# 1 Introduction

Hepatitis C virus (HCV) is among the leading causes for liver disease worldwide. Although the acute HCV infection is often mild, the majority of the patients fail to clear the virus, leading to the establishment of a chronic infection. Chronic infection is associated with a high risk of developing liver cirrhosis and hepatocellular carcinoma. Therapeutic options are still limited as is the knowledge about the molecular biology of virus, highlighting the need for basic research in the field of HCV.

## *1.1 History and classification of HCV.*

With specific diagnostics for hepatitis A and B virus becoming available in the 1970s, it became clear that most of the cases of hepatitis arising from blood transfusion were not caused by infection with these or other known agents (Feinstone et al., 1975). It was therefore referred to as non-A, non-B hepatitis. Despite considerable efforts, classical virological approaches failed to identify the agent that was responsible for non-A, non-B hepatitis – most likely due to the low concentration of viral particles in the infected patients. Finally, in 1989, HCV was identified by screening a cDNA library from an HCV-infected chimpanzee (Choo et al., 1989).

HCV is an RNA virus that belongs to the family of the *Flaviviridae* (from the Latin *flavus* = yellow). The *Flaviviridae* comprise three genera: the flaviviruses, the pestiviruses and the hepaciviruses (Knipe and Howley, 2001). The flaviviruses include the yellow-fever virus, the dengue viruses 1 and 2, the West Nile virus and the Japanese encephalitis virus. The pestiviruses comprise the classical swine fever virus and the bovine viral diarrhea virus. The hepaciviruses include the hepatitis C virus (HCV) and the GB viruses A, B and C, "GB" referring to the initials of a surgeon suffering from acute hepatitis. HCV is subdivided into 6 genotypes and more than 50 subtypes. The genotypes differ by as much as 30-50% in their nucleotide sequence, whereas the subtypes differ by 10-30% (Hoofnagle, 2002).

## *1.2 Epidemiology and natural history of hepatitis C.*

HCV is a global health problem with an estimate of 170 Mio infections worldwide (Poynard et al., 2003). Unlike many other infectious diseases, HCV also affects the so-called developed

countries. This is reflected in the high prevalence of HCV in the USA (1.8% or 3.9 Mio HCV carriers), in Japan (2% or 2 Mio HCV carriers) or in Germany (0.5% or 400 000 HCV carriers) (Higuchi et al., 2002; Kim, 2002; Robert-Koch-Institut, 2004).

Acute HCV infection is often mild or inapparent. As a consequence, patients are usually unaware of the acute HCV infection. However, the majority of the patients fail to clear the virus, leading to chronic HCV infection in 54% to 86% of the cases (Seeff, 2002). Chronic HCV carriers show variable degrees of hepatocellular necrosis and inflammation (referred to as activity of the disease) and fibrosis (referred to as stage of disease). While the activity of inflammation may fluctuate over time, the stage of fibrosis is believed to be progressive and largely irreversible (Marcellin et al., 2002). Fibrosis is characterized by the accumulation of connective tissue in the liver. It is a dynamic process that involves the synthesis and deposition of collagen and other extracellular matrix proteins and their organization in complex polymers which are insoluble and induce a loss of the liver architecture and function. Fibrosis is categorized in four stages with the final stage being cirrhosis. At least 20% of the chronically infected adults develop cirrhosis within 20 years (Seeff, 2002). Patients with cirrhosis may remain clinically stable with good survival rates (compensated cirrhosis). However, if clinical symptoms of cirrhosis become apparent (decompensated cirrhosis), survival rates dramatically decrease (50% mortality within 5 years) (Wright, 2002). Furthermore, cirrhotic patients have a high risk of developing hepatocellular carcinoma (HCC) (annual rate of 1-4%) (El-Serag, 2002). In fact, HCV accounts for a significant portion of all HCC cases, with the numbers ranging from 27% in the USA to 90% in Japan (El-Serag, 2002). Unlike hepatitis B virus, hepatitis C virus does not cause hepatocellular carcinoma (HCC) in the absence of cirrhosis (El-Serag, 2002). In summary, the natural history of an HCV infection is characterized by the absence of symptoms during the acute phase and by the eventual development of cirrhosis or HCC during the chronic phase of the infection.

## *1.3 Immune response.*

As mentioned above, disease progression is characterized by inflammation and fibrosis. HCV itself is a non-cytopathic virus (Hoofnagle, 2002). Liver damage arises mostly as a consequence of the cellular immune response. The extent of liver damage can be assayed by measuring serum levels of alanine aminotransferase (ALT), a liver-specific enzyme that is released to the blood stream as soon as the integrity of the liver is compromised (Fontana and Lok, 2002).

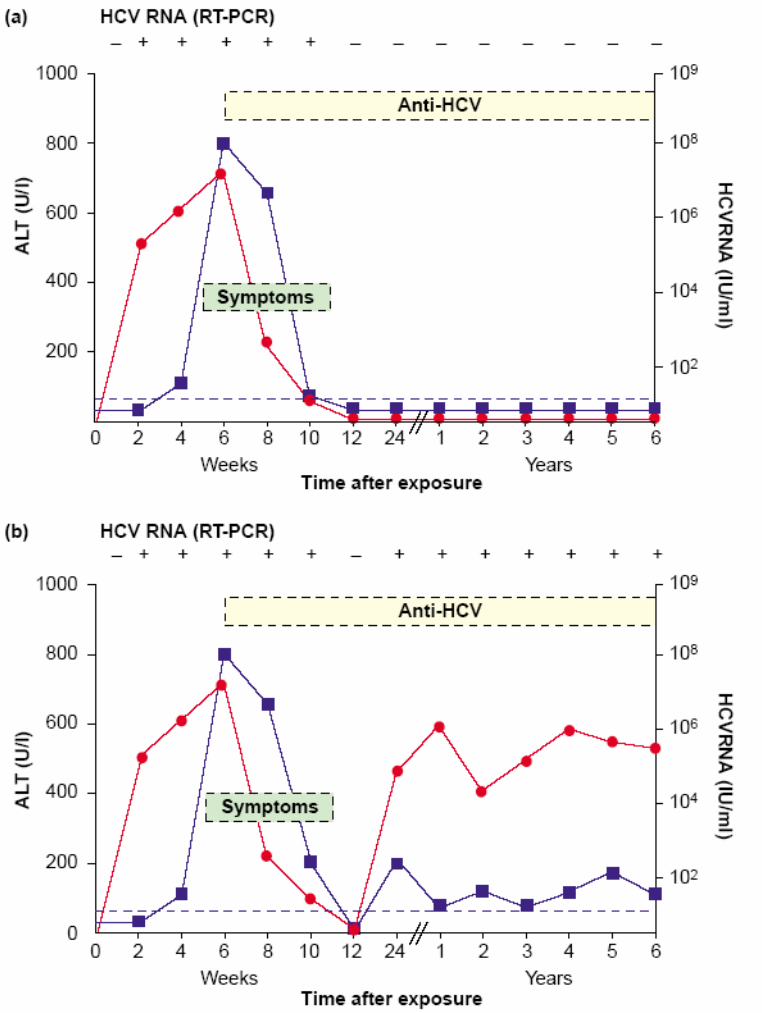


**Fig. 1.1 Typical outcome of an HCV infection.** (a) Course of an acute, resolving HCV infection. HCV RNA (red) is detected in the serum within 2 weeks. ALT levels (blue) rise 2-8 weeks after exposure. As HCV levels decline, symptoms usually subside and HCV-specific antibodies become detectable. (b) Course of an acute, chronically evolving HCV infection. During the evolution of an acute to chronic infection, HCV RNA and ALT levels fluctuate markedly. Reprinted from (Racanelli and Rehermann, 2003).

As seen in Fig. 1.1, HCV RNA is detected in the serum as early as 2 weeks post exposure. ALT levels start rising shortly thereafter. (Hoofnagle, 2002). If the patient resolves the infection, both HCV RNA and ALT levels drop to normal within several weeks. If a chronic infection is established, ALT levels fluctuate markedly as does the viral titre (Hoofnagle, 2002).

Due to the lack of appropriate infection models, the immune response to HCV is still poorly understood. Most of the data available today are derived from experimentally infected

chimpanzees. These experiments suggest that the first line of defense is the T-cell response that is triggered as early as 10-14 days post infection (Racanelli and Rehermann, 2003). Activated HCV-specific T-cells enter the liver through the portal vein. In the liver, contact between T-cells and liver-specific antigen-presenting cells triggers the stimulation of the T-cells. Stimulated T-cells recognize MHC-I-HCV peptide complexes on the surface of infected hepatocytes and respond with cytotoxic effector functions. As a consequence, hepatocytes are killed and release liver-specific enzymes to the blood stream. This is reflected in an elevation in serum ALT levels that coincides with the T-cell response. Antibodies to HCV are detected later during the infection (7-8 weeks). In the absence of an infection model, it is difficult to state whether there are neutralizing antibodies to HCV or not. However, antibodies do seem to adopt a protective role in terms of preventing infection with HCV (Racanelli and Rehermann, 2003).

Establishment of a persistent HCV infection means failure of the immune system to clear the virus. Whereas the majority of the patients fail to clear the virus initially, some patients do resolve the infection even in the absence of therapy. There is accumulating evidence from chimpanzee experiments that suggests that the T-cell response essentially determines the outcome of an acute HCV infection: If there is a robust T-cell response, the patient is likely to resolve the infection. If there is a moderate or weak T-cell response, the infection is likely to progress to chronicity. The viral load is highly (inversely) correlated with the appearance of $CD8^+$ T-cell responses in the liver (Racanelli and Rehermann, 2003). Likewise, vigorous and multispecific $CD4^+$ and $CD8^+$ T-cell responses in the blood of acutely infected patients are associated with recovery (Racanelli and Rehermann, 2003).

To conclude, in the majority of the HCV-infected patients, the immune system fails to clear the virus. Failure to clear the virus is highly correlated with the absence of a sustained T-cell response.

HCV has developed several mechanisms to evade the immune response of the host. (i) HCV replication during the acute phase of the infection is extremely rapid with $10^{10}$-$10^{12}$ virions being produced per day (Racanelli and Rehermann, 2003). Consequently, it has been suggested that HCV outpaces the immune response because maximum viral titres are reached several weeks before humoral or cellular immune responses are detectable. (ii) Since the HCV polymerase lacks a proofreading capacity, HCV persists as a set of quasispecies (Racanelli and Rehermann, 2003). As a result, the host is oftentimes unable to eliminate HCV completely, allowing for the emergence of viral escape variants. (iii) HCV is a non-cytopathic virus that does not infect

antigen-presenting cells. If the infection becomes chronic, HCV replicates at very low levels, thereby limiting the availability of exogenous antigens that could be taken up by antigen-presenting cells. (iv) One important cytokine that limits virus propagation in an infected cell is interferon-α. It is produced in response to double-stranded RNA and it acts by activating/inducing several effector proteins such as protein kinase R (PKR), Mx and 2'-5'-oligoadenylate synthase. Several HCV proteins target this mechanism: Whereas NS3 blocks the phosphorylation of the interferon regulatory factor 3 (IRF3) (Foy et al., 2003), E2 and NS5A interfere with PKR directly (Gale et al., 1998; Taylor et al., 1999).

## *1.4 Transmission, diagnosis and therapy.*

HCV is transmitted by percutaneous or permucosal exposure to infectious blood (Alter, 2002). The incidence of HCV has sharply declined after the discovery of HCV in 1989 (Choo et al., 1989) and the introduction of blood screening measures for HCV in 1992 (Racanelli and Rehermann, 2003). Nowadays, the incidence amounts to 35000 HCV infections per year in the USA (NIH, 2002). The majority (68%) of the newly acquired cases of HCV are related to injecting drug use. A minor percentage relates to sexual activity (18%) or occupational hazards (4%). However, sexual transmission seems to be quite inefficient. This is reflected in a very low rate of transmission (0-0.6% per year) in long-term monogamous relationships where one partner is chronically infected with HCV (Terrault, 2002).

As the acute HCV infection is rarely recognized as such, it is usually diagnosed during routine examinations (Racanelli and Rehermann, 2003). Diagnosis is based on the detection of HCV RNA and/or HCV-specific antibodies in the blood (Pawlotsky, 2002). Other markers that are used for the management of HCV patients include the viral genotype and the HCV core antigen. HCV RNA is detected by quantitative reverse-transcription polymerase chain reaction (RT-PCR). The most sensitive assays that are currently available have a cutoff of 30 IU/ml, ie. they detect HCV in concentrations as low as 30 IU/ml (Pawlotsky, 2002). Antibodies to HCV are detected by enzyme immunoassays (EIA). Whereas HCV RNA is detected as early as 1-2 weeks after exposure, antibodies are detectable 7-8 weeks after the onset of infection (Pawlotsky, 2002).

The current therapy of choice is the combination therapy with pegylated interferon-α and ribavirin (Di Bisceglie and Hoofnagle, 2002). Both of these are broad-band antiviral compounds in that they do not target HCV specifically. Interferon-α is a cytokine that triggers an antiviral

state in the host cell (see above). Ribavirin is a nucleoside analogue that was among the first antiviral compounds to be discovered. However, its mechanism of action is still poorly understood. At least four mechanisms have been proposed to account for the antiviral effect of ribavirin: (i) interference of ribavirin with the T-cell phenotype (ii) inhibition of inosine monophosphate dehydrogenase (IMPDH) (iii) inhibition of the HCV polymerase NS5B (iv) induction of RNA mutagenesis leading to "error catastrophe" (Lau et al., 2002).

The combination therapy with pegylated interferon-α and ribavirin is quite inefficient. This is particularly true for genotype 1, where only 40-45% of the patients respond to therapy (Di Bisceglie and Hoofnagle, 2002). For genotype 2 and 3, response rates are higher (80%) (Di Bisceglie and Hoofnagle, 2002). Furthermore, patients under treatment suffer from severe side-effects, such as fatigue, influenza-like symptoms, hematologic abnormalities and neuropsychiatric symptoms (Fried, 2002). As a consequence, many patients do not comply with the therapeutic regimen or withdraw from therapy completely.

## *1.5 Genome organization and protein biosynthesis*

HCV is a single-stranded RNA virus of positive orientation. Hence, the viral RNA can directly serve as a template for translation. The RNA comprises ~9600 nucleotides that carry one large open reading frame (ORF) (Bartenschlager and Lohmann, 2000). This ORF encodes a polyprotein that is proteolytically cleaved to produce the structural and non-structural proteins of HCV. In addition, an alternate ORF is generated by ribosomal frameshifting, leading to the production of the F protein (F for frameshifting) (Xu et al., 2001). Translation is directed via a ~340 nucleotide long 5' non-translated region (NTR) that functions as an internal ribosome entry site (IRES) (Tsukiyama-Kohara et al., 1992). The 3' NTR has a tripartite structure that includes a variable sequence following the stop codon, a poly(U) tract of heterogenous length and a highly-conserved 98 nucleotide sequence that is essential for replication (Yanagi et al., 1999).

The HCV virion is poorly characterized. The viral RNA is contained in a protein shell that is referred to as the viral capsid. The capsid is formed by homooligomerization of the monomeric core protein (see below). It displays icosahedral symmetry. The capsid is surrounded by a lipid bilayer that contains the viral glycoproteins E1 and E2. It is referred to as the viral envelope.

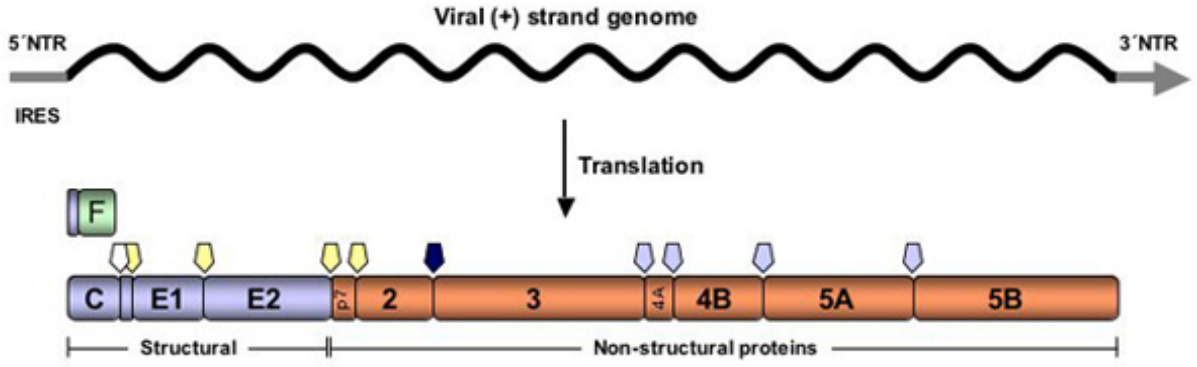


**Fig. 1.2 HCV translation and proteolytic processing.** The viral (+) strand genome is translated to yield a polyprotein of 3010 amino acids and the F-protein (produced by ribosomal frameshifting). The polyprotein is processed by host cell proteases (white and yellow) and viral proteases NS2/3 (dark blue) and NS3/4A (light blue). Reprinted from http://www.molecular-viology.uni-hd.de.

Translation of the viral RNA produces one large polyprotein that is co- and posttranslationally cleaved to yield at least three structural and seven non-structural proteins (Fig. 1.2). Cleavage at the N-terminus is catalyzed by host cell proteases (white and yellow), whereas cleavage at the C-terminus is performed by the viral proteases NS2 and NS3 (dark and light blue). The core protein is necessary and sufficient to build the viral capsid (Baumert et al., 1998). E1 and E2 are glycoproteins that are integrated into the viral envelope (Dubuisson et al., 1994). They form non-covalently linked heterodimers (Bartenschlager and Lohmann, 2000) that are likely to mediate interaction of the virus with its receptor. p7 forms an ion channel of unknown function (Pavlovic et al., 2003). NS2 is a metalloprotease that – together with NS3 – catalyzes the cleavage between NS2 and NS3 (Grakoui et al., 1993b). It has therefore been termed autoprotease. NS3, NS4A, NS4B, NS5A and NS5B form the viral replication complex (Bartenschlager and Lohmann, 2000). NS3 is a bifunctional protein that harbours a serine protease activity at its N-terminus and a helicase activity at its C-terminus (Grakoui et al., 1993a). The serine protease activity is essential for posttranslational processing of the viral polyprotein downstream of NS3. The helicase activity of NS3 mediates RNA unwinding during viral replication. NS4A acts as a cofactor for the NS3 protease. The function of NS4B is unknown although recent data suggest that NS4B nucleates the replication complex (Elazar et al., 2004). NS5A is a pleiotropic protein that modulates signal transduction of the host cell (see below) (Macdonald and Harris, 2004). NS5B is the RNA-

dependent RNA polymerase (Behrens et al., 1996). The function of the F-protein that is encoded by an alternate reading frame is not known (Basu et al., 2004).

## *1.6 Viral life cycle.*

Knowledge about the viral life cycle is very limited. This is mainly due to the fact that until today, infection of cultured cells with HCV is not possible (Pietschmann and Bartenschlager, 2003). Furthermore, there is no convenient small-animal model available either. As a consequence, the putative life cycle presented below is mainly based on the assumption that HCV behaves like other viruses of the family of the *Flaviviridae*.

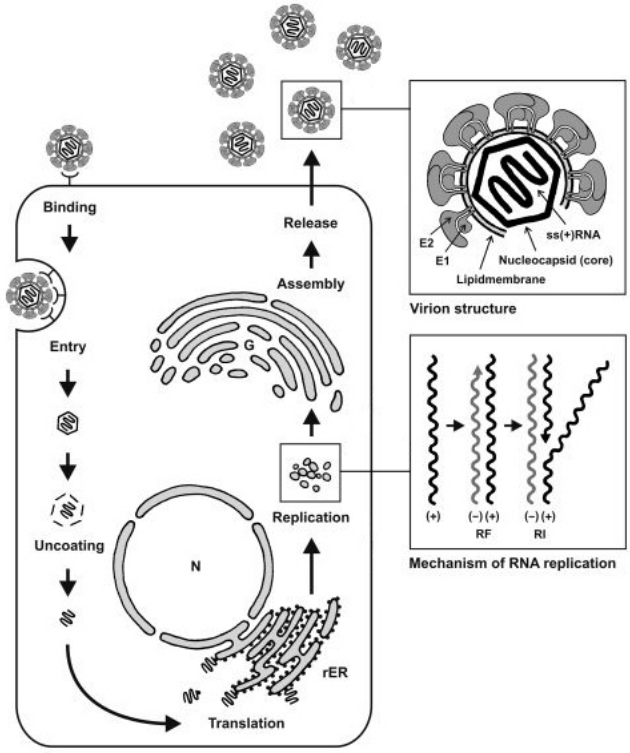


**Fig. 1.3 The putative life cycle of HCV.** HCV binds to its receptor on the surface of the target cell. The virus/receptor complex is internalized by endocytosis. The virus leaves the endosome by membrane fusion. The viral capsid is disintegrated in the cytosol, releasing the viral RNA. The viral RNA is translated at the rough ER to produce the viral proteins. Concomitantly, the viral (+)-strand RNA is replicated by the viral polymerase NS5B. Replication involves a (-)-strand RNA intermediate. Once the viral proteins have been produced and the viral RNA has been replicated, the virus assembles and is released via exocytosis. Reprinted from http://www.molecular-viology.uni-hd.de.

According to the model presented in Fig. 1.3, HCV binds to its receptor (or receptor complex) on the surface of the target cell. HCV primarily infects liver cells, but HCV has also been detected in peripheral blood mononuclear cells (PBMCs) (Bartenschlager and Lohmann, 2000). Upon binding, the virus/receptor complex is internalized via endocytosis. The virus leaves the endosome by membrane fusion. The viral capsid is disassembled in the cytosol, thereby releasing the viral RNA. The viral RNA is translated at the rough ER, producing the viral polyprotein that is co- and posttranslationally processed by host cell and viral proteases (see above). Meanwhile, RNA replication occurs at the endoplasmatic reticulum. It relies on the RNA-dependent RNA polymerase NS5B that is encoded by the virus and it involves a (-)-strand RNA-intermediate. Once the viral proteins have been produced and RNA replication is completed, the virion is assembled and released via exocytosis.

## *1.7 Model systems for the analysis of HCV.*

Two aspects of the viral life cycle are covered by surrogate model systems that have recently become available: Generation of infectious retroviruses that display functional HCV glycoproteins E1 and E2 on the surface (HCV pseudo-particles) allows the analysis of the early steps of infection such as binding and entry (Bartosch et al., 2003a). Establishment of HCV replicons, ie. minimal autonomously-replicating HCV RNAs, provided the means to investigate viral replication (Lohmann et al., 1999).

Although the HCV pseudo-particle system has only recently been established, it has already shed some light on the early steps of HCV infection. Most importantly, it has been used to characterize putative receptors for HCV. Several molecules have been postulated as potential receptor candidates, such as the tetraspanin CD81 (Pileri et al., 1998), the scavenger-receptor class B type I (SR-B1) (Scarselli et al., 2002), the LDL receptor (Monazahian et al., 1999) and L-SIGN/ DC-SIGN (Lozach et al., 2003). However, none of these candidate molecules is sufficient to restore infectivity of HCV pseudo-particles in non-permissive cells (Bartosch et al., 2003b). Nevertheless, infection with HCV pseudo-particles requires a set of coreceptors that include both CD81 and SR-B1 (Bartosch et al., 2003b). Furthermore, infection with HCV pseudo-particles requires the acidification of the endosome, arguing for a classical entry pathway of HCV (endocytosis, acidification of the endosome, membrane fusion) (Bartosch et al., 2003b).

The development of the HCV replicon model has had a profound impact on understanding HCV replication. Since this model system was also used during the course of this work, it will be described in more detail. HCV replicons are partially deleted HCV genomes that are capable of autonomous replication.

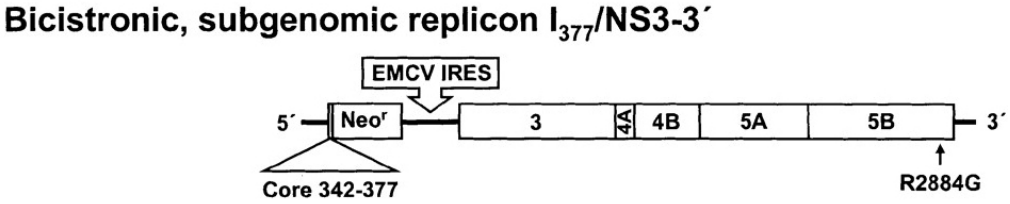


**Fig. 1.4 Structure of the minimal HCV replicon.** The HCV genome was partially deleted and modified to include the neomycin resistance gene as a selection marker. The expression of the neomycin resistance gene is directed by the HCV IRES. HCV gene expression (NS3-NS5B) is driven by the EMCV IRES. Reprinted from (Frese et al., 2003).

The minimal replicon contains the non-structural proteins NS3 to NS5B (Fig. 1.4). Furthermore, it harbours the neomycin resistance gene as a selection marker. Expression of the neomycin resistance gene is directed by the HCV IRES, whereas HCV gene expression is driven by the encephalomyocarditis virus (EMCV) IRES. HCV replicons are transcribed *in vitro*. HuH-7 cells are transfected with the resulting RNA by the means of electroporation. Cells that support HCV replication grow under neomycin selection. HCV replication can be monitored both at the RNA level (Northern blot) or the protein level (Western blot) (Lohmann et al., 1999).

Replication of wild-type HCV replicons in HuH-7 cells is rather poor. However, HCV replicons adapt to the cell culture conditions by acquiring mutations (adaptive mutations). Reintroduction of these mutations into the wild-type genome increases their replication capacity by up to 500-fold (Lohmann et al., 2001). Adaptive mutations are found in almost every non-structural protein, but not in the 5' or 3' non-translated regions. Interestingly, many adaptive mutations affect the phosphorylation state of NS5A (see below) (Blight et al., 2000). Combination of several cell culture-adaptive mutations may either produce a synergistic effect on replication or abrogate replication completely (Lohmann et al., 2003). This argues for the viral proteins being organized highly-structured complex. Introduction of cell culture-adaptive mutations does not only target

the interaction of the virus with its cellular environment, but is likely to affect interactions of viral proteins with each other at the same time.

## *1.8 Role of NS5A for viral replication.*

NS5A is a phosphoprotein of 447 amino acids that migrates as a double-band in an SDS-PAGE (Kaneko et al., 1994). The two NS5A species are referred to as p56 and p58 according to their apparent molecular weight. Both p56 and p58 are phosphorylated proteins. Since p58 disappears after phosphatase digestion, p58 has been named the hyperphosphorylated species, whereas p56 represents the hypophosphorylated or basally phosphorylated variant of NS5A (Kaneko et al., 1994). Whereas hypophosphorylated p56 is always present, hyperphosphorylation only occurs if NS5A is expressed in the context of the HCV polyprotein. The precise requirements for hyperphosphorylation differ between different HCV isolates. The minimal prerequisite is the coexpression of NS4A *in trans* (Tanji et al., 1995), as reported for a Japanese HCV isolate. However, for the HCV BK strain, coexpression of NS3, NS4A, NS4B and NS5A on the same polyprotein (*in cis*) is required to obtain hyperphosphorylated p58 (Neddermann et al., 1999). Although the differential sensitivity of p56 and p58 to phosphatase treatment suggests that p56 and p58 are differentially phosphorylated, it is unknown where phosphorylation of p58 occurs. Mutation of certain serine residues in the central region of NS5A (S2197, S2201, S2204[1]) leads to a loss of NS5A hyperphosphorylation (Tanji et al., 1995). However, this does not mean that these residues are the targets for p58 phosphorylation. The mutated NS5A proteins could simply be unable to bind the kinase that induces hyperphosphorylation.

Why is NS5A hyperphosphorylation so interesting? As mentioned above, most of the cell culture-adaptive mutations lead to a reduction or loss of NS5A hyperphosphorylation in HCV replicons (Blight et al., 2000). This indicates that NS5A hyperphosphorylation negatively affects HCV replication. The positive impact of a loss of NS5A hyperphosphorylation was confirmed by reintroducing adaptive mutations into wild-type (non-adapted) HCV replicons (Lohmann et al., 2001). Interestingly, NS5A hyperphosphorylation is also affected by adaptive mutations outside of NS5A, such as the K1846T mutation in NS4B (Evans et al., 2004). This supports the idea of all HCV proteins being organized in a highly-structured complex where mutation of one protein

[1] Amino acid numbering refers to the complete HCV polyprotein of 3010 amino acids.

is likely to affect the whole complex. Recent experiments confirm the impact of NS5A hyperphosphorylation on HCV replication by using small-molecule inhibitors that affect NS5A hyperphosphorylation (Neddermann et al., 2004). Incubation of HuH-7 cells with these inhibitors leads to a strong increase in replication capacity of wild-type (ie. non-adapted) HCV replicons. However, the mechanistic connection between NS5A hyperphosphorylation and HCV replication remains poorly understood. It has been suggested that hyperphosphorylation of NS5A disrupts binding to a cellular factor (hVAP-A) (Evans et al., 2004) that is required for replication (Gao et al., 2004). However, NS5A binding to hVAP-A is not conserved among all HCV genotypes.

Since NS5A phosphorylation is highly relevant for HCV biology, the search for kinases that phosphorylate NS5A has been a major focus of NS5A research. Several kinases phosphorylate NS5A *in vitro*. Those include protein kinase A (Ide et al., 1997) and casein kinase II (Kim et al., 1999). In addition, a number of candidates have been identified by global screening of yeast-derived kinases capable of phosphorylating NS5A (Coito et al., 2004). Experiments with small-molecule inhibitors of cellular kinases suggest that the NS5A kinase belongs to the CMGC family that includes cyclin-dependent kinases, MAP kinases, GSK3 and casein kinase II (Reed et al., 1997). However, it remains to be demonstrated whether any of these kinases phosphorylate NS5A in a hepatocyte-based setting and whether that affects NS5A function.

## *1.9 NS5A localization and structure.*

NS5A is associated with the endoplasmatic reticulum (ER) (Gosert et al., 2003) where HCV replication takes place. Association of NS5A with the ER membrane is mediated by an amphipathic α-helix that is formed by the amino terminus of NS5A (amino acids 1-30) (Brass et al., 2002). Deletion of this amphipathic α-helix results in nuclear translocation of NS5A. This is mediated by the nuclear localization signal that is present at the C-terminus of NS5A (Ide et al., 1996). However, under physiological conditions, NS5A is not found in the nucleus.

Structural information on NS5A is very limited since no crystal structure of NS5A has been solved yet. Recently, the structure of the membrane anchor domain of NS5A was determined by NMR analysis (Penin et al., 2004). It confirms the previous assumption that this region forms an amphipathic α-helix. Furthermore, the domain organization of NS5A has been analyzed by limited protease digestion (Tellinghuisen et al., 2004). According to these experiments, NS5A can

be divided in three domains (domain I: amino acids 1-213, domain II: amino acids 250-342 and domain III: amino acids 356-447) that are connected by loop regions. The tertiary structure of NS5A is stabilized by four highly conserved cysteine residues in domain I that coordinate one $Zn^{2+}$ ion.

## *1.10 NS5A modulates host cell signal transduction.*

Apart from its role in viral replication, NS5A has been shown to modulate a number of cellular signal transduction pathways (Macdonald and Harris, 2004). Interference with these signal transduction cascades can be categorized according to the potential outcome: (i) NS5A modulates signal transduction pathways that regulate cell growth, cell proliferation and cell survival. (ii) NS5A has an anti-apoptotic potential, ie. it interferes with signal transduction pathways that regulate apoptosis. (iii) NS5A determines the cellular response to the cytokine interferon-α.

The latter has been discovered by clinicians in the mid-1990s who attempted to define sequence elements in the HCV genome that determine the outcome of the interferon-α monotherapy. As mentioned above, many patients fail to respond to interferon-α, arguing that the virus has developed effective strategies to antagonize the interferon response. Comparing the HCV genomes of interferon responders to those of non-responders, a sequence in the central region of NS5A was identified that significantly differed between those two groups of patients (Enomoto et al., 1995). This sequence was therefore named the interferon sensitivity determining region (ISDR). The identification of the ISDR implied that NS5A is the major factor that determines the response of an infected patient to interferon-α. However, later studies showed that NS5A is not the only factor that determines the outcome of an interferon therapy (Reyes, 2002), thereby limiting the use of the ISDR as a prognostic marker. On the molecular level, the interference of NS5A with the interferon response was pinpointed to the interaction with a central effector protein of interferon, namely protein kinase R (PKR) (Gale et al., 1997). PKR is activated by interferon and double-stranded RNA (that functions as a marker for viral replication). Activated PKR phosphorylates the eukaryotic initiation factor 2 (eIF2) that is no longer capable of initiating translation when phosphorylated. This results in the inhibition of general cellular protein synthesis and hence virus replication (Tan and Katze, 2001). NS5A binds to PKR and prevents phosphorylation of eIF2 (Gale et al., 1998). Interestingly, the PKR binding domain corresponds

to the ISDR, lending support to the idea that binding to PKR is causative for the NS5A-mediated antagonism of the interferon response.

Apart from its role in the regulation of the interferon response, NS5A stimulates signal transduction pathways that regulate cell growth, cell proliferation and cell survival. NS5A activates the transcription factors STAT-3 and NF-κB (Gong et al., 2001). Activation of STAT-3 and NF-κB is abrogated in the presence of reducing agents (PDTC and NAC) and calcium chelators, indicating that the NS5A-induced activation of STAT-3 and NF-κB is dependent on reactive oxygen species and intracellular calcium. Microarray analysis of cells expressing NS5A suggests that many genes that are upregulated by NS5A contain NF-κB binding sites within their promoter region (Girard et al., 2004). This argues that the NS5A-mediated NF-κB activation results in an altered gene expression profile in NS5A-producing cells. However, there is conflicting evidence suggesting that NS5A does not activate NF-κB (Miyasaka et al., 2003). Park and coworkers even observed the opposite effect, stating that NS5A inhibits the tumor necrosis factor (TNF)-α-induced activation of NF-κB (Park et al., 2002). Therefore, the role of NS5A in NF-κB signaling is still a matter of debate.

Another pathway that is modulated by NS5A is the mitogenic signaling cascade that is initiated by the epidermal growth factor (EGF). EGF binding to its receptor induces dimerization of the receptor. The dimerized receptor transduces the signal via an adaptor protein (Grb2) and a guanine nucleotide exchange factor (Sos) to Ras. Ras is a small G-protein that activates a number of cellular factors by recruiting them to the plasma membrane. Among those is the kinase c-Raf1 that triggers the kinase cascade c-Raf1/MEK/ERK. ERK in turn activates several transcription factors including the serum response factor (SRF) and the activating protein-1 (AP-1). The role of NS5A in mitogenic signaling is still a matter of debate. Some studies suggest that NS5A activates the transcription factor AP-1 (Polyak et al., 2001; Qadri et al., 2004), claiming that NS5A induces oxidative stress and thereby affects both p38 and c-Jun N-terminal kinase (JNK) activation. Others state that NS5A inhibits mitogenic signaling initiated by EGF (Tan et al., 1999). As a consequence of that, activation of ERK and downstream transcription factors (SRF, AP-1) is largely impaired (Macdonald et al., 2003). Several lines of evidence suggest that NS5A interferes with an early step in the mitogenic signaling cascade. Most importantly, NS5A binds to Grb2 and binding involves a highly-conserved PXXP motif on behalf of NS5A and an SH3 domain on behalf of Grb2 (Tan et al., 1999), lending support to the hypothesis that NS5A perturbs mitogenic signaling by sequestrating Grb2.

However, it seems that the proline-rich region in NS5A can also bind to other SH3 domains, most notably several members of the Src family of tyrosine kinases (Macdonald et al., 2004) and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (He et al., 2002). Interaction of NS5A with PI3K triggers an activation of PI3K, leading to an increased activation of Akt/protein kinase B (Street et al., 2004). Akt is a kinase that is critically involved in apoptosis regulation. Therefore, the NS5A-mediated activation of Akt could provide a signal to prevent apoptosis of HCV-infected cells.

In fact, NS5A has been shown to confer resistance to apoptosis. At least two more mechanisms could account for the anti-apoptotic potential of NS5A. First, it was suggested that NS5A physically interacts with p53 (Lan et al., 2002; Majumder et al., 2001). p53 is a central regulator of apoptosis that is activated by stress signals such as DNA damage or hypoxia (Vousden and Lu, 2002). It acts as a transcription factor that induces expression of genes that promote apoptosis and induce cell cycle arrest. NS5A directly interacts with p53 and thereby sequestrates p53 at the endoplasmatic reticulum (Majumder et al., 2001). As a consequence, p53 cannot translocate to the nucleus and p53-mediated apoptosis induction is strongly diminished.

Second, NS5A protects against TNF-α mediated apoptosis (Ghosh et al., 2000a; Majumder et al., 2002). It has been suggested that NS5A binds to the TNF receptor associated death domain protein (TRADD) (Majumder et al., 2002), but there is no evidence that suggests that NS5A binding to TRADD is causative for apoptosis inhibition.

In summary, NS5A clearly displays an inhibitory effect on apoptosis. However, conclusive evidence that clarifies the underlying mechanisms is scarce.

## *1.11 Cellular interaction partners of NS5A.*

Since NS5A does not possess any known catalytical activity itself, it is likely to exert its effect via protein-protein interaction. Many proteins have been shown to interact with NS5A (Macdonald and Harris, 2004). However, few of these interactions have been reproducible in liver-based systems and even fewer provide an impact on understanding the function of NS5A.

The cellular binding partners of NS5A include transcription factors (SRCAP, PTX1, p53, TBP), regulatory proteins (PKR, Grb2, p85 phosphatidylinositol 3-kinase, TRAF2, TRADD, CdK1) and proteins involved in intracellular membrane reorganization (amphiphysin II, hVAP-A) (Arima et al., 2001; Gale et al., 1998; Ghosh et al., 2003; Ghosh et al., 2000b; He et al., 2002;

Majumder et al., 2001; Majumder et al., 2002; Park et al., 2002; Qadri et al., 2002; Tan et al., 1999; Tu et al., 1999; Zech et al., 2003). It is difficult to validate how individual binding partners contribute to the function of NS5A as long as the function of NS5A remains ill-defined.

The function of individual HCV proteins can only be assessed by appropriate model systems: With the advent of the HCV replicon model, it became possible to study the function of NS5A during HCV replication. On the contrary, many aspects of the viral life cycle are not amenable to analysis due to the lack of appropriate model systems. This is particularly true for the NS5A-mediated deregulation of cellular signaling: Although the alteration of cellular signaling is likely to contribute to HCV-associated liver pathogenesis (most notably the development of hepatocellular carcinoma), it is virtually impossible to substantiate this hypothesis with the model systems that are currently available.

As a consequence, the only function of NS5A that is well documented is its role in replication: HCV does not replicate if the NS5A coding sequence is deleted or mutated at critical positions. The only cellular binding partner that has been shown to affect HCV replication is hVAP-A (Evans et al., 2004; Gao et al., 2004). hVAP-A is a membrane-associated protein that interacts with both NS5A and NS5B (Tu et al., 1999). Interestingly, hVAP-A differentially interacts with hypo- and hyperphosphorylated NS5A: Whereas the p56 species interacts with hVAP-A, binding is disrupted upon NS5A hyperphosphorylation (Evans et al., 2004). hVAP-A seems to induce the formation of the HCV replication complex (Gao et al., 2004). Consequently, siRNA-mediated reduction of hVAP-A expression leads to a decrease in HCV replication.

In addition to its role in HCV replication, NS5A has been speculated to contribute to liver pathogenesis by deregulating signal transduction of the host cell. There is good evidence documenting that NS5A indeed modulates several signaling cascades (see above). Several binding partners of NS5A act as key regulators of signal transduction pathways that are affected by NS5A. Therefore, it is tempting to speculate that these binding partners initiate the NS5A-mediated deregulation of cellular signaling. This is particularly true for p53, PKR, Grb2, p85 (PI3K) and TRAF2/TRADD (see chapter 1.10). However, in most cases, the link between interaction and modulation of cellular signaling remains to be demonstrated.

In summary, there is a growing list of proteins that are capable of interacting with NS5A. However, the significance of most of these interaction partners remains ill-defined.

## *1.12 Core also modulates host cell signal transduction.*

Apart from NS5A, HCV encodes for at least one more protein that interferes with host cell signaling: Core is a potent inducer of cellular signal transduction, ie. core display regulatory functions in addition to its role in virion morphogenesis. Core interacts with the transcription factor STAT3 and activation of STAT3 results in anchorage-independent growth and tumorigenesis (Yoshida et al., 2002). Moreover, core activates NF-κB (Kato et al., 2000; Yoshida et al., 2001) and potentiates the TNF-α- or lymphotoxin-β-induced activation of NF-κB (Chung et al., 2001; You et al., 1999). Core-mediated activation of NF-κB depends on the integrity of TRAF2 and TRAF6 (Yoshida et al., 2001). Furthermore, core also activates the transcription factor AP-1 (Shrivastava et al., 1998). At the same time, core stimulates the MAP kinase signaling cascade, resulting in increased ERK activity in core-producing cells (Erhardt et al., 2002). Altogether, this suggests that core activates a number of transcription factors that are key regulators of cell growth and cell proliferation. Most importantly, core and NS5A target similar signal transduction cascades (compare chapter 1.10). This suggests that activation of these signal transduction cascades is a central regulatory mechanism for HCV that is therefore encoded by two functionally-redundant proteins.

Core-mediated activation of the transcription factors STAT-3, NF-κB and AP-1 is of potential interest for at least two aspects of HCV biology. On the one hand, core-induced activation of cellular signaling could contribute to apoptosis regulation. In fact, HCV core has been shown to interfere with apoptosis regulation. However, it is controversial whether core enhances or diminishes apoptosis (Ray et al., 1998; Zhu et al., 1998). These controversial results may be due to cell-type-specific effects and/or differences in the experimental protocol used for apoptosis induction. Whereas the mechanism of apoptosis induction/enhancement by core remains largely elusive, the anti-apoptotic effect of core could be explained as a consequence of core-mediated activation of NF-κB and AP-1. In fact, the anti-apoptotic effect of core is abolished upon coexpression of IκB (Marusawa et al., 1999), a negative regulator of NF-κB signaling, lending strong support to this hypothesis.

On the other hand, activation of NF-κB and AP-1 may not only be relevant with regard to apoptosis regulation, but also with regard to transformation. HCV is associated with a high risk of developing hepatocellular carcinoma (see chapter 1.13). Analysis of core-transgenic mice suggests that core contributes to HCV-associated liver pathogenesis, most notably to the

development of hepatocellular carcinoma (Moriya et al., 1998). Importantly, AP-1 was found to be activated in the liver of core-transgenic mice (Tsutsumi et al., 2002). This highlights the importance of AP-1 activation for tumorigenesis and supports the notion that development of hepatocellular carcinoma might be attributable to a deregulation of cellular signal transduction cascades.

## *1.13 Liver-pathogenesis in HCV-transgenic mice.*

As mentioned above, model systems for HCV-mediated liver pathogenesis are scarce. This is due to two major reasons: (i) There is no small animal that can be infected with HCV and that shows liver pathogenesis similar to HCV-infection in humans. (ii) HCV-mediated liver pathogenesis is a very slow process with cirrhosis or hepatocellular carcinoma arising decades after the initial infection.

As mentioned above, HCV-mediated liver pathogenesis is characterized by inflammation and fibrosis, ultimately leading to cirrhosis or development of hepatocellular carcinoma (HCC). It is evident that the chronic liver inflammation constitutes one major reason underlying this multi-step disease. However, a chronic liver inflammation alone is not sufficient to induce the development of HCC. This is supported by the observation that patients with autoimmune hepatitis who suffer from severe liver inflammation only rarely develop HCC (Koike et al., 2002). This indicates that there must be viral factors that directly contribute to hepatocarcinogenesis. One potential mechanism of interference with hepatocarcinogenesis could be promotion of tumor growth: Both core and NS5A have been shown to deregulate signaling pathways that control cell growth and proliferation. If these proteins exert their stimulatory effect in a situation of continuing inflammation (ie. cell death and regeneration), transformation may be more likely to occur.

One possibility to analyze the mechanism of HCV-associated liver pathogenesis is the generation and characterization of HCV-transgenic mice. Most of the transgenic mice established so far either contain core alone (Kato et al., 2003; Tsutsumi et al., 2002), the structural region of the HCV genome (Honda et al., 1999; Kawamura et al., 1997; Pasquinelli et al., 1997) or the whole HCV ORF (Blindenbacher et al., 2003; Disson et al., 2004; Lerat et al., 2002). Whereas most transgenic mice express the genes of interest in a constitutive fashion, in some transgenic mice, expression of the HCV ORF is directed by the Cre/*lox*P system, thereby allowing conditional

transgene expression (Machida et al., 2001; Wakita et al., 2000; Wakita et al., 1998). Conditional transgene expression is particularly amenable to analyze the immune response to HCV. However, there are two major drawbacks of the conditional expression mediated by the Cre/*lox*P system: (i) The DNA recombinase Cre is usually administered by infection with a recombinant adenovirus that may by itself induce liver inflammation (Liu et al., 2000). (ii) Conditional gene expression is very unstable over time. HCV-transgenic mice that conditionally express the HCV ORF loose gene expression within one month after administration of the recombinant adenovirus (Cre) (Wakita et al., 2000). This precludes the use of these animals as models for chronic HCV infection and the associated pathogenesis.

Analysis of transgenic mice that express HCV proteins in a constitutive manner has produced mixed results. Most importantly, core-transgenic mice develop liver pathogenesis that is very similar to chronic HCV infection (Moriya et al., 1998). This includes both lipid accumulation (steatosis) and, later on, the development of hepatocellular carcinoma. However, the situation becomes less clear for transgenic mice that express the structural region of the HCV genome (that includes core). Whereas some of these transgenic mice show an altered histology with infiltration of lymphocytes and hepatocyte necrosis at 10 months of age (Honda et al., 1999), others do not show any pathogenic markers at all (Pasquinelli et al., 1997). Transgenic mice expressing the whole ORF show hepatic steatosis and an increased incidence of hepatocellular carcinoma (Lerat et al., 2002). However, fibrosis, a characteristic feature of chronic HCV infection, was not detected. In summary, these data suggest that transgenic mice may at least in part be suitable for the analysis of HCV-associated liver pathogenesis. Conflicting evidence may arise from different genetic backgrounds of transgenic mice or different expression levels of HCV proteins in the liver of transgenic animals.

## *1.14 Introduction to c-Raf1*

c-Raf1 is a kinase that controls cell growth, cell proliferation and apoptosis. Since, during the course of this work, c-Raf1 was identified as a cellular target of NS5A, c-Raf1 shall be introduced in more detail.

c-Raf1 was originally identified as a retroviral oncogene (v-Raf) possessing serine/threonine kinase activity (Moelling et al., 1984). v-Raf-related genes were soon discovered in humans and now comprise a family of three isoforms, namely A-Raf, B-Raf and c-Raf1 (or Raf-1). All Raf

kinases are composed of three highly conserved regions CR1, CR2 and CR3 (Chong et al., 2003). CR1 and CR2 form the regulatory domain whereas CR3 contains the kinase domain with the active center. c-Raf1 is expressed at high levels in most cell types and tissues. In contrast, A-Raf expression is most pronounced in the urogenital system whereas B-Raf is expressed at high levels in neuronal tissues (Mercer and Pritchard, 2003). Nevertheless, both A-Raf and B-Raf expression are also detected in other tissues, albeit at lower expression levels.

Recently, the kinase domain of B-Raf (ΔB-Raf) has been crystallized in the presence of the Raf inhibitor BAY43-9006 (Wan et al., 2004). The crystal structure of ΔB-Raf shows a bilobal architecture of the Raf kinase domain that is very similar to the structure of the c-Abl tyrosine kinase domain. The inhibitor is buried in the interlobal cleft and interacts with various sites including the ATP binding pocket. The sites of interaction are well-conserved in c-Raf1, consistent with the observation that the inhibitor does not distinguish between different Raf isoforms.

## *1.15 Activation of c-Raf1 triggers the MAP kinase cascade Raf/MEK/ERK.*

Activation of c-Raf1 triggers a sequential kinase cascade that is referred to as the mitogen activated protein (MAP) kinase module Raf/MEK/ERK. The fidelity of this cascade is ensured by scaffold proteins like MEK partner 1 (MP1), c-Raf1 kinase inhibitory protein (RKIP) or kinase suppressor of Raf (KSR) (Mercer and Pritchard, 2003; Pouyssegur et al., 2002). Furthermore, specific docking sites specify the interactions with relevant substrates.

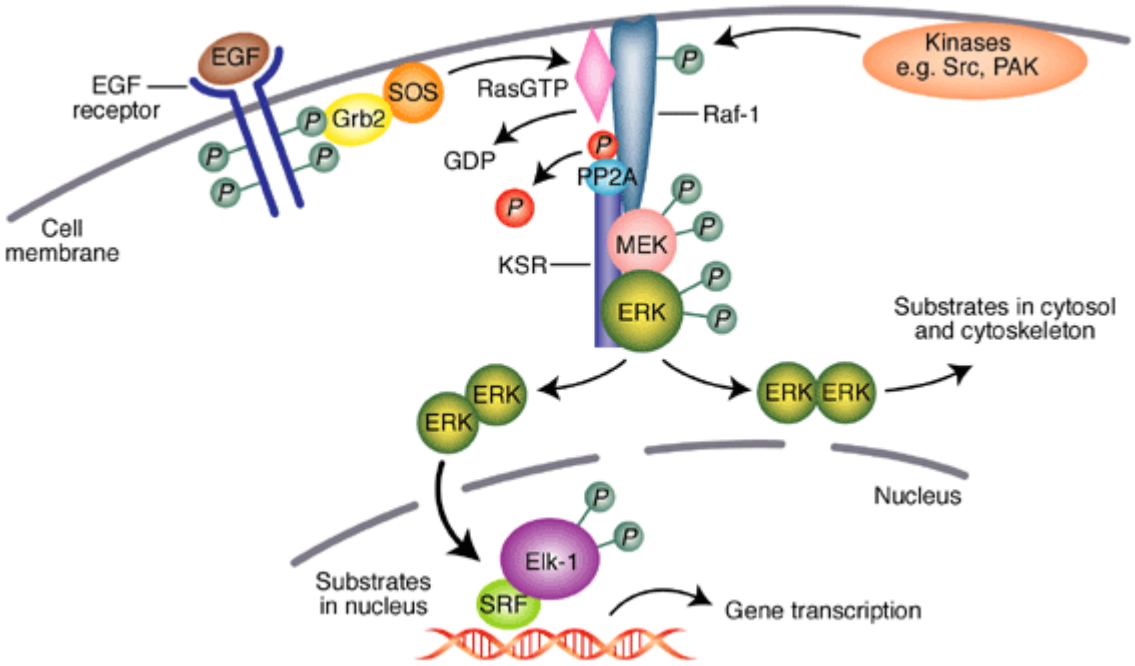


**Fig. 1.5 Organization of the Ras/c-Raf1/MEK/ERK pathway.** Stimulation of the cell by epidermal growth factor (EGF) induces activation of Ras via Grb2 and Sos. Activated GTP-bound Ras recruits c-Raf1 to the plasma membrane. Activation of c-Raf1 involves phosphorylation at distinct serine and tyrosine residues. Activated c-Raf1 triggers a kinase cascade that transmits the signal from c-Raf1 to ERK via MEK. Activated ERK is translocated to the nucleus where it activates several transcription factors including SRF and Elk-1. Reprinted from (Kolch et al., 2002).

As seen in Fig. 1.5, c-Raf1 is usually activated in response to growth factors such as the epidermal growth factor (EGF). EGF binds to the extracellular domain of the EGF receptor and induces dimerization of the receptor. The dimerized receptor is activated via autophosphorylation at conserved tyrosine residues. Those recruit the adaptor protein Grb2 that in turn binds to Sos. Sos acts as a guanine nucleotide exchange factor that catalyzes the GTP/GDP exchange of Ras. GTP-bound Ras activates c-Raf1.

Activation of c-Raf1 involves the interaction of active GTP-bound Ras with the Ras binding domain of CR1. Furthermore, c-Raf1 activity is extensively regulated by phosphorylation. In unstimulated cells, c-Raf1 is located in the cytoplasm and is phosphorylated on two serine residues (S259 and S621). Those serine residues bind to 14-3-3 proteins, thereby retaining c-Raf1 in an inactive state. In stimulated cells, GTP-bound Ras displaces 14-3-3 at S259 and c-Raf1 is

recruited to the plasma membrane where it is dephosphorylated at S259. Furthermore, c-Raf1 becomes phosphorylated at S338 and Y341, thereby establishing the activated conformation of c-Raf1 that is now capable of phosphorylating its substrate MEK (Dhillon and Kolch, 2002).
MEK is the main relevant substrate of c-Raf1. It is activated by phosphorylation of serine residues S218 and S222 (Zheng and Guan, 1994). Activated MEK in turn phosphorylates ERK1 (T202 and Y204) or ERK2 (T185 and Y187) to activate their kinase activity (Chang et al., 2003). ERK has many substrates both in the cytosol and the nucleus (Kolch et al., 2002), most importantly the transcription factors serum response factor (SRF) and Elk-1. Those direct gene expression via serum response elements.

## *1.16 MAP kinase-independent functions of c-Raf1*

It was commonly assumed that activation of the MAP kinase cascade constitutes the major function of c-Raf1. However, recent data indicate that c-Raf1 may have functions that are not related to MAP kinase activation. Those data have mainly been obtained by generation and analysis of *c-Raf1*-deficient mice. c-Raf1 knockout mice die during embryogenesis and show vascular defects as well as increased apoptosis of embryonic tissues (Huser et al., 2001). This argues that for c-Raf1 adopting a central role in apoptosis regulation. Surprisingly, analysis of mouse embryonic fibroblasts revealed that ERK activation is not affected by the deletion of the *c-Raf1* gene. This suggests that (i) c-Raf1 activity is not required for ERK activation and (ii) the role of c-Raf1 during apoptosis regulation is not coupled to the activation of the c-Raf1/MEK/ERK module. The former was attributed to B-Raf compensating for the loss of c-Raf1 in *c-Raf1*-deficient mice. The latter was confirmed by the analysis of "knock-in" mice that express a mutant version of c-Raf1 that does not display MEK kinase activity (Y340F/Y341F) (Huser et al., 2001). These mice survive to adulthood, are fertile and have an apparently normal phenotype. Most importantly, they do not show any defects in apoptosis regulation although ERK activation is largely impaired. This supports the notion that c-Raf1 function is not restricted to MEK/ERK activation. In contrast, c-Raf1-mediated apoptosis regulation seems to be independent of MEK/ERK activation.
Although several alternative c-Raf1 substrates have been described, none has been unequivocally validated yet (O'Neill and Kolch, 2004). Several studies have linked the anti-apoptotic function of c-Raf1 to a localization of c-Raf1 to the mitochondria (Hindley and Kolch, 2002). The first

evidence was provided by reports that c-Raf1 can bind to Bcl-2 (Wang et al., 1996), the prototype of the anti-apoptotic factors. However, the c-Raf1-mediated anti-apoptotic effect is maintained in cells lacking expression of Bcl-2, arguing that Bcl-2 is not essential for c-Raf1-mediated apoptosis regulation (Troppmair and Rapp, 2003).

Another potential target of c-Raf1 that could account for the c-Raf1-mediated anti-apoptotic effect is Bcr-Abl (Hindley and Kolch, 2002). Bcr-Abl induces the translocation of a fraction of endogenous c-Raf1 to the mitochondria. Mitochondrial c-Raf1 stimulates BAD phosphorylation and thereby inactivates the pro-apoptotic factor BAD. However, BAD is no c-Raf1 substrate (Hindley and Kolch, 2002), raising the question of how c-Raf1 interferes with BAD phosphorylation.

In summary, there is good evidence for a role for c-Raf1 in apoptosis regulation that is independent of MEK/ERK activation. However, the mechanism that triggers the anti-apoptotic effect of c-Raf1 is poorly defined.

## *1.17 The role of Raf kinases in cancer.*

Raf kinases were identified as human homologues of the retroviral oncogene product v-Raf. In fact, the v-Raf transducing retrovirus (3611-MSV) was isolated from a mouse with lung carcinoma and peritoneal tumors (Kolch et al., 2002). This suggested that Raf kinases contribute to malignant transformation.

However, transformation is a very complex phenomenon that requires several simultaneous alterations in cell physiology. Those include (i) self-sufficiency to growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evasion of apoptosis, (iv) immortalization, (v) sustained angiogenesis and (vi) potential to metastasise (Hanahan and Weinberg, 2000). As a consequence, in most tumors, more than one mutated gene is necessary to generate the malignant phenotype. A seminal finding was that at least two oncogenes are required to transform primary rodent cells (Land et al., 1983). Human cells, which are much more resilient to transformation, require two oncogenes in addition to an immortalizing event (Hahn et al., 1999). This is in agreement with the requirements postulated by Hanahan and Weinberg (Hanahan and Weinberg, 2000) because most oncogene products confer more than one transforming property.

Raf kinases have been shown to contribute to transformation by a number of mechanisms, most notably by constitutive activation of the Raf/MEK/ERK pathway (Hoshino et al., 1999).

However, there is a mechanism that prevents transformation of cells in which Raf signaling is hyperactive: High-intensity Raf signaling induces cell-cycle arrest (Marshall, 1999). Cell-cycle arrest can be overcome by the presence of a second oncogene product, such as Myc (Bouchard et al., 1999). As a consequence, the deranged cell can now proliferate. This illustrates how different oncogene products cooperate to induce transformation.

The Raf/MEK/ERK pathway is hyperactive in 30% of all human cancers (Hoshino et al., 1999). Although this number is impressive, the *caveat* applies that many results were obtained from tumor cell lines rather than primary tumor tissue. The most frequent lesions seem to be *ras* mutations, *myc* amplification and EGF receptor overexpression. Mutations of the *ras* gene occur in up to 90% of pancreatic carcinomas, 50% of colon carcinomas, 40% of lung carcinomas and 20% of leukemias (Kolch et al., 2002).

Of course, c-Raf1 is activated by mutations that affect the activity of upstream gene products (such as the EGF receptor or Ras). However, there is little evidence for tumor-specific c-Raf1 alterations. In some instances, c-Raf1 overexpression was found, especially in colon cancer and lung cancer, but genetic alterations were essentially absent (Kolch et al., 2002). A different picture emerges for B-Raf that has recently been found to be altered in a number of malignant melanomas and colon cancers (Mercer and Pritchard, 2003). The vast majority of mutations were found at a single site that results in an exchange of valine 599 to glutamate (V599E). This mutation maps to the activation segment of B-Raf and is thought to mimic phosphorylation events that occur at threonine 598 or serine 601. The mutant B-Raf kinase is hyperactive, resulting in increased activation of the MEK and ERK (Wan et al., 2004). Therefore, the malignant phenotype of this mutation is most likely due to hyperactivation of the B-Raf/MEK/ERK pathway.

To conclude, although the *c-Raf1* gene is not genetically altered in human tumors, the Raf/MEK/ERK pathway is frequently activated by upstream oncogene products, most notably Ras.