F-Box-Like Domains are Present in Most Poxvirus Ankyrin Repeat Proteins

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Abstract. Vertebrate poxviruses encode numerous proteins with the ankyrin (ANK) repeat, protein–protein interaction motif but little is known about the role(s) of this large family of poxvirus proteins. We report here that the vast majority of poxvirus ANK repeat proteins share a general molecular architecture that includes a conserved amino acid motif at the carboxyl terminus. This motif is most like the F-box seen in a range of cellular proteins. From 80–100% of the ANK repeat proteins of any one poxvirus have an F-box-like domain and we observed only one poxvirus protein with an F-box-like domain but lacking ANK repeats. The proteins of only one genus of vertebrate poxviruses lack F-box-like domains and this genus does not encode ANK repeat proteins. Many F-box proteins are recognition subunits of ubiquitin ligase complexes in which the F-box binds to core elements of the complex and protein–protein interaction domains in the remainder of the protein bind the substrate protein. These observations suggest a general model of the function of the poxvirus ANK-F-box proteins. We propose that the F-box-like domains in these proteins interact with cellular ubiquitin ligase complexes and thereby direct the ubiquitination of proteins bound to the ANK repeats. The large number of different poxviral ANK-F-box proteins suggests a wide range of cellular proteins might be subjected to ubiquitin-mediated degradation, thereby modulating diverse cellular responses to viral infection.

Key words: ankyrin, F-box, poxvirus, SOCS-box, ubiquitin ligase

The ankyrin (ANK) repeat motif is a sequence of 33 amino acids named after the cytoskeletal protein, ankyrin, which contains 24 copies of the repeat [1]. Although the very many proteins in which the ANK motif occurs have a diverse range of functions, the conserved role of the motif as a mediator of specific protein–protein interactions is well documented [2–4]. ANK repeat proteins are present in bacteria, archebacteria and eukaryotes, but have not been widely reported in viruses. An exception to this generalization is the chordopoxviruses. The members of seven of the eight genera of this subfamily each contain multiple genes encoding ANK repeat proteins and a recent report of the genome of *Canarypox virus* listed 51 ANK repeat genes, representing 21% of the genome [5]. The presence of poxviral ANK proteins was recognized soon after the motif was named [6–9]. Some analyses of the roles of a few of these proteins have been reported. One of the vaccinia virus (VACV) proteins (K1L) recognized as carrying ANK repeats had already been established as a determinant of host range [10] and more recently it has been shown to contribute to the inhibition of NF-kB activation in infected cells [11]. A myxoma virus (MYXV) ANK repeat protein (MYXV 153) that interferes with the inflammatory response has also been suggested to interact with NF-kB [12]. However the mechanisms of actions of these proteins remain unknown. Indeed, surprisingly little is known

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about the possible roles of this largest family of poxvirus proteins. Here we report that an F-box-like domain is conserved in the majority of pox-viral ANK repeat proteins and postulate a general role for this family of proteins. In addition the data suggest a new class of the F-box domain.

We examined the 5 ANK repeat proteins of *Orf virus* (ORFV), the type species of the genus *Parapoxvirus* [13,14], and identified that these proteins show significant sequence similarity to each other (BLASTP scores from 304 to 139), share a similar length (497–525 amino acids) and have a similar number (6–9) of copies of the ANK motif located within the N-terminal two-thirds of each peptide (Fig. 1). Nearer to the C-termini there is no evidence of ANK motifs but the peptides continue to show sequence similarities. This is most evident in a region approximately 30 amino acids from the C-terminus, which includes an octapeptide string that is identical in 3 of the 5 proteins. Searches of protein domain databases revealed a low level match between this region of one of the proteins (ORFV 128) and the F-box domain, prompting us to look more closely at this region of the ORFV proteins. This revealed the presence of an F-box-like domain near the C-terminus of each of the ORFV ANK proteins (Fig. 1). Consensus F-box domains have approximately 50 residues and have few invariant positions [15,16] (Fig. 1). The viral sequences are generally shorter than the established F-box but the consensus ORFV sequence (LPXE[IVL][VL]XX[IV]LXX[VL]XXXXL) conserves significant numbers of the F-box consensus residues. These include positions 1 (Leu), 2 (Pro), 9 (Ile/Val) and 13 (Val/Leu) of the ORFV consensus, which are 4 of the 5 most conserved residues of F-box motifs [16].

We compared the ORFV F-box-like sequence with proteins of other vertebrate poxviruses and found that numerous proteins contained similar sequences. Fig. 2 shows the F-box-like sequences present in proteins of 7 poxviruses, each representing a separate genus. In all cases the motif was located at the C-terminus of the protein and in very nearly all cases the proteins also contained ANK repeats. For example, each of the four myxoma virus (MYXV) ANK proteins contain a C-terminal F-box-like motif (Fig. 2). The same is also true of the ANK proteins of *Swinepox virus* (SWPV) and *Yaba monkey tumor virus* (YMTV).
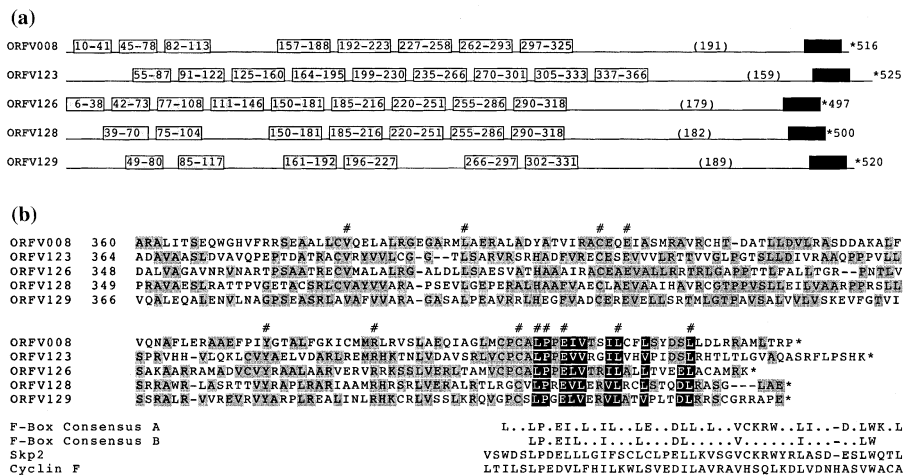

Fig. 1. Analysis of ORFV ANK repeat protein sequences. (a) Schematic representation of the 5 ORFV ANK repeat proteins. The full length of each protein is represented by a black line with the total number of amino acids shown at the right end. Each ANK repeat predicted by SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de) is shown as a shaded box along with its amino acid coordinates. The number of amino acids downstream of the last ANK repeat is shown in brackets. F-box-like motifs are indicated by black boxes. (b) Alignment of the amino acid sequences of the C-terminus of each ORFV ANK repeat protein. Residues present in all 5 sequences are indicated by a cross-hatch (#). Residues shared by the majority of sequences are shaded. The bottom portion of the figure shows 2 published consensus sequences of the F-box (A, [20] and B, [30]) and 2 examples of human F-box proteins, Skp2 and cyclin F. ORFV residues consistent with the consensus sequences are reverse shaded. The amino acid sequences are of proteins of ORFV strain NZ2 (GenBank accession numbers CAD99262, CAD99381, CAD99388, CAD99390 and CAD99391) and have been numbered so as to assist comparisons with published data [14].

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F-Box       ...LP.EIL...L...DL.....V......I.....LW              Size  ANKs  Tail  F-box
POX CON     ...LP.Ei...Ii...i...dl
ORFV008   PCALPPEIVTSILCFLSYDSLLDLRRAMLTRP                       516    8    191    Y
ORFV123   PCALPPEVVRGILVHVPIDSLRHTLTLGVAQASRFLPSHK               525    9    159    Y
ORFV126   PCALPPELVTRILALLTVEELACAMRK                            497    9    179    Y
ORFV128   GCVLPREVLERVIRCLSTQDLRASGLAE                           500    7    182    Y
ORFV129   PCSLEGELVERVLATVPLTDLRRSCGRRAPE                        520    6    189    Y
MYXV151     LP EI   IV  L   EV                                   675    9    170    Y
MYXV152   WGRLPVELQKNILQYLTSYQIKYILCFKKKTRRFFI                   490    8    176    Y
MYXV153   WNDIPPEIKFKILNNLTNKDLVFLNENKVEYNV                      493    5    173    Y
MYXV163   WTRLPVEVRYSVVDYLTDDEIHDLFVKIHA                         483    7    171    Y
SWPV141   WNILPTYVHEKILDCMTLNDIKMLYSLFNRTSVKSLS                  635   10    171    Y
SWPV142   WMRIPIPIQRYILLHLTMKDLSIILK                             485    7    168    Y
SWPV143   WNYLPLEVKMYIMDFLPDTDITNILFMKK                          430    6    165    Y
SWPV144   WNSIPIEIKHKIINNINNHDMYILYKNRKKK                        493    7    180    Y
YMTV011   WFFIPMEIKLKLIKNIEDRYLE                                 637   13    152    Y
YMTV146   WYVIPNEIKIILVNCLSDDDMKKIIYTNVFFKK                      356    3    175    Y
YMTV147   WSIIPIEIKIILCNFLSDNDISILEKKISCRKRV                     497    5    177    Y
YMTV148   WNTVPNEIKLKIISKLSDDEINNIISCVKLKNKTIQCKQVV[6]           483    5    302    Y
LSDV012   FDYLTSLGIDMDETNTSGYNCKDLIKFRNLTFFDKQ                   211    5     23    N
LSDV145   WDILPPEIKDNIFNCMSLRDIRVIFHMFVVYKP                      634    7    200    Y
LSDV147   WNYLPTEIKIHILEYLDFSDFDTIINPKRKSKKYFL                   498    7    180    Y
LSDV148   WNTLPTEIKMYIINFLSDNEIKLLAIK                            447    5    191    Y
LSDV152   WNTIPLEIKFKIVNNLSLNDIEMFLKNNKK                         489    7    172    Y
CMLV003   LSYLPPEIIRNIITKLSDYHLNSMLYGKNHYKYYP                    591    6    220    Y
CMLV004   ISKIPMEIQREILYYSIK                                     672    9    145    Y
CMLV028   IPFIAYFVIMHPDFCKNRRYFTSYKRFVTDYVHEGVSYEVF[4]           446    7     63    N
CMLV180   WACFPNEIKYKILENFNDNELSTYLKIL                           564    7    157    Y
CMLV202   LSRIPNEILLKILYNLSVYDLRKLYTRYMLENETDYHIES[8]            783    5    234    Y
FWPV006   WYTLPIEVKQTILGNMSYEELFNIVRGNIALEEDNEYGCD               418    0     -     Y
FWPV012   WCLLPLKGKLNILSKLNNDNIKKLILNDAIKVNNKTG                  331    4    192    Y
FWPV014   WSDLPLGIKYDILEKIDEEDLPYC                               437    7     74    Y
FWPV018   WWLLSPEIHRVIVSNLSMEDLKTISGYY                           700   12    177    Y
FWPV022   LHMLPLEIRSIICCFLSNNELEKL                               578   10    163    Y
FWPV023   KDIVPDRISSELLDEEYDLDGWRSTSCSIS                         434    6    104    N
FWPV024   INSVPINVKYMIMEMLDNKDLKSIIDSVNK                         596   11    173    N
FWPV026   ITNLPYEVIYIIVEKMTNKLCEIR                               436    6    173    Y
FWPV031   WNNLPNEIKDHIFTYINNDLKIMTGSKT                           341    5    149    Y
FWPV034   EWHLPYSVLTDDKPINYSKIQSKVSHNESRFLPYKKRY[8]              415    9     83    N?
FWPV115   ICKNRQEITQKIIDNLKDIIIYRSFIEKYISRINI                    542    8    135    N?
FWPV162   WNALPIELKYNILAMLGDNDLFNIVRNCS                          603   12    175    Y
FWPV216   ILDIPKTVKVYNTRINMLLDEAIIYNNINIIL                       296    3    160    N
FWPV218   WHDLPYELKHYIIEYINIEFIKSLLEHTNLKNKE                     461    9    149    Y
FWPV219   WKNIPIEVKYMILRYLGKDDLYNIVNSV                           434    7    176    Y
FWPV222   WNLLPVEIKFNILEYLNSKDLISLIHSNTVNEIDLSHIFI               747   15    188    Y
FWPV223   DGLTPLHYAVKYGNISIVKMVIR            fragmented?         141    4      1    N
FWPV224   NINRIDEYYYSALHYAVKSSNLKAVNF       fragmented?         146    3     23    N
FWPV225   WMFLPTEIKFKVLSYLSSKDLYFII                              104    0     -     Y
FWPV227   WSCLPFEIKYKLLENIKDDETLRKI                              361    5    177    Y
FWPV228   FNKLPLDIISMILDFLSDDDLALMAIFN                           525    9    172    Y
FWPV230   LDSNNNTPLIYAVCSVIRLYLKCY          fragmented?         188    3     24    N
FWPV231   *NVLPINIKYVLLEMLDNKDLITLNNT*                           256    4    105    *Y
FWPV232   WYNMPLEIKHDIMYLLDDKSLCNLIVAEYDS                        482    7    185    Y
FWPV233   WNRLPVELQNYIMEYIDDASLKVMHEYEKHKLKY                     512    6    183    Y
FWPV234   WYTLPIEIRWMILTKLDDMILRNLLLQNETNNIKNCKKQ                428    6    190    Y
FWPV240   WNWLPHEIKYNILEYIGNKELDIASMK                            410    7    181    Y
FWPV241   NGASVNELSHTNNTPLHVAVDKNIKP        truncated?          106    2     19    N
FWPV242   GVRLEGDHDYYLQPILLDYSYETYNIIHILLEHGLRITSN[12]           358    7     42    N
FWPV243   WMILEQEIKINLLCYLDNKELDYIYESSLENNKNNTSDKK[5]            262    1    208    Y
FWPV244   WSKLEPDIKLSILEFIGNTELRKICNR                            668   10    166    Y
FWPV245   INEFPIYSMYLVRCLYEYGNILKEMGSCIHNRYKK                    436    8    149    N
FWPV246   WDMLEIEIKNQIVLLLDNTDLSIISNSIQHSQN                      592    8    179    Y
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Fig. 2. Amino acid sequence alignments of the C-termini of ANK repeat proteins of representative mammalian poxviruses and fowlpoxvirus. A consensus F-box sequence [30] is shown along with a consensus of the poxviral F-box-like domain with lower case letters representing variant positions as follows: i, representing I, L or V; d representing D or E; and 1 representing L or I. A dot (.) indicates nonconserved positions. Residues identical with the poxviral consensus are reverse shaded and conservative changes shaded in gray. Also shown for each protein are the total number of amino acids (Size), the number of ANK repeats (ANKs), and the number of amino acids downstream of the last predicted ANK repeat (Tail). The presence (Y) or absence (N) of a predicted F-box is summarised in the right-most column (F-box). Question marks indicate low matches with the consensus sequences that introduce some uncertainty to these predictions. Peptides encoded by adjacent ORFs that might be derived by fragmentation or truncation of a single ORF are indicated by a vertical line that brackets their names and by appropriate text adjacent to their sequences. The sequence shown for FWPV231 is marked with an asterik to indicate that this is from an adjacent, apparently intergenic region (see text). ORFV amino acid sequences are as described in Fig. 1. Other sequences are from the Poxvirus Bioinformatics Resource Center database (http://www.poxvirus.org) and are derived from the following isolates: MYXV strain Lausanne, SWPV isolate 17077-99, YMTV *Yaba monkey tumor virus,* LSDV Neethling isolate 2490, CMLV strain M96, and FWPV strain FCV. In some instances where the amino acid sequences shown have been truncated, the number of residues not included is given in square brackets.

Lumpy skin disease virus (LSDV) also encodes 4 ANK proteins with associated C-terminal F-box-like domains. However, it encodes a further ANK protein (YMTV008 and LSDV012) that does not contain an F-box. A similar situation occurs with the orthopoxvirus, *Camelpox virus* (CMLV) in which one of the ANK proteins (CMLV028) does not contain an F-box. Several small, truncated CMLV ANK proteins (CMLV 015, 016, 017, 199 and 200) also do not have an obvious F-box but the F-box-like motif is apparent in the full-length orthologs of these proteins present in other orthopoxviruses, for example, VACV C9L and B18R (not shown). CMLV030 is a truncated ortholog of one of the most well studied poxvirus ANK repeat proteins, VACV K1L. None of the orthopoxvirus versions of this protein have an F-box-like domain.

This analysis shows that each of the mammalian poxviruses typically encodes 4 or 5 ANK proteins and that 80–100% of these proteins have a C-terminal F-box-like domain. The only genus of the vertebrate poxviruses not to encode ANK repeat proteins is the molluscipoxvirus and we found no evidence of F-box-like motifs in molluscipoxvirus proteins.

Avipoxviruses encode numerous ANK proteins and a search of the fowlpoxvirus (FWPV) sequences detected F-box-like domains in 19 proteins. In all cases the motif is at the C-terminus and in all but two cases the protein also carries ANK repeat motifs. FWPV has been reported to encode 31 ANK proteins and we therefore examined each of these to see if there was an explanation for the apparent lack of an F-box in a significant number of them. The C-terminal regions of all of the FWPV ANK proteins are shown in Fig. 2 with the F-box-like domains marked. Two of the ORFs (223 and 224) that contain ankyrin repeats but not an F-box motif are adjacent and short (141 and 146 amino acids, respectively). The next downstream gene (225) on the other hand does not contain ANK repeats but does have a C-terminal F-box. The DNA sequence of these 3 ORFs would, if combined, encode a peptide of 519 amino acids dominated by ANK repeats and with a C-terminal F-box-like domain. It seems plausible that this region may represent a single gene that has become fragmented. Similar events are likely to explain the lack of F-box domains in ORFs 230 and 231. Together these two ORFs could encode a 433 amino acid peptide and in the adjacent downstream intergenic region, but apparently out of frame, is the potential to code for a strong match with the poxvirus F-box motif. These observations again suggest that this region represents a single ANK-F-box gene that has become fragmented. FWPV ORFs 241, 242 and 243 might also represent a single ORF that once encoded a single large ANK-F-box but has been fragmented. FWPV ORF 034 is 415 amino acids long, contains ANK repeats but no F-box. Intriguingly, the corresponding protein of canarypox virus (CNPV017) is a little longer (486 amino acids) and contains a C-terminal F-box, raising the possibility that a deletion event has truncated FWPV034. Five further FWPV proteins (ORFs 023, 024, 115, 216 and 245) contain ANK repeats but show little evidence of an F-box with no obvious explanation for this absence. In summary there appear to be 27 FWPV ANK genes (some of which have become fragmented), and 22 (81.5%) of these carry an F-box-like domain.

We also identified a FWPV ORF (006 and its counterpart repeated at the other end of the genome, 255) that includes a C-terminal F-box but does not carry ankyrin repeats. This protein shows a general sequence relatedness to a family of poxvirus proteins exemplified by VACV C10L. However other members of the family do not contain F-box-like domains.

This analysis reveals that greater than 80% of poxviral ANK repeat proteins carry a C-terminal F-box-like domain and that this domain is also present in a small number of additional poxvirus proteins. The consensus sequence of the poxviral F-box is LPXE[IVL]XXXI[IVL]XX[IVL]XXX[-DE][IL]. In addition these proteins share the organizational features of the ORFV proteins summarized in Fig. 1a. These features include a size of between 400 and 600 amino acids and the presence of 5–10 ANK repeats. Particularly striking is the clustering of the ANK repeats towards the N-terminus, leaving a region of 150–200 amino acids which lacks ANK repeats but which terminates with an F-box-like domain (Fig. 2).

The widespread distribution of the F-box-like motif in poxviral ANK proteins confirms the significance of the sequence we first detected in ORFV ANK proteins and also raises the possi-

bility that this large family of proteins might form a functionally related group. The presence of an F-box-like motif provides a strong clue as to what that function might be.

Many F-box proteins are the target recognition subunits of ubiquitin ligase complexes. The abundance of numerous cellular proteins is regulated by the ubiquitin-proteasomal degradation system in which polyubiquitin chains are formed on target proteins by the sequential activities of El, E2 and E3 enzymes [17]. The specificity of the ubiquitination process is provided by the ubiquitin ligase, E3, which binds the target protein and completes the transfer of ubiquitin to it. The poly-ubiquitinated substrates are subsequently degraded by the 26S proteasome.

An important class of the E3 ubiquitin ligases is the multi-subunit RING-finger type in which a molecular scaffold is provided by a cullin protein that simultaneously interacts with an adaptor protein and a RING-finger protein, The best understood example is the SCF (Skp1, Cullin1, F-box) complex involving Cullin-1, with Skp1 acting as the adaptor protein and Rbxl as the RING-finger [18,19]. Substrate specificity is provided to this core complex by F-box proteins. These function as a bridge in which the F-box domain binds to Skp1 and other protein–protein interaction domains in the remainder of the F-box protein recruit the substrate protein to the complex [20,21]. An example of such complexes is shown in Fig. 3 in which the F-box protein, Skp2, mediates the ubiquination of P27. The F-box domain of Skp2 binds to Skp1 and the P27 substrate is recruited to the complex via its binding to leucine-rich domains in Skp2. The interchangeable nature of the large number of F-box proteins allows the SCF complexes to target diverse substrates and to control a diverse range of cellular functions [19].

A Trp at position -3 relative to the first Leu is present in the human F-box protein, Skp2, and although this residue is not generally included in F-box consensuses, structural analysis has shown it to be one of the residues that contact Skp1[20]. A Trp at this position is not seen in the parapoxvirus F-box-like domains but is present in many of F-box-like motifs of the other poxvirus genera.

Based on the observations reported here that poxviral ANK proteins carry both the ANK protein–protein interaction domain and a terminal F-box-like motif, we postulate that most members of this family of proteins function in a manner related to that of cellular F-box proteins, interacting via the F-box with cullin-based ubiquitin ligase complexes such as SCF to direct the ubiquitination of cellular proteins that are brought to the E3 complex via their interactions with the ANK repeat motifs within the viral protein. The model we propose is illustrated in Fig. 3 in which the viral F-box interacts with an unidentified adaptor molecule bound to a cullin and a RING protein. The specific protein targeted for ubiquitination and subsequent degradation would be defined by the particular ANK repeat. The large number of different ANK-F-box proteins observed in any one
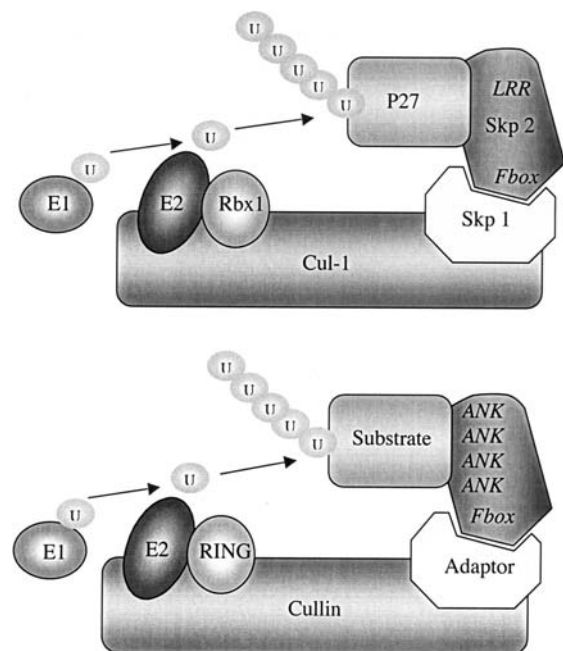

Fig. 3. Comparison of the structure of SCF E3 ubiquitin ligase complexes with a model of the proposed ubiquitin ligase complex incorporating poxvirus ANK-F-box proteins. The top image shows an SCF complex. SCF^{Skp2} directs the formation of polyubiquitin (U) chains on a specific substrate (p27^{Kip1}). Cul-1 provides a scaffold that at one end binds the adaptor protein, Skp1, and at the other end the RING-finger protein, Rbxl. F-box^{Skp2} binds Skpl via its F-box and recruits the substrate via leucine-rich repeat(LRR) domains (20,21), Activated ubiquitin is transferred from El to the carrier, E2, before ligation to the target. The bottom image illustrates the model we propose in which poxviral ANK-F-box proteins bind to an unidentified RING-Cullin-Adaptor complex and bring about polyubiquitination of substrates recruited via binding to the ANK repeat motifs.

poxvirus suggests that a wide range of cellular proteins might be degraded, thereby modulating diverse cellular responses to viral infection. For example, although SCF-F-box ubiquitin ligases have been most studied in the context of cell cycle regulation, these complexes are also active in other processes including regulating apoptotic signalling [22] and interferon receptor turnover [23].

Two features of the viral ANK-F-box-like proteins are not typical of F-box proteins. Firstly, the F-box is typically near the N-terminus of a protein rather than at the C-terminus [24]. Secondly, although F-box proteins typically carry additional protein–protein interaction domains such as WD repeats and leucine-rich repeats, these domains have not been reported to include ANK repeats [24]. On the other hand both of these features occur in a related family of proteins, the SOCS (suppressor of cytokine signaling) box proteins [25]. The SOCS box is approximately 40 amino acids long, typically occurs at the C-terminus and is related in sequence to the F-box. SOCS box proteins also typically carry additional protein–protein interaction domains and in one sub-family of the group this domain consists of ANK repeats. In addition SOCS box proteins have the same target-specificing role as F-box proteins but within ECS (elonginC, cullin2, SOCS-box) E3 ubiquitin ligase complexes and target, for example, components of the cytokine signal transduction pathway [26]. These features make it tempting to speculate that the poxviral ANK proteins might be members of the SOCS box family. However the C-termini of the poxviral proteins are clearly more like the current consensus of F-box domains.

Although F-box motifs are rare among viruses [27], use of the proteasome system to degrade cellular proteins and thereby enhance viral replication is not [28]. Examples include the ubiquitination and degradation of the p53 tumor suppressor directed by the human papillomavirus E6 protein and the targeting of the same cellular protein by adenovirus E4orf6 and E1B 55K. This latter interaction involves viral recruitment of a cullin-based E3 ubiquitin ligase complex similar to SCF [29]. Our model of the action of poxviral ANK-F-box proteins is therefore consistent with types of manipulation of the cellular environment seen with other virus families. It also provides a testable hypothesis for the function of the large group of poxvirus ANK repeat proteins as well as indicating a new F-box-like domain.

Acknowledgments

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