Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1

Ishtiaq QADRI*[1], Mieko IWAHASHI*, Juan M. CAPASSO*†, Matthew W. HOPKEN‡, Sonia FLORES‡, Jerome SCHAACK§ and Francis R. SIMON*

*Department of Medicine, Division of Gastroenterology and Hepatology, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262, U.S.A., †Division of Renal Diseases and Hypertension, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262, U.S.A., ‡Webb-Warring Antioxidant Research Center, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262, U.S.A., and §Department of Microbiology, Division of Gastroenterology and Hepatology, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262, U.S.A.

Activation of cellular kinases and transcription factors mediates the early phase of the cellular response to chemically or biologically induced stress. In the present study we investigated the oxidant/antioxidant balance in Huh-7 cells expressing the HCV (hepatitis C virus) subgenomic replicon, and observed a 5-fold increase in oxidative stress during HCV replication. We used MnSOD (manganese-superoxide dismutase) as an indicator of the cellular antioxidant response, and found that its activity, protein levels and promoter activity were significantly increased, whereas Cu/ZnSOD was not affected. The oxidative stress-induced protein kinases p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase) were activated in the HCV replicon-containing cells and in Huh-7 cells transduced with Ad-NS5A [a recombinant adenovirus encoding NS5A (non-structural protein 5A)], coupled with a 4–5-fold increase in AP-1 (activator protein-1) DNA binding. Ava.1 cells, which encode a replication-defective HCV replicon, showed no significant changes in MnSOD, p38 MAPK or JNK activity. The AP-1 inhibitors dithiothreitol and *N*-acetylcysteine, as well as a dominant negative AP-1 mutant, significantly reduced AP-1 activation, demonstrating that this activation is oxidative stress-related. Exogenous NS5A had no effect on AP-1 activation *in vitro*, suggesting that NS5A acts at the upstream targets of AP-1 involving p38 MAPK and JNK signalling cascades. AP-1-dependent gene expression was increased in HCV subgenomic replicon-expressing Huh-7 cells. MnSOD activation was blocked by inhibitors of JNK (JNKI1) and p38 MAPK (SB203580), but not by an ERK (extracellular-signal-regulated kinase) inhibitor (U0126), in HCV-replicating and Ad-NS5A-transduced cells. Our results demonstrate that cellular responses to oxidative stress in HCV subgenomic replicon-expressing and Ad-NS5A-transduced cells are regulated by two distinct signalling pathways involving p38 MAPK and JNK via AP-1 that is linked to increased oxidative stress and therefore to an increased antioxidant MnSOD response.

Key words: activator protein-1 (AP-1), hepatitis C virus, manganese superoxide dismutase, non-structural protein 5A (NS5A), oxidant/antioxidant balance, stress-activated kinase.

INTRODUCTION

HCV (hepatitis C virus) infection exhibits a prolonged, insidious course, with progressive liver injury leading to fibrosis and eventually hepatocellular carcinoma [1]. Many complex processes play critical roles in the development of liver damage, including apoptosis, necrosis, inflammation, immune response, fibrosis, ischaemia, altered gene expression and regeneration. Although the mechanisms of cell injury and progression are unclear, emerging evidence thus far presented includes enhanced production of free radicals and disease activity in chronic hepatitis C [2–4]. Oxidative stress is a condition linked to the activation of two well characterized transcription factors, NFκB (nuclear factor κB) and AP-1 (activator protein-1) [5,6]. Reports of increases in the levels of malondialdehyde and 4-hydroxynonenal protein adducts during chronic active hepatitis C disease support an *in vivo* role for reactive oxygen species as mediators of liver damage [2,7,8]. The onset of liver damage will depend on the duration of the toxic insult. As a consequence, it is important to understand the oxidative/antioxidative balance in HCV-infected cells.

HCV is a positive-stranded RNA virus distantly related to the flaviviruses and pestiviruses, and has been classified into the genus hepacivirus of the virus family flaviviridae [9,10]. Immediately following the 5′ non-translated region, the order of genes is as follows; 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ [9–11] (where NS denotes non-structural protein). The 5′ non-translated region consists of $\approx$340 nt, and controls the synthesis of the HCV polyprotein through an IRES (internal ribosome entry site) that is highly conserved [12].

Research on HCV replication and pathogenesis, as well as the development of therapeutic strategies, has been severely hampered by the lack of reproducible *in vitro* methods and of an animal

Abbreviations used: Ad-GFP, recombinant adenovirus encoding GFP; Ad-NS5A, recombinant adenovirus encoding NS5A; AP-1, activator protein-1; CMV, cytomegalovirus; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HCV, hepatitis C virus; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NFκB, nuclear factor κB; NS5A, non-structural protein 5A; PKR, double-stranded-RNA-dependent protein kinase; SOD, superoxide dismutase; SRCAP, SNF2-related CBP activator protein [where CBP is CREB (cAMP response element binding protein) binding protein]; STAT5b, signal transduction and activators of transcription 5b; SV40, simian virus 40; TBP, TATA box binding protein; TNF, tumour necrosis factor; TRE, PMA ('TPA')-responsive element.

[1] To whom correspondence should be addressed (e-mail ishtiaq.qadri@uchsc.edu).

model of HCV infection. The majority of attempts to grow HCV in culture have been unsuccessful or have yielded a low virus titre. Recently, an *in vitro* cell culture-based system using HCV subgenomic replicons was developed [13,14]. HCV RNA replicon replication occurs in the human hepatic cell line Huh-7, after transfection of a genotype 1b subgenomic RNA replicon expressing a selectable marker. The HCV subgenomic replicon was able to synthesize minus-strand HCV RNA in a cell line referred to as Huh.8. A mutant Ava.1 cell line (with a deletion of 47 amino acids of NS5A) was also generated that does not support HCV subgenomic replication. To date, these cell lines carrying HCV subgenomic replicons are the most important tools available for understanding HCV replication and pathogenesis associated with the expression of the NS2–NS5B proteins of HCV in a cell-culture system. HCV NS5A is known to possess potent transcriptional transactivator properties [15], and displays a multitude of activities related to enhancement of viral pathogenesis by binding to a repertoire of cellular proteins, including cellular PKR (double-stranded-RNA-dependent protein kinase), phosphoinositide 3-kinase, SRCAP [SNF2-related CBP activator protein, where CBP is CREB (cAMP response element binding protein) binding protein], Grb2 and TBP (TATA box binding protein) [16–23]. We and others have reported functional associations of NS5A with p53 [22,23]. p53 participates in transcription, cell cycle checkpoints, DNA repair and apoptotic pathways [24], and alters the cellular redox state [25]. The p53-dependent induction of $p21^{cip/WAF1}$ protects cells against oxygen-induced toxicity [26].

p38 MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase) and ERK (extracellular-signal-regulated kinase) are stress-inducible kinases that are activated in response to various extracellular and intracellular stimuli, such as UV light, reactive oxygen species, osmotic shock, chemicals and biological agents, including viruses [27–32]. These stress-activated kinases are involved in the regulation of p53 [33,34]. The extent to which activated p38 MAPK, JNK and ERK1/2 influence the functions of p53, including DNA and protein interactions, in the context of oxidative stress and HCV remains to be investigated. To overcome the oxidative burden, the expression of key antioxidant enzymes such as SODs (superoxide dismutases), which are the first enzymes involved in the detoxification of oxygen free radicals, is up-regulated. The human MnSOD gene is highly inducible by various physical, chemical and biological agents that trigger oxidative stress [35–39]. However, it is hypothesized that this activation cannot be sustained throughout the course of the stress, as at some stage the oxidant burden is likely to overwhelm the cells, thus leading to a failure in antioxidant responses. This is evident in a wide variety of tumour cells, where diminished MnSOD activity and mRNA levels occur [35].

In the present study, we investigated cellular oxidant and antioxidant responses during HCV replication. A 5-fold induction of oxidative stress, coupled with activation of stress-inducible p38 MAPK and JNK and transcription factor AP-1, was observed. p38 MAPK and AP-1 are known to activate the key antioxidant gene MnSOD as a response to the burden of oxidative stress. Consistent with this hypothesis, MnSOD enzyme activity, protein expression and promoter activity were induced in cells undergoing HCV replication. HCV NS5A seems to be responsible for these cellular perturbations, as Ad-NS5A (a recombinant adenovirus encoding NS5A) was able to activate p38 MAPK, JNK and AP-1. The expression of c-*fos* in HCV-replicating and Ad-NS5A-transduced cells was not affected, but c-*jun* expression was $\approx$3-fold higher. Inhibitors of JNK and p38 MAPK significantly reduced the activation of MnSOD, while an ERK1/2 inhibitor had no effect, in HCV-replicating and Ad-NS5A-transduced cells. Thus cells expressing an HCV subgenomic replicon respond to stress by activating MnSOD via two distinct signalling pathways involving p38 MAPK, JNK and the transcription factor AP-1.

EXPERIMENTAL

Cell lines and culture conditions

Huh.8 cell lines contain the HCV subgenomic replicon expressing NS components (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [4]. The HCV replication-defective cell line Ava.1 (with a deletion of 47 amino acids within the NS5A region) was also employed. Huh-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing high glucose supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 10 % (v/v) fetal bovine serum. Huh.8 and Ava.1 cells were maintained in complete DMEM supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, non-essential amino acids and 1 mg/ml G418. All cells were maintained at 37 °C in a humidified environment containing 5 % CO_2.

Antibodies

Monoclonal antibodies directed against phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (cat. no. 9216), phospho-SAPK (stress-activated protein kinase)/JNK (Thr^{1803}/Tyr^{185}) (cat. no. 9255) and phospho-p44/p42 MAPK (ERK 1/2) (Thr^{202}/Tyr^{204}) (cat. no. 9106) were from Cell Signaling Technology (Beverly, MA, U.S.A.). Antibodies against MnSOD were purchased from Santa Cruz Labs (San Diego, CA, U.S.A.). An antibody against HCV NS5A was purchased from ID Labs (London, Ontario, Canada; cat. no. IDVG1127). Antibodies recognizing the AP-1 components c-Fos, c-Jun, v-Fos, v-Jun, Jun-B, Jun-D, Fra-1 and Fra-2 were purchased from Santa Cruz Labs.

Oligonucleotides

The AP-1 consensus probe 5′-CGCTTGA**TGAGTCA**GCCG-GAA-3′ (where bold denotes the consensus AP-1 binding site) was used in EMSAs (electrophoretic mobility shift assays). Oligonucleotide 5′-TGGGAACTAGTCC**TGACTCA**GTTAACTGTG-3′, encompassing the AP-1 binding site of the human MnSOD promoter region (− 840 to − 810) [35,39], was used as a competitive DNA in the experiment shown in Figure 7(A).

Plasmids

pAdTrack/CMV (where CMV is cytomegalovirus) and pAdEasy are described in [40]. The *Hin*dIII/*Xba*I fragment from pNeoCMV/NS5A was excised and cloned into pAdTrack/CMV to obtain pAdTrack/CMV-NS5A. pAdTrack/CMV-NS5A was recombined into pAdEasy-1 to obtain AdEasy-1-NS5A, which was cleaved with *Pac*I prior to transfection into HTK293 cells. The full-length 3400 bp MnSOD promoter–reporter construct $MnSOD^{FL}$-LUC and the minimal 210 bp MnSOD promoter–reporter construct $MnSOD^{210}$-LUC have been described previously [41]. The construction of pAP-LUC has been described [6,43], and this plasmid was kindly provided by D. Brenner (Departments of Medicine and Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A.).

Production of Ad-NS5A

The method of He et al. [40] was employed to construct the recombinant adenovirus for expression of HCV NS5A. To generate Ad-NS5A, pAdTrack/CMV-NS5A was cleaved with *Pme*I for recombination in *Escherichia coli* strain BJ334 carrying the pAdEasy-1 vector. Viruses were constructed by transfection of

HTK293 cells with the recombinant pAdEasy-1-NS5A plasmid, digested with *Pac*I. The recombinants were selected for kanamycin resistance, and were confirmed first by restriction endonuclease digestion and then by DNA sequencing. Recombinant adenovirus Ad5TAM, expressing TAM-67, a dominant negative c-Jun (AP-1), was a gift from Dr D. Brenner [43]. To generate adenovirus expressing GFP (green fluorescent protein), pAdTrack/CMV containing the CMV–GFP cassette was introduced into pAdEasy-1 by homologous recombination in *E. coli* strain BJ334. High-titre stocks were dialysed in a buffer containing 10 mM Tris/HCl (pH 8.8), 15 mM NaCl, 10 mM $MgCl_2$ and 10 % (v/v) glycerol, and stored at − 80 °C. The titre of each virus stock was determined by plaque assay using HTK293 cells. Titres were consistently $\approx 1 \times 10^{11}$ plaque-forming units/ml.

Measurement of activation of p38 MAPK, JNK and ERK

Cells were washed three times in ice-cold PBS. Cell lysates were obtained by addition of 0.5 ml of lysis buffer [50 mM β-glycerophosphate, pH 7.2, 0.5 % Triton X-100, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT (dithiothreitol), 2 μg/ml leupeptin and 4 μg/ml aprotinin] and scraping. The lysates were centrifuged at 4 °C for 10 min at 21 000 ***g*** to remove nuclei and cell debris. The supernatants were saved and used for Western blot analysis. Band detection and analysis was performed using a Kodak Image Station CF440. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.).

Western blotting

SDS/PAGE and immunoblotting were carried out using Minigels. Proteins were transferred by the procedures of Towbin et al. [43a] and processed using enhanced chemiluminescence for detection (Amersham) with specific antibodies using 1 % (w/v) non-fat dried milk in Tris-buffered saline. All washes were in 0.5 % Tween/Tris-buffered saline for 5 min. Analyses of autoradiograms were performed using a PhosphoImager.

Preparation of nuclear proteins

Nuclear proteins were prepared from livers using sucrose gradients. Minced liver tissue was homogenized and filtered through several layers of gauze and adjusted to 0.3 % (v/v) Triton X-100. Sucrose gradients were prepared and the sample was centrifuged at 77 000 ***g*** for 50 min at 4 °C. The nuclear pellets were resuspended in buffer and ammonium sulphate was added slowly to a final concentration of 0.4 M. The mixture was centrifuged at 62 000 ***g*** for 60 min. The supernatant was saved and 0.33 g of ammonium sulphate per ml of supernatant was added. The mixture was again centrifuged at 62 000 ***g*** for 60 min, protein pellets were resuspended and dialysed aliquots were snap-frozen at − 70 °C.

Gel EMSA

The oligonucleotides were radiolabelled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Binding reaction mixtures contained 20 mM Hepes (pH 7.9), 4 % Ficoll type 400, 50 mM KCl, 0.5 mM EDTA, 100 ng of poly(dI-dC), 50 ng of pSK$^+$ DNA (Stratagene) and 20 000 c.p.m. of DNA probe. Reaction mixtures were incubated at 25 °C for 30 min with DNA, nuclear extract and bacterially purified proteins. Appropriate antibodies were either purchased commercially or obtained from individual investigators. The complexes were separated by electrophoresis in a 4.5 % (w/v) native polyacrylamide gel at 120 V at 4 °C in 0.5 × TBE buffer (1 × TBE = 45 mM Tris/borate and 1 mM EDTA).

DNA transfections

Cultures were set up 24 h prior to transfections in 35 mm-diam. 6-well plates at 10^6 cells/well in DMEM containing appropriate supplements. Transfections were carried out using Fugene 6 (Roche Diagonistics), at a 1:1 DNA/lipid ratio. At 48 h post-transfection, cells were harvested and assayed for luciferase activity using a dual luciferase reporter assay system (Promega). Luciferase activity was read in a Packard plate reader luminometer.

SOD activity assays

Briefly, cell extracts were incubated with 1 mM diethyldithiocarbamate in 50 mM potassium phosphate (pH 7.8) at 37 °C for 1 h. Cellular extracts were assayed by the cytochrome method originally described by McCord and Fridovich [42]. For the activity gels, a discontinuous buffer system was used. For the separating gel, a native 8 % (w/v) polyacrylamide gel was prepared in a 150 mM Tris/HCl, pH 8.8, buffer. For the stacking gel, a 6 % acrylamide (29:1 monoacrylamide/bisacrylamide) gel in 126 mM Tris/HCl, pH 6.8, was prepared. The upper and lower tank buffers consisted of 37.6 mM Trizma base/40 mM glycine (pH adjusted to 8.89 with HCl) and 63 mM Tris/HCl (pH 7.4) respectively. The gels were pre-electrophoresed at 50 V for 30 min. Total proteins (50 μg) prepared in a loading buffer containing 70 mM Tris/HCl, pH 6.8, 6 % glycerol and 0.24 % Bromphenol Blue were then resolved for 30 min at 50 V, followed by electrophoresis at 100 V for 1.5 h. After electrophoresis, the gels were carefully rinsed and SOD activity was detected by inhibition of NitroBlue Tetrazolium reduction. SOD activity appears as a clear area on a blue background.

RESULTS

Induced oxidative stress during HCV replication

Oxidative stress was measured in Huh-7 (control), Huh.8 (HCV subgenomic replicon) and Ava.1 (non-replicating HCV replicon) cells using flow cytometry (Figure 1). This method involves measuring H_2O_2 production during the oxidative burst using the well known indicator DCFH-DA (2′,7′-dichlorofluorescein diacetate; also known as 2′,7′-dichlorodihydrofluorescin). DCF (2′,7′-dichlorofluorescein) emission is measured at 525 ± 20 nm. H_2O_2 and peroxides are able to oxidize the trapped dye DCFH to DCF, which is highly fluorescent at 530 nm. HCV-replicating Huh.8 cells produced 7-fold more reactive oxygen species (Figure 1, peak C) when compared with Huh-7 cells (peak A). In contrast, HCV replication-defective Ava.1 cells, lacking the 47 amino acids of NS5A, produced comparable amounts of reactive oxygen species as control Huh-7 cells (Figure 1, compare peaks A and B). Many basic events of cell regulation, such as protein phosphorylation and binding of transcription factors to consensus sites in DNA, are driven by physiological oxidants and antioxidant homoeostasis. In particular, key antioxidant SODs (Cu/Zn and Mn) are known to play a major role in counteracting oxidative stress. To understand the relationship between HCV infection and oxidative stress, extracts of HCV-replicating cells were assayed for Cu/Zn and MnSOD activity.

Induced MnSOD activity in HCV-replicating cells

Figure 2(A) shows the levels of SOD activity in Huh-7 (control), Huh.8 (HCV subgenomic replicon) and Ava.1 (non-replicating

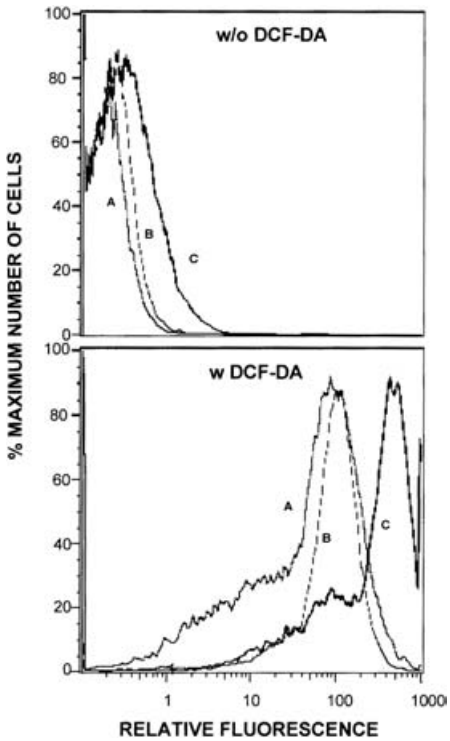

Figure 1 Generation of reactive oxygen species in HCV subgenomic replicon-containing cells

Reactive oxygen species production was measured within control Huh-7 cells (curve A), mutant HCV replicon-containing Ava.1 cells (curve B) and HCV subgenomic replicon-containing Huh.8 cells (curve C) using DCFH-DA as the indicator. DCF emission is measured at 525 ± 20 nm by flow cytometry. Upon cell activation, NADPH oxidase catalyses the reduction of O_2 to O_2^-, which is reduced further to H_2O_2. H_2O_2 and peroxidases are able to oxidize the trapped dye DCFH-DA to DCF, which is highly fluorescent at 530 nm. Approx. 2×10^6 cells were subjected for FACS analysis.

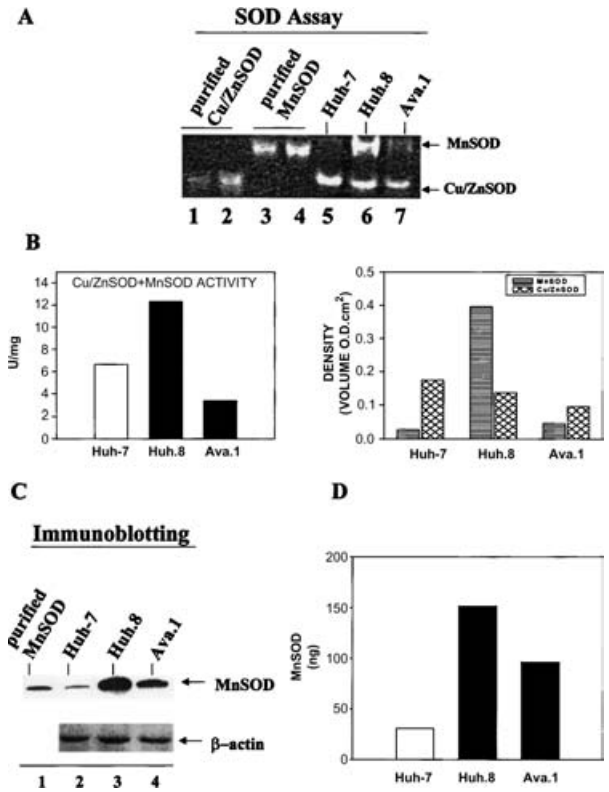

Figure 2 Measurement of SODs in Huh-7 (control), Huh.8 (HCV-replicating) and Ava.1 (HCV mutant) cell extracts

(**A**) SOD activity assay. Purified Cu/ZnSOD standards were loaded in lanes 1 and 2 (0.0212 and 0.0424 unit respectively). MnSOD standards were in lanes 3 and 4 (0.15 and 0.21 unit respectively). Samples of 50 μg of cell extracts from Huh-7 (lane 5), Huh.8 (lane 6) and Ava.1 (lane 7) cells were loaded for the measurement of endogenous MnSOD activity as described in the Experimental section. (**B**) Quantitative analysis of SOD activity in Huh-7, Huh.8 and Ava.1 cell extracts. Total Cu/ZnSOD + MnSOD activity was measured on a diode-array spectrophotometer and plotted. Gels were scanned to measure separately Cu/ZnSOD and MnSOD. Huh.8 cells showed $\approx$2.5-fold more total SOD and $\approx$5-fold more MnSOD activity. (**C**) Immunoblotting of MnSOD. Lane 1, 73.5 ng of MnSOD standard; lane 2, Huh-7 extract; lane 3, Huh.8 extract; lane 4, Ava.1 extract (50 μg of protein used in each lane). β-Actin is shown as a loading control. (**D**) MnSOD present in Huh-7, Huh.8 and Ava.1 cell extracts. Amounts of MnSOD were calculated based on comparisons with purified MnSOD by phosphoimager analysis. HCV-replicating Huh.8 cells contained $\approx$4-fold more MnSOD protein than control Huh-7 cells. Data presented are representative of two independent experiments giving consistent results.

HCV replicon) cell extracts. The positive standards in the activity gel contained 0.0212 and 0.0424 unit of purified Cu/ZnSOD (Figure 2A, lanes 1 and 2 respectively) and 0.11 and 0.21 unit of MnSOD (Figure 2A, lanes 3 and 4 respectively). Total SOD (Cu/Zn + Mn) activity was quantified by diode-array spectrophotometer analysis and the data plotted (Figure 2B). Total SOD activity in Huh.8 cell extracts showed at least a 2-fold increase and that in Ava.1 cells an approx. 50 % decrease when compared with that in control Huh-7 cells. Densitometric analysis of the MnSOD and Cu/ZnSOD bands revealed an approx. 5-fold increase in MnSOD activity in Huh.8 cells when compared with control Huh-7 cells (Figure 2B).

For quantitative analysis of various cell extracts, MnSOD levels were compared with the lane 1 control (1C), in which 73.5 ng of purified MnSOD standard was loaded. The quantitative analysis is presented in Figure 2(D). Immunoblotting of Huh-7, Huh.8 and Ava.1 cell extracts showed an approx. 4-fold increase in the expression of MnSOD protein in Huh.8 cells (HCV replicating) in comparison with control Huh-7 cells (Figure 2C, lanes 2–4). Ava.1 cell extracts showed an approx. 2-fold increase in MnSOD protein expression.

The human MnSOD promoter is activated in Huh.8 cells

To test whether transcriptional regulation is involved in the mechanism of increased MnSOD expression in HCV-replicating cells, DNA transfections were carried out using a 3400 bp human MnSOD promoter–luciferase reporter construct. Transfection of the MnSOD–LUC construct into Huh-7, Huh.8 and Ava.1 cells revealed that the 3400 bp promoter was activated in HCV-replicating cells (Figure 3). An approx. 4-fold increase in MnSOD promoter activity was seen in Huh.8 cells, consistent with increased MnSOD protein and enzyme activity. These results suggested that the MnSOD promoter is activated by HCV replication and protein expression. In the mutant Ava.1 cell line, partial activation (1.7-fold) of the MnSOD promoter was seen (Figure 3). No change in luciferase expression was seen when a heterologous simian virus 40 (SV40)–LUC DNA was transfected into either Huh-7 or Huh.8 cells (results not shown).

p38 MAPK and JNK are activated in HCV-replicating cells

We measured p38 MAPK, JNK and ERK activation in HCV-replicating cells (Figure 4). Immunoblotting of extracts from Huh-7 (lane 1), Huh.8 (lane 2) and Ava.1 (lane 3) cells revealed that p38 MAPK and JNK were activated in HCV-replicating cells. No changes were observed in Ava.1 cells. Whereas JNK and p38

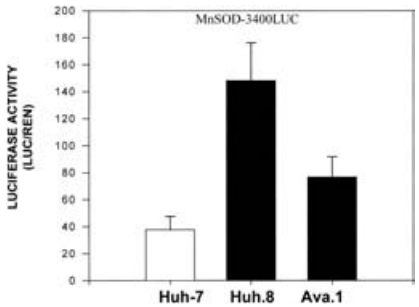

Figure 3 MnSOD promoter activity in HCV subgenomic replicon-containing Huh.8 cells

Huh-7, Huh.8 and Ava.1 cells were transfected with a 3400 bp human MnSOD promoter–LUC construct. Luciferase activity was measured 48 h post-transfection. The means of three independent experiments are presented.

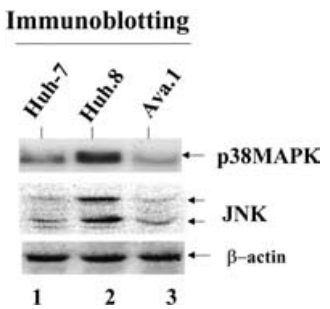

Figure 4 Activation of JNK and p38 MAPK in cells expressing the HCV subgenomic replicon

Samples of 50 μg of protein from Huh-7 (lane 1), Huh.8 (lane 2) and Ava.1 (lane 3) cells were immunoblotted using anti-JNK and anti-p38 MAPK antibodies that recognize only the phosphorylated proteins. β-Actin is shown as loading control.

MAPK were activated in HCV-replicating cells, no changes in ERK1/2 activation were observed (results not shown).

NS5A mediates the induction of JNK and p38 MAPK in Huh-7 cells

We introduced the NS5A cDNA into a replication-defective adenovirus vector (Ad-NS5A) to permit rapid introduction into cells for further analysis of the signalling pathways. As a control, adenovirus encoding GFP (Ad-GFP) was constructed. Huh-7 cells were transduced with either Ad-GFP (Figure 5, lane 3) or Ad-NS5A (lane 4). The expression of NS5A was confirmed by immunofluorescence (results not shown). Western blot analysis was performed using antibodies specific for phosphorylated MAPK and ERK. To serve as a positive control for induction of p38 MAPK and ERK, Huh-7 cells were osmotically shocked with a hypotonic buffer for 15 min (Figure 5, lane 2). Stress-activated signalling cascades play a crucial role during the cellular response to a wide variety of stress-inducing agents through the activation of AP-1 transcription factors [7].

Induction of DNA binding of AP-1 in HCV subgenomic replicon-expressing cells

To test the hypothesis that NS5A contributes towards the accumulation of oxidative stress by activating the key stress-inducible transcription factor AP-1, we measured the DNA binding of AP-1 (Figure 6). AP-1 is an important mediator of tumour promotion. Interactions between c-Jun and c-Fos proteins via leucine zipper

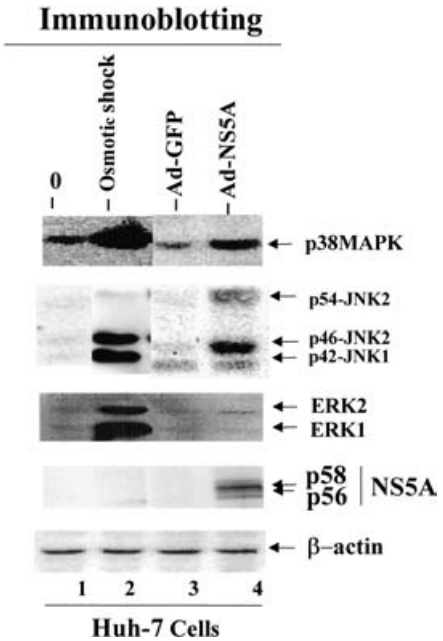

Figure 5 Activation of JNK and p38 MAPK in recombinant Ad-NS5A-transduced Huh-7 cells

Cell extracts prepared after transduction with Ad-GFP (lane 3) or Ad-NS5A (lane 4) were immunoblotted using antibodies to activated p38 MAPK, JNK and ERK. Lane 1, untreated cells; lane 2, cells shocked with hypotonic buffer for 15 min. Osmotic shock is known to induce p38 MAPK, JNK and ERK. No changes in ERK were seen. Anti-NS5A antibodies were used to show the expression of NS5A in Ad-NS5A-transduced cells. β-Actin is shown as loading control.

domains have crucial regulatory implications for the expression of a wide variety of genes. As expected, AP-1 binding to DNA was increased several-fold in HCV-replicating cells (Figure 6, compare lanes 2–4 with lanes 5–7). Unlabelled competitor DNA (lanes 8 and 9) abrogated this interaction, thus establishing the specificity of the AP-1 complex.

Next, we determined whether activation of AP-1 in HCV-replicating cells could be prevented by the antioxidant NAC (*N*-acetylcysteine), which is known to repress stress-induced AP-1 activation [5]. Huh-7 and Huh.8 cells were exposed to 10 μM NAC for 12 h and nuclear extracts were tested for AP-1 binding (Figure 6A). We found that AP-1 binding to DNA was reduced in both control Huh-7 cells (lane 10) and HCV-replicating Huh.8 cells (lane 11), thus establishing the AP-1 specificity of the shifted band. Antibodies to c-Fos or c-Jun were able to supershift the AP-1 complex (lanes 12–15), but neither antibody shifted the AP-1 DNA probe (lane 16).

Activation of AP-1 by NS5A

The participation of c-Fos, c-Jun and JunB in AP-1 complex formation was tested by incubating nuclear extracts with specific blocking antibodies prior to adding the radiolabelled DNA. Nuclear extracts prepared from serum-starved Huh-7 cells were transduced at 25 plaque-forming units/cell with Ad-GFP or Ad-NS5A. Samples of 2, 10, 20, 40 and 60 μg of nuclear extract proteins from cells transduced with Ad-GFP (Figure 6B, lanes 2–6) or Ad-NS5A (lanes 7–11) were used for AP-1 binding by band shift assay using a ^{32}P-labelled AP-1 consensus probe. Nuclear extracts from Ad-NS5A-transduced cells showed increased AP-1 binding when compared with equal amounts of nuclear extracts from Ad-GFP-transduced cells. PMA treatment followed by transduction with Ad-NS5A induced AP-1 binding further (Figure 6B,

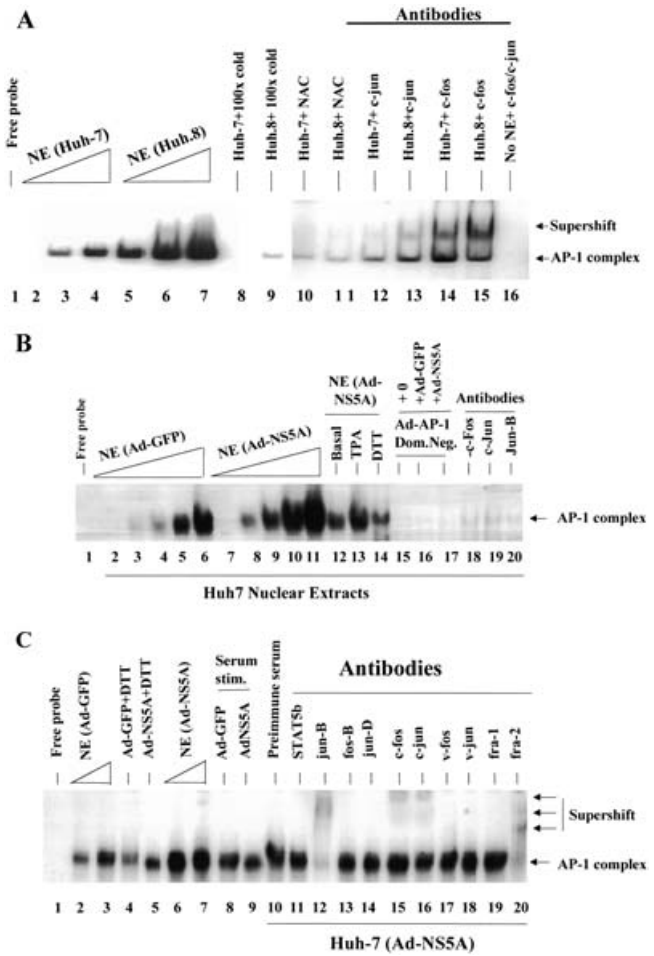

Figure 6 Induction of DNA binding of AP-1 in HCV subgenomic replicon-expressing and Ad-NS5A-transduced Huh-7 cells

(**A**) EMSAs of AP-1 binding using Huh-7 (control) and Huh.8 (HCV-replicating) nuclear extracts (NE). Lane 1, free probe; lanes 2–4 and 5–7, 10, 20 and 40 μg of Huh-7 and Huh.8 nuclear extracts respectively; lanes 8 and 9, 100× unlabelled competitor DNA using Huh-7 and Huh.8 nuclear extracts respectively; lanes 10 and 11, Huh-7 and Huh.8 extracts respectively from cells treated with 10 μM NAC; lanes 12 and 13, Huh-7 and Huh.8 nuclear extracts respectively incubated with anti-c-Fos antibodies; lanes 14 and 15, Huh-7 and Huh.8 nuclear extracts respectively incubated with anti-c-Jun antibodies; lane 16, incubation with anti-c-Fos and anti-c-Jun antibodies in the absence of nuclear extracts to test the mobility shift induced by the antibodies. (**B**, **C**) Changing composition of AP-1 DNA-binding complexes in Ad-NS5A-transduced Huh-7 cells. Cell extracts were assayed for AP-1 DNA binding by EMSA as described in the Experimental section. The participation of c-Fos, c-Jun and Jun-B was tested by incubating nuclear extracts with specific antibodies prior to adding the radiolabelled DNA. Serum-starved cells were transduced with Ad-GFP or Ad-NS5A. Samples of 2, 10, 20, 40 and 60 μg of protein from Ad-GFP-transduced (lanes 2–6) and Ad-NS5A-transduced (lanes 7–11) cells were employed. TPA = PMA. (**C**) Evidence for the participation of c-Fos, c-Jun, Jun-B and Fra-2 in AP-1 complex formation. Samples of 10 and 20 μg of nuclear extract protein from Huh-7 cells transduced with either Ad-GFP (lanes 2 and 3 respectively) or Ad-NS5A (lanes 6 and 7 respectively) were tested. Huh-7 cells were incubated with 10 μM DTT during Ad-GFP (lane 4) or Ad-NS5A (lane 5) transduction. Cells were stimulated with serum followed by Ad-GFP (lane 8) or Ad-NS5A (Lane 9) transduction for 24 h. Preimmune serum (lane 10) and anti-STAT5b (lane 11) were used as controls. Antibodies to Jun-B (lane 12), Fos-B (lane 13), Jun-D (lane 14), c-Fos (lane 15), c-Jun (lane 16), v-Fos (lane 17), v-Jun (lane 18), Fra-1 (lane 19) and Fra-2 (lane 20) were added after the extracts and DNA had been incubated for 30 min at 4 °C.

compare lanes 12 and 13). When cells were exposed to the anti-oxidant DTT (10 μM) during Ad-NS5A transduction, significantly reduced AP-1 binding was seen (Figure 6B, lane 14), indicating the specificity of the AP-1 complex. To further confirm the authenticity of the AP-1 complex, an adenovirus encoding

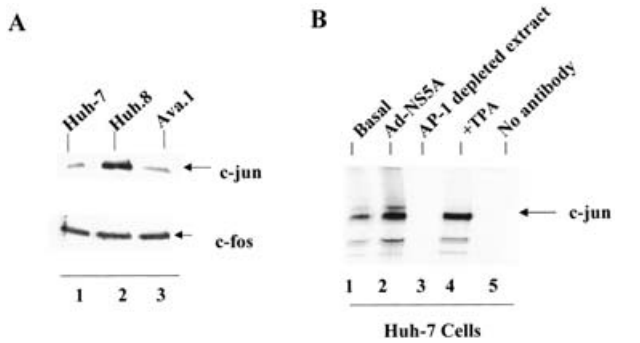

Figure 7 Enhanced accumulation of c-Jun in HCV-replicating Huh.8 cells (A) and Ad-NS5A-transduced Huh-7 cells (B)

(**A**) Lane 1, Huh-7 (control) nuclear extract; lane 2, Huh.8 (HCV-replicating) nuclear extract; lane 3, Ava.1 (ΔNS5A) nuclear extract. (**B**) Serum-starved Huh-7 cells were transduced with Ad-NS5A. Lane 1, basal c-Jun protein expression in Huh-7 cells; lane 2, c-Jun expression in Ad-NS5A-transduced Huh-7 cells; lane 3, anti-c-Jun antibodies were used to deplete AP-1; lane 4, nuclear extracts prepared from cells treated with PMA (TPA); lane 5, no antibody added. Immunoblots were detected by enhanced chemiluminescence using anti-c-Jun.

a dominant negative AP-1 (Ad-DN-AP-1) was transduced into Huh-7 cells (Figure 6B, lane 15) and co-transduced with either Ad-GFP (lane 16) or Ad-NS5A (lane 17). At 24 h post-transduction, no AP-1 binding was seen (lanes 15–17).

Members of the Fos family identified to date are v-Fos, c-Fos, Fos-B, Fra-1 and Fra-2. The Jun family comprises v-Jun, c-Jun, Jun-B and Jun-D. All of the Jun family proteins are capable of forming homo- and hetero-dimers on cognate DNA sites. Fos proteins do not associate with each other, but are capable of associating with any members of the Jun family to form stable dimers that have higher DNA binding activity than Jun dimers [5,29,52].

Huh-7 cells were transduced with either Ad-GFP (Figure 6C, lanes 2 and 3) or Ad-NS5A (lanes 6 and 7) and nuclear extracts were tested for AP-1 activation. Exposure of Huh-7 cells to 10 μM DTT during Ad-GFP or Ad-NS5A transduction resulted in decreased AP-1 activation (lanes 4 and 5 respectively). Serum stimulation of cells transduced with either Ad-GFP (lane 8) or Ad-NS5A (lane 9) resulted in increased AP-1 binding. To identify the individual component of the AP-1 complex, antibodies against the various components of AP-1, including Jun-B, Fos-B, Jun-D, c-Fos, c-Jun, v-Fos, v-Jun, Fra-1 and Fra-2, were added to the reaction (Figure 6C, lanes 12–20). Antibodies to Jun-B (lane 12), Fos-B (lane 13), Jun-D (lane 14), c-Fos (lane 15), c-Jun (lane 16), v-Fos (lane 17, v-Jun (lane 18) Fra-1 (lane 19) and Fra-2 (lane 20) were added after the nuclear extracts and DNA were mixed for 30 min at 4 °C. Only antibodies against Jun-B (lane 12), c-Fos (lane 15), c-Jun (lane 16) and Fra-2 (lane 20) resulted in supershifting of the AP-1 complex. As controls, preimmune serum and a non-specific antibody against STAT5b (signal transduction and activators of transcription 5b) were added (Figure 6C, lanes 10 and 11 respectively). As expected, preimmune serum or anti-STAT5b antibody did not shift the AP-1 complex.

Enhanced accumulation of c-Jun protein in HCV-replicating and Ad-NS5A-transduced Huh-7 cells

Because oxidant stress is known to increase the expression of c-Jun, c-Fos and c-Myc [8,11,51], whole-cell extracts from Huh-7, Huh.8 and Ava.1 cells were prepared and subjected to immunoblotting to determine protein expression of c-Fos and c-Jun (Figure 7A). A substantial increase in the level of c-Jun was observed in HCV-replicating cells (lane 2), while no change in

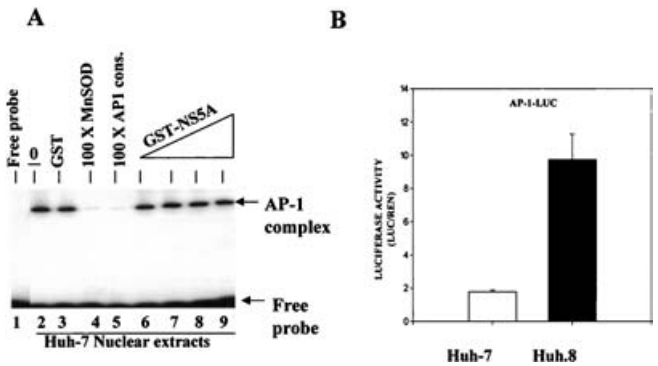

Figure 8 Intracellular activation of AP-1 by NS5A

(**A**) Exogenous GST–NS5A does not induce AP-1 DNA binding. An EMSA of Huh-7 cell nuclear extracts was carried out using an AP-1 consensus probe in the presence of purified GST–NS5A (lanes 6–9; 10, 20, 50 and 100 ng respectively). Lane 1, free probe; lanes 2–9, 20 μg of protein from nuclear extracts added per lane; lane 3, + 100 ng of purified GST; lane 4, + 100× unlabelled MnSOD oligonucleotide encompassing the AP-1 site added; lane 5, + 100× AP-1 consensus oligonucleotide; lanes 6–9, + 10, 20, 50 and 100 ng respectively of purified GST-NS5A. No induction of AP-1 binding was seen on the addition of exogenous NS5A. (**B**) The AP-1 responsive element (TRE) is activated in Huh.8 cells. pAP-LUC was transfected into control Huh-7 and HCV-replicating Huh.8 cells, and luciferase activity was measured. Approx. 5-fold activation of AP-1–LUC was seen in Huh.8 cells.

c-Fos expression was observed (lanes 1–3). Ava.1 cells (lacking the 47 amino acids of NS5A) showed no change in c-Jun expression compared with controls.

Since only c-Jun expression was increased in HCV-replicating cells, we were interested to determine whether Ad-NS5A-transduced cells might also show an increase in c-Jun expression. Serum-starved Huh-7 cells were transduced with Ad-NS5A and lysates were prepared as described. As expected, enhanced expression of c-Jun was seen in Ad-NS5A-transduced Huh-7 cells (Figure 7B, lane 2). As a control, AP-1-depleted cell extracts were employed, and no c-Jun protein band was seen using antibodies to c-Jun (lane 3). Antibodies to c-Fos and c-Jun were used to deplete the extracts. As expected, extracts from PMA-treated cells showed an increase in c-Jun expression (lane 4). These results confirm that the increase in c-Jun expression in HCV-replicating cells is associated with functional NS5A expression.

Exogenous GST (glutathione S-transferase)–NS5A fails to induce AP-1 DNA-binding activity

To determine whether NS5A is physically required in the AP-1 complex for its activation, EMSA was performed with Huh-7 nuclear extracts in the presence of GST–NS5A. GST–NS5A (10, 20, 50 or 100 ng; Figure 8A, lanes 6–9) did not increase AP-1 binding. Since no induction of AP-1 binding by exogenous NS5A was observed, we believe that NS5A is not a component of the AP-1 complex. We propose that NS5A-induced signalling cascades within cells are required for AP-1 activation. To test this hypothesis, we measured the binding activity of the AP-1 responsive element [TRE; PMA ('TPA')-responsive element] within the cell using an AP-1–LUC reporter construct. pAP-1–LUC was transfected into Huh-7 and HCV-replicating Huh.8 cells and luciferase activity was measured (Figure 8B). An approx. 5-fold increase in luciferase activity was observed in HCV replicon-containing cells, suggesting that NS5A-mediated activation of JNK and p38 MAPK is essential to activate AP-1. No difference in luciferase expression was seen when heterologous SV40–LUC DNA was transfected into Huh-7 or Huh.8 cells (results not shown).

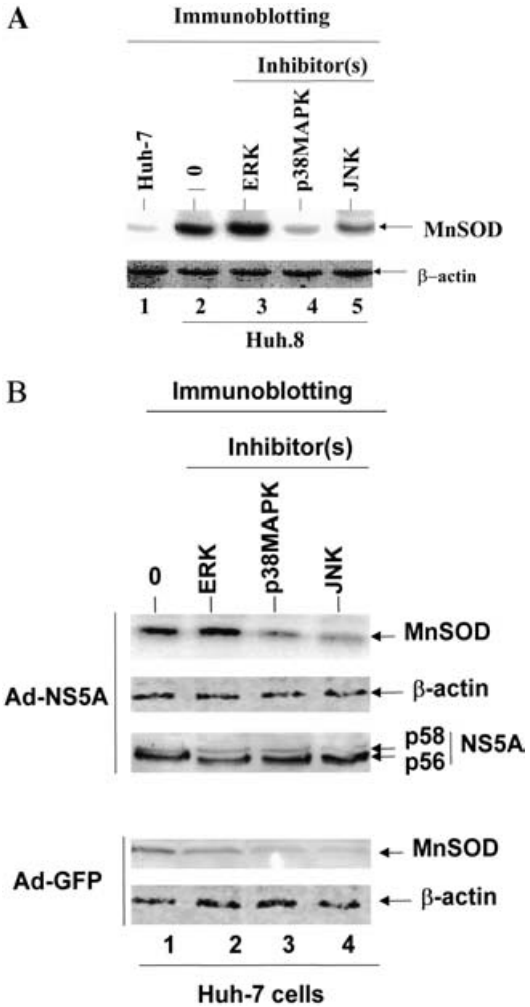

Figure 9 p38 MAPK and JNK inhibitors block the activation of MnSOD in HCV-replicating Huh.8 cells (A) and Ad-NS5A-transduced Huh-7 cells (B)

(**A**) Lanes 1 and 2, Huh-7 and Huh.8 cell extracts respectively; lanes 3, 4 and 5, extracts of Huh.8 cells exposed to the ERK1/2 inhibitor U0126 (20 nM), the p38 MAPK antagonist SB203580 (20 nM) or the JNK blocker JNKI1 (50 nM) respectively. (**B**) Immunoblotting of 50 μg cell extracts, post-transduced with either Ad-NS5A (upper panel) or Ad-GFP (lower panel), using antibodies against MnSOD. Antibodies against NS5A and β-actin were used to show NS5A expression and as the loading control respectively. As expected, Ad-NS5A-transduced cells showed activated MnSOD expression when compared with Ad-GFP-transduced cells. Lane 1, untreated cells; lanes 2–4, ERK, p38 MAPK and JNK inhibitors respectively. p38 MAPK and JNK inhibitors reduced the basal expression (Ad-GFP) and activation (Ad-NS5A) of MnSOD, while no changes were seen with the ERK inhibitor.

Inhibitors of p38 MAPK and JNK block MnSOD activation in HCV-replicating cells

Finally, we investigated possible roles of p38 MAPK, ERK1/2 and JNK in the process of MnSOD activation. Huh.8 cells were exposed to the a p38 MAPK inhibitor (SB203580), a JNK blocker (JNKI1) or an ERK1/2 inhibitor (U0126) for 12 h. Cell extracts were assayed for MnSOD protein expression using anti-MnSOD antibodies (Figure 9A). The p38 MAPK antagonist SB203580 (lane 4) and the JNK blocker JNKI1 (lane 5) blocked MnSOD activation, whereas the ERK1/2 inhibitor U0126 had no effect (lane 3). The observed inhibition is likely to be mediated by the activity of NS5A via activation of AP-1. To confirm this, Huh-7 cells were transduced with either Ad-NS5A (Figure 9B, upper

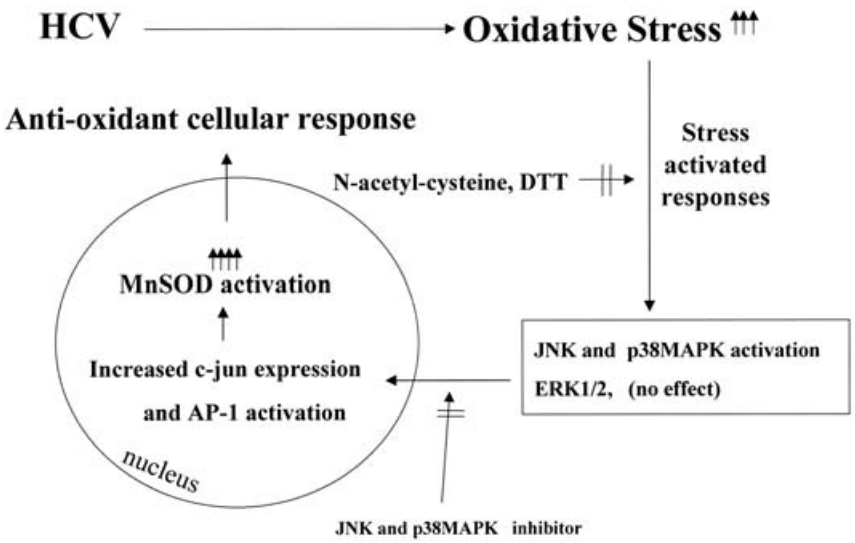

Figure 10 Proposed actions of HCV NS5A in inducing the cascade of stress-activated responses in Huh-7 cells via activation of p38 MAPK, JNK and AP-1 that contribute to increased in MnSOD expression

Both JNK and p38 MAPK inhibitors block the activation of MnSOD expression. The functional inactivation of p53 and down-regulation of p21 by NS5A has been reported, and may contribute to increased oxidative stress.

panel, lane 1) or Ad-GFP (lower panel, lane 1) and incubated with the ERK1/2 inhibitor U0126 (lanes 2), the p38 MAPK inhibitor SB203580 (lanes 3) or the JNK blocker JNKI1 (lanes 4) for 12 h. The expression profile of Ad-NS5A-transduced cells was compared with those of inhibitor-treated and untreated cells by immunoblotting using anti-NS5A antibodies. No changes in NS5A expression were observed following inhibitor treatments (Figure 9B, lanes 1–4). As expected Ad-NS5A-transduced cells showed activated MnSOD expression when compared with Ad-GFP-transduced cells (Figure 9B, compare lanes 1 in upper and lower panels). p38 MAPK and JNK inhibitors reduced the basal expression and activation of MnSOD, whereas the ERK inhibitor had no effect.

DISCUSSION

The results presented here suggest a cascade of signalling events that are triggered by HCV-induced oxidative stress (Figure 10). The HCV protein NS5A seems to play a critical role in the activation of p38 MAPK, JNK and AP-1, leading to increased MnSOD antioxidant responses. The activation of AP-1 and MnSOD by HCV NS5A may be instrumental in the regulation of host oxidant status, and underscores the potential importance of this protein. The observed cellular responses are designed to counteract the oxidative stress induced by HCV protein expression. The first enzymes in the detoxification of oxygen free radicals are the SODs (MnSOD and Cu/ZnSOD). SODs catalyse the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen [35–37]. NFκB, TNFα (tumour necrosis factor α), interleukin 6, the p38 MAPK signalling cascade and the transcription factors AP-1 and AP-2 contribute to the regulation of MnSOD; however, the exact roles of these cellular transcription factors have not been completely defined [35–37,41,44–48]. AP-1 contributes to the control of MnSOD and is activated in response to environmental stress, radiation and growth factors, and is a positive regulator of human and *Drosophila melanogaster* MnSODs [41,45–48]. Exposure to superoxides and secondary oxidants leads to induction of c-Fos, c-Jun, [29,36] and c-Myc [49], increased cellular proliferation [50], and lung hyperplasia and fibrosis in hamsters [36]. Induction of the MnSOD gene by arachidonic acid is mediated by reactive oxygen species and the p38 MAPK signalling pathway in human HepG2 hepatoma cells [37]. The AP-1 activation response was blocked by the known AP-1 inhibitors NAC and DTT [5], thus establishing AP-1 specificity in both control and HCV-replicating cells (Figure 6). Consistent with this observation, a decrease in MnSOD expression following treatment with p38 MAPK and JNK inhibitors was seen in both HCV-replicating cells and Ad-NS5A-transduced cells (Figure 9). Interestingly, the antioxidants NAC and DTT also blocked the replication of HCV in Huh.8 cells, by 75 % and 50 % respectively (results not shown). How these antioxidants may contribute to a decrease in HCV replication remains to be investigated. The basal expression of MnSOD was also blocked in Ad-GFP-transduced Huh-7 cells by p38 MAPK and JNK inhibitors, suggesting the putative involvement of p38 MAPK and JNK in MnSOD regulation. There are no previous results indicating the involvement of JNK in MnSOD regulation, and therefore the present study identifies an additional signalling pathway for antioxidant regulation.

HCV NS5A is a serine phosphoprotein consisting of two isoforms, p56 and p58 [51], which binds to multitude of cellular proteins, including PKR, phosphoinositide 3-kinase, SRCAP, Grb2, TBP and p53 [16–23]. The induction of oxidative stress and the activation of MnSOD by NS5A alone or within the HCV subgenomic replicon via the stress-activated p38 MAPK and JNK pathways may be linked with p53. Diminished p53 function within cells expressing HCV NS5A [22,23] may induce a cascade of events leading to increased stress, as p53 has been linked to oxidative stress and MnSOD expression via JNK, p38 MAPK and ERK1/2 [25,33,34,38,53,54].

Since exogenously added NS5A protein was unable to induce AP-1 binding to DNA, we argue that NS5A is not a component of the AP-1 complex. While NS5A expression induced an approx. 3-fold increase in c-Jun protein levels, it remains to be seen to what extent NS5A exerts its influence on the composition of c-Jun

and c-Fos homo- and hetero-dimers. Our results are consistent with the observation of AP-1 activation by other viruses [44]. Hepatitis B virus HBx protein has been shown to induce AP-1 by activation of ERK and JNK [52], while HIV Tat activates AP-1 through an oxidant-dependent mechanism involving serum response elements and ternary complex factors [30]. It remains to be seen whether NS5A-mediated AP-1 activation also involves these components.

Different viruses have variable responses with regard to superoxide. HIV-1 infection induces the up-regulation of MnSOD transcription in CD4+ lymphocytes and overexpression in purified normal human monocyte-derived macrophages [53]. The up-regulation of MnSOD is concomitant with TNFα and interleukin-6 production. HIV Tat protein represses MnSOD expression in HeLa cells [41]. Rhinovirus infection of respiratory epithelial cells led to an increase in Cu/ZnSOD levels, but not to a change in MnSOD expression [54].

Our observations are novel and provide important clues to the oxidant and antioxidant status of HCV-infected cells that may be relevant for new therapeutic treatments. A role for p38 MAPK in MnSOD expression is consistent with the results of Bianchi et al. [37]. In addition to NS5A, C protein of HCV also activates ERK, JNK and p38 MAPK [32]. The sustained activation of MnSOD may be an acute adaptive response to oxidative stress, and damage may occur when the oxidant burden overwhelms the cell's capacity to detoxify at the late chronic stage of HCV infection. This activation may protect HCV-replicating cells from cell death, as MnSOD and JNK activities mediated by reactive oxygen species are reported to represent a protective mechanism against cell death [53,55,56]. The antioxidant MnSOD responses may collapse after a prolonged battle with the oxidant burden imposed by cytokines and other secondary effects associated with HCV infection. It will be interesting to determine which cytokine and stress-signalling pathways play a role at various stages of HCV infection. This may provide important clues to the mechanisms involved in the development of chronic liver disease with the potential of hepatocellular carcinoma.

We are grateful for the generous help of Dr Charles Rice (Johns Hopkins University, Baltimore, MD, U.S.A.) for providing the Huh.8 and Ava.1 cell lines. We thank Dr Michael Katze (University of Washington, Seattle, WA, U.S.A.) for providing pGST-NS5A. The recombinant adenovirus Ad5TAM and the AP-1–LUC construct were kindly provided by Dr D. Brenner. This work was supported by a grant from the American Liver Foundation, Herman Lopeta Memorial Award, National Institutes of Health grant RO1 DK-15851 and a Veterans' Affairs Merit review grant (to F.R.S.).

REFERENCES

1 Cohen, J. (1999) The scientific challenge of hepatitis C. Science **285**, 26–30
2 De Franceschi, L., Fattovich, G., Turrini, F., Ayi, K., Brugnara, C., Manzato, F., Noventa, F., Stanzial, A. M., Solero, P. and Corocher, R. (2000) Hemolytic anemia induced by ribavirin therapy in patients with chronic hepatitis C virus infection: role of membrane oxidative damage. Hepatology **31**, 997–1004
3 De Maria, N., Colantoni, A., Fagiuoli, S., Liu, G. J., Rogers, B. K., Farinati, F., van Thiel, D. H. and Floyd, R. A. (1996) Association between reactive oxygen species and disease activity in chronic hepatitis C. Free Radical Biol. Med. **21**, 291–295
4 Larrea, E., Beloqui, O., Munoz-Navas, A., Civerir, M. P. and Prieto, J. (1998) Superoxide dismutase in patients with chronic hepatitis C virus infection. Free Radical Biol. Med. **24**, 1235–1241
5 Sen, C. K. and Packer, L. (1996) Antioxidant and redox regulation of gene transcription. FASEB J. **10**, 709–720
6 Xu, Y., Bradham, C., Brenner, D. A. and Czaja, M. J. (1997) Hydrogen peroxide-induced liver cell necrosis is dependent on AP-1 activation. Am J. Physiol. **273**, G795–G803
7 Farinati, F., Cardin, R., Degan, P., de Maria, N., Floy, R. A., van Thiel, D. H. and Naccarato, R. (1999) Oxidative DNA damage in circulating leukocytes occurs as an early event in chronic HCV infection. Free Radical Biol. Med. **27**, 1284–1291
8 Multu-Turkoglu, U., Adenmoglu, E., Turkoglu, S., Badur, S., Uysal, M. and Toker, G. (1997) The effects of interferon-alpha on serum lipid peroxidation and total thiol content in patients with chronic active hepatitis-C. Res. Commun. Mol. Pathol. Pharamcol. **96**, 357–361
9 Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. and Houghton, M. (1999) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244**, 359–362
10 Miller, R. H. and Purcell, R. H. (1990) Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. U.S.A. **87**, 2057–2061
11 Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. and Shimotohono, K. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U.S.A. **87**, 9524–9528
12 Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. and Nomoto, A. (1992) Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66**, 1476–1483
13 Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. and Bartenschlager, R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285**, 110–113
14 Blight, K. J., Kolykhalov, A. A. and Rice, C. M. (2000) Efficient initiation of HCV RNA replication in cell culture. Science **290**, 1972–1974
15 Kato, N., Lan, K.-H., Ono-Nita, K. S., Shiratori, Y. and Omata, M. (1997) Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. J. Virol. **71**, 8856–8859
16 Ghosh, A. K., Steele, R., Meyer, K., Ray, R. and Ray, R. B. (1999) Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth. J. Gen. Virol. **80**, 1179–1183
17 Gale, Jr, M., Kwiecisszwski, B., Dossett, M., Nako, H. and Katze, M. G. (1999) Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. J. Virol. **73**, 6506–6516
18 Ghosh, A. K., Majumade, M., Steele, R. Yaciuk, P., Chrivia, J., Ray, R. and Ray, R. B. (2000) Hepatitis C virus NS5A protein modulates transcription through a novel cellular transcription factor SRCAP. J. Biol. Chem. **275**, 7184–7188
19 Tan, S. L., Nakao, H., He, Y., Vijaysri, S., Neddermann, P., Jacobs, B. L., Mayer, B. J. and Katze, M. G. (1999) NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling. Proc. Natl. Acad. Sci. U.S.A. **96**, 5533–5538
20 He, Y., Nako, H., Tan, S. L., Polyak, S. J., Neddermann, P., Vijaysri, S., Jacobs, B. L. and Katze, M. G. (2002) Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and P85 phosphatidylinositol 3-kinase. J. Virol. **76**, 9207–9217
21 Reyes, G. R. (2002) The nonstructural NS5A protein of hepatitis C virus: an expanding, multifunctional role in enhancing hepatitis C virus pathogenesis. J. Biomed. Sci. **9**, 187–197
22 Majumder, M., Ghosh, A. K., Steele, R., Ray, R. and Ray, R. B. (2001) Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner. J. Virol. **75**, 1401–1407
23 Qadri, I., Iwahashi, M. and Simon, F. (2002) Hepatitis C virus NS5A protein binds TBP and p53, inhibiting their DNA binding and p53 interactions with TBP and ERCC3. Biochim. Biophys. Acta **1592**, 193–204
24 Hupp, T. R., Lane, D. P. and Ball, K. L. (2000) Strategies for manipulating the p53 pathway in the treatment of human cancer. J. Biol. Chem. **352**, 1–17
25 Shatrov, V. A., Ameyar, M., Bouquet, C., Cai, Z., Stancou, R., Haddada, H. and Chouaib, S. (2000) Adenovirus-mediated wild-type-p53-gene expression sensitizes TNF-resistant tumor cells to TNF-induced cytotoxicity by altering the cellular redox state. Int. J. Cancer **85**, 93–97
26 Helt, C. E., Rancourt, R. C., Staversky, R. J. and O'Reilly, M. A. (2001) p53-dependent induction of p21(Cip1/WAF1/Sdi1) protects against oxygen-induced toxicity. Toxicol. Res. **63**, 214–222
27 Cohen, P. (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends Cell Biol. **7**, 592–604
28 Johnson, G. L. and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science **298**, 1911–1912
29 Devary, Y., Gottlieb, R. A., Lau, L. F. and Karin, M. (1991) Rapid and preferential activation of the c-Jun gene during the mammalian UV response. Mol. Cell. Biol. **11**, 2804–2811
30 Gu, Y., Wu, R. F., Xu, Y. C., Flores, S. S. and Terada, L. S. (2001) HIV Tat activates c-Jun amino-terminal kinase through an oxidant-dependent mechanism. Virology **20**, 62–71

31 Kato, N., Yoshida, H., Kioko Ono-Nita, S., Kato, J., Goto, T., Otsuka, M., Lan, K.-H., Matsushima, K., Shiratori, Y. and Omata, M. (2000) Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. Hepatology **32**, 405–412
32 Erhardt, A., Hassan, M., Heintges, T. and Haussinger, D. (2002) Hepatitis C virus core protein induces cell proliferation and activates ERK, JNK, and p38 MAP kinases together with the MAP kinase phosphatase MKP-1 in a HepG2 Tet-Off cell line. Virology **20**, 272–284
33 Zhu, Y., Mao, X. O., Sun, Y., Xia, Z. and Greenberg, D. A. (2002) p38 Mitogen-activated protein kinase mediates hypoxic regulation of Mdm2 and p53 in neurons. J. Biol. Chem. **277**, 22909–22914
34 Kim, H. T., Tasca, S., Qiang, W., Wong, P. K. and Stoica, G. (2002) Induction of p53 accumulation by Moloney murine leukemia virus-ts1 infection in astrocytes via activation of extracellular signal-regulated kinases 1/2. Lab. Invest. **82**, 693–702
35 St Clair, D. K., Porntadavity, S., Xu, Y. and Kiningham, K. (2002) Transcription regulation of human manganese superoxide dismutase gene. Methods Enzymol. **349**, 306–312
36 Nakashima, J. M., Levin, J. R., Hyde, D. M. and Giri, S. N. (1991) Repeated exposures to enzyme-generated oxidants cause alveolitis, epithelial hyperplasia, and fibrosis in hamsters. Am. J. Pathol. **139**, 1485–1499
37 Bianchi, A., Becuwe, P., Franck, P. and Dauca, M. (2002) Induction of MnSOD gene by arachidonic acid is mediated by reactive oxygen species and p38 MAPK signaling pathway in human HepG2 hepatoma cells. Free Radical Biol. Med. **32**, 1132–1142
38 Drane, P., Bravard, A., Bouvard, V. and May, E. (2001) Reciprocal down-regulation of p53 and SOD2 gene expression – implication in p53 mediated apoptosis. Oncogene **20**, 430–439
39 Yeh, C. C., Wan, X. S. and St. Clair, D. K. (1998) Transcriptional regulation of the 5′ proximal promoter of the human manganese superoxide dismutase gene. DNA Cell Biol. **17**, 921–930
40 He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. and Vogelstein, B. (1998) A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. U.S.A. **95**, 2509–2514
41 Flores, S. C., Marecki, J. C., Harper, K. P., Bose, S. K., Nelson, S. K. and McCord, J. M. (1993) Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **90**, 7632–7637
42 McCord, J. M. and Fridovich, I. (1969) The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. J. Biol. Chem. **244**, 6056–6063
43 Bradham, C. A., Hatano, E. and Brenner, D. A. (2001) Dominant-negative TAK1 induces c-Myc and G(0) exit in liver. Am. J. Physiol. Gastrointest. Liver Physiol. **281**, G1279–G1289
43a Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
44 Maeda, H. and Akaike, T. (1991) Oxygen free radicals as pathogenic molecules in viral diseases. Proc. Soc. Exp. Biol. Med. **198**, 721–727
45 Zhao, Y., Xue, Y., Oberley, T. D., Kiningham, K. K., Lin, S. M., Yen, H. C., Majima, H., Hines, J. and St Clair, D. (2001) Overexpression of manganese superoxide dismutase suppresses tumor formation by modulation of activator protein-1 signaling in a multistage skin carcinogenesis model. Cancer Res. **15**, 6082–6088
46 Zhou, W., Zhang, Y., Hosch, M. S., Lang, A., Zwacka, R. M. and Engelhardt, J. F. (2001) Subcellular site of superoxide dismutase expression differentially controls AP-1 activity and injury in mouse liver following ischemia/reperfusion. Hepatology **33**, 902–914
47 Zhang, H. J., Drake, V. J., Xu, L., Hu, J., Domann, F. E., Oberley, L. W. and Kregel, K. C. (2002) Redox regulation of adenovirus-induced AP-1 activation by overexpression of manganese-containing superoxide dismutase. J. Virol. **76**, 355–363
48 Kinscherf, R., Deigner, H. P., Usinger, C., Pill, J., Wagner, M., Kamencic, H., Hou, D., Chen, M., Schmiedt, W., Schrader, M. et al. (1997) Induction of mitochondrial manganese superoxide dismutase in macrophages by oxidized LDL: its relevance in atherosclerosis of humans and heritable hyperlipidemic rabbits. FASEB J. **11**, 1317–1328
49 Crawford, D., Zbinden, I., Moret, R. and Cerutti, P. (1988) Antioxidant enzymes in xeroderma pigmentosum fibroblasts. Cancer Res. **48**, 2132–2134
50 Ensoli, B., Barillari, G., Salahuddin, S. Z., Gallo, R. C. and Wong-Staal, F. (1990) Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature (London) **345**, 84–86
51 Tanji, Y., Kaneko, S., Satoh, S. and Shimotohno, K. (1995) Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. J. Virol. **69**, 3980–3986
52 Benn, J., Su, F., Doria, M. and Schneider, R. J. (1996) Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal mitogen-activated protein kinases. J. Virol. **70**, 4978–4985
53 Raoul, H., Le Naour, R., Blond, D. and Dormont, D. (1998) HIV type 1 infection of human macrophages induces an upregulation of manganese superoxide dismutase gene that may protect cells from death. AIDS Res. Hum. Retroviruses **20**, 427–434
54 Kaul, P., Singh, I. and Turner, R. B. (2002) Effect of rhinovirus challenge on antioxidant enzymes in respiratory epithelial cells. Free Radical Res. **36**, 1085–1089
55 Kinscherf, R., Claus, R., Wagner, M., Gehrke, C., Kamencic, H., Hou, D., Nauen, O., Schmiedt, W., Kovacs, G., Pill, J. et al. (1998) Apoptosis caused by oxidized LDL is manganese superoxide dismutase and p53 dependent. FASEB J. **12**, 461–470
56 Liu, S. L., Lin, X., Shi, D. Y., Cheng, J., Wu, C. Q. and Zhang, Y. D. (2002) Reactive oxygen species stimulated human hepatoma cell proliferation via cross-talk between PI3-K/PKB and JNK signaling pathways. Arch. Biochem. Biophys. **15**, 173–182

Received 16 October 2003/2 December 2003; accepted 10 December 2003
Published as BJ Immediate Publication 10 December 2003, DOI 10.1042/BJ20031587