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Dimerization controls Marburg virus VP24-dependent modulation of host antioxidative stress responses

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Abstract

Marburg virus (MARV), a member of the *Filoviridae* family that also includes Ebola virus (EBOV), causes lethal hemorrhagic fever with case fatality rates that have exceeded 50% in some outbreaks. Within an infected cell, there are numerous host-viral interactions that contribute to the outcome of infection. Recent studies identified MARV viral protein 24 (mVP24) as a modulator of the host antioxidative responses, but the molecular mechanism remains unclear. Using a combination of biochemical and mass spectrometry studies, we show that mVP24 is a dimer in solution that directly binds to the Kelch domain of Kelch-like ECH-associated protein 1 (Keap1) to regulate nuclear factor (erythroid-derived 2)-like 2 (Nrf2). This interaction between Keap1 and mVP24 occurs through the Kelch interaction loop (K-Loop) of mVP24 leading to upregulation of antioxidant response element transcription, which is distinct from other Kelch binders that regulate Nrf2 activity. N-terminal truncations disrupt mVP24 dimerization, allowing monomeric mVP24 to bind Kelch with higher affinity and stimulate higher antioxidative stress response element (ARE) reporter activity. Mass spectrometry-based mapping of the interface revealed overlapping binding sites on Kelch for mVP24 and the Nrf2 proteins. Substitution of conserved cysteines, C209 and C210, to alanine in the mVP24 K-Loop abrogates Kelch binding and ARE activation. Our studies identify a shift in the monomer-dimer equilibrium of MARV VP24, driven by its interaction with Keap1 Kelch domain, as a critical determinant that modulates host responses to pathogenic Marburg viral infections.

Graphical Abstract

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Introduction

Marburg virus (MARV), a member of the family *Filoviridae*, is a filamentous virus that can cause severe hemorrhagic fever with high fatality rates in both humans and nonhuman primates [1, 2]. Filoviral infection is characterized by disseminated viral replication and uncontrolled cytokine secretion in a broad range of cell types [3–7]. Among these, monocytes, macrophages, and dendritic cells are thought to be early sites of replication and important in the dissemination of the virus from the site of infection to the lymph nodes and ultimately into the bloodstream. In response to viral infection and replication, the host responds with changes in gene expression [8]. Host innate antiviral responses are some of the key responses.

Recent studies have shown the upregulation of the antioxidant response element (ARE) pathway during MARV infection [8, 9]. The ARE pathway is regulated by Kelch-like ECH-associated protein 1 (Keap1), a ubiquitin E3 ligase adaptor that links the Cul3/Roc1 ubiquitin E3 ligase activity to the presence of reactive oxygen species (ROS) in the cell [10]. Under homeostatic conditions, Keap1 represses the cellular antioxidant transcriptional program by promoting the ubiquitin-mediated proteasomal degradation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [11–13]. In response to oxidative stress, Keap1/Nrf2 association is disrupted, thereby preventing Nrf2 ubiquitination and degradation. Nrf2 accumulates in the cytoplasm, enters the nucleus where it can interact with coactivator Maf protein, and contributes to the transcriptional activation of genes possessing antioxidant response elements (ARE). These ARE genes encode proteins responsible for detoxification reactions that promote cell survival [8, 9, 14]. In the case of MARV infection, MARV viral

protein 24 (mVP24) is thought to influence cellular redox balance, in part by regulating the ARE pathway. mVP24 directly targets the repressor of the ARE pathway, Keap1, to upregulate the expression of cytoprotective genes [8, 9]. mVP24 binds the Kelch domain of Keap1 to release Nrf2 for nuclear accumulation and ARE-promoter stimulation. The mechanistic details by which mVP24 activates Nrf2, however, are lacking.

Here we describe efforts to determine the molecular mechanism for mVP24 modulation of Keap1 activity through combined biochemical and mass spectrometry (MS) analyses. We identified a monomer-dimer equilibrium for mVP24 and defined a role for the mVP24 N-terminus in dimerization. We mapped the binding interface between mVP24 and the Keap1 Kelch domain, using a combination of MS-based footprinting approaches, including hydrogen-deuterium exchange mass spectrometry (HDX-MS) [15–19], and *N*-ethylmaleimide (NEM) footprinting [20, 21]; both confirmed a role for mVP24 K-Loop (residues 205–212), identified additional elements within mVP24 as critical regulatory determinants, and demonstrate that upon Keap1 binding, mVP24 dimerization is disrupted to form a mVP24-Keap1 2:2 complex. Together, our results reveal how host ARE responses are modulated by mVP24 and provide evidence for a direct competition mechanism by which mVP24 K-Loop directly targets the Nrf2 binding site in the Keap1 Kelch domain.

Results

Loss of mVP24 dimerization results in increased Keap1 binding and mVP24-dependent ARE promoter activity

Most matrix proteins from non-segmented negative strand RNA viruses, including Ebola major matrix protein VP40, have been shown to form homo- and hetero-oligomers. To determine if mVP24 forms oligomers, we characterized mVP24 using a series of hydrodynamic studies. Analysis of the elution profiles of maltose-binding protein (MBP) tagged mVP24 (MBP-mVP24) from a size exclusion chromatography column shows concentration dependent changes over a protein concentration range of 3.00 to 112 μ M (Fig. 1A) that is consistent with self-oligomerization. MBP-mVP24 forms oligomers with increasing protein concentration and shifts back to a monomeric form following dilution. Further characterization of mVP24 using size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) shows that at 60 μ M, MBP-mVP24 elutes at a volume corresponding to an experimental molecular weight of 146 ± 1 kDa, which is approximately twice the theoretical molecular weight (72.6 kDa), indicating that MBP-mVP24 exists in a dimeric form (Fig. 1B). Although we cannot rule out that additional oligomeric states exist [22], we have no evidence to suggest they are present in our protein preparations.

To determine which region on mVP24 is involved in dimerization, we examined the crystal structure of mVP24 (PDB 4OR8) (Fig. S1A) and evaluated the hydrogen bonds and hydrophobic interactions. LigPlot⁺ analysis of the two molecules in the asymmetric unit identifies two sets of contacts, one involving the N-terminal arm of mVP24 (Fig. S1B) and another involving core contacts (Fig. S1C). In addition, we carried out hydrogen-deuterium exchange mass spectrometry (HDX-MS) for mVP24 WT at 5.0 μ M (low concentration) and 90 μ M (high concentration) to determine the differential deuterium uptake of mVP24 in monomeric and predominately dimeric states. Consistent with our hypothesis and that

predicted by the structure of mVP24, N-terminal residues (1–45) and residues involved in contacts with the other monomer in the crystal structure, such as residues within peptides 65–79, 165–180, and 228–253, are protected from hydrogen-deuterium exchange at high mVP24 concentrations as compared to low mVP24 concentrations (Fig. S2). Based upon the hydrogen bonding and hydrophobic contacts involving residues Ala2, Gly3, Leu4, Ser5, Thr6, Arg7, Tyr8, Asn9, and Leu10, we generated an N-terminal truncation construct (mVP24_{Nterm}, residues 22–253) and tested whether the ability of mVP24 to oligomerize was impacted. SEC-MALS analysis of MBP-tagged mVP24_{Nterm} at 60 μ M showed a significant shift in the elution volume (14.4 mL) compared to MBP-tagged mVP24 WT (12.7 mL) (Fig. 1B). The elution volume of MBP-mVP24_{Nterm} corresponds to a calculated molecular weight of 78 ± 6 kDa, which is consistent with the theoretical MW of a monomer (70.1 kDa).

To determine if mVP24 can function without the N-terminal arm, we first measured binding of mVP24 to the Kelch domain of Keap1 using isothermal titration calorimetry (ITC). Analysis of these results show that mVP24_{Nterm} binds with a higher affinity for Keap1 Kelch domain ($K_D = 77.0 \pm 4.0$ nM) than that previously determined for mVP24 WT ($K_D = 160 \pm 20$ nM, [8]) (Fig. 1C). Furthermore, mVP24_{Nterm} in the ARE luciferase reporter gene assay is able to induce ARE reporter activity to levels similar or slightly higher than those observed for the mVP24 WT (Fig. 1D). Altogether, these results suggest that the N-terminus is critical for mediating mVP24-mVP24 interaction. Furthermore, the loss of mVP24 dimerization leads to increased binding affinity to Keap1 and ARE activity.

mVP24 dimerization is disrupted upon Keap1 binding

Keap1 forms a homodimer in solution mediated through its N-terminal BTB domain, allowing two Kelch domains to bind to the DLG and ETGE motifs in the Nrf2 Neh2 domain (Fig. 2A) [23]. To determine the stoichiometry of mVP24 binding to Keap1, we analyzed the interaction of MBP-mVP24 WT with Keap1 Kelch using SEC-MALS. The Keap1 Kelch domain eluted at 16.3 mL, corresponding to the size of a monomer (MW 33.0 ± 3.0 kDa) (Fig. 2B). The MBP-mVP24 WT/Keap1 Kelch complex at 60 μ M eluted at a volume of 14.2 mL, which corresponds to an experimental molecular weight of 106 ± 2.0 kDa and a 1:1 stoichiometry (Fig. 2B). These results suggest that the dimeric form of MBP-mVP24 dissociates into a monomeric form upon Keap1 Kelch binding. However, similar assays to monitor interaction between Keap1 (full length protein) and mVP24 revealed that the 2:2 complex between Keap1 and mVP24 migrate as a single species on SEC-MALS (Fig. S3A), even in the presence of excess mVP24 (Fig. S3B). This data suggests that while mVP24 dimerization is impacted by Keap1 Kelch binding, there is no reciprocal impact of mVP24 on the Keap1 dimerization, which is mediated through the Bric-a-brac Tramtrack Broad-complex (BTB) domain. Native MS analysis of the mVP24/Kelch complex revealed that the major species observed has a MW of 106.4 kDa, consistent with a 1:1 complex. (Fig. 2C).

HDX-MS analysis reveals binding interface between mVP24 and Kelch

As an initial step to define the mVP24/Kelch interface, we carried out HDX-MS for isolated mVP24 and Kelch proteins to capture both fast and slow rates of exchange. On-column

pepsin digestion yielded 97 peptides covering 99% of the sequence of mVP24_{Nterm}, and 126 peptides covering 99% of the sequence of Kelch.

MARV VP24 became >50% labeled after 4 h, indicating a highly dynamic solution conformation (see Fig. S4 for deuterium incorporation of 28 representative peptides). Several mVP24 regions that show rapid exchange correspond to secondary structural elements such as loops and β strands, whereas regions undergoing slow HDX mapped largely to helical regions and other less solvent-exposed residues (Fig. 3A and 3C). The four α -helices (α 3, α 4, α 5, α 6) in the core of the structure remain protected from exchange over several hours of incubation, displaying 10% deuterium incorporation. In contrast, the region viewed as “top of the pyramid” (region between α 5 and α 6, as defined by [24]), undergoes nearly full exchange after 10 s of incubation. The middle region, composed of four antiparallel β -strands, including the acidic mVP24 Kelch interaction loop (K-Loop) (residue 202–219) [β 6 and β 7, exchanges more readily with up to 40% deuterium incorporation at 10 s. The HDX extent of the four β -strands (β 4, β 5, β 6, and β 7) increase over time and reach a maximum amount of labeling after 15 min of incubation, indicating the mobile and dynamic feature of the K-Loop and its adjoining regions.

Next, we examined the Kelch domain of Keap1, which forms a highly symmetric six-bladed β -propeller where each blade is a β -sheet composed of four antiparallel β -strands [25]. Kelch alone undergoes slow deuterium exchange, with most regions becoming only 50% deuterated after 4 h (Fig. 3B and 3D), in part owing to the complex hydrogen-bonding network. However, blades I and II show additional exchange within 6 min of HDX, suggesting that this region possesses a more flexible conformation within the context of the β -propeller.

Next, we compared the HDX-MS data for peptides from mVP24 free in solution and in complex with Kelch, and we identified six peptides from three regions with significant protection in HDX (Fig. 4A and 4B): (1) β -strands in K-Loop (residues 201–221) (β 6 and β 7), (2) β -strands close to K-Loop (residues 80–89 (β 3) and 181–187 (β 5)), and (3) residues 227–253 (β 7). In the presence of Kelch, the mVP24 K-Loop (peptides 201–212 (β 6), 213–217 (β 7), and 216–221 (β 7)) is most protected, with HDX substantially reduced at the earliest labeling time (10 s). These residues correspond to the previously identified Kelch interacting loop (K-Loop) (amino acids 202–219) [8], which contains the sequence DIEPCCGE [26]. Moreover, peptides 80–89 (β 3) and 181–187 (β 5) undergo slower HDX in the presence of Kelch at long incubation times (Fig. 4C; full dataset shown in Fig. S4). These regions are composed of antiparallel β -sheets and are spatially close to the K-Loop, suggesting possible conformation changes due to allosteric effects induced by Kelch binding. In addition to regions surrounding the K-Loop binding site, several regions distal to the K-Loop (peptides 30–45, 227–243, and 244–253 α 7) also exhibit minor protection from HDX in the Kelch-bound state, in relation to the mVP24_{Nterm} alone, at long incubation times. Coincidentally, these regions are also suggested to be the dimer interface of mVP24. That minor protection occurs in regions distal from the binding site indicates a possible conformation change upon binding that results in decreased flexibility of those regions.

mVP24 and Nrf2 binds an overlapping site on Kelch

We next characterized the mVP24 binding sites on Keap1 Kelch domain with differential HDX, and we identified two regions within the Kelch domain, represented by residues 335–342 (bottom of blade I) and 375–393 (bottom of blade II), with pronounced protection in the mVP24_{Nterm}-bound complex (Fig. 4D, E, and F; full dataset shown in Fig. S5A). These peptides are protected at the earliest time points (10 s labeling), suggesting an immediate occlusion from the solvent upon mVP24_{Nterm} binding. These β -strand regions are dynamic prior to ligand binding, and blade II has an additional β -strand extending from the bottom of the structure. Thus, the protection in this region in the mVP24_{Nterm}-bound complex indicates a direct electrostatic interaction with the acidic K-Loop on mVP24. This result is consistent with recent co-immunoprecipitation results that show Keap1 mutants S383P and Y334A disrupt the binding to VP24 [9]. In addition, all peptides with reduced deuterium incorporation upon mVP24_{Nterm} binding are located at the basic pocket of the Kelch propeller structure and are in close proximity to the binding sites.

mVP24 is known to upregulate expression of Nrf2-dependent genes by competing for Nrf2 binding sites and preventing Keap1 from sequestering Nrf2 [9]. *Tong et al.* [27] identified an Nrf2 acidic ETGE motif that is required for binding to Keap1 Kelch domains. It is likely that Keap1 recruits Nrf2 by binding to the ETGE motif, through electrostatic interaction with the positively charged binding sites on Kelch, in a way similar to Kelch binding with mVP24. To test this hypothesis, we incubated Kelch with the Nrf2 peptide and analyzed the complex by HDX. Nearly all peptides exhibiting protection in the ETGE peptide-bound complex of Kelch show a similar level of protection in the mVP24_{Nterm}-bound complex. These results confirm that mVP24 binds to Keap1 Kelch domain and the binding sites of Nrf2 and mVP24 overlap, as illustrated in Fig. 4F and 4G (full dataset shown in Fig. S5B).

C209 and C210 of the mVP24 K-Loop are critical for Keap1 Kelch binding

mVP24 K-Loop forms a β -turn secondary structure [24], whereas the corresponding residues of Ebola virus VP24 (eVP24) form a loop region [8]. We previously demonstrated that eVP24 does not interact with Kelch domain of Keap1. When the K-Loop is swapped into the eVP24, however, the chimera gains Keap1 Kelch binding ability and functions to activate the ARE luciferase reporter gene assay [8]. Moreover, the mVP24 K-Loop peptide (202-RRIDIEPCCGETVLSV-219) does not bind Kelch, suggesting that the structural context of the K-Loop is important (Fig. 5A). To define further key determinants within the K-Loop, we assessed the role of conserved cysteine residues in the mVP24 K-Loop. Specifically, we tested binding of mVP24 C209A/C210A and C209M/C210K mutants to Keap1 Kelch by ITC. Compared to that of mVP24 WT, mVP24 C209M/C210K does not bind Keap1 Kelch (Fig. 5B).

The binding affinity for mVP24 C209A/C210A is substantially reduced ($K_D = 550 \pm 26$ nM) (Fig. 5C). A previously characterized mutant with loss of function in the ARE reporter assay, G211A/E212A, also lost binding (Fig. 5D). In order to ensure that mutants of mVP24 used in these studies did not lose function due to structural destabilization, we conducted a series of circular dichroism studies, which reveals that there are no observable changes for the K-loop mutations used in our studies (Fig. S6). Moreover, mVP24 C209/C210 mutants

do not induce ARE reporter activity in luciferase reporter gene assay (Fig. 5E). This observation is consistent with a key role for mVP24 C209 and C210 of the K-Loop in Keap1 Kelch binding and Nrf2 activation.

To elucidate further the role of cysteine in the interaction with Kelch, we utilized a *N*-ethylmaleimide (NEM) chemical footprinting strategy [21]. This approach measures changes in reactivity/solvent-accessibility of cysteine side chains. In the absence of Kelch, mVP24_{Nterm} C209 and C210 react rapidly with NEM to take up both +1 NEM (+125 Da) and +2 NEM (+250 Da) modification products after 10 s of incubation (Fig. 6A and Fig. S7). The unmodified protein nearly disappears after 6 min of incubation. Remarkably, a significant decrease in labeling kinetics was observed in Kelch-bound mVP24_{Nterm}, with 40 % of unmodified protein remaining at longer incubation times (30 min and greater) (Fig. 6B). Therefore, the K-Loop residues Cys 209 and 210 are the key residues that contribute to the binding with Kelch.

Discussion

Filoviral infections result in a large number of changes in host gene expression, and MARV infections are associated with changes in the antioxidant response element pathways that appear to facilitate MARV replication [9]. Previous studies identified a role for mVP24 in direct modulation of Keap1-Nrf2 association [8, 9], but specificity of the interaction and the molecular mechanism by which mVP24 promote ARE activity, however, remain unclear. Using a combined biochemical and mass spectrometry study, we show that mVP24 directly engages the Kelch domain of Keap1. mVP24 binding to Kelch results in a transition from dimer to monomer for mVP24, and the resulting complex displays 1:1 stoichiometry. Consistent with our results, we propose a model where mVP24 oligomerization, driven by its N-terminus, controls the role of mVP24 in infected cells. In support of this model, we show that the N-terminal truncation elicit higher levels of ARE promoter activation and mutation of key cysteine residues within the K-Loop abrogate mVP24 interaction with Keap1 as well as loss of mVP24-dependent Nrf2 activation.

HDX-MS reveals that regions involved in the binding interface show higher levels of HDX, suggesting that dynamics in these regions may contribute to binding. Evaluation of mVP24 binding interface on Keap1 Kelch with that for Nrf2 binding on Kelch also reveals that Nrf2 and mVP24 binds Kelch at a similar site. Our results suggest that the conformation of Nrf2 in the Kelch bound form is significantly different as the K-Loop alone is unable to bind the Kelch domain. Importantly we observe that two highly conserved cysteine residues, C209 and C210, are not only major contributors to binding, but these residues may be poised as oxidative stress sensors. Importantly, substitution of alanines at these positions also result in loss of ARE activation, consistent with a functional role for the cysteine residues. Together, our results point to a highly correlated/coupled regulation between the mVP24 oligomeric state and ARE activity modulation by mVP24 during viral infection and indicate that the shift in the dimerization equilibrium is modulated by Keap1 Kelch binding.

In addition to regulation of antioxidant responses, Keap1 regulates other pathways through interactions via its Kelch domain with factors, including IKK β , p62, and Bcl2. Keap1 targets

IKK β , a factor of the NF- κ B pathway, to suppress its expression through interactions with the IKK β ETGE motif. Others have proposed that mVP24 disrupts the Keap1-IKK β interaction, leading to expression of genes with roles in apoptosis, stress responses, and immune responses [28]. This present study suggests that mVP24 directly outcompetes IKK β for Keap1 Kelch binding as a mechanism of NF- κ B pathway activation. However, the binding mode of mVP24 with Keap1 Kelch is distinct from those previously observed for other Keap1 binding proteins. Specifically, unlike previous Kelch binders, such as Nrf2, that bind via an extended conformation, we find that mVP24 binding is controlled by the structural scaffold of the filoviral VP24 proteins. The significance of this finding is that the VP24 structural scaffold controls specificity of the interaction. This conclusion is further supported by the lack of binding of a peptide corresponding to the isolated K-Loop. However, the same sequence when fused to eVP24 can confer Keap1 Kelch binding. Together, these results provide new insights into a highly specific interaction between mVP24 and Keap1 Kelch domain that controls host oxidative responses.

In addition to regulation of Keap1 function, mVP24 is also the minor matrix protein and is essential for the virus life cycle. Specifically, mVP24 is a component of the ribonucleoprotein complex in the Marburg virion [29] and is required for the formation of fully infectious virus particles [22]. mVP24 also contains membrane binding abilities and functions in the release of viral particles. We suggest that VP24 functions in the transport of nucleocapsid and facilitates interaction between the nucleocapsid and plasmid membrane. Furthermore, the N-terminus of Ebola virus VP24 is important for nucleocapsid formation and oligomerization [30, 31]. We provide evidence that mVP24 homo-oligomerization is largely dependent on the N-terminus. Thus, mVP24 likely functions in assembly and budding as higher order oligomers. Our results provide a mechanism by which mVP24 can partition into ARE regulation or matrix function by shifting the dimer to monomer equilibrium. Additionally, our results identify a novel target for antiviral development whereby small molecules that can modulate ARE activity, or prevent mVP24 from modulating ARE activity, can limit high levels of virus replication.

Experimental Procedures

Cloning, Protein Expression, and Purification

The coding region of full length human Keap1 (accession no. NP_036421), human Keap1 Kelch (residues 322–624; Accession # NP_0364210), and full length mVP24 (Marburg strain; Accession # YP_001531158) were subcloned into a modified pET15b vector (Novagen, Madison, WI) containing a maltose binding protein (MBP) fusion tag. Mutant mVP24 constructs were generated by overlap PCR and confirmed by sequencing. Proteins were expressed in BL21(DE3) cells, cultured in Luria Broth media at 37 °C, induced at an OD₆₀₀ of 0.6 with 0.5 mM IPTG, and grown for 12–15 h at 18 °C. Cells were lysed using a cell disrupter (Avestin) and centrifuged at 47,000 g to remove debris. Proteins were purified to homogeneity using affinity and ion-exchange chromatographies (GE Healthcare, Pittsburgh, PA), prior to a final gel filtration step into buffer containing 10 mM HEPES pH 7.5, 150 mM NaCl, and 2 mM *tris*(2-carboxyethyl)phosphine. Protein purity was assessed by Coomassie staining of SDS-PAGE and mass spectrometry.

Isothermal Titration Calorimetry

Binding assays were performed on a VP-isothermal titration calorimeter (VP-ITC) (MicroCal, Northampton, MA). Protein samples were dialyzed against buffer (10 mM HEPES pH 7.5, 150 mM NaCl, and 2 mM tris(2-carboxyethyl)phosphine (TCEP)) for 12 h at 25 °C. Titrations were set up with 50–100 μ M protein solution in the syringe and 4–10 μ M protein solution in the cell. A reference power rate of 3 μ cal/s was used, and the resulting ITC data were processed and fit to a one-site binding model to determine *n* (number of binding sites) and *K_D* (dissociation constant) using ORIGIN 7.0 software. All experiments were performed at least in duplicate.

Multi-angle Light Scattering

Standard multi-angle light scattering experiments were carried out with a Superdex 200 (10/300 GL) connected in-line to a Dawn Heleos II multi-angle light-scattering (MALS) detector (Wyatt Technologies, Santa Barbara, CA). 100 μ L samples (2 mg/mL or 4 mg/mL) were injected at a flow rate of 0.3 mL/min in a column equilibrated in 10 mM HEPES, pH 7.5, 150 mM NaCl, and 2 mM *tris*(2-carboxyethyl)phosphine (TCEP) buffer. Molecular weights and standard deviations were determined using Astra software package version 6.1 (Wyatt Technology Corporation, Santa Barbara, CA). All experiments were performed at room temperature and at least in duplicate.

Luciferase Reporter Assay

HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY) with the antioxidant response element (ARE) firefly luciferase reporter plasmid (pGL4.37[luc2P/ARE/Hygro] Promega), a constitutively expressed *Renilla luciferase* reporter plasmid (pRLTK, Promega) and Flag-Nrf2, Flag-Keap1, HA-mVP24 and HA-mVP24 mutants as indicated. A dual luciferase assay (Promega) was performed 18 h post transfection. Firefly luciferase values were normalized to *Renilla* luciferase values. Error bars represent the standard error of the mean (SEM) of triplicate samples. Statistical significance was assessed by independent, unpaired t-tests as indicated; * *p*<0.05, ** *p*<0.01, *** *p*<0.001 and **** *p*<0.0001.

Hydrogen deuterium exchange mass spectrometry (HDX-MS)

The mVP24, Kelch, and mVP24/Kelch complex were buffer-exchanged with PBS buffer (pH 7.4). Deuterium labeling was initiated by diluting the mVP24, Kelch, and mVP24/Kelch complex (30 μ M, 2 μ L) 10-fold with D₂O buffer, or H₂O buffer for samples measured for no deuterium control. As a control, mVP24 and Kelch (30 μ M, 2 μ L each) were incubated alone in D₂O buffer and labeled under identical conditions. At different time intervals (10, 30, 60, 360, 900, 3600, and 14400 s), the labeling reaction was quenched by adjusting rapidly the pH to 2.5 with 30 μ L of quench buffer (3 M urea, 1% trifluoroacetic acid, H₂O) at 4 °C. For mVP24 dimerization experiments, deuterium labeling of mVP24 samples was initiated by diluting mVP24 at 30 μ M (2 μ L) and 222 μ M (2 μ L) in 3 μ L D₂O buffer or H₂O buffer for no deuterium control. At time intervals specified above, the labeling reaction was quenched by adjusting rapidly the pH to 2.5 with 145 μ L quench buffer. Following the quench and mixing, the protein mixture was immediately injected into a custom-built HDX device and

passed through a column containing immobilized pepsin (2 mm × 20 mm) at a flow rate of 200 µL/min in 0.1% formic acid, and the resulting peptic peptides were captured on a ZORBAX Eclipse XDB C8 column (2.1 mm × 15 mm, Agilent, Santa Clara, CA) for desalting (3 min). The C8 column was then switched in-line with a Hypersil Gold C18 column (2.1 mm × 50 mm, Thermo Fisher, Waltham, MA), and a linear gradient (4% to 40% acetonitrile, 0.1 % formic acid, 50 µL/min flow rate, over 5 min) was used to separate the peptides and direct them to a LTQ-FTICR mass spectrometer (Thermo Fisher, Waltham, MA) equipped with an electrospray ionization source. Valves, columns, and tubing for protein digestion and peptide separation were submerged in an ice-water bath to minimize back-exchange.

Resulting data were processed and peptides identified by exact mass analysis and LC-MS/MS using Mascot (Matrix Science, London, UK) and manual inspection as previously described [32]. Raw HDX spectra and peptide sets were also submitted to HDX Workbench for calculation and data visualization in a fully automated fashion [33]. All HDX data were normalized such that the deuterium content of the sample reaction is considered 100% and differences in deuterium uptake level following all incubation time points were calculated and mapped onto the protein 3D structure for data visualization.

Circular Dichroism

All protein samples were prepared in 25 mM NaPO₄ pH7, 150 mM NaCl, 2mM TCEP at 10 µM. CD spectra were acquired in triplicate using a Chirascan CD spectrometer (Applied Photophysics). Wavelength scans from 200–280 nm were performed to monitor the change in molar ellipticity of each protein at 4°C and at a 40 nm/min scan rate.

NEM chemical footprinting of proteins

All protein samples were prepared in PBS buffer (pH 7.4) at 60 µM concentrations. NEM labeling experiments were performed as described before [21]. Briefly, 100 µl of mVP24/Kelch was mixed with NEM (60 µM, 100 µl) and incubated at 25 °C with aliquots removed at different time points. As a control, the footprinting of mVP24 alone was performed under identical conditions. At certain selected time point (0, 30, 60, 120, 360, 900, 1800, 3600 and 14400s), 4-µL fractions were removed and added to a series of microcentrifuge tubes containing 4 µL of DTT (20 mM) to quench the labeling reaction. Following quenching, 2 µl of each sample was diluted with water and injected into a maXis 4G Q-TOF mass spectrometer (Bruker, Billerica, MA) coupled to an Agilent 1200 HPLC (Agilent, Santa Clara, CA) for mass measurement of the intact protein. Protein samples were loaded on a ZORBAX Eclipse XDB C8 column (2.1 mm × 15 mm, Agilent) for desalting (3min) and then eluted to the mass spectrometer by using 50% (v/v) acetonitrile with 0.1% formic acid (FA) at a flow rate of 10 µl/min.

NEM chemical footprinting data processing

Spectral deconvolution was performed using MagTran program. Both mVP24/Kelch and mVP24 control samples at t₀ showed a single peak feature of mVP24 at the global mass level, suggesting no additional oxidation. We then examined the mass spectra of mVP24 in the presence and absence of Kelch at the protein level, and two features with 125 Da and 250

Da mass shifts were assigned as mVP24:NEM and mVP24: 2NEM, respectively. Quantification was achieved by dividing the ion peak signal intensity of NEM labeled proteins by the sum of the peak intensities of modified and unmodified proteins at the global MS level. The sums of intensities of different NEM adducts of mVP24 were normalized to 1. The fraction of all species of VP24: Kelch and VP24 control proteins was plotted against the incubation time for comparison.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Antioxidative response mechanisms modulated by viruses are not well understood.
- Marburg virus VP24 protein oligomerization modulates antioxidative responses.
- Combined biophysical and cell biology study defines a novel mechanism.
- Conformational changes provides a novel target for antiviral development.

Figure 1. Dimerization of mVP24 is not required for Keap1 interactions

(A) Elution profiles of MBP-mVP24 on a Superdex 200 column at 3 (black), 6 (navy), 11 (royal blue), 22 (green), 38 (orange), 59 (magenta), 112 (yellow) μ M concentrations. (B) SEC-MALS elution profiles of 60 μ M MBP-mVP24 WT (purple) and MBP-mVP24_{N-term} (black) from a Superdex 200 column. The theoretical monomeric molecular masses for MBP-mVP24 WT and MBP-mVP24_{N-term} are 72.6 and 70.1 kDa, respectively. Molecular weights determined by SEC-MALS of the major peaks of MBP-mVP24 WT and MBP-mVP24_{N-term} are 146 ± 1 and 78 ± 6 kDa, respectively. The minor peaks of MBP-mVP24 WT and MBP-mVP24_{N-term} correspond to MBP contaminant and dimeric MBP-mVP24_{N-term}, respectively. (C) ITC raw data and binding isotherm for MBP-mVP24_{N-term} bound to Keap1 Kelch. Measured values are $K_D = 77 \pm 4$ nM, $H = -2.0 \pm 0.06 \times 10^{-4}$ cal/mol, $S = -37$ cal/mol/deg, and n (no. of sites) = 0.90 ± 0.02 . (D) HEK293T cells were transfected with ARE luciferase reporter plasmid, a constitutively expressed Renilla

luciferase plasmid, and pCAGGS (empty vector) or decreasing concentrations of mVP24 WT or mVP24_{Nterm}. At 18 h posttransfection, luciferase activity was assayed. Western blots of HA and β -tubulin are indicated.

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Figure 2. mVP24 binds Keap1 Kelch domain with 1:1 stoichiometry

(A) Domain organization of Nrf2 and Keap1. Numbers and residues corresponding to Nrf2 Neh2 domain from the human species are shown. Regions containing the DLG and ETGE motifs are underlined. Keap1 is composed of N-Terminal Region (NTR), Bric-a-brac Tramtrack Broad-complex (BTB), intervening region (IVR), and Kelch/C-Terminal Region (CTR) domains. (B) Elution profiles of MBP-mVP24 (purple), Keap1 Kelch domain (blue), and MBP-mVP24/Keap1 Kelch domain complex (red) on a Superdex 200 column couple to MALS. Molecular weights determined by SEC-MALS of MBP-mVP24, Keap1 Kelch

domain, and MBP-mVP24 WT/Kelch complex are 146 ± 1 , 33 ± 3 and 106 ± 2 kDa, respectively. The theoretic monomeric molecular masses for Keap1 Kelch domain and MBP-mVP24 are 33.3 and 72.6 kDa, respectively. (C) Native mass spectra. The colored dots denote peaks for MBP-mVP24 monomer (green, +17 to 14), Keap1 Kelch domain (blue, +11 to 9), and MBP-mVP24/Keap1 Kelch domain complex (red, +21 to 16) corresponding to calculated MWs of 73, 34, and 106 kDa, respectively. Peaks surrounded by a blue box denotes peaks arising from the mVP24 dimer.

Figure 3. HDX-MS identifies solvent accessible regions within mVP24 and Keap1 Kelch domain
 Cartoon representations of (A) Phyre2 model of mVP24 threaded with PDB 4OR8 and (B) Keap1 Kelch domain structure (PDB 1U6D). The percentage of deuterium incorporation for selected peptides of (C) mVP24 and (D) Kelch are mapped onto the structures for five incubation times (10 s, 1 min, 6 min, 15 min, 4 h); the color code legend is shown to the right of panels (C)-(D). Sequences that could not be detected by HDX-MS are colored in gray.

Figure 4. The molecular interface between mVP24 and Keap1 Kelch domain is defined by HDX-MS

(A) Differences in deuterium uptake induced by Kelch binding are displayed as a color gradient for each peptide at the indicated time points. The color code (see legend) represents the differential HDX; regions colored in white are not detected by HDX. (B) Comparison of deuterium uptake curves of mVP24 (black) and mVP24-Kelch complex (red). (C) Important binding regions are highlighted in the cartoon representation of mVP24. The color gradient represents the average amount of deuterium uptake of mVP24-Kelch complex subtracted

from that of free mVP24 and is the same as in (A). **(D)** Differences in deuterium uptake induced by mVP24 (left column) and ETGE peptide (right column) binding are displayed as a color gradient for each peptide at the indicated time points. The color code (see legend) represents the differential HDX; regions colored in white are not detected by HDX. **(E)** Comparison of deuterium uptake curves of Kelch upon binding with mVP24 (top row, black and red curve) and ETGE peptide (bottom row, blue and purple curve), respectively. The average amount of deuterium uptake of **(F)** mVP24-Kelch and **(G)** Kelch-Nrf2 ETGE peptide complexes were subtracted from that of free Kelch and mapped onto the crystal structure of Kelch. Coloring follows the legend in (D). mVP24 and Keap1 Kelch structures have the same orientation as Fig. 3A and Fig. 3B, respectively.

Figure 5. Conserved cysteine residues within the K-Loop are critical for Kelch binding
(A) Representative ITC data for Keap1 Kelch binding to mVP24 K-Loop (K_D = not determined). Representative ITC data for Keap1 kelch binding to **(B)** MBP-mVP24_{Nterm} C209M/C210K (K_D = not determined) **(C)** MBP-mVP24_{Nterm} C209A/C210A (K_D = 550 ± 26 nM, $H = -1.45 \pm 0.07$ E4 cal/mol, $S = -20.1$ cal/mol/deg, and $n = 0.850 \pm 0.03$) and **(D)** MBP-mVP24_{Nterm} G211A/E212A (K_D = not determined). Representative raw heats of reaction versus time (top panel) and the integrated heats of reaction versus molar ratio of ligand to receptor (bottom panels) are shown. **(E)** HEK293T cells were transfected with ARE luciferase reporter plasmid, a constitutively expressed Renilla luciferase plasmid, and pCAGGS (empty vector) or increasing concentrations of mVP24 WT, mVP24 C209A/C210A, or mVP24 C209M/C210K. At 18 h posttransfection, luciferase activity was assayed. Western blots of HA and β -tubulin are indicated.

Figure 6. NEM labeling reveals differential labeling of the mVP24 K-Loop

(A) mVP24 and (B) mVP24/Kelch complex at different labeling times. The relative fraction of unmodified mVP24 (black dots), + 1 NEM adduct (blue dots) and +2 NEM adduct (red dots) were calculated as the ratio of the intensity of each species to the sum of intensities of all labeling products. The sums of intensities of different NEM adducts of mVP24 were normalized to 1.