Viruses are known to exploit the host cell machinery for their benefit during different stages of their life cycle within the infected host. One of the major challenges for a virus during the early stages of infection is to escape recognition by the host immune system. Viruses have adopted many novel strategies to evade the host immune response or to create an immune suppressed environment. An earlier study in our laboratory has demonstrated that the ORF3 protein of Hepatitis E virus expedites the secretion of α1 microglobulin, an immuno-suppressant molecule. Based on this observation, we proposed that enhanced secretion of α1 microglobulin may help maintain an immuno-suppressed milieu around the infected hepatocyte (Tyagi et al., 2004 J. Biol. Chem. 279:29308-29319). In the present study, we discovered that the ability of the ORF3 protein to expedite α1 microglobulin secretion is attributed to the PSAP motif present at the C-terminus of the former. The ORF3 protein was able to associate with the tumor susceptibility gene 101 (TSG101) through the PSAP motif. Further, a PSAP motif mutated ORF3 protein was unable to associate with TSG101 and also lost its ability to enhance the secretion of α1 microglobulin. In addition, the ORF3 protein was found to associate simultaneously with TSG101 and α1 microglobulin since all 3 of them were co-precipitated as a ternary complex. Finally, a dominant negative mutant of the VPS4 protein was shown to block the enhanced α1 microglobulin secretion in ORF3 expressing hepatocytes. These results suggest a mechanism by which the ORF3 protein exploits the endosomal sorting machinery to enhance the secretion of an immuno-suppressant molecule (α1 microglobulin) from the cultured hepatocytes.

The endosomal protein sorting pathway is a highly dynamic system in cells. It coordinates protein transport from both the biosynthetic and endocytic pathways and hence plays an important role in sorting proteins destined to be degraded, recycled or secreted. The machinery responsible for this tightly regulated protein sorting process in the endosomal pathway consists of a series of proteins including three protein complexes - ESCRT-I, II and III (endosomal sorting complex required for transport) (1). In recent years, several RNA viruses have been demonstrated to re-program the host endosomal protein sorting machinery towards their benefit (2). For example, viruses such as HIV-1 and Ebola do not encode their own machineries for budding. Instead they bear two proline-rich motifs in their late domain: P(S/T) AP and PPXY (where X is any amino acid), which have been proved to be essential for viral budding (3, 4). The P(S/T) AP motif of HIV-1 Gag late domain has been shown to bind to TSG101, in a manner analogous to that of HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), and hijacks the ESCRT complex for virus budding (5). Similarly, the VP40 gene product of Ebola virus binds TSG101 via the PSAP motif (6) for virus budding. TSG101 is a central mediator of the endosomal trafficking process. It associates with HRS through the PSAP motif (HRS recognizes ubiquitinated cargo in normal cellular context). This association enables the cargo to be targeted to the late endosomes and subsequently get sorted into lysosomes or recycled back. Therefore, TSG101 has been an attractive target for viruses to exploit the host endosomal trafficking machinery to bud off the host cell.

Like P(S/T)AP, the PPXY motif present in the late domain of capsid proteins has also been shown to be important for viral budding. This motif is a preferred binding motif for WW domains found in the Nedd4 family of HECT domain containing ubiquitin ligases (7). Both HIV-1 Gag and VP40 protein of Ebola virus binds NEDD4 and get ubiquitinated which aid in
recognition by HRS/TSG101 and virus budding (8, 9).

Another crucial function for which viruses hijack the endosomal sorting machinery is to subvert the immune surveillance process. An example of this phenomenon is provided by the k3 gene product of Kaposi sarcoma associated Herpes virus. The k3 protein is an ubiquitin ligase that ubiquitinates newly synthesized MHC class I molecules, thus targeting them for lysosomal degradation in a TSG101 dependent manner (10). Similarly, the M153R gene product of myxoma virus ubiquitinates CD4 molecules and causes its lysosomal degradation (11). Therefore, viral exploitation of the endosomal sorting machinery for immune evasion appears to be a prevalent strategy.

In this report we provide yet another example of a viral protein exploiting the endosomal sorting pathway that may help create an immuno-suppressed milieu surrounding the infected cell. The ORF3 protein of Hepatitis E virus was observed to associate with TSG101 through the PSAP motif present at its C-terminus and expedite the secretion of an immuno-suppressant molecule called α1 microglobulin (α1m). α1m is a member of the lipocalin superfamily, found both in blood and in most tissues (12). Its amino acid sequence shows 80% homology from rodents to humans (13). α1m is a general immuno-suppressant and inhibits the antigen induced proliferation of peripheral lymphocytes, IL-2 production of T cells and the migration and chemotaxis of granulocytes (14, 15, and 16). Previous studies done in our laboratory have shown that ORF3 interacts with α1m and expedites its export and secretion from hepatocytes. This enhanced transport of α1m in ORF3 expressing cells was sensitive to both brefeldin A and monensin. The above observation coupled with sub-cellular co-localization studies further confirmed that the transport is expedited from the trans golgi region of hepatoma cells (17).

In an effort to understand the mechanism by which the ORF3 protein might be expediting the secretion of α1m, we found the former to interact with TSG101. A PSAP mutant form of the ORF3 protein was unable to associate with TSG101; neither could it expedite the secretion of α1m. Moreover, a dominant negative mutant of the VPS4 protein could also block the enhanced α1m secretion in wild-type ORF3 expressing cells, further confirming the above observation. Further, ORF3 was found to simultaneously associate with both α1m and TSG101 in a ternary complex from hepatocyte extracts. All these observations suggest that the ORF3 protein associates with TSG101 in a manner analogous to late domain bearing proteins and expedites the secretion of α1m. The possible significance of this phenomenon during viral pathogenesis is discussed.

**EXPERIMENTAL PROCEDURES**

*Plasmid constructs—* Full-length ORF3 gene of HEV was excised from pSGORF3 (18) using NdeI and PstI and cloned into the pGBK7 vector cut with the same. pAS2 1-79 ORF3 and pAS2 81-123 ORF3, Mexican ORF3 and Ser80Ala ORF3 have been described earlier (19, 20). All DNA manipulations were carried out as described by Sambrook *et al.* (21). pGADT7 ORF3 RLIA construct was prepared by two rounds of cloning. First, oligo bearing the mutant sequence were cloned into pGADT7 at the Clal/Xhol site. Upper strand and lower strand sequences are: 5’CGATTGATCGCTCCGCTGCCTCACGTCGTAGACCTACCACAGCTGGGCCGCGGTAAAC 3’ and 5’TCGAGTTAGCGCGGCCCCAGCTGTGGTAGGTCTACGACGTGAGCAGCGGAGCGATCAAT 3’ respectively. This construct was denoted as pGADT7 309-370 RLIA ORF3. Next, ORF3 sequence from amino-acids 1 to 308 was PCR amplified using pSGI ORF3 as template. The PCR product was excised and ligated into SmaI/ClaI site of pGADT7 309-370 RLIA ORF3 to get pGADT7 309-370 RLIA ORF3. Forward and reverse primer sequences are 5’CCCGGGAGAGCTCGATTC 3’ and 5’ATCGATCCTGGTCGCGCCAAGCG3’ respectively. pGADT7 101, pGADT7 101 M95A, pGBKT7 101, pGBKT7 101 M95A, wild-type and dominant negative VPS4a and 4b were kind gifts from Dr. Wesley I Sundquist (22, 23).

*Yeast two-hybrid techniques—* The Matchmaker GAL-4 based yeast two-hybrid system III (Clontech) was used. Yeast two-hybrid study was performed as described previously (24). Briefly, competent AH109 cells were transformed with respective activation domain and binding domain constructs and cotransformed cells were selected by growing them on Leu’, Trp’ medium for 3 days. Next, for each sample, 10 clones were selected at random and checked for their ability to grow on Leu’, Trp’ and His’ plates with up to 50mM 3-AT. Colonies were assayed for β-galactosidase activity by both

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filter lift and liquid assays as described previously (17).

Cell culture and transfection-- Huh7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum and penicillin, streptomycin. Cells were transfected at 60% confluency with FuGene 6 reagent (Roche Biochemicals) according to manufacturer’s instructions. Cells were transfected in either a 60 mm culture dish (for immunoprecipitation) or a 6-well culture plate on glass cover slips (for immuno-fluorescence microscopy).

Metabolic labeling and immunoprecipitation-- At 44 hour post-transfection cells were washed twice with 1X PBS and starved for 1hour in cysteine/methionine deficient medium and were then labeled with 100µCi/ml of 35S cys/met promix (NEN) for 4hour. After 4hour, labeling media was collected (in case secreted α1m was to be detected). The cells were washed once with 1X PBS and lysed in 500µl immunoprecipitation buffer (20mM Tris-Cl, pH 7.5; 150mM NaCl; 1% Triton X-100; 1mM EDTA; 1mM EGTA; 2.5 mM sodium pyrophosphate; 1mM β-glycerol phosphate; 1mM Na3VO4) supplemented with protease inhibitor cocktail. The lysates were collected and clarified by centrifugation for 10 min at 13,000 rpm. For immuno-precipitation, an equal amount of protein was incubated with 1µg of corresponding antibody overnight at 40C, followed by incubation with 100µl of 10% protein A sepharose suspension for 1hour. Beads were washed three times in the lysis buffer, boiled in 2X SDS dye and resolved on SDS-PAGE followed by immunobohtting or fluoroigraphy.

Immunoblotting--The immuno-precipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA for 1hour and then incubated with respective primary antibodies, again washed and incubated with appropriate HRPO conjugated secondary antibodies. The proteins were then detected by the ECL® (Enhanced chemiluminescence) detection method (Cell Signaling Technology) according to manufacturer’s protocol.

Immuno-fluorescence assay--Immuno-fluorescence staining was done as described by Tyagi et al., (17). The ORF3 protein was stained with anti-mouse Texas red and α1m or TSG101 was stained by anti-rabbit FITC. Nucleus was stained by DAPI where indicated. Images were acquired using a NIKON TE 2000U immuno-fluorescence microscope. Superimposition of images was done using Adobe Photoshop 6.0 software.

RESULTS

ORF3 protein of Hepatitis E virus interacts with TSG101--Earlier studies in our laboratory have proved that the ORF3 protein of Hepatitis E virus (HEV) binds to α1m and expedites its anterograde transport from the trans golgi region of cultured hepatoma cells. The observed phenomenon was sensitive to both brefeldin A and monensin, indicating that the ORF3 protein executes its effect at some step beyond the golgi region (17). We hypothesized that ORF3 might be associating with some crucial intermediate factor of the cellular cargo transport machinery and manipulating the entire pathway towards the benefit of the virus, as has been observed for many other viral gene products. Thus we analyzed the ORF3 protein sequence for any known motifs that might interact with a cellular protein trafficking factor. Interestingly, we observed the presence of a PSAP motif between 104-107 amino acids of the ORF3 protein. A homology search revealed that the PSAP motif is conserved among different isolates of HEV. It was also conserved in avian HEV isolate, which shows maximum diversity from all other HEV isolates (Fig 1). The PSAP motif has earlier been reported to be present in the late domain containing proteins of many RNA viruses such as HIV-1 and Ebola (2). PSAP motif present in these late domain proteins has been shown to interact with the tumor susceptibility gene 101 (TSG101) and usurp the host ESCRT machinery for budding of viral particles. Hence, we tested whether the ORF3 protein too associated with TSG101.

A yeast two-hybrid assay was conducted to check the interaction between ORF3 and TSG101. Results of this assay revealed that a Gal4 DNA-binding domain (BD) or activation domain (AD) fused ORF3 interacted with AD or BD fused TSG101, respectively. In addition, the M95A mutant TSG101 (designated as 101A) that has been well documented to lose its ability to bind the PSAP motif (23) was unable to interact with ORF3 (Fig. 2a). Among the human HEV isolates, the Mexican strain shows maximum diversity from others in the amino acid sequence of ORF3 protein. Thus, we also tested the Mexican ORF3 protein interaction with TSG101 in the yeast
two-hybrid system (BD-MexO3), which showed a positive result. Further, the Mexican ORF3 protein was unable to associate with the M95A mutant TSG101, thus indicating that the observed interaction was a specific property of the former. This result came as no surprise since the PSAP motif was found conserved in the Mexican isolate of the ORF3 protein as well (Fig. 1). As a positive control for the assay, self-association of TSG101, which has been reported earlier (25), was observed in a parallel set of experiments. Co-transformed empty BD/AD vectors and ORF3 co-transformed with M95A mutant TSG101 served as negative controls in the experiment. A quantitative estimation of the relative strengths of interaction between ORF3 and TSG101 in comparison to controls as judged by liquid β-galactosidase assay is also shown graphically in Fig. 2A. As seen in the graph, ORF3 was found to strongly interact with wild type TSG101 which was evident from approximately 7-8 fold more β-galactosidase activity in cells co-transformed with both ORF3 and TSG101. Further, strength of interaction between the M95A mutant TSG101 with ORF3 protein was significantly lower than that of wild-type TSG101 with ORF3 and comparable to that of controls.

To confirm the results obtained from the yeast two-hybrid assay, we performed a co-immunoprecipitation assay to check the association of the ORF3 protein with TSG101 in a mammalian cell environment. Huh7 cells were transfected with pSGI (Fig. 2B, lane1) or pSGI ORF3 (lane2, 3, 4). 44 hour post-transfection, cells were labeled with 100µCi 35S cys/met labeling mix for 4 hour and harvested in immunoprecipitation (IP) buffer. Equal amount of samples were immuno-precipitated using anti-ORF3 or anti-TSG101 antibody. Half of the sample was resolved by 12% SDS-PAGE and immuno-blotted with anti-TSG101 antibody (Fig. 2B, upper panel), the remaining half was resolved on 15 % SDS-PAGE followed by fluorography to detect ORF3 expression (Fig. 2B, lower panel). TSG101 was found to be co-immunoprecipitated with ORF3 antibody (upper panel, lane 2). Similarly TSG101 antibody could co-precipitate ORF3 protein (lower panel, lane4). As a negative control, mock (denoted by M) transfected cell lysate was immuno-precipitated with anti-ORF3 antibody and immuno-blotted with anti-TSG101 antibody (lane 1). In order to further confirm that ORF3 antibody specifically co-precipitated TSG101 protein, one set of lysate was subjected to two rounds of immuno-depletion using anti-ORF3 antibody and the resulting supernatant was again immuno-precipitated using anti-ORF3 antibody and immuno-blotted with anti-TSG101 antibody. As expected, both ORF3 and TSG101 band intensity was significantly reduced (lane 3), thus indicating that both of them existed as a complex in the cell lysate. This experiment confirmed that the ORF3 protein associates with TSG101 in a cellular milieu.

The PSAP motif of ORF3 is essential for interaction with TSG101—In an effort to further prove the involvement of the PSAP motif for interaction with the ORF3 protein; we adopted a site-directed mutagenesis approach. All four amino acids P-S-A-P were modified to R-L-I-A so as to destroy the PSAP motif. Care was taken to minimize nucleotide alteration during mutagenesis. The modified gene was cloned into the yeast two-hybrid vectors as a fusion with the GAL4 activation domain. Expression of the mutant protein was verified using an in-vitro transcription translation system (TNT kit, Promega). Transcription of the PSAP mutant ORF3 gene in the TNT reaction was driven by the T7 promoter located between the GAL4 activation domain and the ORF3 coding sequence, thus producing a HA tagged protein. Wild-type ORF3 in pSGI vector was used as a control. Translation in the TNT system was driven by the protein synthesis machinery present in the reticulocyte lysate. The protein thus produced was radiolabeled with 35S cys/met. Reticulocyte lysates expressing pGADT7, pSGI wild-type ORF3 or pGADT7 PSAP mutant ORF3 protein (ORF3M) were immuno-precipitated using anti-ORF3 antibody and the expression of ORF3 protein was detected by fluorography (Fig. 3A). Both wild-type and ORF3M protein were efficiently expressed (lane 2 and 3) and recognized by the anti-ORF3 antibody. The ORF3M protein migrated at a higher position than the wild-type protein due to the presence of the HA tag attached to the former. Mock translated lysate did not show any band corresponding to the ORF3 protein (lane 1) thus confirming that the observed bands were specific to ORF3 protein.

The ORF3M protein was tested for interaction with TSG101 in a yeast two-hybrid assay. As expected, this protein lost its ability to interact with TSG101 (Fig. 3B). As a further proof, we also tested the ability of two deletion mutants of ORF3 gene to interact with TSG101. These constructs have earlier been used in yeast two-hybrid assays (19). In agreement with our
proposed hypothesis, the ORF3 deletion expressing 1-79 amino-acids of the ORF3 protein did not show interaction with the TSG101 protein; where as the deletion expressing 81-123 amino-acids was capable of interacting with TSG101 as efficiently as the full-length wild-type ORF3 protein (Fig. 3B). However, none of the mutants showed any significant interaction with the TSG101 M95A mutant protein.

We next investigated whether phosphorylation of the ORF3 protein was a determinant factor for its association with TSG101. It has been reported earlier that the ORF3 protein is phosphorylated at a single serine residue located at the 80th amino acid from the N-terminal of the protein. Changing this serine to alanine abolishes phosphorylation of ORF3 (26). We used this S80A ORF3 mutant yeast two-hybrid construct (pAS2 S80AORF3) (20) as a bait to check its interaction with TSG101. The S80A ORF3 protein showed efficient interaction with TSG101 as judged by the histidine prototrophy exhibited by cotransformants, as well as relative liquid β-galactosidase activity (Fig. 3B). Therefore, the interaction of the ORF3 protein with TSG101 is independent of the phosphorylation status of ORF3.

The yeast two-hybrid data was further confirmed by a co-immunoprecipitation assay. The PSAP mutant ORF3 (ORF3M) gene was cloned into the pSGI expression vector (here no epitope tag is present). Expression of the ORF3M protein in Huh7 cells was verified by immuno-precipitation analysis of the transfected cell lysate (data not shown). Next, Huh7 cells were transfected with pSGI (Fig. 3C, M, lane 1), pSGI wild-type ORF3 (O3, lane 2) or pSGI PSAP mutant ORF3 (O3M, lane 3), labeled with 35S cys/met promix and immuno-precipitated using anti-ORF3 antibody followed by immuno-blotting using anti-TSG101 antibody. As expected, the ORF3M protein was unable to associate with TSG101, whereas wild-type could efficiently pull out the TSG101 product (Fig. 3C, upper panel, lane 2 and 3). The lower panel shows the expression of the ORF3 protein in corresponding samples. This observation was further confirmed by reversing the assay condition i.e. detecting the level of ORF3 protein that can be pulled down using anti TSG101 antibody. As seen in figure 3D, TSG101 antibody could effectively pull down the wild type ORF3 protein (lane 3) where as it failed to pull down detectable amount of ORF3M protein (lane2). Lane 1 shows rabbit preimmune serum immunoprecipitated sample as a control to verify the specificity of anti TSG101 antibody. Together, these experiments confirm that the PSAP motif of the ORF3 protein is functionally active and confers upon the ORF3 protein the ability to associate with TSG101.

**Wild-type ORF3 protein co-localizes with the cellular TSG101 protein**—Having proved that wild-type ORF3 and the TSG101 protein could associate with each other in a cellular environment, we subsequently checked whether the two proteins co-localize with each other. Huh7 cells were cultured on cover slips and transfected with the wild-type ORF3 or ORF3M expression constructs. 48 hour post-transfection, cells were fixed and processed for immuno-fluorescence assay. Expression of the ORF3 protein was visualized by staining with Texas red (Fig. 4, panel IV and VII) and that of TSG101 was visualized by staining with FITC (Fig. 4, panel II, V and VIII). As expected, the wild-type ORF3 protein co-localized with TSG101 whereas the ORF3M mutant was unable to do so (Fig. 4, compare panel VI with IX). Effective co-localization resulted in the production of golden yellow color upon superimposition of Texas red over FITC, as seen in the case of wild-type ORF3 protein (panel VI). However, in case of the ORF3M protein, no yellow color was observed (panel IX). In wild-type ORF3 expressing cells, TSG101 was found prominently in the cytoplasm, whereas, in mock-transfected cells the distribution was diffused and a significant fraction was present in the nucleus (panel II). However, in mutant ORF3M expressing cells, no difference was observed from that of mock-transfected cells.

**The PSAP motif of ORF3 is essential for enhanced secretion of α1 microglobulin from ORF3 expressing hepatocytes**—The above experiments confirmed that association with TSG101 is a specific property of the ORF3 protein. Since TSG101 is a key component in assembly of the ESCRT complex, we reasoned that by associating with it, ORF3 might be promoting the assembly of the ESCRT complex which may carry α1m as a cargo, leading to its secretion. This assumption was based on previous reports that association of a viral late domain protein (e.g. - HIV-1 gag) with TSG101 is able to recruit ESCRT machinery to promote virus budding. Thus, we asked whether this property enables the ORF3 protein to expedite the secretion of α1m. To test this possibility, we expressed the ORF3M protein in Huh7 cells and
scoring α1m leaching from ORF3M transfected cells as compared to wild-type ORF3 expressing cells. A representative image is shown in Fig. 5. ORF3 and α1m are stained with Texas red and FITC, respectively. Wild-type ORF3 could efficiently produce the α1m leaching effect as reported earlier (17) (panel AII and AV). ORF3 expressing cells are marked with white arrows and non-transfected cells that behave as controls are marked with red arrow. In cells expressing the ORF3M protein, no α1m leaching effect was observed. The protein level of α1m in the ORF3M expressing cells was comparable to that of non-transfected cells (panel BII and BV). This result clearly indicated that the ability of the ORF3 protein to expedite the secretion of α1m from hepatocytes was a property dictated by the PSAP motif present within the ORF3 protein. A total of two hundred cells were counted for each sample and α1m leaching was scored. This provided a quantitative account of the observed phenomenon (Table 1). Approximately 84% of cells expressing the wild-type ORF3 protein showed the leaching phenomenon. In cells expressing the mutant ORF3 protein, the leaching phenomenon was significantly blocked. However, around 1.5% of cells expressing the ORF3M protein showed leaching. The reason behind this remains unclear. We feel this may be due to the expression of α1m being regulated by some other unknown mechanism.

In order to further confirm the immuno-fluorescence data, we checked the protein levels of secreted α1m from hepatoma cells expressing wild-type ORF3 or ORF3M proteins. Huh7 cells were transfected with pSGI (Fig. 6A, lane 1), pSGI ORF3 (lane 2) or pSGI ORF3M (lane 3). 44 hour post-transfection, cells were labeled with 35S cys/met promix and both the culture medium and cells were harvested. Culture media was immuno-precipitated with anti-α1m antibody and the total cell lysate was immuno-precipitated using anti-ORF3 antibody. As shown in the figure, expression of the wild-type ORF3 resulted in increased α1m protein levels in the medium, whereas, this phenomenon was not observed in samples expressing the ORF3M protein. The graph represents a quantitative estimation of relative band intensities of α1m as recorded from three sets of the same experiment.

Above experiments indicated that the ORF3 protein probably recruits the ESCRT complex by binding to TSG101 so as to enhance the trafficking of α1m from the trans golgi network. In order to further substantiate this hypothesis, we checked α1m secretion in wild-type ORF3 expressing cells co-expressing a dominant negative mutant of VPS4. VPS4 is an AAA family ATPase that functions at the final stage of the endosomal sorting pathway by catalyzing the release of different components from the membrane. An ATP binding mutant of VPS4 is catalytically inactive and thus behaves as a dominant negative mutant against the wild-type VPS4 protein (27, 28). Our reason for co-expressing the VPS4 dominant negative mutant along with wild type ORF3 was that if ORF3 exploits the endosomal sorting pathway to enhance the secretion of α1m, then the VPS4 dominant negative mutant should be able to block it since VPS4 acts downstream of TSG101 in this pathway. To test this hypothesis, Huh7 cells were co-transfected with wild-type ORF3 and wild-type or dominant negative mutant of VPS4 a and b (VPS4a and VPS4b are the 2 isoforms of VPS4 expressed in mammalian cells). Cells were labeled with 35S cys/met promix for 4 hour and the levels of secreted α1m were checked by immuno-precipitation. As shown in Fig. 6A, and the accompanying graph, levels of α1m were found to be significantly reduced in cells expressing the VPS4a dominant negative construct (lane 4 and 5), whereas cells expressing the VPS4b dominant negative construct did not show a strong difference in the levels of secreted α1m protein (lane 6 and 7). Expression of ORF3 in these samples was confirmed by immuno-precipitating the total cell lysate with anti-ORF3 antibody (Fig. 6A, lower panel). Expression of wild-type and dominant negative VPS4a and 4b was checked by visualizing cells under a fluorescence microscope, since these constructs express EGFP and dsRed fused proteins respectively. As a control to check whether α1m secretion in normal course is regulated by VPS4 activity, we simultaneously transfected cells with wild-type or dominant negative mutant constructs of VPS4a and 4b and monitored the levels of secreted α1m. There was minimal change in the levels of α1m in cells expressing VPS4a dominant negative mutant construct (Fig. 6B, lane 2) whereas no significant change was observed in cells expressing VPS4b dominant negative expression construct (Fig. 6B, lane 4). As a normalization control, total cell lysate was immuno-blotted using anti-P38 antibody (Fig. 6B, lower panel). Graph was plotted after normalizing α1m band intensity to that of total...
α was immuno-blotted using anti-ORF3 antibody and half of the lysate possibility, we tried to co-precipitate the ORF3-α1m-TSG101 complex from human hepatoma cells. ORF3 expressing Huh7 cells were labeled with 35S cys/met promix, immuno-precipitated with anti-ORF3 antibody and half of the lysate was immuno-blotted using anti-α1m antibody (Fig. 7, 2nd panel). The other half was resolved on 15% SDS-PAGE followed by fluorogrographic detection of ORF3 protein (Fig. 7, 3rd panel). The α1m blot was stripped and reprobed with anti-TSG101 antibody to check the presence of TSG101 (Fig. 7, 1st panel). As shown in the second panel of the figure, both wild-type ORF3 (O3) and ORF3M (O3M), could associate with α1m. However, ORF3M was unable to co-precipitate TSG101 (Fig. 7, 1st panel, lane 3). The fact that all proteins, ORF3, TSG101 and α1m, were forming a ternary complex was further confirmed by the disappearance of both α1m and TSG101 proteins from samples immuno-depleted with anti-ORF3 antibody (Fig. 7, lane 4). Therefore, we conclude that the ORF3 protein simultaneously binds to both α1m and TSG101 and drives the assembly of the ESCRT complex. This leads to enhanced surface trafficking and secretion of α1m in ORF3 expressing cells.

DISCUSSION

In the present study, we report that the ORF3 protein of Hepatitis E virus associates with TSG101 and exploits the cellular ESCRT machinery to expedite the secretion of an immuno-suppressant protein, α1m, from the human hepatoma cells. This study provides a mechanistic view to our earlier observation that the ORF3 protein interacts with α1m and expedites its transport from the trans Golgi region of hepatoma cells. In addition, this study also uncovers a novel example of a viral protein utilizing the endosomal sorting pathway to enhance the secretion of an immuno-suppressant molecule which may help in viral pathogenesis during its natural course of infection. The fact that the observed phenomenon was not an artifact of the experimental design was ruled out in many ways. For example, a PSAP mutant form of ORF3 protein was unable to associate with TSG101 as well as incapable of expediting the secretion of α1 microglobulin; a dominant negative mutant of VPS4 protein, which acts independently downstream of TSG101 activity in the endosomal sorting pathway, could efficiently block the effect of wild type ORF3. Hence, the observed phenomenon is a specific property of the ORF3 protein.

The ORF3 protein is translated from the smallest ORF of the Hepatitis E virus. Although it is expressed in Hepatitis E patients, the role of ORF3 protein during viral pathogenesis is unknown. HEV per se is a poorly studied virus. It causes acute self-limiting hepatitis in much of the developing world without any serious side effects. Therefore studies related to its replication, pathogenesis etc. has been overlooked for years. In addition to that, lack of a model animal system or efficient in vitro infection system has hampered studies related to its viral life cycle. However, HEV infection in pregnant women has been reported to be associated with a high mortality rate (~ 20%), due to fulminant hepatic failure. Hence it is important to study the life-cycle and pathogenesis of this virus.

ORF3 has earlier been shown to be a phosphoprotein that associates with the cytoskeleton (26). It also interacts with the major capsid protein (ORF2) of the virus (20), based on which, it has been predicted to be a structural component of the virus particle. However, no direct proof exists for this hypothesis. Further, it has been shown to interfere with different cell signaling intermediates leading to increased survival potential of cells expressing the ORF3 protein (29). In an effort to further understand the functional properties of this protein, a human liver library was screen using ORF3 protein as bait in a yeast two-hybrid system in our laboratory. This study identified ORF3 to interact with AMBP (α1 microglobulin bikunin precursor protein) as well as its processed product α1m. Also it was observed that over expression of the ORF3 protein expedited the secretion of α1m from the hepatoma cells, based on which it was proposed that the ORF3 protein may help maintain an immuno-suppressed milieu surrounding the infected hepatocytes (17).
defining a mechanism for this process in this report, we further prove that this phenomenon might be physiologically relevant during the natural course of infection. However further experiments using an infectious virus system need to be done to prove this hypothesis.

Many viral gene products have earlier been shown to bind TSG101, which is a central component of the ESCRT machinery. TSG101 recognition motif (i.e. the PSAP motif) is present in late domains of many RNA virus capsid proteins such as that of HIV, Ebola and Vesicular Stomatitis Virus (2). It has been experimentally proven that the PSAP motif of these proteins recruits the ESCRT machinery by binding TSG101, which is essential for viral budding. However, recruitment of ESCRT machinery for accelerating the transport of cellular proteins by viral factors has not been reported earlier. Hence the ORF3 protein provides the first example of a viral protein that utilizes the ESCRT machinery for accelerating cellular protein trafficking. Nevertheless, viral proteins have earlier been reported to exploit the endosomal sorting pathway to attenuate host immune response. One example includes down-regulation of MHC class I surface expression by the k3 gene product of kaposi sarcoma herpes virus (30). ORF3, though utilizing a different mode of action, appears to produce a similar effect by creating an immuno-suppressed milieu.

TSG101 is known to recognize both ubiquitinated and non-ubiquitinated proteins. Ubiquitination of HIV Gag protein has been shown to enhance its binding with TSG101. On the other hand, VP40 protein of Ebola virus was shown to enhance viral budding both by association with TSG101 through a PSAP motif or independent of TSG101 by the PPXY motif in a ubiquitination dependent manner (31). Ubiquitination of late domain proteins is brought about by the presence of a PPXY motif in them which interacts with the Ned4 family of ubiquitin ligases and gets mono-ubiquitinated. As found in the VP40 gene product of Ebola virus, the ORF3 protein also bears an overlapping PSAP/PPXY motif, though the tyrosine residue is substituted with proline. Bioinformatics analysis suggests that this PPLP motif may bind to WW domain present in Ned4 family of ubiquitin ligases. But we failed to detect a mono-ubiquitinated species of ORF3 protein in human hepatoma cell extracts, despite repeated attempts. Nonetheless, failure might be attributed to limitations of our assay system or instability of the mono-ubiquitinated ORF3 protein. It may also be possible that the event of mono-ubiquitination is spatially and temporally regulated in the case of the ORF3 protein, making it difficult to capture. Other in vitro approaches may help find out whether the PPXP motif is functional in ORF3 protein.

Two isoforms of the VPS4 protein exist in humans, known as VPS4a and VPS4b. Among them, only the dominant negative mutant of VPS4a could effectively block the effect of ORF3 on α₁m. Although we do not have a direct explanation for this observation, it might be possible that ORF3 specifically induces VPS4a-dependent enhancement of α₁m secretion.

One question that persistently strikes our mind from this study is whether expedited α₁m secretion is the only job for which ORF3 protein harbors a PSAP motif. It is important to note here that this motif is conserved among all isolates of human and animal HEV although the PPXP motif is not. In all other RNA viruses, where the PSAP motif is present in the capsid protein, it helps in budding of the virus. Hence, it may be possible that in addition to expediting α₁m secretion, ORF3 acts as an accessory structural protein and its association with TSG101 recruits the ESCRT machinery for efficient budding of progeny HEV particles. Further experiments using a model culture system might clarify this possibility.

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FOOTNOTES

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The abbreviations used are: ORF: open reading frame; aa, amino acids; AD, activation domain; BD, binding domain; 3-AT, 3-Amino-1,2,4-Triazole. TSG101, tumor susceptibility gene 101; α1m, α1 microglobulin; ESCRT, endosomal sorting complex required for transport.
Keywords: Hepatitis E virus, endosomal sorting, HEV ORF3 protein, TSG101, α1 microglobulin, PSAP motif.

FIGURE LEGENDS

FIG. 1. ORF3 amino acid sequence alignment of different HEV isolates. First, second and third columns represent the NCBI accession number, name of different isolates and corresponding amino acid sequences, respectively.

FIG. 2. The ORF3 protein interacts with TSG101. A, Yeast two-hybrid analysis of ORF3 protein interaction with TSG101. L- represents Synthetic Dextrose complete media (SD) lacking Leucine, T- represents SD lacking Trpophane and LTH + 3-AT represents SD lacking, Leucine, Tryptophan and Histidine with 3-Amino 1, 2, 4-Triazole (3-AT) concentrations used. The horizontal bar graph represents relative β-galactosidase units from the liquid β-gal assay. AH109 is the untransformed yeast host strain. BD-TSG101+AD-TSG101 is the positive control cotransformant used in the study. 101A represents the M95A mutant TSG101. B, The ORF3 protein co-precipitates with TSG101 in mammalian cells. Huh7 cells were transfected with empty vector (M, lane 1) or pSGI ORF3 expression construct (lanes 2-4) and labeled with 35S cys/met promix. 48 hours post-transfection, cells were harvested in IP buffer and the cell lysate was immuno-precipitated with anti-ORF3 antibody (lanes 1 and 2) or with anti-TSG101 antibody (lane 4). One set of sample was immuno-depleted with anti-ORF3 antibody (lane 3). Aliquots of the samples were resolved by 10% SDS-PAGE and immuno-blotted with anti-ORF3 antibody (upper panel). The other half of the sample was resolved on 15% SDS-PAGE and the bands were detected by fluorography (lower panel).

FIG. 3. The PSAP motif of ORF3 is essential for interaction with TSG101. A, Gene expression was verified for the PSAP mutant ORF3 protein by in vitro coupled transcription-translation (TNT) system. pGADT7 (M, lane 1), pSGI ORF3 (O3, lane 2) or pGADT7 PSAP mutant ORF3 (O3M, lane 3) translated lysates were immuno-precipitated with anti-ORF3 antibody, resolved on 15% SDS-PAGE and bands were detected by fluorography. B, Yeast two-hybrid analysis of the interaction of different ORF3 mutants with TSG101. L- represents Synthetic Dextrose complete media (SD) lacking Leucine, T- represents SD lacking Trptophane and LTH + 3-AT represents SD lacking, Leucine, Tryptophan and Histidine with 3-Amino 1, 2, 4-Triazole (3-AT) concentrations used. The horizontal bar graph represents relative β-galactosidase units from the liquid β-gal assay. AH109 is the untransformed yeast host strain. C, The PSAP mutant ORF3 (ORF3M) is unable to associate with TSG101. Huh7 cells were transfected with pSGI (M, lane 1), pSGI ORF3 (O3, lane 2) or pSGI PSAP mutant ORF3 (O3M, lane 3), labeled with 35S cys/met promix and harvested in IP buffer. Half of the cell lysate were immuno-precipitated with anti-ORF3 antibody and immuno-blotted with anti-TSG101 antibody (upper panel). The other half of the lysate was resolved on 15% SDS-PAGE and ORF3 expression was checked by fluorography (lower panel). D, TSG101 does not associate with the PSAP mutant ORF3 (ORF3M). Huh7 cells were transfected with pSGI (M, lane 1), pSGI ORF3 (O3, lane 3) or pSGI PSAP mutant ORF3 (O3M, lane 2), labeled with 35S cys/met promix and harvested in IP buffer. Cell lysate was immuno-precipitated with rabbit preimmune serum (lane 1, PS) or anti-TSG101 antibody (lane 2 &3) and immuno-blotted with anti-TSG101 antibody (upper panel). The same blot was dried and ORF3 expression was checked by autoradiography (lower panel).

FIG. 4. The wild-type ORF3 protein co-localizes with TSG101. Huh7 cells grown on cover slips were transfected with wild-type and mutant ORF3 constructs. 48 hours post-transfection, immuno-fluorescence analysis was done. The wild-type and PSAP mutant ORF3 expression was detected by staining with anti-mouse Texas red (panel IV and VII). TSG 101 expression was detected by staining with anti-rabbit FITC (panel II, V and VIII). Panel III, VI and IX show superimposition of Texas red image over FITC image. Panel I shows pSGI transfected (mock) cell stained with anti ORF3 followed by anti mouse Texas red.

FIG. 5. α1-microglobulin leaching is blocked by PSAP mutant ORF3 protein. Huh7 cells were transfected with wild-type or PSAP mutant ORF3 expression constructs (panel A and B respectively). 48 hour post-transfection, cover slips were processed for immuno-fluorescence assay. Wild type ORF3,
mutant ORF3 and $\alpha_1m$ was detected by staining with anti-mouse Texas red and anti-rabbit FITC respectively. Image I and IV of panel A and B show wild-type and mutant ORF3 expression respectively. Image II and V of panel A and B show $\alpha_1m$ expression. Image III and VI of panel A and B show superimposition of Texas red stained image over FITC stained image respectively. White arrow shows ORF3 transfected cells and red arrow shows non-transfected control cells in all images.

FIG. 6. The PSAP mutant ORF3 protein is unable to expedite the secretion of $\alpha_1$ microglobulin. A, Huh7 cells were transfected with pSGI only (M, lane 1), pSGI ORF3 (O3, lane 2) or pSGI PSAP mutant ORF3 (O3M, lane 3), pSGIORF3 plus VPS 4A or 4B wild-type (4AW and 4BW; lanes 4 and 6) or pSGIORF3 plus VPS 4A or 4B dominant negative (4AD and 4BD; lanes 5 and 7) expression constructs. 44 hour post-transfection, cells were labeled with 100 $\mu$Ci $^{35}$S cys/met labeling mix for 4 hour. Labeling media was immuno-precipitated with anti-$\alpha_1$m antibody (upper panel) and cell lysate was immuno-precipitated with anti-ORF3 antibody (lower panel). Graph represents ± S.E.M. of relative band intensities from three independent experiments. Band intensities were normalized with reference to that of ORF3 protein band intensity except for lane 1 where ORF3 band is absent. Lane 1 represents band intensity of $\alpha_1$m level. B, Huh7 cells were transfected with wild type VPS 4a and 4b (4AW and 4BW; lane 1 and 3) or dominant negative VPS 4a and 4b (4AD and 4BD; lane 2 and 4) expression constructs. 44 hour post-transfection, cells were labeled with 100 $\mu$Ci $^{35}$S cys/met labeling mix for 4 hour, media was immuno-precipitated with anti-$\alpha_1$m antibody (upper panel) and total cell lysate was immuno-blotted with Total P38 antibody (lower panel). Graph represents normalized relative band intensity in different samples.

FIG. 7. ORF3, $\alpha_1$ microglobulin and TSG101 associate with each other in a ternary complex. Huh7 cells transfected with pSGI (M, lane 1), pSGIORF3 (lane 2 and 4) or pSGI PSAP mutant ORF3 (lane 3) were labeled with 100 $\mu$Ci $^{35}$S cysteine/methionine labeling mix for 4 hour followed by lysis in immuno-precipitation buffer. Equal amount of lysate was used for immuno-precipitation with anti-ORF3 antibody (lanes 1-3). One set of sample was immuno-depleted twice with ORF3 antibody and the supernatant was again immuno-precipitated with anti-ORF3 antibody (lane 4). Half of the sample was resolved by 12% SDS-PAGE followed by immuno-blotting with anti-$\alpha_1$m antibody (second panel). The same blot was stripped and reprobed with anti-TSG101 antibody (first panel). Other half of the lysate was resolved by 15% SDS-PAGE and the ORF3 band was detected by fluorography (third panel).

Legend to Table 1
Cells transfected with pSGI (mock), pSGI ORF3 (wild-type ORF3) or pSGI PSAP mutant ORF3 were harvested at 48 hour post-transfection and processed for immuno-fluorescence assay (as described in Figure 5). A total of 200 cells were counted in different fields. Of them, cells expressing ORF3 protein were scored for $\alpha_1$m leaching. Only cells showing clear disappearance of $\alpha_1$ microglobulin were counted as positives. Data shown are ± S.E.M. of 3 independent experiments. Approximate percentage was calculated from total number of cells expressing ORF3 versus number of cells showing leaching.
| Accession | Description                  | Sequence                                      |
|-----------|------------------------------|-----------------------------------------------|
| X98292    | Fulminant human liver        | PLGVTRPSAPPLPHVDLPQLGLRR                      |
| M74506    | Mexican isolate             | PLGEIRPSAPPLLPPVADLPQPGLRR                   |
| AF051830  | Nepali isolate              | PLGVTRPSAPPLPHVDLPQLGLRR                     |
| AJ272108  | HEV genotype 4              | PLGVTSAPPLLPPVADLPQLGLRR                     |
| AF076239  | Indian isolate              | PLGATRPSAPPLPHVDLPQLGLRR                     |
| AY043166  | Avian isolate               | NNAPREPSAPPLSQTLSPRQVLARYQM                  |
| AY594199  | Swine isolate               | PLGATRPSAPPLPPVDDLPOQLGRR                    |
| AY230202  | Morocco isolate             | PLGVTRPSAPPLPHVDLPQLGLRR                     |
| D10330    | Myanmar isolate             | PLGVTRPSAPPLPHVDLPQLGLRR                     |
| AY204877  | Algeria & Chad isolate      | PLGVTRPSAPPLPHVDLPQLGLRR                     |
| AB197674  | Japanese isolate            | PLGATNPSSAPPLPPVDDLPOQLGRR                   |
| AB189074  | Wild boar, deer isolate     | PLGVTSAPPLLPPVDDLPOQLGRR                     |
| Two-Hybrid Co-transformants | L- | T- | LTH+3-AT | Relative β-galactosidase units |
|---------------------------|----|----|----------|-------------------------------|
| AH109                     |    |    |          |                               |
| BD-                       |    |    |          | 0.18                          |
| AD-                       |    |    |          | 0.20                          |
| BD- + AD-                 |    |    |          | 0.20                          |
| BD-ORF3 + AD-101          |    |    |          | 1.80                          |
| BD-101 + AD-ORF3          |    |    |          | 1.75                          |
| BD-ORF3 + AD-101A         |    |    |          | 0.42                          |
| BD-101A + AD-ORF3         |    |    |          | 0.40                          |
| BD-MexO3 + AD-101         |    |    |          | 1.62                          |
| BD-MexO3 + AD-101A        |    |    |          | 0.33                          |
| BD-101 + AD-101           |    |    |          | 1.82                          |

Fig. 2a
| Antibody | O3 | O3 | O3 | 101 |
|----------|----|----|----|-----|
| Plasmid  | M  | O3 | O3 | O3  |

- **TSG 101**
- **ORF3**

1  2  3  4
A

| Antibody | O3  |
|----------|-----|
| Plasmid  | M   | O3 | O3M |

ORF3

Fig 3a
| Two-Hybrid Co-transformants | L' T' | LTH + 3-AT | Relative β-galactosidase units |
|---------------------------|-------|-----------|-----------------------------|
| AH109                     |       |           | 0.19                        |
| BD- + AD-                 |       |           | 0.25                        |
| BD-ORF3 + AD-101          | 1 123 |           | 1.85                        |
| BD-1-79 ORF3 + AD-101     | 1 79  |           | 0.44                        |
| BD-81-123 ORF3 + AD-101   | 1 81  |           | 1.79                        |
| BD-S80A ORF3 + AD-101     | 1 S80 |           | 1.75                        |
| BD-ORF3M + AD-101         | 1 M   |           | 0.41                        |
| BD-101 + AD-ORF3M         | 1 123 |           | 0.40                        |
| BD-1-79 ORF3 + AD-101A    | 1 79  |           | 0.46                        |
| BD-81-123 ORF3 + AD-101A  | 1 81  |           | 0.40                        |
| BD-S80A ORF3 + AD-101A    | 1 S80 |           | 0.43                        |
| BD-101A + AD-ORF3M        | 1 M   |           | 0.36                        |
C

| Antibody | O3 |
|----------|----|
| Plasmid  | M  | O3 | O3M |

![Image of gel with bands labeled TSG101 and ORF3]

D

| Antibody | PS | TSG101 |
|----------|----|--------|
| Plasmid  | M  | O3 M   | O3   |

![Image of gel with bands labeled TSG101 and ORF3]
Mock

Wild type ORF3

Mutant ORF3

TSG 101

Merge

Fig. 4
A Wild type ORF3  \( \alpha_1 \) microglobulin Merge
A. A table showing the plasmid constructs with relative intensities for ORF3, α1 microglobulin, and α1m conjugates. The plasmids include pSGI, 4AW, 4AD, 4BW, 4BD, and their corresponding constructs labeled M, O3, O3M, O3, O3, O3.

B. A graph showing the relative intensity of total p38 and α1 microglobulin across different plasmid constructs (4AW, 4AD, 4BW, 4BD).
| Antibody | O3 | O3id |
|----------|----|------|
| Plasmid  | M  | O3   | O3M  | O3  |

- TSG101
- α₁ microglobulin
- ORF3
Table I

| Cells expressing | Mock | Wild type ORF3 | PSAP mutant ORF3 |
|------------------|------|----------------|-----------------|
| Number of cells  |      | 140±5          | 146±4           |
| Expressing ORF3  | 0    |                |                 |
| Number of Cells  |      | 118±4.6        | 2.6±1           |
| showing leaching | 0    |                |                 |
| % age of cells   |      | ~84            | ~1.5            |
| Showing leaching | 0    |                |                 |
Enhanced α1 microglobulin secretion from hepatitis E virus ORF3 expressing human hepatoma cells is mediated by the tumor susceptibility gene 101
Milan Surjit, Ruchi Oberoi, Ravinder Kumar and Sunil K Lal

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