CHAPTER 22

Biological Warfare: Infectious Disease and Bioterrorism

SUMMARY

Many types of biological warfare exist. The goal is not just to infect a large number of individuals with bacteria or viruses, but it could also mean contamination of the food supply or water. Regardless, biological warfare has been practiced for many centuries by humans but is not unique to the human species. Bacteria produce bacteriocins (colicins) against other bacteria to help eliminate competition for resources. Toxins are also produced by bacteria, but unlike colicins, toxins act on higher organisms. Some single-celled, eukaryotic protozoans, such as *Paramecium*, release symbiotic bacteria called kappa particles. Any *Paramecium* that is not already protected by its own stash of kappa particles will be killed if it consumes the kappa particle. The etiologic agent of diphtheria, *Corynebacterium diptheriae*, produced a toxin that is encoded by an integrated virus. Eukaryotes, such as spiders, snakes, caterpillars, and maggots, have devised ingenious ways to protect themselves through biowarfare.

In medical microbiology, the war between humans and microbes often takes a hit when the microbe has developed resistance to antibiotics. Resistance occurs because antibiotics are misused, overprescribed, and available over the counter in some countries without prescriptions. Widespread agricultural use is also problematic. In addition to the well-known methicillin-resistant *Staphylococcus aureus* (MRSA), the CDC reports that antibiotics resistance is occurring for *Clostridium difficile;* some Enterobacteriaceae, including *Klebsiella* and *Escherichia coli;* and *Neisseria gonorrhoeae*. Additionally, some strains of *Mycobacterium tuberculosis* appear to be resistant to all treatment. With all these antibacterial-resistant microbes, certainly the identification of novel antibiotics and new targets is sought. Some strategies include targeting siderophores, which mammals do not have and would therefore be specific to the microbe. Another strategy involves identification of novel antibiotics from microbes or the identification of novel antimicrobial biosynthetic pathways. Finally, existing antibiotic resistance mechanisms can be disrupted. Other methods to combat bacterial infections include the use of phage therapy, genetic engineering, and nanotechnology.

Phage therapy is the use of a bacteriophage to stop a bacterial infection. This therapy was used with some success by the French, Polish, Russians, and Georgians to treat ailments such as dysentery, typhoid fever, colitis, skin infections, and others. Genetic engineering has been employed to target bacterial adhesion proteins or the production of altered toxins to interfere with natural analogs of toxins. Nanotechnology research has provided bactericidal surfaces coated with either metal ions or "black silicon" composed of "nanopillars" or nanocarpets. Some surfaces prevent the attachment of bacteria and therefore improve sanitation in health-care settings.

Human biological warfare often involves the destruction of crops or water sources. During the days of the bubonic plague, the corpses of people who had died from the plague were catapulted over the walls of cities in an attempt to infect the inhabitants. Unfortunately, the goals of the aggressors were rarely accomplished because the plague is mostly spread by fleas and rats, and not by contact with corpses. The Germans used biological agents against horses during WWI. The French did the same in WWII. During WWII the Japanese infected Chinese prisoners with diseases such as cholera, hemorrhagic fever, and venereal diseases. In addition, the United States released a relatively harmless strain of *Serratia marcescens* over American cities to study the spread of disease. The Russians managed to greatly increase their biological weapons program despite signing treaties prohibiting their use. Although unconfirmed, the Russians weaponized the Marburg virus, a hemorrhagic fever.

Bioterrorism is a tactic used to scare people. Disruption of major services, such as the health, postal, or military services, is a key goal of biological warfare. The psychological impact usually outdoes the pathological impact, however. The costs associated with preventing infection are great, even when troops from an industrialized country enter into combat in a third-world country. There is a great financial strain when distributing antimalarial drugs or antibiotics.

Research in counter-bioterrorism involves thinking like a bioterrorist. From a bioterrorist's perspective, several major factors influence the choice of germ weapon: preparation, weaponization, dispersal, persistence, incubation time, and laboratory space. Preparation involves the growth of the agent. Some organisms are easier to grow than others, and viruses require a host cell. Therefore, cell culture techniques would be employed. Weaponization is the ability to prepare the agent in such as a way as to make delivery more effective. Also, dispersal of the infective agent needs to be considered. To infect the largest number of people, the germ would need to be aerosolized. Large cities and urban areas might seem like good targets for a bioterrorist attack, but these locations are actually somewhat protected … by air pollution! The pollutants have been shown to kill some aerosolized bacteria in controlled studies. Also, desiccation and UV light exposure can kill a potential biowarfare germ. Persistence is the most difficult factor. The agent must persist in weaponized format for long periods of time, in addition to persisting in the environment long enough to infect people. If the agent persists for too long, it might infect the invaders. The incubation period for most biowarfare organisms is slow, which means that humans can survive the initial infection and continue to fight or function during that time period, unlike traditional brute-force type weapons. Not only incubation period and delivery of the agent, but a bioterrorist also needs to worry about storage and preparation, which also involve high-containment facilities (biosafety level four for some agents) and cautious practices by trained staff. Anthrax, plague, brucellosis, tularemia, and others have been suggested as potential biowarfare bacterial agents, whereas smallpox, Ebola fever, and Lassa fever are potential viral agents.

Anthrax is the disease caused by a common soil bacterium, *Bacillus anthracis*, that often infects livestock. It has the ability to form spores when conditions are not ideal for growth, which will germinate when conditions improve. Anthrax can cause three types of infections in humans. Cutaneous anthrax is a skin infection. Gastrointestinal anthrax is caused by ingestion of the spores or bacteria. The third method of infection is by inhalation. Inhalational anthrax has a high death rate associated with it. Anthrax is a weapon of choice because it is easy to propagate, can cause high mortality, and the spores are persistent. Another bacterial agent that is potentially a good biowarfare agent is *Yersinia pestis*, which causes bubonic plague. The plague is spread easily and kills quickly, thus, making it a choice biological agent. Other bacterial agents include *Brucella*, *Francisella tularensis*, and *Bukholderia pseudomallei*.

Variola is the smallpox virus that was eradicated from the world in 1980, although some samples remain in research facilities. It is highly infectious and can cause a moderate amount of mortality. Most people are not vaccinated anymore, which means this is an excellent potential agent.

Other viral agents include filoviruses, such as Marburg and Ebola, which cause hemorrhagic fevers. They are spread via contaminated blood and body fluids and can be very lethal, with upwards of 90% mortality. Yellow fever, dengue fever, and Lassa fever are also possible viral agents for bioterrorists. Yellow fever and lassa fever are lethal, but dengue fever is not usually lethal. Dengue is, however, painful and incapacitating.

Targets for bioterrorists include not only humans, but also agriculture. The goal is to cause a food shortage by specifically targeting livestock and crops. Fungi, such as rusts, smuts, and mold, could potentially be used to destroy crops. The fungi are pathogenic to the plants, and some even produce toxins that could be harmful to animals, including humans, if consumed.

Purified toxins, such as botulinum toxin or ricin, are also potential agents. *Clostridium botulinum* is the bacterium that causes botulism by producing a neurotoxin. This toxin has also been marketed in cosmetics as Botox. Ricin, a ribosome-inactivating protein, is extracted from seeds of the castor bean plant and can be weaponized for biological warfare. Conotoxin is produced by cone snails and causes muscle paralysis in its victims.

With the many advances in molecular biology and biotechnology, some minor concerns have been raised that a more deadly biological agent could be engineered to work faster, spread farther, and cause more damage and/or death. Researchers have attempted to create viruses that are more capable of eliciting an immune response, presumably for more efficient vaccination. However, in one case, the manipulations of mousepox actually lead to an increased virulence. Genetic engineering might also be used to create camouflaged viruses, that is, pathogenic viruses hiding in nonpathogenic bacterial cells.

Molecular diagnostics analyzes biomolecules, such as DNA or RNA, to identify a pathogen. Fluorescence *in situ* hybridization allows samples to be directly probed with fluorescent DNA oligonucleotides to a specific pathogen. Peptide nucleic acid (PNA) replaces the DNA backbone with a neutral peptide. This enables probes to enter cells more easily. PCR and variations of PCR offer multiple methods as diagnostic tools. One such variation called PLEX-ID employs PCR and mass spectrometry to identify pathogens in a sample within 8 hours.

Biosensors could be developed through biotechnology applications to one day sense the presence of biowarfare agents such as toxins, viruses, and bacteria. Although biosensors are currently used in the clinical and food and drug industries, they might one day be expanded to counter bioterrorism.

Case Study The Marburg Virus VP24 Protein Interacts with Keap1 to Activate the Cytoprotective Antioxidant Response Pathway

Megan R. Edwards et al. (2014). *Cell Reports* 6, 1017–1025.

Kelch-like ECH-associated protein 1 (Keap1) targets transcription factor nuclear factor (Nrf2) for ubiquitination and degradation by interacting directly with Nrf2-ECH homology-2 (Neh2) domain through the Kelch domain. Any disruption in Nrf2-Keap1 leads to expression of antioxidant response elements (AREs), which include genes involved in detoxification, cell survival, and immune modulation.

Both Marburg viruses and Ebola viruses are members of *Filoviridae* and cause hemorrhagic fevers with high human mortality rates. Both viruses are zoonotic pathogens that likely reside in bats as the host reservoirs. VP24 proteins in filoviruses are involved in the formation of nucleocapsids, release of the virus particles from the host cells, and modulation of RNA synthesis. Marburg mVP24 has recently been shown to bind the Kelch domain of Keap1, thus activating Nrf2. Upon activation, cytoprotective responses are induced during infection.

In this study, the authors determined that mVP24, but not eVP24 (Ebola VP24), interacts with both human and bat Keap1 and activates Nrf2, which in turn causes an upregulation of cytoprotective responses.

What technique was utilized to determine that Keap1 interacts with mVP24 but not eVP24? What was the outcome?

Coimmunoprecipitation (coIP) assays were performed with Flag-tagged Keap1 and HA-mVP24 or HA-eVP24. The lysate from cells containing both proteins (Keap1 and either mVP24 or eVP24) were incubated with magnetic beads. The precipitates were then eluted from the beads and analyzed. Through the coIP assays, the authors determined that Keap1 interacts with HA-mVP24 only and not with HA-eVP24.

Several previously determined domains are present on Keap1. How were the authors able to locate the specific domain of Keap1 that interacts with mVP24? Were the authors able to identify specific amino acid residues involved in the interaction?

Keap1 contains several domains, including N-terminal region (NTR), intervening region (IVR), Bric-a-Brac, Tramtrack, Broad complex, and Kelch/C-terminal domain (CTR). Domain deletion mutants were utilized to determine if mVP24 still interacted with mVP24 in coIP assays. Loss of interaction was observed in Kelch/CTR domain mutants. The authors determined the arginine residue at position 415 was responsible for not only Keap1 interaction, but also Nrf2 interaction.

What specific region within mVP24 interacts with Keap1?

The authors utilized solved structures of VP24 from the Zaire strain of Ebola virus to compare with predicted mVP24 structures from the Phyre2 software package. From this comparison, an exposed K-loop was identified between residues 202 and 212. The sequence of the loop is not conserved with other filoviruses and resembles Nrf2 binding motifs used to interact with the Kelch domain of Keap1. Several mutants with altered K-loop sequences were constructed and investigated. Through coIP assays, the authors determined that the K-loop of the Keap1 Kelch domain is responsible for mVP24-Keap1 interaction.

The interaction between Keap1 and Nrf2 inhibits ARE gene expression. How did the authors establish that Nrf2 gene expression is determined by the interaction of mVP24 with the Kelch domain of Keap1?

Nrf2 fusion proteins to green fluorescent protein (GFP) were constructed and expressed with Keap1 and wild-type or mutant versions of mVP24 and eVP24. Nuclear localization was observed for GFP-Nrf2 when Nrf2 was overexpressed. Coexpression of Keap1 with GFP-Nrf2 resulted in Nrf2 remaining in the cytoplasm. Upon addition of mVP24 or eVP24 mutant, GFP-Nrf2 nuclear localization returned.

Nuclear localization of GFP-Nrf2 returned upon addition of mVP24 or a mutant eVP24 (carries a K-loop sequence that is the same as the mVP24 K-loop sequence). Additionally, ARE-luciferase reporter genes are activated in the presence of wild-type mVP24. What is the significance of these results in terms of Marburg viral infection?

Upon viral infection, host cells respond by inducing a cytoprotective state, specifically by upregulating ARE promoters. AREs are antioxidant response elements. This means that upon infection with Marburg virus, the host cell's antioxidant response is activated directly through the initial interaction of Keap1 and mVP24 followed by upregulation of ARE transcription through the action of Nrf2. In this case, viral strategy is not only to replicate more virus particles, but also to activate the antioxidant response of the host cell. Interaction with mVP24 disrupts Keap1 interactions with several proteins and inhibits apoptosis, activates cell survival pathways, and moderates autophagy.

The authors determined that Marburg virus VP24 protein interacts with Keap1 protein from both human and bat. This interaction disrupts the Keap1-Nrf2 binding and leads to accumulation of Nrf2 in the nucleus, followed by activation of the antioxidant response. Specifically, the interaction occurs at the K-loop on the Kelch domain of Keap1. An R415A amino acid substitution abolished Keap1-mVP24 interaction.

Marburg virus and Ebola virus are members of *Filoviridae*. When investigating the interactions of VP24 to human and bat Keap1 protein, the authors noted the differences in binding of Keap1 to either mVP24 or eVP24. The amino acid sequence on a portion of the K-loop is different between mVP24 and eVP24. The sequence on the mVP24 is more similar to other Keap1-binding motifs.

In conclusion, the authors investigated a direct binding mechanism of a viral protein that results in the upregulation of antioxidant response in the host cell.

Cell Reports
Report

The Marburg Virus VP24 Protein Interacts with Keap1 to Activate the Cytoprotective Antioxidant Response Pathway

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http://dx.doi.org/10.1016/j.celrep.2014.01.043

SUMMARY

Kelch-like ECH-associated protein 1 (Keap1) is a ubiquitin E3 ligase specificity factor that targets transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) for ubiquitination and degradation. Disrupting Keap1-Nrf2 interaction stabilizes Nrf2, resulting in Nrf2 nuclear accumulation, binding to antioxidant response elements (AREs), and transcription of cytoprotective genes. Marburg virus (MARV) is a zoonotic pathogen that likely uses bats as reservoir hosts. We demonstrate that MARV protein VP24 (mVP24) binds the Kelch domain of either human or bat Keap1. This binding is of high affinity and 1:1 stoichiometry and activates Nrf2. Modeling based on the Zaire ebolavirus (EBOV) VP24 (eVP24) structure identified in mVP24 an acidic loop (K-loop) critical for Keap1 interaction. Transfer of the K-loop to eVP24, which otherwise does not bind Keap1, confers Keap1 binding and Nrf2 activation, and infection by MARV, but not EBOV, activates ARE gene expression. Therefore, MARV targets Keap1 to activate Nrf2-induced cytoprotective responses during infection.

INTRODUCTION

Kelch-like ECH-associated protein 1 (Keap1) is a cellular adaptor protein that links the Cul3/Rbx1 (Roc1) ubiquitin E3 ligase to the oxidative stress response through its interaction with the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (reviewed in Copple, 2012). Under homeostatic conditions, Keap1 suppresses the cellular antioxidant transcriptional program by directing the ubiquitin-mediated degradation of Nrf2 (Itoh et al., 1999; McMahon et al., 2003). Keap1 interacts, via its Kelch domain, with two sites located in the Nrf2-ECH homology-2 (Neh2) domain of Nrf2 (Itoh et al., 1999; Tong et al., 2006). Disruption of Nrf2-Keap1 interaction leads to transcription of genes possessing antioxidant response elements (AREs) (Tong et al., 2007). The upregulated ARE genes encode proteins involved in detoxification reactions, cell survival, and immune modulation (reviewed in Baird and Dinkova-Kostova, 2011; Ma, 2013).

ARE responses impact the outcome of viral infections. For example, the Nrf2 pathway inhibits influenza virus and respiratory syncytial virus replication in cell culture and in vivo (Cho et al., 2009; Kesic et al., 2011). In contrast, for hepatitis B virus, hepatitis C virus, and human cytomegalovirus, induction of ARE responses may protect infected cells from oxidative damage and influence immune responses by modulating immunoproteasome function (Burdette et al., 2010; Ivanov et al., 2011; Lee et al., 2013; Schaedler et al., 2010).

Marburg viruses (MARVs) and Ebola viruses (EBOVs), members of the family *Filoviridae*, are emerging, zoonotic pathogens that likely use bats as reservoir hosts. Filoviruses are of concern because they cause hemorrhagic fever with a high fatality rate in humans (reviewed in Brauburger et al., 2012). Filoviruses encode multifunctional VP24 proteins, which play important roles in the formation of viral nucleocapsids, release of infectious virus particles, and modulation of viral RNA synthesis (Bamberg et al., 2005; Beniac et al., 2012; Bharat et al., 2011, 2012; Hoenen et al., 2006; Huang et al., 2002; Mateo et al., 2011; Noda et al., 2006; Watanabe et al., 2007; Wenigenrath et al., 2010). In addition, EBOV VP24 (eVP24) disrupts interferon (IFN) signaling pathways and interacts with select karyopherin α proteins (KPNAs), thereby blocking nuclear accumulation of tyrosine-phosphorylated STAT1 (Mateo et al., 2010; Reid et al., 2006, 2007). In contrast, MARV VP24 (mVP24) neither interacts with KPNAs nor inhibits IFN signaling, and functionally relevant interactions with host factors have not previously been defined (Valmas et al., 2010). However, a recent mass spectrometry screen identified Keap1 as a potential mVP24 binding partner (Pichlmair et al., 2012).

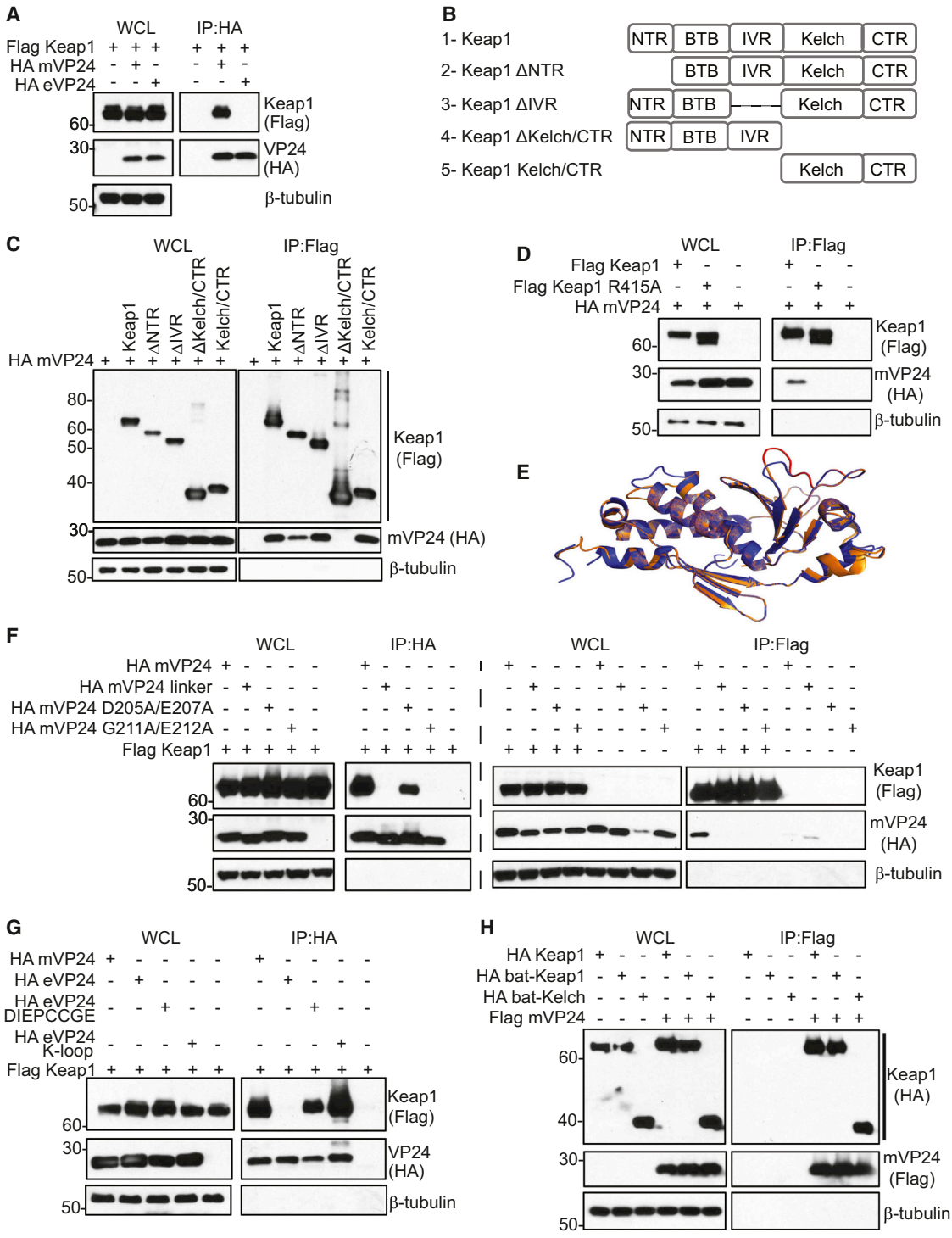


Figure 1. mVP24 Interacts with Keap1 in CoIP Assays

(A) coIPs with HA antibody were performed on lysates of HEK293T cells cotransfected with plasmids for Flag-Keap1 and HA-mVP24 or HA-eVP24. Western blots were performed for Flag and HA. WCL, whole cell lysate; IP, immunoprecipitation.

(B) Schematic diagram of Flag-tagged Keap1 domain deletion mutants used in (C).

(C) Flag-Keap1 domain deletion mutant constructs were coexpressed in HEK293T cells with HA-mVP24 and analyzed by coIP with Flag antibody.

(D) HA-mVP24 and either Flag-Keap1 or Flag-Keap1 R415A were analyzed by coIP as in (C).

(E) Overlay of the mVP24 structural model (orange) on the determined eVP24 structure (purple). The mVP24 K-loop (amino acids 205–212) is indicated in red.

(legend continued on next page)

To date, the described mechanisms by which viruses engage the ARE response do not involve direct interaction with components of the signaling pathways. Rather, viruses are demonstrated to activate other signaling pathways or induce oxidative stress, indirectly activating antioxidant responses. Here, we demonstrate that mVP24 but not eVP24 directly interacts with the human and bat Keap1 proteins. We further define the basis of the interaction and demonstrate that expression of mVP24 but not eVP24 activates Nrf2, triggering cytoprotective responses. Correspondingly, MARV but not EBOV infection activates ARE gene expression. Collectively, these data suggest that MARV evolved to specifically target a host cytoprotective gene expression program to facilitate its replication.

RESULTS

mVP24 Interacts with Keap1

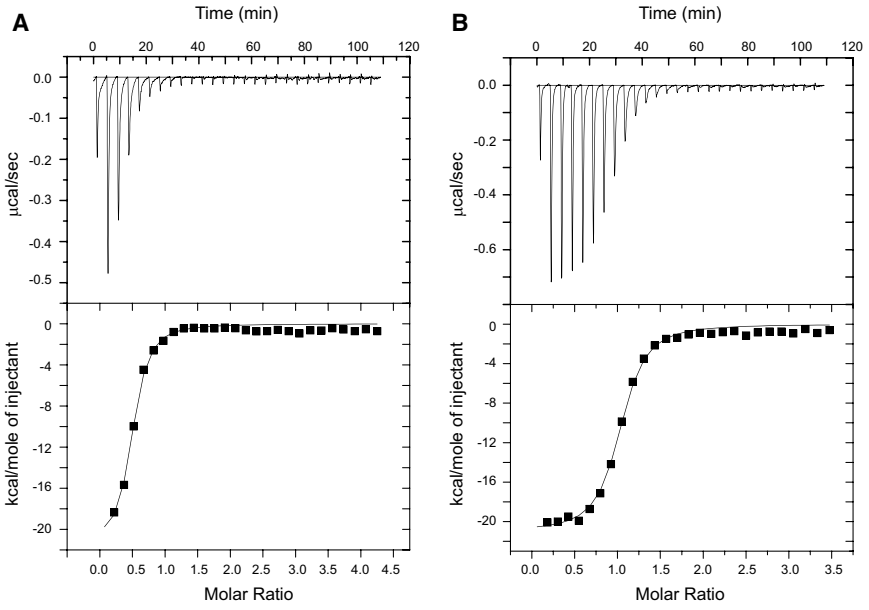
Coimmunoprecipitation (coIP) assays demonstrated that Flag-tagged Keap1 interacts with HA-mVP24, but not with HA-eVP24 (Figure 1A). Keap1 contains several previously defined domains: the N-terminal region (NTR); the Bric-a-Brac, Tramtrack, Broad complex (BTB) domain; the intervening region (IVR); and the Kelch domain/C-terminal region (CTR) (Komatsu et al., 2010). Domain deletion mutants of Keap1 and a construct comprising only the Kelch domain/CTR were tested for mVP24 interaction by coIP (Figure 1B). The NTR and IVR deletion mutants retained interaction, whereas deletion of the Kelch/CTR resulted in loss of interaction (Figure 1C). The isolated Kelch/CTR domain also interacted with mVP24 (Figure 1C). Therefore, the Kelch/CTR domain is necessary and sufficient to interact with mVP24 (Figure 1C). The mutation to alanine of Keap1 Kelch domain residue R415 disrupts interaction with Nrf2 (Lo et al., 2006). Similarly, Keap1 R415A did not coprecipitate with mVP24 (Figure 1D), suggesting that Nrf2 and mVP24 interact with the Keap1 Kelch region in a similar fashion.

To gain insight into the region(s) of mVP24 required to interact with Keap1, we used our recently solved structure of VP24 from Zaire EBOV, which is very similar to the structures of Sudan and Reston eVP24s (Zhang et al., 2012) (see Supplemental Experimental Procedures, Supplemental Results, and Table S1), and the Phyre2 software package to obtain a molecular model of mVP24 (Kelley and Sternberg, 2009). The resulting structural model identified a loop (the K-loop, amino acids 202–212) that is likely solvent exposed (Figure 1E). The sequence near the K-loop is not well conserved among filoviral VP24 proteins. This loop contains a sequence DIEPCCGE that is reminiscent of the high-affinity binding motif of DXXTGE, used by Nrf2 to interact with the Keap1 Kelch domain (Lo et al., 2006). Among the several Keap1 Kelch domain binding determinants, "GE" motifs appear to be the most highly conserved, with nearby upstream acidic residues also playing an important role for several interacting partners (Komatsu et al., 2010; Padmanabhan et al., 2008). Given this similarity, we made three HA-tagged mVP24 constructs (Figure 1F). In "mVP24 linker," the 205-DIEPCCGE-212 sequence was replaced with a serine-glycine linker. "mVP24 D205A/E207A" and "mVP24 G211A/E212A" were designed based on analogous loss-of-binding mutants described for cellular Keap1-interactor p62 (Komatsu et al., 2010). By coIP, wild-type mVP24 strongly interacted with Keap1, mVP24 D205A/E207A interacted weakly, and no interaction was detected with either mVP24 linker or mVP24 G211A/E212A (Figure 1F). To assess the role of the DIEPCCGE motif for interaction with Keap1, DIEPCCGE was swapped in place of the corresponding residues within eVP24, creating "eVP24 DIEPCCGE." We also replaced the loop of eVP24 (202-QEPDKSAMDIRHPGPV-217) with the mVP24 K-loop (202-RRIDIEPCCGETVLSESV-219), creating the "eVP24 K-loop." eVP24 DIEPCCGE and eVP24 K-loop interacted with Keap1, with the full K-loop appearing to confer better binding, whereas wild-type eVP24 once again did not interact with Keap1 (Figure 1G). These results demonstrate that the DIEPCCGE sequence and the K-loop, when placed in the context of the VP24 structural scaffold, play a critical role for mVP24-Keap1 interaction.

MARVs likely use bats as reservoir hosts (Amman et al., 2012; Towner et al., 2009). Therefore, a specific viral interaction with Keap1 likely evolved and should be conserved in bats. Alignment of human Keap1 and two divergent bat species, a microbat (*Myotis lucifugus*) and a megabat (*Pteropus alecto*), revealed 97% amino acid identity between human and microbat Keap1 and 98% amino acid identity between human and megabat Keap1 (data not shown). Full-length Keap1 (bat-Keap1) and Kelch domain (bat-Kelch) constructs were generated from an available microbat (*Myotis velifer incautus*) cell line. Both coprecipitate with mVP24 with efficiencies similar to that of human Keap1 (Figure 1H).

Keap1 inhibits ARE gene expression through its interaction with Nrf2 (McMahon et al., 2003). When Keap1 repression is relieved, which can be due to posttranslational modification of Keap1 or interaction with select Kelch domain binding partners such as p62, Nrf2 translocates to the nucleus and activates ARE gene expression (Itoh et al., 1999; McMahon et al., 2003). To determine whether the interaction of mVP24 with the Keap1 Kelch domain activates Nrf2, a GFP-Nrf2 fusion protein was expressed alone or in the presence of Flag-Keap1 and HA-tagged wild-type mVP24, mutant mVP24 or wild-type, or chimeric eVP24s. Overexpression of Nrf2, which is known to overwhelm the available endogenous Keap1, resulted in nuclear localization of GFP-Nrf2, as expected (Figure S1). Coexpression of Keap1 retained most of the Nrf2 in the cytoplasm. Additional expression of mVP24 and eVP24-K-loop restored Nrf2-GFP nuclear localization, whereas mVP24 mutants and eVP24-DIEPCCGE, which do not interact efficiently with Keap1, did not (Figure S1; see Supplemental Results for details).

(F) Flag-Keap1 and HA-mVP24 wild-type or mutants were analyzed by coIP as in (A) and (C).
(G) Flag-Keap1 and HA-mVP24, eVP24, eVP24 DIEPCCGE, or eVP24 K-loop were coexpressed in HEK293T cells and analyzed by coIP as in (A).
(H) Flag-mVP24 and HA-Keap1, bat-Keap1, and bat-Kelch were coexpressed in HEK293T cells and analyzed by coIP as in (C).
See also Figure S1.



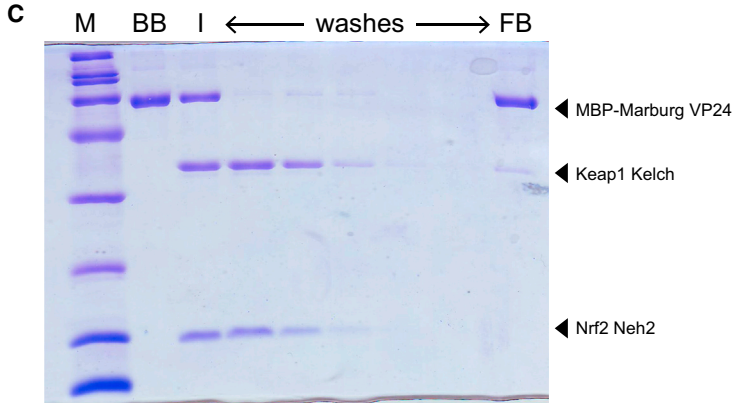


Figure 2. mVP24 Binds to Keap1 Kelch Domain with High Affinity and Specificity

(A and B) Representative ITC data for Kelch domain of Keap1 binding to (A) Nrf2 Neh2 domain and (B) mVP24. Raw heats of reaction versus time (top panels) and the integrated heats of reaction versus molar ratio of ligand to receptor (bottom panels) are shown. Thermodynamic binding parameters of $K_D = 170 \pm 60$ nM, $\Delta H = -1.96 \pm 0.1 \times 10^4$ kcal/mol, $T\Delta S = -10.4$ kcal/mol, and n (no. of sites) = 0.49 ± 0.02 for (A) and $K_D = 158 \pm 20$ nM, $\Delta H = -2.10 \pm 0.03 \times 10^4$ kcal/mol, $T\Delta S = -11.7$ kcal/mol, and n (no. of sites) = 1.00 ± 0.01 for (B) were obtained.

(C) mVP24 binding to Kelch prevents Nrf2-Neh2 interaction. Coomassie blue-stained SDS-PAGE of a pull-down assay where MBP-mVP24 was immobilized on amylose resin (BB, bound beads) is shown. Keap1 Kelch and Nrf2 Neh2 domain were subsequently added to the resin (I, input), and the resin was washed with buffer (washes). The final bound bead sample (FB, final beads) is indicated. M, molecular weight marker.

See also Figure S2.

mVP24 Binds the Keap1 Kelch Domain with High Affinity and Specificity

Binding of mVP24 to Keap1 Kelch was further evaluated by isothermal titration calorimetry (ITC), which measures heat generated by these exothermic interactions. ITC results confirmed that Keap1 Kelch binds the Nrf2 Neh2 domain with high affinity (K_D = 170 ± 60 nM) and stoichiometry (n = 0.46) (Figure 2A) and support a stoichiometry of 2:1 for Kelch binding to Neh2 with thermodynamic parameters similar to those previously reported by Tong et al. (2006). Assays under similar conditions for Kelch-mVP24 resulted in a K_D of 158 ± 20 nM (Figure 2B) with a binding stoichiometry of 1:1.

To gain additional mechanistic insight, we performed competition pull-down experiments using wild-type mVP24, eVP24, and eVP24 K-loop, which were designed based on the mVP24 structural model (Figures S2A–S2C). We established the basal binding conditions for the Kelch and Neh2 interaction by pull-down (Figure S2D) as well as Kelch binding to mVP24 (Figure S2E) and examined the ability of recombinant eVP24 (Figure S2F) and eVP24 K-loop (Figure S2G) to bind the Keap1 Kelch domain. Next, we assessed whether mVP24 can outcompete Neh2 binding to the Kelch domain. A complex between the Kelch domain and Neh2 was preformed, and the ability of an immobilized mVP24 protein to displace Neh2 from the Kelch/Neh2 complex was assessed. Despite similar affinities of Neh2 and mVP24 for Kelch domain, mVP24 can bind the Kelch domain in the presence of a 2-fold excess of Neh2 (Figure 2C). Therefore, in the absence of other factors, mVP24 displaces Nrf2 from Keap1. This provides a biochemical explanation as to how the mVP24-Keap1 interaction triggers Nrf2 nuclear localization.

mVP24 Expression Activates ARE-Directed Gene Expression

Stimuli that disrupt the Nrf2-Keap1 interaction and promote Nrf2 nuclear localization activate expression of ARE genes (reviewed in Magesh et al., 2012). We therefore assessed the ability of wild-type or mutant mVP24s to activate an ARE luciferase reporter gene. Cellular Keap1-interacting protein p62, a previously described activator of Nrf2, served as a positive control (Komatsu et al., 2010; Lau et al., 2010). Expression of mVP24 induced the ARE reporter to similar levels as p62 (Figure 3A). In contrast, mVP24 linker mutant and mVP24 G211A/E212A did not activate the ARE promoter. mVP24 D205A/E207A did activate the ARE promoter but to a lesser extent than wild-type mVP24, reflecting the residual binding activity of this mutant for Keap1 (Figure 3A). Therefore, Nrf2 activation correlates with Keap1-mVP24 binding activity (Figure 1F). In a separate experiment, expression of Nrf2 alone resulted in

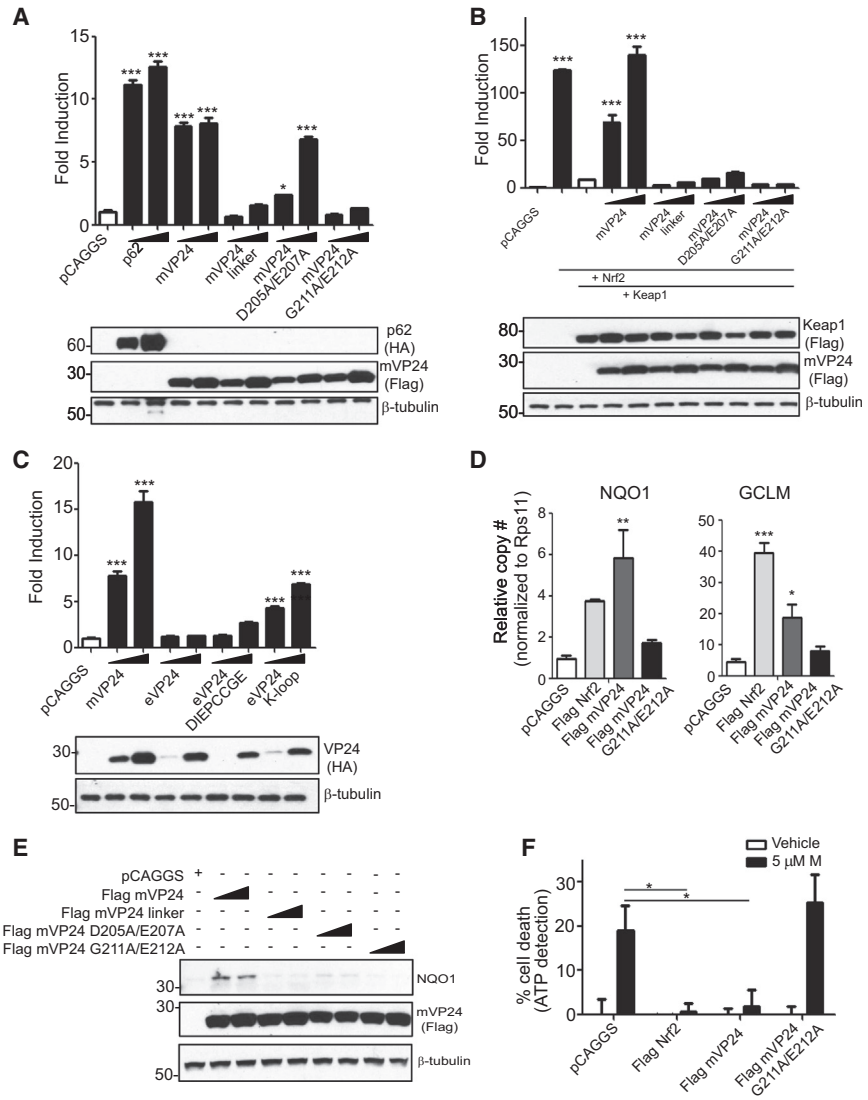


Figure 3. mVP24 Activates Expression of ARE Genes

(A and B) HEK293T cells were transfected with the ARE luciferase reporter plasmid, a constitutively expressed *Renilla* luciferase plasmid, and pCAGGS (empty vector) or increasing concentrations of HA-p62, Flag-wild-type mVP24, or mVP24 mutants. (B) Same as (A), with the additional overexpression of Flag-Nrf2 and Flag-Keap1. At 18 hr posttransfection (hpt), luciferase activity was assayed for (A) and (B). Western blots performed for HA and Flag are indicated.

(C) Same assay protocol as (A) but transfected with HA-mVP24, eVP24, or eVP24 mutants.

(D) pCAGGS, Flag-Nrf2, mVP24, or mVP24 G211A/E212A was transfected in triplicate in HEK293T cells. At 24 hpt, qRT-PCR was performed to quantify mRNAs for the indicated genes, normalized to the *RPS11* mRNA.

(E) HEK293T cells were transfected with the indicated plasmids, and 18 hpt, endogenous NQO1 was measured by western blot.

(F) Cell viability assay. HEK293T cells were transfected with pCAGGS, Flag-Nrf2, mVP24, or mVP24 G211A/E212A and 24 hpt were treated with vehicle control (ethanol) or 5 μM menadione (M) for 3 hr.

In (A)–(D), values represent the mean and SEM of triplicate samples, and statistical significance was assessed by a one-way ANOVA comparing columns to the control (white bar): ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$. Samples in (F) represent the mean and SEM of six samples, and significance was assessed by a one-way ANOVA: *$p < 0.05$.

See also Figure S3.

greater than 100-fold ARE reporter activation (Figure 3B). Keap1 coexpression inhibited the activation. mVP24 expression relieved the repression of Nrf2, resulting in ARE gene expression (Figure 3B). None of the mutant mVP24s induced significant ARE activation, despite expression comparable to that of wild-type mVP24 (Figure 3B). This suggests that the residual binding of mVP24 D205A/E207A is not sufficient to disrupt the repressive activity of the overexpressed Keap1 (Figure 3B). Although expression of eVP24 did not activate the ARE reporter, expression of the mutant eVP24-DIEPCCGE resulted in a slight increase in reporter activity, and eVP24 K-loop significantly induced ARE reporter expression (Figure 3C). Similarly, bat-Keap1 inhibited the activation of the ARE reporter by overexpressed human Nrf2 (Figure S3A), and mVP24 expression relieved the repression mediated by bat-Keap1 on the ARE reporter (Figure S3A). Therefore, mVP24 interaction with Keap1 has functional consequences because it can trigger Nrf2-dependent transcriptional activity in a K-loop-dependent manner.

mVP24 expression also induced expression of the endogenous ARE genes, NAD(P)H quinone oxidoreductase 1 (*NQO1*) and glutamate-cysteine ligase, modifier subunit (*GCLM*) (Lau et al., 2010), as assessed by quantitative RT-PCR (qRT-PCR) (Figures 3D and S3B). Neither the mVP24 mutants nor eVP24 induced expression of these genes (Figure S3). In contrast, eVP24 DIEPCCGE and eVP24 K-loop did induce significant levels of *GCLM* mRNA (Figures 3D and S3B). Correspondingly, NQO1 protein levels increased in the presence of wild-type but not mutated mVP24s, eVP24, or the eVP24 chimeras (Figures 3E and S3C). Interestingly, the eVP24 chimeras did not induce *NQO1* and induced *GCLM* mRNA to a lesser extent than did mVP24. This may reflect in part an as yet uncharacterized inhibitory activity of eVP24 on Nrf2-induced transcription responses that can be seen in ARE reporter gene assays (Figure S3D). Consistent with the ARE induction, cells transfected with Nrf2 (a positive control) or mVP24 were protected from killing by menadione, a compound that induces oxidative damage. In contrast, significant cell death was detected in the pCAGGS and mVP24 G211A/E212A-transfected cells (Figure 3F).

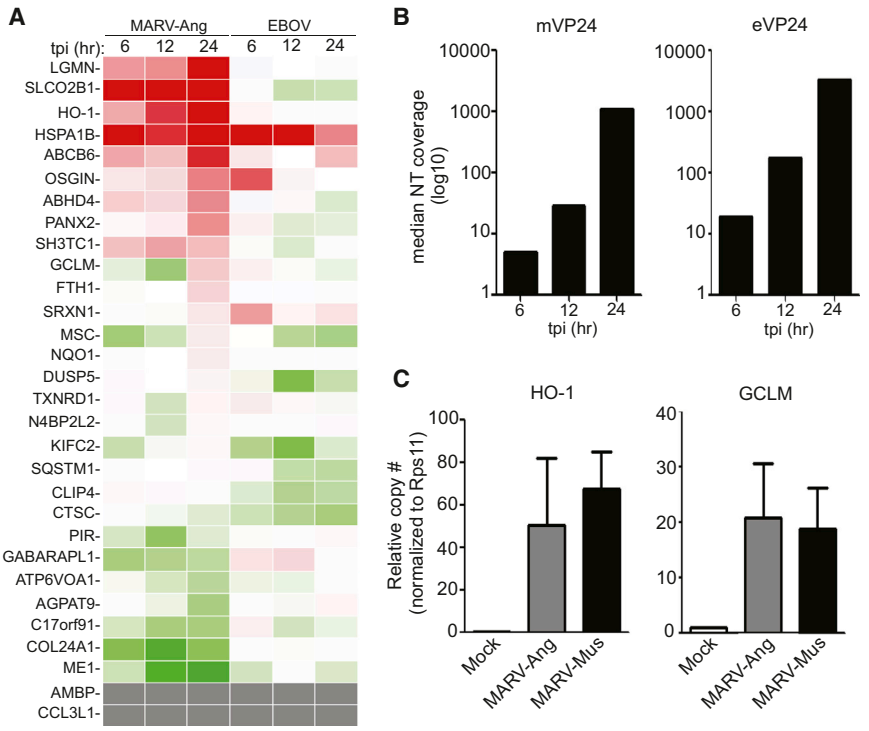


Figure 4. MARV Infection Upregulates the Nrf2 Antioxidant Pathway

(A and B) THP-1 cells were infected with MARV-Ang or Zaire EBOV (moi = 3) and subjected to expression analysis by mRNA sequencing (mRNA-seq).

(A) Heatmap displaying the expression profile of 30 Nrf2-activated genes (Chorley et al., 2012). Red indicates upregulated genes (maximum induction, 8.55-fold relative to mock-infected cells). Green indicates downregulated genes (lowest value, 0.2-fold relative to mock-infected cells). Gray indicates genes undetected in the mRNA-seq.

(B) mVP24 and eVP24 mRNA expression levels represented as median nucleotide coverage.

(C) THP-1 cells were infected with MARV-Ang or MARV-Mus (moi, 1) and subjected to qRT-PCR. Values were normalized to *RPS11*. Mock sample contains a single replicate; MARV-Ang and MARV-Mus represent the mean and SEM of triplicate samples.

See also Figure S4.

MARV Infection Induces the Expression of Nrf2-Responsive Genes

mVP24 activates Nrf2 via interaction with Keap1, but eVP24 does not, suggesting that MARV but not EBOV infection should induce an ARE response. To test this hypothesis, we profiled the expression of select ARE genes in THP-1 cells following MARV Angola strain (MARV-Ang) or Zaire EBOV infection (multiplicity of infection [moi], 3). A substantial number of ARE genes were upregulated in MARV-infected THP-1 cells as the infection progressed and mVP24 mRNA levels increased (Figures 4A and 4B). Although a few ARE genes were upregulated by EBOV infection, the response was not as global as was seen with MARV, and the response did not correlate well with eVP24 expression (Figures 4A and 4B). The mVP24 K-loop sequence is conserved among MARV strains, suggesting that ARE activation should also be shared between MARV strains. Indeed, induction of two representative ARE genes, heme oxygenase 1 (*HO-1*) and *GCLM*, was demonstrated by qRT-PCR following infection of THP-1 cells with MARV-Ang or Musoke (MARV-Mus) (Figure 4C). Interestingly, HO-1 is highly upregulated during MARV infection (Figure 4A), and a recent study has indicated that EBOV replication/transcription is inhibited by HO-1 expression (Hill-Batorski et al., 2013). However, using a MARV minigenome assay, we did not detect any inhibition following HO-1 overexpression (Figure S4; see Supplemental Results for further details), suggesting that upregulation of this ARE may not impair MARV replication.

DISCUSSION

The host antioxidant response has been increasingly recognized as relevant to virus infections. Here, we demonstrate a direct, high-affinity interaction between mVP24 and the Kelch domain of the human and bat Keap1, a major negative regulator of antioxidant responses (see also Supplemental Discussion on bat Keap1). This interaction, for which we define a critical role for the mVP24 K-loop sequence, can disrupt Nrf2-Keap1 interaction and induce a cytoprotective state through transcriptional activation of the ARE promoter. Although other viruses have previously been demonstrated to activate antioxidant responses, the mechanisms of activation appear indirect, with virus infection triggering oxidative stress or other cellular signaling pathways that stimulate Nrf2 nuclear accumulation (Burdette et al., 2010; Cho et al., 2009; Ivanov et al., 2011; Kesic et al., 2011; Lee et al., 2013; Schaedler et al., 2010). In contrast, the direct interaction between mVP24 and Keap1 provides compelling evidence that viruses have evolved mechanisms to engage the cellular antioxidant response as part of their replication strategy.

Keap1-Nrf2 interaction is required for negative regulation of the antioxidant response. A number of stimuli, such as oxidative stress, that perturb the Keap1-Nrf2 interaction stabilize Nrf2, allowing it to accumulate in the nucleus where it binds AREs and cooperates with other factors to activate ARE-containing promoters (Dinkova-Kostova et al., 2002; Zhang and Hannink, 2003). In addition, the interaction of the Keap1 Kelch domain with p62, an autophagy factor that functions in the clearance of polyubiquitinated complexes, activates Nrf2 through the disruption of binding via the lower-affinity Keap1 binding site on Nrf2 (Komatsu et al., 2010; Lau et al., 2010). We demonstrated that the mVP24-Keap1 interaction requires the Keap1 Kelch domain, as is true for many other Keap1 interactors (Kim et al., 2010; Komatsu et al., 2010; Lo and Hannink, 2006; Niture and Jaiswal, 2011). Our data further suggest that the interaction of mVP24 with Keap1 can disrupt the high-affinity Nrf2-Keap1 binding site, leading to the subsequent nuclear localization of Nrf2 and activation of the antioxidant response.

The structural basis for the Keap1 Kelch interaction with peptides derived from several cellular Keap1 binding partners, including Nrf2, p62, and prothymosin α, was previously described by Komatsu et al. (2010), Lo et al. (2006), and Padmanabhan et al. (2008). These peptides bind the bottom of the Keap1 β sheet propeller, which forms a basic pocket, in part through electrostatic interactions with Keap1 arginine residues. Common features of the binding peptides include acidic residues along with a GE motif (Komatsu et al., 2010; Lo and Hannink, 2006). Data obtained with mutated mVP24 K-loop acidic residues and the GE motif support a similar mode of binding for mVP24, although we cannot exclude a contribution of other parts of mVP24. Consistent with a model where the mVP24 loop and the acidic residues within the loop make analogous contacts with the Keap1 Kelch domain, substitution of Keap1 R415 to alanine abrogated Keap1-mVP24 interaction.

It is striking that MARVs and EBOVs differ in their interaction with the ARE response (see Supplemental Discussion for details). Although there are no structures of mVP24, several structures of eVP24s, including Sudan and Reston EBOVs (sVP24 and rVP24) (Zhang et al., 2012) as well as Zaire EBOV (eVP24), are available (Figure 2; PDB 4M0Q). In order to evaluate the mVP24 structure, we used the eVP24 structure, which was most complete as the basis for the Phyre2-threading model of mVP24. In the mVP24 model, the K-loop contains the DIEPCCGE sequence, a sequence that is not conserved between mVP24 and eVP24 but shows similarity to motifs of other Keap1-interacting "GE motifs." Replacement of the K-loop residues with a heterologous linker sequence or mutation to alanine of the D205 and E207 or of G211 and E212 was sufficient to greatly reduce or abrogate binding, although it should be acknowledged that the nuclear localization confounds interpretation of the G211A/E212A mutant data. That the DIEPCCGE loop is central to binding is confirmed by the fact that transfer of the loop to eVP24, which otherwise does not interact with Keap1, confers binding activity. Furthermore, wild-type mVP24 effectively competes with Nrf2 for binding to Keap1 in vitro and dissociates GFP-Nrf2 from Flag-Keap1 in a K-loop-dependent manner. These observations suggest a mechanism by which mVP24 activates an ARE transcriptional response. Interestingly, the mVP24 DIEPCCGE sequence diverges from other Keap1 binding motifs, such as the so-called ETGE motif of Nrf2 (DEETGE), with "PCC" inserted between "GE" and more amino-terminal acidic residues. The presence of the Cys residues is intriguing given that Keap1-Nrf2 interactions are regulated by oxidation. Whether these residues, which are not present in other Keap1-interacting motifs, play an important role in the mVP24-Keap1 interaction will be the subject of future studies.

In addition to the ARE response, Keap1 regulates other stress-induced cell survival pathways through interaction of its Kelch domain with a variety of proteins, including PGAM5, IKKβ, and p62 (Kim et al., 2010; Komatsu et al., 2010; Lau et al., 2010; Lee et al., 2009; Lo and Hannink, 2006; Niture and Jaiswal, 2011). mVP24 disruption of these Keap1 interactions could inhibit apoptosis, activate NF-κB-mediated cell survival pathways, and influence autophagy (Fan et al., 2010; Kim et al., 2010; Lee et al., 2009; Niture and Jaiswal, 2011). Furthermore, the stable interaction of mVP24 and Keap1, which did not detectably influence mVP24 expression levels, might allow the recruitment of Keap1 and binding partners for new functions. Further study is therefore required to fully elucidate the impact of the mVP24-Keap1 interaction upon MARV infection.

EXPERIMENTAL PROCEDURES

CoIP

Twenty-four hours posttransfection with the indicated plasmids, HEK293T cells were lysed in NP-40 lysis buffer (50 mM Tris [pH 7.5], 280 mM NaCl, 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, and protease inhibitor [cOmplete; Roche]). Anti-FLAG M2 magnetic beads or anti-HA beads (Sigma-Aldrich) were incubated with lysates for 1 hr at 4°C, washed five times in NP-40 lysis buffer, and eluted using either 3× FLAG peptide (Sigma-Aldrich) or by boiling in sample loading buffer.

Activation of Nrf2

For ARE reporter gene assays, a commercially available reporter gene, pGL4.37[luc2P/ARE/Hygro] (ARE) (Promega), was cotransfected with a constitutively expressed *Renilla* luciferase reporter plasmid (pRL-tk; Promega), and the indicated protein expression plasmids. At 18 hr posttransfection, a dual luciferase reporter assay (Promega) was performed in triplicate, and firefly luciferase values were normalized to *Renilla* luciferase values. Statistical significance was assessed with one-way ANOVA using Tukey's test for comparisons to the control. Protein expression levels were assessed by western blot. Levels of endogenous *NQO1*, *GCLM*, or *HO-1* mRNAs were assessed by qRT-PCR, and NQO1 protein levels were assessed by western blot using a commercially available antibody (Santa Cruz Biotechnology).

Virus Infections

The following infections were performed under BSL-4 conditions at the Galveston National Laboratory. THP-1 cells were differentiated overnight with 100 nM PMA and infected with MARV-Ang (moi = 3 or 1), MARV-Mus (moi = 1), or EBOV (moi = 3). Viral total RNA was extracted with TRIzol at the indicated time points for analysis by deep sequencing or qRT-PCR. For deep sequencing, mRNA was purified with Oligo(dT) magnetic beads (Invitrogen). cDNA libraries were generated (NEBNext; New England Biolabs) and sequenced on the Illumina HiSeq 2500 platform, and relative expression for each gene of interest was determined. For qRT-PCR, cDNA was generated with Oligo(dT) primers, and relative expression for each gene of interest was determined by normalizing to the indicated housekeeping gene. Refer to Supplemental Experimental Procedures for additional details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Discussion, Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.01.043.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI059536 (to C.F.B.) and AI081914 (to G.K.A.), DTRA grant HDTRA1-12-1-0051 (to C.F.B. and G.K.A.), and NSF graduate fellowship DGE-1143954 (to B.J.). All microscopy studies were performed with the generous assistance of the Icahn School of Medicine at Mount Sinai Microscopy Shared Resource Facility. Sequencing was performed at the Genomics Sequencing Facility at Mount Sinai. We thank Hardik Shah and the Bioinformatics Group of the Icahn Institute for Genomics and MultiScale Biology for help with sequence analysis. We thank Drs. S. Ginell, N. Duke, and J. Lazarz at the Structural Biology Center (Advanced Photon Source) and Dr. J. Nix at Beamline 4.2.2 (Advanced Light Source) for data collection support. Use of Argonne National Laboratory SBC beamlines at APS was supported by the U.S. D.O.E. contract DE-AC02-06CH11357.

Received: August 2, 2013
Revised: December 12, 2013
Accepted: January 30, 2014
Published: March 13, 2014

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