6 Discussion

6.1 NS5A purification

Purification of NS5A turned out to be quite difficult. In fact, several purification strategies were tried, including the purification by nickel chelating chromatography (using a hexa-histidine-tagged version of NS5A) (data not shown), the conventional purification by ion exchange chromatography and subsequent gel filtration (data not shown) and the purification by *Strep*Tactin Sepharose chromatography (Fig. 5.6). The latter proved to be the most successful. This is most likely due to the comparably high affinity of the *Strep* tag for the *Strep*Tactin material (K_D=0.1μM).

Furthermore, purification of NS5A was complicated by two major factors: (i) The expression level of full-length NS5A was quite low. Even if produced in bacteria, the NS5A protein was not detected in the lysate by coomassie or silver staining, although the Western blot suggested that the induction was successful. (ii) Full-length NS5A is a membrane associated protein that interacts with cellular membranes by the means of its amphipathic helix (amino acids 1-30). Hence, solubility of this protein in the absence of detergent is low.

In order to optimize solubility of full-length NS5A, the purification was performed in the presence of 0.5% NP-40 as recently suggested (Huang et al., 2004). Whereas purification of full-length NS5A in the absence of detergent was essentially not possible, significant amounts of NS5A were purified in the presence of 0.5% NP-40.

For the isolation of the NS5A deletion mutant NS5A(211-449), addition of detergent was not necessary since NS5A(211-449) lacks the amphipathic helix that is responsible for membrane association. NS5A(211-449) was expressed at high levels and readily accessible for purification.

6.2 Interaction of c-Raf1 with NS5A

Interaction of NS5A with c-Raf1 was observed in several experimental settings. First, c-Raf1 derived from human hepatoma cells bound to immobilized NS5A. This was true for both bacterially- and Sf9 cell-derived NS5A (data not shown), arguing that the interaction is not dependent on posttranslational modification of NS5A (such as phosphorylation). Second, NS5A was coimmunoprecipitated with c-Raf1 from baculovirus-infected Sf21 cells (Fig. 5.8). Third,

NS5A was found to colocalize with endogenous c-Raf1 in human hepatoma cells (Fig. 5.9) and HCV replicon cells (Fig. 5.10). Fourth, NS5A deletion mutants that were localized in the nucleus caused a nuclear translocation of endogenous c-Raf1 (Fig. 5.12).

However, NS5A and c-Raf1 were not coimmunoprecipitated using either transiently transfected HuH-7 cells or HCV replicon cells (data not shown). The most obvious interpretation of this experiment is that NS5A and c-Raf1 do not interact in human hepatoma cells. Although this cannot be fully excluded, the immunofluorescence data with truncated NS5A mutants (Fig. 5.12) provide strong evidence that the NS5A/c-Raf1 interaction is indeed relevant in these cells. In fact, the interaction seems strong enough to alter the localization of endogenous c-Raf1, leading to the translocation of c-Raf1 to a cellular compartment that c-Raf1 is usually not found (nucleus) (O'Neill and Kolch, 2004).

Therefore, alternative explanations for the inability to coprecipitate NS5A and c-Raf1 should be considered: (i) The experiments shown in chapter 5.5 indicate that NS5A is a substrate of c-Raf1. If c-Raf1 is an enzyme that modifies NS5A by the means of phosphorylation, it is of critical importance that the interaction is short-lived. Otherwise, c-Raf1 could not act as a catalytical factor. Hence, the coprecipitation experiment might have failed due to the temporary nature of the interaction. (ii) Alternatively, in cultured human hepatoma cells, NS5A may bind to c-Raf1 with low affinity. Consecutively, the coprecipitation experiment might have failed due to the competition of other NS5A-interacting proteins with c-Raf1. (iii) Interaction of NS5A with c-Raf1 in human hepatoma cells might depend on the phosphorylation status of NS5A or c-Raf1. Phosphorylation of NS5A or c-Raf1 might require the stimulation of human hepatoma cells with certain cues (such as growth hormones or cytokines). Therefore, the coprecipitation might have failed due to the incorrect posttranslational modification of NS5A or c-Raf1. In fact, it has been shown that interaction of c-Raf1 with its endogenous substrate MEK is strongly enhanced upon phosphorylation of c-Raf1 on distinct serine and tyrosine residues (Xiang et al., 2002). (iv) As mentioned in chapter 6.1, solubilization of full-length NS5A requires the addition of detergent. However, addition of detergent might either dissociate the NS5A/c-Raf1 complex or it might weaken the antigen/antibody interaction during the immunoprecipitation.

6.3 Localization of NS5A and c-Raf1

Concerns may be raised as to whether the ER-associated protein NS5A is capable of interacting with c-Raf1 that requires the recruitment to the plasma membrane for proper activation. In other words, how can active c-Raf1 interact with NS5A if activation of c-Raf1 requires plasma membrane localization?

First, c-Raf1 displays a certain activity *ex vivo* even in the absence of membranes, e.g. if immunoprecipitated from mammalian cells or if purified from Sf9 cells by the means of affinity chromatography. This argues that membrane association may not be required for basal activation of c-Raf1. Second it seems that, in mammalian cells, the requirement for plasma membrane association is not as strict as originally formulated (Marshall, 1995). In fact, c-Raf1 may associate with other membranous compartments including the endoplasmatic reticulum and nevertheless trigger activation of downstream substrates (Chiu et al., 2002; O'Neill and Kolch, 2004). This suggests that it is not necessarily the *plasma* membrane that c-Raf1 has to associate with in order to be activated.

Therefore, it is conceivable that a fraction of cytosolic c-Raf1 associates with NS5A at the endoplasmatic reticulum (ER) and that this fraction of c-Raf1 is nevertheless active.

6.4 MAP kinase signaling and HCV replication

6.4.1 Advantages and drawbacks of small-molecule kinase inhibitors

The use of small-molecule inhibitors to assay the impact of cellular factors on HCV replication is a powerful approach because it is not limited by transfection efficiency. Furthermore, it allows a dose-dependent reduction of activity rather than a complete loss of activity. Finally, the use of small-molecule inhibitors allows a detailed kinetic analysis since the onset of inhibition can be monitored. However, there are two major drawbacks of this approach:

First of all, the specificity of small molecule inhibitors is hard to assess. It is possible to assess whether one specific target (c-Raf1) is inhibited, but it is virtually impossible to state whether other targets are also compromised. The human proteome comprises about 500 kinases (Manning et al., 2002), some of which are strikingly similar with regard to the active center and/or mechanisms that regulate kinase activity. As a consequence, most kinase inhibitors are likely to be less specific than commonly stated. This is particularly true for inhibitors that

compete with ATP because ATP is used by virtually all kinases. BAY43-9006 is a competitive inhibitor of c-Raf1. In fact, BAY43-9006 has been shown to effectively bind to B-Raf as well (Wan et al., 2004). On the other hand, this is not too surprising given the fact that the kinase domain is highly conserved between c-Raf1 and B-Raf (Chong et al., 2003).

Second, cytotoxicity of small-molecule inhibitors poses a potential problem. In most of the cases, toxicity arises as an unforeseen side effect. However, for some target molecules, loss of function (by the means of a small-molecule inhibitor) may be deleterious by itself. This may be the case for c-Raf1: c-Raf1-deficient mice die early during embryogenesis (Mikula et al., 2001), suggesting that the loss of c-Raf1 function severely compromises the integrity of living cells.

6.4.2 Impact of c-Raf1- and MEK-inhibition on HCV replication

With all drawbacks in mind, small-molecule inhibitors that target c-Raf1 (BAY43-9006) or MEK (PD98059, U0126) were used to study the impact of a loss of c-Raf1/MEK function for viral replication. Incubation of HCV replicon cells with 10μM BAY43-9006 lead to a decrease in viral replication within 6h (Fig. 5.17), arguing that a loss of c-Raf1 function negatively affects HCV replication. The MEK inhibitors PD98059 and U0126 produced controversial results: Whereas 20μM PD98059 increased the rate of viral replication (Fig. 5.20), 10μM U0126 did not affect replication whatsoever (Fig. 5.21). This is surprising, given the fact that PD98059 and U0126 bind to MEK in a mutually exclusive fashion (Favata et al., 1998), implying that PD98059 and U0126 share a common binding site on MEK. Furthermore, both inhibitors blocked ERK phosphorylation to the same extent (Fig. 5.15). This means that although PD98059 and U0126 share similar characteristics with regard to MEK inhibition, they differentially modulate viral replication. This indicates that the inhibition of MEK *per se* does not affect viral replication. Moreover, it supports the notion that the PD98059-mediated increase in viral replication is an artefact that is not related to MEK inhibition.

Since the use of small-molecule inhibitors raised some concerns, the impact of c-Raf1 on viral replication was analyzed in an alternative experimental design, ie. by reducing c-Raf1 expression by RNA interference. Fig. 5.19A shows that c-Raf1 expression is reduced as a consequence of the RNA interference. At the same time, viral replication is diminished (Fig. 5.19B), essentially corroborating the results generated with the small molecule inhibitor of c-Raf1. However, the reduction in viral replication is rather subtle (~50%). Several reasons could account for that: (i)

One limit of the siRNA-mediated reduction of c-Raf1 expression is transfection efficiency. This is particularly true for the transfer of the siRNA by lipofection. Moreover, transfection efficiency is hard to assess as long as fluorescent siRNAs are unavailable. (ii) The degree of reduction of c-Raf1 expression in the individual cell is hard to assess. Furthermore, it is not known whether all c-Raf1 molecules that are present in the cell are indeed necessary. A reduction of c-Raf1 expression by 80% might leave the cell with 20% of c-Raf1 that is still sufficient to sustain c-Raf1 function, thereby attenuating the outcome of the RNA interference. (iii) The kinetics of this kind of experiment are very tricky. First of all, the onset of c-Raf1 reduction is not evident. Second, the cell might compensate for the loss of c-Raf1 by upregulating c-Raf1. Therefore, the levels of c-Raf1 might fluctuate over time and the time point that was chosen to harvest the cells and determine the outcome on viral replication (48h post transfection) might have been unfavourable.

Nevertheless, the results obtained with the c-Raf1-specific siRNAs are in agreement with the results obtained for the small-molecule inhibitor of c-Raf1 (BAY43-9006). They suggest that an inhibition of c-Raf1 results in a decrease in HCV replication.

6.4.3 Kinetics of HCV replication and viral gene expression

Using the luciferase replicon, it is difficult to distinguish between viral replication (RNA level) and viral gene expression (protein level). Looking at drugs that slow down or abrogate viral replication such as BAY43-9006, both the half-life of the viral RNA and the half-life of the luciferase protein are limiting. The half-life of the viral RNA is surprisingly long and lies in the range of 14h (Johnston et al., 2003). The half-life of the luciferase protein amounts to 3h (Thompson et al., 1991). Therefore, the reduction that is measured after incubation of HCV replicon cells with the c-Raf1 inhibitor for 6h most likely reflects a block in viral gene expression rather than a block in RNA replication. Nevertheless, it suggests that c-Raf1 activity is needed for viral replication since viral gene expression is the prerequisite for autonomous RNA replication. In other words, RNA levels will necessarily drop as a consequence of reduced viral gene expression, most notably as a consequence of reduced NS5B expression.

6.4.4 HCV replication is coupled to host cell proliferation

What happens if HCV replicon cells are incubated with the small-molecule inhibitors for longer than 6h? When HCV replicon cells were incubated with the small-molecule inhibitors for 16h, the outcome on viral replication (and/or gene expression) was the same (data not shown). However, there is one *caveat* that applies more so for extended time periods: HCV replication in HCV replicon cells is tightly coupled to host cell proliferation (Pietschmann et al., 2001), ie. HCV replicates in proliferating cells and replication is essentially abolished once the cells stop dividing. As a consequence, HCV replication is very sensitive to alterations in host cell proliferation.

BAY43-9006 has been shown to block proliferation of human melanoma cells when applied at concentrations above 5μM (Karasarides et al., 2004). Fig. 5.18B indicates that it also blocks proliferation of HuH-7 cells. Therefore, it was crucial to limit the exposure of HCV replicon cells to BAY43-9006 to 6h in order to minimize the effect of BAY43-9006 on cell proliferation.

At the same time, HCV replicon cells were incubated with 1mM hydroxyurea in order to analyze how the hydroxyurea-induced block in cell proliferation affects viral replication/ gene expression within 6h. As shown in Fig. 5.18A, 1mM hydroxyurea does not affect viral replication whereas 10μM BAY43-9006 does, arguing that the BAY43-9006 mediated reduction of replication is not due to a block in cell proliferation. However, it seems that 1mM hydroxyurea does not inhibit cell proliferation to the same extent as BAY43-9006 (Fig. 5.18B).

Therefore, in summary, it can not be excluded that the BAY43-9006-mediated reduction of replication is at least in part due to an interference of BAY43-9006 with host cell proliferation.

6.4.5 HCV replicons as model systems for HCV replication

The establishment of the HCV replicon model has been a major breakthrough of HCV research. Nevertheless, it seems that the HCV replicon system differs quite significantly from the situation of an authentic HCV infection: Most importantly, the HCV genome is altered by the occurrence and selection of cell culture-adaptive mutations. Cell culture-adapted HCV genomes are no longer infectious in the chimpanzee model although they replicate very efficiently in cell culture (Bukh et al., 2002). Furthermore, certain combinations of cell culture-adaptive mutations either synergistically enhance or completely abolish replication, arguing that these mutations induce very complex alterations that affect the interaction of HCV proteins with the cellular environment and with each other.

Therefore, it would be interesting to repeat the above mentioned experiments in several adaptive backgrounds and – most notably – in a wild-type situation. The latter is particularly difficult and requires very sensitive methods of detection because wild-type HCV genomes replicate very poorly in cultured cells. This problem was recently overcome by the establishment of transient replication assays (Krieger et al., 2001; Neddermann et al., 2004). It seems most promising to use this novel technology to evaluate the contribution of c-Raf1 in the wild-type situation.

6.5 Impact of NS5A on c-Raf1-induced signal transduction.

6.5.1 NS5A does not modulate the MAP kinase cascade c-Raf1/MEK/ERK

NS5A is said to be a pleiotropic molecule that modulates a number of signal transduction pathways of the host cell (Macdonald and Harris, 2004). The majority of these signaling cascades regulate cell growth, cell proliferation and cell survival, such as the MAP kinase pathway, the NF-κB pathway, the PI3K pathway or the p53 pathway (Gong et al., 2001; He et al., 2002; Lan et al., 2002; Macdonald et al., 2003). On the other hand, c-Raf1 is a key player in some of these pathways, in particular the MAP kinase pathway and the NF-κB pathway (Kolch, 2000). Therefore, it was reasonable to hypothesize that interaction of NS5A with c-Raf1 alters host cell signal transduction.

The most obvious target downstream of c-Raf1 is the MEK/ERK module that activates the transcription factors SRF and AP-1. However, Fig. 5.22 shows that NS5A does not interfere with ERK phosphorylation. Likewise, NS5A does not alter the level of basal transcriptional activation of SRF and AP-1 (Fig. 5.23). Furthermore, basal activation of SRF and AP-1 is essentially similar in HCV replicon cells and naïve HuH-7 cells (Fig. 5.25). This argues that NS5A does not modulate the activity of the MAP kinase cascade.

Given the fact that the role of NS5A for AP-1 signaling is still a matter of debate, the data presented in chapter 5.4 lend support to the hypothesis that NS5A by itself does not significantly alter AP-1 activation. Furthermore, the data document that NS5A does not alter the PMA-induced stimulation of the MAP kinase cascade. Nevertheless, it cannot be excluded that NS5A attenuates the EGF-induced stimulation of the MAP kinase cascade (Macdonald et al., 2003). However, if NS5A interferes with the EGF-induced stimulation of the MAP kinase cascade and does not affect the PMA-induced stimulation of this same cascade, NS5A must interfere with an

early step in the MAP kinase cascade (downstream of EGF, but upstream of protein kinase C/ c-Raf1). It is commonly assumed that binding to Grb2 is causative for the interference of NS5A with the MAP kinase pathway (Tan et al., 1999). Whether or not NS5A interferes with MAP kinase cascade at the level of Grb2, the experiments presented in chapter 5.4 indicate that NS5A does not interfere with the MAP kinase pathway *at the level of c-Raf1*.

6.5.2 NS5A might alter MAP kinase-independent functions of c-Raf1

c-Raf1 is key regulator of the MAP kinase cascade c-Raf1/MEK/ERK. This module is often perceived as a linear pathway in which ERK is the effector. However, recent advances have unveiled a role of c-Raf1 outside the established MAP kinase cascade. (Hindley and Kolch, 2002). The strongest evidence that caused this change of paradigms stems from the analysis of c-Raf1-deficient mice which die early during embryogenesis. Surprisingly, the lethal phenotype of c-Raf1-deficient mice is reverted by reintroduction of a c-Raf1 mutant that fails to trigger signaling via the MAP kinase cascade (Huser et al., 2001). This argues that initiation of the MAP kinase cascade is not constitutive for c-Raf1 function and it implies that there are other targets of c-Raf1 that are not identified by now. Since c-Raf1 is a pleiotropic molecule itself, it is conceivable that NS5A modulates c-Raf1 function with respect to other targets than MEK.

One major aspect of c-Raf1 function that seems to be independent of MAP kinase activation is the role of c-Raf1 in apoptosis regulation. However, the underlying mechanism is poorly understood and alternative c-Raf1 substrates (other the MEK) are scarce. Given the fact that NS5A acts as an anti-apoptotic factor, it might be fruitful to analyze whether the anti-apoptotic potential of NS5A is explainable in terms of the NS5A/c-Raf1 interaction.

6.5.3 NS5A does not activate NF-κB signaling

An alternative strategy to determine the outcome of the NS5A/c-Raf1 interaction on the level of host cell signal transduction was the analysis of pathways that have been shown to be activated by NS5A. As mentioned above, the most prominent target of NS5A that might be attributable to the interaction with c-Raf1 (apart from the MAP kinase cascade) is the NF-κB pathway. Therefore, it was analyzed whether NS5A affects the basal transcriptional activation of NF-κB and STAT-3. STAT-3 was included because it has been suggested that the NS5A-mediated

activation of NF-κB and STAT-3 rely on a common mechanism (Gong et al., 2001). As seen in Fig. 5.23, NS5A did not induce a significant activation of NF-κB or STAT-3. Furthermore, there was no significant difference between HCV replicon cells and naïve HuH-7 cells with regard to NF-κB or STAT-3 activation (Fig. 5.25). This is in contradiction to published data (Girard et al., 2004; Gong et al., 2001) and suggests that NS5A does not activate NF-κB or STAT-3. On the other hand, HCV core protein did induce NF-κB activation in this experimental setting (data not shown), arguing that the experimental conditions were suitable for the analysis of NF-κB activation.

One explanation for the observed discrepancy lies in the experimental design. This study capitalized on a reporter assay that has only recently become available. Apart from the reporter gene whose expression is directed by the transcription factor of interest (e.g. NF-κB), it includes a reporter gene that is constitutively expressed. This allows the exclusion of artefacts that arise from differences in transfection efficiency. These differences were rather subtle in experiments in which the same cell line was transfected with different inducers (such as the experiment shown in Fig. 5.23). However, they were substantial in experiments where different cell lines were compared (Fig. 5.25). In fact, for NF-κB, the difference between HCV replicon cells and naïve HuH-7 cells amounted to 200%, ie. HCV replicon cells displayed a 3 fold reduction of NF-κB activation in comparison to naïve HuH-7 cells (data not shown). However, this difference was also seen for the constitutively active reporter gene and therefore reflects a difference in transfection efficiency rather than an authentic difference in NF-κB activation.

This interpretation is supported by experiments in which HuH-7 cells that stably express NS5A were compared to naïve HuH-7 cells with regard to NF-κB activation using the reporter assay described above (Miyasaka et al., 2003). Although NS5A expression was detected by Western blotting, there was no detectable NF-κB activation in these cells.

However, the conflicting evidence might be attributable to other factors as well: (i) NS5A expression levels might vary in different experimental designs, ie. the expression levels seen in Fig. 5.24 and Fig. 5.26 might have been insufficient to induce a sustained activation of NF-κB and STAT-3. (ii) Some effects might be cell type-specific. It seems that NS5A does not affect basal NF-κB activation in HEK293 cells (Park et al., 2002) whereas it has been shown to activate NF-κB in HuH-7 cells (Gong et al., 2001).

In summary, the experiments presented in chapter 5.4 cast a doubt on previously published data which suggest that NS5A interferes with a multitude of signaling pathways. Furthermore, the data suggest that the signaling pathways downstream of c-Raf1 that were investigated in this study are not affected by the presence of NS5A. This is most notably true for the MAP kinase pathway. However, it cannot be ruled out that MAP kinase-independent functions of c-Raf1 are altered by the interaction with NS5A.

6.6 NS5A phosphorylation by c-Raf1.

6.6.1 c-Raf1 is an NS5A kinase

Since NS5A is phosphorylated on serine and threonine residues and c-Raf1 is a serine/ threonine kinase, it was obvious to analyze whether NS5A is phosphorylated by c-Raf1. To that end, NS5A and c-Raf1 were purified by affinity chromatography and phosphorylation was reconstituted *in vitro*. Initial experiments with c-Raf1 and an inactive c-Raf1 mutant (K375W) showed that both of these were capable of phosphorylating NS5A *in vitro* (Fig. 5.29), arguing that the c-Raf1 preparations were contaminated with other kinases. Therefore, the purification protocol for c-Raf1 and the inactive c-Raf1 mutant was modified to include an additional washing step in order to remove kinases associated with c-Raf1. Using these highly-purified c-Raf1 preparations, NS5A was specifically phosphorylated by c-Raf1 and not by the inactive c-Raf1 mutant (Fig. 5.30). This indicates that c-Raf1 is an NS5A kinase. To analyze the impact of c-Raf1 on NS5A hyperphosphorylation, HCV replicon cells were incubated with the c-Raf1 inhibitor BAY43-9006. As seen in Fig. 5.31, inhibition of c-Raf1 results in a loss of hyperphosphorylation, arguing that c-Raf1 contributes to NS5A hyperphosphorylation.

Purification of GST-Raf turned out to be quite difficult. This was essentially due to the fact that under native conditions, other kinases associate with c-Raf1 (Janosch et al., 1996). To remove those kinases, an additional washing step was included as suggested by Janosch. This washing step relied on RIPA buffer that contains a mixture of different detergents (0.1% SDS, 1% triton X-100, 1% deoxycholate). It seems that this washing step was quite efficient in terms of removing contaminating kinase activity (compare Fig. 5.29 with Fig. 5.30). However, c-Raf1 kinase activity was also reduced, arguing that these buffer conditions might at least partially compromise the tertiary structure of c-Raf1. As a consequence, the phosphorylation signals were

quite weak, given the fact that purified proteins were incubated in an *in vitro* reaction. Nevertheless, phosphorylation signals were specific with the inactive c-Raf1 mutant showing no catalytical activity towards NS5A whatsoever. Therefore, these results can be interpreted such that c-Raf1 phosphorylates NS5A *in vitro*.

However, several kinases have been shown to phosphorylate NS5A *in vitro* (Coito et al., 2004; Ide et al., 1997; Kim et al., 1999). In fact, it is commonly observed that kinases are quite promiscuous *in vitro*. In contrast, this promiscuity is significantly restrained in the *in vivo* situation. Several aspects might limit phosphorylation *in vivo*: (i) Certain cofactors that increase the specificity *in vivo* might be lacking *in vitro*. (ii) The kinase and its *in vitro* substrate might be localized in distinct cellular compartments *in vivo*. (iii) Expression levels of both the kinase and its *in vitro* substrate might be very different in the *in vivo* situation. As a consequence, there might be competing substrates that prevent the kinase from phosphorylating its *in vitro* substrate *in vivo*. Hence, the question arose whether c-Raf1 has an impact on NS5A phosphorylation *in vivo*.

One obvious way to analyze NS5A phosphorylation *in vivo* is to look at NS5A hyperphosphorylation. NS5A hyperphosphorylation can be visualized by Western blotting: If hyperphosphorylation of NS5A occurs, an additional NS5A-specific band occurs in the Western blot (p58). Incubation of HCV replicon cells with the c-Raf1 inhibitor BAY43-9006 lead to a reduction in NS5A hyperphosphorylation (Fig. 5.31).

Several mechanisms could account for this observation. Of course, the most obvious interpretation of this experiment is that c-Raf1 is the kinase that induces NS5A hyperphosphorylation. However, hyperphosphorylation could be a multi-step process with certain kinases inducing basal phosphorylation and other kinases binding to basally phosphorylated serine residues in order to induce hyperphosphorylation. If this scenario holds true, c-Raf1 could also contribute by catalyzing an early phosphorylation event that paves the way for hyperphosphorylation. Alternatively, it cannot be excluded that a kinase downstream of c-Raf1 induces NS5A hyperphosphorylation. Incubation of HCV replicon cells with the MEK inhibitor PD98059 does not reduce hyperphosphorylation (data not shown), arguing that neither MEK nor ERK contribute to NS5A hyperphosphorylation *in vivo*.

One potential target downstream of c-Raf1 is casein kinase II: Casein kinase II is associated with c-Raf1 and its activation status is regulated by c-Raf1 *in vivo* (Janosch et al., 2000). At the same time, casein kinase II phosphorylates NS5A *in vitro* (Kim et al., 1999). Hence, c-Raf1 could act as

a scaffolding protein to recruit casein kinase II, thereby inducing phosphorylation of NS5A *in vivo*.

To further corroborate the impact of c-Raf1 on NS5A phosphorylation *in vivo*, several experiments should be considered – most notably the *in vivo* labelling of NS5A with [^{32}P]orthophosphate and the two-dimensional gel electrophoresis. The latter seems particularly suited because it provides information on the isolelectric point of NS5A. Given the fact that phosphorylation alters the isoelectric point of a protein, basally phosphorylated and hyperphosphorylated NS5A should be detected as distinct entities in the two-dimensional gel. Furthermore, the isolelectric focusing should clarify whether p56 and p58 represent distinct phosphoproteins or clusters of phosphoproteins.

Given the fact that kinases which phosphorylate NS5A *in vivo* have been long sought and remain largely unidentified, the identification of c-Raf1 as an NS5A kinase is of great importance. Future experiments should delineate the precise role of c-Raf1 in NS5A hyperphosphorylation.

6.6.2 NS5A hyperphosphorylation and HCV replication

On the first glance, there seems to be discrepancy with regard to the effect of BAY43-9006 on HCV replication and NS5A hyperphosphorylation: BAY43-9006 reduces both HCV replication and NS5A hyperphosphorylation. However, an inhibition of NS5A hyperphosphorylation should result in an increased replication rate. In fact, loss of hyperphosphorylation is among the most prominent adaptive mutations and has been shown to increase replication by more than 10 fold (Lohmann et al., 2003). One simple explanation for this discrepancy might be the fact that differentially adapted HCV replicons were used in this study: Whereas hyperphosphorylation was investigated in HCV replicon cells that retain the hyperphosphorylated species p58, replication was studied in an adaptive background that leads to a loss of hyperphosphorylation. In other words, it was impossible to observe any secondary effects on HCV replication that may arise from a loss of hyperphosphorylation because there was no hyperphosphorylation in these cells at the first place.

All replicon cell lines that contain autonomously-replicating HCV RNA are cell culture-adapted to some extent, ie. they accumulate characteristic cell culture-adaptive mutations. Many of these cell culture-adaptive mutations lead to a loss of NS5A hyperphosphorylation. Reintroduction of these mutations into a wild-type HCV genome leads to increased replication capacity. However,

introduction of these mutations into a cell culture-adapted background does not necessarily produce an additional increase in replication capacity but rather completely abolishes replication (Lohmann et al., 2001). This means that certain adaptive mutations are incompatible with each other, thereby causing a complete loss of HCV replication. The opposite has also been observed: Some mutations act synergistically with regard to their impact on HCV replication (Krieger et al., 2001). Likewise, it has been shown that a mutation in NS3 (E1202G) synergistically enhances the rate of replication of an HCV replicon that has lost NS5A hyperphosphorylation due to an adaptive mutation in NS5A (S2197P). This illustrates that the impact of a loss of hyperphosphorylation on replication is strongly dependent on the adaptive background.

This notion is supported by a recent study that identified small-molecule inhibitors which inhibit NS5A hyperphosphorylation (Neddermann et al., 2004). Whereas in wild-type replicons, as expected, a decrease in hyperphosphorylation stimulated HCV replication, the opposite effect was observed for adapted replicons which had lost NS5A hyperphosphorylation as a consequence of cell culture-adaptation. In these adapted replicons, incubation with kinase inhibitors that induce a loss of hyperphosphorylation strongly diminished HCV replication. This is in perfect agreement with what was observed for the c-Raf1 inhibitor BAY43-9006: Incubation with BAY43-9006 reduced NS5A hyperphosphorylation (Fig. 5.31), but it lead to a decrease in HCV replication in the adapted situation (Fig. 5.17). Therefore, it seems most promising to investigate the impact of c-Raf1 inhibition in the situation of a wild-type replicon or an adapted replicon that contains mutations that synergize with a loss of hyperphosphorylation (such as the NS3 mutation described above).

6.7 Kinase inhibitors as therapeutic agents

Protein kinases are promising targets for the treatment of human diseases. The most prominent examples of kinases inhibitors as therapeutic agents arise from the field of cancer. In fact, a number of kinases have been targeted by the means of small-molecule inhibitors in order to attenuate kinase hyperactivity in cancer. Those include receptor tyrosine kinases, non-receptor tyrosine kinases and cyclin-dependent kinases (CdKs) (Noble et al., 2004). The most prominent example of a kinase inhibitor with therapeutic potential is Gleevec, an inhibitor of the non-receptor tyrosine kinase Abl that was approved in May 2001 for therapy against chronic myeloid leukemia (CML). Crystallization of the catalytical domain of Abl in complex with Gleevec shows

that Gleevec binds to the ATP binding site of Abl and locks the molecule in its inactive conformation (Nagar et al., 2002).

MAP kinases have also been exploited as pharmacological targets. The most prominent targets include p38 and MEK1/MEK2. p38 adopts a central role in inflammation and autoimmune reactions. Hence, p38 inhibitors are promising drugs for the treatment of rheumatoid arthritis and Crohn's disease (English and Cobb, 2002; Noble et al., 2004). Second-generation inhibitors of MEK1/MEK2 have been shown to inhibit the growth of colon carcinoma cells and are currently being evaluated in Phase I clinical oncology trials (English and Cobb, 2002).

The most advanced c-Raf1 inhibitors have been developed in collaboration between Bayer and Onyx. They were based on a bis-aryl urea backbone that was modified using a combinatorial chemistry approach. The most potent compound was BAY43-9006 which acts as a competitive inhibitor of c-Raf1. Preclinical data indicated that BAY43-9006 significantly inhibited tumor growth in a mouse xenograft model (Lyons et al., 2001). Phase I clinical studies documented the low toxicity of the compound. BAY43-9006 is currently evaluated in Phase II clinical trials for the treatment of hepatocellular cancer and colorectal cancer (Lee and McCubrey, 2003).

Small-molecule inhibitors that target cellular kinases have essentially not been exploited as antiviral drugs. There are some preclinical data on the use of p38 and CdK inhibitors in HIV-1 infection (de la Fuente et al., 2003; Muthumani et al., 2004). However, the use of kinase inhibitors is limited by several aspects: (i) Many kinase inhibitors that are currently available show a low degree of specificity, ie. they inhibit several kinases and not just the one they nominally target. (ii) Many kinase inhibitors display a certain level of cytotoxicity, leading to a number of side-effects, in particular in long-term therapies. Cytotoxicity may either arise as a consequence of low specificity or as a consequence of the loss of function of the particular kinase of interest in a given tissue. (iii) Monotherapy with kinase inhibitors is likely to trigger the emergence of viral escape variants – as observed for other monotherapies as well.

Nevertheless, a combination therapy that includes low-dose kinase inhibitors may represent a fruitful approach for novel antiviral therapies. This is more so true for HCV since the current therapeutic strategies rely on broad-band antiviral compounds (interferon-α and ribavirin) rather than on molecules that specifically target HCV. The impact of BAY43-9006 on HCV replication was quite substantial given the short incubation time of 6h. However, in order to evaluate the potential of BAY43-9006 as an anti-HCV drug, it has to be evaluated how BAY43-9006 affects

viral replication in different adaptive backgrounds and – most importantly – in the wild-type situation.

6.8 Impact of c-Raf1 on HCV-associated liver pathogenesis

c-Raf1 has been identified as a novel cellular target of NS5A. Although the data presented in chapter 5.4 suggest that NS5A does not activate the transcription factors SRF, AP-1, NF-κB or STAT-3, the NS5A/c-Raf1 interaction might alter other aspects of c-Raf1-mediated signaling, most notably apoptosis regulation. This is supported by the notion that NS5A displays an anti-apoptotic potential (Ghosh et al., 2000a; Majumder et al., 2002).

Ongoing apoptosis and necrosis are typical features of the inflammatory process that accompanies the acute and the chronic phase of an HCV infection (Marcellin et al., 2002). Cell death arises from the T-cell response that targets HCV-infected hepatocytes. Therefore, it makes perfect sense that HCV has developed strategies to antagonize apoptosis and thereby prevents the host from clearing the virus. One such strategy could rely on the interaction of NS5A with a cellular factor that is capable of antagonizing apoptosis, such as c-Raf1.

However, interference with apoptosis regulation is not only important with regard to viral clearance, but also with regard to cancer development (Hanahan and Weinberg, 2000). In fact, one hallmark of cancer is resistance to apoptosis, thereby preventing the elimination of transformed cells by apoptosis. Therefore, the NS5A/c-Raf1 interaction might also contribute to the cancerous phenotype of HCV.

However, the impact of NS5A on HCV-associated liver pathogenesis is difficult to study since there are no appropriate model systems available. This may at least in part be overcome by the transgenic mouse model that was established during the course of this work. As demonstrated in chapter 5.6, these NS5A-transgenic mice display robust expression of NS5A (Fig. 5.35) and expression is liver-specific (Fig. 5.36). Future experiments should focus on the histopathological analysis of these mice. Since many features of HCV-associated liver pathogenesis do not become obvious until the infected patient ages considerably, particular focus should be laid on the analysis of aged mice (>15 months of age).

Recently, the first study on NS5A-transgenic mice was published (Majumder et al., 2003). These mice express NS5A under the control of the regulatory sequence of the human apoE gene, leading to constitutive liver-specific expression of NS5A. The NS5A-transgenic mice were

phenotypically similar to their non-transgenic littermates and do not exhibit major histological changes within the liver up to 24 months of age. This indicates that NS5A by itself might not be sufficient to induce hepatocellular carcinoma in mice. However, this is not unexpected given the fact that most of the HCV-transgenic mice published so far lack a striking phenotype (compare chapter 1.13).

The major reason for the absence of a phenotype is likely to lie in the fact that these mice constitutively express the HCV proteins of interest. As a consequence, HCV-transgenic mice are immunotolerant with regard to the otherwise foreign HCV proteins. Hence, liver inflammation which constitutes a hallmark of the HCV infection does not develop. However, liver inflammation, ie. repeated hepatocyte injury and regeneration, is one important event that precedes the development of hepatocellular carcinoma (HCC). In fact, for hepatitis B virus (HBV), it has been shown that chronic immune-mediated liver cell injury can trigger the development of HCC in absence of insertional mutagenesis or genotoxic chemicals (Nakamoto et al., 1998). This highlights the role of the immune response for the ultimate outcome of chronic HCV infection.

However, NS5A might still act as a co-carcinogen by promoting tumor growth and/or preventing the apoptosis of transformed cells. Therefore, it may be fruitful to treat NS5A-transgenic mice with a carcinogen (UV irradiation, benzpyrene, carbon tetrachloride) and analyze the frequency of cancerous lesions in the livers of these mice. If NS5A acts as tumor promoter, the lesions should occur more frequently in the livers of NS5A-transgenic mice and tumor development should be more pronounced. In fact, similar studies have been conducted for the HCV core protein and it has been shown that core promotes tumorigenesis in a situation of repeated hepatocyte injury (Kato et al., 2003).

6.9 Integrity of the c-Raf1/MEK/ERK pathway affects viruses other than HCV.

The results presented in chapter 5.3 indicate that integrity of c-Raf1 is an essential prerequisite for HCV replication. Although the underlying mechanism remains ill-defined, it seems that the integrity of the MAP kinase cascade downstream of c-Raf1 (c-Raf1/MEK/ERK) is not required since inhibition of MEK (U0126) does not affect HCV replication (Fig. 5.21).

This is surprising given the fact that there are a number of viruses that interfere with the MAP kinase pathway. Likewise, blockade of the MAP kinase cascade oftentimes disrupts the viral life cycle. Hepatitis B virus (HBV) contains two transcriptional activator proteins (LHBs and HBx) which independently activate the MAP kinase pathway. Disruption of the MAP kinase cascade by expression of dominant-negative c-Raf1 or MEK abrogates both viral replication and viral gene expression (Stockl et al., 2003), leading to a sustained decrease in the *de novo* synthesis of viral compounds.

For the human immunodeficiency virus type 1 (HIV-1), the outcome of MAP kinase inhibition is still a matter of debate. Expression of a dominant-active (plasma membrane-targeted) version of c-Raf1 leads to an increase in HIV-1 gene expression, resulting in increased virus production and secretion (Flory et al., 1998). This indicates that c-Raf1 activity is essential for viral gene expression, similar to the mechanism discussed for HBV. On the other hand, inhibition of the MAP kinase cascade has been reported to induce a loss of HIV-1 infectivity without affecting viral gene expression (Yang and Gabuzda, 1999). Loss of HIV-1 infectivity was attributed to differential modification of HIV-1 proteins by MAP kinase-induced phosphorylation. So whichever mechanism holds true, integrity of the MAP kinase cascade is a prerequisite for the formation of infectious HIV-1.

A distinct mechanism has been described for influenza A virus: MAP kinase activation seems to be essential for nuclear export of viral ribonucleoproteins (RNPs) in the late stages of the replication cycle (Pleschka et al., 2001). As a consequence, inhibition of the MAP kinase pathway leads to a loss of influenza A virus production because viral RNPs are trapped in the nucleus.

Altogether, these findings highlight the importance of the MAP kinase cascade in the life cycle of different viruses. Although the common theme seems to be the MAP kinase-induced activation of viral gene expression, there are quite remarkable examples of different viral strategies to exploit this ubiquitous pathway.

6.10 Working model and future perspectives

The experiments presented so far suggest that c-Raf1 is a cellular binding partner of NS5A. Binding of NS5A to c-Raf1 does not affect c-Raf1-mediated signal transduction. However, it affects the phosphorylation status of NS5A. Disruption of c-Raf1 impairs HCV replication,

whereas disruption of MEK leaves replication unaltered. The following schematic (Fig. 6.1) illustrates how these observations can be integrated into a consistent working model.

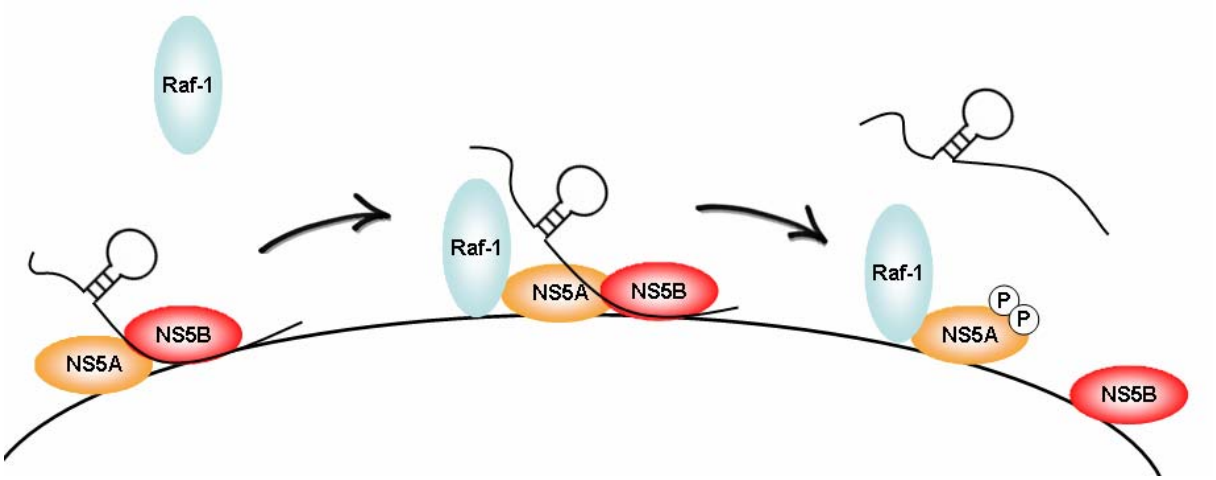

Fig. 6.1 c-Raf1 regulates HCV replication by affecting NS5A phosphorylation. c-Raf1 binds to NS5A that is physically associated with the HCV replication complex and functionally required for HCV replication. c-Raf1 phosphorylates NS5A upon binding, leading to the production of the hyperphosphorylated NS5A species. HCV replication is modulated by hyperphosphorylated NS5A.

Replication takes place in the HCV replication complex that is associated with the endoplasmatic reticulum. It requires the association of HCV non-structural proteins NS3-NS5B. c-Raf1 binding to NS5A induces NS5A phosphorylation, ultimately leading to the formation of the hyperphosphorylated NS5A species. The rate of viral replication is modulated by the presence of hyperphosphorylated NS5A. This could be achieved by hyperphosphorylated NS5A dissociating the replication complex or by differential binding of hyperphosphorylated NS5A to cellular factors as recently suggested (Evans et al., 2004).

Future experiments should focus on three topics. First of all, it should be analyzed how c-Raf1 inhibition affects HCV replication in the wild-type (non-adapted) situation. As mentioned above, this was hampered by the low replication levels of wild-type HCV genomes. However, with the advent of transient replication assays, the answer to this question seems within reach. Second, the biochemical properties of the NS5A protein should be characterized. This includes the detailed analysis of NS5A phosphorylation and the precise role of c-Raf1 in this process. Finally, with the NS5A-transgenic mice at hand, it seems promising to investigate whether NS5A contributes to HCV-associated liver pathogenesis.