Poxvirus ankyrin repeat proteins are a unique class of F-box proteins that associate with cellular SCF1 ubiquitin ligase complexes

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F-box proteins direct the degradation of an extensive range of proteins via the ubiquitin-proteasome system. Members of this large family of proteins are typically bipartite. They recruit specific substrates through a substrate-binding domain and, via the F-box, link these to core components of a major class of ubiquitin ligases (SCF1). F-box proteins thus determine the specificity of SCF1-mediated ubiquitination. F-box-like motifs were recently detected in poxvirus ankyrin repeat (ANK) proteins but clear compositional differences to typical F-box proteins raise questions regarding the classification and function of the motif. Here we show that all five ANK proteins of a representative poxvirus, Orf virus, interact *in vivo* with core components of the SCF1 ubiquitin ligase complex. Interaction is dependent on the poxviral F-box-like motif and the adaptor subunit of the complex (SKP1). The viral protein does not block enzymatic activity of the complex. These observations identify the poxviral motif as a functional F-box. They also identify a new class of F-box that in contrast to cellular counterparts is truncated, has an extreme C-terminal location and is paired with an ANK protein-binding domain. ANK proteins constitute the largest family of poxviral proteins but their function and the significance of their abundance have remained an enigma. We propose that poxviruses use these unique ANK/F-box proteins to dictate target specificity to SCF1 ubiquitin ligases and thereby exploit the cell's ubiquitin-proteasome machinery.

E3 ligase | Orf virus | proteasome | viral pathogenesis | Vaccinia virus

Polyubiquitination and subsequent proteasomal degradation is an ancient eukaryotic regulatory system by which the cell removes unwanted proteins (1). The sequential activity of three classes of enzymes marks proteins for proteasomal degradation. The ubiquitin activating enzyme (E1) facilitates the ATP-dependent activation of ubiquitin, which is then transferred to a ubiquitin conjugase (E2). Ubiquitin ligases (E3) then facilitate the transfer of ubiquitin onto specific lysine residues of the target protein. Specificity in this process is provided by the large family of E3 enzymes, each of which recognizes particular substrates. A major group of the E3 family is formed by the cullin-RING complexes, and of this group, the SCF1 complexes have been the most extensively studied (2, 3). SCF1 complexes contain the RING-domain protein RBX1 (RING-box 1), CUL1 (cullin 1), and SKP1 (S-phase-kinase associated protein 1). They also include one of a family of proteins with a distinctive motif, the F-box (4, 5), that binds SKP1 (6, 7). F-box-containing proteins also typically have a second protein-binding domain such as a leucine rich repeat located C-terminal to the F-box. It is this second domain that binds substrates and recruits them to the E3 complexes. The many different F-box proteins, around 70 have been identified in humans, thus provide specificity to the complexes and to the ubiquitination process (5).

Many viruses exploit the cellular ubiquitination system by various mechanisms (8, 9). However, poxviruses, an extensive family of large DNA viruses that includes the devastating human pathogen, Variola virus, appear an exception. Despite expressing an impressive array of immune modulators and other factors that enhance viral replication in a hostile cellular environment (10), there has been only limited evidence of poxviral modulators of the ubiquitination system (11). Ankyrin repeat (ANK) proteins form the largest family of poxvirus proteins and are encoded by almost all chordopoxviruses. Generally each virus encodes 4 or 5 ANK proteins, although some avipoxviruses encode over 50 (12). These proteins are composed largely of multiple copies of the ANK motif that in many eukaryotic proteins mediates protein-protein interactions (13). Poxviral ANK proteins were first recognized in 1990 (14), but direct evidence of their functions is limited and the significance of their abundance in the chordopoxvirus subfamily has remained an enigma. In this study we investigate a possible role of poxvirus ANK proteins in the manipulation of the host cell's ubiquitination system.

Results

Poxvirus ANK Proteins Display a Distinctive Domain Organization and an F-Box-Like Domain. Amino acid sequence alignments of poxviral ANK proteins revealed a C-terminal motif shared by many of these proteins. In addition, searches of protein domain databases using low cut-off settings detected a possible match between this region of one of the poxviral proteins and the F-box motif. Further manual inspection revealed the presence of this F-box-like domain in more than 80% of poxvirus ANK proteins (Fig. 1*A*). As examples, the F-box-like domains of all five ANK proteins encoded by one representative poxvirus, Orf virus, as well as ANK proteins of viruses representing six other chordopoxvirus genera are shown in Fig. 1*B* and compared with established F-box domains. Orf virus is a parapoxvirus that causes localized skin infections in sheep, goats and humans (15, 16). Typical of poxviral ANK proteins, the five Orf virus ANK proteins display only limited (27–45%) overall amino acid sequence identity to each other (not shown) but are similar in size and share a bipartite, ANK–F-box domain organization (Fig. 1*B*). The poxviral F-box-like domains contain a number of the most conserved amino acids of cellular F-boxes. Amongst these are Pro at position 5, Glu at position 7, as well as Val, Leu, or Ile at positions 4, 8, 9, 12, 13, 16, and 21 (Fig. 1*B*). These include residues reported to mediate contact between SKP1 and the cellular F-box proteins, β-TrCP1 (β-transducin repeat containing protein 1) and SKP2 (S-phase kinase associated protein 2). However, the poxviral F-box domain is shorter than the typical cellular F-box and lacks helix 3 (Fig. 1*B*). Furthermore, the

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A
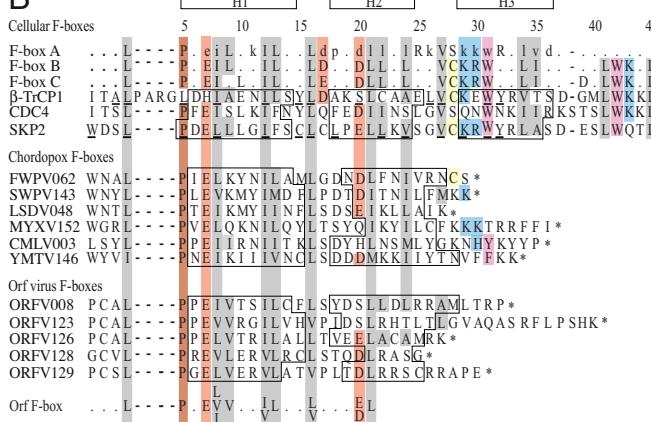
Chordopoxvirus genera	Type species	Ankyrin repeat proteins in genome	Ankyrin proteins with putative F-box
Avipoxvirus	FWPV	33	67 %
Suipoxvirus	SWPV	4	100 %
Capripoxvirus	LSDV	5	80 %
Leporipoxvirus	MYXV	4	100 %
Orthopoxvirus	CMLV	5	80 %
Yatapoxvirus	YMTV	4	100 %
Parapoxvirus	ORFV	5	100 %
Molluscipoxvirus	MOCV	0	-

B

C
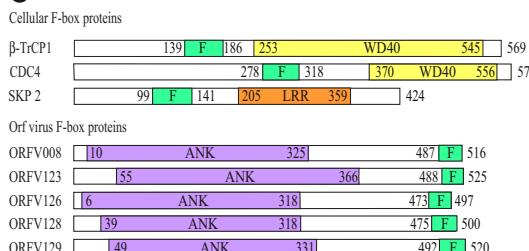
Fig. 1. Poxvirus ANK proteins display a distinct domain organization and contain a shortened F-box domain. (*A*) Summary of ANK (ankyrin repeat) proteins encoded by chordopoxviruses. The type species of each chordopoxvirus genus, except for CMLV which is used to represent orthopoxviruses, is listed along with the number of ANK proteins a representative strain of each species encodes and the percentage of these proteins containing a putative F-box-like domain. The specific virus strains analyzed were Fowlpox virus (FWPV) strain FCV, Swinepox virus (SWPV) strain Nebraska 17077–99, Lumpy skin disease virus (LSDV) strain Neethling, Myxoma virus (MYXV) strain Lausanne, Camelpox virus (CMLV) strain M96, Yaba monkey tumor virus (YMTV) strain RYP, Orf virus (ORFV) strain NZ2, and Molluscum contagiosum virus (MOCV) strain subtype 1. (*B*) Amino acid alignment of the F-box domains of β-TrCP1, CDC4, and SKP2 with the five putative Orf virus F-box domains and six other chordopoxvirus F-box domains each representing a chordopoxvirus genus. The three F-box consensus sequences F-box A, B, and C were adapted from Kipreos and Pagano (32), Willems *et al.* (18), and Schulman *et al.* (7), respectively. In the F-box A consensus sequence capital letters indicate amino acids conserved in >40% of the 234 F-box domains used to generate the Pfam F-box profile, while the lowercase letters indicate amino acids conserved in 20–40% of these F-box domains. The residues conserved in at least two consensus sequences were marked with colored columns and used for the analysis of the poxvirus F-box domains. Residues in colored columns are grouped as follows: H, K, R = blue; D, E = red; A, V, L, I, M = gray; F, Y, W = purple; P, G = brown, and C = yellow. A dot indicates any amino acid, a dash indicates a space and an asterisk indicates the end of the protein. The position of the first listed amino acid shown is indicated in brackets. The F-box α helixes H1, H2, and H3 of the three cellular F-box proteins β-TrCP1, CDC4, and SKP2 have been determined from their crystal structures (7, 33, 34), while the poxvirus α helixes were determined by PROF analysis using the Predict Protein program (35). The general locations of the helices are shown at the top and the specific residues involved are boxed. Residues of β-TrCP1 and SKP2 that contact SKP1 are underlined with black bars. Orf F-box is the F-box consensus sequence of the five Orf virus ANK proteins. (*C*) Comparison of the domain arrangement of the cellular F-box proteins CDC4,

poxviral F-box-like domain is located at the C terminus of each ANK protein whereas cellular F-boxes are typically located in the N-terminal half of the protein with a second protein interaction domain C-terminal to it (Fig. 1*C*). In addition, the ANK domain has not been reported in cellular F-box proteins. These differences raise the question of whether the C-terminal domain of most poxvirus ANK proteins is a functional F-box. We used the Orf virus set of five ANK proteins to address this question.

The Orf Virus ANK Protein 008 Interacts with the SCF1 Complex Component SKP1 in an F-Box–Dependent but Ankyrin-Independent Manner. F-box proteins connect with SCF1 primarily by the binding of their F-box to SKP1. We therefore tested the ability of Orf virus ANK protein 008 to interact with SKP1 *in vivo* using N-terminal Flag fusions with full length Orf 008 (Flag-008), an F-box deletion construct (Flag-008ΔFbox), and a construct lacking all ankyrin repeats (Flag-008ΔANK; Fig. 2*A*). The constructs were transiently coexpressed with GST-SKP1 and immunoprecipitates prepared with anti-Flag-agarose analyzed by SDS/PAGE/ Western blotting. GST-SKP1 but not the GST control coprecipitated with Flag-008. The same pattern of coprecipitation was observed with Flag-008ΔANK but not with the construct lacking the F-box-like domain (Fig. 2*B*). Reciprocal immunoprecipitations confirmed these interactions [supporting information (SI) Fig. S1]. Orf virus protein 008 therefore interacted with SKP1 in a poxviral-F-box-dependent and ANK domain-independent manner.

The Interaction of Orf Virus Protein 008 with the SCF1 Complex Depends on the Presence of SKP1. Flag-008 coprecipitated with GST-Cul1, an interaction presumably mediated by endogenous SKP1 (Fig. S1). To examine this interaction 293EBNA1cells were transiently transfected with GST-Cul1 and either Flag-008, Flag-008ΔANK, or Flag-008ΔFbox. Proteins were immunoprecipitated with anti-Flag-agarose and analyzed by SDS/PAGE/ Western blotting. GST-CUL1 coprecipitated with Flag-008 or Flag-008ΔANK and endogenous SKP1 was present in these precipitates (Fig. 3*A*). Neither GST-CUL1 nor endogenous SKP1 co-precipitated with Flag-008ΔFbox. We next investigated the effect of SKP1 abundance on the interaction of Orf virus protein 008 with the SCF1 complex. We compared coprecipitation of SCF1 components with Flag-008 in the presence and absence of overexpressed GST-SKP1. Each of GST-SKP1, GST-CUL1, and GST-RBX1 robustly coprecipitated with Flag-008 when all were coexpressed (Fig. 3*B*). However, when GST-SKP1 was substituted with GST, both GST-CUL1 and GST-RBX1 failed to coprecipitate with Flag-008 to a comparable level (Fig. 3*B*). Only a very low level of coprecipitating GST-CUL1, relying on endogenous SKP1, could be observed. When both GST-SKP1 and GST-CUL1 were substituted with GST, coprecipitation of GST-Rbx1 with Flag-008 was not detected. The same pattern of coprecipitation was observed when using the cellular F-box protein Flag-SKP2, which is known to interact primarily with SCF1 by directly binding Skp1. We conclude that Orf virus F-box protein 008 associates with a complete SCF1 complex and does so primarily through SKP1.

All Five Orf Virus ANK Proteins Interact with Endogenous SCF1 Complex Components SKP1, CUL1, and RBX1. To determine whether all Orf virus ANK proteins were able to interact with the SCF1 complex, we first transiently coexpressed Flag-tagged versions of

β-TrCP1, and SKP2 with the five ANK proteins of Orf virus. F-box domains are green, WD40 yellow, LRR (Leucine rich repeats) orange, and ANK domains are purple. The first and last amino acid of each domain as well as the last residue of the protein are indicated.
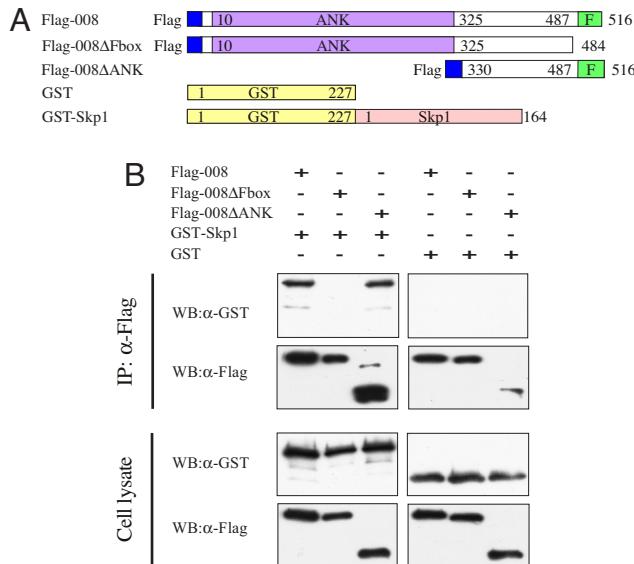
Fig. 2. Orf virus ANK protein 008 interacts with SKP1 through its F-box domain but not its ANK domain. (*A*) Schematic representation of the constructs used in the immunoprecipitation assays. The positions of the first and last amino acid of the ANK domain (purple) and the F-box domain (green) are indicated. The N-terminal Flag-tag is indicated in blue and the N-terminal GST-tag in yellow. (*B*) 293EBNA1 cells were transiently transfected with the indicated (+) combinations of plasmids, harvested after 24 h and cell lysates immunoprecipitated with anti-Flag-agarose beads. The samples were analyzed by SDS/PAGE/Western blot for the presence of coprecipitating proteins. IP, immunoprecipitation; WB, Western blot; α Flag, anti-Flag-antibody; α GST, anti-GST-antibody. Antibodies used for immunoprecipitation and Western blot are indicated to the left.

Fig. 3. The interaction of Orf ANK protein 008 with the SCF1 complex depends on the presence of SKP1. (*A*) Endogenous SKP1 is present in the Flag-008 - GST-CUL1 coprecipitate. (*B*) Overexpression of SKP1 greatly increases the level of coprecipitation of GST-CUL1 and GST-RBX1 with Flag-008. 293EBNA1 cells were transiently transfected with the indicated (+) plasmids. Flag-tagged proteins were immunoprecipitated with anti-Flag-agarose and samples analyzed by SDS/PAGE and Western blotting. End. Skp1, endogenous SKP1; α Skp1, anti-SKP1-antibody. Other abbreviations and labels are as described for Fig. 2.

the four remaining Orf virus ANK proteins with either GST-SKP1 or GST and immunoprecipitated with anti-Flag-agarose beads. GST-SKP1 but not GST coprecipitated with all four Orf virus proteins (Fig. 4*A*). We next examined the interaction between the Orf virus ANK proteins and endogenous components of the SCF1 complex in cells overexpressing only the viral protein. Endogenous SKP1, CUL1 and RBX1 coprecipitated with each of the Orf virus ANK proteins but not with Flag-008ΔFbox (Fig. 4*B*, Fig. S2).

Thus far we had used specific antibodies as a targeted approach to identify proteins that interact with Orf virus ANK proteins. We next used liquid chromatography and tandem mass spectrometry (LC-MS/MS) as an untargeted approach to identify proteins that interact with one of the Orf virus ANK proteins. 293T cells stably expressing Flag-tagged Orf virus ANK protein 129 (293T-Flag-129) were lysed and immunoprecipitated with anti-Flag-agarose. SDS/PAGE revealed four unique bands that were detectable by colloidal Coomassie blue staining in the Flag-129 precipitate compared to a 293T control precipitate (Fig. 4*C*). These were excised, digested and analyzed by LC-MS/MS. The four bands corresponded to either Flag-129 or SCF1 complex associated proteins. A band of 55 kDa size was identified as the bait protein 129. Bands of 21 kDa and 75 kDa were identified as human SKP1 and CUL1, respectively (Fig. 4*C*, Fig. S3). A 120 kDa band corresponded to CAND1 (Cullin-associated and neddylation-dissociated 1), a known regulator of the SCF complex. This observation is consistent with the model that the poxvirus F-box ANK proteins can exist as a component of endogenous multiprotein SCF complexes.

Orf Virus ANK Protein 008 Does Not Inhibit the Activity of Its Associated E3 Ligase. To determine if the enzymatic activity of SCF1 complexes assembling with the Orf virus F-box proteins

might be inhibited by the viral protein, we tested their activity in a polyubiquitination assay. Flag-tagged Orf virus protein 008 and the three core components of the SCF1 complex, CUL1, SKP1, and RBX1, all GST-tagged, were coexpressed in 293EBNA1 cells. Anti-Flag agarose was used to immunoprecipitate Flag-008 and coprecipitate SCF1 components (Figs. 3*B* and 5). Using a nonspecific *in vitro* ubiquitination assay we showed that complexes coprecipitating with 008 were able to form polyubiquitin chains at comparable levels to that seen with complexes coprecipitating with the cellular F-box protein SKP2, which was used as a positive control (Fig. 5 *Left*). Immunoprecipitation of the Flag-tagged vector resulted in limited background ubiquitin polymerization and when enzymes E1 and E2 were omitted from the reaction no polyubiquitin was detected (Fig. 5 *Right*). Immunoprecipitation and cell lysate controls showed expression and immunoprecipitation had occurred at similar levels (Fig. 5). We conclude that the association of the poxviral F-box protein 008 with SCF1 complexes does not block the ubiquitin ligase function of the complexes.

Discussion

This work presents the first functional evidence that poxviral ANK proteins can link to the SCF complex through their distinctive C-terminal F-box domain. We thus provide a putative functional role for the largest protein family of poxviruses. To direct proteins for ubiquitination, poxviruses appear to have evolved a unique ANK/F-box architecture that exploits the

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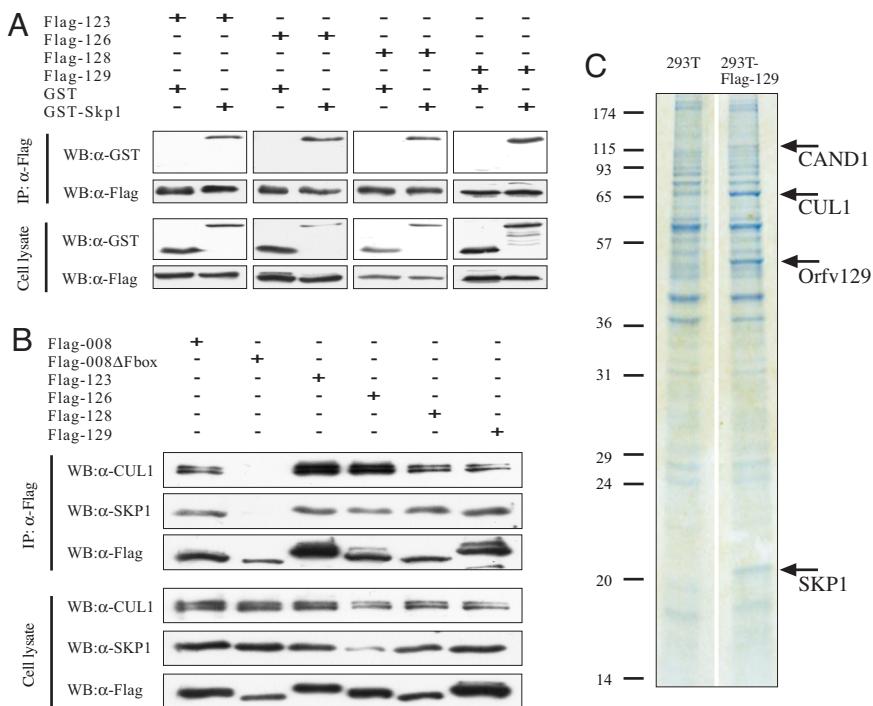
Fig. 4. All five Orf virus ANK proteins interact with the endogenous SCF1 complex components SKP1 and CUL1. (*A* and *B*) The indicated (+) plasmids were transiently expressed in 293EBNA1 cells. Lysates were prepared after 24 h and immunoprecipitated with anti-Flag-agarose. The samples were analyzed by SDS/PAGE / Western blot for the presence of coprecipitating proteins. Abbreviations and labels are as described for Fig. 2 and α-Cul1 indicates anti-CUL1-antibody. (*C*) Lysates of 293T control cells or cells stably expressing Orf virus ANK protein 129 (293T-Flag-129) were immunoprecipitated with anti-Flag-agarose and samples resolved by SDS/PAGE. Proteins were visualized by Coomassie blue staining. The unique bands of the 293T-Flag-129 co-precipitate were excised and analyzed by liquid chromatography- tandem mass spectrometry. Protein sizes (kDa) are indicated to the left and the identity of the unique bands to the right.

versatile ANK repeat motif as a protein-interaction module to dictate targeting specificity.

We examined the set of five ANK proteins encoded by Orf virus for the functionality of their F-box-like domains. All five ANK proteins efficiently interacted with the SCF1 complex. The interaction of Orf virus ANK protein 008 with SCF1 depended on the poxviral F-box-like motif and on the presence of Skp1, but not on the ANK domain of 008. The untargeted approach of using LC-MS/MS also identified SCF1 proteins as associating with an Orf virus ANK protein.

These observations allow us to conclude that all five Orf virus ANK proteins contain a functional F-box domain. Similar domains are present in the vast majority of ANK proteins encoded by vertebrate poxviruses (17). The extent of sequence variation seen among the F-box domains of the five Orf virus proteins is representative of the variation seen among these other chordopoxviruses, allowing us to predict that these too are functional F-boxes. We have therefore identified a common mechanism of action for most ANK proteins of poxviruses: to manipulate the cellular ubiquitination machinery.

The poxviral F-box proteins contain less than 30 residues, while the cellular F-box consensus sequence spans 45. As a consequence of this truncation, only the first two of the three α-helices of cellular F-boxes are predicted to be present in poxviral F-boxes. The most extensive contacts in the interface between SKP2 and SKP1 are made by F-box residues of the loop 1-helix 1 region (7). Also, the 4-helix cluster (2 from the F-box and 2 from SKP1) at the core of the SKP1-SKP2 interface is a shared feature of substrate adaptors in other cullin-based ubiquitin ligases (18). These observations are consistent with the short poxviral F-box retaining the ability to interact with SKP1.

It is intriguing that CAND1 coprecipitated with Orf virus ANK/F-box protein 129. Although CAND1 is a recognized associate of SCF1 complexes it is generally thought that binding of CAND1 to CUL1 prevents binding of SKP1 to CUL1. However the interactions between CAND1-CUL1 binding, CUL1 neddylation, CUL1 dimerization, and SCF complex assembly are not fully understood, and there are indications that CAND1 may play a positive role in SCF complex formation (19–22).

F-box proteins typically have a bipartite composition with an N-terminal F-box paired with a C-terminal substrate-binding domain. The presence of a second protein–protein interaction domain in F-box proteins is thought to be predictive of their ability to assemble into cullin-based E3 ligases (3). Poxvirus F-box proteins share this bipartite structure notwithstanding their reversed domain order. The absence of cellular F-box/ ANK domain proteins make the poxviral pairing of these two motifs unique. ANK domains are present in some SOCS-box proteins, which are the substrate receptor subunits of SCF2/5 (3). The C-terminal motif of the poxviral proteins is, however, clearly most like an F-box motif and we have no evidence of SOCS-box related activity in these proteins.

We considered the possibility that poxviral F-box proteins function as inhibitors of SCF. While we cannot completely exclude this possibility we believe it to be a less likely role of this group of proteins. The encoding of multiple ANK/F-box proteins by individual viruses (from 4 to more than 50) as well as the presence of a variant ANK domain in each protein would seem redundant if their role was simply to competitively inhibit the binding of cellular F-box proteins to SKP1. In addition our data show that the SCF1 complexes bound by a representative of the poxviral F-box proteins mimics the ability of SCF1^{SKP2} complexes to direct the formation of polyubiquitin chains in an *in vitro* assay.

Flag-008	+	-	-	+	-	-
Flag	-	+	-	-	+	-
Flag-Skp2	-	-	+	-	-	+
GST-Cul1	+	+	+	+	+	+
GST-Skp1	+	+	+	+	+	+
GST-Rbx1	+	+	+	+	+	+
E1/E2	+	+	+	-	-	-
ATP	+	+	+	+	+	+
Ubiquitin	+	+	+	+	+	+

ubiquitination reaction

WB:α-ub — poly-ubiquitin

WB:α-ub — monoubiquitin

IP / WB:α-Flag

Cell lysate

WB:α-Flag — input controls

WB:α-GST
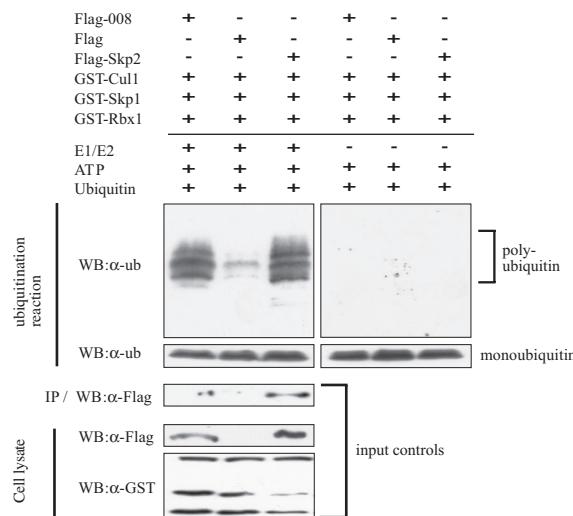
Fig. 5. The Orf virus F-box protein 008 associates with a functional SCF1 E3 ligase complex. SCF1 complex core components (GST-CUL1, GST-SKP1, GST-RBX1) and Flag-008, Flag-SKP2 or Flag were transiently coexpressed in 293EBNA1 cells. The Flag-proteins were immunoprecipitated with anti-Flag-agarose and extensively washed. The precipitates were divided and used for the ubiquitination reaction or as immunoprecipitation controls. For the ubiquitination reaction, E1, E2, ATP and ubiquitin were added to the precipitated complexes and incubated at 37°C for 1.5 h. The reactions were stopped by addition of SDS-loading dye. Samples were resolved by SDS/PAGE and analyzed by Western blotting with antibody against ubiquitin (*Upper*, ubiquitination reaction) or against Flag and GST (*Lower*, input controls). Other abbreviations and labels are as described for Fig. 2.

Identification of the substrates of poxviral F-box proteins will be the primary focus of future work. The substrate(s) of specific poxviral ANK/F-box proteins may only be present in particular cell types and their recognition may require concurrent viral infection or an external trigger to initiate essential posttranslational modifications such as phosphorylation (3).

Published data suggest possible targets for two poxvirus ANK proteins that we now recognize as F-box proteins. Myxoma virus protein MYX153 colocalizes with NF-κB in TNFα- stimulated cells (23). Another myxoma virus ANK protein, MYX163, has been shown to interact with CUL1 and was proposed to do so via a putative F-box domain (24).

Only few other viruses have been shown to encode functional F-box proteins. Two plant virus proteins, CLINK of faba bean necrotic yellow virus and P0 of poleroviruses, have been shown to contain F-box-like motifs and bind to plant homologs of SKP1 (25, 26). In addition, ovine and bovine atadenoviruses have been shown through bioinformatic analysis to contain four putative F-box proteins (27).

Many viruses manipulate the ubiquitination system by other means (8, 9). HIV-1 Vif (viral infectivity factor), for example, acts as a specificity factor, directing polyubiquitination of two antiviral factors via the SCF5 E3 ligase complex. Human papilloma virus-16 protein E6 recruits p53 to the HECT-type E6-AP E3 ligase where it is polyubiquitinated. Other viruses, such as KSHV, express proteins with intrinsic E3 ligase activity that targets antiviral factors of the cell. Viruses thus use the cellular ubiquitination system to remove antiviral factors and thereby facilitate viral replication.

We propose that most poxviral ANK proteins share the function of targeting proteins, including antiviral factors, to the ubiquitination pathway. This is achieved via a new class of F-box, defined by its C-terminal location, truncated nature, and pairing with an N-terminal ANK domain as a probable substrate-binding region.

Materials and Methods

Cell Lines. Human embryonic kidney 293 cells expressing Epstein-Barr virus-encoded nuclear antigen (293EBNA1) or the SV40 large T antigen (293T) were grown in DMEM (GIBCO). Media were supplemented with 10% FCS, 2 mM glutamine, 500 units/ml penicillin, 0.5 mg/ml streptomycin, 1% kanamycin, and 40 μg/ml gentamycin.

Plasmid Constructs. Coding regions were amplified by PCR and incorporated into expression vectors based on pApex-3 (28). The cloning strategies used resulted in expressed proteins that were tagged at the N terminus with either the Flag octapeptide or with GST. Details of each construct are provided in *SI Materials and Methods*.

Transfection and Immunoprecipitation. Cells were transfected with 0.5 μg (ubiquitination assays and SKP1-dependent immunoprecipitations) or 1 μg of each plasmid per well of a 6-well dish using FuGENE 6 (Roche) according to the manufacturer's instructions. Cells were harvested and lysed with the appropriate lysis buffer containing protease inhibitor mixture at 1× strength (Roche) as detailed in *SI Materials and Methods*. Lysates were cleared by centrifugation and the supernatants incubated with either anti-Flag M2 agarose (Sigma) or Glutathione-Sepharose 4B (Amersham). Beads were washed with lysis buffer before being resuspended in 10 μl SDS–polyacrylamide gel electrophoresis (SDS/PAGE)-loading buffer with 10% β-mercaptoethanol, boiled, and analyzed by SDS/PAGE and immunoblotting.

Immunoblotting. Samples were separated on SDS/PAGE gels and transferred to nitrocellulose membranes using standard methods. Flag-tagged proteins were detected with anti-Flag M2 antibody conjugated to horseradish peroxidase (HRP) (Sigma) and GST-tagged proteins were detected with anti-GST antibody conjugated to HRP (Amersham). Endogenous SKP1, CUL1, and RBX1 were detected using as primary antibodies rabbit-anti-SKP1 (Santa Cruz, clone H-163), rabbit-anti-CUL1 (Santa Cruz, clone H-213), and rabbit-anti-RBX1 (Lab Vision, RB-069-P1), respectively, and goat-anti-rabbit-HRP (Sigma, A6154) as the secondary antibody. Bands were visualized with SuperSignal West Pico chemiluminesce substrate (Pierce).

Liquid Chromatography and Tandem Mass Spectrometry. HEK293T cells were transfected with pApex-Flag129 DNA and stable cell lines generated through hygromycin selection. Cells were lysed with 1% Nonidet P-40 supplemented with 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 10 mg/ml leupeptin, and 10 μg/ml aprotinin. Lysates precleared with agarose beads were incubated with anti-Flag agarose beads, washed with lysis buffer, and resolved by SDS/PAGE. Gels were stained with GelCode colloidal Coomassie blue reagent (MJS BioLynx). Excised protein bands were digested according to standard protocol (29) and analyzed by liquid chromatography-tandem mass spectrometry with an HP 1100 HPLC System (Agilent) connected to an LCQ-Deca Mass Spectrometer (ThermoElectron). MS/MS spectra were searched using the MASCOT program (30) against NCBI nonredundant DataBase (NCBInr; www.matrixscience.com). The nonhuman orf virus sequences were also included to enable identification of the orf virus bait protein.

Ubiquitination Assays. Ubiquitin ligation assays were adapted from published protocols (31) using specific immunocomplexes as the E3 ligase. 293EBNA cells (10^6) were transfected with plasmids encoding Flag-008, Flag-SKP2 or Flag and GST-CUL1, GST-SKP1, and GST-RBX1 (0.5 μg of each plasmid). Lysates were prepared with TBS (20 mM Tris base pH 7.6, 500 mM NaCl), 1% Nonidet P-40 and 5 mM NaF. Flag-tagged proteins and coprecipitating components of SCF1 complexes were immunoprecipitated with anti-Flag agarose and washed extensively. Packed beads (25 μl) were resuspended with a 5-μl mixture containing 150 ng E1 (Sigma), 645 ng E2 (UbcH5b, Affinity BioReagents), 12.5 mM ATP, and 1.25 μg/μl ubiquitin. Reaction mixtures were incubated for 1.5 h at 37°C before analysis by SDS/PAGE and immunoblotting with anti-ubiquitin-HRP (Santa Cruz).

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