Fusion Proteins with COOH-terminal Ubiquitin Are Stable and Maintain Dual Functionality in Vivo*

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The ubiquitin (Ub) fusion degradation pathway functions to degrade fusion proteins containing a nonremovable Ub moiety at their NH2 terminus (Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17456). Here we show that ubiquitin fusion degradation also targets proteins for proteasomal degradation when Ub is present in the middle of fusion proteins (X-Ub-Y), in a process that entails poly-ubiquitylation of Ub Lys48. By contrast, fusion proteins bearing COOH-terminal Ub (X-Ub) are metabolically stable. Such fusion proteins, either newly biosynthesized or generated by Ub hydrolyses, are reversibly conjugated to heterogeneous target proteins in a manner similar to wild-type Ub. Most importantly, the NH2-terminal fusion partner (X) can maintain its structure and function in the formed X-Ub conjugates as inferred from the fluorescence of green fluorescent protein-Ub conjugates and the incorporation of human immunodeficiency virus type 1 Gag-Ub into viral particles. These findings strongly suggest that 26S proteasomes exhibit spatial discrimination of Ub-conjugated proteins, sparing domains extended from the NH2 terminus of Ub from unfolding and degradation. The multifunctionality of X-Ub fusion proteins opens the possibility for a number of novel practical applications, including the imaging of Ub conjugate formation in living cells.

Ubiquitin (Ub)1 is a 76-residue protein present in eukaryotic cells in extremely high amounts. Ubiquitylation is a posttranslational modification in which Ub chains or single Ub moieties are attached to target proteins, creating poly- or mono ubiquitylated proteins, respectively (1, 2). Typically, Ub is linked to substrates through an isopeptide bond between its COOH-terminal Gly76 and an ε-amino group of a Lys residue on the target protein. Ub can also be attached to free NH2 termini via a standard peptide bond (3). Polyubiquitin (polyUb) chains are created by adding Ubs to a single Ub attached to a target protein. PolyUb chains consisting of Lys48-Gly76-linked subunits are the principal signal for proteasome-mediated proteolysis (4, 5). Chains of four or more Ubs bind to the 19S regulatory subunits of 26S proteasomes with sufficient avidity to enable the 19S subunit to unfold the attached substrate and thread it into the interior of 20S proteasomes, where the substrate is cleaved into oligopeptides (6, 7), which are a principal source of peptides for class I molecules of the major histocompatibility complex. During this process, polyUb chains are removed from the substrate and depolymerized, and Ub subunits rejoin the free Ub pool.

The idealized ubiquitylation signal is bipartite, consisting of a recognition element and a Ub attachment site (8), which need not be in close proximity in the primary sequence and may even be present on different subunits (9). Varshavsky and co-workers (10, 11) have shown that nonremovable Ub situated at the NH2 terminus of linear fusion proteins provides both a recognition and attachment site for ubiquitylation and mediates the subsequent degradation of fusion proteins by 26S proteasomes. This degradation pathway, called Ub fusion degradation, entails ubiquitylation of Lys48 of fused NH2-terminal Ub (10).

What happens, however, when Ub is located other than at the NH2 terminus of fusion proteins? Can Ub function as a cis-acting degradation signal when located anywhere in a protein? PolyUb chain addition sites are predominantly located near the NH2 terminus of substrates (3, 5, 11–13, 17), suggesting a preference for NH2 termini by the ubiquitylation machinery. Fusion proteins with Ub located at the COOH terminus (X-Ub) have been used to demonstrate proteasome-independent roles for monoubiquitin in membrane protein trafficking and viral budding (18, 19). However, in most studies utilizing fusion proteins with COOH-terminal Ub, Lys48 of Ub is altered to Arg to prevent both ubiquitylation and degradation of the target protein (18–20, 22). Because recombinant Ub proteins that have NH2-terminal epitope extensions, such as His6-Ub, myc-Ub, and glutathione-S-transferase-Ub, can substitute for wild-type Ub in a variety of Ub conjugation reactions (24–28), there is a largely untested assumption that wild-type Ub situated at the COOH terminus will trigger the ubiquitin fusion degradation pathway, resulting in degradation of X-Ub fusions by proteasomes. This issue is complicated by the conjugation of the X-Ub to target proteins via Ub Gly76, which is available for isopeptide bond formation (22).

In the present study, we further define the ubiquitin fusion degradation pathway by examining the fate of fusion proteins containing internally and COOH-terminally situated Ub. Our findings have important implications for the recognition of substrates by the ubiquitylation machinery and proteasomes and have a number of potential and realized practical applications.

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‡ The abbreviations used are: Ub, ubiquitin; GFP, green fluorescent protein; GFP*, SHINFEKLE-tagged green fluorescent protein; NP, influenza nucleoprotein; polyUb, polyubiquitin; X-Ub, fusion proteins bearing COOH-terminal ubiquitin; zLLL, cbz-Leu-Leu-leucinal; HIV-1, human immunodeficiency virus type 1; TBS, Tris-buffered saline; TX-100, Triton X-100; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

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**EXPERIMENTAL PROCEDURES**

**Materials**—HeLa-Kb cells were established by transfection of Kb cDNA into HeLa cells, and stable cell clones were isolated after selection with G418. The expression of Kb molecules on the cell surface was determined by flow cytometry. Proteasome inhibitor cbz-Leu-Leu-leucinal (zLLL) was purchased from Sigma. Ub aldehyde was purchased from Affiniti (Manhead, UK), polyclonal antibody against GFP was purchased from Clontech, and anti-poly-Ub mouse monoclonal antibody FK2 was purchased from Nippon Bio-Test Laboratories. Antibodies to a synthetic peptide corresponding to influenza virus nucleoprotein (NP) 428–441 (anti-NP$_{428-441}$) and 488–498 (anti-NP$_{498}$) were produced in rabbits and affinity-purified against the peptide coupled to Sepharose. The 25-D1.16 antibody was coupled to Cy5 (Amersham Biosciences) according to the manufacturer’s instructions. Recombinant vaccinia viruses encoding NP fusion proteins have been described previously (30).

**DNA Constructs—**GFPS was produced using PCR by incorporating a sequence encoding LEQLESINKFLKTLWTS corresponding to chicken ovalbumin residues 252–269 preceding the stop codon of GFP. Ub-GFP was first constructed by inserting Ub lacking a stop codon into ApaI and BamHI sites of pEGFP-N1 (Clontech). Unchloral fusion protein Ub$^{76}$-GFP was then created by altering Gly ty 69 of Ub to Val with the use of enhanced chemiluminescence (Pierce). To detect Ub, Ub-GFPS fragment was directionally ligated to the 3′-terminus of GFPS, PCR-amplified Ub or G76V mutant was ligated into pCDNE3.1 (Invitrogen). To introduce an in-frame Ub fusion into the COOH terminus of GFPS, PCR-amplified Ub or G76V mutant was ligated into pcDNA3.1 digested with ApaI and NotI, generating GFPS–Ub or GFPS–Ub$^{76}$ (Fig. 1B). In the case of NP fusion proteins, a NP of Ub-GFPS was used, and the stop codon was first amplified and cloned into pcDNA3, and then Ub-GFPS fragment was directionally ligated to the 3′ end of NP (Fig. 7A). To introduce the K48R mutation into Ub, site-directed mutagenesis was carried out using the QuikChange site-directed mutagensesis kit (Stratagene, La Jolla, CA). The construct was changed to Ub$^{76}$-GFP by replacing GFP fragment with GFPS. Both GFPS and Ub$^{76}$-GFPS were cloned into pcDNA3.1 (Invitrogen). To introduce an in-frame Ub fusion into the COOH terminus of GFPS, PCR-amplified Ub or G76V mutant was ligated into pcDNA3.1 digested with ApaI and NotI, generating GFPS–Ub or GFPS–Ub$^{76}$ (Fig. 1B). In the case of NP fusion proteins, a NP of Ub-GFPS was used, and the stop codon was first amplified and cloned into pcDNA3, and then Ub-GFPS fragment was directionally ligated to the 3′ end of NP (Fig. 7A).

**Cell Culture, Transfection, and Flow Cytometry**—HeLa-Kb cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Recombinant vaccinia viruses encoding NP fusion proteins have been described previously (30).

**RESULTS**

**Ub in the Middle Position of Fusion Proteins (X-Ub-Y) Acts as a Degradation Signal**—To investigate the properties of fusion proteins bearing Ub at different positions, we designed a series of plasmids encoding an antigenic peptide-tagged GFP fusion protein (GFPS) fused with wild-type or modified Ub at the NH$_2$ or COOH termini or Ub fusions sandwiched between NP and GFPS (Fig. 1A). The Ub located at the NH$_2$ terminus or in the middle position was rendered insensitive to Ub hydrolases by mutating Gly$^{76}$ to Val. To gain a measure of proteasome-mediated protein degradation in mammalian cells, we tagged GFPS with a peptide (SIINFEKL) that forms a complex with a mouse class I molecule (Kb). The Kb-SIINFEKL complex can be quantitated on the cell surface using Cy5-conjugated 25-D1.16 monoclonal antibody (32).

HeLa cells expressing Kb molecules (HeLa-Kb) were transiently transfected for 36 h with the various plasmids, and the amounts of GFPS and Kb-SIINFEKL complexes were determined by flow cytometry. We validated the system by examining the behavior of the Ub$^{76}$-GFPS fusion protein. As expected, cells expressing this protein exhibited weak GFP fluorescence and high Kb-Ova257–264 complex expression (Fig. 1B), effects that were reversed by treating cells for the last 12 h after transfection with the proteasome inhibitor zLLL or by mutating Lys$^{48}$ to Arg.

These observations were consistent with Western blots of the corresponding whole cell lysates using anti-GFP polyclonal antibodies (Fig. 2A, lanes 4 and 5). Little Ub$^{76}$-GFPS was detected in the absence of proteasome inhibitors, which rescued a species of the expected molecular weight as well as two minor species. The more abundant species co-migrates with GFPS and is probably formed by Ub hydrolases incompletely inhibited by Gly$^{76}$ to Val substitution. The less abundant species is detected in all Western blots from proteasome inhibitor-treated cells that express GFP in all contexts, ruling out downstream initiation. Rather it almost certainly represents a proteasome-degraded proteolytic fragment produced by a zLLL-resistant protease. We did not detect obvious polyUb species of Ub$^{76}$-GFPS after proteasome inhibition. We cannot distinguish whether this is due to the rapid deubiquitylation by deubiquitylation enzymes or to the poor reactivity of the antibodies with ubiquitylated species of Ub$^{76}$-GFPS.

To determine whether Ub$^{76}$-GFPS is ubiquitylated, we turned to *in vitro* translation, using rabbit reticulocyte lysate with $^{35}$S Met to identify translation products. Radiolabeled translation products were analyzed by SDS-PAGE and visualized by fluorography. As shown in Fig. 2B, noncleavable, NH$_2$-terminal Ub efficiently initiates polyUb chain formation in Ub$^{76}$-GFPS, resulting in a clear Mr, 8,000 ladder of higher molecular weight bands.
molecular weight species pathognomonic of ubiquitylation (lane 5) that is not seen when GFP S is translated (lane 4). Substituting Arg for Lys48 blocked ubiquitylation, demonstrating that the Lys 48 of NH2-terminal Ub is the site of ubiquitylation (Fig. 2B, lane 6).

The NP-UbY76–GFP Ub sandwich chimera is also degraded by proteasomes (Fig. 2A, lanes 8 and 9), resulting in low GFP fluorescence and high generation of Kβ-SIINFEKL complexes (Fig. 1B) that are blocked by zLLL. Targeting of the protein for degradation requires polyUb chain formation initiated from Lys48 of Ub because Lys48Arg substitution inhibits degradation in cells (Fig. 2A, lanes 10 and 11) and polyubiquitylation after cell free translation (Fig. 2B, lanes 11 and 12). This demonstrates that the ubiquitin fusion degradation pathway is not limited to proteins expressing NH2-terminal Ub but encompasses proteins with internally situated Ub, even when located 498 residues from the NH2 terminus. Furthermore, it shows that the presence of substantial protein in the form of NP at the NH2 terminus does not prevent ubiquitylation of Ub Lys48.

Fusion Proteins with COOH-terminal Ub Are Metabolically Stable—What is the effect of placing Ub at the COOH terminus of GFP? Wild-type Ub had only a minor destabilizing effect on GFP8 as measured by Kβ-Ova257–264 complex expression or zLLL-induced enhanced fluorescence (Fig. 1B). Modification of Ub residues Gly76 or Lys48 had only minor effects on the fluorescence of the fusion protein or the generation of Kβ-SIINFEKL complexes. Consistent with these findings, zLLL exerted similar minor effects on fusion proteins with wild-type versus mutated Ub. These results demonstrate that COOH-terminal Ub does not act as a degradation signal when fused to GFP8.

It is interesting that altering Lys48 did not affect the stability of GFP8–Ub. One explanation is inefficient polyUb chain formation initiated at the Lys48 of the COOH-terminal Ub. In vitro translation revealed that this was the case (Fig. 2B, lane 7) because we failed to detect ladder formation. Although we do detect a species of lower mobility, we believe that this represents di-ubiquitylation occurring in GFP8 because (i) it is not affected by modification of Lys48 or Gly76 (Fig. 2B, lanes 8–10), and (ii) a band with a similar mobility shift appears after translation of GFP8 (Fig. 2B, lane 4). As expected, in vitro translation of His6- or HA epitope-tagged Ub resulted in labeling of the protein itself and a heterogeneous smear that represents addition of the labeled Ub to proteins in the rabbit reticulate lysate mixture (Fig. 2B, lanes 2 and 3). Inspection of the top of lane 7 of Fig. 2B also reveals the presence of high molecular weight material. This is not affected by substituting Val for Gly76 (lanes 9 and 10). The latter finding indicates that this material represents the posttranslational addition of GFP8–Ub to heterogeneous proteins in the rabbit reticulate lysate mixture. Therefore, this X-Ub fusion protein maintains its conjugation capability if the Gly76 residue is available at the COOH terminus.

Formation of X-Ub Conjugates in Mammalian Cells: Biochemical Evidence—We have found that most Ub conjugates generated by cells are Triton X-100 (TX-100)-insoluble after
incubation of cells with proteasome inhibitors. We used this observation to examine GFP-Ub conjugate formation in HeLa-Kb cells using GFP-specific polyclonal antibodies in Western blots of TX-100 fractions (Fig. 3A). In cells expressing GFP-Ub, we detected a species migrating with the molecular weight expected for the fusion protein (designated Ub fusion). We also detected antibody-reactive species migrating with a much higher molecular weight that represents GFP-Ub-conjugated substrates. A number of findings indicate that GFP-Ub is conjugated to proteins in a dynamic manner similar to wild-type Ub. First, the amount of high molecular weight material increases when cells are incubated with zLLL, and, concomitantly, the level of unconjugated GFP-Ub decreases. This is exactly as reported for free Ub after exposure of cells to proteasome inhibitors (33). Second, the high molecular weight forms of GFP-Ub were exclusively recovered from TX-100-insoluble fractions; although most free GFP-Ub fusion proteins remain TX-100-soluble. Third, substituting Val for Gly76 results in the loss of the high molecular weight material (Fig. 3B), whereas substituting of Lys48 with Arg does not significantly affect recovery of high molecular weight material. This indicates that GFP-Ub is added to proteins via Gly76. It further suggests, in conjunction with the cell free translation data, that Lys48 in GFP-Ub may not efficiently promote chain elongation, implying that GFP-Ub addition may frequently cause chain termination.

**Formation of X-Ub Conjugates in Mammalian Cells: Cytotoxic Evidence**—We next determined the intracellular distribution of GFP-Ub conjugates by laser scanning confocal microscopy in cells incubated with or without zLLL (Fig. 4). GFP (depicted in green) was detected by direct fluorescence, and Ub conjugates (depicted in red) were detected by indirect immunofluorescence staining using the FK2 monoclonal antibody, which binds ubiquitylated proteins but not free Ub (34). We determined the solubility of Ub conjugates in TX-100 by treating cells with TX-100 before fixation. The behavior of GFP-Ub was not affected by substitution of Lys48 with Arg but was greatly altered by substitution of Gly76 with Val. GFP-UB76 did not relocalize to the cytoplasm upon zLLL treatment but remained largely in the cytoplasm.

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nucleus. Under these conditions, FK2 staining was dissociated from GFPS-Ub, demonstrating that the co-incident staining of FK2 with GFPS-Ub Gly76 is not based on binding of the monoclonal antibody to the fusion protein itself. Notably, substituting Gly76 with Val resulted in the loss of 90% of green fluorescence when cells were treated with TX-100 before fixation. These findings confirm the Western blot results, demonstrating that GFPS-Ub conjugates selectively resist TX-100 extraction, in a manner similar to wild-type Ub conjugates, and that this requires that Ub support conjugation via its COOH terminus but not via Lys48. Importantly, this establishes that GFPS-Ub provides a *bona fide* means for visualizing Ub conjugates in living cells.

Generalizing the Properties of X-Ub Fusion Proteins—We next sought to demonstrate the conjugation of GFPS-Ub to a specific proteasome substrate, in this case, Ub genetically fused to NP with Arg replacing NP Met1, a well-defined “N-end rule” substrate. We infected HeLa-Kb cells expressing GFPS-Ub fusion proteins with a recombinant vaccinia virus expressing Ub-(Arg)NP (30). Cells treated with or without proteasome inhibitors were extracted with SDS to solubilize Ub conjugates. TX-100 was then added to the extracts to reduce the effective SDS concentration, enabling the collection of proteins reactive with anti-GFP antibodies. After Western blotting with NP-specific antibodies, we could detect high molecular weight species (Fig. 5). Based on the following observations, we believe that these represent GFPS-Ub-NP conjugates. First, detection required the addition of proteasome inhibitors (compare lanes 3 and 4). Second, alteration of Gly76 to Val in GFPS-Ub prevented their formation. Third, these species were not detected in cells infected with a control recombinant vaccinia virus that does not express Ub-Arg-NP (data not shown). Note that the high molecular weight smears representing NP-Ub conjugates are better resolved than the equivalent GFPS-Ub conjugates (Fig. 3A). This reflects the lower percentage of acrylamide used in separating gel in Fig. 5 (10%) as compared with that in Fig. 3 (14%). GFPS-Ub conjugates demonstrate a similar mobility in 10% gels (data not shown).

Ellison *et al.* have shown that myc-Ub conjugation inhibited substrate proteolysis to some extent in *Saccharomyces cerevisiae* (25). We did not detect a significant difference for the half-life of (Arg)NP in HeLa-Kb cells expressing either GFPS-Ub or GFP* (data not shown). However, we cannot distinguish whether GFPS-Ub supports (Arg)NP degradation, or whether remodeling of Ub by deubiquitylation enzymes occurs with sufficient rapidity to mask the potential inhibitory effects of GFPS-Ub.

X-Ub Fusion Proteins Liberated by Ub Hydrolases Retain Conjugation Function—If X-Ub fusion proteins are generally functional *in vivo*, this could affect the Ub protein reference technique in which internally situated Ubs are used as a means of generating co-translationally cleaved fragments of fusion proteins. Levels of NH₂-terminal fragments as determined by SDS-PAGE are used as an internal reference (Ref) for translation rates of COOH-terminal products (37, 38). The accuracy of this method could be compromised if Ref is attached to substrates via its COOH-terminal Ub.

We examined this question by generating a plasmid encoding a protein consisting of Ub sandwiched between NH₂-terminal NP and COOH-terminal GFP (Fig. 6A). HeLa-Kb cells trans-
fected with the plasmid were incubated in the presence or absence of zLLL, and whole cell extracts and TX-100-soluble and -insoluble fractions were probed in immunoblots using antibodies specific for NP (Fig. 6B, top panel) or GFP (Fig. 6B, bottom panel). GFP was exclusively detected in material migrating with the expected molecular weight, demonstrating that NP-Ub is released from the fusion protein with near 100% efficiency. In the presence of zLLL, the amount of NP-Ub migrating with the predicted molecular weight decreased concomitant with a large increase in the amount of high molecular weight material representing NP-Ub conjugates. Virtually all of the conjugates are TX-100-insoluble (NP is also largely insoluble, which we previously attributed to its nuclear localization (39)). These findings demonstrate that X-Ub proteins liberated by Ub hydrolases behave like X-Ub proteins directly synthesized by ribosomes. To the extent that these proteins are conjugated to substrates, this will result in an underestimation of their levels and compromise the Ub protein reference method.

Notably, as with GFP S-Ub, the formation of NP-Ub conjugates was not significantly affected by altering Lys 48, demonstrating that the high molecular weight material represents addition of NP-Ub via Gly 76.

**X-Ub Maintains Dual Functionality**—The behavior of GFP S-Ub in HeLa-Kb cells already indicates that the NH2-terminal fusion partner (GFP S) not only remains stable but also remains in a folded conformation, as inferred from GFP fluorescence. To further test the functionality of fusion partner X in X-Ub fusion proteins, we investigated the properties of HIV-1 Gag bearing wild-type (Gag-Ub) or modified Ub (Gag-UbA76) at the COOH terminus (Fig. 7A). It has been shown that Ub lacking Gly 76 fused to the COOH terminus of Rous sarcoma virus Gag suffices to assist the assembly function of Rous sarcoma virus Gag (19). Given the ability of X-Ub fusion proteins to attach to heterogeneous target proteins, it is interesting to demonstrate whether Gag-Ub conjugation will interfere with Gag-mediated virus budding. 293T cells were transfected with HIV-1 NL4-3 proviral DNA encoding hybrid Gag-Ub fusions or wild-type Gag as a control. Lysates of released virions isolated by centrifugation onto a sucrose cushion were analyzed by Western blotting using Gag-specific antibodies directed against p24 capsid (anti-CA) or p6Gag (anti-p6) or a nonspecific antibody (anti-p9). Neither of the HIV-1 Gag-Ub-expressing plasmid constructs expresses the viral protease, yet budding of immature virus particles consist-
ing of nonprocessed Gag-Ub molecules was observed. Whereas the standard profile of viral proteins was detected for wild-type HIV-1 NL4-3 (Fig. 7B, lanes 2 and 6), a heterogeneous population of high molecular weight species was detected with Gag-specific antibodies in addition to the Mr/7H11 of 63,000 Gag-Ub fusions (lanes 3 and 7). These high molecular weight molecules most likely represent Gag-Ub covalently bound to various substrate proteins, as demonstrated by the absence of such material in virus expressing Gag-UbA76 virions (Fig. 7B, lanes 4 and 8).

Because we detect similar high molecular weight conjugates in host cell lysates (data not shown), it is possible, and indeed likely, that the conjugates were formed before viral budding. However, the efficient incorporation of Gag-Ub conjugates into released HIV-1 viruses strongly indicates that both partners of Gag-Ub fusion proteins retain their function.

**DISCUSSION**

Based on the properties of nonremovable Ub located at the NH₂ terminus of fusion proteins, it is generally believed that a nonremovable Ub moiety in a linear fusion protein provides both a recognition and attachment site for ubiquitylation and subsequent degradation by 26S proteasomes (10, 11). We extend this rule to centrally located noncleavable Ub, which we demonstrate to efficiently trigger degradation of fusion proteins by initiating polyUb chain formation at Lys48. Most importantly, we also have found a previously unrecognized loophole in this rule: a COOH-terminal Ub moiety does not mediate fusion proteins themselves for destruction by 26S proteasomes, even when blocking Ub conjugation by mutating Gly76 to Val. One possible mechanism is the inefficient polyUb chain formation from the COOH-terminal Ub. The manner by which cells determine which monoubiquitylated proteins should be further ubiquitylated remains a mystery. A short sequence at the COOH termini of endocytic proteins Eps15 and Eps15R is known to induce monoubiquitylation (42), but additional information regarding the recognition of monoubiquitylated proteins by ubiquitin ligases is lacking. This issue is complicated by an important role for deubiquitylating enzymes in regulating the level of ubiquitylation of any given substrate.

Rather than induce degradation of the NH₂-terminal domain, the COOH-terminal Ub participates in ubiquitylation and indeed behaves very much like free Ub in its dynamic addition and removal from substrates. Based on lack of effect of the Lys⁴⁸ to Arg substitution on the properties of both GFP⁵⁵-Ub and NP-Ub, it is possible that the X-Ub fusion proteins do not support further ubiquitylation via Lys⁴⁸. We also fail to observe a difference in conjugation formation when Lys 29 is substituted, suggesting that Lys 29 does not participate in chain elongation.

It is believed that binding of substrates to 19S proteasome is mediated by tetrubiquitin (41). Even if X-Ub fusion proteins do not support chain elongation, they should be incorporated into tetrubiquitin as the terminal Ub. Even if this occurs relatively infrequently, the high basal rate of degradation of polyubiquitylated proteins in HeLa cells (36, 40) should mean that X-Ub fusion proteins have ample opportunity to associate with 26S proteasomes as the terminal chain of Ub₄-conjugated substrates.

³ S.-B. Qian, unpublished results.
Our findings therefore suggest that in the process of unfolding and degrading proteins attached to the COOH terminus of Ub4, 26S proteasomes ignore NH2-terminal extensions of Ub, which is reflected in the metabolic stability of X-Ub constructs. In the case of GFPS-Ub, we can even infer that the NH2-terminal extension maintains its native structure because the protein remains fluorescent even when present in conjugates, as determined by immunofluorescence of TX-100-extracted cells (Fig. 4).

The 19S complex of 26S proteasomes mediates polyUb chain recognition and substrate unfolding (35). Pickart and co-workers (29) have shown that Ub4 interacts with the base of the 19S complex in a manner that places a critical region of the chain in close proximity to the S6/H11032 ATPase. Mutational analysis suggests that binding is mediated by Ub located in positions 1 and 3 from the substrate attachment point, making it unlikely that terminal X-Ub in Ub4 chains would interfere with association. We propose a model in which the association of Ub4-conjugated substrates with the 19S subunit aligns the complex in a fixed orientation such that 19S subunit can only unfold proteins attached to the COOH terminus of the Ub that initiates the polyUb chain (Fig. 8).

The dual functionality of X-Ub fusion proteins was further confirmed by the behavior of HIV-1 Gag-Ub. We show that there is a significant accumulation of Gag-Ub conjugates inside the released HIV-1 virus particles. The substrates attached to Gag-Ub await identification. If Gag-Ub is only conjugated to proteins in the cytosol, it is likely that many of the substrates represent cellular proteins. In this case, we presume there would be selection for proteins that do not prevent virus morphogenesis and release. Alternatively, if Ub carrier protein (E2) or Ub-protein isopeptide ligase (E3) are incorporated into virions or recruited to areas of virion release, Gag-Ub may largely be conjugated to viral proteins. Indeed there is evidence that both E2 and E3 may be recruited by Gag itself (16, 21, 23). In any event, the presence of conjugates must owe their delivery to virions based on the self assembly of Gag, providing additional evidence that domains appending to NH2-terminal Ub retain their native structure or something rather close to it.

Based on the dual functionality of X-Ub fusion proteins, our findings have a number of practical applications. First, GFP-Ub enables real-time fluorescence microscopy to visualize the dynamics of Ub redistribution in living cells under various conditions. Using these conjugates, it should be possible to perform fluorescent recovery after photobleaching and fluorescence resonance energy transfer to measure, respectively, the mobility of Ub conjugates and the interaction of Ub with targets labeled with suitable fluorescent partners. Second, it
should be possible to use Ub to target therapeutic molecules to Ub-containing aggregates. The formation of such aggregates appears to contribute to a number of pathological conditions (14, 15). It may be possible to help cells clear these aggregates by expressing hybrid proteases or molecular chaperones containing COOH-terminal Ub.

REFERENCES
1. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
2. Weissman, A. M. (2001) Nat. Rev. Mol. Cell. Biol. 2, 169–178
3. Breitschopf, K., Bengal, E., Ziv, T., Admon, A., and Ciechanover, A. (1998) EMBO J. 17, 5964–5973
4. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
5. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583
6. Pickart, C. M. (2000) Trends Biochem. Sci. 25, 44–48
7. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061
8. Bachmair, A., and Varshavsky, A. (1989) Cell 56, 1019–1032
9. Johnson, E. S., Gonda, D. K., and Varshavsky, A. (1990) Nature 346, 287–291
10. Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17456
11. Johnson, E. S., Bartel, B., Seufert, W., and Varshavsky, A. (1992) EMBO J. 11, 497–505
12. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11259–11263
13. Sokolik, C. W., and Cohen, R. E. (1992) J. Biol. Chem. 267, 1067–1071
14. Sherman, M. Y., and Goldberg, A. L. (2001) Neuron 30, 15–32
15. Kopito, R. R. (2000) Trends Cell Biol. 10, 524–530
16. VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J., and Carter, C. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7724–7729
17. Yu, H., and Kopito, R. R. (1999) J. Biol. Chem. 274, 36852–36858
18. Terrell, J., Shih, S., Dunn, R., and Hicine, L. (1998) Mol. Cell 1, 193–202
19. Patnaik, A., Chau, V., and Wills, J. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13069–13074
20. Shih, S. C., Sloper-Mould, K. E., and Hicine, L. (2000) EMBO J. 19, 187–198
21. Garaus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszaka, S. D., and Sandquist, W. I. (2001) Cell 107, 65–65
22. Roth, A. F., and Davis, N. G. (2000) J. Biol. Chem. 275, 8143–8153
23. Strack, B., Calistrri, A., Acola, M. A., Pahu, G., and Gottlinger, H. G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13063–13068
24. Hicine, L., and Riezman, H. (1996) Cell 84, 277–297
25. Ellisson, M. J., and Hochstrasser, M. (1999) J. Biol. Chem. 274, 21150–21157
26. Roth, A. F., and Davis, N. G. (1998) J. Cell Biol. 134, 661–674
27. Bears, E. P., and Callis, J. (1993) J. Biol. Chem. 268, 21645–21649
28. Schefmer, M., Hui, R., Mierst, J., Vierstra, R. D., and Howie, P. M. (1993) Cell 75, 455–465
29. Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (2002) Nature 416, 763–767
30. Townsend, A., Bastin, J., Gould, K., Brownlee, G., Andrew, M., Coupar, B., Boyle, D., Chan, S., and Smith, G. (1988) J. Exp. Med. 168, 1211–1224
31. Ott, D. E., Coren, L. V., Chertova, E. N., Gagliardi, T. D., and Schubert, U. (2000) Virology 278, 111–121
32. Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R., and Germain, R. N. (1997) Immunity 6, 715–726
33. Schubert, U., Ott, D. R., Chertova, E. N., Welker, R., Tressler, U., Princiotta, M. F., Bennink, J. R., Krausslich, H. G., and Yewdell, J. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13057–13062
34. Fujimuro, M., Sawada, H., and Yoskawa, H. (1994) FEBS Lett. 349, 173–180
35. DeMartino, G. N., and Slaughter, C. A. (1999) J. Biol. Chem. 274, 22125–22126
36. Yewdell, J. W. (2001) Trends Cell Biol. 11, 294–297
37. Levy, F., Johnson, N., Rumenapf, T., and Varshavsky, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4907–4912
38. Varshavsky, A. (2000) Methods Enzymol. 327, 578–593
39. Anton, L. C., Schubert, U., Bare, J. P., Princiotta, M. F., Wearsch, P. A., Gibbs, J., Day, P. M., Reali, C., Rechsteiner, M. C., Bennink, J. R., and Yewdell, J. W. (1999) J. Cell Biol. 146, 113–124
40. Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000) Nature 404, 770–774
41. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) EMBO J. 19, 104–110
42. Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P. P. (2002) Nature 416, 451–455
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