The adenovirus E4-ORF3 protein functions as a SUMO E3 ligase for TIF-1γ sumoylation and poly-SUMO chain elongation

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The adenovirus (Ad) early region 4 (E4)-ORF3 protein regulates diverse cellular processes to optimize the host environment for the establishment of Ad replication. E4-ORF3 self-assembles into multimers to form a nuclear scaffold in infected cells and creates distinct binding interfaces for different cellular target proteins. Previous studies have shown that the Ad5 E4-ORF3 protein induces sumoylation of multiple cellular proteins and subsequent proteasomal degradation of some of them, but the detailed mechanism of E4-ORF3 function remained unknown. Here, we investigate the role of E4-ORF3 in the sumoylation process by using transcription intermediary factor (TIF)-1γ as a substrate. Remarkably, we discovered that purified E4-ORF3 protein stimulates TIF-1γ sumoylation in vitro, demonstrating that E4-ORF3 acts as a small ubiquitin-like modifier (SUMO) E3 ligase. Furthermore, E4-ORF3 significantly increases poly-SUMO3 chain formation in vitro in the absence of substrate, showing that E4-ORF3 has SUMO E4 elongase activity. An E4-ORF3 mutant, which is defective in protein multimerization, exhibited severely decreased activity, demonstrating that E4-ORF3 self-assembly is required for these activities. Using a SUMO3 mutant, K11R, we found that E4-ORF3 facilitates the initial acceptor SUMO3 conjugation to TIF-1γ as well as poly-SUMO chain elongation. The E4-ORF3 protein displays no SUMO-targeted ubiquitin ligase activity in our assay system. These studies reveal the mechanism by which E4-ORF3 targets specific cellular proteins for sumoylation and proteasomal degradation and provide significant insight into how a small viral protein can play a role as a SUMO E3 ligase and E4-like SUMO elongase to impact a variety of cellular responses.

adenovirus | SUMO | E3 ligase | TIF-1γ | proteasome degradation

A denoviruses (Ads) are ubiquitous pathogens that infect a wide range of vertebrates. Ad infection is generally associated with mild disease, but Ads have been increasingly recognized as significant pathogens in infants, the elderly, and immunocompromised patients (1). Ads have evolved diverse mechanisms to counteract host antiviral responses during infection (2). Successful Ad replication relies on functions provided by early region 4 (E4). The highly conserved E4-ORF3 protein assembles into a multimeric nuclear network, referred to as tracks (3), in infected cells (4, 5). The Ad5 E4-ORF3 protein recruits numerous cellular proteins into nuclear tracks including promylocytic leukemia (PML) nuclear body components (3), the Mre11-Rad50-Nbs1 (MRN) complex (6, 7), small ubiquitin-like modifier (SUMO) proteins (8), transcription intermediary factor (TIF)-1α (9), TIF-1γ (10), and TFII-I (11). This event causes sequestration of the target proteins inhibiting their antiviral functions. Relocalization of TIF-1γ and TFII-I by Ad5 E4-ORF3 results in their proteasomal degradation (12, 13). We previously showed that Ad5 E4-ORF3 mediates sumoylation of multiple cellular proteins (8, 11, 13) and suggested that E4-ORF3–induced sumoylation triggers ubiquitination and proteasomal degradation of some substrates. Relocalization of target proteins into E4-ORF3 nuclear tracks is required for substrate sumoylation and proteasomal degradation (8, 11, 13).

Significance

Viruses interplay with the host sumoylation system to manipulate diverse cellular responses. The adenovirus (Ad) early region 4 (E4)-ORF3 protein forms a dynamic nuclear network to interfere with and exploit host processes. Here we provide the first direct evidence to our knowledge that the Ads E4-ORF3 protein is a new type of viral small ubiquitin-like modifier (SUMO) E3 ligase that promotes substrate sumoylation and poly-SUMO3 chain formation. This explains the interrelationship between E4-ORF3–mediated substrate sumoylation and proteasomal degradation. Our study reveals that E4-ORF3 functions directly to regulate cellular enzymatic processes. Because E4-ORF3 does not share homology with any known ubiquitin or SUMO E3 ligases, these results confer an important clue to understanding the mechanism by which E3 ligases transfer SUMOs from charged E2 to selected substrates and mediate chain elongation.

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TIF-1γ (TRIM33) is a ubiquitous nuclear protein that belongs to the tripartite motif (TRIM) family, containing a conserved N-terminal region composed of a RING domain, two B boxes, and a coiled-coil domain. TIF-1γ functions in various cellular processes including transcriptional regulation, TGF-β signaling, and DNA damage repair (reviewed in ref. 21). The RBCC domain of TIF-1γ has ubiquitin ligase activity that targets Smad4 for ubiquitin-dependent proteasomal degradation (22). Functioning as a chromatin reader, the PHD/Bromo domain positions TIF-1γ on chromatin of target genes and activates (23) or represses transcription (24). TIF-1γ also acts as an E3 ligase for inhibitory monoubiquitination of Smad4 (25). TIF-1γ regulates epithelial-mesenchymal transition by functioning as a SUMO E3 ligase for the transcriptional regulator, SnoN1 (26). Finally, ectopic TIF-1γ expression reduces Ad early and late gene expression, whereas TIF-1γ knockdown augments viral gene expression (12). These latter results show that TIF-1γ has antiviral activity and help to explain why TIF-1γ is a target of Ad E4-ORF3 activity.

Here we confirm that Ad5 E4-ORF3 is sufficient to mediate sumoylation and proteasomal degradation of TIF-1γ in vivo. Using an in vitro assay system with purified components, we show for the first time to our knowledge that the Ad5 E4-ORF3 protein is an E3 ligase for TIF-1γ sumoylation and poly-SUMO chain elongation. The ability of the E4-ORF3 protein to form higher order oligomers is essential for these activities. These results are consistent with the hypothesis that E4-ORF3 protein functions to recruit the SUMO machinery and its substrates in proximity of one another using its polyvalent scaffold to assemble higher order protein complexes and facilitate SUMO conjugation.

**Results**

The Ad5 E4-ORF3 Protein Is Sufficient to Promote TIF-1γ Sumoylation and Proteasomal Degradation. Our previous study showed that TIF-1γ sumoylation is induced by infection with wild-type Ad5, but not by an E4-ORF3-deficient virus (11). To further verify the role of E4-ORF3, we infected HeLa cells with recombinant Ad5-E4-ORF3 expression vector and examined TIF-1γ sumoylation. In infected cells, basal levels of TIF-1γ (sumoylated) and TFI-I remained unaltered, whereas ubiquitination of Nbs1 was undetectable (Fig. 1A and B). In addition, TIF-1γ and TFI-I were co-induced in HeLa and A549 cells using the proteasome inhibitor MG132 (Fig. 1C). These results demonstrate that E4-ORF3 targets TIF-1γ for sumoylation and proteasomal degradation and suggest that E4-ORF3 regulates cellular target proteins through distinct posttranslational modifications.

TIF-1γ Sumoylation Is Increased by Ad5 E4-ORF3 in Vitro. Ad5 E4-ORF3 induces sumoylation of multiple cellular proteins (8, 11, 13). It remained unclear whether E4-ORF3 is a SUMO E3 ligase or employs a cellular E3 ligase. To test this, we prepared recombinant proteins and performed in vitro sumoylation assays. Purified recombinant GST-TIF-1γ protein was incubated with SUMO E1 (SAE1/SAE2), SUMO E2 (Ubç9), and His-SUMO3 in the presence of ATP and sumoylation levels were determined by Western blot. TIF-1γ was efficiently conjugated by SUMO3 in vitro in the absence of SUMO E3 ligase. We optimized the reaction conditions such that weak TIF-1γ sumoylation was evident with SUMO E1 and E2 enzymes alone. We then incubated reaction mixtures with increasing concentrations of purified Ad5 E4-ORF3 protein. Strikingly, we observed a significantly enhanced ladder of high molecular weight species of TIF-1γ and SUMO3 in the presence of wild-type E4-ORF3, whereas only one shifted band was observed in the control reaction (Fig. 2A), demonstrating that TIF-1γ sumoylation was enhanced by E4-ORF3 in the absence of any cellular SUMO E3 ligase. We also tested the E4-ORF3 L103A mutant, which is defective in formation of nuclear tracks and subsequent E4-ORF3 functions, including the sumoylation of target proteins (7, 8). Interestingly, TIF-1γ sumoylation remained unchanged by addition of E4-ORF3 L103A in the same range of concentrations as wild-type, suggesting that E4-ORF3 self-assembly is required for this activity. Slightly increased higher molecular weight species of SUMO3 were evident at the highest concentration of L103A (Fig. 2A), showing that the mutant protein has residual activity. It is noteworthy that we did not observe any change in the mobility of the E4-ORF3 protein in these assays, indicating that E4-ORF3 is not a SUMO substrate, although it contains a consensus sumoylation site at K8. These results led us to hypothesize that E4-ORF3 is a viral SUMO E3 ligase.

E4-ORF3 Is a SUMO Ligase for TIF-1γ. A previous study showed that TIF-1γ has SUMO E3 ligase activity in its C-terminal domains (26). Therefore, we could not exclude the possibility that E4-ORF3 stimulates TIF-1γ autosumoylation. To test this, we purified a C-terminal truncated TIF-1γ protein (TIF-1γΔC). TIF-1γΔC contains all four known sumoylation sites (K776, 793, 796, and 839) (28) and the RBCC domain, but not the PHD domain and bromodomain

![Fig. 1. E4-ORF3 enhances TIF-1γ sumoylation and proteasomal degradation.](Image)
E4-ORF3 has been shown to associate with TIF-1γ via the coiled-coil domain (10). Similar to the full-length protein, TIF-1γΔC sumoylation was significantly increased by wild-type E4-ORF3, but very weakly by the L103A mutant (Fig. 2B). The basal level of sumoylation of TIF-1γ and TIF-1γΔC with E1 and E2 enzymes alone varied slightly between experiments (Fig. 2A and B, lanes 2), but E4-ORF3 mutant protein L103A did not behave differently with the two substrates. We examined TIF-1γΔC sumoylation at a higher concentration of E4-ORF3 L103A protein and observed minor, but detectable, activity (Fig. S1). This result is consistent with the conclusion that the L103A mutant protein has residual activity. These data demonstrate that the SUMO E3 ligase activity of TIF-1γ is dispensable for its enhanced sumoylation mediated by the E4-ORF3 protein.

We compared the kinetics of TIF-1γΔC sumoylation in the absence or presence of E4-ORF3 (Fig. 2C). TIF-1γ sumoylation was observed as early as 5 min after incubation with E4-ORF3. It took 15 min to generate multiply sumoylated TIF-1γΔC with the presence of wild-type E4-ORF3, whereas 120 min in the absence of E4-ORF3 and 60 min in the presence of the L103 mutant. After a 120-min incubation with E4-ORF3, only multiply sumoylated TIF-1γΔC protein was detected, demonstrating that E4-ORF3 markedly accelerates the rate of TIF-1γ sumoylation. We confirmed that all of the high molecular weight bands were SUMO3 conjugates by incubating the reaction mixture with the SUMO protease, SENP1 (Fig. 2D). These results confirm that E4-ORF3 promotes TIF-1γ sumoylation along with E1 and E2 enzymes in vitro and suggest that E4-ORF3 has intrinsic SUMO E3 ligase activity.

We investigated the assembly of SUMO3 chains with E4-ORF3 (Fig. 3). We detected poly-SUMO3 conjugates that were not present in the absence of E4-ORF3 (Fig. 3A and B). However, the formation of poly-SUMO3 conjugates was not affected by the presence of E4-ORF3 L103A (Fig. 3B). These data suggest that E4-ORF3 promotes the assembly of SUMO3 conjugates in vitro and that E4-ORF3 L103A is capable of promoting this activity.

We also investigated the role of E4-ORF3 in the poly-SUMO3 conjugate assembly in the absence of E4-ORF3 (Fig. 3C). We found that the formation of poly-SUMO3 conjugates was not affected by the presence of E4-ORF3 L103A (Fig. 3C). These data suggest that E4-ORF3 promotes the assembly of SUMO3 conjugates in vitro and that E4-ORF3 L103A is capable of promoting this activity.

In conclusion, our results demonstrate that E4-ORF3 promotes the SUMO E3 ligase activity of TIF-1γ and that E4-ORF3 L103A has residual activity. These findings provide new insights into the mechanisms of SUMOylation and suggest that E4-ORF3 is a potential therapeutic target for treating diseases associated with SUMOylation.

Fig. 2. In vitro sumoylation of TIF-1γ with E4-ORF3. (A) GST-tagged recombinant TIF-1γ protein (100 nM) was incubated with 50 nM E1, 250 nM E2, 50 μM His6-SUMO3, and the indicated concentrations of His6-E4-ORF3-WT or His6-E4-ORF3-L103A proteins at 37 °C for 60 min. Reaction mixtures were analyzed by Western blot with anti-TIF-1γ, anti-SUMO2/3, and anti–E4-ORF3 antibodies. (B) GST–TIF-1γΔC (100 nM) was used as a substrate and sumoylation was analyzed as described in A. (C) GST–TIF-1γΔC was incubated with (+WT and +L103A) or without (−) His6–E4-ORF3 for the indicated time periods. (D) GST–TIF-1γΔC was sumoylated at 37 °C for 60 min and incubated with 10 nM SENP1 catalytic domain at 20 °C for 10 min. A total of 3 μM His6–E4-ORF3 was used in C and D. (E) Schematic representation of TIF-1γ and TIF-1γΔC. RBCC contains the RING finger, B box, and coiled-coil domain; PB contains the plant homeodomain (PHD) and bromodomain.

Fig. 3. Poly-SUMO3 chain assembly with E4-ORF3. (A) The indicated concentrations of His6–E4-ORF3-WT or His6–E4-ORF3-L103A proteins were added to reaction mixtures described in Fig. 2A and incubated at 37 °C for 60 min. Products were analyzed by Western blot with anti-SUMO2/3 and anti–E4-ORF3 antibodies. The two images in the panel separated by a white space were obtained from the same source file. (B) The E1/E2/His6–SUMO3 mixture was incubated with (+E4-ORF3) or without (−) His6–E4-ORF3 for the indicated time periods and products were analyzed by Western blot with anti-SUMO2/3 antibody. (C) E1/E2/His6–SUMO3 and His6–E4-ORF3 were incubated at 37 °C for 60 min and further incubated with 10 nM SENP1 catalytic domain at 20 °C for 10 min. (D) E1 or E2 enzyme was left out of the reaction mixtures described in Fig. 2A, incubated at 37 °C for 60 min. A total of 1.5 μM His6–E4-ORF3 was used in B–D.
Remarkably, E4-ORF3 dramatically promoted poly-SUMO3 chain formation depending on the E4-ORF3 concentration (Fig. 3A). In contrast, the L103A mutant showed no effect in this assay (Fig. 3A). Poly-SUMO chain formation reactions were performed as described in Fig. 2A using 50 μM SUMO proteins and 1.5 μM His6-E4-ORF3 at 37 °C for 60 min or 180 min.

**E4-ORF3 Promotes Poly-SUMO3 Chain Formation.** In anti-SUMO2/3 blots of in vitro sumoylation products, we observed SUMO conjugates of lower molecular weight than unmodified substrate proteins in the presence of E4-ORF3 (Fig. 2A and B). SUMO E3 ligases are generally thought to induce poly-SUMO chain formation (often referred as SUMO E4 elongase activity), but the detailed mechanisms are not well understood. Recent studies identified E4 elongase activities of plant SUMO E3 ligases PIAL1 and 2 (29) and human SUMO E3 ligase ZNF451 (30). To determine if E4-ORF3 has SUMO E4 elongase activity, we performed an in vitro sumoylation assay without substrate protein. Remarkably, E4-ORF3 dramatically promoted poly-SUMO3 chain formation depending on the E4-ORF3 concentration (Fig. 3A) and incubation time (Fig. 3B). In contrast, the L103A mutant showed no effect in this assay (Fig. 3A). SUMO E1 and E2 enzymes alone induced the formation of polymeric SUMO3 chains consistent with a previous report (31). The addition of SENP1 resulted in breakdown of E4-ORF3-induced SUMO3 chains into free SUMO3 (Fig. 3C). We removed E1 or E2 enzyme from the reaction and detected only free SUMO3 protein (Fig. 3D). These data demonstrate that E4-ORF3 functions as a SUMO E4 elongase.

**E4-ORF3 Induces the Initial Acceptor SUMO3 Conjugation to TIF-1γ as Well as SUMO3 Chain Elongation.** We next sought to determine if E4-ORF3 promotes the initial SUMO3 conjugation to lysine residues in the substrate protein, chain elongation of SUMO3 conjugates already linked to substrate, or both. We generated a mutant SUMO3, K11R, which is unable to form polymeric chains (31) and tested it for in vitro sumoylation with E4-ORF3. TIF-1γΔC was incubated with SUMO3 wild-type or K11R for 60 min or 180 min. In the absence of E4-ORF3, minimal conjugation of wild-type SUMO3 was observed at 60 min and several higher molecular weight bands were evident at 180 min. TIF-1γΔC was conjugated to SUMO3 K11R at 180 min in the absence of E4-ORF3, indicating that TIF-1γΔC is modified at multiple lysine residues by only SUMO E1 and E2 enzymes. E4-ORF3 increased TIF-1γΔC modification by the SUMO3 K11R mutant as well as by wild-type SUMO3 (Fig. 4A), but the high molecular weight ladder observed with wild-type SUMO3 was not evident with K11R. We also examined SUMO3 chain formation using the K11R mutant in the absence of substrate. The SUMO3 K11R mutant was unable to form a poly-SUMO chain in the presence or absence of E4-ORF3 (Fig. 4B). These results demonstrate that E4-ORF3 mediates polymeric SUMO3 chain assembly via the consensus K11 conjugation site but not at nonconsensus sites in SUMO3. This result confirms that all of the SUMO conjugates from the reaction with K11R in Fig. 4A correspond to mono-SUMO conjugation of TIF-1γΔC. These results demonstrate that E4-ORF3 promotes initial SUMO3 conjugation to TIF-1γ as well as SUMO3 chain extension on multiply monosumoylated TIF-1γ protein.

**Interaction Between E4-ORF3 and the SUMO Machinery Proteins.** In agreement with a recent report (32), we did not see relocalization of the SUMO E2 enzyme Ubc9 into E4-ORF3 nuclear tracks in Ad5-infected HeLa cells. However, E4-ORF3 would only need to recruit a subpopulation of Ubc9 to use its catalytic activity. We examined Ubc9–E4-ORF3 interaction by communoprecipitation. Lysates containing Myc-tagged Ubc9 and HA-tagged E4-ORF3 were prepared by transient transfection and viral vector infection of HeLa cells, respectively. Interestingly, we could pull down Myc-Ubc9 with wild-type E4-ORF3, but not with the L103A mutant (Fig. 5A), demonstrating that E4-ORF3 binds exogenously expressed Ubc9 in cells.

E4-ORF3 has a consensus sumoylation site at position K8 and is predicted to have a SUMO-interaction motif (SIM) (33) at...
were incubated with 5 μM HA-ubiquitin, 100 nM His6-Ube1, and 200 nM UbcH5a in the presence of 0.5 or 2.5 μM His6-E4-ORF3 or 0.5 μM His6-RNF4 at 37 °C for 90 min. Ubiquitination of SUMO chains was analyzed by Western blot using anti-SUMO2/3 and anti-ubiquitin antibodies. Input His6-tagged proteins were visualized using anti-His antibody. (b) His6-E4-ORF3, His6-RNF4, or BSA was incubated with recombinant poly-SUMO chains and pulled down using NTA-NTA beads. Bound proteins were analyzed by Western blot with antibodies against SUMO2/3, RNF4, and E4-ORF3. A total of 5% of the input reaction is shown in lane 1.

Fig. 6. In vitro STUbL assay. (A) Mixtures of di- to octa-SUMO3 chains (50 ng) were incubated with 5 μM HA-ubiquitin, 100 nM His6-Ube1, and 200 nM UbcH5a in the presence of 0.5 or 2.5 μM His6-E4-ORF3 or 0.5 μM His6-RNF4 at 37 °C for 90 min. Ubiquitination of SUMO chains was analyzed by Western blot using anti-SUMO2/3 and anti-ubiquitin antibodies. Input His6-tagged proteins were visualized using anti-His antibody. (b) His6-E4-ORF3, His6-RNF4, or BSA was incubated with recombinant poly-SUMO chains and pulled down using NTA-NTA resin. Bound proteins were analyzed by Western blot with antibodies against SUMO2/3, RNF4, and E4-ORF3. A total of 5% of the input reaction is shown in lane 1.

Discussion

We investigated the role of the Ad5 E4-ORF3 protein in the regulation of protein sumoylation. Using in vitro reactions with purified proteins, we found that E4-ORF3 dramatically stimulates SUMO conjugation of TIF-1γ as well as poly-SUMO chain formation (Figs. 2-4). These results demonstrate that Ad5 E4-ORF3 is a viral SUMO E3 ligase and E4 elongase. This is the first demonstration to our knowledge of a viral SUMO E4 elongase. Only a limited number of SUMO E3 ligases have been identified and much less is known about them compared with ubiquitin E3 ligases. The ubiquitin E3 ligases are divided into two major classes: the RING and HECT families (34). The RING family enzymes transfer ubiquitin directly from the E2 enzyme to the substrate, whereas the HECT family enzymes form an intermediate thioester bond between a cysteine residue and ubiquitin, and then transfer ubiquitin to the substrate. One of the best-characterized SUMO E3 ligase families, the Siz/PIAS proteins, transfer SUMO proteins through a similar mechanism to that used by ubiquitin RING-type E3 ligases. Another SUMO E3 ligase, RanBP2, differs from RING-type and HECT-type ubiquitin ligases and its E3 domain is proposed to enhance sumoylation by tethering the substrate in an optimal alignment with the E2-SUMO complex to promote catalysis (35). Most SUMO E3 ligases are known to provide interfaces between substrates and the SUMO machinery through their RING domains, SIMs, or both. The structure of the E4-ORF3 protein has been solved (4). Based on this structure, E4-ORF3 does not have features associated with SUMO or ubiquitin ligases. E4-ORF3 residues 103-107 are a predicted SIM, but these sequences are not critical for E4-ORF3 SUMO ligase activity (Fig. 5).

E4-ORF3 regulates posttranslational modification of target proteins by distinct mechanisms during Ad infection. Mre11 and Nbs1 sumoylation is triggered by E4-ORF3 (8) and these proteins are then targeted for proteasomal degradation by other viral proteins, E1B-55K and E4-ORF6, in association with a cellular ubiquitin E3 ligase complex (6). On the other hand, E4-ORF3 is sufficient to mediate sumoylation and proteasomal degradation of TIF-1γ in infected cells (11-13). Mre11 and Nbs1 sumoylation is transient (8), whereas TIF-1γ (13) and TIF-1γ (Fig. 1) remain sumoylated until their protein levels are undetectable. A separation of function mutant, E3-ORF3 D105A/L106A, relocalizes and stimulates sumoylation of cellular STUbL to selectively target substrate proteins for ubiquitination and subsequent proteasomal degradation.

E4-ORF3 does not have STUbL activity in vitro. TIF-1γ sumoylation and proteasomal degradation is induced by E4-ORF3 (Fig. 1) (12). We recently showed that E4-ORF3 targets TIF-1γ for sumoylation and proteasomal degradation (13). However, it remained unclear whether E4-ORF3 itself is a STUbL or uses a cellular STUbL for degradation of sumoylated proteins. We examined ubiquitination of poly-SUMO3 chains in vitro. Because the cellular STUbL RNF4 (34) and a viral STUbL, K-Rta (20), prefer UbcH5a as a ubiquitin E2 enzyme for ubiquitination of poly-SUMO2, we adopted the same reaction conditions to assay E4-ORF3 activity. Heterologous recombinant poly-SUMO3 chains (di- to octa-SUMO3 molecules) were incubated with ubiquitin, ubiquitin E1 (UBE1), and ubiquitin E2 (UbcH5a) in the absence or presence of E4-ORF3 (Fig. 6A). RNF4 was used as a positive control of STUbL activity. RNF4 strongly increased ubiquitination of poly-SUMO3 chains, whereas E4-ORF3 had no effect, even at the five times higher protein concentration than RNF4, indicating that E4-ORF3 does not exhibit STUbL activity in these reaction conditions. We examined interaction between E4-ORF3 and poly-SUMO3 chains, a property of STUbLs. Recombinant poly-SUMO3 chains were incubated with His6-E4-ORF3, His6-RNF4, or control BSA and His6-tagged proteins were captured using NTA-NTA resin (Fig. 6B). Poly-SUMO3 chains were efficiently pulled down by RNF4, but not by E4-ORF3, indicating that E4-ORF3 is unable to bind poly-SUMO3 chains. Overall, these data suggest that E4-ORF3 is unlikely to have STUbL activity and may recruit a cellular STUbL to selectively target substrate proteins for ubiquitination and subsequent proteasomal degradation.

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E4-ORF3-mediated sumoylation of these substrates does not affect proteasomal degradation of these proteins.

Recent studies have demonstrated that SUMO chains act as a degradation tag upon various cellular stress responses and that STUbLs mediate ubiquitination of proteins conjugated with poly-SUMO chains. In our in vitro ubiquitination assay (Fig. 6), we could not detect any STUbL-like activity of E4-ORF3. This result suggests several possibilities. First, E4-ORF3 may be a STUbL that has different preference for the ubiquitin E2 enzyme from RNF4. There are ~40 E2 enzymes in the human genome (34). Second, E4-ORF3 may recruit a cellular STUbL to polysumoylated substrates such as TIF-1γ and TIF-1β. Third, another Ad protein may function as a STUbL. E4-ORF3 is sufficient to induce degradation of target proteins, so this possibility is unlikely. Because E4-ORF3–mediated sumoylation does not always induce proteasomal degradation, we speculate that a cellular STUbL may be involved to target selective proteins for degradation.

Previously, we proposed that relocalization of target proteins into E4-ORF3 nuclear tracks is required for sumoylation and subsequent degradation in cells (8, 11, 13). Here we show that E4-ORF3 self-assembly is critical for SUMO E3 ligase activity in vitro, further substantiating that E4-ORF3 function requires higher-order multimerization, which creates dynamic interfaces with ubiquitin-like proteins, substrates, and enzymes (4, 5). This study provides the first direct evidence to our knowledge that E4-ORF3 plays a role in cellular enzymatic processes. We hypothesize that E4-ORF3 functions in an analogous manner to RanBP2 to recruit the SUMO machinery and its substrates in proximity of one another, using its polyvalent scaffold to assemble higher order protein complexes. Finally, we still do not exclude the possibility that a cellular SUMO E3 ligase may be involved in E4-ORF3–mediated sumoylation for some substrates. In a recent report, exogenously expressed PIAS3, a known cellular SUMO E3 ligase, was shown to colocalize with E4-ORF3 (32). Further studies are required to completely understand the molecular mechanism(s) of E4-ORF3 activity.

Materials and Methods

Virtu Sumoylation/Desumoylation Assay. SUMO3 conjugation reactions were performed using 100 nM substrate protein, 50 μM SUMO3, 20–50 nM E1, 150–250 nM E2, and 0.1–1.0 μM E4-ORF3 in a in sumoylation reaction buffer (20 mM Hepes pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.5% Tween-20, 2 mM ATP, and 5 mM MgCl2) at 37 °C for the indicated time periods. For the desumoylation assay, 10 nM SENP1 catalytic domain (amino acids 419–644) was added to the sumoylation reaction mixture and incubated at 20 °C for 10 min. Poly-SUMO3 chain assembly/disassembly reactions were performed as described above minus substrate protein.

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