CHAPTER 4: RESULT
4.1 Result I

I have used the yeast three-hybrid system to detect RNA binding properties of the two structural proteins of HEV and map the interaction domains for the interacting protein and genomic RNA. The yeast three-hybrid system is a genetic assay in which specific RNA-protein interactions can be detected rapidly in yeast, in a fashion that is independent of the biological role of the RNA or protein (Fig. 4.1.1).

Fig. 4.1.1 Schematic representation of yeast three hybrid assay and the vectors used for the experiments.

This approach is based on the yeast two-hybrid system, in principle, which detects protein-protein interactions. The three-hybrid system allows simple phenotypic properties of yeast, such as the ability to grow on auxotrophic medium or to metabolize a chromogenic compound, to be used to detect and analyze an RNA-protein interaction. In the co-transformed yeast cell, a fusion RNA molecule bridges two hybrid proteins, one containing a DNA-binding domain and the other containing a transcriptional activation domain resulting in the transcriptional activation of \textit{HIS3} and \textit{lacZ} reporter genes.
Results

downstream of the binding site for the DNA-binding domain. To apply this system to HEV, we designed constructs fusing MS2-RNA with the 5' HEV (1-910nt) genome and the 3' HEV (6807-7184nt) genome in two separate constructs (Fig. 4.1.2). The HEV ORF2 and ORF3 genes were cloned in-frame with the Gal4 activation domain (Gal4AD), in two separate constructs (Fig 4.1.2).

Fig. 4.1.2 Schematic representation of HEV genome and hybrid RNA and protein constructs. A, Genes and genome organization of the hepatitis E virus. Start sites for all three ORFs and both 3' and 5' UTRs are shown (red). A(n) represents the polyA tail (blue). Predicted stem-loop structures (SL) are shown schematically and numbered across the HEV genome. B, Fusion RNA constructs designed to express fusion transcripts within the yeast cell. MS2 RNA coding region (yellow) was cloned with two different HEV genomic regions (green). Predicted mRNA stem-loop structures are shown schematically as fusion transcripts. C, Hybrid protein constructs to test RNA binding activity of the ORF2 and ORF3 proteins of HEV using the yeast three-hybrid system. Schematic diagram shows the Gal4 Activation Domain (Gal4 AD) fused in-frame to the ORF2 and ORF3 genes of HEV thus expressing fusion proteins in yeast cells.
4.1.1 The 5' end of the HEV genomic RNA interacts with the ORF2 protein.

Different regions of the HEV genome were cloned into yeast three-hybrid vectors so as to express fusion RNA transcripts in yeast cells. The 5' end (base 1-910) of the HEV genome was cloned into the pIIIMS2-2 yeast three-hybrid vector (Table A). Similarly the 3' end (base 6807-7184) and the 572nt from the middle region (5108-5680) of the HEV genome were cloned into the pIIIMS2-2 yeast three-hybrid vector (Table A). All the constructs were transformed into the L40-coat, yeast three-hybrid host strain and grown on respective auxotrophic medium. Independent transformants were picked and their total RNA was isolated. Transcription of the fusion RNA was checked by RT-PCR analysis. On the other hand, full-length ORF2 and ORF3 genes were cloned in-frame with the Gal4 activation domain to express hybrid proteins in the yeast cells.

Single and co-transformants were obtained in different combinations and tested for RNA-protein interactions (Fig. 4.1.1.1). The host yeast strain (L40-coat) showed negligible background His\(^+\) phenotype. When singly transformed host cells were analyzed, low background reporter gene activity was detected on His\(^+\) 5mM 3-AT media and \(\beta\)-galactosidase assays. The MS2-5'HEV / AD-ORF2 co-transformants clearly showed strong His\(^+\) prototrophy up till 15mM 3-AT concentration. This clone was a strong positive when tested for \(\beta\)-galactosidase activity, in both filter and liquid assays. However, the MS2-3'HEV / AD-ORF2 co-transformants showed no reporter gene activity. The HEV genomic RNA (5108nt to 5680nt) was also tested for interaction with AD-ORF2 and AD-ORF3. Both assays gave negative results. Similarly, AD-ORF3 when co-transformed separately with MS2-5' HEV and MS2-3'HEV, failed to show increased reporter gene activity.
Results

Fig. 4.1.1.1 Results from the three-hybrid analysis showing 5’ HEV genomic RNA interacting with the ORF2 protein. YPD: yeast extract, peptone, dextrose media (non-selective); Leu’, Ura’, LU’ represent SD-Leu’ (Synthetic Dextrose complete media lacking Leucine), SD-Ura’ (Synthetic Dextrose complete media lacking Uracil) and SD-Leu’Ura’ synthetic growth media. LUHis’ + 3-AT (Synthetic Dextrose complete media lacking Histidine, Leucine and Uracil with 3-Aminotriazole) represents SD-Leu’Ura’His’ synthetic media with 0, 5, 10 and 25 mM 3-Amino Triazole (3-AT) added. βF represents results from the β-galactosidase filter assay and the bar graph represents relative β-galactosidase units from the liquid β-gal assay. L40-coat is the untransformed yeast host strain. MS2-IRE / AD-IRP are the positive control used in the assay.

The protein binding domain was subsequently shortened to 1-250nt from 1-910nt and subcloned into the pIIIMS2-2 vector (Table 1) and checked for interaction with ORF2. Since this shorter RNA region showed positive interaction with the ORF2 protein, almost of equal strength, subsequent EMSA experiments were performed using this 1-250nt 5’ HEV RNA. The 5’ and 3’ regions of the HEV genome were transcribed as ^32P labeled transcripts (Fig. 4.1.1.2).
Fig. 4.1.1.2 Schematic representation of RNA probe preparation and in vitro protein production.

As described in methods for EMSA, hot RNA probe were incubated with unlabeled ORF2 protein, in separate tubes. As negative controls, the $^{32}$P labeled transcripts from the 5' and 3' genomic regions of HEV were analyzed separately on a 6% non-denaturing polyacrylamide gel. Clearly, the 5' HEV genomic transcript containing 1-250nt showed mobility shift in presence of ORF2 (lane2) indicating that the ORF2 protein was interacting with it (Fig. 4.1.1.3) as compared with lane 1, where no protein was present. On the other hand the 3' genomic region of HEV showed no binding to the ORF2 protein (lane 3, 4). Similar experiments were repeated with the ORF3 protein, which showed a negative interaction with both the 5' and 3' genomic regions of HEV.
Fig.4.1.1.3 EMSA showing interaction between wild type ORF2 protein and HEV 5'1-250 nucleotide sequence. 5' HEV (1-250) RNA and 3' HEV (6807-7184) were $^{32}$P radiolabelled. ORF2 protein was produced using a coupled transcription-translation system. Arrow shows ORF2 protein bound to $^{32}$P labeled RNA (lanes 2). Lanes 1 and 3 are negative controls. * refers to $^{32}$P labeled transcript.

A competitor-binding assay was performed to study the specificity of the ORF2 protein interaction with 5' HEV (1-250) RNA. In this experiment, unlabeled 3' HEV (6807-7184) RNA was used in 100 fold higher molar concentration, as a non-specific competitor (Fig. 4.1.1.4). The 3' HEV RNA (6807-7184) did not compete for binding with the labeled 5' HEV RNA (1-250) - ORF2 complex which was visible on the autoradiogram. When un-labeled 5' HEV (1-250) RNA at 100 fold higher molar concentrations was incubated with labeled 5'HEV (1-250) and unlabeled ORF2 protein, it competed with the labeled 5'HEV (1-250) RNA. This was evident by the complete disappearance of signal (Fig. 4.1.1.4, lane 2). Appropriate positive and negative controls
Results

are shown in Fig. 4.1.1.4, lane 3 and 4 respectively. This experiment showed clearly that the RNA-protein interaction was specific to the 5' HEV (1-250) transcript and full-length ORF2 protein. We further designed deletions to identify the interaction domain of ORF2, responsible for RNA binding.

Fig.4.1.1.4 Competitior binding assay showing specificity of 5' HEV RNA and wild type ORF2 protein interaction. ORF2 protein being used in this experiment is unlabeled. Lane 1 contains a non-specific competitor, 3' HEV (6807-7184). Lane 2 contains a 100 fold excess of unlabeled 5'HEV (1-250) RNA transcript. Lanes 3 and 4 are positive and negative controls, respectively. * refers to $^{32}$P labeled transcript. Non-radioactive transcripts 3' HEV (6807-7184) and 5' HEV (1-250) were used in 100-fold higher molar concentrations.
4.1.2 The N-terminal 111 amino-acid deleted ORF2 protein interacts with 5' HEV genomic RNA.

The full-length ORF2 protein was subject to a series of deletions. These deletions were subsequently cloned in-frame with the Gal4 activation domain using the vector pACT2, as described in Table 1. When tested for yeast three-hybrid interactions with 5' HEV (1-910) RNA, the constructs, AD-ORF2 (1-227), AD-ORF2 (1-358), AD-ORF2 (1-586) and AD-ORF2 (228-660) showed negative. Only AD-ORF2 (112-660), when tested with 5' HEV (1-910) RNA showed positive on the three-hybrid analysis. All three-hybrid co-transformants were tested on increasing levels of 3-AT on His' media, and on β-galactosidase filter and liquid assays (Fig. 4.1.2.1).

![Yeast three hybrid assay showing interaction of ORF2 deletions with 5'HEV RNA.](image)

**Results**

Amino acids 112-660 from the ORF2 protein are required for interaction with the 5' HEV (1-910) RNA region. Left side schematic boxes represent the activation domain regions which were fused in-frame with the ORF2 protein (full-length or deletions). Right side schematic boxes show the MS2 regions fused with the 1-910 5' HEV RNA. Open boxes represent regions that were deleted from ORF2. The numbers above the boxed regions represent the first and last nucleotide of the regions included in the ORF2 deletion constructs. YPD: yeast extract, peptone, dextrose media (non-selective); LU represent SD-Leu'Ura' synthetic growth media. LUHis' +3-AT represent SD-Leu'Ura' His' synthetic media with 0, 5, 10 and 25 mM 3-Amino Triazole (3-AT) added. BF represents results from the β-galactosidase filter assay and the bar graph represents relative β-galactosidase units from the liquid β-gal assay.
Results

Although 5' HEV (1-910) interactions with the AD-ORF2 (1-227), AD-ORF2 (1-358) and AD-ORF2 (1-586) point towards the importance of the C-terminal region of the protein, AD-ORF2 (228-660) was unable to show a positive interaction. This result points to an observation made in the past (Tyagi et al., 2001) regarding the ORF2 protein that its properties are lost when it is truncated beyond the amino acid 111 from the N-terminal end.

Results obtained from the yeast three-hybrid system were verified by conventional in-vitro techniques. All ORF2 deletions were expressed in a coupled transcription-translation system and checked for expressed protein as shown in Fig. 4.1.2.2.

Fig. 4.1.2.2. Expression analysis of different ORF2 deletion mutants. A control gel showing ORF2 deletions expressed using a coupled transcription-translation expression system. Major bands show expressed protein of interest and correspond to their calculated molecular weights. Weaker bands in each lane show non-specific translation of rabbit reticulocyte proteins.

Different ORF2 deletions were used for EMSA with the 5' HEV RNA transcript (Fig. 4.1.2.3). Results obtained from EMSA matched exactly with our observations from
the yeast three-hybrid system proving that only the full-length and the ORF2 (112-660) were capable of interacting with the 5' genomic RNA. Due to the inherent property of the ORF2 protein losing its RNA binding activity when truncated beyond the N-terminal 111 amino acid, it was impossible to perform a finer mapping of the interaction domain for the ORF2 protein.

Fig. 4.1.2.3. EMSA showing interaction of ORF2 deletion mutants with 5' HEV RNA.

4.1.3 A 76nt conserved domain from the 5' HEV genomic RNA interacts with the ORF2 protein.

The 5' HEV (1-910) RNA was subcloned into smaller fragments. 5' HEV (1-250), 5'HEV (1-130), 5' HEV (130-250) and 5' HEV (130-206) were deletions of the
full-length 5' HEV (1-910) RNA as described in Table I. Each of these deletions was individually tested with the AD-ORF2 protein in the yeast three-hybrid assay/EMSA, for interaction. The 5' HEV (1-250) RNA transcript showed a positive interaction as had been observed in EMSA results described in the previous experiments. Subsequently, the two deletion transcripts, 5' HEV (1-130) and 5' HEV (130-250), that split the 1-250nt region, were tested for interaction with ORF2 (Fig. 4.1.3.1). From this pair, the 5' HEV (130-250) RNA transcript interacted with ORF2. The 5' HEV 130-250nt region, consisting of 120 bases, showed a considerably stronger interaction with the ORF2 protein, as compared to the 5' HEV 130-206nt region, consisting of only 76 nucleotides (Fig. 4.1.3.2). Hence, the 130-206 region (76nt) of the HEV genome may contain the major interaction domain required for binding to ORF2 however; genomic sequences 44 nucleotides downstream of this region contribute towards increasing the strength of this RNA-protein interaction significantly.

Fig 4.1.3.1 Schematic representation of RNA deletions and summary of yeast three hybrid study. Mapping of the interaction domain of the 5' HEV (1-910) RNA region. Thatched box represent alphavirus consensus sequence. Right panel shows the result of yeast three-hybrid assay. βF represents filter β-galactosidase assay result.
Fig. 4.1.3.2 EMSA showing interaction of different RNA deletions with wild type ORF2 protein.
4.2 RESULT II:

Previous reports on studies of ORF2 distribution using human hepatocyte derived cell line (HepG2 cells) has shown that this protein initially accumulates in the endoplasmic reticulum (ER) as a N-linked glycosylated protein, which is gradually converted into a non-glycosylated form that stably exists in the cytoplasm (Torresi et al., 1999). Some fraction of the protein is also expressed on the cell surface (Jameel et al., 1996). However, the functional significance and the pathway responsible for redistribution of this protein remain unknown. In an attempt to understand the above process and gain functional insight towards the role of the ORF2 protein in HEV pathogenesis, we asked whether ORF2 protein accumulation in the ER induces ER stress. Thus, experiments were designed to check the markers of ER stress in ORF2 transfected cells.

4.2.1 Heterologous expression of the ORF2 protein inhibits NFκB activity.

ER stress has been shown to be manifested mainly through the unfolded protein response (UPR) and ER overload response (EOR) (Pahl, 1999). The hallmark of UPR includes transcriptional upregulation of ER chaperones driven by activation, nuclear translocation and ER stress response element (ESRE) binding of activated transcription factor 6 (ATF6). EOR is marked by a significant increase in the activity of NFκB. A chloramphenicol acetyltransferase (CAT) based reporter assay system was used to check induction of ER stress in ORF2 expressing Huh7 human hepatoma cells.

UPR was measured using an ESRE driven CAT reporter (ESRE-CAT) and EOR was measured using an IL-2 receptor promoter driven reporter that is strongly responsive to cellular NF-κB activity (NF-κB CAT). Transient transfection of ORF2 into Huh7 cells was found to upregulate ESRE-CAT (Fig. 4.2.1.1.A), while NF-κB reporter activity was found to be significantly downregulated (Fig. 4.2.1.1.B). Although, up-regulation of ESRE-CAT activity was probable owing to ER localization of over-expressed ORF2 protein, the inhibition of NF-κB activity was in contrast to the reports of NF-κB signaling in response to ER stress. Cycloheximide and tunicamycin were used as negative and
Results

positive controls respectively to monitor GRP94 promoter activity. In order to confirm whether ORF2 mediated inhibition of NF-κB activity was an artifact of the transient transfection system, cells were treated with Phorbol 12-myristate 13-acetate (TPA) for 30 minutes, which is a known inducer of NF-κB activity (Rebois and Patel, 1985) or with an expression construct of IκB kinase β, which is the catalytic subunit of the IKK complex and is known to act as a constitutively active inducer of NF-κB activity (Brummelkamp, 2003). TPA treatment was found to increase NF-κB activity of mock transfected cells by approximately 4 folds whereas ORF2 expressing cells did not show any significant increase in NF-κB activity. Similarly, ORF2 and IKKβ cotransfected cells did not show any upregulation of NF-κB activity (Fig. 4.2.1.1.B). Hence, it was clear that the inhibition of NF-κB activity by the ORF2 protein is a specific property of the latter.

Fig. 4.2.1.1 CAT assay showing upregulation of ATF6 and downregulation of NF-κB activity in ORF2 expressing cells. Huh7 cells were cotransfected in duplicate with ESRE (A) or NF-κB (B) reporter alone or along with ORF2 and/or IKKβ expression plasmids and 48 hour post transfection, CAT activity was assayed by measuring acetylation of chloramphenicol by thin layer chromatography. TPA, cycloheximide and tunicamycin was added at a final concentration of 100nM, 10μg/ml and 10μg/ml for 30, 180 and 180 minutes respectively. Equal amount of DNA was transfected in each sample by adjusting the total concentration with empty vector (pSGI). Spot intensity was quantified using NIH Image program and percentage CAT activity was calculated.
assuming the highest value as 100%. Graph represents ± SEM of 3 independent sets of experiments.

The major cellular factor regulating the activity of NF-κB is IκBα, which binds to and masks the nuclear localization signal of NF-κB, thus sequestering it in the cytoplasm. However, NF-κB activity may also be inhibited following its nuclear translocation by regulating acetylation, phosphorylation and other cofactor recruitment (Hayden, 2004). TPA or IKKβ induce proteasomal degradation of IκBα thereby enabling NF-κB to translocate to the nucleus and activate the transcription machinery. Hence, we looked at the turnover of IκBα in IKKβ and ORF2 co-expressing cells. During a pulse chase assay using 35S-cys/met labeling mix, the half-life of IκBα in IKKβ expressing cells was found to be approximately closer to 90 minutes, whereas in cells expressing both IKKβ and ORF2, approximately 15% of IκBα was found to be degraded at a chase period of 90 minutes (Fig. 4.2.1.2).

![Graph showing increased half-life of IκBα in ORF2 and IKKβ overexpressing cells. Huh7 cells were cotransfected with IKKβ and pSGI or pSGI ORF2, pulse labeled for 20 minutes with 35S cys/met promix and chased for indicated time periods in complete medium. Total IκBα was immunoprecipitated from equal amount of sample, resolved by 12% SDS-PAGE, band intensities quantified using NIH Image program and graph plotted assuming protein level at zero time to be 100%. In the graph, dotted line represents mock transfected sample and solid line represents ORF2 transfected samples. The graph represents quantitative measurement of band intensities of IκBα at the indicated time points assuming the band intensity at the onset of the chase was 100%.

Fig. 4.2.1.2 Graph showing increased half-life of IκBα in ORF2 and IKKβ overexpressing cells. Huh7 cells were cotransfected with IKKβ and pSGI or pSGI ORF2, pulse labeled for 20 minutes with 35S cys/met promix and chased for indicated time periods in complete medium. Total IκBα was immunoprecipitated from equal amount of sample, resolved by 12% SDS-PAGE, band intensities quantified using NIH Image program and graph plotted assuming protein level at zero time to be 100%. In the graph, dotted line represents mock transfected sample and solid line represents ORF2 transfected samples. The graph represents quantitative measurement of band intensities of IκBα at the indicated time points assuming the band intensity at the onset of the chase was 100%.
This result suggests that the ORF2 protein inhibits NF-κB activity by stabilizing the cellular IκBα pool.

To further confirm whether IκBα stabilization actually blocked nuclear translocation of NF-κB in ORF2 expressing cells, nuclear and cytoplasmic fractions were separated in mock and ORF2 transfected cells and samples were immunoprecipitated with anti-p65 antibody (P50 and P65 are the two subunits of NF-κB). As seen in Fig. 4.2.1.3, ORF2 transfected cells were found to accumulate more p65 protein in the cytoplasmic fraction than in the nuclear fraction (lane 2 and 4). Alternatively, mock transfected cells were found to contain more p65 protein in the nuclear fraction (lane 1 and 3). This is attributed to the fact that basal NF-κB activity is higher in Huh7 cells. Aliquots of the lysate were also immunoblotted with anti-calnexin antibody (middle panel) and anti-phospho c-jun antibody (lower panel) to prove that there was no cross-contamination between the nuclear and cytoplasmic fractions.

![Fig. 4.2.1.3 Nuclear fractionation of p65 subunit of NF-κB showing decreased nuclear presence in ORF2 expressing cells. Mock or ORF2 transfected cell lysate was separated into cytoplasmic (lane 1 and 2) and nuclear fraction (lane 3 and 4), immunoprecipitated with anti-p65 antibody, resolved in 12% SDS-PAGE and bands were detected by western blotting with anti-p65 antibody using ECL method (upper panel). A fraction of the lysate was western blotted with calnexin (middle panel) and phospho c-jun (lower panel) antibody.](image-url)
Results

Next, we checked the levels of phosphorylated IκBα (ser32) and total IκBα in ORF2 expressing cells. ORF2 expression was found to mildly increase the levels of total IκBα (Fig.4.2.1.4, middle panel, lane 1 and 2) as well as the level of phosphorylated IκBα (upper panel, lane 1 and 2). In IKKβ and ORF2 co-expressing cells, the level of total as well as phosphorylated IκBα was found to be significantly higher compared to only IKKβ expressing cells (lane 3 and 4). In order to check that the decreased band intensity in IKKβ expressing cells was due to accelerated degradation of IκBα, one set of cells was also treated with the proteasome inhibitor MG132 for 2 hours, resulting in equal protein levels in both the samples (lane 5 and 6). Aliquots of the sample were immunoblotted with anti-calnexin antibody to check equal loading.

![Image](image_url)

**Fig. 4.2.1.4 Level of phosho and total IκBα in ORF2 expressing cells.** Huh7 cells were transfected with vector (pSGI, lane2), ORF2 (lane 1), IKKβ (lane 4 and 6), ORF2+IKKβ (lane 3 and 5) and the cell lysate was immunoblotted with phospho-IκBα, ser 32 (upper panel); total IκBα (middle panel); and calnexin (lower panel).MG132 was added 2 hours prior to harvesting the cells.

The fact that ORF2 did not interfere with phosphorylation of IκBα by the IKK complex was further confirmed by checking the activity of the IKK complex. The IKK complex is known to be active when IKKα and β are phosphorylated at ser176 and ser180 (for IKKα) and ser177 and ser181 (for IKK β) residues respectively (Delhase, 1999). The ORF2 expressing cell lysate was immunoblotted using an antibody that specifically recognizes phosphorylated IKKα/β (ser180/181). As expected, ORF2 expression did not modulate the levels of pIKK α/β (Fig. 4.2.1.5, lane 2 and 3). Aliquots of the lysate were
immunoblotted with anti-calnexin antibody to ensure equal loading (lower panel). From the above experiments it was confirmed that ORF2 did not inhibit IκBα phosphorylation.

Fig. 4.2.1.5 Level of phospho IKK α/β in ORF2 expressing cells. pSGI only or full length ORF2 (pSGI ORF2, lane 2 and 3) expressing cell lysate was immunoblotted with phospho IKKα/β (ser 180/181) antibody (upper panel) or, calnexin antibody (Lower panel). 2μg & 4μg denotes the respective amount of DNA transfected. 4μg of pSGI DNA was transfected.

4.2.2 ORF2 protein interferes with IκBα ubiquitination.

Proteasomal degradation of IκBα is preceded by its ubiquitination, which occurs by the association of phosphorylated IκBα with the SCFβTRCP complex (Hayden, 2004). In order to check whether ORF2 inhibits IκBα ubiquitination, we checked for the levels of ubiquitinated IκBα in ORF2 expressing cells. Mock or ORF2 transfected cells were treated with MG132 for 2 hours, IκBα was immunoprecipitated and western blotting was done using anti-ubiquitin antibody. The levels of ubiquitinated IκBα were found to be significantly decreased in full-length ORF2 expressing cells as compared to control cells (Fig. 4.2.2.1 top panel, lane 1 and 2). A similar effect (lane 3) was observed following expression of a signal sequence deleted mutant of the ORF2 protein (Δ35) that constitutively localizes to the cytoplasm (Zafullah et al., 1999).
Results

Fig. 4.2.2.1 Level of ubiquitinated IKBα in ORF2 expressing cells. Mock (lane1), full-length ORF2 (fl, lane2) and Δ35 ORF2 (Δ35, lane 3) expressing cells were treated with MG132 for 2 hours prior to lysis, immunoprecipitated with anti-IKBα antibody and immunoblotted with anti-ubiquitin antibody (upper panel). Same blot was stripped and blotted with IKBα antibody (middle panel). An aliquot of the lysate was immunoprecipitated with anti-ORF2 antibody (lower panel).

Since IKBα ubiquitination was found to be directly inhibited by ORF2 expression, we reasoned that the ORF2 protein may interfere with assembly of the IKBα ubiquitination machinery. Hence, we checked the effects of the ORF2 protein, if any, on IKBα association with the different subunits of the SCF^TRCP complex by coimmunoprecipitation assay.

Fig. 4.2.2.2 Effect of ORF2 association on the assembly of SKP1-CUL1-IKBα ubiquitination complex. Mock (pSGI, 3μg DNA, lane 1) or full-length ORF2 (1, 2, 3 μg DNA, lane 2, 3, 4 respectively) transfected cells were labeled with ^35S labeling mix, immunoprecipitated with IKBα and aliquots of sample were immunoblotted with SKP1.
(1\textsuperscript{st} panel), CUL1 (2\textsuperscript{nd} panel) and IKB\(\alpha\) (3\textsuperscript{rd} panel). An aliquot of lysate was immunoprecipitated with anti-ORF2 antibody and radioactive bands were detected by fluorography (4\textsuperscript{th} panel). Equal amount of DNA was transfected in each sample by adjusting the total concentration with empty vector.

The expression of ORF2 was found to inhibit the association of IKB\(\alpha\) with SKP1 (Fig. 4.2.2.2, top panel) and CUL1 (2\textsuperscript{nd} panel) in a dose-dependent manner (compare lane 1 with lanes 2, 3 and 4). Aliquots of the sample were immunoblotted with anti-IKB\(\alpha\) antibody to check the levels of IKB\(\alpha\) (3\textsuperscript{rd} panel). Aliquots of the lysate were immunoprecipitated with anti-ORF2 antibody to check the expression of ORF2 (4\textsuperscript{th} panel). We further checked whether the ORF2 protein expressed in these cells inhibited the association of IKB\(\alpha\) with the F-box protein \(\beta\) TRCP. The ORF2 and myc tagged \(\beta\) TRCP co-expressing cell lysate was immunoprecipitated with anti-myc antibody and immunoblotted using anti-IKB\(\alpha\) antibody. ORF2 expression was found to inhibit IKB\(\alpha\) association with full-length \(\beta\) TRCP when compared to control cells (Fig. 4.2.2.3, lane 3 and 2, respectively). However, IKB\(\alpha\) association with an F-box deleted mutant \(\beta\) TRCP (\(\Delta F-\beta\) TRCP) was found to be unaffected despite the presence of ORF2 (lane 5). Same

<table>
<thead>
<tr>
<th>IP sample</th>
<th>IKB(\alpha)</th>
<th>Myc</th>
<th>Myc</th>
<th>Myc</th>
<th>Myc</th>
<th>ORF2</th>
<th>ORF2</th>
<th>ORF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\Delta)TRCP + ORF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\Delta F)TRCP + ORF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta) TRCP + ORF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4.2.2.3 Association of IKB\(\alpha\) with \(\beta\) TRCP. Huh7 cells expressing the indicated protein were immunoprecipitated with the respective antibodies and immunoblotted with anti-IKB\(\alpha\) antibody (upper panel). Same blot was stripped, cut into halves and immunoblotted with anti myc (lower pane, lane 1-5) or anti ORF2 (lane 6-8) antibodies. Size of ORF2 is larger than that of \(\beta\) TRCP, however the two figures has been aligned such that it appears parallel.
Results

blot was stripped and reprobed with anti myc antibody to check the expression of full length and ΔF-β TRCP (Fig. 4.2.2.3, lower panel, lane 1-5). As expected mock transfected cells did not show any β TRCP band and level of full length and ΔF-β TRCP appeared equal in corresponding lanes. Other half of the blot was immunoblotted with anti ORF2 antibody to check the expression of ORF2 protein (Fig. 4.2.2.3, lower panel, lane 6-8).

β TRCP is the F-box receptor of the SCFβTRCP complex that simultaneously interacts with IκBα through the WD40 motif (present in ΔF-box β TRCP) and SKP1, CUL1, and other subunits through the F-box motif. Since the presence of ORF2 could inhibit IκBα association with full-length β TRCP but not with ΔF-β TRCP, we postulated that the ORF2 protein either interacts with IκBα and sequesters it away from β TRCP or interacts with other subunits of the SCF complex thereby modulating β TRCP binding to IκBα, or it binds to β TRCP itself and blocks the formation of the SCF complex. We thus systematically checked the association of ORF2 with the above components. Initially, we tested the possibility of ORF2 interacting with IκBα using a coimmunoprecipitation assay. No interaction was detected between ORF2 and IκBα (Fig. 4.2.2.3, lane 6). The possibility that β TRCP over expression might be promoting ORF2 association with IκBα was ruled out by testing ORF2 and IκBα association in full-length or ΔF-β TRCP overexpressing cells (lane 7 and 8). Lane 1 shows the level of IκBα in control cells. Subsequently, we checked whether ORF2 interacts with SKP1 and CUL1. The ORF2 protein did not associate with either SKP1 or CUL1 (Fig. 4.2.2.4, lane 1 and 2) under conditions where IκBα associated with these proteins (lane 3).

Fig. 4.2.2.4 Test for interaction of ORF2 with SKP1/ CUL1 subunits. Mock (lane 3) or full-length ORF2 (lane 1 and 2) expressing cells were immunoprecipitated with anti-IκBα antibody, pre-immune serum (PS), or anti-ORF2 antibody respectively and aliquots of the lysate was immunoblotted with SKP1 (upper panel) and CUL1 (middle panel).
Results

Same blot was air dried and exposed to X-ray film to check for ORF2 expression (lower panel).

We then tested whether ORF2 directly associated with β TRCP. Lysates of cells expressing myc tagged β TRCP and ORF2 were coimmunoprecipitated and ORF2 was found to associate with full-length β TRCP (Fig. 4.2.2.5, top panel). The same blot was air-dried and the expression of ORF2 was checked (4th panel). We subsequently used the F-box deleted mutant β TRCP (ΔF-β TRCP) and F-box only mutant β TRCP (F-box β TRCP) and checked for their association with the ORF2 protein. However, neither of these was found to coprecipitate with the ORF2 protein (2nd and 3rd panels respectively). The 3rd lane in each panel shows the expression of the respective plasmids as judged by immunoprecipitation using anti-myc antibody.

Fig. 4.2.2.5 ORF2 association with wild type β TRCP. Mock (lane 1), full-length ORF2 + full length β TRCP (lane 2), or full-length β TRCP alone (lane 3) expressing cells were labeled with $^{35}$S promix, lysate pre-cleared and immunoprecipitated with the anti-ORF2 antibody and immunoblotted with anti-Myc antibody (1st panel, fl β TRCP). Same blot was air dried and exposed to X-ray film to check for ORF2 expression (4th panel). 2nd and 3rd panel represent samples processed as above barring that ΔF box β TRCP and F box β TRCP was transfected respectively in place of full-length β TRCP.
Results

Since none of the β TRCP mutants were able to interact with ORF2, we reasoned that ORF2 association with β TRCP might be specific for the overall conformation of the SCF complex, or the former might be interacting with other non-significant or overlapping motifs of the F-box protein β TRCP. Also, the possibility exists that the ORF2 protein might be associating with all the F-box proteins through some common motif or modular structure. In order to check this possibility, we looked for the association of ORF2 with another F-box protein, SKP2. SKP2 is known to be the receptor for a wide range of proteins for ubiquitination like p27, cyclin D, Notch, myc, E2F1 etc. (Jackson & Eldridge, 2002). We could not detect any significant interaction between ORF2 and SKP2 as judged by coimmunoprecipitation assay (Fig. 4.2.2.6, 1st panel, lane 2). In order to ensure that SKP2 was indeed functional in these experiments the same blot was stripped and reprobed with anti-myc antibody. Endogenous SKP2 was able to coprecipitate endogenous c-myc (Fig. 4.2.2.6, middle panel, lane 3). As a positive control for coimmunoprecipitation, cells were transfected with human c-myc expression plasmid and immunoblotted with anti-SKP2 antibody (4th lane). The same blot was air dried and the autoradiogram was checked for expression of ORF2 protein (3rd panel). These experiments proved that ORF2 specifically interacts with the F-box protein β TRCP.

![Fig. 4.2.2.6 Testing the association of ORF2 with SKP2.](image)

Mock (lane 1), full-length ORF2 (lane 2 and 3), c-myc (lane 4) expressing cells were immunoprecipitated with the indicated antibodies and immunoblotted with anti-SKP2 antibody (1st panel) or Myc antibody (2nd panel). Same blot was air dried and exposed to X-ray film to check for ORF2 expression (3rd panel).
Results

We next checked whether the signal sequence deleted mutant ORF2 (Δ35 ORF2) and a C-terminal KDEL fused mutant ORF2 (KDEL-ORF2), that acts as an ER resident protein was capable of interacting with β TRCP. As seen in Fig. 4.2.2.7, upper panel, both of these mutant proteins were capable of associating with β TRCP. The lower panel shows the expression of the ORF2 protein in the same blot.

![Fig. 4.2.2.7 Association of ORF2 mutants with β TRCP.](image)

Fig. 4.2.2.7 Association of ORF2 mutants with β TRCP. Full-length ORF2 (fl, lane 1), Δ35 ORF2 (Δ35, lane 2) or KDEL ORF2 (KD, lane 3) and full-length β TRCP co-expressing cells were immunoprecipitated with anti-ORF2 antibody and immunoblotted with anti-Myc antibody (upper panel). Same blot was air dried and exposed to X-ray film to check for ORF2 expression (lower panel).

Direct interaction between ORF2 and β TRCP was further confirmed using an in-vitro pull-down assay. $^{35}$S labeled β TRCP protein was expressed using a coupled in-vitro transcription translation kit. ORF2 and its mutant proteins were expressed in Huh7 cells and immunoprecipitated using anti-ORF2 antibody or pre immune serum. After washing 4 times with lysis buffer, the ORF2 protein bound to protein-A sepharose beads was mixed with the β TRCP protein, incubated for 4 hours at 4°C and the samples again washed 4 times in lysis buffer. Any β TRCP protein associating with ORF2 was then detected by fluorography. ORF2 as well as its mutants were able to pull down β TRCP (Fig. 4.2.2.8, lane 3, 4 and 5). As a control, an ORF2 transfected sample was immunoprecipitated with pre-immune serum and processed simultaneously (lane 6) or mock translated lysate was incubated with ORF2 protein bound to beads (lane 2). Lane 1 shows the input amount of β TRCP protein used in each reaction mix. To prove that the observed interaction is not an experimental artifact, the ORF3 protein was checked for its
Results

ability to interact with β TRCP. However, no interaction was observed between them (lane 3).

Fig. 4.2.2.8 In-vitro pull down assay showing interaction of ORF2 with β TRCP. Cell lysate expressing full-length ORF2 (Mock, fl, lane 2, 3 and 6 respectively), Δ35 ORF2 (Δ35, lane 4) and KDEL ORF2 (KD, lane 5) was pulled with anti-ORF2 antibody or pre immune serum (PS); mixed with in-vitro translated full-length β TRCP protein (lane 3, 4, 5, 6) or mock translated lysate (lane 2), washed in immunoprecipitation buffer and bound β TRCP protein band was visualized by fluorography. Lane 1 represents total β TRCP protein used in each sample. Lane 7 shows ORF3 expressing cells immunoprecipitated with anti-ORF3 antibody and processed as above.

Since both Δ35 ORF2 and KDEL-ORF2 were capable of interacting with β TRCP, we asked next whether they were also capable of inhibiting NF-κB activity. An NF-κB reporter assay using the different ORF2 mutant constructs revealed that both Δ35-ORF2 and KDEL-ORF2 were capable of blocking NF-κB activity as effectively as the full-length ORF2 (Fig. 4.2.2.9).
Fig. 4.2.29 Inhibition of NF-κB reporter activity by ORF2 mutants. Huh7 cells expressing reporter alone (mock) or along with different ORF2 mutants were assayed for CAT activity as shown in the graph. Y axis represents percentage CAT activity of different samples assuming highest value to be 100%. Data represents ± SEM of 3 independent sets of experiments.

4.2.3 A fraction of the ORF2 protein is dislocated from the ER to the cytoplasm.

As Δ35 ORF2 protein effectively blocked NF-κB activity, it ruled out the possibility of involvement of signals generated in the ER for inhibition of NF-κB activity. β TRCP localizes in the cytoplasm and if ORF2 binding to β TRCP is crucial for NF-κB inhibition, then ORF2 needs to be translocated to the cytoplasm. There are 3 possible ways for an N-linked glycoprotein to get access to the cytoplasm: (i) through retrograde transport from the cell surface (ii) by exploiting the trans-Golgi to the lysosomal transport pathway and (iii) by direct retro-translocation from ER into the cytoplasm. Earlier it has been shown that non-glycosylated ORF2 protein is stably present in the cytoplasm, which may be possible if it is dislocated from the ER by retro-translocation machinery or if ORF2 somehow exploits the anterograde transport pathway to get localized in the cytoplasm. However, the KDEL-ORF2 mutant, that behaves like an ER resident protein was also capable of inhibiting NF-κB activity. Hence, the probability of ORF2 getting retro-translocated to the cytoplasm was more promising. Retro-translocation is a cellular quality control mechanism that selectively eliminates misfolded or improperly assembled protein from the ER by dislocating them to the cytoplasm followed by degradation by the proteasome. This process is described as ER associated degradation (ERAD). However, extra-cellular pathogens have been reported to exploit this pathway to deliver their toxic proteins to the cytoplasm. Thus, I first checked the possibility of full-length ORF2 protein gaining access to the cytoplasm via direct retro-translocation from the ER.

To test this hypothesis, we separated cytoplasmic and membrane fractions from ORF2 expressing cells and examined each fraction for the presence of the ORF2 protein at different time points. ORF2 expressing cells were pulse-labeled for 20 minutes with $^{35}$S-cysteine methionine labeling mix in the presence of monensin (to block anterograde
transport beyond trans-Golgi) and chased for 2 hours in complete medium. Cell lysate was separated into membrane and cytoplasmic (100,000xg supernatant) fractions and immunoprecipitated with anti-ORF2 antibody. In the beginning of the chase period, all the ORF2 protein was found to be accumulated in the membrane fraction (M) with no detectable ORF2 protein in the cytoplasmic fraction (C) (Fig. 4.2.3.1, lane 1 and 2). After a 2hour chase period, a fraction of the ORF2 protein was detected in the cytoplasmic fraction (lane 3).

![Fig. 4.2.3.1 Pulse chase assay showing ORF2 translocation from the ER to the cytosolic fraction.](image)

As a control, one set of ORF2 expressing membrane and cytoplasm fractionated lysate was immunoprecipitated with rabbit pre immune serum. No bands corresponding to the size of ORF2 protein were detected either in membrane or in cytoplasmic fraction (lane 7 and 8) thus proving that the observed bands are specific for the ORF2 protein. However, a strong band was found to migrate below the ORF2 band in all the samples (marked with star) which might represent a non specific protein pulled by protein A sepharose beads. The fact that monensin actually blocked anterograde transport was ensured by checking α1-microglobulin secretion, which was found to be blocked in
Results

presence of the inhibitor (Fig. 4.2.3.2). This proved that the ORF2 protein present in the membrane fraction represents the protein inside the ER compartment.

![Effect of monensin on α1 microglobulin secretion](image)

Fig. 4.2.3.2 Effect of monensin on α1 microglobulin secretion. Huh7 cells were treated with 10μM monensin and labeled with $^{35}$S cysteine/methionine promix for 2 hours. Media containing equal amount of protein was immunoprecipitated with anti-α1 microglobulin antibody, resolved by 10% SDS-PAGE and bands detected by fluorography.

To further prove that ORF2 is dislocated directly from the ER to the cytoplasm, KDEL-ORF2 was expressed in huh7 cells and a pulse-chase experiment followed by cytoplasm/membrane fractionation was done, as described previously for the full-length ORF2 protein. As expected, the KDEL-ORF2 protein was detected in the cytoplasmic fraction after the 2 hour chase (Fig. 4.2.3.1, lane 5). As a control to check cross-contamination between membrane and cytoplasmic fractions, aliquots of the control cell lysate were immunoblotted with anti-calnexin antibody. Calnexin was localized in the membrane fraction only (Fig. 4.2.3.3) ruling out the possibility of cross-contamination between the two fractions. Hence, it was clear that ORF2 was capable of retro-translocating from the ER into the cytoplasm.
Results

**Fig. 4.2.3.3 Calnexin and PDI immunoblot showing purity of membrane fraction.** Mock cell lysate was processed simultaneously and immunoblotted with anti-calnexin (upper panel) or anti PDI (lower panel) antibody. M denotes membrane and C denotes cytoplasmic fraction.

In normal course, retro-translocated substrates display a very short half-life in the cytoplasm since they are very efficiently degraded by the 26S proteasome (Tsai et al., 2002). However, the ORF2 protein was stably present in the cytoplasm even after the 2 hour chase period. Hence we subsequently designed experiments to check if ORF2 was a substrate of the 26S proteasome.

A pulse-chase assay was conducted to determine the half-life of the ORF2 protein. Immunoprecipitation, using anti-ORF2 antibody, of pulse-labeled (20 minute) ORF2 expressing cell lysate at different time points indicated its half-life to be approximately 5-6 hours (Fig. 4.2.3.4). At 8 hours of chase period, all of the pulse labeled ORF2 protein was found to be completely degraded.

**Fig. 4.2.3.4 Pulse chase assay showing half life of ORF2 protein.** ORF2 expressing cells were pulse labeled for 20 minutes with $^{35}$S cysteine/methionine promix, chased for the indicated time period in complete medium, immunoprecipitated with anti-ORF2 antibody and radioactive bands were visualized by fluorography. Band intensities were quantified using NIH Image program and graph plotted. Band intensity at zero time point was treated as 100%.
Results

Next, we checked the effect of different lysosomal and proteasomal inhibitors on the stability of the ORF2 protein. At 8 hours of chase, the proteasome inhibitor (MG132) had no effect (Fig. 4.2.3.5, lane 3), however of the different lysosomal proteases, acid protease (NH₄Cl and pepstatin) and serine protease (aprotinin) inhibitors were able to stabilize approximately 30-50% of the total protein (lane 5, 6 and 7), whereas the cysteine protease inhibitor (chloroquine) had no effect on the stability of the ORF2 protein (lane 4).

![Graph showing effect of protease inhibitors on ORF2 protein stability](image)

**Fig. 4.2.3.5 Effect of different protease inhibitors on the half life of ORF2 protein.** ORF2 expressing cells were pulse labeled for 20 minutes with ³⁵S promix, chased for 8 hours in complete medium in the presence of respective inhibitors, immunoprecipitated with anti-ORF2 antibody and radioactive bands were visualized by fluorography. Band intensities were quantified using NIH Image program and graph plotted. Data represents ± SEM of 3 independent sets of experiments.

The above observation suggested that ORF2 degradation was independent of proteasomal activity. This observation was further confirmed by checking for ubiquitination of the ORF2 protein since normally ubiquitination serves as the signal for substrate recognition by the proteasome complex. For this purpose, ORF2 expressing cells were treated with MG132 for 3 hours followed by immunoprecipitation using anti-ORF2 antibody and immunoblotting with anti-ubiquitin antibody. We were unable to detect any ubiquitinated species of the ORF2 protein (Fig. 4.2.3.6, lane 4). The same blot
Results

was air dried and exposed to X-ray film to check the expression of ORF2 (Fig. 4.2.3.6, lower panel). Lane 3 shows 30μg of total cell lysate used as a positive control for the ubiquitin antibody.

![Image]

**Fig. 4.2.3.6 Testing ubiquitination of ORF2 protein.** $^{35}$S promix labeled Mock (lane 1), ORF2 (lane 2 and 4) expressing cells were immunoprecipitated with anti-ORF2 antibody and immunoblotted with anti-ubiquitin antibody. Bands were detected by ECL method. 3rd lane represents total cell lysate. All samples were maintained with MG132 for 3 hours prior to lysis. PS denotes pre-immune serum immunoprecipitated sample. Lower panel shows ORF2 expression in the same gel.

Thus, it was confirmed that ORF2 protein is not a substrate of proteasome and hence the classical ERAD pathway is not responsible for its degradation.

4.2.4 ORF2 protein exploits the ERAD pathway to enter the cytoplasm and inhibit NF-κB activity.

To gain further insight into the mechanism of retro-translocation of the ORF2 protein, we utilized many known biochemical and dominant negative inhibitors of the ERAD pathway and monitored ORF2 appearance in the cytoplasmic fraction by pulse-chase analysis. Tunicamycin is known to inhibit glycosylation of ER proteins thereby interfering with their interaction with lectin binding chaperones, which aid in glycoprotein folding. In a 2 hour chase of the ORF2 protein in tunicamycin treated cells,
no ORF2 protein was detected in the cytoplasmic fraction, indicating that ORF2 dislocation was dependent on the glycosylation status of the ORF2 protein (Fig. 4.2.4.1, lane 3 and 4). The fact that tunicamycin was active was reflected by the faster migration of ORF2 as a single band (lane 1).

![Fig. 4.2.4.1 ORF2 translocation assay in presence of different inhibitors of glycosylation pathway.](image)

Fig. 4.2.4.1 ORF2 translocation assay in presence of different inhibitors of glycosylation pathway. Full-length ORF2 expressing cells were pulse chased for 2 hours in the presence of vehicle only (lane 1 and 2) or respective inhibitors (lane 3-8), membrane and cytoplasmic fractions were isolated and immunoprecipitated with anti-ORF2 antibody; radioactive bands were visualized by fluorography (upper panel). Star denotes a non specific band pulled by ORF2 antibody. M denotes membrane and C denotes cytoplasmic fraction.

We subsequently conducted a pulse-chase assay in the presence of castanospermine and kifunensine, the inhibitors of glucosidase II and mannosidase I, respectively. These inhibitors have been shown to block degradation of some ERAD substrates (Tokunaga et al., 2000). Both inhibitors were found to block ORF2 dislocation (Fig. 4.2.4.1, lane 6 and 8, respectively).

Valosin containing protein (VCP)/p97 (mammalian homolog of yeast CDC 48 AAA ATPase) has been shown to be crucial for the translocation of ERAD substrates from the ER lumen to the cytoplasm (Ye et al., 2001) and has been reported to co-precipitate with ERAD substrates (Rabinovich et al., 2002). Further, an ATPase deficient mutant p97 protein has been shown to behave in a dominant negative manner to block ERAD (Ye et al., 2001). In order to check whether ORF2 retro-translocation is mediated in a p97 dependent manner, we looked for interaction of ORF2 with p97 in a coimmunoprecipitation assay. Both full-length ORF2 and KDEL-ORF2 protein was found to interact with p97 (Fig. 4.2.4.2, upper panel, lane 2 and 3). The interaction was further confirmed by immunoprecipitation from an aliquot of the same sample with anti-
Results

p97 antibody and immunoblotting with anti-ORF2 antibody (middle panel). The same blot was stripped and probed using anti-p97 to check for p97 levels (lower panel).

**Fig. 4.2.4.2 Association of ORF2 with p97/VCP.** Full-length ORF2 or KDEL-ORF2 expressing (lane 1, 2 and 3 respectively) or control cell (lane 4) lysate was immunoprecipitated with pre-immune serum (PS, lane 1), anti-ORF2 antibody (lane 2 and 3) or anti-p97 antibody (lane 4) and immunoblotted with anti-p97 antibody (upper panel). Mock (lane 1) or ORF2 (lane 2 and 4) or KDEL-ORF2 (lane 3) transfected cells were immunoprecipitated with anti-p97 antibody (lane 1, 2 and 3) or anti-ORF2 antibody (lane 4) and immunoblotted with anti-ORF2 antibody (middle panel). Same blot was stripped and probed with anti-p97 antibody (lower panel).

Having shown that p97 can co-precipitate ORF2, we checked whether a dominant negative mutant of the p97 protein could block ORF2 retro-translocation. Full-length ORF2 and the KDEL-ORF2 protein were expressed along with myc tagged wild-type (WT-P97) or dominant negative p97 (QQ-p97). The appearance of ORF2 in the cytoplasm was monitored by pulse-chase analysis as described before. Full-length ORF2 expressing cells were also treated with monensin to block anterograde transport. As expected, QQ-p97 could block dislocation of both full-length as well as KDEL-ORF2 (Fig. 4.2.4.3, lane 4 and 8). Hence, it was confirmed from these experiments that a fraction of the ORF2 protein was retro-translocated from the ER to the cytoplasm in a p97 dependent manner.
Fig. 4.2.4.3 ORF2 translocation assay in presence of QQ p97 mutant. Huh7 cells were cotransfected with full-length ORF2 or KDEL-ORF2 and wild type p97 (WT P97) or mutant P97 (QQ P97) expressing plasmids, pulse chased for 2 hours in the presence or absence of monensin respectively. Membrane and cytoplasmic fractions were immunoprecipitated with anti-ORF2 antibody and the radioactive bands were visualized by fluorography.

We next asked whether ORF2 retro-translocation and its ability to inhibit NF-κB activity were coupled. For this, we conducted an NF-κB reporter assay of ORF2 expressing cells in the presence of different retro-translocation inhibitors. Δ35 ORF2 was used as a control to ensure that these inhibitors per se did not modulate NF-κB activity. As expected, treatment with tunicamycin, castanospermine and kifunensine could restore NF-κB activity to near basal levels whereas treatment of Δ35-ORF2 expressing cells with the same set of inhibitors showed no effect (Fig. 4.2.4.4).

Fig. 4.2.4.4 Effect of different glycosylation inhibitors on NFκB reporter activity in ORF2 expressing cells. NF-κB reporter assay of mock (lane 1) or full-length ORF2 (lane 2, 3, 4 and 5) and Δ35 ORF2 mutant (lane 6, 7, 8 and 9) expressing cells in the presence of respective inhibitors. Data represents ± SEM of 3 independent sets of experiments.
Results

This experiment clearly proved that NF-κB inhibitory activity of ORF2 was dependent on its ability to exploit the ERAD pathway. However, the p97 dominant negative expression construct could not be used to monitor NF-κB activity in ORF2 expressing cells since expression of p97 dominant negative itself could inhibit NF-κB activity significantly.

4.2.5 The ORF2 protein down regulates the expression of NF-κB targets.

NF-κB is known to regulate the transcription of a large number of genes during different cellular conditions. One of the most critical events for initiation of immune response against viral antigens is the presentation of viral peptide epitopes over the infected cell surface, which can then be recognized by cytolytic T cells. The viral antigens are presented through their association with the major histocompatibility complex-I (MHC-I). As MHC-I is a known target of NF-κB, we checked expression levels of the MHC-I heavy chain in ORF2 expressing cells stimulated with bacterial lipopolysacharides (LPS). EGFP (lane 1), full-length ORF2 (lane 2) or Δ35 ORF2 transfected cells were treated with LPS for 45 minutes and total cell lysate was immunoprecipitated and immunoblotted with anti-MHC-I antibody (Fig. 4.2.5.1, upper panel). Protein level of MHC-I was found to be decreased in both full-length and Δ35 ORF2 expressing cells in comparison to EGFP expressing cells. An aliquot of the lysate was immunoblotted with anti-calnexin antibody to ensure equal loading of the sample (lower panel).

Fig. 4.2.5.1 Protein level of MHC-I heavy chain in ORF2 expressing cells. EGFP, full-length (fl) or Δ35 ORF2 (lane 1, 2 and 3 respectively) expressing cell lysate was
Results

immunoprecipitated and immunoblotted with anti-MHC-I antibody (upper panel). An aliquot of the lysate was immunoblotted with anti-calnexin antibody (lower panel).

Levels of the MHC-I protein were further checked by an indirect immunofluorescence assay (Fig. 4.2.5.2).

![Immunofluorescence assay showing surface as well as intracellular protein level of MHC I heavy chain.](image)

Fig. 4.2.5.2 Immunofluorescence assay showing surface as well as intracellular protein level of MHC I heavy chain. Mock (I, II, V, VI) or ORF2 (III, IV, VII, VIII) transfected huh7 cells were stained with anti-ORF2 and MHC-I antibody followed by secondary staining with anti-rabbit FITC and anti-mouse Texas red. Permeabilization (V to VIII) was done by incubating cover slips in 0.01% saponin for 30 minutes. Images were acquired using the Nikon TE 2000u fluorescence microscope.
Results

Surface expression of MHC-I was checked in mock transfected non-permeabilized cells stained with anti-MHC-I heavy chain antibody (image II). The same cells were also stained with anti-ORF2 antibody which acts as a negative control (image I). MHC-I was found to be distributed all over the cell surface. However, in ORF2 expressing cells, no significant MHC-I surface staining was observed (image IV). Similarly, in permeabilized cells, intracellular MHC-I levels were found to be reduced (image VIII) in comparison to the control (image VI). 200 cells were counted for each sample and the levels of MHC-I down regulation were scored. Only those cells showing prominent MHC-I down regulation were scored as positive. As shown in Fig. 4.2.5.3, an average of 133 and 129 non-permeabilized and permeabilized cells respectively, were found to be expressing ORF2 and all of them showed a clear cut down regulation of MHC-I expression.

<table>
<thead>
<tr>
<th>Mock Transfected</th>
<th>ORF2 Transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/o ORF2 staining</td>
</tr>
<tr>
<td>MP</td>
<td>0</td>
</tr>
<tr>
<td>p</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4.2.5.3 Quantitative estimation of MHC-I protein level in ORF2 expressing cells. Statistical analysis of ORF2 mediated down regulation of MHC-I protein expression. Mock or ORF2 transfected cells were stained with Texas red (MHC-I staining) or FITC (ORF2 staining) and the level of MHC I was detected by fluorescence microscopy. A total of 200 cells were counted for each sample and out of that ORF2 expressing cells were scored for the down regulation of MHC-I. Only cells showing significantly decreased fluorescence intensity of MHC-I was counted as positives. Data shown is ± SEM from 3 independent sets of experiments.

Finally, NF-κB recruitment to the MHC I promoter was checked in LPS stimulated ORF2 expressing cells by a chromatin immunoprecipitation (CHIP) assay. Immunoprecipitation was conducted using antibody specific for the p65 subunit of the NF-κB complex. As seen in Fig. 4.2.5.4 first panel, EGFP expression did not alter p65
recruitment to MHC-I promoter (1st panel, lane 3 and 5). However, full-length or Δ35-ORF2 expression decreased p65 recruitment to MHC-I promoter. 30% of the total lysate was used as input in each sample. As a control to check whether the observed phenomenon was specific for NF-κB, aliquots of the lysate were immunoprecipitated with anti-SP1 antibody (specific for SP1 transcription factor, which also binds to MHC-I promoter) and amplified the DNA using same set of primers. As expected, SP1 recruitment to MHC-I promoter was not altered in ORF2 expressing cells (Fig. 4.2.5.4, 3rd panel, lane 9-14). I also checked p65 recruitment to interleukin 8 (IL-8) promoter which is also a target of NF-κB. CHIP assay was conducted using anti-p65 antibody which revealed a similar pattern as observed for the MHC-I promoter (Fig. 4.2.5.4, 2nd panel). This confirmed that ORF2 expression indeed inhibited NF-κB translocation to the nucleus and activation of its cognate targets.

![CHIP assay](image)

Fig. 4.2.5.4 CHIP assay showing recruitment of p65 and SP1 to MHC I promoter and p65 recruitment to IL-8 promoter. PCR amplification of immunoprecipitated chromatin using respective antibodies. Cells were transfected with EGFP, full-length or Δ35 ORF2 (GFP, fl, Δ35) expression plasmids. 48 hours post-transfection, cells were formalin fixed and pursued for CHIP assay. Lane 7 shows EGFP expressing cells immunoprecipitated with rabbit pre-immune serum. Lane 8 shows mock immunoprecipitation using p65 antibody. 30% of the pre-immune serum
immunoprecipitated supernatant was used as input in each reaction. 1st and 3rd panels show PCR using MHC-I primers. 2nd panel shows PCR using IL-8 primers.

4.2.6 The ORF2 protein upregulates expression of ER chaperones.

Preliminary experiments described earlier demonstrated that the ORF2 protein upregulated ESRE-CAT activity, indicating that the latter induced ER stress. This observation was further verified using a GRP 94 promoter driven CAT reporter. Expression of full-length ORF2 and KDEL-ORF2 was found to activate the GRP94 promoter (Fig. 4.2.6.1). As expected, Δ35-ORF2 was unable to activate the promoter owing to the fact that it is a cytoplasm localized protein (Fig. 4.2.6.1). As negative and positive controls for promoter regulation, reporter transfected cells were treated with cycloheximide and tunicamycin respectively (Fig. 4.2.6.1).

![Graph showing effect of different ORF2 mutants on GRP 94 promoter activity](image)

**Fig. 4.2.6.1** Effect of different ORF2 mutants on GRP 94 promoter activity. Huh7 cells were transfected in duplicate with GRP 94 reporter construct alone or along with full-length ORF2 or its mutant expression constructs (Δ35 and KD, respectively) and CAT activity was assayed by measuring acetylation of 14C chloramphenicol. CAT activity was quantified and graph plotted assuming highest activity as 100%. Data represents ± SEM of 3 independent sets of experiments.
Results

Upregulation of GRP 94 promoter activity was reflected at the protein level too. Protein levels of chaperones like GRP 94 and protein disulfide isomerase (PDI) were found to be upregulated in ORF2 expressing cells (Fig. 4.2.6.2, 1st and 2nd panel respectively). However, protein levels of calnexin were not altered (3rd panel). Actin was used as a loading control (4th panel).

Fig. 4.2.6.2 Protein level of different ER chaperones in ORF2 expressing cells. Mock (lane 1) or full-length ORF2 (lane 2) expressing cell lysate was immunoblotted with anti-GRP 94 (1st panel), anti-PDI (2nd panel), anti-calnexin (3rd panel) and anti-actin (4th panel) antibody.

Since ORF2 bears 23 cysteine in the central 250 amino acids region, up regulation of PDI suggested that the former might be getting misfolded in the ER. This hypothesis was further verified by monitoring the duration of ORF2 interaction with the lectin binding chaperon calnexin. Calnexin is known to bind nascent glycoprotein and assist in their folding. Only properly folded substrates can be released from the calnexin cycle to move beyond the ER. Normal proteins are known to interact with the calnexin cycle for a very short duration. However, misfolded or incompletely assembled proteins remain in the calnexin cycle until they attain proper conformation (Parodi, 2000.). To monitor ORF2 interaction with calnexin, cells were pulse-labeled for 20 minutes and chased for 1 or 2 hours in complete medium. Cell lysate were immunoprecipitated with anti-calnexin antibody followed by a second round of immunoprecipitation with anti-ORF2 antibody. Mock transfected samples were not subjected to the 2nd immunoprecipitation (Fig. 4.2.6.3, lane 2). A significant amount of ORF2 protein was found to associate with
calnexin after 1 hour of chase (lane 3). Even after a 2hr chase, detectable amounts of ORF2 protein were found to be associated with calnexin (lane 4). Lane 1 shows ORF2 associated with calnexin at the start of the pulse-chase experiment. This data clearly suggests that the ORF2 protein gets misfolded in the ER.

Fig. 4.2.6.3 Pulse chase assay showing prolonged association of ORF2 with calnexin. Mock (lane 2) or full-length ORF2 transfected (lane 1, 3 and 4) cells were pulse labeled with $^{35}$S promix for 20 minutes and chased for indicated time period. Control cell were immunoprecipitated with anti-calnexin antibody only. ORF2 expressing cell lysate was immunoprecipitated with anti-calnexin antibody followed by 2$^{nd}$ immunoprecipitation with ORF2 antibody. Samples were resolved by 8% SDS-PAGE and bands were detected by fluorography.

Thus, in summary, the ORF2 protein gets misfolded in the ER, mimics ER stress and exploits the ERAD pathway to gain access into the cytoplasm to inhibit NF-κB activity leading to down regulation of NF-κB targets.