The Viral E3 Ubiquitin Ligase mK3 Uses the Derlin/p97 Endoplasmic Reticulum-associated Degradation Pathway to Mediate Down-regulation of Major Histocompatibility Complex Class I Proteins

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Ubiquitin E3 ligases are important cellular components for endoplasmic reticulum (ER)-associated degradation due to their role in substrate-specific ubiquitination, which is required for retrotranslocation (dislocation) of most unwanted proteins from the ER to the cytosol for proteasome degradation. However, our understanding of the molecular mechanisms of how E3 ligases confer substrate-specific recognition, and their role in substrate retrotranslocation is limited especially in mammalian cells. mK3 is a type III ER membrane protein encoded by murine γ herpesvirus 68. As conferred by its N-terminal RING-CH domain, mK3 has E3 ubiquitin ligase activity. In its role as an immune evasion protein, mK3 specifically targets nascent major histocompatibility complex class I heavy chains (HC) for rapid degradation. The mechanism by which mK3 extracts HC from the ER membrane into the cytosol for proteasome-mediated degradation is unknown. Evidence is presented here that HC down-regulation by mK3 is dependent on the p97 AAA-ATPase. By contrast, the kK5 protein of Kaposi’s sarcoma-associated herpesvirus is p97-independent despite the fact that it is highly homologous to mK3. mK3 protein was also found in physical association with Derlin1, an ER protein recently implicated in the retrotranslocation of HC by immune evasion protein US11, but not US2, of human cytomegalovirus. The mechanistic implications of these findings are discussed. 

To exist in the presence of an active host immune system, herpesviruses in particular have elaborate mechanisms of immune evasion. For example, to specifically block CD8 T cell detection of infected cells, murine γ herpesvirus 68 (γHV68) expresses the mK3 protein. The mK3 protein is a viral RING-CH type ubiquitin E3 ligase (1) that specifically ubiquitinites MHC2 class I heavy chains (HC) causing their rapid degradation in a proteasome dependent manner (2, 3). Thus mK3 inhibits surface class I expression and its antigen presentation function required for cytolytic T cell detection of virus infected cells. Consistent with these findings, the lack of mK3 expression by the virus results in the detection of more virus-immune CD8 T cells and a lower level of latency (4).

Comparisons of coding sequences revealed that mK3 is highly homologous to both kK3 and kK5 gene products of Kaposi’s sarcoma-associated herpesvirus (KSHV) (1, 5). The kK3, kK5, and mK3 proteins share a common topology including a highly conserved, N-terminal RING-CH domain, followed by two predicted transmembrane segments and a C-terminal domain varying in sequence and length (3, 6, 7). Immunofluorescence studies confirmed that these K3 homologs are type III membrane proteins with both N- and C termini on the cytosolic face of the ER membrane (3, 6). The RING-CH domain possesses ubiquitin E3 ligase activity as demonstrated by its ability to carry out autoubiquitination in vitro and substrate ubiquitination in cells (3, 7, 8). Additional K3 homologs are also found in poxviruses, such as M13R from myxoma virus (9). Cellular K3 homologs have also been detected, suggesting appropriation of the viral K3 homologs from their eukaryotic host (10, 11).

Despite similar domain organization, mK3 appears to be unique among the K3 homologs in terms of its subcellular site of degradation. More specifically, kK3, kK5, and the other K3 family members defined thus far target their respective substrates for endocytosis at the plasma membrane resulting in degradation in the lysosome (9–14). By contrast, mK3 targets newly synthesized HC in the ER leading to their degradation in a proteasome-dependent manner. Defining differences in the host proteins recruited by mK3 versus kK3/kK5 will provide key insights into disparate pathways of ubiquitin-dependent protein degradation.

Although molecular details of how mK3 induces rapid degradation of HC are incompletely understood, intriguing features have been recently defined. For example, mK3 induces the polyubiquitination of glycosylated, membrane-bound HC. More specifically, the mK3-targeted HC are associated with the transmembrane proteins TAP and tapasin, two ER proteins involved in peptide binding by class I proteins (15). Unexpectedly, lysine to arginine mutations in HC tails did not alter the mK3-induced polyubiquitination pattern of HC or its rapid degradation (16). Furthermore, cysteine residues in the HC tail are also not required for mK3 down-regulation of HC, as they are required for kK3 regulation of HC (17). Thus, ubiquitination of the HC tail is not the initiating event for mK3-induced ERAD. Alternatively these findings support the model that mK3 induces partial dislocation of ER membrane-bound HC to the cytosol prior to mK3-induced ubiquitination (18).
Similar to our findings, tail lysines are also dispensable for ERAD of HC induced by human cytomegalovirus immune evasion proteins US2 and US11 as well as other ERAD substrates (19–21). Like mK3, US2 and US11 induce rapid degradation of nascent class I molecules through ubiquitination-dependent proteasome proteolysis (22, 23). Yet, distinct from mK3, US2 and US11 are type I membrane proteins with their major domains located in the ER lumen, and they are not ER ligases (22, 23). Furthermore, US2, and presumably US11, bind directly to class I molecules and induce their dislocation from the ER (24, 25). Interestingly, despite functional and predicted structural homology, US2 and US11 appear to utilize different mechanisms to dislocate HC into the cytosol. For example, the cytoplasmic tail of US2, but not US11, is required for ERAD of HC (26). In addition, it has been recently reported that Derlin1 (mammalian homolog of yeast Der1p) is essential for the degradation of class I HC catalyzed by US11 but not by US2 (27). The interaction of US11 with Derlin-1 and VIMP (a novel Derlin-1-associated ER membrane protein) recruits the p97 AAA-ATPase together with cofactors Ufd1 and Npl4 (28, 29). Furthermore, it has been proposed that this p97 complex “pulls” HC into the cytosol in an ATP-dependent manner by interacting with segments of the HC backbone as well as attached polyubiquitin chains (29). Whether US2 function depends on p97 has not been reported. The disparity between the US2-versus US11-recruited cellular proteins for dislocation suggests that there may be multiple pathways by which ER proteins are dislocated to the cytosol. More specifically, these multiple ERAD pathways in mammals could involve several distinct dislocation channels associated with disparate ATPases and E3 ligases.

We now investigate whether the Derlin1/p97 pathway is involved in mK3-mediated HC degradation and surface down-regulation. We now show that mK3, while interacting with class I, is physically associated with Derlin1 and p97. Furthermore, using dominant negative forms of p97 known to ablate ATP binding or hydrolysis, HC down-regulation by mK3 was found to be p97-dependent, whereas kk5-mediated substrate degradation was p97-independent.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Murine B6/WT3 (WT3, H-2d) and L cell (H-2b) fibroblasts as well as their transductants (Ld, human B7.2, mK3, and kK5) have been described previously (8). 293T cells (30) were used for production of ecotropic retrovirus (to transduce B6/WT3 cells) and amphotropic retrovirus (to transduce L cells). All cells were maintained in complete RPMI 1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HyClone, Logan, UT), as described (15). Retrovirus-containing supernatants were produced as described (15) using Vpack vector system (Stratagene, La Jolla, CA) with transient transfection of 293T cells. Cells transduced by pMIP-containing virus were confirmed by DNA sequencing.

**DNA Constructs**—Mouse Derlin1 (NCBI ID: NP_77169) and mouse VIMP (NCBI ID: NP_077759) cDNA were amplified from total cDNA of L cells by PCR. The necessary flanking restriction endonuclease sites on each end of the cDNA were incorporated into the primers. Retroviral expression constructs pMIP.mDerlin1-HA or pMIP.myc-mVIMP that express C-terminal HA-tagged mouse Derlin1 or N-terminal myc-tagged mouse VIMP, respectively, were created by subcloning mouse Derlin1 or VIMP cDNA together with the sequence encoding HA or myc epitope tag into a pMSCV.IRES.Puro (pMIP) retroviral expression vector (gift of Dr. L. Lybarger, University of Arizona). The retroviral constructs pMSCV.IRES.GFP (pMIG), HA-mK3, pMIG.HA-mK3 RING (mK3 C48G/C51G mutant which abolishes the E3 ligase activity of mK3), and pMIG.myc-mK3 were generated by inserting a HA or myc tag sequence in front of mK3 in the corresponding constructs as described previously (8). The correct sequences for all of the constructs were confirmed by DNA sequencing.

**p97 Adenoviruses and Cell Infection**—Recombinant adenoviruses that express RGS-His tagged wild type (WT), ATP binding mutant K524A (KA), and ATP-hydrolysis mutant E305Q/E578Q (QQ) p97 under the control of tetracycline (tet-off) have been described previously by Dr. Lencer and colleagues (31). Cells were co-infected with p97 and tet-transactivator tTA adenoviruses. The amount of each virus applied to B6/WT3 or L cells was determined empirically by establishing equivalent viral p97 expression while not causing cytotoxic effects. Where indicated, 1–2 ng/ml doxycycline was added 4 h postinfection to specifically suppress viral p97 expression. Cells were collected for analysis after incubation with viruses for 24 h for B6/WT3 cells or 48–72 h for L cells.

**Antibodies**—Rabbit antisera to Derlin1 and VIMP were described previously by Ye et al. (28). Rabbit anti-mK3, hamster anti-tapasin, ubiquitin antibodies, and mAbs to MHC class I (including 30-5-7, 64-3-7 to Ld, 15-5-5 to Dd, and B8-24-3 to Kd have been described previously (15). RGS-His antibodies, p97 antibodies (58.13.3), and ß-actin (AC-74) antibodies were purchased from Qiagen, Research Diagnostics Inc. (Flanders, NJ), and Sigma, respectively. Antibodies to HA (16B12), myc (9E10), and GFP (B34) were obtained from Covance Research Products (Berkeley, CA). Rabbit antibodies to calreticulin (SPA600) and Sec61a were obtained from Upstate Biotechnology (Lake Placid, NY) and Stressgen (Victoria, British Columbia, Canada).

**Flow Cytometry and Immunofluorescence Staining**—All flow cytometric analyses were performed as described previously (15). For immunofluorescence microscopy, cells were plated into 24-well plates and fixed with 2% paraformaldehyde at room temperature for 20 min. After blocking in phosphate-buffered saline containing 0.5% saponin and 3% bovine serum albumin, fixed cells were then incubated with mAb to RGS-His (0.1 μg/ml, Qiagen) and Alexa 594 goat anti-mouse IgG1 (6.5 μg/ml, Molecular Probes, Inc., Eugene, OR) sequentially.

**Immunoprecipitation and Immunoblots**—For co-immunoprecipitation (co-IP), cells were lysed in buffer containing 1% digitonin (Wako, Richmond, VA), 20 mM Tris-HCl (pH 7.4), 150 mM potassium acetate, 4 mM magnesium chloride, 1 mM ATP, 20 mM iodoacetamide (Sigma), and protease inhibitors (Complete mini, Roche). Postnuclear lysates were then incubated with immobilized anti-HA or anti-myc beads (ProFound e-Myc tag/HA tag co-IP kit, Pierce) and 1 mg/ml bovine serum albumin. Beads were washed four times with 0.05% Tween 20-Tris-buffered saline (pH 7.4) and proteins were eluted with an excess of HA.11 peptide (Covance Research Products) for anti-HA beads or by boiling in non-reducing sample buffer (Pierce) for anti-myc beads. For immunoblots of cell lysates, cells were lysed in 1% Nonidet P-40, TBS (pH 7.4). Postnuclear lysates were mixed with LDS sample buffer (Invitrogen), and 2-mercaptoethanol was added to a final concentration of 2%. Immunoblotting was performed following SDS-PAGE separation of precipitated proteins or cell lysates as described previously (2). Specific proteins were visualized by chemiluminescence using the ECL system (Amersham Biosciences).

**Metabolic Labeling and Pulse-Chase**—After 30 min of preincubation in cysteine- and methionine-free medium (Dulbecco’s modified Eagle’s medium with 5% dialyzed fetal calf serum), cells were pulse labeled with Express [35S]Cys/Met labeling mix (PerkinElmer Life Sciences) at 150
μCi/ml for 10–15 min. Chase was initiated by the addition of an excess of unlabeled Cys/Met (5 μM each). Immunoprecipitation and endoglycosidase H (Endo H; ICN Pharmaceuticals, Costa Mesa, CA) treatment were performed as described (32). Samples were subjected to SDS-PAGE, and gels were treated with Amplify (Amersham Biosciences), dried, and exposed to BioMax-MR film (Eastman Kodak Co.).

RESULTS

p97 ATPase Is Required for mK3-induced Degradation and Surface Down-regulation of MHC Class I HC—To test whether p97 ATPase function is required by mK3-mediated degradation of HC, dominant negative forms of p97 were employed. Previous studies have shown that p97 mutants QQ (E305Q/E578Q) or KA (K524A) do not affect the binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29). Since binding of substrates and cofactors but significantly reduce the ATPase activity by either blocking ATP hydrolysis or ATP binding (29).

To probe p97 involvement in mK3-induced turnover of HC, mouse WT3-L4 fibroblasts carrying empty vector (WT3+V) or mK3 (WT3+mK3) were infected with adenovirus expressing WT or dominant negative (QQ or KA) forms of p97. Twenty-four hours postinfection, the total level of p97 protein detected by Western blotting increased ~2-fold compared with non-infected cells (Fig. 1A). To monitor MHC class I HC turnover, infected cells were pulse-labeled with [35S]Cys/Met and chased for 0, 30, 60, and 90 min. Class I HC (Kb) was immunoprecipitated, resolved in SDS-PAGE, and visualized by autoradiography (Fig. 1B). In the cells without mK3, expression of p97 QQ had no significant effect on oligosaccharide maturation or turnover of HC compared with p97 WT. Indeed, in cells lacking mK3 the majority of HC acquired mature (Endo H-resistant (r)) oligosaccharides by 60 min in the presence of either WT or QQ p97 (Fig. 1B, upper left panel). By contrast in cells expressing mK3, HC turned over more rapidly in the presence of WT p97 with a half-life of less than 60 min; and as expected, the majority of HC retained immature (Endo H-sensitive (s)) oligosaccharides, consistent with their ER residency. Dominant negative p97 was found to prolong the half-life (>90 min) of HC in mK3-expressing cells and increase the detection of HC with matured oligosaccharides (Fig. 1B, lower right and upper right panels). A similar reversal of mK3 function by dominant negative forms of p97 was observed monitoring the turnover of Ld HC expressed by these cells (data not shown). Furthermore, this kinetic data were corroborated by comparisons of surface expression of MHC class I by these cells. As shown in Fig. 1C, expression of WT p97 did not interfere with the ability of mK3 to down-regulate surface Kb expression to about 7% of that of cells without mK3. By contrast, expression of dominant negative forms of p97 resulted in a greater than 2-fold increase in class I expression as measured by mean fluorescence intensity (Fig. 1C). Similar results were obtained using either the KA or QQ dominant negative forms of p97 (Fig. 1C) and monitoring surface expression of Kb (Fig. 1C) or Ld (data not shown).
Thus, dominant negative forms of p97 clearly impair the ability of mK3 to rapidly degrade class I HC and thereby decrease surface class I expression. These findings indicated mK3 regulation of class I HC is functionally dependent upon p97, thus providing additional evidence that mK3 exploits a physiological ERAD pathway.

The p97 Dependence of mK3 Does Not Extend to the Highly Homologous kk5 Immune Evasion Protein of KSHV—To characterize the specificity of the interaction of mK3 with p97, we also tested whether kk5 is functionally dependent upon p97. The kk5 protein of KSHV is highly homologous to mK3 including a RING-CH domain and two transmembrane segments (6). Despite this homology to mK3, kk5 does not induce ERAD but acts at the cell surface to accelerate endocytosis and lysosomal degradation of its substrates (12–14). In human cells, kk5 substrates include HC and other immune regulatory receptors such as the T cell co-stimulation protein B7.2 (CD86). However, importantly for these experiments, kk5 down-regulates B7.2 but not mouse class I HC in mouse cells (Fig. 2, panels a and b). By contrast kk3 down-regulates class I but not B7.2 (Fig. 2C, panels c and d). This reciprocal substrate specificity allowed us to directly compare the p97 dependence of substrate down-regulation by mK3 versus kk5 in the same cell type. For these experiments, mouse fibroblast L cell lines co-expressing human B7.2 (huB7.2) and kk3 or kk5 were used. Because these cells were found to be relatively resistant to cytopathic effects of adenovirus infection, we expressed different doses of dominant negative forms of p97 (data for the QQ p97 mutant are shown, but comparable results were obtained with the KA mutant (data not shown)). As visualized by immunofluorescence using an antibody to the His tag, the number of infected cells with high levels of expression of His-p97 protein increased commensurately with increases in adenovirus infection (Fig. 2A). In agreement with the cell staining, the protein levels of p97 in whole lysates from cells infected with increasing doses of dominant negative p97 also showed ~2-, 3-, and 4-fold enhancement over the level of endogenous p97 (Fig. 2B, p97 blot, lanes 2–4 and 6–8 compared with lanes 1 and 5). It should be noted that the steady-state levels of mK3 protein in cells infected with different doses of dominant negative p97 were unchanged, demonstrating that mK3 turnover is p97-independent (Fig. 2B, mK3 blot).

To compare the p97 dependences of the substrate interactions of mK3 versus kk5, we monitored surface expression of huB7.2 and mouse class I in the aforementioned L cell lines (Fig. 2). For surface expression of MHC class I, we tested both Kk and Dk proteins, and similar results were obtained. Thus, only Dk expression is shown. As expected, expression of mK3 or kk5 induced a profound (>10-fold) down-regulation of their respective substrates class I (Fig. 2C, panel d) and huB7.2 (Fig. 2C, panel a). However, expression of dominant negative forms of p97 affected only substrate down-regulation by mK3 and not kk5. Indeed, compared with normal levels of class I (i.e. no mK3), the percentage of down-regulation increased from 24 to 72% as the dose of p97 QQ was raised from 1 to 3 (Fig. 2C, panel d, and Fig. 2D). By contrast kk5-induced down-regulation of huB7.2 remained unchanged in the presence of dominant negative p97 (Fig. 2C, panel a). Furthermore, the effect of dominant negative p97 on mK3 down-regulation of HC was reversed by treatment with doxycycline during p97 adenovirus infection (Fig. 3B), which correlated with the complete shut down of virally expressed His-p97 protein (Fig. 3A). This control establishes that the effects seen with dominant negative p97 are not secondarily caused by the adenovirus infection. Thus dominant negative forms of p97 specifically interfere with ubiquitin-proteasome-dependent class I down-regulation by mK3 but not ubiquitin-lysosome-dependent B7.2 down-regulation by kk5. This indicates that substrate down-regulation by mK3 and not kk5 is dependent upon p97 ATPase activity.

Accumulation of Polyubiquitinated HC in the Presence of Dominant Negative p97—Another known effect of dominant negative p97’s, which links p97 to the ubiquitin-proteasome pathway, is the accumulation of polyubiquitinated proteins in cells (29, 34). Polyubiquitination is required for the retrotranslocation of almost all ERAD substrates. Depletion of ubiquitin or overexpression of mutant ubiquitin incapable of forming polyubiquitin chains causes substrates to be retained in the ER (29, 35, 36). Indeed, polyubiquitination is also an absolute requirement for mK3-mediated degradation of HC. In support of this conclusion, a RING-CH domain mutant of mK3 abolishes its E3 ligase activity in vitro; and HC molecules are not polyubiquitinated and not rapidly degraded in cells expressing RING-CH mutants of mK3 (3, 8). Here we also observed a dose-dependent accumulation of polyubiquitinated proteins in lysates of cells infected with dominant negative p97 (Fig. 4A).

To test the effect of dominant negative p97 on ubiquitination of HC in the presence of mK3, cells expressing mK3 and dominant negative p97 were lysed, and HC was immunoprecipitated and visualized by immunoblotting. As expected, compared with cells infected with WT p97, cells infected with dominant negative p97 showed more intact HC (Fig. 4B). Coincident with the increase in HC detection, more glycosylated, polyubiquitinated HC accumulated in cells expressing both dominant negative p97 and mK3 proteins (Fig. 4B). This finding demonstrates that mK3-induced polyubiquitination of HC occurs independently of the functional requirement for p97.

mK3 Is Physically Associated with the Derlin1/p97 Complex—It is important to note that ubiquitin conjugation of HC happens while it is membrane-bound and glycosylated (15, 16). This raises the intriguing question of how cytosolic p97 complexes are recruited to the membrane and whether membrane bound mK3 is detected in physical association with p97. Recently two independent groups reported that in mammalian cells, a Der1p-like ER membrane protein designated Derlin1 (for Der1p-like protein) is involved in US11-induced ERAD of HC (27, 28). Derlin1 is predicted to span the membrane four times and has been speculated to be a component of a putative dislocation channel. Furthermore, Derlin-1 may be involved in recruiting p97 through interacting with another newly defined ER membrane protein termed VIMP (28). To test whether there is a physical association between mK3 and Derlin1/p97, a co-IP experiment was conducted. Comparable levels of mK3 protein were expressed with either a HA- or myc-tag (mK3 blot of input lysate), whereas only HA-mK3 was immunoprecipitated by anti-HA antibody (Fig. 5A). This comparison was used to confirm the specificity of mK3-associated proteins. As shown in Fig. 5A, low levels of p97, Derlin1, and class I HC were observed only in HA-mK3 precipitates but not in the mK3-negative or myc-mK3 control precipitates. It should also be noted that the association of mK3 with p97 and Derlin1 was not detected with Derlin1-HA and myc-mK3. The precipitates were then blotted to detect associated proteins. As shown in Fig. 5B, Derlin1 was detected in association with p97 and VIMP as reported previously (28). More importantly, mK3 displayed a clear and specific association with Derlin1. As further evidence for the specificity of the mK3 interactions, the ER proteins calreticulin and Sec61α did not co-precipitated with Derlin1 (Fig. 5B). In addition the GFP protein co-expressed with mK3 was only detected in whole cell lysates and not in immunoprecipitates (Fig.
FIGURE 2. Effect of dominant negative forms of p97 on mK3-mediated MHC class I down-regulation is dose-dependent and specific for mK3 but not its homolog kK5. A, $5 \times 10^5$ L-kK5 or L-mK3 cells expressing human B7.2 (huB7.2) were infected with 0.7 µl (dose1), 1.4 µl (dose2), or 2.8 µl (dose3) of concentrated p97 QQ adenovirus. Forty-eight hours postinfection, virally expressed p97 was examined by immunofluorescence with mAb to the RGS-His epitope. Images from both lines were indistinguishable so only those from L-mK3 line are shown here. B, cells from the same experiment as in A were lysed and subjected to immunoblot for p97 and mK3 proteins. An actin blot was also included as a loading control. C, surface expression of huB7.2 and MHC class I Dk on cells from the same experiment as in B was analyzed by FACS. The shaded peaks represent staining of cells expressing kK5 (panel a) or mK3 (panel d). The black lines represent staining of cells without kK5 (panel a) or mK3 (panel d). The colored lines indicate the staining of cells infected with various doses of p97 QQ. Cells without adenovirus infection are presented as shaded peaks. Surface Dk rescued in L-mK3 cells infected with different dose of p97 QQ was calculated as a percentage of specific counts in the indicated M1 region (individual plots in panel d). D, the percentages of class I rescued by different doses of p97 QQ as determined in C. Note: comparable results were obtained with p97 KA mutant (data not shown).
To determine whether mK3 was also associated with VIMP, we used a similar strategy as above but employed cells co-expressing myc-VIMP and HA-mK3. Again, p97 and Derlin1 were found to co-precipitate with VIMP as expected from previous reports (28). Meanwhile, a weaker mK3 band was also detected only in the cells co-expressing VIMP and mK3, indicating a specific interaction between mK3 and VIMP (Fig. 5C). In sum mK3 has a weak, but specific, physical association with p97, Derlin1, and VIMP. It should be noted that these interactions are considerably weaker than the interaction of mK3 with TAP or tapasin (Fig. 5A and Ref. 2). Indeed TAP appears to be the primary binding partner of mK3, whereas tapasin is required for mK3 stabilization (15, 37). By comparison, the interaction of mK3 with p97/Derlin1 is substantially weaker suggesting it maybe indirect or transient.

**DISCUSSION**

Ubiquitination is required for retrotranslocation (dislocation) of most luminal ERAD substrates including integral ER membrane proteins. Covalent conjugation of ubiquitin is carried out by sequential actions of three enzymes, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). The E3 ligases catalyze the attachment of ubiquitin to the lysine residue of substrate or in rare cases to the N terminus of substrate. It is the E3 that directly interacts with the substrate and therefore plays a central role in conferring substrate specificity of ubiquitination (38). Furthermore, in contrast to the more uniformly distributed ubiquitin, E1 and E2, E3 are most often localized in distinct subcellular structures or compartments (39). The compartmentalization provides another layer of regulation, enhancing the specificity and timing the proteolysis. Thus studying the mechanisms underlying the role of E3 in ERAD should provide important insights into how ERAD is regulated. So far only a few E3 have been
localized to the ER and have been unequivocally implicated in ERAD pathways. Examples include Hrd1p/Der3p (40) and Doa10p (41) in yeast and HRD1 (homolog of yeast Hrd1p/Der3p) (42) and gp78 (originally recognized as tumor autocrine motility factor receptor) in humans (43). Hrd1p and Doa10p are responsible for ubiquitination of distinct subsets of ERAD substrates that may contain misfolded domains on the luminal or cytosolic faces of the ER membrane respectively (41, 44). This distinction suggests these two yeast E3 may be involved in different ERAD pathways (45). In mammals, HRD1 and gp78 appear to have overlapping substrate specificity (42, 43), and interestingly HRD1 and gp78 co-precipitate demonstrating they can co-exist within the same complex (46). More recently, both HRD1 and gp78 have been found in the complex consisting of Derlin1 and Derlin2 (two of the three yeast Der1p homologs in mammals), VIMP and p97 (46, 47).

It remains to be determined how any of these E3 ligases are recruited to the Derlin/VIMP/p97 complex. Mammalian Derlin proteins have been proposed to function as components of a putative retrotranslocation channel, and different Derlin proteins may process different types of substrates in mammalian cells (47). Similarly, yeast Derlp and Doa10p are responsible for degradation of a subset of substrates (40, 44). Given that ERAD associated E3 defined so far are also multispanning membrane proteins, it is attractive to speculate that these E3 may also be components of a channel for retrotranslocation. All of these outstanding issues raise the intriguing question of the role of E3 ligases in the specific recognition, dislocation, and ubiquitination of ERAD substrates. mK3 is a particularly attractive probe for these E3-related questions because of its substrate specificity and structural organization is very similar to Doa10p in yeast. They both have an N-terminal RING-CH domain in the cytosol followed by multi-
transmembrane fragments (2 in mK3 and 13 in Doa10p). It has been shown that typical substrates of Doa10p are integral ER membrane proteins with large cytosolic domains, and Doa10p-mediated ERAD is independent of Sec61 (41). In a recent study, a human homolog of Doa10p, named TEB4/MARCH VI has been identified, located in the ER and shown to have in vitro ubiquitin ligase activity (50). Its substrates, however, remain to be defined.

p97 is one of the most abundant intracellular proteins involved in multiple basic cell biological functions, including membrane fusions of ER, Golgi apparatus, and nuclear envelopes, as well as retrotranslocation of ubiquitinated ERAD substrates. In these processes, p97 differentially utilizes its cofactors such as p47 in the process of membrane fusion and Ufd1/Npl4 in ERAD (51). Here we show that dominant negative forms of p97 do not affect kK5-mediated down-regulation of B7.2, which occurs via a lysosomal degradation pathway. This comparison thus defines a key molecular interaction that differentiates the ubiquitination pathway induced by these two highly homologous RING-CH domain-containing viral E3 ligases. The block of mK3 function by dominant negative forms of p97, however, was incomplete in our assays which may be due to the fact that endogenous p97 is in such abundance that it is difficult to completely inhibit with dominant negative forms. This explanation is supported by the fact that the extent of accumulation of ubiquitinated protein in whole cell lysates and the surface class I rescue is dose dependent and positively correlates with the expression level of dominant negative p97. Alternatively, we do not exclude the possibility that other ATPases may be redundantly involved in mK3-induced ERAD. Indeed this conclusion is consistent with the observation that similar other ATPases may be redundantly involved in mK3-induced ERAD. Furthermore demonstrate that partial dislocation and ubiquitination of ERAD substrates not requiring tail lysines (21). In this model, partial dislocation via a lysosomal degradation pathway. This comparison thus defines a partial negative p97. Alternatively, we do not exclude the possibility that other ATPases may be redundantly involved in mK3-induced ERAD. Indeed this conclusion is consistent with the observation that similar other ATPases may be redundantly involved in mK3-induced ERAD.

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