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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 (C_3HC_4) at their carboxyl-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 polyubiquitylation 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. 1*A*). 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 LD_{50} (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 (C_3HC_4) at its carboxyl-terminal domain (4). It is highly conserved within the genus *Orthopoxvirus* and to a certain extent within the genera *Leporipoxvirus* and *Suipoxvirus* (Fig. 1*A*). 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. 1*B*). Interestingly, the p28 gene products in culture-adapted VACV strains WR, COP, Ankara, MVA, and TIA are either truncated or interrupted (see Fig. 1*B*) (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

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[1] The abbreviations used are: VARV, variola virus; ECTV, ectromelia virus; EVP28, ECTV p28; SP28, VARV p28; KRAB, Krüppel-associated box; KAP1, KRAB-associated protein-1; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; Ubc, ubiquitin-conjugating E2 enzyme; Uev, ubiquitin-conjugating E2 enzyme variant; TRAF6, tumor necrosis factor receptor-associated factor 6; APC, anaphase-promoting complex; STAT, signal transducers and activators of transcription; HMM, hidden Markov model; GST, glutathione *S*-transferase; ORF, open reading frame; TCEP, Tris(2-carboxyethyl)phosphine.

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 m^7G 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 KAP1/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 ubiquitylation 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 Ubc4 and UbcH5c, the E2 ubiquitin-conjugating enzymes known to be involved in polyubiquitylation for proteasome-mediated protein degradation, but also works with Ubc13/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 was built by choosing MDM2 as the seed sequence with four refinement iterations. The query sequences were aligned to the profile HMM afterward.

Cloning and Mutagenesis—Synthetic oligonucleotides were designed to assemble the genes encoding EVP28 (p28 of the ectromelia virus, GenBank™ accession code NC_004105) or SP28 (p28 of the variola virus, GenBank™ accession code L22579). The synthetic EVP28 and SP28 genes were cloned into the TA vector (Invitrogen). The FLAG tag was then introduced by PCR to engineer amino-terminal-tagged EVP28 or SP28. Point mutations were introduced at the predicted zinc-coordinating residues (C172A or H198A for EVP28; C173A and/or H199A for SP28) using a Gene Tailor mutagenesis kit (Invitrogen). For mammalian cell expression, genes for FLAG-tagged EVP28 or SP28 were cloned into vector pNIG, in which the expression of p28 was driven by the EF-1α promoter, one of the strongest promoters identified in the literature (15).

Recombinant Protein Expression and Purification—The wild-type EVP28, SP28, and their respective RING finger mutants (EVP28 C172A, EVP28 H198A, SP28 C173A, and SP28 H199A) were cloned into the pFast-Bac expression vector (Invitrogen) with an amino-terminal GST tag. Baculovirus production, protein expression, and purification were performed according to the manufacturer's protocols. The following is a brief description of the protocol for the purification of the GST·EVP28 fusion protein. High Five cells (Invitrogen) were inoculated with GST·EVP28 baculovirus at a multiplicity of infection of 1–10. The infected cells were grown for 40 h, collected by a 5-min centrifugation at 2,500 $\times$ *g* at 4 °C, and then lysed briefly at 4 °C in buffer A (20 mM Tris-HCl, 15% glycerol, 0.5 M NaCl, 2.5 mM EDTA, and 1 mM TCEP at pH 8.0) containing protease inhibitors (20 μg/ml phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 μM pepstatin A). The lysate was sonicated and then clarified by centrifugation at 12,000 $\times$ *g* at 4 °C for 40 min. The supernatant was mixed with glutathione-agarose beads for 1 h. The pellet beads with bound GST·EVP28 were washed with buffer B (20 mM Tris-HCl, 15% glycerol, 0.5 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and 1 mM TCEP at pH 8.0) followed by elution with buffer C (20 mM Tris-HCl, 20 mM glutathione, 15% glycerol, 0.05 M NaCl, 1 mM EDTA, 0.01% Nonidet P-40, and 1 mM TCEP at pH 8.0). The eluted protein was further purified by Q-Sepharose chromatography, and the purity of each fraction was analyzed by SDS-PAGE. Pure p28 fractions were pooled, dialyzed, and stored in aliquots at −80 °C. The same protocol was used to purify SP28 and its respective mutants.

Human ubiquitin E1 was expressed as a His tag fusion protein in insect cells using the same Bac to Bac system (Invitrogen). Different human E2s were expressed as amino-terminal GST-tagged fusion proteins in *Escherichia coli* using the pGex-6p system (Amersham Biosciences). GST was cleaved following protein purification through a glutathione column. Ubiquitin was expressed with an amino-terminal FLAG epitope using the expression vector pFLAGMac (Sigma) and then purified from *E. coli*.

Cell Fractionation and Western Blot—Approximately 10^7 HEK 293 cells transfected with pNIG vector or pNIG-SP28 were washed once with phosphate-buffered saline and incubated with 1 ml of phosphate-buffered saline containing 0.5% Triton X-100 and 1× protease inhibitor mixture (Roche Applied Science) on ice for 15 min. The cellular membrane was disrupted gently with a Dounce tissue grinder (without breaking the nuclear membrane), the lysate was centrifuged at 2,000 rpm for 5 min, and the supernatant was collected as the cytoplasmic fraction. The nuclear pellet was washed twice with ice-cold phosphate-buffered saline and then resuspended in 1 ml of phosphate-buffered saline containing 0.5% Triton X-100 and 1× protease inhibitor mixture. The nuclear membrane was disrupted by sonication for 20 s, and the lysate was further incubated on ice for 10 min and centrifuged at 14,000 rpm for 5 min. The supernatant was collected as the nuclear fraction. To analyze the level of expression and the intracellular distribution of p28, both the cytoplasmic and the nuclear fractions were immunoprecipitated with the anti-FLAG antibody as described above, the proteins on beads were eluted with 100 μl of FLAG peptide (100 μg/ml, Sigma), and 20 μl of each eluted sample was loaded on a gel and blotted with anti-FLAG antibody (1:2000, Sigma), or an anti-p28 polyclonal antiserum (3).

In Vitro Ubiquitin Ligase Assay—E1 (10 ng), E2 (25 ng), and SP28 or EVP28 (100 ng) were added to a ubiquitin ligase reaction mixture that contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.6 mM dithiothreitol, 2 μM ATP, and 100 ng of FLAG-ubiquitin. Each 100-μl reaction mixture was incubated at room temperature for 1 h, and the reaction was stopped by the addition of 4× Laemmli loading buffer followed by standard SDS-PAGE and Western blot analysis with anti-FLAG or anti-GST antibodies.

RESULTS

Phylogenetic Analysis of Poxvirus p28 RING Finger Proteins—As shown in Fig. 1*A*, 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 homologues encoded by genera *Leporipoxvirus* and *Suipoxvirus*. In particular, the RING finger domains of the p28 proteins of the wild-type orthopoxviruses are almost identical. In contrast, the p28 gene products of culture-adapted VACV isolates contain large deletions that disrupt the RING finger domain (Fig. 1*B*). 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 10^6 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-

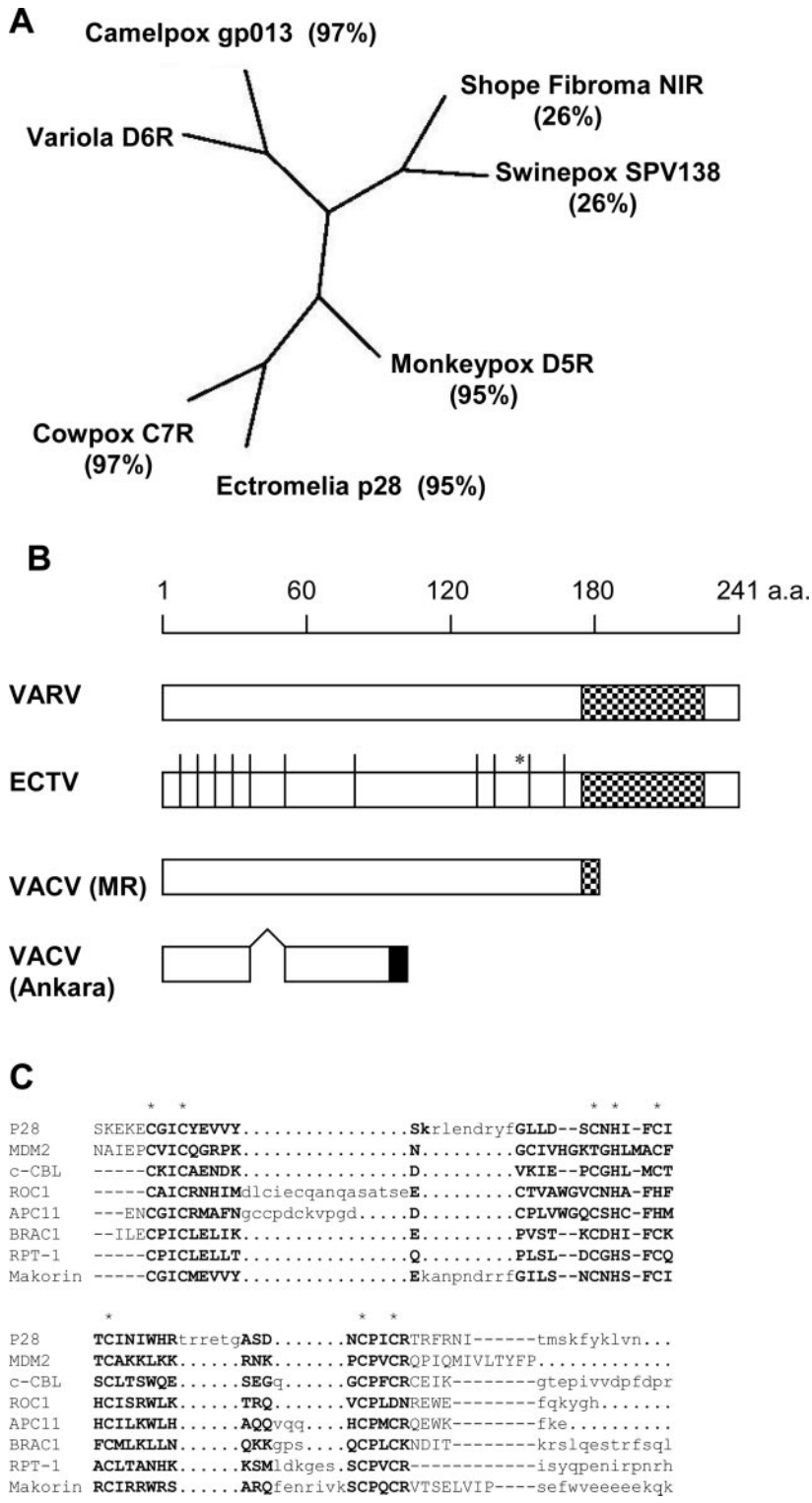

FIG. 1. **Sequence analysis.** *A*, phylogenetic tree of p28 proteins encoded by poxvirus genus *Orthopoxviruses* (VARV ORF D6R, Monkeypox D5R, ECTV p28, Cowpox ORF C7R, and Camelpox ORF gp013), and p28 homologues encoded by poxvirus genera *Leporipoxvirus* (Shope fibroma virus ORF N1R) and *Suipoxvirus* (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.

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. 1*C*). 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-

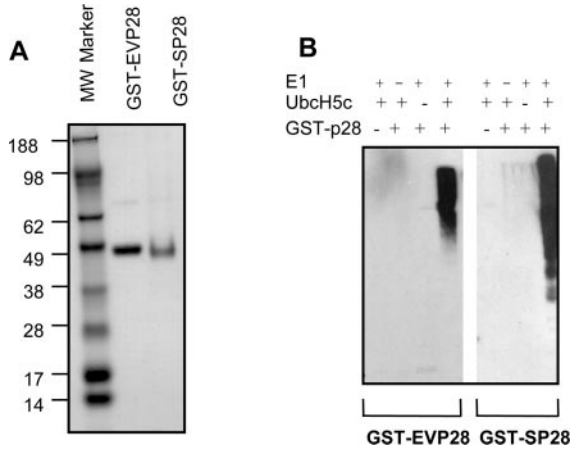

FIG. 2. **Both EVP28 and SP28 are RING finger E3 ubiquitin ligases.** *A*, purification of recombinant GST·EVP28 and GST·SP28 expressed using the baculovirus expression system. The purity of the proteins was verified by SDS-PAGE analysis and Coomassie Blue staining. *MW Marker*, molecular mass marker. *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.

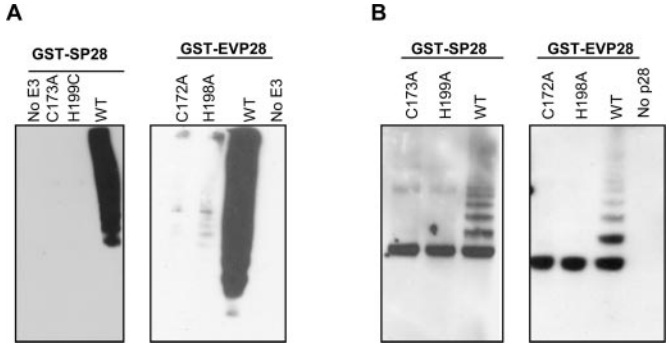

FIG. 3. **p28 E3 ligase activity is RING-dependent.** *A*, equal amounts of the wild-type (*WT*) SP28, EVP28 and their respective mutants were incubated with E1 and UbcH5c in an *in vitro* ubiquitylation assay as described under "Experimental Procedures" and analyzed by Western blot analysis with anti-FLAG antibody. The p28 wild type, but not the RING mutants, showed E3 ligase activity. *B*, SP28 and EVP28 catalyze self-ubiquitylation. Wild-type SP28 or EVP28, as well as their respective mutants (all contain a GST tag at their amino termini), were tested in the *in vitro* ubiquitylation reaction and analyzed by Western blot using an anti-GST antibody to monitor the formation of polyubiquitin chains on SP28, EVP28, and the mutants.

nus. Following differential centrifugation, glutathione-agarose bead affinity purification, and Q-Sepharose chromatography, the p28 proteins of ECTV and VARV, referred to herein as EVP28 and SP28, respectively, were obtained. The purity of the proteins was verified by SDS-PAGE analysis to be greater than 90% (Fig. 2*A*).

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. 2*B*, 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. 2*B*).

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 H199A in SP28; C172A or H198A in EVP28). GST-fused mutant SP28 and EVP28 proteins were purified to near homogeneity using glutathione affinity enrichment followed by Q-Sepharose 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. 3*A*).

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. 3*B*). Consistent with the null phenotype of RING finger point mutants, p28 bearing either C172A/C1733A or H198A/H199A mutations showed no auto-ubiquitylation activity (Fig. 3*B*).

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 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/APC11 did not (Fig. 4*C*). 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/UevA E2 complex, the *in vitro* ligase assay was performed using different forms of ubiquitin. As shown in Fig. 5, with Ubc13/UevA 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

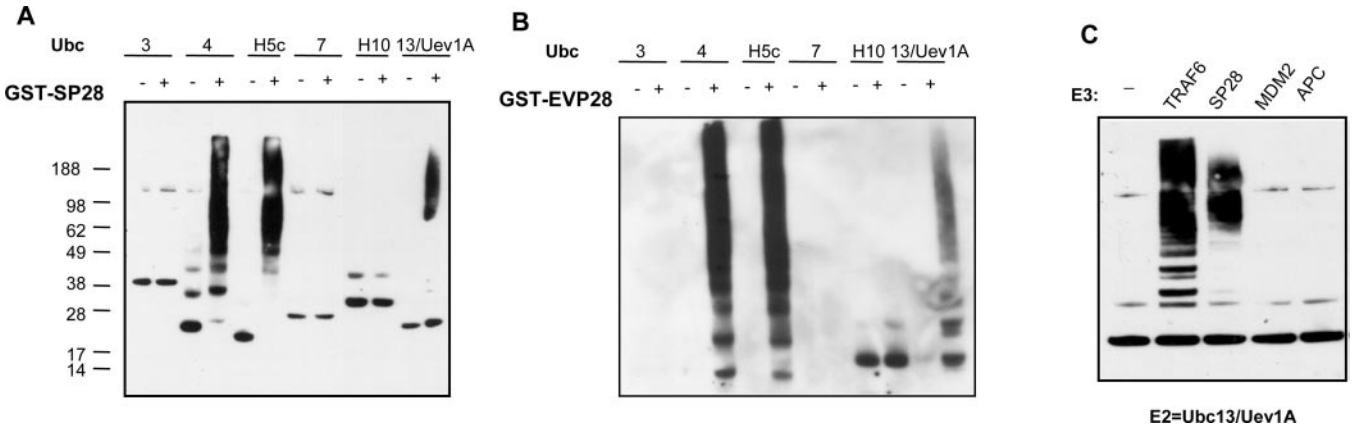

FIG. 4. **SP28 and EVP28 cooperates with Ubc4, UbcH5c, and Ubc13/Uev1A to catalyze ubiquitylation.** *A* and *B*, SP28 (*A*) and EVP28 (*B*) have the same E2 selectivity profile. An equal amount of the indicated recombinant E2 was incubated with E1, FLAG-ubiquitin, and ATP, in the absence or presence of GST·SP28 or GST·EVP28, and the formation of polyubiquitin chains was examined by Western blot with an anti-FLAG antibody. *C*, a comparison of SP28 and a few known E3 ligases for compatibility with the Ubc13/Uev1A E2 heterodimer. TRAF6, SP28, MDM2, and APC2/APC11 in equal quantities were added to an *in vitro* reaction containing Ubc13/Uev1A, E1, FLAG-ubiquitin, and ATP and incubated at room temperature for 1 h. All the reaction products were analyzed by Western blot with an anti-FLAG antibody. The data suggest that SP28 and TRAF6, but not MDM2 or APC2/APC11, catalyze polyubiquitylation in cooperation with Ubc13/Uev1A.

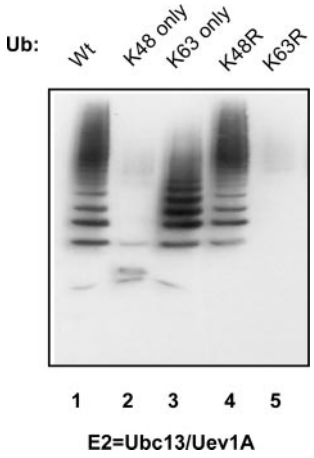

FIG. 5. **SP28 catalyzes the formation of Lys-63-linked polyubiquitin chains.** The wild-type (*Wt*, *lane 1*) and mutant ubiquitins (*lanes 2–5*), all tagged with a FLAG epitope at the amino terminus, were added to a substrate-independent *in vitro* ubiquitylation reaction mixture containing human E1, Ubc13/Uev1A as E2, GST·SP28, and ubiquitylation buffer. *K48 only* (*lane 2*) and *K63 only* (*lane 3*) denote ubiquitin mutants containing Lys-48 or Lys-63 as the sole Lys residue, respectively. *K48R* (*lane 4*) and *K63R* (*lane 5*) denote ubiquitin mutants with a single lysine-to-arginine substitution at amino acid position 48 and 63, respectively. With Ubc13/Uev1A as E2, SP28 selectively catalyzed the formation of polyubiquitin chains via residue Lys-63 of ubiquitin.

reduced in the same reaction using a mutant ubiquitin containing a single Lys residue at position 48 (Lys-48 only). Furthermore, a Lys-63 to Arg substitution in ubiquitin (K63R) abolished the generation of polyubiquitin chains by SP28, whereas replacing Lys-48 with an Arg residue had no significant effect (K48R). EVP28 displayed the same ubiquitin recognition profile in assays using Ubc13/Uev1A heterodimeric E2 (data not shown). A similar experiment using UbcH5c as an E2 failed to yield a conclusive result (data not shown). This is probably due to the fact that UbcH5c appears to be a highly robust E2 that has little ubiquitin substrate stringency in *in vitro* ligase assays. Our observations demonstrate that the poxvirus p28 E3 ligase is capable of catalyzing Lys-63-linked polyubiquitylation in concert with Ubc13/UevA. It raises the possibility that p28 ligases not only mediate the proteolytic destruction of their target protein(s) but may also play a role in functional regulation through a unique Lys-63-linked polyubiquitylation, as has been reported for a few other E3 ubiquitin ligases (19, 22, 23).

p28 E3 Ligase Activity in Transfected Mammalian Cells—To verify that SP28 has ubiquitin E3 ligase activity *in vivo*, HEK293 cells were transfected with a FLAG-tagged SP28 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 SP28. The lysate was further fractionated by differential centrifugations 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 SP28. As shown in Fig. 6*A*, SP28 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 SP28 (Fig. 6*B*). 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. 6*B*). A similar effect of MG132 on EVP28 was also observed (data not shown). The pronounced stabilization of wild-type p28 by MG132 suggests that SP28 is an active E3 ubiquitin ligase in cells. To further prove that SP28 expressed in mammalian cells encodes E3 ubiquitin ligase activity, HEK293 cells were transfected with pNIG vectors expressing either wild-type SP28 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 SP28, but not the C173A/H199A mutant, forms ubiquitin ladders in transfected cells (Fig. 6*C*). 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-

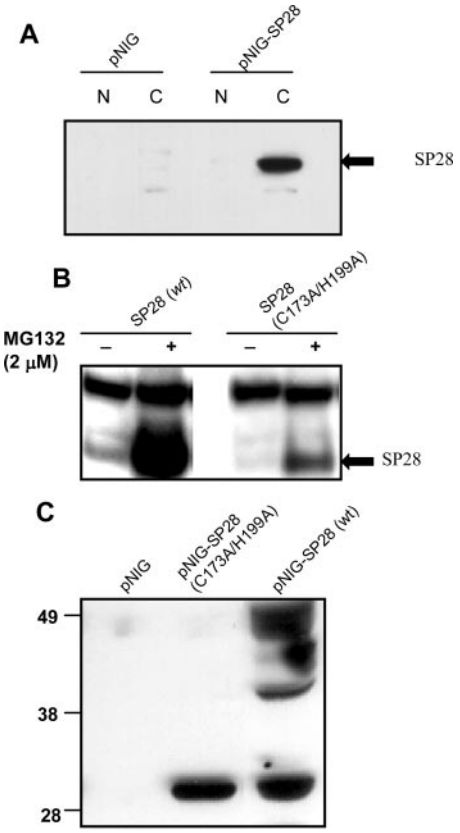

FIG. 6. **Characterization of SP28 transiently expressed in mammalian cells.** *A*, subcellular localization of SP28. HEK293 cells were transfected with pNIG vector or pNIG-SP28 for 24 h, treated with 2 μM MG132 for 6 h, and then homogenized in lysis buffer (see "Experimental Procedures"). Each cell lysate was fractionated into cytoplasmic (*C*) and nuclear (*N*) fractions by differential centrifugation and analyzed by Western blot using an anti-FLAG antibody. SP28 was mainly detected in the cytoplasmic fraction of the cell lysate. *B*, SP28 undergoes proteasome-dependent degradation. HEK 293 cells transfected with either SP28 wild-type (*wt*) or the mutant (C173A/H199A) were treated for 6 h with Me_2SO control or 2 μM MG132 at 24 h after transfection. The cells were lysed, and each lysate was subjected to electrophoresis in denaturing gels and probed with the anti-FLAG antibody. MG132 stabilizes both the wild-type and the mutant SP28, but the stabilizing effect on the wild-type is more pronounced, suggesting that SP28 catalyzed self-ubiquitylation in cells. The FLAG antibody reacts with a nonspecific protein above SP28, which serves here as a loading control. *C*, evidence of SP28 self-ubiquitylation in mammalian cells. HEK 293 cells transfected with either the wild-type SP28 or the C173A/H199A mutant were treated with 2 μM MG132 for 6 h before harvest. Boiling 1× lysis buffer was added to cell pellets, mixed, and immediately heated at 95 °C for 5 min. Genomic DNA in the samples was subsequently sheared with 251/2 gauge needles. The cell lysates were analyzed by Western blot probed with p28 polyclonal antibody (3).

tions in a host and are termed host response modifiers. A significant portion of host response modifier activity is involved in antagonizing host antiviral responses (2). The ECTV p28 is a unique host response modifier that dramatically enhances the lethality of the virus in a murine infection model, with an unknown mechanism of action (4). The importance of p28 in poxvirus/host interactions is suggested by the virtual identity among p28 proteins encoded by ECTV, VARV, and the majority of the wild-type orthopoxviruses (Fig. 1). Elucidating the biochemical activity of p28 would provide clues regarding the biological function of the protein during poxvirus infection. The results described here demonstrate that the poxvirus p28 virulence factor encodes an E3 ubiquitin ligase. p28 mutants lacking one of the proposed zinc-binding residues (C173A or H199A in SP28; C172A or H198A in EVP28) lose their ligase activity, confirming the crucial role of the RING finger domain 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 polyubiquitylation 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 proteasome 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 antipoxvirus 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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