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Ubiquitin Recognition by the Human TSG101 Protein

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Summary

The UEV domain of the TSG101 protein functions in both HIV-1 budding and the vacuolar protein sorting (VPS) pathway, where it binds ubiquitylated proteins as they are sorted into vesicles that bud into late endosomal compartments called multivesicular bodies (MVBs). TSG101 UEV-ubiquitin interactions are therefore important for delivery of both substrates and hydrolytic enzymes to lysosomes, which receive proteins via fusion with MVBs. Here, we report the crystal structure of the TSG101 UEV domain in complex with ubiquitin at 2.0 Å resolution. TSG101 UEV contacts the Ile44 surface and an adjacent loop of ubiquitin through a highly solvated interface. Mutations that disrupt the interface inhibit MVB sorting, and the structure also explains how the TSG101 UEV can independently bind its ubiquitin and Pro-Thr/Ser-Ala-Pro peptide ligands. Remarkably, comparison with mapping data from other UEV and related E2 proteins indicates that although the different E2/UEV domains share the same structure and have conserved ubiquitin binding activity, they bind through very different interfaces.

Introduction

Ubiquitin (Ub) is a small 76 residue protein that serves as a covalent modifier of other proteins, usually through attachment of the Ub C terminus to a lysine side chain of the target protein. This process of ubiquitylation directs proteins into a variety of important biological pathways, including degradation by the 26 proteasome (Hershko and Ciechanover, 1998), endocytosis (Hicke and Dunn, 2003), and lysosomal targeting (Katzmann et al., 2002). Consequently, ubiquitylation helps to control a number of cellular processes including cell cycle progression (King et al., 1996), protein quality control (Kostova and Wolf, 2003), signaling (Di Fiore et al., 2003), receptor downregulation (Katzmann et al., 2002), and budding of HIV and other viruses (Pornillos et al., 2002c; Vogt, 2000). Given this widespread importance, it is not surprising that ubiquitylated proteins can be recognized by a variety of different Ub binding proteins and motifs, including UIM, CUE, VHS, UBA, and UEV domains (Buchberger, 2002; Hicke and Dunn, 2003; Katzmann et al., 2002).

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One function of ubiquitylation is to tag proteins for sorting into vesicles that bud into the interior of multivesicular bodies (MVBs) (Katzmann et al., 2002; Raiborg et al., 2003). These organelles can subsequently fuse with lysosomes, thereby delivering both hydrolytic enzymes and substrate proteins into the lysosome/vacuole. MVB biogenesis requires the actions of the class E (VPS) proteins, many of which are subunits of three distinct soluble complexes, called the ESCRT complexes (endosomal sorting complexes required for transport). These complexes are sequentially recruited to the endosomal membrane where ubiquitylated proteins are sorted and vesicles formed (Katzmann et al., 2002). TSG101 (Vps23p in yeast) is the central component of ESCRT-I (Katzmann et al., 2001), which is recruited to the membrane through an interaction with the upstream HRS complex (Bache et al., 2003; Bilodeau et al., 2003; Katzmann et al., 2001, 2003; Lu et al., 2003). TSG101 functions both to recognize ubiquitylated protein cargo (Bilodeau et al., 2003; Bishop et al., 2002; Katzmann et al., 2001), and to help recruit the downstream ESCRT-II and ESCRT-III complexes (Babst et al., 2002; Katzmann et al., 2001; Martin-Serrano and Bieniasz, 2003; von Schwedler et al., 2003). TSG101 and other class E proteins also function in the Ub-dependent process of HIV budding, where they are apparently coopted to help virus particles bud from plasma and endosomal membranes (Pornillos et al., 2002c).

In general, monoubiquitylation appears to be sufficient for lysosomal protein targeting, although at least some targeted proteins have polyubiquitin chains, in which a lysine side chain of one Ub is attached to the C terminus of another (Dai et al., 2003; Katzmann et al., 2003; Pickart, 2000). TSG101 binds Ub directly through its N-terminal UEV domain (Garrus et al., 2001; Katzmann et al., 2001; Pornillos et al., 2002b), which is homologous to the E2 enzymes that ligate Ub to substrate proteins, but lacks the catalytic cysteine residue of authentic E2 enzymes (Koonin and Abagyan, 1997; Ponting et al., 1997). TSG101 UEV-Ub interactions have been shown to be essential for the trafficking of several ubiquitylated cargoes to MVBs (Bilodeau et al., 2003), including carboxypeptidase S (Katzmann et al., 2001) and the epidermal growth factor receptor (EGF-R) (Lu et al., 2003). It is therefore likely that TSG101 recognition is a critical step in the lysosomal targeting of most, if not all, ubiquitylated protein cargoes. In order to learn how TSG101 recognizes ubiquitylated proteins, we have determined the crystal structure of a complex between ubiquitin and the human TSG101 UEV domain.

Results and Discussion

TSG101 UEV-Ub Structure

The TSG101 UEV domain was cocrystallized with Ub and the complex structure determined by anomalous diffraction and refined against 2.0 Å data to an R_{free} value

Table 1. Crystallographic Statistics

Crystal Parameters	
Space group	C2
Unit cell dimensions	a = 143.6 Å, b = 59.2 Å c = 94.0 Å, β = 128.7
Data Collection Statistics	
Wavelength (Å)	0.97979
Resolution range (Å)	75-2.0 (2.1-2.0)
Unique reflections	41,841 (6,080)
Completeness (%)	97.8 (96.2)
I/σ(I)	9.4 (2.1)
R_{merge} (%)	5.8 (33.5)
Refinement Statistics	
R_{factor} (%)	19.9 (25)
R_{free} (%)	23.5 (28)
Rms deviations	
Bond lengths (Å)	0.016
Bond angles (°)	1.463
Average B factors (Å^2)	
Main chain (UEV/Ub)	28.3/31.2
Side chain (UEV/Ub)	30.8/33.3
Solvent	36.4

Numbers in parenthesis are for the high-resolution bin.
$R_{merge} = \Sigma \mid I - \langle I \rangle \mid / \Sigma I$ where I is the intensity of an individual measurement and $\langle I \rangle$ is the average intensity from multiple observations.
$R_{factor} = \Sigma ||F_{obs}| - k|F_{calc}|| / \Sigma |F_{obs}|$.
R_{free} equals the R_{factor} against 5% of the data removed prior to refinement.

of 24.0% (Table 1) (Figure 1). The crystal structure contains two essentially identical copies of the TSG101 UEV-Ub heterodimer in the asymmetric unit (r.m.s. deviation = 0.24 Å over all Cα atoms). The TSG101 UEV and Ub structures seen in the complex are also very similar to those of the unbound proteins. Ub forms a five-stranded mixed β sheet packed against a helix, as in the unbound protein (Vijay-Kumar et al., 1987), and the C-terminal four residues are flexible (disordered) in both free and UEV-bound structures. The UEV domain adopts the characteristic α/β fold of cannonical E2 enzymes but has an additional N-terminal helix and lacks the two C-terminal helices (Pornillos et al., 2002b). The C-terminal helices are also missing in Mms2, which is the only other UEV domain of known structure (Moraes et al., 2001; VanDemark et al., 2001). The major difference between TSG101 UEV in its Ub-bound and free forms (Pornillos et al., 2002b) is an ~6 Å displacement of residues 43-49, which form an extended "β-tongue" (S1-S2) that participates directly in Ub binding (Figure 1). This apparent shift is probably best viewed as an ordering of these inherently flexible residues upon complex formation.

UEV-Ub Interface

The structure reveals how TSG101 recognizes its ubiquitylated protein targets (Figure 1). The TSG101 UEV β sheet forms a concave surface from which residues of the β-tongue and the loop that follows S4 contact residues primarily from the C-terminal half of Ub. This elongated interface buries Ub Ile44 and surrounding residues, which comprise the "Ile44 surface" of Ub that functions in endocytosis, proteasome-mediated proteolysis (Sloper-Mould et al., 2001) and HIV release (Strack et al., 2002). Contacts are also made to residues in the loop between S4 and S5 of ubiquitin. The interface buries a total of ~1,250 Å^2 of the proteins' solvent accessible surface area away from bulk solvent, and incorporates 15 ordered interface water molecules (Figure 2). This high degree of solvation is consistent with the weak Ub binding affinity of the TSG101 UEV domain (K_d ~500 μM) (Pornillos et al., 2002b).

There is no obvious reason why TSG101 UEV could not bind polyubiquitin in the same manner as seen here for monoubiquitin, because neither lysine residues nor the C terminus of Ub are buried at the UEV interface. Two Ub lysine side chains, Lys48 and Lys63, which are well-characterized sites of polyubiquitin linkage, lie adjacent to the UEV interface (Figure 2). Both of these residues retain significant solvent accessibility in the complex, however, and simple model building suggests that attachment of another Ub moiety could be accommodated.

Relevance for Biological Function

The TSG101 UEV-Ub structure has relevance for numerous cellular functions owing to the fundamental importance of the VPS pathway in development (Kramer, 2002), receptor downregulation (Katzmann et al., 2002), and enveloped virus budding (Pornillos et al., 2002c). Several observations confirm that TSG101 UEV and Ub form the same complex in solution as seen in the crystal. First, there is excellent agreement between the crystal structure and maps of the TSG101 UEV and Ub interaction surfaces obtained by solution NMR chemical shift perturbation (Pornillos et al., 2002b) (see Supplemental Data at http://www.molecule.org/cgi/content/full/13/6/783/DC1). Second, alanine substitution mutations in the TSG101 UEV domain that diminish binding affinity: Val43, Asn45, Asp46, and Phe88 all map to the Ub binding interface seen in the complex structure (Figures 1 and 2D) (Pornillos et al., 2002b). Finally, alanine substitutions in Ub that diminish binding affinity to Vps23 (yeast TSG101): Ile44, Gln62, and Val70, also map to the interface (Bilodeau et al., 2003). Collectively, the mutations in Ub or UEV that diminish binding affinity cover almost the entire interface seen in the crystal structure (Figure 1).

The TSG101 UEV-Ub complex also rationalizes the effects of a series of mutations that have been shown to inhibit the proper sorting of ubiquitylated protein cargoes (Katzmann et al., 2001). The Asn45Ala TSG101 mutation causes an 8-fold reduction in Ub binding affinity (Pornillos et al., 2002b) and inhibits downregulation of the EGF receptor (Lu et al., 2003), presumably because the ubiquitylated receptor is not properly trafficked to the lumen of the MVB. These observations are explained by the extensive interactions seen between Asn45 and Ub His68 (Figure 2D). Similarly, the Ub Gln62Ala and Glu64Ala double mutant blocks Vps23 binding and inhibits the entry of cargo into MVBs (Bilodeau et al., 2003). These observations are also explained by the structure, because the side chain of Ub Gln62 forms hydrogen bonding interactions with the main chain of UEV Ile97 and with a bridging water molecule. Finally, it has also been suggested that the TSG101 UEV-Ub interface functions in HIV budding, because viral

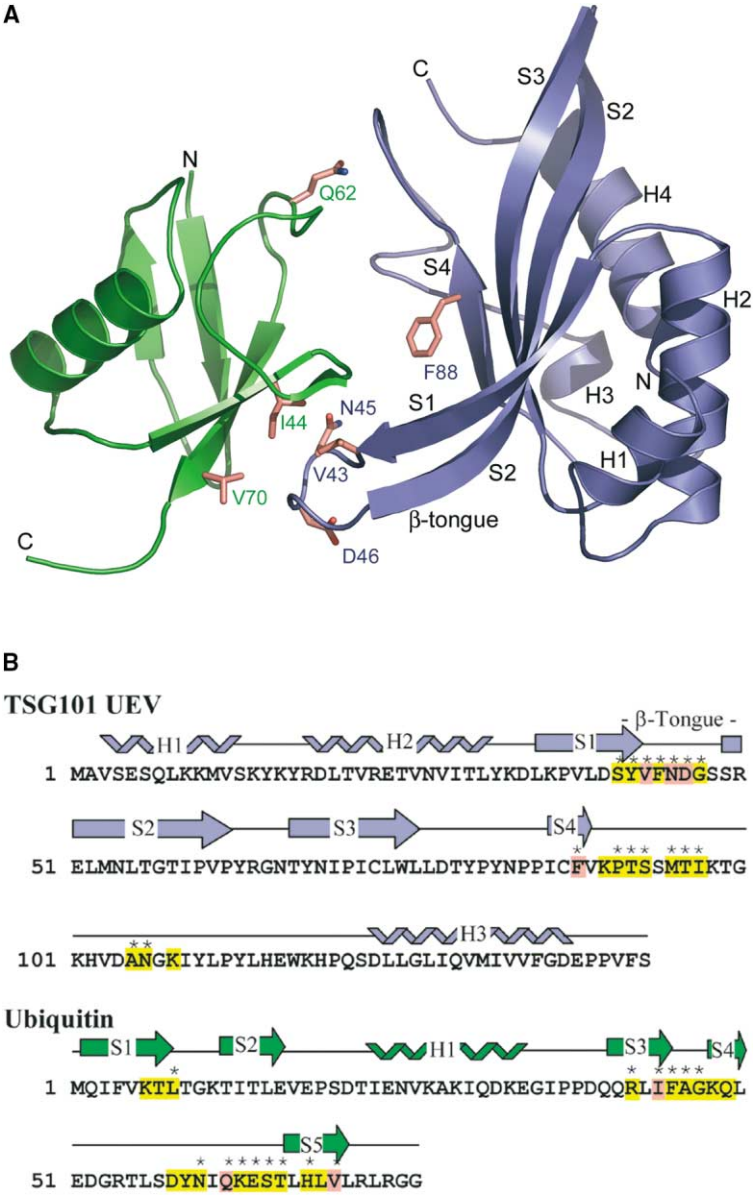

Figure 1. Structure of the Complex between the TSG101 UEV Domain and Ub

(A) Ribbon diagram of the TSG101 UEV domain (slate) in complex with Ub (green). Residues of Ub or TSG101 UEV that diminish Ub binding when substituted with alanine (Bilodeau et al., 2003; Pornillos et al., 2002b) are shown explicitly and colored pink. All of these side chains mediate direct interactions, except for UEV Asp46, whose carboxylate forms a hydrogen bonding interaction that stabilizes the β-tongue conformation. Secondary structural elements of UEV are indicated.

(B) Amino acid sequences and secondary structures. Residues shown explicitly in (A) are shown on a pink background. Additional residues that lose at least 20% of their solvent accessible surface area upon complex formation are shown on a yellow background. Residues that make direct protein-protein contacts across the interface are indicated with an asterisk.

trafficking and release are disrupted by expression of a TSG101 construct that lacks residues 41-43 and is therefore unable to bind Ub (Goff et al., 2003). This deletion mutation is expected to disrupt the β-tongue structure and thereby inhibit Ub binding, although it could also cause a more global disruption of the UEV fold.

Ub binding domains generally seem to bind isolated ubiquitin with relatively weak affinities (Hicke and Dunn, 2003). This is presumably because these domains frequently function as biological switches, rather than as permanent Ub binding partners, and because they cooperate with other interactions to achieve the appropriate overall affinity and specificity. For example, an enzyme that deubiquitylates p53 recognizes Ub via inherently weak and solvated interactions much like those seen here for the TSG101 UEV-Ub complex. In that case, additional binding affinity and specificity are provided by a second binding interaction between the enzyme and p53 (Hu et al., 2002). In the VPS pathway itself, there are at least three other Ub-sensing proteins that utilize three different motifs to bind Ub: HRS/Vps27 (UIM motifs [Mueller and Feigon, 2003; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002; Swanson et al., 2003]), Vps36 (NZF motifs [Alam et al., 2003]), and Vps9 (CUE domains [Davies et al., 2003; Donaldson et al., 2003; Kang et al., 2003; Katzmann et al., 2002; Prag et al., 2003; Shih et al., 2003]). All of these motifs bind with micromolar dissociation constants to the Ile44 surface of isolated Ub proteins. Thus, weak, cooperative binding interactions may facilitate the sequential recognition of overlapping Ub surfaces as ubiquitylated protein cargoes are passed along the VPS pathway.

In addition to binding Ub, the TSG101 UEV domain also binds P(T/S)AP sequence motifs, which are found in both viral and cellular proteins, including TSG101 itself (Lu et al., 2003), HRS (Lu et al., 2003; Pornillos et al.,

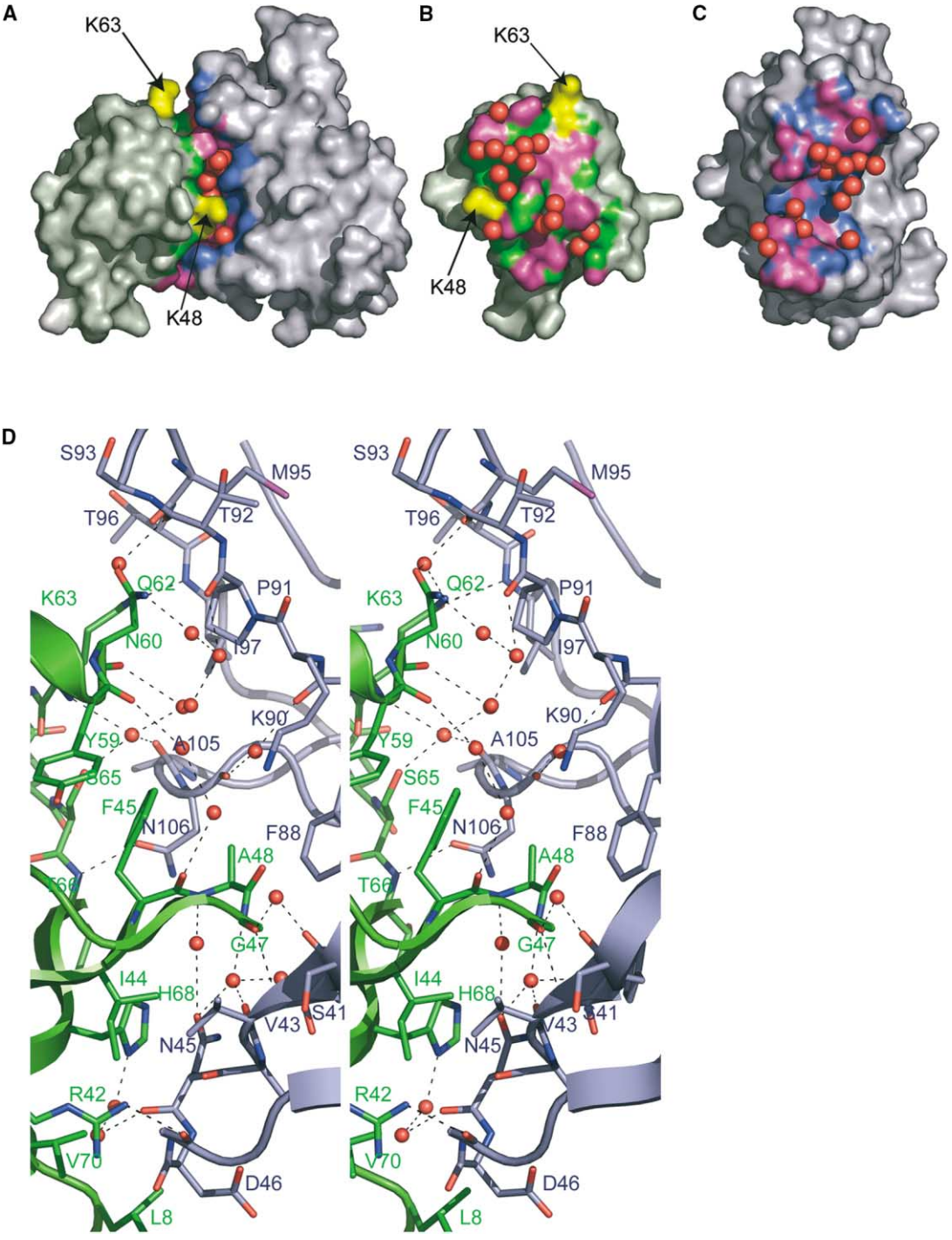

Figure 2. TSG101 UEV-Ub Interface

(A) Molecular surface of the complex viewed in the same orientation as Figure 1.

(B and C) Interaction surfaces of the Ub and UEV interfaces, respectively. Interface water molecules (red) are defined as those that make hydrogen bonding interactions with both proteins (6 waters) or those that hydrogen bond one protein directly and the other protein via another water molecule (9 waters). Of these 15 interface water molecules, one buries ~70% and the other 14 bury at least 90% of their solvent accessible surface in the complex structure. Protein atoms are colored magenta if they participate in direct protein-protein contacts. Additional protein atoms that lose solvent accessibility upon complex formation are colored blue (UEV) and green (Ub). Ub Lys48 and Lys63 are shown with yellow surfaces.

(D) Stereoview showing details of interactions at the interface. Hydrogen bonds are shown as dashed lines.

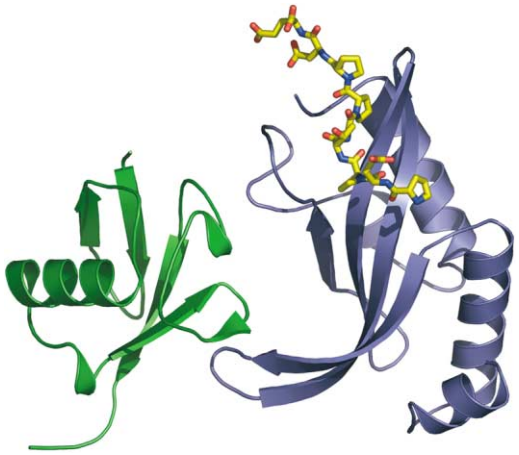

Figure 3. UEV Can Bind Ub and PTAP Peptides Independently

Same as Figure 1 but with the PTAP peptide shown as seen in a TSG101 UEV-peptide complex (Pornillos et al., 2002a).

2002a), AIP1 (von Schwedler et al., 2003), and the HIV-1 Gag p6 protein (Martin-Serrano et al., 2001; Pornillos et al., 2002c; VerPlank et al., 2001). P(T/S)AP-containing polypeptides bind TSG101 UEV in a groove between the S2-S3 hairpin, the N-terminal third of the extended loop between S4 and H3, and the C-terminal end of H4 (Pornillos et al., 2002a). The TSG101 UEV-Ub crystal structure indicates how the UEV domain can bind both Ub and PTAP peptides simultaneously (Figure 3). We favor the possibility that the UEV domain binds P(T/S)AP and Ub motifs located on different proteins, thereby linking them together (e.g., HRS and ubiquitylated cargo) (Bache et al., 2003; Bilodeau et al., 2003; Bishop et al., 2002). It remains possible, however, that Ub and P(T/S)AP motifs reside on the same binding partner and thereby collaborate to provide enhanced binding affinity. In either case, these interactions might function to organize the TSG101/ESCRT-I complex (Katzmann et al., 2001), recruit cargo, and/or compete with the binding

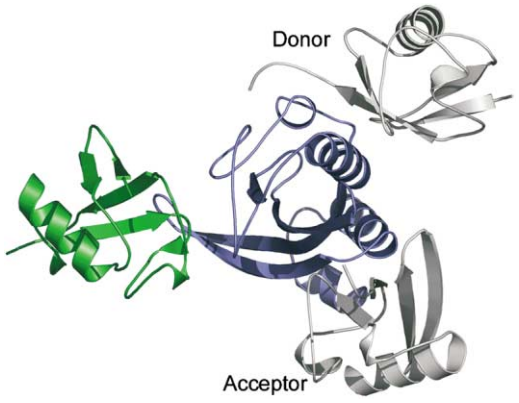

Figure 4. Interactions of E2/UEV Domains with Ub

The TSG101 UEV-Ub crystal structure is shown viewed from the top in Figure 1. The donor and acceptor Ub molecules are shown in white after overlap of the E2/UEV domains of E2 (donor) (Hamilton et al., 2001; VanDemark et al., 2001) and Mms2 UEV (acceptor) (VanDemark et al., 2001) on the TSG101 UEV domain structure.

of the UEV domain to TSG101's own autoinhibitory PSAP motif, thereby activating TSG101 for further protein recruitment.

Comparison with Other E2/UEV-Ub Interactions

Ours is the first report of a three-dimensional structure of a UEV or E2 complex with Ub. Others, however, have reported mapping data that define regions of contact between various UEV or E2 proteins and Ub. One example is Mms2-Ubc13, a heterodimeric complex composed of both a catalytically active E2 (Ubc13) and a UEV domain (Mms2). In the process of making poly(Ub) chains, the Mms2-Ubc13 complex must bind two different types of Ub molecule: an "acceptor" Ub, which is bound by Mms2 so that its Lys63 side chain can form an isopeptide bond with the "donor" Ub C terminus, which is activated by formation of a thiolester bond with the active site cysteine of Ubc13. The binding site of the donor Ub on Ubc13 has been mapped by chemical shift perturbation and modeling (Hamilton et al., 2001; VanDemark et al., 2001), and is located primarily along the helix equivalent to H3 of TSG101 UEV. The binding site of the acceptor Ub on Mms2 was similarly mapped using mutagenesis and modeling (VanDemark et al., 2001), and is located primarily on regions equivalent to TSG101 UEV H2 and the N-terminal residues of S1. Surprisingly, the mode of Ub recognition seen in the TSG101 UEV-Ub structure differs significantly from both the characterized donor and acceptor modes for E2/UEV-Ub protein interactions (Figure 4). In separate studies, the approximate binding surface of the ubiquitin-like protein SUMO/Smt3 was mapped on the E2 enzyme Ubc9 by chemical shift perturbation (Liu et al., 1999) and mutagenesis (Bencsath et al., 2002). This surface overlaps that of the acceptor Ub of the Mms2 complex shown in Figure 4 and again appears to be distinct from that of the TSG101 UEV complex. Hence, there appears to be a remarkable variety of ways in which E2 fold proteins can recognize ubiquitin-like molecules, and the precise binding mode used in each case presumably reflects the functional requirements of each specific system.

Experimental Procedures

Expression, Purification, and Crystallization

Human Ub (Beal et al., 1996) and TSG101 UEV (Pornillos et al., 2002b) were expressed and purified as described. TSG101 UEV and Ub solutions (UEV-100 mM NaCl, 5 mM BME, and 10 mM Tris [pH 8.0]; Ub-150 mM NaCl and 50 mM Tris [pH 7.5]) were mixed in a 1:1 ratio at 0.7 mM and the complex crystallized at 21°C in hanging drops by mixing 2 μl of the protein solution with 3 μl of the well solution (1.4 M ammonium sulfate and 0.1 M sodium acetate [pH 4.6]) and 0.5 μl 100 mM cupric chloride. Crystals grew in 3–5 days and belong to space group C2 with two heterodimers per asymmetric unit. Isomorphous crystals were obtained of selenomethionine-substituted UEV and Ub proteins that were produced using the methionine inhibition method (Van Duyne et al., 1993).

Data Collection and Model Refinement

Crystals were transferred to a solution of the well condition made up with 25% glycerol and cooled by plunging into liquid nitrogen. Data were collected at the Advanced Light Source, beamline 8.3.1. The structure was determined using the Elves package (Holton and Alber, 2004), in which data were processed and scaled using MOSFLM (Powell, 1999). Ten anomalously scattering atom sites

(nine selenium and one copper atoms) were identified and phases calculated using SOLVE (Terwilliger, 2002). Phases were refined by solvent flattening using DM (Cowtan, 1994). Subsequent model building and refinement were performed with O (Jones et al., 1991) and REFMAC5 (Murshudov et al., 1997). The refined model includes four copper atoms, four sulfate molecules, and two acetate ions, none of which approach the UEV-Ub interface. The TSG101 UEV molecules appear to have been modified at residue Cys73 by a BME molecule to yield the modified s,s-(2-hydroxyethyl)thiocysteine residue.

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Accession Numbers

Coordinates and structure factor amplitudes have been submitted to the Protein Data Bank with accession code 1S1Q.