the HCV IRES using a range of H_2O_2 concentrations in order to reflect the situation *in vivo*. Concentrations of H_2O_2 that did not generate elevated intracellular ROS levels had no effect on HCV IRES activity. This is in agreement with a previous study in which treatment of genomic and subgenomic replicons with levels of H_2O_2 that did not deplete intracellular glutathione or induce cell death had no effect on HCV IRES activity (Choi *et al.*, 2004). We demonstrated that, at increased concentrations of H_2O_2, the response varied according to the sensitivity of each cell type to H_2O_2, highlighting the spectrum of responses exhibited by different cell lines. The results in HepG2 and HuH7 cells, two more relevant cell lines in which to study HCV IRES activity, indicated that HCV IRES activity may also be influenced by cell type. HepG2 cells are very resistant to H_2O_2, perhaps reflecting a more robust anti-oxidant defence in these cells. At concentrations of H_2O_2 that increased levels of ROS but failed to induce a cell response that affected cell viability, HCV IRES activity was upregulated. By contrast, HuH7 and HeLa cells exhibited a more H_2O_2-sensitve phenotype and demonstrated a dose-dependent decrease in cell viability. Cell-type specificity was also observed in IRES response to HCV core protein expression (Li *et al.*, 2003). This also underscores the importance of using relevant cell lines in such studies.

In H_2O_2-sensitive cells, such as HeLa cells, translation was repressed with H_2O_2 treatment, whereas in H_2O_2-resistant cells, such as HepG2 cells, translation was upregulated. Irrespective of whether translation was repressed or upregulated, both HCV IRES-mediated and cap-dependent translation responded in a similar way. This is in contrast to the regulation of many other viral and cellular IRESs. An essential step during productive picornaviral infection is the viral protease-mediated shut-off of host translation
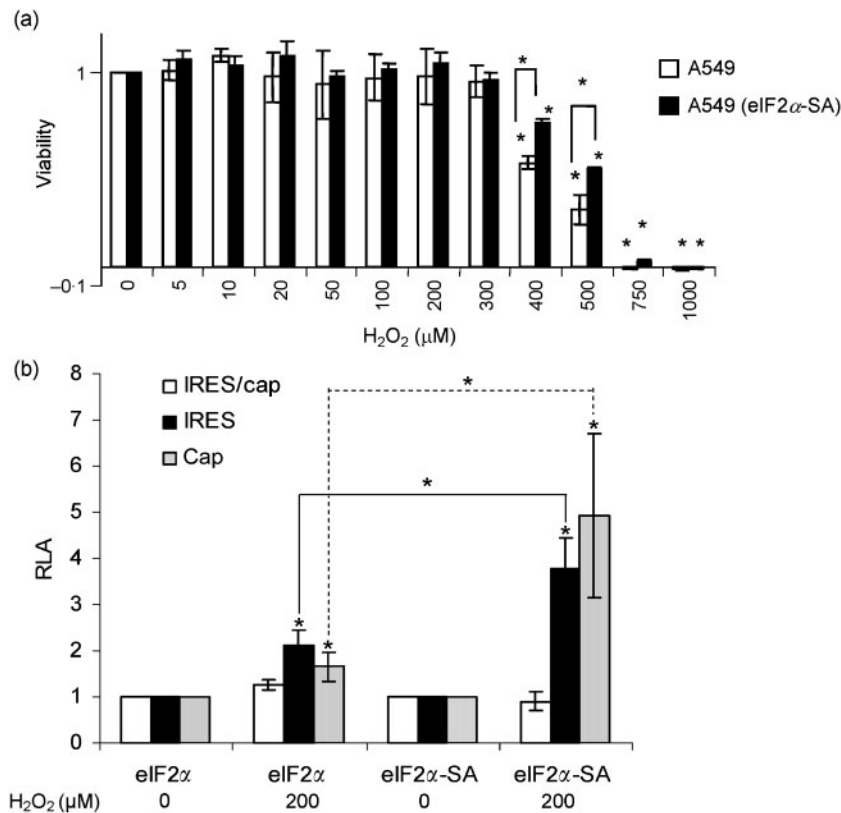
Fig. 7. Enhanced translation in A549 cells stably expressing eIF2α-SA and treated with H_2O_2. (a) XTT assay showing the viability of A549 and A549 (eIF2α-SA) cells (6000 cells per well) after treatment with 0–1000 μM H_2O_2 for 24 h. (b) Relative HCV IRES- and cap-dependent translational activities in A549 and A549 (eIF2α-SA) cells transiently transfected with the bicistronic construct pRFHCV1b for 16 h and then treated with H_2O_2 for 5 h. For both cell types, translational activities at 200 μM H_2O_2 were expressed relative to those of their respective untreated controls. *$P < 0.05$; RLA, relative luciferase activity.

(Belsham & Sonenberg, 1996). The picornaviral IRES element enables virus translation to proceed whilst that of the host cell has been shut off. An increasing number of eukaryotic mRNAs, often encoding regulatory proteins and possessing an IRES within their 5′ NTR, have recently been identified (Holcik et al., 2000). Some of these have been shown to function preferentially when cap-dependent translation is impaired under various stress conditions (Holcik et al., 2000). The HCV IRES differs in both length and structure from other IRESs and has a simpler requirement for translational factors (Beales et al., 2003; Pestova et al., 1998). Additionally, in contrast to picornaviruses, HCV establishes a chronic infection and, consequently, the role of the IRES may be functionally different to other viral elements. We speculate that it is necessary to confer translational competence to cellular genes in order to maintain a chronic infection; thus, HCV IRES-mediated and cap-dependent translation are regulated in a similar way under oxidative stress.

One exception to this rule of co-regulation of HCV IRES-mediated and cap-dependent translation is the preferential translation by the HCV IRES during exposure of HuH7 cells to a low (physiological) level of H_2O_2, suggesting that regulation of HCV IRES translation may be different at physiological and cytotoxic levels of H_2O_2. Moreover, it is tempting to speculate that molecules activated during physiological H_2O_2 signalling may be utilized to facilitate HCV IRES translation under such conditions.

Our results suggest that both HCV IRES-mediated and cap-dependent translation are modulated by phosphorylation of eIF2α. Phospho-eIF2α negatively regulates global cellular cap-dependent translation; however, increasing evidence suggests that it is also a positive regulator of certain viral and cellular IRES elements (Fernandez et al., 2002a, b; Gerlitz et al., 2002). For example, under certain conditions of cellular stress when most translational activities are shut off, translation from IRES elements is differentially upregulated. This is selectively advantageous to the life cycle and survival of some viruses and is essential for the regulation of specific genes involved in cellular processes such as survival and differentiation (Fernandez et al., 2002a, b; Gerlitz et al., 2002). It is not known whether all IRES elements are positively regulated by phospho-eIF2α. In this study, we have shown that the induction of oxidative stress repressed HCV IRES-mediated translation in HeLa cells. The observed reduction coincided with the increased phosphorylation status of eIF2α, suggesting a negative regulatory role for phospho-eIF2α in HCV translation. This finding was further supported by phosphomimetic eIF2α studies in HeLa cells and the rescue of translational activities in HepG2 cells and in A549 cells expressing a non-phosphorylatable eIF2α. In HuH7 cells, the reduction in the degree of translational activation at 100 μM H_2O_2 (compared with that at 20 μM H_2O_2) coincided with an elevation in the level of phospho-eIF2α, although we cannot currently link the two events together in the absence of additional mechanistic studies. Negative regulation of viral IRES elements has been
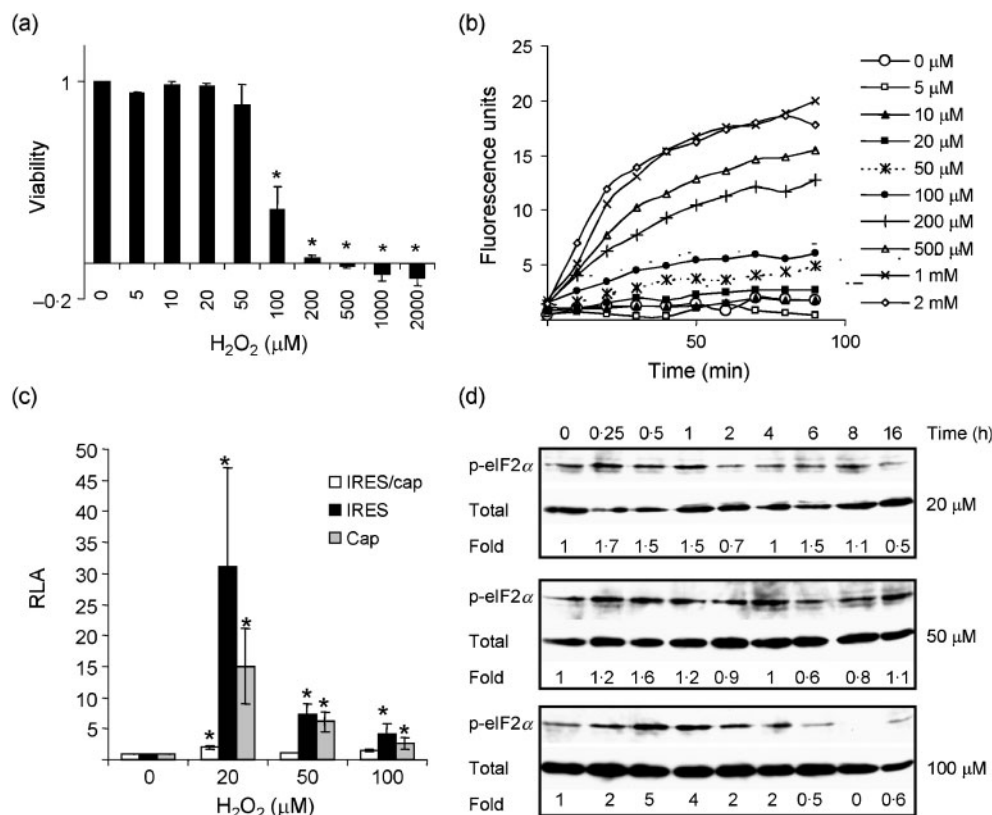
Fig. 8. H_2O_2 activates HCV IRES-mediated and cap-dependent translation in HuH7 cells at physiological and cytotoxic levels. (a) XTT assay showing the viability of HuH7 cells (10 000 cells per well in 96-well plates) after treatment with 0–2 mM H_2O_2 for 24 h. (b) DCFH-DA fluorometric assay showing the kinetics of ROS generation in HuH7 cells (10 000 cells per well in 96-well plates) after treatment with 0–2 mM H_2O_2. (c) Relative IRES- and cap-dependent translational activities in HuH7 cells transfected with the bicistronic RNA transcript from pRL for 4 h, followed by treatment with the indicated doses of H_2O_2 for 4 h. (d) Western blots showing the levels of eIF2α phosphorylation in HuH7 cells after treatment with the indicated doses of H_2O_2 over a time course of 16 h. $*P < 0.05$; RLA, relative luciferase activity.

demonstrated previously. Increased HCV IRES activity has been observed with reduced levels of phospho-eIF2α in replicon cells (He *et al.*, 2003). In contrast, RNA-activated protein kinase-induced HCV IRES translation seems to be dependent on phospho-eIF2α (Rivas-Estilla *et al.*, 2002).

Together these results suggest strongly that not all IRES elements are subject to positive regulation by phospho-eIF2α and that HCV IRES activity may be dependent on the cell type and the type of stimuli.

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