Epitope-tagged Ubiquitin

A NEW PROBE FOR ANALYZING UBIQUITIN FUNCTION*

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In the present work, a method based on an epitope-tagged ubiquitin derivative is described that allows for the unambiguous detection of ubiquitin-protein conjugates formed in vivo or in vitro. Expression in the yeast Saccharomyces cerevisiae of ubiquitin that has been tagged at its amino terminus with a peptide epitope results in the formation of tagged ubiquitin-protein conjugates that are detectable by immunoblotting with a monoclonal antibody that recognizes the tag. The expression of tagged ubiquitin has no adverse effect on vegetative growth and, moreover, can suppress the stress-hypersensitive phenotype of yeast lacking the polyubiquitin gene UBI4. We also show that tagged ubiquitin is correctly conjugated in vivo and in vitro to a short-lived test protein and can be covalently extended into the multimeric ubiquitin chain that is normally required for the degradation of this protein. Surprisingly, however, conjugation of tagged ubiquitin inhibits proteolysis. These and related results suggest that the amino-terminal region of ubiquitin is important in protease-substrate recognition and that the multiquitin chain is a dynamic transient structure. The potential of tagged ubiquitin for the identification and isolation of ubiquitin-protein conjugates and ubiquitin-related enzymes, and as a tool in mechanistic studies is discussed.

Ubiquitin (Ub)† is a small protein found in all eukaryotes that becomes covalently coupled to other proteins post-translationally. The enzymatic mechanisms of Ub-protein conjugation and the role of ubiquitination in protein degradation have been largely established through studies using purified components from cell-free extracts (see Rechsteiner, 1988, for review).

In recent years considerable light has been shed on the biological function of Ub from molecular genetic studies of the yeast Saccharomyces cerevisiae. To date, at least 13 yeast genes involved in Ub metabolism have been cloned and characterized. They include the genes encoding the four Ub precursors, UBI1-4 (Özkaynak et al., 1987), the Ub-activating enzyme UBA1 (McGrath et al., 1991), the seven Ub conjugating or E2 enzymes, UBC1-7 (Jentsch et al., 1990), and a Ub-protein ligase (UBR1) involved in the targeting of proteins for degradation via the N-end rule pathway (Bartel et al., 1990). Selective elimination or mutation of several of these genes has directly demonstrated the involvement of Ub in a broad spectrum of cellular activities. The polyubiquitin precursor, UBI4, and the Ub-conjugating enzymes UBC4 and UBC5, for example, are required for resistance to various forms of environmental stress (Finley et al., 1987; Seufert and Jentsch, 1990). The Ub-conjugating enzyme RAD6 (UBC2) plays a critical role in post-replicative DNA repair, meiotic recombination, and strongly influences spontaneous and DNA damage induced mutagenesis (reviewed in Friedberg, 1988). The CDC34 conjugating enzyme (UBC3) is required for cell cycle progression from G1 phase into S phase (Goebel et al., 1988). It has also been known for some time that Ub is involved in the turnover of damaged and naturally short lived proteins; the UBC4 and UBC5 enzymes are largely responsible for a sizeable fraction of Ub-dependent protein degradation in yeast (Seufert and Jentsch, 1990).

Although the covalent ligation of Ub to other proteins has been linked to a diverse range of biological phenomena, very little is known about the precise mechanistic role of Ub in many of these processes. This lack of understanding can be largely attributed to the fact that the specific protein targets of ubiquitination associated with each cellular function have not yet been identified. In the past, antibodies that recognize Ub have proven to be valuable tools for the detection and monitoring of Ub-protein conjugates (reviewed in Haas, 1988). The usefulness of any antibody, however, is limited by its specificity towards the desired target antigen. With antibodies raised against an electrophoretically pure protein, the level of antibody cross-reactivity is readily determined from the number of additional electrophoretic bands detected by immunoblotting or immunoprecipitation. While anti-Ub antibodies react with free Ub, they are also expected to react with Ub-protein conjugates which constitute a diverse and largely uncharacterized class of proteins in total cell extracts. It is therefore difficult to determine by immunological methods alone which bands from a complicated pattern of bands are conjugates or cross-contaminants without the laborious process of protein isolation and sequencing. Although preimmune controls are often sufficient for the identification of cross-contaminating species, they cannot be relied upon to distinguish between the primary antigen and secondary reactivities that are not directed against the primary immunogen, but have nonetheless been induced in immunized animals. In the present work, we demonstrate that the expression of an im-
munologically tagged derivative of Ub in yeast can be used to detect the targets of ubiquitination sensitively and unambiguously. The use of tagged Ub in the in vivo and in vitro studies presented here also helped reveal novel aspects of multoubiquitin chain dynamics on the substrate protein and its recognition by proteolytic enzymes.

**MATERIALS AND METHODS**

Expression Vectors—YPE96 was a gift from D. Ecker (University of Pennsylvania). YEp96 was derived from YEp46 (Ecker et al., 1987a) by deletion of the smaller Psul-I11a fragment. The tagged Ub expression vector YEp105 was constructed from YEp96 by inserting a synthetic DNA fragment (encoding the myc epitope) between the EcoRI and BglII sites that flank the initiating codon of the Ub gene cassette (Fig. 1). YEp96A was constructed from YEp96A by eliminating the U coding fragment between the BglII and Xhol restriction sites. The galactose inducible expression vectors pUb23M (expressing Ub-Link-β-Gal) and pUb23L (expressing Ub-Link-β-Gal) have been described previously (Bachmair et al., 1986).

The Escherichia coli mycUb expression plasmid, pMycUb, was constructed from the heat inducible Ub expression plasmid, pNMBUb (Ecker et al., 1987b) by substituting the Ndel-BglII DNA fragment of pNMBUb which contains the Met-1 codon of Ub with a synthetic Ndel-BglII DNA fragment containing a similar peptide sequence as the fragment used to create YEp105 (in this case Cys-2 was replaced by Pro-1). The oligonucleotides used to create the synthetic myc fragment were 5'-TTGGAGCAAAAGCTCATTTCTGAAGAGGACTTTGGTATGCA and 5'-GATCTGCATACCCAAGTCCTCTTCAGAAATGAGC. MycUb was purified further by a modification of a previously reported method (Wessel and Flugge, 1984). Four volumes of methanol and 1 volume of chloroform were added to pooled DEAE fractions followed by precipitation with 50% (v/v) ethanol prior to their addition to water. Phenylmethylsulfonyl fluoride was added to the cell lysate buffer to inhibit the action of endogenous Ub isopeptidases.

Purification of Ub and MycUb Expressed in E. coli—Cells were grown at 30 °C in Luria broth containing ampicillin (50 mg/ml) to an optical density of 1.0 at 550 nm followed by induction at 43 °C for 2 h. Cells were then harvested and lysed with lysozyme as previously described. In vivo pulse labeling of cellular proteins was accomplished by the addition of [3H]leucine to the cultures at the time of induction. The tagged Ub was identified by immunoprecipitation with the monoclonal antibody Myc1-9E10 anti-IgG LiK (Evan et al., 1985). Anti-myc antibody was prepared prior to use by dialysis into HAN buffer (1.5, v/v) the tissue culture supernatant derived from D. Ecker (University of Pennsylvania). YEp96 was a derivative of YEp46 (Ecker et al., 1987a, 1987b) by deletion of the smaller PuuII-SmaI fragment. The tagged Ub expression plasmid YEp96 was constructed from YEp96 by inserting a synthetic DNA fragment (encoding the myc epitope) between the EcoRI and BglII sites that flank the initiating codon of the Ub gene cassette (Fig. 1). YEp96A was constructed from YEp96A by eliminating the U coding fragment between the BglII and XhoI restriction sites. The galactose inducible expression vectors pUb23M (expressing Ub-Link-β-Gal) and pUb23L (expressing Ub-Link-β-Gal) have been described previously (Bachmair et al., 1986).

**Results**

Epitope-tagged Ubiquitin—The possibility that Ub could function with a short NH2-terminal peptide tag was suggested by two observations. First, the NH2-terminal methionine of Ub is exposed on the surface of the protein and is located at maximal distance from the carboxyl terminus, which serves as the site of coupling to other proteins (Vijay-Kumar et al., 1987). Although Met-1 is apparently hydrogen bonded to Lys-63 (Vijay-Kumar et al., 1987), model

ashes for 10 min each at room temperature in HAN buffer (50 mM HEPES, pH 7.0, 50 mM NaCl, 0.02% NaN), the filter was blocked for 1 h at room temperature in HAN buffer plus 20% fetal calf serum (Sigma). The filter was washed four times for 10 min each in ANT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NaN3) and placed in a solution containing the antibody Myc1-9E10 anti-IgG LiK (Evan et al., 1985). Anti-myc antibody was prepared prior to use by dialysis into HAN buffer (1.5, v/v) the tissue culture supernatant derived from D. Ecker (University of Pennsylvania). YEp96 was a derivative of YEp46 (Ecker et al., 1987a, 1987b) by deletion of the smaller PuuII-SmaI fragment. The tagged Ub expression plasmid YEp96 was constructed from YEp96 by inserting a synthetic DNA fragment (encoding the myc epitope) between the EcoRI and BglII sites that flank the initiating codon of the Ub gene cassette (Fig. 1). YEp96A was constructed from YEp96A by eliminating the U coding fragment between the BglII and XhoI restriction sites. The galactose inducible expression vectors pUb23M (expressing Ub-Link-β-Gal) and pUb23L (expressing Ub-Link-β-Gal) have been described previously (Bachmair et al., 1986).

In Vivo β-Gal Degradation Experiments—KY117 strains carrying plasmids were grown to midexponential phase (~1.0) in supplemented minimal medium containing galactose in the presence and absence of CsUo, as described above. In vivo pulse labeling of cellular proteins was accomplished by the addition of [3H]leucine to the cultures at the time of induction. The tagged Ub was identified by immunoprecipitation with the monoclonal antibody Myc1-9E10 anti-IgG LiK (Evan et al., 1985). Anti-myc antibody was prepared prior to use by dialysis into HAN buffer (1.5, v/v) the tissue culture supernatant derived from D. Ecker (University of Pennsylvania). YEp96 was a derivative of YEp46 (Ecker et al., 1987a, 1987b) by deletion of the smaller PuuII-SmaI fragment. The tagged Ub expression plasmid YEp96 was constructed from YEp96 by inserting a synthetic DNA fragment (encoding the myc epitope) between the EcoRI and BglII sites that flank the initiating codon of the Ub gene cassette (Fig. 1). YEp96A was constructed from YEp96A by eliminating the U coding fragment between the BglII and XhoI restriction sites. The galactose inducible expression vectors pUb23M (expressing Ub-Link-β-Gal) and pUb23L (expressing Ub-Link-β-Gal) have been described previously (Bachmair et al., 1986).

In Vitro β-Gal Conjugation Experiments—The conjugation assay and the reagents used for these experiments have been previously described in detail (Gonda et al., 1989). Briefly, purified Ub or MycUb (100 μg/ml final) were added to rabbit reticulocyte Fraction II containing purified [35S]Met radiolabeled Ub-M-β-Gal or Ub-L-β-Gal in the presence or absence of an ATP-regeneration system. Samples were incubated at 37 °C for 60 min, after which time aliquots were removed for electrophoresis on SDS-polyacrylamide gels and subsequent autoradiography. ATP depletion of appropriate incubated samples was accomplished by the addition of hexokinase (Sigma, 0.05 units/ml final) and d-glucose (10 mM final) followed by a further incubation for 20 min at 37 °C prior to electrophoretic analysis.

RESULTS

Construction and Expression of Tagged Ubiquitin.—The possibility that Ub could function with a short NH2-terminal peptide tag was suggested by two observations. First, the NH2-terminal methionine of Ub is exposed on the surface of the protein and is located at maximal distance from the carboxyl terminus, which serves as the site of coupling to other proteins (Vijay-Kumar et al., 1987). Although Met-1 is apparently hydrogen bonded to Lys-63 (Vijay-Kumar et al., 1987), model
building indicated that amino acids could be added without significant steric interference. Second, chemical modification studies of Ub have shown that the oxidation of Met-1 has no apparent effect on the ability of Ub to be coupled or decoupled to other proteins in vitro (Breslow et al., 1986).

The epitope tag selected for these studies was a 10-residue sequence encoding a portion of the c-myc proto-oncogene product recognized by the sensitive generally available monoclonal antibody, 9E10 (Evan et al., 1985; Munro and Pelham, 1986). Three additional residues were incorporated into the peptide: a glycine between the COOH-terminal end of the myc sequence and Met-1 of Ub, which we rationalized might minimize interference between the tag and Ub, and a Met-Cys dipeptide at the NH2-terminal side of the myc epitope, to provide an initiating codon and to facilitate purification of Ub conjugates by thiol affinity chromatography.

The approach we adopted to express tagged Ub in yeast utilizes the expression plasmid YEp96, previously constructed by Ecker et al. (1987a). This yeast-E. coli shuttle vector contains an artificially constructed yeast Ub gene cassette that has been placed under the control of the yeast copper metallothionein (CUP1) promoter. Fusion of the Ub cassette in YEp96 to the myc tag was achieved by oligonucleotide-directed mutagenesis, as shown in Fig. 1, to create the plasmid YEp105. Induction of the CUP1-driven Ub and mycUb genes with CuSO4 resulted in levels of expression 25-50 times greater than uninduced levels and an estimated 50-100 times greater than endogenously produced levels of Ub (Ecker et al., 1987a). MycUb produced under these conditions showed no obvious signs of proteolysis.

**Detection of Tagged Ub-Protein Conjugates**—MycUb-protein conjugates formed in vivo can be visualized by immunoblot analysis with the anti-myc antibody. In the experiment shown in Fig. 2, extracts from CuSO4 induced and uninduced yeast cells that carried either the Ub expression plasmid YEp96 or the mycUb expression plasmid YEp105 were electrophoresed on an SDS-polyacrylamide gel, and the proteins were transferred to a membrane and immunostained with the 9E10 antibody ("Materials and Methods"). Extracts from yeast expressing mycUb revealed a complex spectrum of mycUb-protein conjugates extending up from the position of free mycUb, whereas extracts from cells expressing only Ub showed no immunostaining.

Induction of mycUb expression with copper led to an increase in the amount of conjugates owing to an increase in the pool of free mycUb relative to the natural pool of free Ub. In uninduced cells, the levels of mycUb were ~2-5 times higher than endogenous Ub, which are apparently insufficient to quantitatively convert conjugate species to the tagged form. Evidence showing that induced levels of mycUb are sufficient to bring about nearly quantitative conversion of at least two known Ub-protein conjugates to the tagged form is presented below (Fig. 4) and elsewhere (Hochstrasser et al., 1991).

The pattern of mycUb-protein conjugates visible in immunoblots prepared from copper-induced cells was similar but more intensely stained when compared to its uninduced counterpart. One notable difference, however, was the appearance of a major 18-kDa band upon copper induction. NH2-terminal sequencing and amino acid analysis of the purified 18-kDa protein identified it as a conjugate between two mycUb moieties (di-mycUb; data not shown).

A major advantage of using the epitope-tagged Ub approach for detecting Ub-protein conjugates is the ability to discern true Ub-protein conjugates from bands that are simply cross-reactive with the antibody probe. In the case of the anti-myc immunoblot shown in Fig. 2, the presence of any given band in lanes from yeast expressing mycUb coupled with the absence of a corresponding band in lanes from yeast expressing unmodified Ub, identifies that band unambiguously as a bona fide Ub-protein conjugate. With our immunoblotting conditions, we estimate the lower limit of detection in the range of 0.01-0.1 pmol of mycUb-protein conjugate.

**MycUb Can Substitute for Normal Ub during the Stress Response**—The usefulness of mycUb for the investigation of Ub function in yeast depends on the degree to which yeast cells can tolerate conjugation of mycUb to physiologically

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**Fig. 1. Structure of the mycUb gene cassette.** MycUb was created by inserting a synthetic DNA fragment encoding a portion of the c-myc proto-oncogene between the EcoRl (R1) and BglII (B2) sites of YEp96. The peptide sequence used to generate the anti-myc monoclonal antibody is underlined. The Ub protein sequence begins at the second Met.

**Fig. 2. Immunodetection of tagged Ub conjugates.** Proteins were isolated and electrophoresed from cultures of yeast carrying either plasmid YEp96 (Ub) or YEp105 (mUb) that were grown exponentially in the absence or presence of CuSO4. Proteins were electroblotted onto polyvinylidene difluoride membrane and probed using the anti-myc antibody ("Materials and Methods").
important substrates of the Ub system. Several experiments were performed to assess the phenotypic consequences of mycUb overexpression. The growth rates of yeast cells carrying YEp96, YEp105, or a derivative of YEp96 in which the Ub gene was deleted (YEp96Δ), were compared both in the presence and absence of CuSO4, in defined minimal media. Overexpression of Ub or mycub did not affect the doubling time (2.5 h) of exponentially growing cultures. Overexpression of Ub or mycUb also had little or no effect on the ability of cells to enter or exit stationary phase under these growth conditions (data not shown).

We also tested whether mycUb expression can substitute for Ub expression in various specific Ub-dependent processes. It is known that yeast cells carrying a deletion in the stress-inducible gene UBI4, which encodes polyubiquitin, are extremely sensitive to several types of environmental stress including chronic exposure to elevated temperature and growth on amino acid analogues such as canavanine (Finley et al., 1987). If expression of mycUb could complement the stress-sensitive phenotype of ubi4 mutants, this would provide evidence that mycub could substitute for Ub in at least some Ub-dependent processes. Yeast bearing a deletion for UBI4 and carrying the plasmids YEp96Δ, YEp96, or YEp105 were first tested for survival in the presence or absence of CuSO4 on plates containing the amino acid analogue canavanine (Table I). The survival of the ubi4 deletion mutant expressing either tagged or untagged forms of plasmid-derived Ub is enhanced more than 100-fold relative to mutant cells carrying YEp96Δ (Ub deleted). The ability of the Ub or mycUb expression vectors to complement the canavanine sensitivity phenotype of the UBI4 deletion was largely independent of the presence of CuSO4, suggesting that sufficient Ub or mycub is produced even in the uninduced state to compensate for the loss of UBI4 function.

These strains were also tested for their ability to survive prolonged exposure to an elevated growth temperature of 39 °C, as previously described (Finley et al., 1987) (Table I). ubi4 mutants expressing either the tagged or untagged forms of Ub showed a greater than 100-fold increase in survival relative to the ubi4 cells carrying YEp96Δ. As in the case of growth on canavanine, this increase in viability does not require addition of CuSO4, to the growth medium. Taken together, these experiments provide strong evidence that mycub can functionally substitute for wild-type Ub in the response of yeast to these environmental stresses.

### Table I

Mycub complements the canavanine and chronic heat-sensitive phenotypes of a ubi4 mutant

| Plasmid | Percentage viability | 39°C viability |
|---------|----------------------|----------------|
|         | -Can | +Can | -Cu | +Cu | -Can | +Can | -Cu | +Cu |
| YEp96Δ (Ub deleted) | 100 | 133 | <0.02 | <0.02 | <0.1 | <0.1 |
| YEp96 (Ub) | 100 | 99 | 62 | 48 | 10 | 17 |
| YEp105 (mycUb) | 100 | 114 | 47 | 26 | 21 | 11 |

**Fig. 3.** The effect of mycUb expression on L-β-Gal degradation. Cells were pulse labeled with [35S]Met for 5 min and chased with unlabeled Met for the chase times indicated. Shown is the disappearance of L-β-Gal and the appearance of p90 by comparison with its stable M-β-Gal counterpart upon CuSO4 induction. Also shown is a ladder of bands that correspond to L-β-Gal-mycUb, conjugate species.
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tatively by an endogenous ubiquitin processing activity (Bachmair et al., 1986; Gonda et al., 1989; Bachmair and Varshavsky, 1989). Consequently, only L-β-Gal and Met-β-Gal (M-β-Gal) are detected at t = 0. In cells expressing Ub, formation of the multi-Ub chain on L-β-Gal is readily apparent at t = 0 as a “ladder” of higher molecular weight bands above the nonubiquitinated form of L-β-Gal. A ladder of comparable intensity is also evident at t = 0 in lanes from cells overproducing mycUb. In contrast, no ladder is evident in lanes containing M-β-Gal.

The L-β-GalUb, ladder is readily distinguishable from the L-β-Gal-mycUb, ladder on the basis of the decrease in electroforetic mobility that accompanies the addition of a mycUb moiety relative to its Ub counterpart (Fig. 4). The virtual absence of Ub ladder bands in the mycUb lane (Fig. 4, lane B) indicates that mycUb competes effectively for conjugate formation with the endogenous Ub synthesized from the Ub genes, UBI1–4. That a mycUb chain can be assembled onto L-β-Gal indicates that mycUb is an effective substrate for the enzymatic components involved in this pathway.

An analysis of the effect of mycUb expression on L-β-Gal turnover is complicated by the observation that two proteolytic pathways contribute to the degradation of short-lived β-Gal test proteins in vivo (Bachmair et al., 1986). The pathways apparently share the steps involved in NH₂-terminal recognition and substrate ubiquitination but differ at a proteolytic step. The predominant pathway (pathway 1) may eliminate short-lived β-Gal derivatives by a processive mechanism since no degradative intermediates are detectable. The minor pathway (pathway 2), however, gives rise to a 90-kDa degradation product that is considerably more stable than its ~120-kDa precursor. This 90-kDa product (p90) constitutes a major band in lanes from yeast expressing L-β-Gal and either Ub or mycUb but is virtually absent in lanes from yeast expressing M-β-Gal and either form of Ub (Fig. 3).

The contribution of pathways 1 and 2 to L-β-Gal degradation and the relative effect of mycUb expression on these pathways can be determined by monitoring the amounts of L-β-Gal and p90 as a function of time (Fig. 5). In cells expressing Ub, ~87% of L-β-Gal is degraded within the first hour. By comparison, there is little loss of M-β-Gal over the same period of time. The relative contribution of pathway 1 to L-β-Gal degradation can be estimated by combining the counts present in the L-β-Gal and p90 bands at each time point (L-β-Gal + p90). In cells expressing Ub, the rate of degradation of L-β-Gal + p90 when compared to L-β-Gal alone indicates that under these conditions, pathway 1 is the predominant route of destruction, as previously observed (Bachmair et al., 1986; Gonda et al., 1989).

The expression of mycUb strongly inhibits L-β-Gal turnover with little or no effect on the appearance of p90 (Fig. 5). The similarity in the degradation curves showing the sum of L-β-Gal and p90 (L-β-Gal + p90) versus M-β-Gal indicates that virtually all L-β-Gal loss in this case can be accounted for by its conversion to p90 by pathway 2. Thus, mycUb expression strongly inhibits degradation of L-β-Gal by the normally predominating pathway 1 but has little or no effect on pathway 2.

The inhibition of pathway 1 by mycUb is underscored by the turnover kinetics of the mycUb ladder relative to the Ub ladder (Fig. 3). After 60 min of incubation with unlabeled methionine, the multi-Ub ladder in cells expressing L-β-Gal is no longer detectable. In contrast, the intensity of the mycUb ladder changes very little over the same time period.

The Mult ubiquitin Chain Is in Rapid Equilibrium with Free Ubiquitin—Inhibition of L-β-Gal proteolysis by mycUb might be expected to produce an increase in the levels of mycUb-L-β-Gal conjugate species at the expense of free L-β-Gal, as a function of time. The finding that the levels of mycUb-L-β-Gal species do not increase during the chase in the experiment shown in Fig. 3 may be due to the removal of mycUb moieties from the growing chain by Ub-protein isopeptidases (see Rose, 1988, for review). This suggestion is borne out by a comparison of the multiply ubiquitinated L-β-Gal species formed in cells expressing mycUb in the absence or absence of CuSO₄ (Fig. 6). In the presence of CuSO₄, the level of mycUb expression is sufficient to ensure that mycUb is virtually the sole constituent of the chain. As was seen in Fig. 3, the mycUb ladder persists through the chase period. In the absence of CuSO₄, the synthesis of mycUb and endogenously supplied Ub are balanced such that either monomer has a similar probability of becoming incorporated into the growing chain. The result is a complex spectrum of ladder bands whose composition represents all permutations and combinations of mycUb and Ub that can be resolved on the gel. Interestingly, all chain species decay uniformly during the chase, including those composed entirely of mycUb, in contrast to the result obtained in Fig. 3. The most straightforward explanation for this observation is that the branched multi-mycUb chain is actually a dynamic structure in which the competing processes of ubiquitination and deubiquitination rapidly alter its composition.
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The Behavior of MycUb in Vitro Parallels Its Behavior in Vivo—Several envisioned applications involving mycUb require that mycUb be utilized effectively by the conjugation apparatus in vitro (see “Discussion”). We therefore examined whether or not mycUb could be correctly targeted to L-β-Gal in a rabbit reticulocyte cell-free extract that was conjugation-proficient but which lacked an endogenous source of ubiquitin (Fraction II, Hershko et al., 1980). Gonda et al. (1989) have shown that the addition of purified radiolabeled Ub-X-β-gal derivatives to Fraction II result in the rapid processing of these proteins to the X-β-gal form. In the presence of ATP and an exogenously added source of Ub, X-β-gal versions with a destabilizing residue at their amino termini are ubiquitinated and degraded by a mechanism that parallels the turnover of these proteins in yeast. Interestingly, the degradative pathway that gives rise to the 90-kDa intermediate, p90, is not in evidence in rabbit reticulocyte-derived extracts.

In the present work, an E. coli plasmid expressing the human version of mycUb was first constructed using a strategy previously reported by Ecker et al. (1987b, see also, “Materials and Methods”). Next, a rapid two-step procedure for the purification of mycUb was devised that yielded homogeneously pure protein (Fig. 7, “Materials and Methods”).

The selective conjugation of mycUb to L-β-Gal in Fraction II is shown in Fig. 8 (left panel). As expected, the conjugation of Ub or mycUb to either M-β-Gal or L-β-Gal does not occur in the absence of ATP. In the presence of ATP, however, both Ub and mycUb form multimeric conjugates with L-β-Gal but not with M-β-Gal. When ATP is subsequently depleted from the L-β-Gal reactions containing either Ub or mycUb, the conjugated forms of L-β-Gal disappear (Fig. 8, right panel). These results demonstrate that mycUb is selectively targeted to a metabolically unstable derivative of β-Gal in an ATP dependent manner and that these conjugate species are effectively acted on by the Ub isopeptidases in the absence of ATP. Moreover, examination of the ATP dependent turnover rates of L-β-Gal in Fraction II reveal that the degradation of L-β-Gal is inhibited 3-4-fold in the presence of mycUb when compared with reactions containing Ub (results not shown). Taken together with the results of Fig. 8, these experiments illustrate that the behavior of mycUb with respect to the targeting and degradation of unstable β-Gal derivatives in mammalian extracts closely parallels the behavior of mycUb observed in yeast.

DISCUSSION

A General Method for Identifying and Isolating Ubiquitin Conjugates—In the present work we describe a useful tool for detecting Ub-protein conjugates formed in yeast and in rabbit reticulocyte extracts. This strategy relies upon the overexpression of an immunologically tagged derivative of Ub which when coupled to other proteins can be detected on immunoblots of protein gels when an anti-tag antibody is used as the probe. The major advantage offered by this approach over conventional strategies that use antibodies raised against Ub (or some portion of its sequence) is that it is both sensitive and unambiguous. Protein gel bands are assigned as conjugates not only on the basis of immunostaining, but also by their absence or altered mobility in corresponding gel lanes from yeast expressing the untagged form of Ub, a comparison which permits the discrimination between true conjugate species and cross-reactive species. Recently, the tagged Ub approach has been used successfully to show that the yeast α2 mating type repressor is degraded in a Ub-dependent manner in vivo (Hochstrasser et al., 1991).

An immediate potential application of the tagged Ub approach is in the assignment of specific Ub conjugates to each of the Ub-conjugating enzymes and ligases characterized in yeast to date. This could be accomplished simply by comparing the electrophoretic pattern of tagged Ub species obtained from wild-type yeast with currently existing yeast strains carrying deletions or mutations in each of the UBC or UBR genes (Jentsch et al., 1990; Bartel et al., 1990).

The presence of the tag on Ub-conjugated proteins should facilitate their affinity purification using the anti-tag antibody. The inclusion of the cysteine residue at position 2 of the appended tag sequence will ultimately be useful as a first step in the purification of these conjugate species by thiol affinity chromatography and could also be useful for the attachment of radioactive or fluorogenic chemicals.

Tagged Ub may also be useful for the rapid identification

FIG. 6. Assembly of transient multi-Ub/mycUb chains on L-β-Gal. Yeast cells co-expressing mycUb and L-β-Gal were pulsed with [35S]methionine and chased with unlabeled methionine for the times indicated as described in the legend to Fig. 3. Left three lanes: protein derived from yeast induced for mycUb expression by the addition of CuSO4. Right three lanes: protein derived from yeast cultures from which CuSO4 was omitted. Relevant protein bands are identified as described in the legends to Fig. 3.

FIG. 7. SDS-polyacrylamide gel electrophoresis of bacterially expressed Ub and mycUb purified as described under “Materials and Methods”. The gel was stained with Coomassie Blue.

![Diagram of SDS-polyacrylamide gel electrophoresis of ubiquitin conjugates](image-url)
Radiolabeled Ub-M-P-Gal were added to Fractioning absence the disappearance of L-β-Gal-Ub or L-β-Gal-mycUb, species when extracts supplemented with ATP (left two lanes) are depleted of ATP (right two lanes). Following electrophoresis, radiolabeled species were visualized by autoradiography.

and purification of Ub-dependent enzymes that become coupled to Ub covalently. The Ub-activating enzyme (E1), and the Ub-conjugating enzymes (E2s) are known to form thiol esters with Ub at their active site cysteines (Pickart, 1988). In addition, the Ub isopeptidases can be trapped as stable intermediates with Ub by treatment with sodium borohydride (Rose, 1988). Using tagged Ub in such reactions may therefore provide an effective and convenient way of identifying other Ub-related proteins in addition to Ub-protein conjugates.

A number of observations reported here and elsewhere make it likely that the tagged Ub approach can be applied to the identification of conjugates in higher organisms as well. There is a high degree of mechanistic and structural conservation displayed between the Ub systems of yeast and mammals. Yeast Ub, for example, shares 96% identity with its counterpart in plants and animals (see Wilkinson, 1988, for review). A high degree of similarity has also been found to exist for the yeast Ub-conjugating enzyme UBC2 (RAD6) and Ub-conjugating enzymes isolated from Schizosaccharomyces pombe, wheat germ, and humans (Reynolds et al., 1990; Sullivan and Vierstra, 1989; Schneider et al., 1990). In addition, it has recently been shown that the turnover of metabolically unstable test proteins by the Ub-dependent "NH₂-end rule pathway" is mechanistically similar in yeast and rabbit reticulocytes (Gonda et al., 1989). Based on these evolutionary similarities and on our observations of the behavior of tagged Ub in yeast and mammalian cell-free extracts, the prospect of extending the tagged Ub approach into higher organisms appears promising.

Tagged Ubiquitin and Protein Turnover—In 1980 Hershko et al. proposed that Ub conjugation functioned as a signal for protein degradation. From a mechanistic standpoint, this proposal has given rise to two variations: either Ub itself is recognized directly by some component of the proteolytic apparatus or alternatively, Ub acts indirectly by changing the structure of the substrate to a proteolytically labile form (Haas and Bright, 1985). To date neither of these mechanisms has been proven.

The turnover of L-β-Gal by two independent pathways may represent an example where both mechanisms are in operation on the same substrate. In the first pathway, the inhibition of L-β-Gal turnover by mycUb illustrates that while the NH₂-terminal region of Ub is not an essential factor in Ub conjugation, it plays a critical role at the proteolytic step of pathway 1. We propose, therefore, that the amino-terminal region of Ub forms a portion of a structural element that is recognized specifically by the Ub/ATP-dependent proteolytic complex involved in this pathway. It is noted that the amino termini of Ub molecules sequestered within the multi-Ub chain constitute the most accessible portion of this structure for intermolecular interaction.

Although the expression of mycUb effectively inhibits L-β-Gal turnover via pathway 1, it has no effect on pathway 2 (the minor pathway) which accounts for the accumulation of the 30-kDa polypeptide. This result demonstrates that the turnover of L-β-Gal is indeed the result of two separable proteolytic mechanisms that are nonetheless Ub dependent. Whether or not different Ub-dependent proteolytic routes are exploited for the turnover of naturally occurring protein substrates remains to be seen. The notable absence of pathway 2 in rabbit reticulocyte cell-free extracts (Gonda et al., 1989) may either reflect a true evolutionary difference between yeast and mammals or simply the inability to recover this pathway in vitro.

There is an apparent paradox in the observation that mycUb inhibits proteolysis yet produces no adverse effects when overexpressed and even complements the stress-sensitive phenotypes of a poly-Ub gene mutant. There are two reasonable resolutions to this contradiction. It is possible that under most circumstances, the dominant effects of mycUb overexpression are "leaky." While the conversion of Ub conjugates to the tagged form appears to be virtually quantitative for L-β-Gal (Fig. 4), it may not be so for all proteins. A residual level of ubiquitination coupled with the rapid exchangeability of the tagged and untagged forms could result in less efficient protein turnover (particularly of substrates whose degradation is less dependent on Ub chain length), which is nonetheless sufficient to meet the needs of the cell. Our finding that mycUb overexpression only inhibits the degradation of the MATα2 transcriptional regulator by 2-fold (Hochstrasser et al. 1991) is consistent with this idea.

Finally, we have presented evidence (Fig. 6) that the multi-Ub chain formed on targeted substrates is a transient dynamic structure in which individual Ub molecules, regardless of their position within the chain, are rapidly exchanged with free Ub. The repeated assembly and breakdown of these chains is energetically inefficient. We believe, however, that mult ubiquitination represents a logical balance between the "proof-reading" activity of isopeptidases whose functions may include the removal of Ub from mistargeted substrates (Haas and Bright, 1987; Hough and Reichsteiner, 1988) and the components of the Ub ligase system whose function is to selectively target proteins for ubiquitination. Based upon these opposing tendencies of ubiquitination and deubiquitination, we propose that the multi-Ub chain fulfills two functions. First, the formation of such a chain on a targeted substrate should increase the period of time that the substrate exists in a ubiquitinated state, thereby increasing the likelihood of being recognized by the proteolytic component of the.
pathway. Implicit in this mechanism is that isopeptidases are constrained to removing Ub molecules from the chain one at a time from the chain terminus towards the substrate anchor. Second, the chain may serve to locally amplify the structural signal contained within Ub (in this case the amino-terminal region) that is recognized by the protease. These hypotheses are conceptually distinct although not necessarily contradictory to the recent proposal by Chau et al., (1989) who have suggested that a novel structural determinant is created by the linkage of one Ub to another, thereby creating a protease-recognition site.

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