Mapping the ubiquitin-binding domains in the p54 regulatory complex subunit of the *Drosophila* 26S protease

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Abstract Short-lived intracellular proteins, after being marked by multiubiquitination, are degraded by the 26S protease. This large ATP-dependent protease is composed of two multiprotein complexes: the regulatory complex and the 20S proteosome. The selective recognition of ubiquitinated proteins is ensured by the regulatory complex. Using an overlay assay a single 54-kDa multiubiquitin-chain-binding subunit was detected in the regulatory complex of the *Drosophila* 26S protease. Overlay assay with the recombinant p54 subunit confirmed its ubiquitin-binding property. The recombinant protein showed pronounced preference for higher ubiquitin multimers, in agreement with the known preference of the 26S protease for multiubiquitinated proteins as substrates. To map the ubiquitin-binding domain of the p54 subunit different segments of the recombinant protein were expressed in *E. coli* and tested by the overlay assay. The p54 subunit carries two independent ubiquitin-binding domains. The central domain carries two highly conserved sequence blocks: the FGVDP sequence (at position 207), which is 100% conserved from yeast till human, and the DPELALALRVSMEE sequence (at position 214), which is 100% conserved in higher eukaryotes with two amino acid changes in yeast. In the C-terminal ubiquitin-binding domain the GVDP sequence motif is repeated and 100% conserved in higher eukaryotes. This domain, however, due to the shorter size of the yeast multiubiquitin-binding subunit, is present only in higher eukaryotes.

Key words: 26S Proteasome; Regulatory complex; Ubiquitin; Ubiquitin-binding subunit

1. Introduction

Degradation of ubiquitinated proteins, which is one of the key events in the controlled proteolysis of intracellular proteins, is performed by a multicomponent, ATP-dependent protease, the 26S protease. The catalytic core of the 26S protease is the 20S proteosome, a barrel-shaped multiprotein complex, capable of degrading any non-ubiquitinated protein in the absence of ATP. The selectivity of the 26S protease is ensured by a large multiprotein complex, called the regulatory complex, which can bind to the bases of the barrel-shaped 20S proteosome in an ATP-dependent reaction, and restricts its catalytic activity to ubiquitinated proteins. Several different functions have been attributed to the regulatory complex. From the viewpoint of the selectivity, its most important function is the selective recognition and binding of ubiquitinated proteins. All the other presumed functions of the regulatory complex – removal of the ubiquitin tag, unfolding of the protein and transporting it through the regulatory complex to the catalytic centers within the internal channel of the 20S proteosome – are not directly related to the selectivity of the intracellular protein degradation. Among the 15 or more subunits of the human regulatory complex, in vitro a single polypeptide was found to be able to bind ubiquitinated proteins selectively [1]. Homolog of the human ubiquitin-binding regulatory complex subunit has also been identified and characterized from *Arabidopsis* [2]. These subunits, which have high degree of sequence homology [3], have pronounced affinity for multiubiquitinated proteins [1,2]. This observation further supports the assumption that a single multiubiquitin-chain-binding subunit ensures the selectivity of the 26S protease, as multiubiquitinated proteins, compared with their monoubiquitinated forms, are more favored substrates of the 26S protease [4,5]. The *Saccharomyces cerevisiae* homolog of this multiubiquitin-chain-binding subunit, designated Mcb1, has recently been cloned [6].The recombinant protein also binds multiubiquitinated proteins in vitro, and exhibits binding preference for longer ubiquitin chains. Yeast mcb1 deletion mutants, however, were viable, grew at near-wild-type rates and only modest disturbance of short-lived protein degradation was detected. These data suggested that in yeast Mcb1 is not the sole factor involved in ubiquitin recognition by the 26S protease. Sequence comparison of the yeast, human and *Arabidopsis* multiubiquitin-chain-binding subunits, however, revealed a significant difference. While the N-terminal segments of these proteins are highly homologous, the yeast protein is shorter, and this is due to the lack of a C-terminal segment carrying highly homologous sequences in the human and *Arabidopsis* proteins. The C-terminal highly homologous sequence lacking in the yeast protein is one of the repeated elements proposed to be responsible for the multiubiquitin-chain recognition in the human regulatory subunits [3].

We have cloned and sequenced a regulatory complex subunit of the *Drosophila* 26S protease, which is highly homologous with the human, yeast and *Arabidopsis* multiubiquitin-chain-binding subunits [7]. The C-terminal segment of the human or *Arabidopsis* subunit, which is missing in yeast, is present in the *Drosophila* protein and carries the second conserved repeated element. In this study we present evidences that the cloned protein is the multiubiquitin-chain-binding subunit of the *Drosophila* 26S protease. Analyzing the different segments of the recombinant protein in an in vitro multiubiquitin-chain-binding assay revealed that there are two multiubiquitin-binding domains in this subunit. One of these domains, present in all four species, is localized on a 115 amino acid long central segment of the subunit, carrying one of the highly conserved repeated sequence elements proposed earlier to be responsible for the multiubiquitin-chain recognition [3].The second domain on the C-terminal part of the protein carries the second highly conserved repeated se-

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quence element, present only in the human, plant and fly 26S protease subunit.

2. Materials and methods

2.1. Construction of a full-length p54 cDNA

Primer extension analysis revealed that the first 3 amino were missing in our longest cDNA clone [7]. To construct a full-length p54 cDNA the 5′-GATCCCATGGTTCTGGAGAGT-3′ and 3′-GGTACCAAGACCTCTCA-5′ oligonucleotides were synthesized and annealed. This was ligated to the first RSA I site present close to the 5′ site of our cDNA and to the *Bam*HI site of the pET3-His expression vector ([8], kind gift of Dr. T. Hai). The proper reading frame was checked by sequencing the recombinant clones. Recombinant DNA manipulations and DNA sequencing were done by standard procedures [9].

2.2. Construction of recombinants expressing different segment of the p54 subunit

Different segments of the cDNA, corresponding to internal restriction fragments, were cloned into the pET3-His vector. To maintain the correct reading frame an appropriate restriction site from the polylinker region of the vector was cleaved and the cDNA fragment was ligated. If blunt-end ligation was required for the proper reading frame, the protruding ends of the restriction fragments were filled up or removed by Klenow polymerase before ligation. The orientation of the ligated fragment and the proper reading frame were checked by DNA sequencing. The recombinant plasmid DNA was transformed into *E. coli* BL21(DE3), the transformed culture was induced at an $OD_{550} = 0.5$ by the addition of 1 mM isopropyl-β-thiogalactoside and the culture was grown at 37°C for an additional 4 h after induction. The cells were disrupted by ultrasonication in phosphate buffered saline containing 1 mM phenylmethyl-sulphonyl fluoride, and the protein extract was used for gel electrophoresis.

2.3. Preparation of radiolabelled multiubiquitin chains

Ubiquitin activating enzyme (E1 enzyme) was prepared from a hydroxylapatite fraction of a *Drosophila* embryonic extract [10] ny ubiquitin affinity chromatography [11]. The ubiquitin carrier protein (E2 enzyme) was expressed from a recombinant plasmid encoding an E2 enzyme from maize (kind gift of Dr. R.D. Vierstra) and purified by DEAE-cellulose chromatography. Ubiquitin (Sigma) was radiolabelled by iodination using standard procedures [9]. Radiolabelled multiubiquitin chains were prepared by the method of Pickart et al. [12].

2.4. Conjugate binding assay

The partially purified hydroxylapatite or DEAE-cellulose fractions of a *Drosophila* embryonic extract and the *Drosophila* 26S protease were prepared as described earlier [10]. The purified 26S protease, or recombinant derivatives of the p54 subunit were fractionated on SDS-polyacrylamide gel and blotted to nitrocellulose filter. After blocking the nitrocellulose filters in 5% non-fat dried milk, 10 mg/ml bovine serum albumin dissolved in 10 mM Tris-Cl pH 8.0, 150 mM NaCl (TBS) overnight at 4°C, the filters were rinsed in TBS and incubated with the radiolabelled multiubiquitin-chain mixture in TBS+10 mg/ml BSA for 4 h at 4°C. The filters were washed 3 times for 10 min in TBS at 4°C, once in TBS+0.05% triton X-100 and analyzed in a Molecular Dynamics Phosphor Imager. Radiolabelled multiubiquitin chains bound by the recombinant p54 derivatives were recovered by boiling the nitrocellulose filter piece corresponding to the radioactive band in SDS sample buffer. The recovered radioactive proteins were fractionated on SDS-polyacrylamide gel and analyzed in a Molecular Dynamics Phosphor Imager.

3. Results

3.1. Identification of the ubiquitin-binding subunit in the Drosophila 26S protease regulatory complex

Regulatory complex subunits of the purified *Drosophila* 26S protease were separated on a denaturing polyacrylamide gel, blotted to nitrocellulose membrane and the ubiquitin-binding ability of the subunits was tested in an overlay assay using radiolabelled multiubiquitin chains [1]. As shown in Fig. 1B, the 54-kDa subunit is the only polypeptide of the regulatory complex which can bind the radiolabelled multiubiquitin chains in this in vitro overlay assay. The presence of a single multiubiquitin-binding subunit in the regulatory complex is not due to the loss of other subunits with binding potency, because similar binding pattern was obtained during all purification steps of the 26S protease (Fig. 1).

Earlier we have cloned a 54-kDa regulatory complex subunit of the *Drosophila* 26S protease [7]. The cloned p54 subunit showed high degree of sequence homology with the multiubiquitin-chain-binding subunits of the human, plant and yeast regulatory complexes [3]. To verify that the cloned p54 protein is really the multiubiquitin-chain-binding subunit of the *Drosophila* regulatory complex, the cloned P54 protein was expressed in *E. coli* and the recombinant protein was tested in a conjugate binding assay. As shown in Fig. 2C, the full-length recombinant protein (p54/1–396/) actively bound the radiolabelled multiubiquitin chains. The size of the recombinant p54 protein is slightly larger compared with the authentic multiubiquitin-binding subunit of the regulatory complex, which is due to the his-tag present in the recombinant protein. Immunoblotting the filters, used for the conjugate binding assay, with a monoclonal anti-p54 antibody clearly indicated that p54 is the conjugate binding subunit of the regulatory complex (data not shown).

To prove the binding preference of p54 protein for multiubiquitin chains, radiolabelled proteins recovered from the area of the nitrocellulose filter occupied by the full-length recombinant protein were analyzed by SDS-gel electrophoresis and autoradiography. As shown in Fig. 3, besides the E2-ubiquitin thiol ester adduct, mono- and diubiquitin chains are the major radiolabelled compounds in our preparation used for the overlay assay. Among the radiolabelled proteins bound by p54 and recovered from the nitrocellulose filter, the E2-ubiquitin thiol ester adduct was completely missing and only trace amount of monoubiquitin was present. The major bound components were the di-, tri- and higher multimers of the multiubiquitin chains. Thus, the preference of p54 for the multiubiquitin chains is obvious. Nevertheless, the affinity of the *Drosophila* subunit for diubiquitin is more pronounced compared with the human, plant and yeast proteins [1–3,6].

3.2. Mapping the ubiquitin-binding domain within the cloned p54 subunit

To localize the ubiquitin-binding domain within the subunit, different segments of the cloned *Drosophila* p54 protein were expressed in *E. coli* and tested in the conjugate binding assay. The truncated derivatives of the cloned p54 subunit are depicted in Fig. 2A. The 206 amino acid long N-terminal half of the recombinant p54 cannot bind the multiubiquitin chains (54/1–206/, Fig. 2B and C). The high degree of sequence conservation, from yeast till human, in this N-terminal segment of the protein (Fig. 2A) suggests that it is responsible for an other conserved function of the subunit. The C-terminal half of the recombinant protein p54/207–396/ (carrying a segment of the subunit between amino acids 207 and 396) strongly bound the multiubiquitin chains (Fig. 2B and C). This segment carries both of the repeated, highly conserved sequence elements. Removal of the C-terminal repeated element, however, neither abolished, nor diminished significantly the bind-

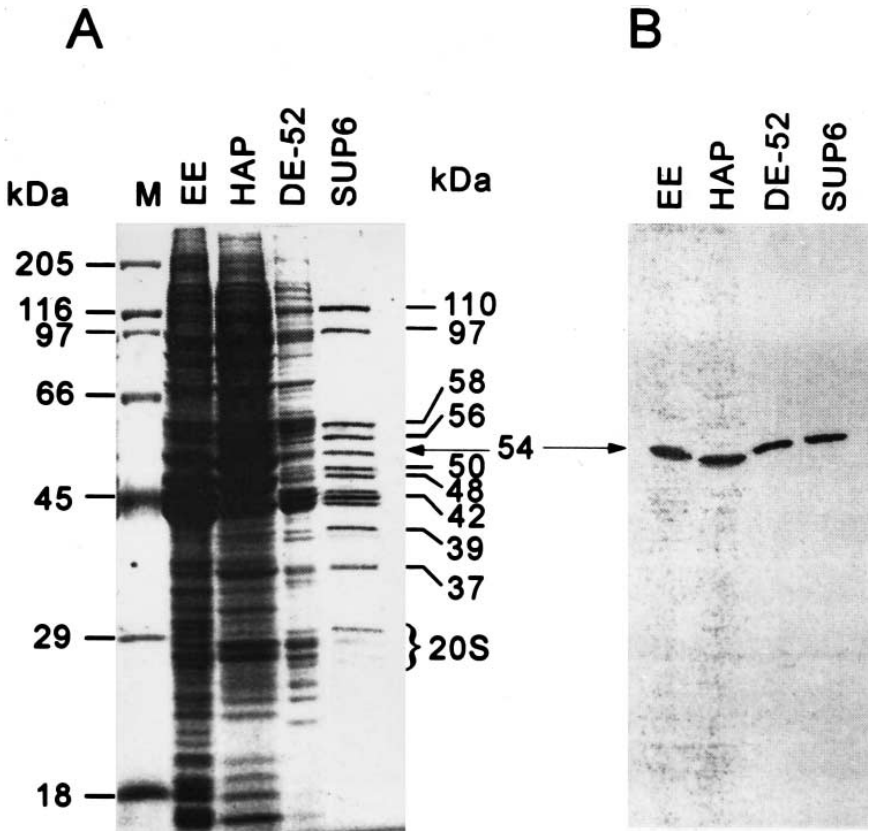
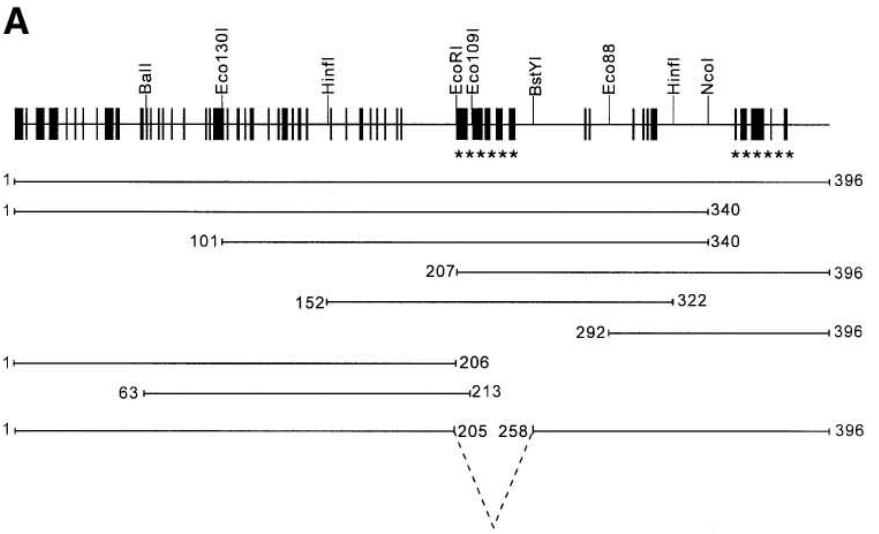


Fig. 1. Identification of the multiubiquitin-chain-binding subunit of the *Drosophila* 26S protease. An aliquot of the different purification steps of the *Drosophila* 26S protease was separated on a 10% denaturing polyacrylamide gel and stained with Coomassie-blue (A) or tested for multiubiquitin-chain binding in an overlay assay (B). *Drosophila* embryonic extract (EE), hydroxylapatite fraction (HAP), DEAE-cellulose fraction (DE-52) and Superose 6 FPLC fraction (SUP6) were tested. The molecular weight of the marker fragments are indicated on the left side of A, and the molecular weight of the regulatory complex subunits are shown on the right side of A.

ing activity. This is shown in Fig. 2B and C for three recombinant derivatives: the C-terminally truncated p54/1–340/, the C- and N-terminally truncated p54/101–340/ and a shorter version of the C- and N-terminally truncated protein p54/152–322/. All three proteins exhibited strong binding activity. The overlapping parts of the strongly binding p54/207–396/ and p54/152–322/ truncated proteins further narrow the boundaries of the central multiubiquitin-binding domain to a 115 amino acid long segment between amino acids 207 and 322. There are two highly conserved blocks in this segment: the FGVDP sequence (at position 207), which is 100% conserved from yeast till human, and the DPELALALRVSMEE sequence (at position 214), which is 100% conserved in higher eukaryotes with two amino acid changes in yeast [3]. It is reasonable to suppose that these sequence motifs are directly involved in the multiubiquitin-chain recognition and binding.

In the C-terminal part of the subunit the GVDP sequence motif is repeated and 100% conserved in higher eukaryotes. This is part of a longer consensus sequence: FXXSVLXXL-PGVDPXXXAXXXXXXSL conserved in higher eukaryotes [3]. To prove the involvement of this second repeated, conserved sequence element in the multiubiquitin-chain-binding, a 105 amino acid long C-terminal segment of p54 was expressed in *E. coli* and tested in the overlay assay. As shown in Fig. 2B and C, p54/292–396/ is capable of binding the radiolabelled multiubiquitin chains. In repeated experiments, however, this binding was not absolutely reproducible. As the binding properties of all the longer truncated p54 derivatives shown in Fig. 2 was absolutely reproducible, it was reasonable to suppose that the renaturation of the C-terminal p54/292–396/ was inappropriate due to its short size. To improve the chance of an appropriate renaturation of the C-terminal part of the subunit, the central segment of the cloned p54 gene between the *Eco*RI and *Bst*YI restriction sites was deleted, the N- and C-terminal segments of the subunit was fused in-frame, and the fusion protein (p54/205–258/) was tested in an overlay assay. This deletion completely removes the central conserved sequence elements between amino acids 205 and 258. The N-terminal half of the subunit which is retained in this fusion protein cannot bind the multiubiquitin chains, whereas the C-terminal domain present in this fusion protein is 34 amino acids longer than the p54/292–396/. In higher eukaryotes this 34 amino acid long segment is the least conserved part of the multiubiquitin-chain-binding subunits [3]. As shown in Fig. 2B and C this fusion protein, carrying a longer C-terminal segment of p54 exhibit strong multiubiquitin-chain-binding, and this binding is reproducible.

Although GVDP is the most conserved sequence motif present both in the central and in the C-terminal ubiquitin-binding domains of p54, this sequence alone cannot ensure the ubiquitin-binding function. This follows from the observation that the truncated recombinant protein p54/63–213/, which carries the central GVDP sequence motif, cannot bind the multiubiquitin chains (Fig. 2B and C).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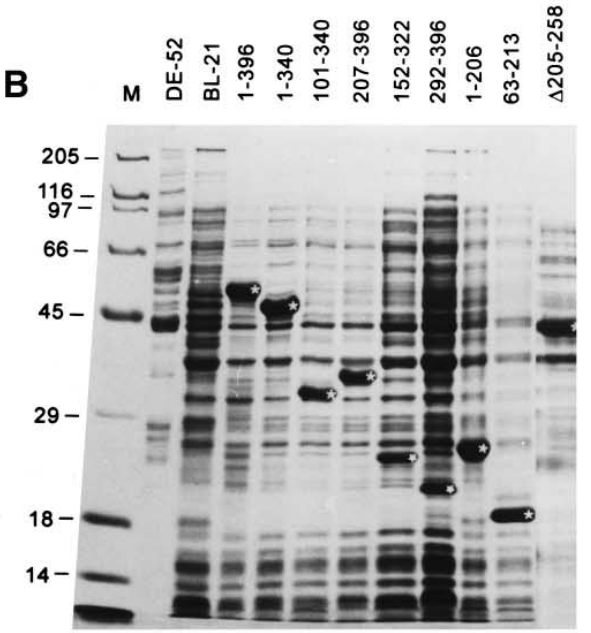
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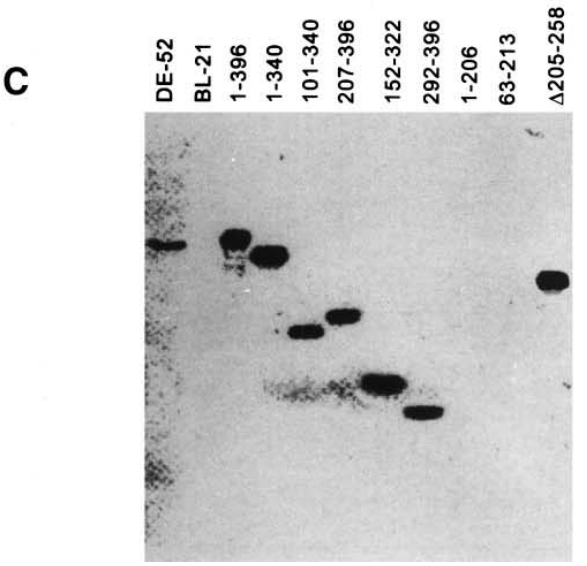
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Fig. 2. Mapping the multiubiquitin-chain-binding domains of the p54 regulatory complex subunit. (A) In the upper part of the figure the relevant restriction sites of the cloned p54 subunit are shown. The black lines superimposed on the map represents the 100% homologous blocks conserved between human, *Arabidopsis*, *Drosophila* and *Saccharomyces*. Homology search was done by the (GCG) PileUp software of the Wisconsin Sequence Analysis Package (Gap creation penalty = 3.0, Gap extension penalty = 0.1). The asterisks mark the conserved sequences in the central and C-terminal ubiquitin-binding domains. In the lower part of the panel the different truncated derivatives of the clones p54 subunit are shown, the numbering of the deletion derivatives corresponds to the amino acid sequence of p54. (B) Deletion derivatives of the clones p54 subunit were expressed in *E. coli* BL-21, the cell extracts were fractionated on a 12% SDS-polyacrylamide gel and stained with Coomassie-blue. The molecular weight of the marker proteins in lane M are shown on the left side. DE-52 is a partially purified 26S protease (DEAE-Tris-cellulose fraction shown in Fig. 1). BL-21 is a cell extract of an *E. coli* BL-21 strain transformed only with the vector. The truncated derivatives of the p54 subunits are marked as described in A. The expressed proteins are marked by asterisks. (C) The same gel shown in B was blotted to nitrocellulose filter, incubated with a radiolabelled multiubiquitin-chain mixture and the deletion derivatives capable of multiubiquitin-chain binding was detected by autoradiography.
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4. Discussion

In this paper we presented evidences that p54 is a multiubiquitin-chain-binding subunit of the *Drosophila* regulatory complex, and the only subunit of the complex which can bind the multiubiquitin chains in vitro in an overlay assay. Although p54 has stronger affinity for diubiquitin chains compared with the human, plant or yeast homologs, this subunit has clear preference for longer ubiquitin multimers. Recent observations on the 26S protease catalysed degradation of monoubiquitinated and diubiquitinated-globin [13] support the physiological relevance of diubiquitin binding. The detectable binding of diubiquitin chains may be due to a better renaturation of the *Drosophila* protein.

The multiubiquitin-chain-binding subunit of the 26S protease should have at least two independent functions: recognition and binding of ubiquitinated proteins, and the establishment of specific protein-protein interactions required for the proper assembly of the regulatory complex. It is reasonable to suppose that separate domains of the subunit are responsible for these functions. Sequence comparison of the human, plant, fly and yeast proteins seems to favor this assumption. The N-terminal segment of these proteins (amino acids 1–197), which carries several highly homologous blocks but has no any conjugate binding activity, may be the particle-binding domain. The ubiquitin-binding domain is located in the central and the C-terminal segment of the *Drosophila* subunit. These is a long, almost 100% conserved sequence in the central segment of these proteins, and a part of this conserved sequence is repeated in higher eukaryotes in the C-terminal part of the protein. These segments were supposed to be responsible for the multiubiquitin-chain recognition [3]. Our conjugate binding assays support this assumption.

These are several alternative scenarios to explain the role of multiplicity of ubiquitin-binding domains. The assumption that they exert cooperative action and in this way they are involved in the preferred binding of multiubiquitin chains [3] is not very likely, as no C-terminal domain is present in the yeast protein which, however, exhibits a strong binding preference for longer multiubiquitin chains [6]. Our p54 deletions which carry only a single ubiquitin-binding domain also retained their preference for longer multiubiquitin chains. It is more reasonable to suppose that either the ubiquitin-binding domains function independently, in which case they simply enhance the efficiency of conjugate binding, or each domain may have preferred affinity for certain types of multiubiquitinated substrates or different multiubiquitin linkages [14], and could only partially substitute each other.

Considering the lethal phenotype of all ubiquitin mutations which interfere with the formation of the multiubiquitin chain [15], the near-wild-type growth property of the yeast mcb1 deletion mutant is a serious enigma [6]. The wild-type rate degradation of N-end rule substrates but the selective accumulation of ubiquitin fusion degradation substrate in the mcb1 deletion mutant indicates that the deletion impairs only a certain part of the ubiquitin recognition function of the yeast 26S protease. The phenotype of the mcb1 deletion mutant suggests that the yeast regulatory complex has at least two different ubiquitin-binding subunits. The detection of a single ubiquitin-binding subunit in the *Drosophila* regulatory complex may reflect an inherent technical limitation of the in vitro overlay assay: only those subunits will exhibit multiubiquitin binding in this assay, which can easily refold on the nitrocellulose filter after denaturation. Alternatively, it may indicate a genuine difference in the organization of the yeast and the higher eukaryotic regulatory complexes. Considering the high degree of conservation of both the central and the C-terminal ubiquitin-binding domains in higher eukaryotes, it

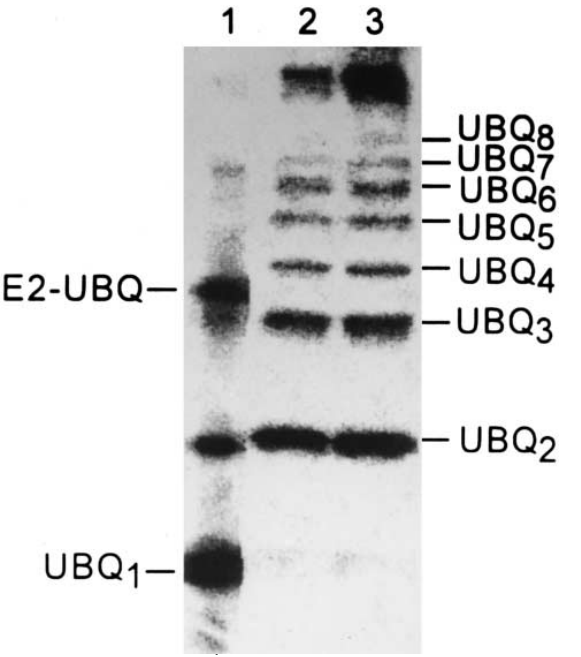

Fig. 3. Multiubiquitin-chain-binding preference of the cloned p54 subunit. The radiolabelled multiubiquitin-chain mixture used for the overlay assay was separated on a 15% SDS-polyacrylamide gel and analyzed in the Phosphor Imager (lane 1). Radiolabelled proteins bound from this multiubiquitin-chain mixture by the full-length recombinant p54 protein (lane 2) or the C-terminally shortened p54/1–340/ derivative (lane 3) were eluted from the nitrocellulose filter, separated and analyzed. The repeat length of the multiubiquitin chains is indicated on the right side. E2-UBQ marks the position of the E2 enzyme-ubiquitin thiol ester adduct.

is reasonable to suppose that the C-terminal ubiquitin-binding domain may be present on a separate subunit in the yeast regulatory complex. To detect additional ubiquitin-binding subunits a systematic analysis of the different purification steps of the yeast regulatory complex will be required. As p54 is encoded by a single copy gene in *Drosophila* [7], we have started to construct a p54 deletion mutant. The phenotype of this deletion will unambiguously indicate if there are several ubiquitin-binding subunits in the *Drosophila* regulatory complex.

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