The Poxvirus p28 Virulence Factor Is an E3 Ubiquitin Ligase*

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A majority of the orthopoxviruses, including the variola virus that causes the dreaded smallpox disease, encode a highly conserved 28-kDa protein with a classic RING finger sequence motif (C4HC4) at their carboxy-terminal domains. The RING domain of p28 has been shown to be a critical determinant of viral virulence for the ectromelia virus (mousepox virus) in a murine infection model (Senkevich, T. G., Koonin, E. V., and Buller, R. M. (1994) Virology 198, 118–128). Here, we demonstrate that the p28 proteins encoded by the ectromelia virus and the variola virus possess E3 ubiquitin ligase activity in biochemical assays as well as in cultured mammalian cells. Point mutations disrupting the RING finger domain of p28 completely abolish its E3 ligase activity. In addition, p28 functions cooperatively with Ubc4 and UbcH5c, the E2 conjugating enzymes involved in 26 S proteasome degradation of protein targets. Moreover, p28 catalyzes the formation of Lys-63-linked polyubiquitin chains in the presence of Ubc13/Uev1A, a heterodimeric E2 conjugating enzyme, indicating that p28 may regulate the biological activity of its cognate viral and/or host cell target(s) by Lys-63-linked ubiquitin multimers. We thus conclude that the poxvirus p28 virulence factor is a new member of the RING finger E3 ubiquitin ligase family and has a unique polyubiquitination activity. We propose that the E3 ligase activity of the p28 virulence factor may be targeted for therapeutic intervention against infections by the variola virus and other poxviruses.

Genus Orthopoxvirus of virus family Poxviridae encompasses such complex double-stranded DNA viruses as variola virus (VARV),1 one of the most virulent human pathogens that causes smallpox, and vaccinia virus (VACV), the virus utilized in the smallpox vaccine. VARV is highly transmissible and causes up to 40% lethality, which puts it on the list of top high threat agents (1). Since the eradication of smallpox in 1980, our understanding of VARV replication has relied primarily on biological research involving VACV. However, VACV, the origin and natural host of which are unknown, has apparently lost some viral genes that are not essential for its replication in cell culture but required for infection of a natural host (2). One such example is the poxvirus gene encoding a 28-kDa RING finger protein, which is highly conserved among the wild-type orthopoxviruses, such as ectromelia virus (ECTV, or mousepox virus), monkeypox virus, and VARV (see Fig. 1A). Previous studies suggest that an ECTV mutant lacking the p28 RING finger domain fails to replicate in murine peritoneal macrophages in vitro, although it replicates normally in other culture systems (3). Interestingly, this mutant ECTV was found to be highly attenuated in a murine infection model, with an LD50 (the viral dose that kills 50% of the infected animals) more than 6 orders of magnitude higher than that of wild-type ECTV (4). It has been postulated that the attenuation of this mutant ECTV is due to its inability to replicate in macrophage lineage cells at all successive steps in the spreading of the virus from the skin to its target organ, the liver (3). Additional insight regarding the mechanism of p28-mediated viral virulence derives from studies of both Shope fibroma virus and ECTV and indicates that p28 is involved in the inhibition of viral infection-induced apoptosis, thereby preventing abortive viral replication (5, 6). Further attempts to study the biological activity of the p28 virulence factor have been hampered by the fact that recombinant p28 shows poor solubility in the bacterial host, as well as cytotoxicity and low expression in eukaryotic cells. As a result, the molecular mechanism of p28-mediated viral virulence remains elusive.

Poxvirus p28 virulence factor is a protein of ~240 amino acids, containing a RING finger motif (C4HC4) at its carboxy-terminal domain (4). It is highly conserved within the genus Orthopoxvirus and to a certain extent within the genera Leporipoxvirus and Suipoxvirus (Fig. 1A). For instance, the ECTV- and VARV-encoded p28 proteins have greater than 95% amino acid sequence identity, and their RING finger domains are identical (see Fig. 1B). Interestingly, the p28 gene products in culture-adapted VACV strains WR, COP, Ankara, MVA, and TIA are either truncated or interrupted (see Fig. 1B) (7, 8). The expression of ECTV p28 is driven by an early/intermediate viral promoter. The protein is translated in the early phase of viral replication, persists throughout the viral life cycle, and is localized to cytoplasmic virus factories in poxvirus-infected cells (3). Given the significant effect of the p28 RING finger on ECTV virulence and the absolute sequence conservation among the p28 RING domains of ECTV, VARV, and other wild-type...
orthopoxviruses, the p28 virulence factor could be an attractive antiviral target. The identification and characterization of the biochemical activity of p28 is critical for inhibitor screening and subsequent drug development.

The p28 RING finger motif shares the highest homology with the RING finger region of the makorin protein family, the biological function of which is unknown (9). RING fingers are Cys-rich zinc-binding domains identified in over 200 proteins to date. A few discrete biochemical activities have been attributed to RING finger domains. For example, the RING domains of the promyelocytic leukemia protein and the arenavirus-encoded Z protein bind the translation initiation factor eukaryotic initiation factor 4E (eIF4E) and significantly reduce the affinity between eukaryotic initiation factor 4E and the m7G cap of mRNA, thereby inhibiting mRNA transport and causing translational repression (10). In addition, the RING finger domain of KAP1 is critical to the formation of the KAPI/KRAB complex, which mediates transcriptional repression (11). Significantly, a growing number of RING finger proteins have been identified as RING-class E3 ubiquitin ligases. The RING finger domain of E3 protein constitutes the binding site for its E2 ubiquitin-conjugating enzyme, facilitating the transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme to a protein target (12, 13). An increasing amount of evidence supports the notion that viruses commonly utilize the cellular ubiquitinylation pathway to regulate viral replication (Ref. 14 and references therein), and thus, the inhibition of a virus-specific ligase activity provides a new avenue for antiviral discovery.

Here, we describe results from biochemical and cell culture analysis, demonstrating that the poxvirus p28 virulence factor is a new member of the RING class of E3 ubiquitin ligases. We have shown that mutagenesis of the zinc-coordinating residues of the carboxyl-terminal RING finger domain ablates the E3 ligase activity of p28. Moreover, the p28 E3 ligase not only cooperates with Ub4 and Ubch5c, the E2 ubiquitin-conjugating enzymes known to be involved in polyubiquitylation for proteasome-mediated proteolysis degradation, but also works with Ubcl3/Uev1A, a heterodimeric E2 complex that has been shown to mediate the formation of unique Lys-63-linked polyubiquitin chains important for functional regulation of cellular signal transduction.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis**—Sequences of the poxviruses p28 and related RING finger proteins were extracted from NCBI. Alignment of the three closely related poxvirus sequences was generated by ClustalW. Alignment of the distantly related RING finger proteins was constructed by the profile hidden Markov model (HMM) alignment package SAM. The profile HMM afterward. Cloning and Mutagenesis—Sequences of the poxviruses p28 and related RING finger proteins were extracted from NCBI. Alignment of the three closely related poxvirus sequences was generated by ClustalW. Alignment of the distantly related RING finger proteins was constructed by the profile hidden Markov model (HMM) alignment package SAM. The profile HMM afterward. Phylogenetic Analysis of Poxvirus p28 RING Finger Proteins—As shown in Fig. 1A, the p28 proteins encoded by genus Orthopoxviruses (except those of the tissue culture-adapted VACV strains) have a high degree of sequence conservation at amino acid level. This homology is also found in the p28 homologs encoded by genera Leporipoxvirus and Suipoxivirus. In particular, the RING finger domains of the p28 proteins of the genotypes are almost identical. In contrast, the p28 gene products of culture-adapted VACV isolates contain large deletions that disrupt the RING finger domain (Fig. 1B). Coincidentally, VACV infection causes very mild symptoms in humans, a property that enables its use as a live vaccine for smallpox. Interestingly, the RING finger domain of p28 in ECTV has been found to enhance the virulence of the virus by over 106 times in a mouse infection model (4), suggesting that the RING domain of p28, although redundant in cell culture, is associated with ECTV pathogenesis in the host. Although there has been no previously demonstrated evidence of p28-depend-
ent virulence in other orthopoxviruses, it is very likely that, based on the high level of sequence conservation, p28 plays a similar role for this family of poxviruses, including VARV.

The poxvirus p28 RING finger domain aligns with several well characterized cellular E3 ubiquitin ligases, with the most significant homology found between p28 and the makorin RING finger proteins, the biologic function of which is unknown (9). The conservation of residues between p28 and the cellular RING finger domains is relatively poor with the exception of the 7 cysteine and 1 histidine residues that chelate two zinc atoms (Fig. 1C). Despite the low homology between poxvirus p28 and host RING finger ligases, they may share a similar RING finger structural architecture (16, 17). It is likely that the dissimilarity in the RING finger may provide the molecular basis for specific recognition of these RING finger ligases with their respective E2s and protein substrates.

Both ECTV- and VARV-encoded p28 Proteins Possess E3 Ubiquitin Ligase Activity—Attempts to express recombinant p28 in bacterial hosts failed due to the toxicity and poor solubility of the protein, as reported previously (3). p28 expression was achieved by using the baculovirus expression system in which the protein was fused to a GST tag at its amino termi-

Fig. 1. Sequence analysis. A, phylogenetic tree of p28 proteins encoded by poxvirus genus Orthopoxviruses (VARV ORF D6R, Monkeypox D5R, ECTV p28, Cowpox ORF C7R, and Camelpox ORF gp013), and p28 homologues encoded by poxvirus genera Leporipoxviruses (Shope fibroma virus ORF NIR) and Suipoxviruses (Swinepox virus ORF SPV138). Amino acid sequence identities (aligned with VARV ORF D6R) are shown in parentheses. B, diagram of p28 gene products encoded by VARV, ECTV, and two culture-adapted vaccinia virus strains (MR and Ankara). The RING finger domains of ECTV or VARV p28 are shown as shaded bars. ECTV p28 amino acids (a.a.) differing from those of VARV p28 are indicated by vertical lines, and a missing amino acid is indicated by an asterisk in the ECTV p28 diagram. p28 equivalent gene products encoded by MR or Ankara strains of VACV have truncations and disruptions in the carboxyl domain, lacking an intact RING domain. The short filled bar at the end of p28 of VACV Ankara represents a sequence with no homology to other p28 sequences. C, multiple sequence alignment of poxvirus p28 RING with distantly related cellular RING finger proteins. The RING domain is printed in bold. A profile HMM for the RING domain was built based on the MDM2 seed sequence, and each individual sequence was aligned with the profile HMM. The signature of the RING is labeled with asterisks over the actual residues. Uppercase letters designate the sequence segment that can be aligned to the HMM, and lowercase letters denote the part that does not fit to the HMM.
Both EVP28 and SP28 are RING finger E3 ubiquitin ligases. A, purification of recombinant GST-EVP28 and GST-SP28 expressed using the baculovirus expression system. The purity of the proteins was verified by SDS-PAGE analysis and Coomassie Blue staining. MW Marker, molecular mass marker. B, EVP28 and SP28 possess E3 ubiquitin ligase activity in vitro. Recombinant GST-EVP28 or GST-SP28 was added to an in vitro ubiquitylation reaction containing purified human E1, UbcH5c (E2), and FLAG-ubiquitin in the presence of ATP. The formation of polyubiquitin chains by p28 was examined by Western blot using an anti-FLAG antibody.

Because a significant subset of the RING finger family of proteins has been found to be ubiquitin E3 ligases, we examined whether or not the poxvirus p28 virulence factors encoded E3 ligase activity. A substrate-independent in vitro ligase assay was used to monitor the formation of polyubiquitin chains in the presence of p28, a method commonly applied to analyze E3 ligases with unknown substrates (18). As shown in Fig. 2B, both GST-EVP28 and GST-SP28 strongly promoted the formation of a high molecular weight ladder/smear on the blot, an indication of ubiquitin polymerization. As expected, the ubiquitin polymerization was not detected in control reactions in which E1, E2, or p28 was excluded (Fig. 2B).

The p28 E3 Ligase Activity Is RING Finger-dependent—To verify the importance of the RING finger in the E3 ligase activity of p28, point mutations were generated at the proposed zinc-binding residues of the RING domain (C173A or H198A in SP28; C172A or H198A in EVP28). GST-fused mutant SP28 and EVP28 proteins were purified to near homogeneity using glutathione-agarose chromatography and were tested for their E3 ligase activity in vitro. It was found that a single amino acid substitution at either residue Cys-172/3 or residue His-198/9 completely abolished the E3 ubiquitin ligase activity of the p28 proteins, suggesting that the integrity of the RING finger domain is critical for its ligase activity (Fig. 3A).

It has been shown that RING finger E3 ligases can mediate auto-ubiquitylation and substrate ubiquitylation, as well as the formation of unanchored polyubiquitin chains in vitro (19). Although the natural substrate(s) of p28 are not known, Western blot using a GST antibody was performed to determine whether p28 underwent auto-ubiquitylation in the biochemical reaction. Indeed, the formation of high molecular weight GST-SP28 or GST-EVP28 was readily detected in the blot, suggesting that a substantial portion of the polyubiquitin chains formed in the biochemical assay was the product of p28 auto-ubiquitylation (Fig. 3B). Consistent with the null phenotype of RING finger point mutants, p28 bearing either C172A/C173A or H198A/H199A mutations showed no auto-ubiquitylation activity (Fig. 3B).

p28 Cooperates with Different E2s to Catalyze Ubiquitylation—There are over 30 E2 ubiquitin-conjugating enzymes in humans (20), with a similar number of E2 homologues expected in mouse. To determine which E2s could cooperate with SP28 or EVP28, six different recombinant E2s were tested in the in vitro ligase assay. It was found that three of the E2s, Ubc4, UbcH5c, and Ubc13/Uev1A, were capable of working with p28 to promote polyubiquitin chain formation (Fig. 4, A and B). Ubc4 and UbcH5c share about 97% identity in protein sequence and have been shown to participate in catalyzing Lys-48-linked polyubiquitylation for proteasome recognition and subsequent protein degradation (21). The heterodimeric Ubc13/Uev1A has been shown to work with a smaller subset of E3 ligases and plays a unique role in promoting the formation of Lys-63-linked polyubiquitylation for proteasome recognition (22). Among these ligases, TRAF6 and SP28 have been shown to work with a smaller subset of E3 ligases and play a unique role in promoting the formation of Lys-63-linked multi- or polyubiquitin chains for a variety of cellular functions such as kinase activation (19), regulation of ribosome activity (22), and endocytosis (23). We thus compared SP28 and several well characterized RING finger E3 ligases for their compatibility with Ubc13/Uev1A. Among these ligases, TRAF6 and SP28 cooperated well with Ubc13/Uev1A, whereas MDM2 and APC2/11 did not (Fig. 4C). The finding that SP28 and EVP28 can cooperate with different E2s suggests that p28 may be involved in both Lys-48- and Lys-63-linked ubiquitin polymerization and therefore may impact multiple cellular activities.

p28 Catalyzes the Formation of Lys-63-linked Polyubiquitin Chains—Ubiquitin is one of the most highly conserved proteins in eukaryotes, and the 76-amino-acid-long human and mouse isoforms are identical. There are 7 Lys residues in human/mouse ubiquitin, including Lys-48 and Lys-63. Polyubiquitin chains linked via Lys-48 are structurally distinct from those linked via Lys-63, which might be the basis for their differential biological functions (24). To further investigate the possibility of Lys-63-linked ubiquitin chain formation by p28 E3 ligase and the heterodimeric Ubc13/Uev1A E2 complex, the in vitro ligase assay was performed using different forms of ubiquitin. As shown in Fig. 5, with Ubc13/Uev1A as an E2, SP28 catalyzed polyubiquitin chain formation in the presence of either wild-type ubiquitin or a mutant ubiquitin that contains only a single Lys residue at position 63 (Lys-63 only). In contrast, the formation of polyubiquitin chains was dramatically
Poxvirus p28 Encodes E3 Ubiquitin Ligase Activity

The poxvirus genome contains ~200 distinct open reading frames (ORFs), 25% of which are “nonessential” for virus replication in cell culture (25); however, these ORFs are believed to play an important role in establishing productive viral infection in vivo. Here, we report that p28 is expressed as a cytosolic protein during ECTV infection (2), suggesting that p28 has a role in viral pathogenesis. To verify that p28 has ubiquitin E3 ligase activity in vivo, HEK293 cells were transfected with a FLAG-tagged p28 expression vector, pNIG-FLAG-SP28. Twenty-four hours after transfection, cells were treated with 2 μM MG132, a proteasome inhibitor, for 6 h and then lysed in a buffer containing 10 μM MG132 to reduce 26 S proteasome-mediated degradation of the lysate. The lysate was further fractionated by differential centrifugation into cytoplasmic and nuclear fractions. The samples were immunoprecipitated with an anti-FLAG antibody, separated on an SDS-PAGE gel, and blotted with anti-p28 antibody (3) to reveal the subcellular localization of p28. As shown in Fig. 6B, p28 was mainly detected in the cytoplasmic fraction of the cell lysate, an observation confirming a previous report that p28 is expressed as a cytosolic protein during ECTV infection (3). Moreover, treating SP28-expressing cells with the proteasome inhibitor MG132 remarkably enhanced the stability of wild-type p28 (Fig. 6B). The SP28 mutant with C173A/H199A substitutions, shown to have a null phenotype in E3 ligase assays, was also stabilized by MG132, although to a much lesser extent (Fig. 6B). A similar effect of MG132 on SP28 was also observed (data not shown). The pronounced stabilization of wild-type p28 by MG132 suggests that p28 is an active E3 ubiquitin ligase in cells. To further prove that p28 expressed in mammalian cells encodes E3 ubiquitin ligase activity, HEK293 cells were transfected with pNIG vectors expressing either wild-type p28 or its RING finger mutant (SP28 C173A/H199A), both tagged with an amino-terminal FLAG epitope. The cells were lysed in boiling lysis buffer to preserve polyubiquitin chains from degradation by deubiquitinating enzymes or proteases. The lysates were then analyzed by Western blot using a p28 polyclonal antibody. The results demonstrate that wild-type p28, but not the C173A/H199A mutant, forms ubiquitin ladders in transfected cells (Fig. 6C). Thus we conclude that the poxvirus p28 virulence factor encodes an E3 ubiquitin ligase that has activity in mammalian cells, and we have demonstrated that there is substantial SP28 self-ubiquitylation in cells as well as in biochemical assays.

**DISCUSSION**

The poxvirus genome contains ~200 distinct open reading frames (ORFs), 25% of which are “nonessential” for virus replication in cell culture (25); however, these ORFs are believed to play an important role in establishing productive viral infec-
in the enzymatic process. Although previous reports demonstrated that ECTV expressing a RING-deleted p28 has a highly attenuated phenotype in mice (4), ongoing virological experiments seek to address the question of whether p28 E3 ligase activity per se is essential for ECTV virulence. If p28 E3 ligase is indeed essential for ECTV virulence, this would provide a strong argument for targeting poxvirus p28 ligase for the discovery of antivirals against smallpox.

p28 is capable of catalyzing the formation of both Lys-48 and Lys-63-linked polyubiquitin chains, a unique feature found in few E3 ligases (Ref. 26 and references therein). The fact that p28 triggers Lys-63-linked polyubiquityation in concert with Ubc13/Uev1A heterodimeric E2 suggests that the protein may have functions beyond mediating protein degradation (19, 22, 23). One possibility is that p28 may interfere with cellular antiviral mechanisms, such as immune/inflammatory responses and apoptosis, through Lys-63-linked ubiquitylation of itself or its protein substrate(s). It is of particular interest to examine whether p28-mediated Lys-63-linked ubiquitylation has any effects on the innate antiviral signaling pathways of the host, such as those activating interferons in response to viral infections. At present, the actual protein substrate(s) for p28 ligase are not known. The identification of its natural substrate(s), either viral or host, will facilitate our understanding of p28-host interactions, in particular the mechanism of p28-mediated virulence in virus infection.

A growing body of evidence suggests that many viruses hijack the cellular ubiquitylation pathway to establish a successful infection (14). For example, the herpes virus immediate-early protein ICP0 is a well characterized viral ubiquitin E3 ligase that has been shown to be essential for efficient initiation of lytic infection as well as reactivation from latency in herpes virus infections. It is hypothesized that in the absence of ICP0, a host cellular mechanism suppresses viral transcription. ICP0 counteracts this process by directing the proteolytic degradation of several cellular proteins via the ubiquitin-proteasome pathway (27). Additionally, myxoma virus, a poxvirus that causes rabbit myxomatosis, encodes a membrane-bound ubiquitin ligase M153R that conjugates ubiquitin to CD4 and MHC-I molecules and down-regulates their cell surface expression, constituting a possible viral mechanism of immune evasion (28, 29). As has been observed for p28 in ECTV infection, the M153R ligase was identified to be a myxoma virus virulence factor. An M153R deletion mutant of myxoma virus showed an attenuated phenotype in animals (28). In the case of paramyxovirus infection, viral protein V has been shown to specifically target STATs for proteasomal degradation, thereby impairing the interferon-induced innate antiviral response (30). Finally, it has been reported recently that the Vif protein of the human immunodeficiency virus promotes ubiquitylation and the subsequent proteasomal degradation of APOBEC3G, a cellular deaminase that confers innate resistance to retroviral infection by catalyzing the deamination of deoxycytidine to deoxyuridine in viral cDNA replication intermediates (31, 32). It is conceivable that finding inhibitors that block a viral-specific ubiquitylation process may be an effective approach for antiviral drug development.

VARV, an orthopoxvirus and the causative agent of smallpox, is a major concern because of its potential use in bioterrorism (1). Recently, sporadic cases of monkeypox infection have also drawn significant public attention (33). There are currently no effective antiviral therapies for these poxvirus infections. The discovery of druggable antiviral targets (e.g. essential viral enzymes) and the development of effective antiviral poxvirus therapies is of increasing importance (1). The identification of poxvirus p28 as a highly effective virulence factor...
that possesses E3 ubiquitin ligase activity makes it an attractive viral enzyme target for anti-poxvirus drug discovery.

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