CHAPTER 5: DISCUSSION
5. Discussion:

My research work was focused on characterizing two different properties of the major capsid (ORF2) protein of Hepatitis E virus (HEV). First, I proved that ORF2 binds to the 5' end of genomic RNA. Secondly, I have proved that expression of ORF2 protein in cultured hepatocytes induces ER stress and inhibits NF-κB activity. Inhibition of NF-κB activity resulted in decreased expression of its targets, one of the most crucial during early infection stage being MHC class I. While investigating the mechanism of NF-κB inhibition, I discovered a novel pathway utilized by ORF2 protein. ORF2 protein was able to retrotranslocate back to the cytoplasm in an ubiquitination independent manner, binds to the F box protein β TRCP and inhibits the degradation of IκBa, leading to diminished NF-κB activity in the host cell. This is the first report of a viral protein exploiting the above pathway to inhibit NF-κB activity.

5.1 Genomic RNA binding by ORF2 protein.

I have used the yeast three-hybrid system to show that the ORF2 protein of HEV is capable of binding its viral genome, at the 5' end. The ORF3 protein does not possess this activity. Also, the ORF2-RNA interaction is specific to the 5' end of the HEV genome and not to the 3' or any other region tested. Finally, we have mapped the ORF2 protein binding region on the RNA genome to a 76nt region (5' HEV 130-206). This 76nt region of the viral genome is capable of binding the full-length ORF2 protein and its 1-111 N-terminal deletion as well. Previous observations from our laboratory on the dimerization of ORF2 (Tyagi et al., 2001) and its heterotypic interaction with ORF3 (Tyagi et al., 2002), have shown similar observations suggesting that the ORF2 protein loses its activity when truncated beyond amino-acid 111 from the N-terminus.
Fig. 5.1.1 Predicted secondary structure of the ORF2 binding genomic sequence.

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The mfold program was used to predict the RNA secondary structure for the HEV genomic region 130-250nt (which includes the 76nt region + downstream 44nt), based on minimum free energy calculations (Fig. 5.1.1). Interestingly enough, the 76nt region that we have shown to be responsible for binding the ORF2 protein, completely encompasses the alphavirus consensus conserved 51nt sequence (base 150-208) (Niesters et al., 1990). This 51nt conserved region is highlighted and is part of 2 stem-loop structures (SLI and SLII) similar to other alphaviruses viz. Sindbis virus (Niesters et al., 1990), Highlands J virus and Semliki Forest virus (Strauss et al., 1986). Looking at the secondary structures it becomes obvious that sequences downstream of base 208 contribute to the strength of stem-loop SLII. Our experimental data on the HEV RNA-ORF2 protein interaction falls in line with the in-silico secondary structure prediction, suggesting that sequences downstream of base 208 contribute to increased strength of the stem-loop SLII thus strengthening the RNA-protein interaction. The 4nt region (209-212nt, SLII) may not be essential for the RNA-protein interaction, but may contribute towards increasing the binding strength of the RNA-protein interaction in question. The 5' HEV 130-250nt region also forms a third stem-loop structure (SLIII). Although not essential, this stem-loop structure may have significant contributions towards increasing the overall strength of the HEV genomic RNA-ORF2 protein interaction.

HEV being a plus-stranded RNA virus, will require one of its structural proteins to show RNA binding activity for two essential viral functions such as viral replication.
and packaging of its genome into the capsid during viral assembly. ORF2 being the major capsid protein, it would be the most likely candidate to bind the genomic RNA for viral packaging. We have experimentally shown that the ORF2 protein binds to the 5' terminal region of the viral genome, thus becoming the most likely candidate to perform this biological function.

HEV is postulated to form subgenomic RNA transcripts (~3.7 kb and ~2kb) from the 3' (structural) region of the genome (Li et al., 1994). Hence it seems like a good strategic option for the virus to have its RNA encapsidation signal at the 5' end of the genome. This will result in only the full-length genomic RNA (~7.2 kb) to get differentially recognized by the capsid protein ORF2 for headfull packaging during viral assembly in the hepatocyte.

Although data obtained form this work points towards a fundamental viral process, viz. genome encapsidation, and strongly indicates the possibility that the ORF2 protein may be responsible for bringing the genomic RNA into the capsid during assembly, direct biological evidence is difficult to obtain due to the absence of an in vitro culture system for HEV. Indirect approaches using mutational knock-off of the identified interaction domain and restoration by complementary mutations may be used for further understanding of this RNA-protein interaction.

5.2 NF-κB inhibitory property of the ORF2 protein.

This study uncovers an interesting mode of a viral protein exploiting the cellular machinery to its benefit. A fraction of the major capsid protein of the hepatitis E virus, i.e. ORF2 protein, was found to be retro-translocated from the ER to the cytoplasm in a glycosylation dependent manner where it was able to interact with the F-box protein β TRCP blocking ubiquitination and proteasomal degradation of IκBα with a concomitant reduction in NF-κB activity. This result defines a possible regulatory role of the ORF2 protein during the viral life cycle besides forming the core of the virus particle. The fact that the observed phenomenon was not an artifact of the experimental setup was evident from multiple experiments. First, Δ35 ORF2, a signal sequence deleted mutant that is unable to translocate to the ER was also capable of inhibiting NF-κB activity thus ruling out the effect to be due to a protein over-expression induced ER stress. Moreover, if at all
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ER stress induction is an experimental artifact it should lead to upregulation, not downregulation of NF-κB activity as observed in our study. Second, ORF2 was capable of physically binding to the F-box protein β TRCP, a cytoplasmic protein. Third, inhibition of ORF2 retro-translocation by different biochemical inhibitors was capable of reversing the ORF2 mediated block of NF-κB activity.

HEV is the causative agent of an acute, self-limiting and icteric disease that is prevalent in much of the developing world. Although self-limiting infection occurs in adults with mortality rate ~1-2%, a high 10-20% mortality rate is observed during pregnancy (Bradley, 1990; Khuroo et al., 1994; Purcell et al., 1988). Due to the lack of a small animal or replication competent cell culture model for the propagation of HEV, the basic mechanism of pathogenesis mediated by the virus remains poorly understood. Attempts to understand the biology of HEV pathogenesis has been limited to functional studies of the property of individual HEV proteins expressed in different recombinant gene expression systems (Korkaya et al., 2001; Tyagi et al., 2004). The HEV genome codes for a 7.2 kb positive-strand RNA that is translated into 3 different open reading frames (ORFs). ORF1 (5079 bases) is at the 5' end of the genome and is predicted to code for putative nonstructural proteins with sequences homologous to those encoding viral methyltransferase, protease, helicase, and RNA-dependent RNA polymerase. ORF2 codes for the major capsid protein and ORF3 codes for a phosphoprotein predicted to be important for host cell survival during HEV infection (Korkaya et al., 2001).

ORF2 is an N-linked glycoprotein that is co-translationally translocated into the ER and is also expressed on the cell surface (Jameel et al., 1996). The ORF2 protein has been shown to be present initially as a glycosylated protein which gradually becomes unglycosylated. The majority of this unglycosylated protein resides in the cytoplasm (Torresi et al., 1999). Although, it has been suggested by Torresi et al. (1999) that a fraction of the ORF2 protein is not translocated into the ER; that possibility is unlikely since co-translational translocation per se would send ORF2 protein into the ER compartment even before the complete protein is synthesized. Moreover, membrane fractionation study has shown that initially all the ORF2 protein is localized in the membrane fraction only. Hence, a more feasible alternative could be that the protein accumulates in the ER, induces ER stress which, activates the cellular quality control
mechanism to remove it from the ER by the retro-translocation pathway. Accordingly the levels of the ER chaperones were found to be up-regulated in ORF2 expressing cells. Upregulation of HSP 70 (e.g. GRP 78) and HSP 90 (e.g. GRP 94) family of chaperones is known to be associated with the unfolded protein response, which binds to and retains the misfolded protein in the ER. This in turn releases their interaction with ATF6 which subsequently gets cleaved to produce active form of the transcription factor ATF6 that transmits the stress signal to the nucleus (Yoshida et al., 2000). This leads to the activation of ATF6 responsive promoters like the GRP 94 promoter. Thus, upregulation of GRP 94-CAT activity in ORF2 expressing cells indicated that the ORF2 protein induces ER stress. Upregulation of the levels of protein disulfide isomerase (PDI) suggest that the ORF2 protein is misfolded in the ER since the latter bears 26 cysteine residues, 23 of them being clustered at the central 250 amino acids (247-513 amino acids) and thus PDI help is essential for rearrangement of disulfide bonds in order to attain proper conformation of the ORF2 protein. Alternatively, it may also be possible that ORF2 exploits PDI activity to get unfolded so as to get retro-translocated as has been demonstrated for cholera toxin (Tsai et al., 2001).

Further, the ORF2 protein was found to interact for a prolonged period with the lectin binding chaperone calnexin. It is known that misfolded glycoproteins are anchored by calnexin and undergo repeated cycles of glycosylation and deglycosylation until they attain proper conformation (in normal course, proteins transiently interact with calnexin making it difficult to co-precipitate them). If proteins fail to be folded in a limited period, they become the target of mannosidase I which is a slow acting enzyme. Removal of the terminal mannose residue from the aberrant protein allows it to be recognized by an intermediate factor called EDEM (ER degradation enhancing mannosidase like protein) that promotes retro-translocation and degradation of the substrate by the 26S proteasome (Oda et al., 2003). ORF2 was found to follow the same pathway as judged by its inability to dislocate in the presence of biochemical inhibitors of glucosidase II and mannosidase I. However, the property that was peculiar to ORF2 was its ability to avoid proteasomal degradation in the cytoplasm since deglycosylated ORF2 could be readily detectable in the cytosolic fraction. This may be due to the fact that the ORF2 protein was not ubiquitinated and hence not a target of the proteasome (however, proteasomal
degradation may occur in the absence of ubiquitination too). Alternatively, it may be possible that after getting retro-translocated, ORF2 protein promptly refolds into such a conformation that the protease sensitive or ubiquitination sites are masked. The protease resistant property of ORF2 protein has been described earlier, whereby in-vitro expressed ORF2 protein was found to be partially trypsin resistant despite having 50 trypsin cleavage sites (Zafrullah et al., 1999). Retro-translocation of non-ubiquitinated substrates has been shown earlier by using proteins that are engineered to be ubiquitination deficient (Ye et al., 2003). However, to the best of our knowledge, ORF2 is the first example of a protein that naturally demonstrates this ability.

The ability of ORF2 to retro-translocate was found to be dependent on its glycosylation status. The ORF2 protein bears 3 N-linked glycosylation sites at amino acid residues 137, 310 and 562 (Zafrullah et al., 1999). Although these sites along with the signal sequence are conserved in all isolates of human as well as swine HEV, no functional significance of ORF2 glycosylation is yet defined. Based on our observation that glycosylation is an absolute requirement for the NF-kB inhibitory property of ORF2, it is tempting to speculate that the observed phenomenon will bear significance during viral infection.

MHC class I, which is known to be a transcriptional target of NF-kB, was found to be down-regulated in ORF2 expressing cells presumably due to reduced promoter occupancy of p65, which is crucial for NF-kB dependent transcriptional activation. In an infected cell, by default viral antigens are processed by the proteasome and presented by the MHC class I molecules so that the former can be recognized by cytolytic T cells. This helps in virus clearance at an early stage of infection. Thus, it is beneficial for any virus to escape this step. In fact, downregulation of MHC class I both at transcriptional and post-translational level is observed in many cases of viral infection (Hazes et al., 1997; Hirst, 1995; Hwang et al., 1992; Jakob et al., 1999). Since NF-kB is the major transcription factor induced by virus infection that enhances gene expression of many chemokines and class I molecules in the infected cell, inhibition of the former by virus encoded proteins will ensure evasion of host immune response at an early stage, thus providing the pathogen a time window to establish successful infection. However, prolonged NF-kB inhibition itself is known to promote programmed cell death of the
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host. Clinical data pertaining to HEV pathogenesis in human volunteers falls in congruence with this hypothesis where by virus shedding in the faecal matter of infected patient precedes by 1-2 weeks from the onset of icterus and elevation of liver enzymes (Panda et al., 1997). However, assessing the exact functional relevance of these results will only be possible by studying it in an infected animal model.

Nevertheless, these studies reveal a novel mechanism of NF-κB inhibition mediated by a viral protein. Earlier, several other viral proteins have been shown to inhibit NF-κB activity. For example, African swine fever virus encodes an IκBα homolog that can bind to and block relA mediated transcriptional activation. The Vpu protein of HIV-1 has been shown to bind the WD40 motif of F-box protein β TRCP, thus blocking IκBα ubiquitination. Here, we find ORF2 to follow an analogous mechanism. However, the characteristic property of ORF2 in rendering this function underlies its ability to remain protease resistant in the cytoplasm after getting dislocated from the ER. Although this effect may be a novel property of the ORF2 protein, this provides another example that a retro-translocated substrate can be functionally active in the cytosol. Earlier, cholera toxin was shown to get dislocated and remain active in the cytosol. Hence, the possibility remains that this transport pathway might be utilized by cellular proteins under certain conditions to gain access to the cytoplasm.