or inactive conformation because of the nonequivalence of the H-y3 and H-y5 conformations.

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A Multiubiquitin Chain Is Confined to Specific Lysine in a Targeted Short-Lived Protein

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The ubiquitin-dependent degradation of a test protein β-galactosidase (βgal) is preceded by ubiquitination of βgal. The many (from 1 to more than 20) ubiquitin moieties attached to a molecule of βgal occur as an ordered chain of branched ubiquitin-ubiquitin conjugates in which the carboxyl-terminal Gly^{76} of one ubiquitin is joined to the internal Lys^{48} of an adjacent ubiquitin. This multiubiquitin chain is linked to one of two specific Lys residues in βgal. These same Lys residues have been identified by molecular genetic analysis as components of the amino-terminal degradation signal in βgal. The experiments with ubiquitin mutated at its Lys^{48} residue indicate that the multiubiquitin chain in a targeted protein is essential for the degradation of the protein.

UBIQUITIN, A 76-RESIDUE PROTEIN, IS PRESENT IN EUkaryotes either free or covalently joined, through its carboxyl-terminal glycine residue, to various cytoplasmic, nuclear, and integral membrane proteins (*1*). The coupling of ubiquitin to other proteins is catalyzed by a family of ubiquitin-conjugating enzymes (also called E2 enzymes) (*1, 2*). In the yeast *Saccharomyces cerevisiae*, two of the approximately six such enzymes present have been identified as products of the genes *RAD6*, whose

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functions include DNA repair, and *CDC34*, which is required for the transition from the G1 to the S phase of the cell cycle (*3*).

Biochemical studies of mammalian reticulocyte extracts (*1, 4*) and molecular genetic studies in mammals and yeast (*1, 5*) have indicated that selective protein degradation requires a preliminary step of ubiquitin conjugation to a targeted proteolytic substrate. Thus, one function of ubiquitin is to mark proteins destined for selective elimination. Another role for ubiquitin, in which its reversible joining to an acceptor protein modulates protein function without metabolically destabilizing the acceptor protein, has also been suggested (*1*). This hypothesis accounts for the existence of metabolically stable ubiquitin-protein conjugates (*1–3*), but poses the problem of how ubiquitinated proteolytic substrates are distinguished from other ubiquitinated proteins in the same intracellular compartment.

Experiments with engineered proteolytic substrates such as β-galactosidase (βgal), have shown that an important component of the degradation signal in a short-lived protein is the amino-terminal residue of the protein (*6–8*). The code or rule that relates the metabolic stability of the protein and the nature of its amino-terminal residue has been called the N-end rule (*6*), and has been found to be conserved between yeast and mammals (*7*). To produce otherwise identical βgal proteins bearing different amino-terminal residues, we used a set of constructs that encode ubiquitin-βgal fusion proteins (Ub-X-βgal). The nascent Ub-X-βgal is rapidly and precisely deubiquitinated by an endogeneous processing protease present in both yeast and mammalian cells to yield an X-βgal test protein bearing the desired residue, X, at the βgal amino terminus. Depending on the nature of X, the X-βgal proteins are long-lived or metabolically unstable, with destabilizing amino-terminal residues conferring short half-lives on the corresponding X-βgal's (*6–8*).

The degradation of short-lived X-βgal proteins is accompanied by their extensive ubiquitination both in yeast cells (*6*) and in mammalian reticulocyte extracts (*7*). At least in the reticulocyte extract, the degradation of X-βgal depends on the presence of adenosine triphosphate (ATP) and ubiquitin. Multiply ubiquitinated X-βgal proteins contain from 1 to more than 20 ubiquitin moieties per molecule of βgal. Apparent half-lives of these ubiquitinated X-βgal species were shown to be at least as short as the half-life of a parental, nonubiquitinated X-βgal (*6, 7*). These findings (*1, 4, 5*) suggest that the multiply ubiquitinated derivatives of X-βgal are obligatory intermediates in the pathway of X-βgal degradation.

Covalent conjugation of ubiquitin to other proteins involves formation of an isopeptide bond between the carboxyl-terminal Gly residue of ubiquitin and the ε-amino group of a Lys residue in an acceptor protein (*1–4*). Since an X-βgal contains 22 Lys residues in each of its four identical ~1000-residue subunits (*9*) (Fig. 1), the ubiquitin moieties of multiply ubiquitinated X-βgal could be envisioned to exist as monoubiquitin adducts distributed over the many Lys residues of X-βgal. However, when ubiquitin is conjugated to other acceptor proteins, such as lysozyme, multiubiquitinated derivatives are produced in which the number of attached ubiquitin moieties can exceed the number of Lys residues in the acceptor protein (*1, 10*). Thus, at least some multiply ubiquitinated proteins contain ubiquitin-ubiquitin linkages. Furthermore, chemical modification of Lys residues in ubiquitin does not prevent conjugation of the modified ubiquitin to lysozyme, but it does prevent formation of those multiubiquitinated lysozyme species in which the number of ubiquitin moieties exceeds the number of Lys residues in lysozyme (*11*). Neither the structure or function of putative multiubiquitin chains nor their location within acceptor proteins has yet been addressed.

We now show that all ubiquitin moieties in a targeted, short-lived X-βgal protein occur as a multiubiquitin chain in which the carboxyl-terminal Gly^{76} of one ubiquitin is joined to Lys^{48} of an adjacent ubiquitin. This multiubiquitin chain is linked to one of the two specific Lys residues in X-βgal that have been identified by molecular genetic analysis as components of the amino-terminal degradation signal in X-βgal (*8*). We also present biochemical and genetic evidence bearing on the function of ubiquitin-ubiquitin conjugates in selective protein degradation.

Multiple ubiquitination of X-βgal occurs within the first 177 residues of X-βgal. Since multiply ubiquitinated, ^{35}S-labeled X-βgal proteins can be isolated in radiochemically pure form, we used sequence-specific chemical cleavage of X-βgal to map its ubiquitin moieties. There are seven Asn-Gly sequences in X-βgal (Fig. 1). The Asn-Gly peptide bond can be selectively cleaved with hydroxylamine (*12*). Cleavage of an [^{35}S]methionine-labeled X-βgal at these seven

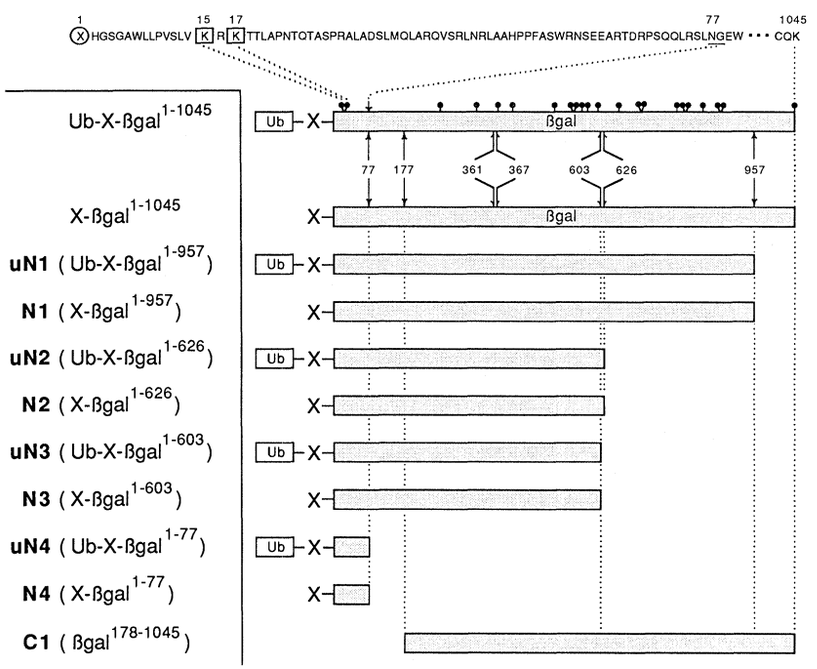

Fig. 1. Asn-Gly sequences (hydroxylamine cleavage sites) in X-βgal and Ub-X-βgal. Arrows and the corresponding numbers indicate the positions of Asn residues in the seven Asn-Gly sequences within X-βgal and Ub-X-βgal; see main text for other designations. Shown here are only those N, uN, and C fragments that are explicitly mentioned in the discussion of experimental data. Because of the design of the original βgal expression vector (*6–8, 14*), our X-βgal test proteins differ from the wild-type *E. coli* βgal (*9*) in having a 45-residue amino-terminal extension derived in part from an internal sequence of the *lac* repressor (*8*). The sequence of an amino-terminal region of X-βgal is shown at the top. The wild-type *E. coli* βgal sequence starts at position 46 of X-βgal (position 24 of *E. coli* βgal). There are 22 Lys residues in an X-βgal (indicated by black circles), with the first two lysines (Lys^{15} and Lys^{17}) located within its *lac* repressor-derived amino-terminal extension. Single letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

sequences should generate up to 35 fragments, all of which contain at least one Met residue (*9*). Most of the a priori possible fragments were actually produced because of incompleteness of hydroxylamine digests (Fig. 2, C, D, I, and J).

In our notation (Fig. 1), hydroxylamine-produced fragments of X-βgal are denoted as $\beta gal^{y\text{-}z}$ where y and z are, respectively, the numbers of the first and the last residue of the fragment. In abbreviated designations of these fragments, the letter N denotes those fragments that retained the amino-terminal residue X, and the letter C indicates those that did not. Since there are no Asn-Gly sequences in ubiquitin (*1*), hydroxylamine-produced fragments of Ub-X-βgal that retained the residue X invariably retained the amino-terminal ubiquitin moiety as well; these fragments are denoted as uN (or Ub-X-$\beta gal^{1\text{-}z}$) (Fig. 1). Of the Ub-X-βgal fragments, only those that retained the amino-terminal ubiquitin moiety (uN-type fragments) should be different from their counterparts in a hydroxylamine digest of X-βgal (Figs. 1 and 2, C and D). Thus, the comparison of electrophoretic mobilities of βgal fragments from hydroxylamine digests of X-βgal and Ub-X-βgal allows identification of the N-type X-βgal fragments. Of the seven N-type fragments expected, at least four could be identified (Figs. 1 and 2, C, D, I, and J). An independent confirmation of these assignments was obtained by immunoblotting with antibody to ubiquitin (*6, 13*) to show that the Ub-X-βgal–derived counterparts of the N-type X-βgal fragments did contain their expected ubiquitin moieties. These one-dimensional electrophoretic comparisons also identified the second largest C-type fragment, $\beta gal^{178\text{–}1045}$ (C1 in Fig. 1), which lacks the amino terminus of X-βgal and is therefore present in hydroxylamine digests of both X-βgal and Ub-X-βgal (Figs. 1 and 2, C and D). The largest C-type fragment, $\beta gal^{78\text{–}1045}$, was not detected, apparently because of a relatively infrequent cleavage at the corresponding Asn-Gly sequence. That this cleavage by hydroxylamine could occur was indicated by the presence of the uN4 fragment (produced by the same cleavage) in the more extensive digests of Ub-Pro-βgal (Fig. 2, I and K). Since there are no Lys residues between positions 78 and 178 of X-βgal, our conclusions do not depend on either the presence or assignment of the $\beta gal^{78\text{–}1045}$ fragment.

We then examined the arrangement of ubiquitin moieties within multiply ubiquitinated X-βgal. Purified, [^{35}S]methionine-labeled Ub-Gln-βgal (*14*) was incubated in ATP-supplemented reticulocyte extract, yielding, initially, Gln-βgal (*6, 7*). In both yeast and mammalian cells, the amino-terminal Gln (a tertiary destabilizing residue) is rapidly and selectively deamidated to yield Glu, a secondary destabilizing residue; this step is followed by the conjugation of a primary destabilizing residue, Arg, to the new, Glu-bearing amino terminus (*7*). The resulting short-lived Arg-Glu-βgal protein (denoted as Arg-βgal) was isolated (together with its multiply ubiquitinated derivatives) from ATP-supplemented reticulocyte extract, and separated by first-dimension SDS–polyacrylamide gel electrophoresis (PAGE) (*14*). The Arg-βgal and its separated ubiquitin-containing derivatives were then treated with hydroxylamine in the gel, and analyzed by second-dimension SDS-PAGE (Fig. 2E).

Three classes of fragments could be distinguished. (i) The relatively mild digestion with hydroxylamine (*15*) left a significant proportion of Arg-βgal and its ubiquitin-containing derivatives uncleaved; these species produced an upper diagonal set of spots. (ii) The second set of spots corresponded to the previously identified N-type βgal fragments, N1, N2, and N3. (iii) The third set of spots corresponded to the previously identified C1 fragment ($\beta gal^{178\text{–}1045}$) which, unlike the N-type fragments, lacks the amino-terminal region

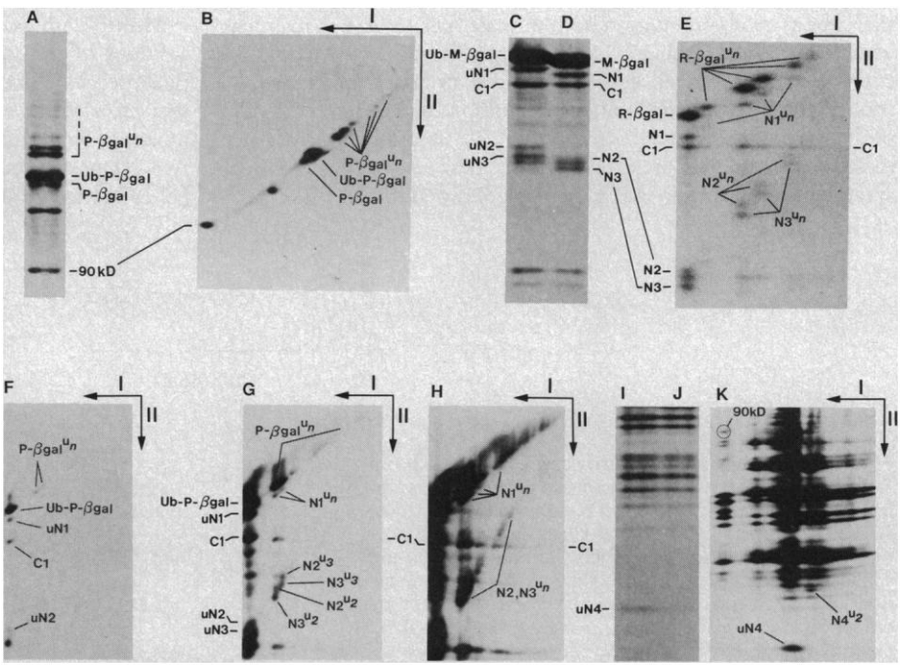

Fig. 2. Two-dimensional electrophoretic mapping of hydroxylamine-produced fragments of X-βgal, Ub-X-βgal, and their multiply ubiquitinated derivatives. (**A**) Ub-Pro-βgal, expressed in *S. cerevisiae* and labeled with [^{35}S]methionine (*14*). The two bands beneath the band of Pro-βgal are those of metabolically stable, apparently in vivo–produced (*6*), cleavage products of short-lived X-βgal proteins (the smaller, about 90 kD, is indicated by an arrow). (**B**) The same set of separated proteins as in (A) was subjected to a second-dimension electrophoresis in a 6 percent SDS-polyacrylamide gel. (**C** and **D**) Comparison by SDS-PAGE of hydroxylamine-treated Ub-Met-βgal (C) and Met-βgal (D) (*14, 15*). (**E**) Two-dimensional mapping of hydroxylamine-treated Arg-Glu-βgal and its ubiquitinated derivatives. The ^{35}S-labeled Ub-Gln-βgal was incubated in ATP-supplemented reticulocyte extract, yielding Arg-Glu-βgal (denoted as Arg-βgal). The Arg-βgal, together with its ubiquitinated derivatives, was isolated by immunoprecipitation (*14*), followed by first-dimension electrophoresis in a 6 percent SDS-polyacrylamide gel, treatment with hydroxylamine in the gel, and second-dimension SDS-PAGE in a 6 percent gel. (**F**) Two-dimensional mapping of hydroxylamine-treated Pro-βgal and its ubiquitinated derivatives. Ub-Pro-βgal and Pro-βgal^{u_n} were isolated from yeast cells as in (A), and subjected to the two-dimensional SDS-PAGE, with hydroxylamine treatment between the first- and second-dimension fractionations as in (E). (**G** and **H**) Same as (F) but longer fluorographic exposures (2 and 10 days, respectively). (**I** and **J**) Same as (C) and (D), respectively, except that hydroxylamine treatment (*15*) was extended to 8 hours and SDS-PAGE was carried out in a 15 percent gel to retain and resolve smaller hydroxylamine-produced fragments of βgal. (**K**) Same as (F) to (H) but hydroxylamine treatment (*15*) was extended to 8 hours, and the second-dimension electrophoresis was carried out in a 15 percent SDS-polyacrylamide gel. A spot corresponding to uN4 is present, as expected (I), among the fragments of Ub-Pro-βgal but is not found among the fragments derived from multiply (posttranslationally) ubiquitinated derivatives of Pro-βgal. However, a distinct species is present among the hydroxylamine-produced fragments derived from Pro-βgal^{u_2} that is absent from the Ub-Pro-βgal–derived fragments; its apparent molecular mass is consistent with it being $N4^{u_2}$. As is also characteristic of the other (larger) N-type βgal fragments (E, G, and H), the spot corresponding to $N4^{u_2}$ is absent from the fragments of more heavily ubiquitinated Pro-βgal^{u_n} species, implying that the size of N4 is a function of the number of ubiquitin moieties in the parental Pro-βgal^{u_n}. P-, M-, and R-βgal denote, respectively, Pro-, Met-, and Arg-βgal.

of Arg-βgal. Strikingly, the C1 fragments produced from either Arg-βgal or Arg-βgal^{u_n} formed a horizontal (rather than a diagonal) streak of spots (Fig. 2E). Thus, the size of the C1 fragments was independent of n (the number of ubiquitin moieties in an Arg-βgal^{u_n}), in contrast to the dependence on *n* that was characteristic of the N-type fragments. The C1 fragment (βgal$^{178-1045}$) encompasses all but the first 177 residues of an X-βgal (Fig. 1). This, and the fact that the size of C1 fragment was independent of the number of ubiquitin moieties in an Arg-βgal^{u_n}, directly located these ubiquitin moieties within the first 177 residues of Arg-βgal.

The same conclusions were reached from the analogous two-dimensional mapping of hydroxylamine-produced fragments of Ub-Pro-βgal and its multiply ubiquitinated derivatives isolated from *S. cerevisiae*. In particular, the Pro-βgal^{u_n} species, separated and treated with hydroxylamine between the first- and second-dimension electrophoresis (Fig. 2, F to H and K), yielded the sets of N1, N2, and N3 fragments and their ubiquitinated counterparts that had already been observed with Arg-βgal^{u_n} (Fig. 2E). A horizontal streak of spots corresponding to the C1 fragment was also observed (Fig. 2, G and H) (*16*).

Previous evidence indicated that the ε-amino groups of Lys residues, and possibly also the amino-terminal α-amino groups, are the only functional groups in proteins that can be conjugated to ubiquitin either in vivo or in cell-free systems (*1–4*). This constraint, and our finding that all ubiquitin moieties in a multiply ubiquitinated X-βgal are attached within its first 177 residues, limited the number of possible ubiquitin attachment sites within X-βgal to three—Lys^{15}, Lys^{17}, and the amino-terminal α-amino group. Since the number of ubiquitin moieties within an X-βgal^{u_n} can greatly exceed three, our results also indicated that the multiple ubiquitin moieties of an X-βgal^{u_n} must be organized into a chain or chains of ubiquitin-ubiquitin conjugates.

Attachment of a multiubiquitin chain to either Lys^{15} or Lys^{17} of X-βgal. Possible arrangements of a multiubiquitin chain within X-βgal not precluded by the above data are the amino-terminal location of a multiubiquitin chain, with its last ubiquitin moiety joined to the amino-terminal α-amino group of X-βgal (Fig. 3B), and the arrangement whereby a multiubiquitin chain is attached to one of the two closely spaced lysine residues, Lys^{15} or Lys^{17} (Fig. 3A), the only Lys residues within the first 177 residues of X-βgal. These models are not mutually exclusive (Fig. 3C). A fourth possibility of two multiubiquitin chains being attached to both Lys^{15} and Lys^{17} is not considered explicitly for reasons given below.

To decide among these models, we used site-directed mutagenesis to construct a Ub-Gln-βgal variant, Ub-Gln-(Xa)-βgal, which contained a four-residue sequence recognized by factor Xa, one of proteases in the blood clotting cascade (*17, 18*). A seven-residue insertion that contains the four-residue factor Xa cleavage site was positioned between the Gln residue of Ub-Gln-βgal and the first Lys residue (Lys^{15}) of Gln-βgal (*18*). Cleavage at the factor Xa site has different consequences in each of the three models for the retention of a multiubiquitin chain within X-βgal.

The purified, [^{35}S]methionine-labeled Ub-Gln-(Xa)-βgal was incubated in ATP-supplemented reticulocyte extract. This incubation converted Ub-Gln-(Xa)-βgal into a short-lived Arg-Glu-(Xa)-βgal, which, together with its ubiquitin-containing derivatives, was isolated from reticulocyte extract, digested with factor Xa (*18*), and analyzed by SDS-PAGE (Fig. 4A). Cleavage by factor Xa should remove ten residues from the amino terminus of Arg-Glu-(Xa)-βgal, resulting in an approximately 1.1-kD decrease in the molecular mass of the approximately 116-kD Arg-Glu-(Xa)-βgal protein (*18*) (Fig. 1). This change was detectable as a small but distinct and reproducible increase in electrophoretic mobility that accompanied the factor Xa-mediated conversion of Arg-Glu-(Xa)-βgal into the ten-residue shorter βgal$^{3-1045}$ (Fig. 4A, lanes e to h). These data, while confirming that the cleavage by factor Xa did occur, did not in themselves bear on the specific models of Fig. 3; these are addressed in Fig. 4B. As has been seen with the initial, nonubiquitinated Arg-Glu-(Xa)-βgal, digestion with factor Xa resulted in small but detectable increases in electrophoretic mobility of ubiquitin-containing Arg-Glu-(Xa)-βgal species (Fig. 4B).

Independent evidence that factor Xa did cleave both the Arg-Glu-(Xa)-βgal and its multiply ubiquitinated derivatives was obtained by incubating purified, unlabeled Ub-Gln-(Xa)-βgal in ATP-supplemented reticulocyte extract in the presence of [^{3}H]arginine (*14*). The labeled product, Arg-Glu-(Xa)-βgal, was isolated, treated with

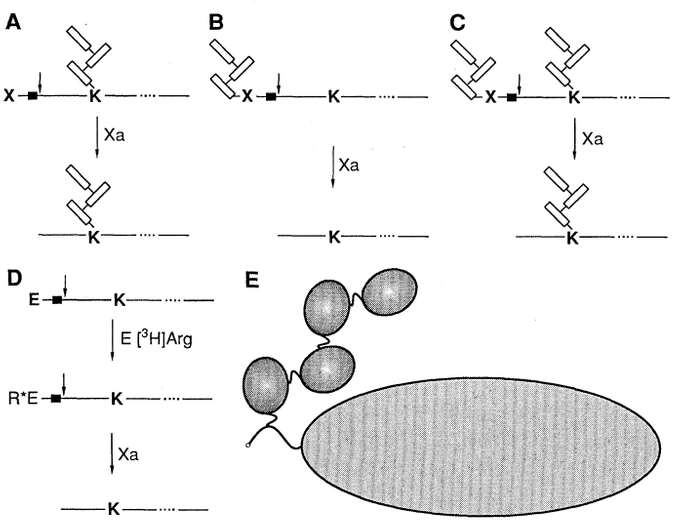

Fig. 3. Distinguishing between amino-terminal and amino terminus-proximal locations of ubiquitin moieties in multiply ubiquitinated X-βgal. A Ub-Gln-βgal variant, Ub-Gln-(Xa)-βgal, containing a cleavage site for the factor Xa protease (*17*) between the Gln residue and the first Lys (K) residue of Gln-βgal (the Lys^{15} residue) (Fig. 1), was constructed (*18*). The four-residue Xa cleavage site (*17*) is indicated by a black rectangle. (**A** to **C**) Possible locations of a multiubiquitin chain within the amino-terminal region of X-βgal, and the predicted outcomes of cleavage at the factor Xa site. Open rectangles denote the ubiquitin moieties. K is either Lys^{15} or Lys^{17} of an X-βgal. Common to all three models is the constraint (Figs. 1 and 2) that ubiquitin moieties are present as a multiubiquitin chain whose attachment point or points in an X-βgal must lie within the first 177 residues of the 1045-residue subunit of X-βgal and therefore must be either at its amino terminus (A) or at one of its two Lys residues in this region, Lys^{15} or Lys^{17} (B). The models in (A) and (B) are not mutually exclusive (C). (**D**) Use of Arg-Glu-(Xa)-βgal to map the location of an enzymatically conjugated Arg residue. In the first step of the scheme shown, [^{3}H]Arg is conjugated to unlabeled Glu-(Xa)-βgal [derived from Gln-(Xa)-βgal by selective deamidation in ATP-supplemented reticulocyte extract(7)]. Subsequent cleavage by factor Xa is expected to release the incorporated radioactivity if the conjugated [^{3}H]Arg is located exclusively at the amino terminus of a test protein. (**E**) A schematic, roughly to scale representation of a single 1045-residue subunit in the X-βgal homotetramer that contains an attached multiubiquitin chain. The carboxyl-terminal Gly^{76} of one ubiquitin in the chain is joined to the internal Lys^{48} of an adjacent ubiquitin (Fig. 5). The flexible carboxyl terminus of ubiquitin (the last ~4 residues) is located approximately at 90° relative to the Lys^{48} residue that is exposed on the surface of the ubiquitin molecule (*1, 27*). The multiubiquitin chain—shown here containing four ubiquitin moieties, while the actual number for X-βgal varies from 1 to more than 20 (*6, 7*)—is attached to either Lys^{15} or Lys^{17} within the amino-terminal region of X-βgal. This ~45-residue region (Fig. 1), which is absent from the wild-type *E. coli* βgal, is likely to be conformationally unstable (segmentally mobile), in contrast to the rest of the X-βgal subunit within the tetramer (*8*). The individual βgal subunits within the βgal homotetramer, whose detailed three-dimensional structure is unknown, are likely to be arranged with the D2 symmetry (*9*). An open circle at the X-βgal's amino terminus denotes a destabilizing residue X.

factor Xa, and analyzed by SDS-PAGE (Figs. 4C, lanes a to d, and 3D). The otherwise identical Ub-Gln-βgal protein, which lacked the factor Xa cleavage site, was processed under the same conditions. The products, Arg-Glu-βgal and Arg-Glu-βgal^{u_n}, were isolated and treated with factor Xa alongside the Arg-Glu-(Xa)-βgal protein (Fig. 4C, lanes a to h). While the treatment of Arg-Glu-(Xa)-βgal and its ubiquitinated derivatives with factor Xa led to the gradual disappearance of ^{3}H-labeled βgal species, the identical treatment of Arg-Glu-βgal, which lacked the factor Xa cleavage site, did not lead to detectable changes in the electrophoretic pattern (Fig. 4C, lanes e to h). These results provided independent evidence that the specific cleavage of Arg-Glu-(Xa)-βgal by factor Xa did occur. Moreover, since the [^{3}H]Arg-derived label decreased during the digestion with factor Xa not only in the band of Arg-Glu-(Xa)-βgal but also in the bands of its multiply ubiquitinated derivatives as well, we conclude that the presence of a multiubiquitin chain in Arg-Glu-(Xa)-βgal did not prevent its cleavage by factor Xa.

Thus, both the Arg-Glu-(Xa)-βgal and its multiply ubiquitinated derivatives could serve as specific substrates for the factor Xa protease. Furthermore, the small changes in electrophoretic mobility of both the initial and the ubiquitinated Arg-Glu-(Xa)-βgal species after digestion with factor Xa were consistent with the expected, approximately 1.1-kD decrease in their molecular mass (*18*). The lack of stronger changes in electrophoretic mobility of the multiubiquitinated Arg-Glu-(Xa)-βgal^{u_n} species, and lack of significant changes in their relative abundance after digestion with factor Xa excluded all but one of the models in Fig. 3, A to C, because only in model A are all of the ubiquitin moieties of an X-βgal retained in βgal$^{3-1045}$.

We conclude that a multiubiquitin chain is attached to X-βgal at either Lys^{15} or Lys^{17} (out of the total of 22 Lys residues in an X-βgal). Although the possibility that at least some X-βgal^{u_n} species contained two multiubiquitin chains attached to both Lys^{15} and Lys^{17} cannot be ruled out by the foregoing data, such an arrangement is unlikely in view of the proximity of the two lysines (see below). Regardless of these considerations, an arrangement in which two multiubiquitin chains are attached to both Lys^{15} and Lys^{17} cannot be functionally essential because either Lys^{15} alone or Lys^{17} alone can serve as the second determinant of the amino-terminal degradation signal (*8*).

Structure of the multiubiquitin chain. If a ubiquitin moiety within the multiubiquitin chain is linked to an adjacent ubiquitin moiety via an isopeptide bond between the carboxyl-terminal Gly^{76} of ubiquitin and an internal Lys residue in its nearest ubiquitin neighbor, the structure of the chain could be established by determination of which (among the seven) Lys residues of ubiquitin are involved in the linkage.

Trypsin cuts after every one of the seven Lys residues in ubiquitin, and after its Arg residues as well (*19*) (Fig. 5B). Thus, a comparison between the lysine-containing tryptic peptides derived from free ubiquitin with those derived from ubiquitin moieties within the multiubiquitin chain should pinpoint a peptide that encompasses the ubiquitin-ubiquitin junction because tryptic cleavage at the corresponding Lys residue would be inhibited by the presence of isopeptide bond (Fig. 5, B and C).

To implement this mapping approach, we introduced into *Escherichia coli* a gene encoding human ubiquitin [mammalian ubiquitins have identical amino acid sequences (*1*)]. The overexpressed ubiquitin was metabolically labeled with [^{3}H]lysine, purified to homogeneity (*20*), and added to a fraction of rabbit reticulocyte extract (fraction II) that lacks most of the extract's free ubiquitin but retains its deubiquitinating activity and the ability to carry out ubiquitin-dependent protein degradation if supplemented with ubiquitin and ATP (*1, 4, 7*). Addition of the purified, unlabeled Ub-Arg-βgal (*7, 14*) to fraction II supplemented with ATP and [^{3}H-Lys]ubiquitin (*20*) yielded a short-lived Arg-βgal protein, which was isolated (together with its multiply ubiquitinated derivatives) by immunoprecipitation, and thereafter fractionated by SDS-PAGE (Fig. 5A). Since the Arg-βgal itself was unlabeled, and since the bulk of ubiquitin in fraction II was provided by the added [^{3}H-Lys]ubiquitin, only ubiquitin moieties within a multiply ubiquitinated Arg-βgal^{u_n} contained ^{3}H, and moreover, only Lys residues within these ubiquitin moieties were labeled. The individual species of electrophoretically resolved, multiply ubiquitinated Arg-βgal (Arg-βgal^{u_1} to Arg-βgal^{u_4}; Fig. 5A) were isolated, and then digested with

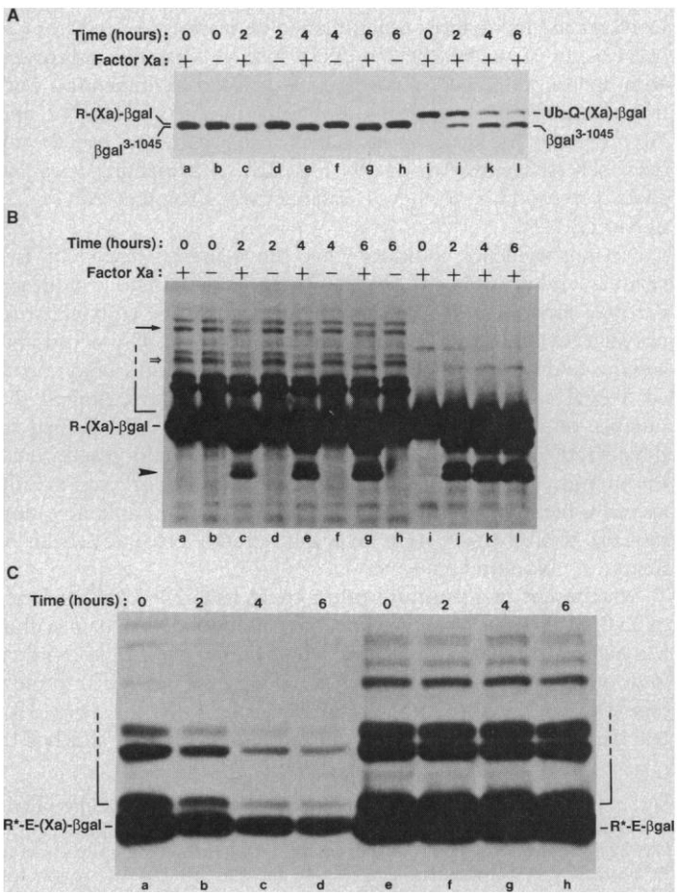

Fig. 4. Ubiquitin moieties in a multiply ubiquitinated X-βgal are confined to its Lys^{15} or Lys^{17} residues. (**A**) ^{35}S-labeled Ub-Gln-(Xa)-βgal (*18*) was incubated in ATP-supplemented reticulocyte extract to yield Arg-Glu-(Xa)-βgal and its multiply ubiquitinated derivatives. These proteins were isolated by immunoprecipitation and digested with factor Xa (lanes a, c, e, and g) for the times indicated, followed by SDS-PAGE and fluorography (*14*). The samples in lanes b, d, f, and h were treated identically, except that no factor Xa was added. In lanes i to l, the initial construct, Ub-Gln-(Xa)-βgal (*14*), was digested with factor Xa directly, without prior incubation in reticulocyte extract. (**B**) A longer fluorographic exposure to bring out the multiply ubiquitinated derivatives of Arg-Glu-(Xa)-βgal. A double arrow points to one set of ubiquitinated βgal species which undergo a readily detectable change in electrophoretic mobility upon treatment with factor Xa. In some cases (denoted by an arrow; especially lanes e and g), closely spaced doublets of ubiquitinated βgal derivatves could be seen after, but not before, the digestion with factor Xa, indicating incomplete cleavage by factor Xa. An arrowhead indicates two minor, aberrant products of digestion with factor Xa. (**C**) SDS-PAGE analysis of the products of reactions considered in Fig. 3D. The unlabeled Ub-Gln-(Xa)-βgal (lanes a to d) and the unlabeled Ub-Gln-βgal (lacking the factor Xa cleavage site) (lanes e to h) were incubated in ATP-supplemented reticulocyte extract in the presence of [^{3}H]arginine. The reaction products were reisolated from the extract by immunoprecipitation, digested with factor Xa (*18*) for the times indicated, and fractionated by SDS-PAGE. The half-open square brackets indicate the bands of multiply ubiquitinated βgal containing the conjugated [^{3}H]Arg.

trypsin. A sample of the free [^{3}H-Lys]ubiquitin was similarly treated with trypsin. Each of these near-complete tryptic digests was mixed with an excess of equally extensive tryptic digest of the unlabeled free ubiquitin, and the samples were fractionated by reversed-phase, high-performance liquid chromatography (HPLC). Of the Lys-containing tryptic peptides of ubiquitin (K1 to K7), and the arginine-containing peptides (R1 to R4), only the K peptides were labeled with ^{3}H (Fig. 5, B and D). Because of incomplete cleavage at Lys^{33}, the HPLC profiles yielded eight instead of seven K-type peptides in all of the samples examined (*19*). Comparison of the relative yields of the K peptides in tryptic digests of individual Arg-βgal^{U_n} species showed that peptide K6 (which contains Lys^{48} of ubiquitin) was the only one whose relative yield was reproducibly low in multiply ubiquitinated Arg-βgal^{U_n} species (Fig. 5D). Since in this analysis *all* of the ubiquitin moieties in a given Arg-βgal^{U_n} species contributed to the final composition of a tryptic digest, the relative yield of peptide K6 was expected to be a function of the number of ubiquitin moieties in an Arg-βgal^{U_n} even if the ubiquitin-ubiquitin linkages within a multiubiquitin chain were exclusively of the Gly^{76}-Lys^{48} type. Specifically, for a monoubiquitinated Arg-βgal species (Arg-βgal^{U_1}), the relative yield of peptide K6 should be the same as that for free ubiquitin. The relative yield of peptide K6 for a diubiquitinated Arg-βgal (Arg-βgal^{U_2}) should decrease to 50 percent of its value for either Arg-βgal^{U_1} or free ubiquitin, with further decreases to 33 percent and 25 percent for Arg-βgal^{U_3} and Arg-βgal^{U_4}, respectively (Fig. 5, A to C). The observed differences in the relative yield of peptide K6 between free ubiquitin, Arg-βgal^{U_1}, Arg-βgal^{U_2}, Arg-βgal^{U_3}, and Arg-βgal^{U_4} were in good agreement with the above predictions (Fig. 5D).

Thus, the major, if not the only junctional structure within the βgal-attached multiubiquitin chain is one in which the carboxyl-terminal Gly^{76} of one ubiquitin is joined to the internal Lys^{48} of an adjacent ubiquitin. This conclusion directly follows from the data of Fig. 5D provided that the following assumptions are valid: (i) Ubiquitin-ubiquitin linkages within the βgal-attached multiubiquitin chain are indeed the isopeptide bonds of the type known to exist in monoubiquitinated proteins (*1*). Although very likely correct (*1, 2, 4, 10*), this assumption remains to be directly proved. (ii) No proteins other than ubiquitin are present as components of the multiubiquitin chain. Indeed, a multiubiquitin chain (of unknown

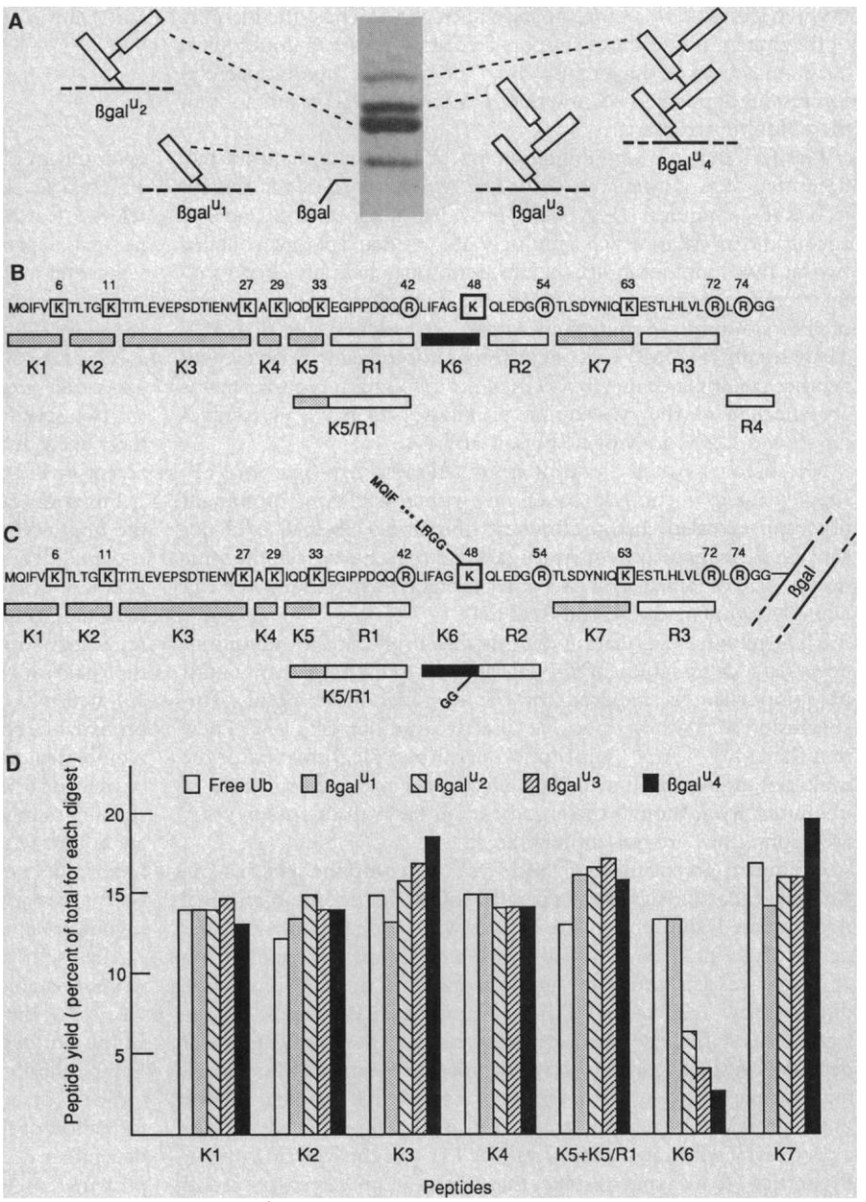

Fig. 5. In a multiubiquitin chain, the carboxyl-terminal Gly^{76} of one ubiquitin is joined to Lys^{48} of an adjacent ubiquitin. (**A**) The [^{3}H]lysine-labeled ubiquitin was conjugated to the unlabeled Arg-βgal in ATP-supplemented reticulocyte fraction II. Arg-βgal and its ubiquitinated derivatives were separated by SDS-PAGE in a 6 percent gel, followed either by fluorography, as shown, or by electroblotting onto nitrocellulose filters for subsequent digestion with trypsin (*19*). Since the initial, nonubiquitinated Arg-βgal lacks the ^{3}H label, its band is absent from the fluorogram. Open rectangles denote ubiquitin moieties. (**B**) The amino acid sequence of mammalian ubiquitin and the set of its tryptic fragments. The seven Lys and four Arg residues in ubiquitin are highlighted by the square and round frames, respectively. The corresponding tryptic peptides containing a carboxyl-terminal Lys (stipled rectangles) are denoted as K1 to K7, and those containing a carboxyl-terminal Arg (open rectangles) are denoted as R1 to R4. Peptide K6 is indicated by a black rectangle (see below). Incomplete cleavage at Lys^{33} resulted in the presence of both K5 and K5/R1 peptides (in approximately equal amounts) in the extensive tryptic digests (*19*) of both free and conjugated ubiquitin. Similarly, Arg^{74} was recovered largely as a component of the partial peptide R4 (*19*). (**C**) Same as (B) but for a ubiquitin moiety within a multiubiquitin chain in which the ε-amino group of the Lys^{48} in one ubiquitin is joined to the carboxyl-terminal Gly^{76} of an adjacent ubiquitin. Although the carboxyl-terminal Gly^{76} of the ubiquitin moiety shown is joined to X-βgal, the mapping data would be the same for a ubiquitin moiety embedded between two other ubiquitins within the multiubiquitin chain. (**D**) Yields of tryptic K peptides of free ubiquitin relative to those for ubiquitin moieties within individual Arg-βgal^{U_n} species. Electrophoretically resolved Arg-βgal^{U_n} proteins (n = 1 to 4; see A) in which only ubiquitin moieties, but not Arg-βgal, were labeled with [^{3}H]lysine and digested with trypsin; the resulting peptides were separated by reversed-phase HPLC (*19*). The relative yield of a given K peptide in a digest of an individual Arg-βgal^{U_n} species (expressed as percentage of the total yield for each digest) was determined by dividing the amount of ^{3}H in the peptide by the total amount of ^{3}H recovered in all of the K peptides of the digest.

junctional structure) has been produced in vitro with a purified ubiquitin-conjugating enzyme from reticulocytes, purified ubiquitin, and purified histone H2A as an acceptor protein (*21*).

A multiubiquitin chain in a targeted protein is essential for protein degradation. To assess the functional significance of the multiubiquitin chain, we first converted, by site-directed mutagenesis (*22*), the Lys^{48} residue of ubiquitin into Arg, a residue that is also positively charged but cannot serve as acceptor of ubiquitin (*1, 2, 4*). The mutant ubiquitin (Ub-R48) was expressed in *E. coli*, purified (*20, 22*), and added to ubiquitin-depleted, ATP-supplemented fraction II, which contained an ^{35}S-labeled Arg-βgal. While the addition of the wild-type ubiquitin (Ub-K48) to ATP-supplemented fraction II resulted in the formation of multiply ubiquitinated derivatives of Arg-βgal (Fig. 6B, lane c), addition of the mutant ubiquitin (Ub-R48) yielded only monoubiquitinated Arg-βgal (Fig. 6B, lane b). The Arg-βgal monoubiquitinated with Ub-R48 could be deubiquitinated by incubating it in ATP-depleted fraction II (*23*). Hydroxylamine mapping (*15*) of the Ub-R48 moiety within Arg-βgal^{U1} yielded results indistinguishable from those obtained with Arg-βgal^{U1} that contained wild-type ubiquitin (*23*). These results indicated that the conservative replacement of Lys^{48} with Arg^{48} in ubiquitin (*22*) did not perturb its structure enough to make it unrecognizable by ubiquitin-specific enzymes. Thus, the inability of the mutant ubiquitin to support formation of the multiubiquitin chain is likely to be due exclusively to the lack of a (ubiquitinatable) Lys residue at position 48, and not to a more general perturbation of the ubiquitin structure.

The fact that a monoubiquitinated, but not a diubiquitinated, derivative of Arg-βgal was formed in the presence of the mutant (Ub-R48) ubiquitin (Fig. 6B), provided independent evidence against the model in which a multiply ubiquitinated X-βgal contains two or more multiubiquitin chains, with one chain attached to X-βgal's amino terminus, and at least one more chain attached to one of the two amino terminus–proximal Lys residues (see Fig. 3C). These results (Fig. 6B) were inconsistent, for the same reasons, with another "multiple-chain" model in which two multiubiquitin chains are attached to the two amino terminus–proximal Lys residues (Lys^{15} and Lys^{17}) in X-βgal (Figs. 1 and 3).

Measurements of the rate of degradation of Arg-βgal in ATP-supplemented fraction II containing either wild-type or mutant ubiquitin revealed that the mutant ubiquitin (Ub-R48) did not support the degradation of Arg-βgal (Fig. 6A). Essentially the same results were obtained with two other proteolytic substrates, hen egg white lysozyme and Arg-Glu-βgal (*24*).

Thus, monoubiquitination of X-βgal is insufficient for ubiquitin-dependent degradation of X-βgal, and the presence of the multiubiquitin chain is required for the degradation to occur. This conclusion would have followed directly from our data if we knew that the $Lys^{48} \rightarrow Arg^{48}$ substitution perturbed the formation of the multiubiquitin chain but did not affect other relevant properties of ubiquitin. Even though the arguments in favor of this assumption are strong, they are not entirely direct.

Ubiquitin-accepting Lys^{15} and Lys^{17} are components of the X-βgal degradation signal. In a parallel study (*8*), the amino-terminal degradation signal present in X-βgal was examined in molecular genetic experiments in which the 38-residue amino-terminal region of X-βgal was attached to the amino terminus of the ~20-kD mouse dihydrofolate reductase (DHFR). Mutational analysis of this X-βgal–derived DHFR extension showed that the amino-terminal degradation signal comprises two distinct determinants. One determinant (manifested as the N-end rule) is the amino-terminal residue of the protein. The second determinant is a specific lysine residue of a proteolytic substrate (either Lys^{15} or Lys^{17} in the case of X-βgal). Properties of a lysine residue that make it an effective second determinant of the degradation signal do not depend on the unique amino acid sequences in the vicinity of the lysine. Instead, the relevant features of the second-determinant lysine appear to include its spatial proximity to the amino terminus of the protein and segmental mobility of the lysine-containing region (*8*).

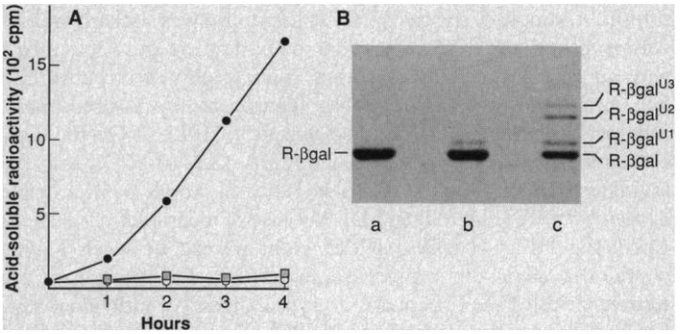

Fig. 6. Replacement of Lys^{48} in ubiquitin with Arg allows monoubiquitination of X-βgal but neither its degradation nor formation of the multiubiquitin chain. For these experiments, the Lys^{48} of ubiquitin was replaced, by site-directed mutagenesis, with the Arg residue (*22*). (**A**) Purified, ^{35}S-labeled Arg-βgal was added to ATP-supplemented reticulocyte fraction II and incubated at 37°C for the times indicated. The degradation of [^{35}S]Arg-βgal was assayed by measuring acid-soluble radioactivity (*7, 14*). (Open circle) No ubiquitin added to fraction II; (stippled square) mutant ubiquitin (Ub-R48) added; (filled circle) wild-type ubiquitin (Ub-K48) added. (**B**) The 0.5-hour samples from the experiment in (A) were subjected to SDS-PAGE and fluorography. (Lane a) No ubiquitin added to fraction II. (Lane b) Same as lane a, but mutant ubiquitin (Ub-R48) was added. (Lane c) Same as lane a, but wild-type ubiquitin (Ub-K48) was added. Individual species of Arg-βgal^{Un} are indicated on the right.

Our structural approach pinpoints the same residues, Lys^{15} and Lys^{17}, in X-βgal, and shows that either Lys^{15} or Lys^{17} serves as a specific acceptor of the multiubiquitin chain (Figs. 1 to 4). Thus, the genetically identified requirement for the presence of at least one of the two specific Lys residues in the X-βgal degradation signal (*8*) is most likely due to the function of these residues as specific ubiquitin acceptors in the targeted X-βgal protein.

Function of the multiubiquitin chain. Our results suggest that the multiubiquitin chain in a targeted protein is essential for the protein's degradation. Specifically, monoubiquitination of a protein is not sufficient for its degradation (Fig. 6). We propose that the multiubiquitin chain provides a set of binding sites (formed by the repeating, structurally distinct ubiquitin-ubiquitin junctions within the chain) for the "downstream" ubiquitin-dependent protease (*1, 10*) which is responsible for the degradation of a ubiquitinated proteolytic substrate. This hypothesis not only explains the observed requirement for the multiubiquitin chain in ubiquitin-dependent protein degradation, but also accounts for the relative metabolic stability of monoubiquitinated intracellular proteins, most of which are not made short-lived by ubiquitination (*1, 3*). According to this model, the presence of the substrate-attached multiubiquitin chain is both necessary and sufficient for the binding and activation of the ubiquitin-dependent protease.

The use of mutant ubiquitin whose Lys^{48} was replaced by a non-ubiquitinatable Arg residue has been extended to experiments in vivo (*25*). The mutant ubiquitin, which can form monoubiquitinated but not multiubiquitin chain-containing ubiquitin adducts (Fig. 6), specifically inhibited degradation of (at least) abnormal, canavanine-containing proteins in the yeast *S. cerevisiae* (*25*). Thus, formation of the multiubiquitin chain is required for the ubiquitin-dependent degradation of a broad class of short-lived intracellular proteins.

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14. The set of 20 *E. coli* expression vectors (pKKUb-X-βgal) encoding Ub-X-βgal fusion proteins was constructed as described (7). Either the unlabeled or [^{35}S]Met-labeled Ub-X-βgal proteins (~2×10^5 cpm/μg) were purified from *E. coli* by affinity chromatography. Deubiquitination of Ub-X-βgal fusion proteins in ATP-depleted extract from rabbit reticulocytes was used to produce the corresponding X-βgal test proteins. Multiply ubiquitinated X-βgal proteins were generated by incubating a short-lived X-βgal in ATP-supplemented reticulocyte extract (7). Fraction II was prepared by DEAE chromatography of ATP-depleted reticulocyte extract (4). Both the initial X-βgal proteins and their multiply ubiquitinated derivatives were isolated from either reticulocyte extract or yeast (*S. cerevisiae*) by immunoprecipitation with a monoclonal antibody to βgal as described (6) except that *N*-ethylmaleimide (NEM, 10 m*M*) was added to extracts for 10 minutes before the addition of antibody to prevent deubiquitination of βgal. The excess NEM was inactivated (before the addition of antibody) by the addition of 10 m*M* cysteine. Unless stated otherwise, the first-dimension SDS-PAGE was done in a 6 percent SDS-polyacrylamide gel (26), with subsequent fluorography.
15. The ^{35}S-labeled Ub-Met-βgal and Met-βgal (14) were subjected to electrophoresis in adjacent lanes of a 6 percent SDS-polyacrylamide gel; when samples were treated with hydroxylamine, heating before the electrophoresis (6, 26) was omitted. The bands of βgal were excised, and the gel slices were washed for 1 hour, with shaking, in 8*M* urea, 0.2*M* tris-HCl (*p*H 9.2). The slices were transferred to several volumes of 2*M* hydroxylamine in the same buffer, incubated with shaking for 2 hours at 37°C (12), washed in 0.1*M* tris-HCl (*p*H 6.8), equilibrated with an SDS-containing sample buffer, and positioned on top of a 7 percent SDS-polyacrylamide gel, and subjected to electrophoresis (Fig. 2, C and D). For two-dimensional fractionations, an entire lane of a first-dimension 6 percent SDS-polyacrylamide gel was excised, treated with hydroxylamine as above, and positioned on top of a second-dimension 6 percent SDS-polyacrylamide gel.
16. The relative abundance of the inefficiently deubiquitinatable Ub-Pro-βgal species (Fig. 2, A and F) prevents detection of a branch-conjugated Pro-βgal^{ui}. Although several lines of indirect evidence (6) suggest that the multiply ubiquitinated Pro-βgal^{un} species lack the amino-terminal ubiquitin moieties, this remains to be verified directly.
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18. The small Bam HI fragment of pKKUb-Gln-βgal (7, 14) was subcloned into M13mp19 (28, 29), where a sequence encoding Gly-Ser-Gly-Ile-Glu-Gly-Arg was inserted into the gene for Ub-Gln-βgal by oligonucleotide-directed mutagenesis (28) with a Mutagene kit (Bio-Rad). This seven-residue insertion, in which the last four residues form the recognition site for the factor Xa protease, was introduced after the second (His) residue in X-βgal (Fig. 1). The final M13mp19-based construct and the pKKUb-Gln-βgal vector were both cut with Bam HI, and the small Bam HI fragment of the M13mp19-based construct was ligated to the large Bam HI fragment of pKKUb-Gln-βgal to yield pKKUb-Gln-(Xa)-βgal. The overexpressed, purified Ub-Gln-(Xa)-βgal (either unlabeled or metabolically labeled with [^{35}S]methionine) (7, 14) was incubated at room temperature for 2 hours at 0.1 to 0.3 mg/ml in 12 percent glycerol, 0.1*M* NaCl, 0.25 m*M* $MgCl_2$, 0.11*M* tris-HCl (*p*H 8.3) in the presence of bovine serum albumin (1 mg/ml) and factor Xa (5 U/ml) from human plasma (Boehringer). The Arg-Glu-(Xa)-βgal [derived from Ub-Gln-(Xa)-βgal by processing in reticulocyte extract] and its multiply ubiquitinated derivatives were isolated from ATP-supplemented reticulocyte extract by immunoprecipitation (7). The βgal proteins (~30 μg), while still bound to protein A-Sepharose beads via the antibody to βgal (6), were washed with 0.1*M* NaCl, 0.1 M tris-HCl (*p*H 8.3) containing bovine serum albumin at 1 mg/ml, resuspended in 50 μl of the same buffer, and then digested with 0.2 U of factor Xa at room temperature, with gentle shaking.
19. Purified, [^{3}H]lysine-labeled human ubiquitin (~1.5×10^5 cpm) (20) was added to 30 μl of ATP-supplemented reticulocyte fraction II containing ~6 μg of unlabeled Arg-βgal. The Arg-βgal and its [^{3}H]lysine-labeled, multiply ubiquitinated derivatives were isolated by immunoprecipitation (14) and subjected to electrophoresis in a lane of a 6 percent SDS-polyacrylamide gel that was flanked by lanes containing the otherwise identical species of ubiquitinated Arg-βgal that had been produced with ^{125}I-labeled ubiquitin (1, 4). Separated proteins were electroblotted onto a nitrocellulose filter (pore size 0.2 μm), and the lanes with ^{125}I-labeled proteins were visualized by autoradiography. Regions of the filter that contained the separated, [^{3}H]lysine-labeled Arg-βgal^{un} species ($n = 1 - 4$) (Fig. 5A) were pinpointed with the use of the otherwise identical, ^{125}I-labeled Arg-βgal^{un} species in adjacent lanes as guides. These regions were individually excised, blocked with polyvinylpyrrolidone (30), and incubated with TPCK (tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin (Sigma) in the presence of 6*M* urea (31) and 5 percent (v/v) acetonitrile for 0, 2, and 8 hours. Soluble products of digestion were added to 50 μg of an extensive tryptic digest of unlabeled ubiquitin (31) (used as a carrier), injected into a TSK ODS-T120 column (LKB), and eluted with a gradient of 0 to 40 percent acetonitrile in 0.1 percent trifluoroacetic acid (31).
20. Exponentially growing *E. coli* AR58 bearing the plasmid pNMHUB which contains a synthetic human ubiquitin gene under control of the heat-inducible P_L promoter (32) were shifted to 42°C, and incubated in the presence of [^{3}H]lysine (>15 Ci/mmole, Amersham) for 1 hour. The [^{3}H]lysine-labeled ubiquitin was purified to homogeneity by a procedure that omits heat treatment and acid precipitation steps (23).
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22. A pUC plasmid bearing a synthetic human ubiquitin gene (32) was cut with Bsm I and Xho I. The larger of the two fragments was ligated with a synthetic double-stranded DNA identical to the originally excised small Bsm I–Xho I fragment, except for a base change that converted the sequence from the one coding for Lys^{48} of ubiquitin into one encoding Arg at the same position. An Hpa I–Kpn I fragment of the final pUC-based construct was exchanged with its counterpart in the P_L promoter-containing plasmid pNMHUB (32) to yield the plasmid pNMHUB-R48. The mutant ubiquitin was overexpressed with the use of pNMHUB-R48 and purified as described (20, 32).
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