

Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains

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Ubiquitination, a modification in which single or multiple ubiquitin molecules are attached to a protein, serves as a signalling function that controls a wide variety of cellular processes. To date, two major forms of polyubiquitin chain have been functionally characterized, in which the isopeptide bond linkages involve Lys48 or Lys63. Lys48-linked polyubiquitin tagging is mostly used to target proteins for degradation by the proteasome, whereas Lys63-linked polyubiquitination has been linked to numerous cellular events that do not rely on degradative signalling via the proteasome. Apparently linkage-specific conformations of polyubiquitin chains are important for these cellular functions, but the structural bases distinguishing Lys48- and Lys63-linked chains remain elusive. Here, we report NMR and small-angle X-ray scattering (SAXS) studies on the intersubunit interfaces and conformations of Lys63- and Lys48-linked di- and tetraubiquitin chains. Our results indicate that, in marked contrast to Lys48-linked chains, Lys63-linked chains are elongated molecules with no stable non-covalent intersubunit interfaces and thus adopt a radically different conformation from that of Lys48-linked chains.

Introduction

Chains or single molecules of ubiquitin, a 76-amino acid protein that is highly conserved throughout eukaryotes, can be covalently attached to various cellular proteins (Pickart 2000; Weissman 2001; Marx 2002). Chains of ubiquitin molecules are generated through isopeptide bonds formed between the C terminus of one ubiquitin molecule and a specific lysine residue in the next. Ubiquitin has seven Lys residues, which are all used for polymerization (Peng *et al.* 2003), but polyubiquitin chains formed via Lys at 48 or 63 have been extensively studied so far. These two types of chain share a common specific enzymatic cascade consisting of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme)

and E3 (ubiquitin-protein ligase), of which E2 seems to define the topology of the chains. Whereas several E2 enzymes have been identified for Lys48-linked chains, the only known E2 enzymes responsible for conjugating Lys63-linked chains are the yeast Mms2/Ubc13 heterodimer and its human counterpart Uev1A/Ubc13 (Deng *et al.* 2000; Hofmann & Pickart 2001). Mms2 and Uev1A, a ubiquitin E2 variant (UEV) protein, resemble E2 enzymes for Lys48-linked chains, but lack the active cysteine residue that forms a thioester bond with the C terminus of ubiquitin. Structural studies have shown that UEV proteins act as an adaptor that directs Lys63 of the acceptor ubiquitin to an active cysteine on Ubc13 (Moraes *et al.* 2001; VanDemark *et al.* 2001; McKenna *et al.* 2003).

A polyubiquitin chain formed via Lys48 functions mainly as a marker for proteolytic attack by the 26S proteasome (a eukaryotic ATP-dependent proteolytic

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complex) (Coux *et al.* 1996; Baumeister *et al.* 1998). In contrast, Lys63-linked polyubiquitin tagging is involved in a variety of cellular events that do not rely on degradative signalling via the proteasome (Weissman 2001; Marx 2002). Of these, the best-characterized is post-replicative DNA repair, which restarts replication machinery that is stalled at a damaged template DNA by repairing the damaged site (Friedberg *et al.* 1995). In *Saccharomyces cerevisiae*, this repair process is carried out through the *RAD6* pathway, which involves several proteins, including the E2 complex for Lys63-linked chains, Mms2/Ubc13. Thus, the modification of nuclear factors by Lys63-linked polyubiquitin has been implicated in post-replicative DNA repair (Ulrich & Jentsch 2000). Recently, proliferating cell nuclear antigen (PCNA), a DNA polymerase sliding clamp that is required for replication and several DNA repair pathways, has been shown to be a substrate of Mms2/Ubc13-mediated Lys63-linked polyubiquitination (Hoege *et al.* 2002). The same lysine residue was found to be the target of modification by monoubiquitin or a small ubiquitin-related modifier (SUMO), and it has been suggested that these different modifications switch the function of PCNA (Hoege *et al.* 2002; Stelter & Ulrich 2003). Although the Lys63-linked chain was shown to signal substrate degradation *in vitro*, proteasomal degradation seems not to be required for error-free post-replicative repair (Hofmann & Pickar 2001). Lys63-linked polyubiquitination has also been shown to be involved in activation of I κ B kinase, ribosome function, endocytosis, inheritance of mitochondrial DNA, transcriptional regulation of HIV-1 and the mitotic checkpoint (Galan & Haguener-Tsapis 1997; Fisk & Yaffe 1999; Springael *et al.* 1999; Deng *et al.* 2000; Spence *et al.* 2000; Bothos *et al.* 2003; Bres *et al.* 2003).

Structural information on polyubiquitin chains is needed to understand the signals that polyubiquitin tags provide, because their linkage-specific conformations present different surfaces to interacting proteins that mediate the signals. Structural studies on Lys48-linked di- and tetraubiquitins have been reported. The crystal structure of the diubiquitin shows that a hydrophobic patch, composed of Leu8, Ile44 and Val70, in each ubiquitin molecule comprises the interface between its two subunits (Cook *et al.* 1992). Consistent with this, NMR studies indicate that the diubiquitin adopts a closed conformation at neutral pH in which the hydrophobic patch is sequestered at the interface, but undergoes a conformational change to an open form at lower pH (Varadan *et al.* 2002). For Lys48-linked tetraubiquitin, two crystal forms have been reported in which the conformations are significantly different, suggesting that the polyubiquitin

chains take a conformation depending on crystal packing and possess a high degree of conformational flexibility (Cook *et al.* 1994; Phillips *et al.* 2001). Such a dynamic property has also been suggested for Lys48-linked diubiquitin (Varadan *et al.* 2002). NMR chemical shift perturbation experiments suggest that the distal two subunits of Lys48-linked tetraubiquitin may adopt a closed conformation in solution, which is similar to that of the Lys48-linked diubiquitin (Varadan *et al.* 2002).

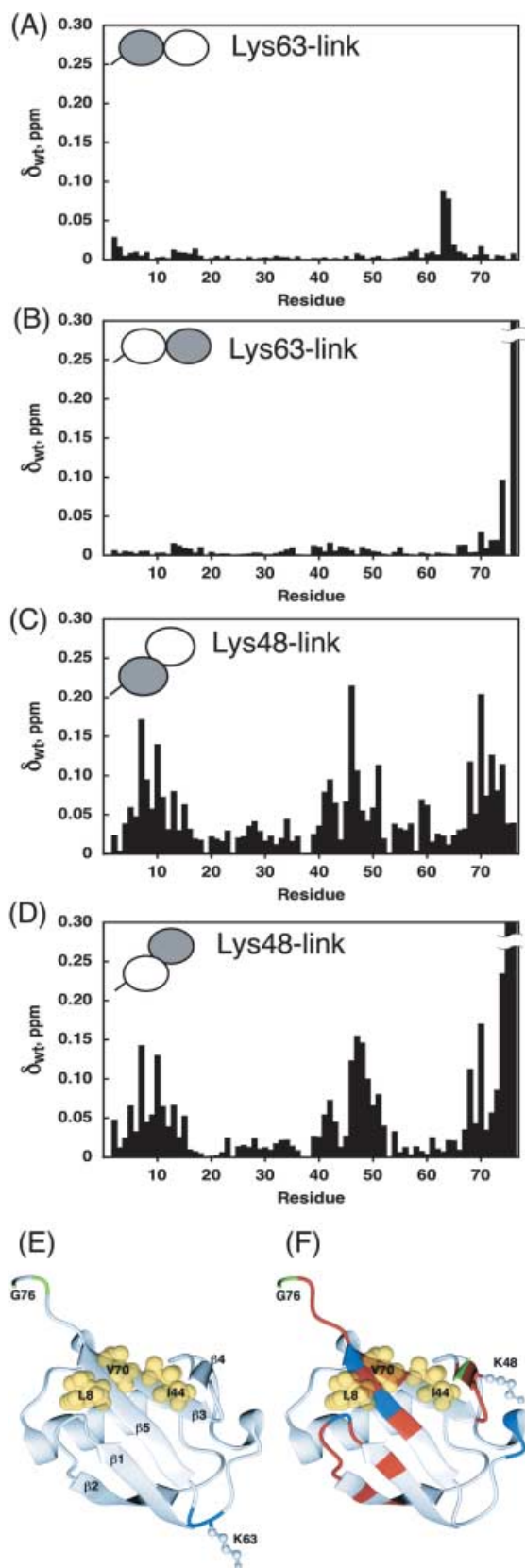
In contrast to the Lys48-linked form, structural studies of Lys63-linked chains have not been reported so far. Here, we have carried out NMR and small-angle X-ray scattering (SAXS) studies of the intersubunit interfaces of the Lys63-linked di- and tetraubiquitin chains and have compared them with those of Lys48-linked chains. Our results indicate that Lys63-linked di- and tetraubiquitins are much more elongated than Lys48-linked di- and tetraubiquitins, and thus the intersubunit interfaces of Lys63-linked polyubiquitin chains are distinct from those of Lys48-linked chains, suggesting that these forms of polyubiquitin chain adopt radically different conformations.

Results

Chemical shift mapping of the intersubunit interfaces of Lys63- and Lys48-linked ubiquitin chains

To identify the intersubunit interface of Lys63- and Lys48-linked diubiquitin, we compared the ^{15}N and ^1H chemical shifts of the main-chain amide groups of these diubiquitins with those of the unlinked ubiquitin derivatives (see Experimental procedures). Such chemical shift perturbation experiments have been frequently used to identify protein-protein interfaces. For the analyses, we labelled only one subunit of diubiquitin with ^{15}N nuclei (see Experimental procedures) so that a [$^1\text{H},^{15}\text{N}$]-correlation spectrum of the subunit-specific labelled sample would give cross-peaks attributable to the amide nuclei of the labelled subunit. Hereafter, the subunits of the polyubiquitin chains are numbered according to the convention of Thrower *et al.* (2000), that is, the subunit that contains a free main-chain carboxyl terminus is denoted subunit 1; the one whose C-terminal Gly76 is linked to a lysine of subunit 1 through the isopeptide bond is denoted subunit 2; and so on.

The two subunits of Lys48-linked diubiquitin displayed similar patterns of chemical shift perturbation at pH 6.8, except for the C-terminal three residues of subunit 2, which contain the isopeptide bonding site, Gly76 (Fig. 1C,D). Residues in strands $\beta 1$ – $\beta 5$ ($\beta 1$: residues 1–6, $\beta 2$: 12–17, $\beta 3$: 41–45, $\beta 4$: 48–49, $\beta 5$: 66–71), and



loops between strands $\beta 1$ and $\beta 2$, and between strands $\beta 3$ and $\beta 4$ of both ubiquitin subunits showed significant differences in their chemical shifts when compared with the unlinked ubiquitin derivatives. These residues are all confined to a surface of ubiquitin that centres on a hydrophobic patch composed of the side chains of Leu8, Ile44 and Val70 on the β -sheet (Fig. 1F). This observation suggests that a similar protein surface on each subunit interacts with each other at the interface.

We then analysed the residues at the intersubunit interface of Lys63-linked diubiquitin by chemical shift perturbation using subunit 1- and subunit 2-selective [^{15}N]-labelled diubiquitin samples. Chemical shift perturbation of either subunit of Lys63-linked diubiquitin was much less than that of either subunit of Lys48-linked diubiquitin (Fig. 1A,B). A significant chemical shift perturbation was observed only for the following residues, which are located at or adjacent to the Gly76–Lys63 isopeptide bond: Lys63 and Glu64 of subunit 1, and Arg74 and Gly76 of subunit 2 (Fig. 1E). Changes in these residues can be attributed simply to the chemical modification associated with formation of the isopeptide bond, and unlikely to non-covalent intersubunit contacts. Apart from these residues, the weighted chemical shift differences, δ_{wt} , associated with the isopeptide bond formation were less than 0.03 p.p.m. This is in clear contrast to Lys48-linked diubiquitin, whose 44 residues of subunit 1 and 32 of subunit 2 exhibited weighted chemical shift differences larger than 0.03 p.p.m. upon isopeptide bond formation. These results indicate that Lys63-linked diubiquitin does not have a well-defined non-covalent intersubunit interface distal from the linkage site. Furthermore, the chemical shift values were essentially invariant from pH 4.5 to pH 7.5, suggesting the

Figure 1 Chemical shift perturbations of Lys63- and Lys48-linked diubiquitin chains compared with those of the unlinked ubiquitin derivatives. (A, B) Lys63-linked diubiquitin; (C, D) Lys48-linked diubiquitin. A and C correspond to ^{15}N -labelled (indicated in grey) subunit 1, B and D to ^{15}N -labelled subunit 2 (see text for subunit naming convention). The weighted chemical shift differences, δ_{wt} , are shown (see Experimental procedures). (E, F) Ribbon representation of ubiquitin (PDB code: 1D3Z), with colour coding indicating the chemical shift perturbations observed for Lys63-linked diubiquitin (E) and Lys48-linked diubiquitin (F). Residues that exhibited weighted chemical shift differences (δ_{wt}) of more than 0.05 p.p.m. are coloured blue if they showed this difference only in subunit 1, green if only in subunit 2 and red if they showed this difference in both subunits. The side chains of the linkage-site residues in the Lys48- and Lys63-linked polyubiquitin chains and the hydrophobic patch-forming residues are also indicated.

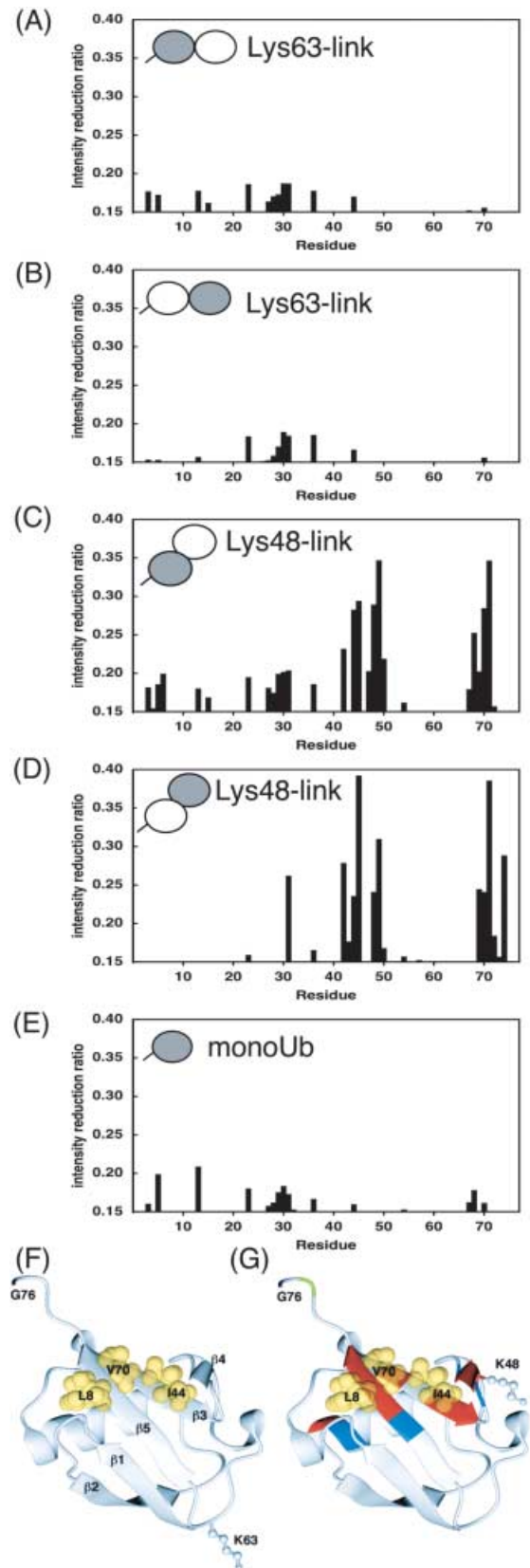
absence of conformational differences in this pH range (data not shown). This is also in clear contrast to Lys48-linked diubiquitin, whose subunit arrangement varies markedly over this pH range: namely, its conformation switches from closed to open with increasing pH (Varadan *et al.* 2002).

Cross-saturation experiments

Differences between the intersubunit interfaces of Lys63- and Lys48-linked diubiquitin chains were also examined by the cross-saturation method (Takahashi *et al.* 2000), using the subunit-selective [^{15}N , ^2H]-labelled diubiquitin samples. In this type of experiment, the aliphatic proton resonances of the non-labelled subunit of diubiquitin are irradiated using a radiofrequency field that saturates not only the aliphatic protons but also the amide protons of this subunit in a uniform manner, due to the spin diffusion effect. By contrast, the [^{15}N , ^2H]-labelled subunit is not directly irradiated by this radiofrequency field because the aliphatic protons are substituted by deuterium; however, amide proton resonances located at the subunit interface will be subsequently saturated through saturation transfer from the non-labelled subunit during the irradiation period. Therefore, signals of interfacial residues of the labelled subunit in the [^1H , ^{15}N]-correlation spectra should show time-dependent saturation effects.

Consistent with the chemical shift perturbation data, these cross-saturation experiments indicated that Lys63-linked diubiquitin has no stable non-covalent intersubunit interface (Fig. 2A,B). The effects of irradiation on the intensity of the backbone signals in [^1H , ^{15}N]-correlation spectra for Lys63-linked diubiquitin were smaller and

Figure 2 Cross-saturation experiments of Lys63- and Lys48-linked diubiquitin chains. (A–E) Plots of the reduction ratio of signal intensities originating from irradiation by a radiofrequency field (Nishida *et al.* 2003). Data arising from an irradiation time of 2.5 s are shown. (A, B) Lys63-linked diubiquitin; (C, D) Lys48-linked diubiquitin; (E) [^{15}N , ^2H]-labelled unlinked D77 (see text). (A) and (C) correspond to [^{15}N , ^2H]-labelled (indicated in grey) subunit 1 (B) and (D) to [^{15}N , ^2H]-labelled subunit 2. (F, G) Ribbon representation of ubiquitin (PDB code: 1D3Z), with colour coding indicating the cross-saturation effects observed for Lys63-linked diubiquitin (F) and Lys48-linked diubiquitin (G). Residues that exhibited a reduction rate of more than 0.2 are coloured blue if they showed this difference only in subunit 1, green if only in subunit 2 and red if they showed this difference in both subunits. The side chains of the linkage-site residues of the Lys48- and Lys63-linked polyubiquitin chains and the hydrophobic patch-forming residues are also indicated.



more uniform between residues than those observed for the Lys48-linked form (Fig. 2C,D). For both subunits of Lys63-linked diubiquitin, small but systematic effects of irradiation were observed for residues 23–35, which comprise the unique α helix (residues 23–34) of ubiquitin. This effect is unlikely to originate from cross-saturation between subunits, because similar effects were observed for residues in a [^{15}N , ^2H]-labelled unlinked ubiquitin derivative (Fig. 2E). These small effects of irradiation in Lys63-linked diubiquitin and the unlinked ubiquitin derivative are probably due to cross-saturation via water molecules or saturation from the residual aliphatic protons of [^{15}N , ^2H]-labelled subunit (Takahashi *et al.* 2000). Such effects were also observed for residues in the helix of subunit 1 of Lys48-linked diubiquitin (Fig. 2C). In summary, the results of both the chemical shift perturbation and the cross-saturation experiments indicate the absence of stable, non-covalent intersubunit contacts in Lys63-linked diubiquitin (Fig. 2F).

The cross-saturation experiments showed that, in contrast to Lys63-linked chains, Lys48-linked diubiquitin chains have a non-covalent intersubunit interface. Each subunit of the diubiquitin exhibited a similar pattern of cross-saturation effects, consistent with the results of the chemical shift perturbation experiment (Fig. 2C,D). These observations suggest that the same residues in each of the two subunits make up the interfacial contact site (Fig. 2G).

Intersubunit interfaces of Lys63-linked tetraubiquitin

We also analysed the intersubunit interactions of Lys63- and Lys48-linked tetraubiquitin chains by chemical shift perturbation experiments. We synthesized Lys63- and Lys48-linked tetraubiquitin chains in which either subunit 1 or 3 was specifically labelled with ^{15}N and measured the [^1H , ^{15}N]-correlation spectra, which enabled observation of only the labelled subunit in the tetraubiquitin chain.

These experiments indicate that neither subunit 1 nor subunit 3 of Lys63-linked tetraubiquitin possesses a stable non-covalent intersubunit interface. The positions of cross-peaks observed in the spectrum of tetraubiquitin with ^{15}N -labelled subunit 1 were nearly identical to those of Lys63-linked diubiquitin with ^{15}N -labelled subunit 1 and thus very similar with those of the unlinked ubiquitin derivative, except for Lys63 and Glu64 (Fig. 3A). None of the observed cross-peaks exhibited a weighted chemical shift difference of more than 0.03 p.p.m. between subunit 1 of the diubiquitin and subunit 1 of the tetraubiquitin, suggesting that the

conformation and contact surface of subunit 1 are nearly invariant in these chains.

Similar results were obtained for subunit 3 of the same chain. In the spectrum of tetraubiquitin with ^{15}N -labelled subunit 3, no cross-peak exhibited a weighted chemical shift difference of more than 0.03 p.p.m. as compared with the unlinked ubiquitin derivative, except for Lys63, Glu64, Arg74 and Gly76, which surround the linkage sites (Fig. 3B). The perturbations observed at or near Lys63 and Gly76 were similar to those observed for subunits 1 and 2 of Lys63-linked diubiquitin, respectively (Fig. 1A,B), suggesting that these effects are probably as a result of the chemical modification associated with formation of the isopeptide bond and unlikely to non-covalent intersubunit contacts. In summary, the results of chemical shift perturbation experiments indicate that, as in the diubiquitin chain, subunits 1 and 3 of Lys63-linked tetraubiquitin do not form a stable interface with any other subunit in the chain (Fig. 3E).

In contrast to the results for Lys63-linked chains, chemical shift perturbation experiments showed that Lys48-linked tetraubiquitin chains possess non-covalent intersubunit interfaces. Cross-peaks of several residues in both subunits 1 and 3 displayed much larger chemical shift perturbations than did the corresponding subunits of the Lys63-linked tetraubiquitin chain, indicating that the subunits of Lys48-linked chains have non-covalent contact areas (Fig. 3C,D,F). The patterns of chemical shift perturbations observed for subunits 1 and 3 were similar to each other and to the two subunits of Lys48-linked diubiquitin (Fig. 1C,D,F). In particular, the [^1H , ^{15}N]-correlation spectra of subunit 1 of the diubiquitin and subunit 1 of the tetraubiquitin chains were nearly identical: all of the main-chain cross-peaks, except for Arg74, showed a weighted chemical shift difference of less than 0.03 p.p.m. (data not shown). Taken together, these observations indicate that the intersubunit surfaces and modes of interaction are similar in the Lys48-linked chains of di- and tetraubiquitin.

Structural parameters and distance distribution functions of polyubiquitin chains from small-angle X-ray scattering

In order to obtain structural parameters such as the radius of gyration (R_g) and the maximum molecular distance (D_{max}), we measured small angle X-ray scattering (SAXS) intensities of the di- and tetraubiquitins linked through Lys63 or Lys48 in solution (Table 1). All R_g values estimated from the Guinier plots were in good agreement with those estimated from the distance distribution function, $P(r)$. This indicates that the R_g values were

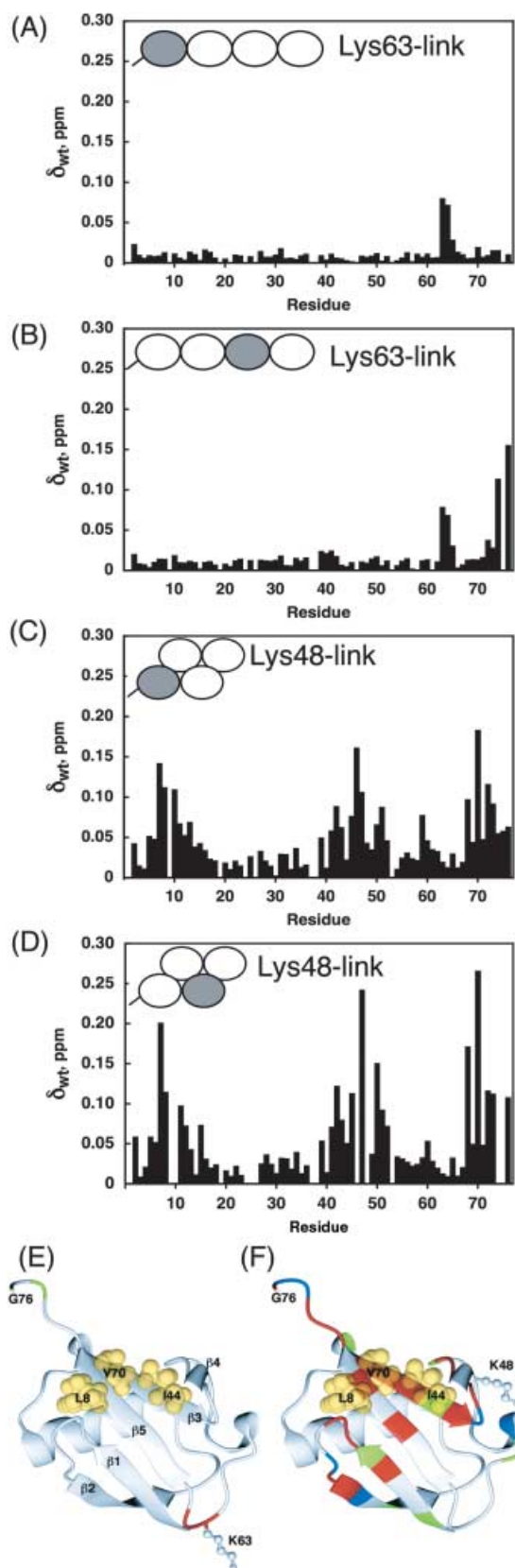


Table 1 R_g and D_{max} values estimated from Guinier plots and $P(r)$ functions

| | $P(r)$ function | | Guinier plot |
|-----------------------------|------------------|----------------------|------------------|
| | $R_g/\text{\AA}$ | $D_{max}/\text{\AA}$ | $R_g/\text{\AA}$ |
| Lys63-linked diubiquitin | 19.0 ± 0.3 | 62 ± 3 | 19.0 ± 0.8 |
| Lys48-linked diubiquitin | 17.4 ± 0.3 | 52 ± 2 | 17.4 ± 0.8 |
| Lys63-linked tetraubiquitin | 29.1 ± 0.5 | 95 ± 2 | 29.1 ± 0.8 |
| Lys48-linked tetraubiquitin | 25.8 ± 0.7 | 90 ± 4 | 25.7 ± 0.8 |

correctly estimated and that all of the polyubiquitin chains were mono-dispersed in solution.

R_g and D_{max} , estimated from the $P(r)$ function of Lys63-linked diubiquitin were 19.0 ± 0.3 and 62 ± 3 Å, respectively. These values were larger than those of Lys48-linked diubiquitin (17.4 ± 0.3 and 52 ± 2 Å, respectively), indicating that Lys63-linked diubiquitin is more elongated than Lys48-linked diubiquitin. This elongation was more apparent in Lys63-linked tetraubiquitin: the respective R_g and D_{max} of Lys63-linked tetraubiquitin were 29.1 ± 0.7 and 95 ± 2 Å, which were much larger than the respective values of Lys48-linked tetraubiquitin (25.8 ± 0.5 and 90 ± 4 Å).

Figure 4 shows the $P(r)$ functions of the di- and tetraubiquitins linked through Lys63 and Lys48. The distance distributions of the Lys48-linked di- and tetraubiquitin and Lys63-linked diubiquitin chains showed single Gaussian profiles with respect to the distance r . These profiles are characteristic of globular proteins. In contrast, the distance distribution of the Lys63-linked tetraubiquitin chain had two broad peaks at $r = 25$ Å and $r = 50$ Å, with a skirt extending to 95 Å. These

Figure 3 Chemical shift perturbations of Lys63- and Lys48-linked tetraubiquitin chains as compared with the unlinked ubiquitin derivatives. (A, B) Lys63-linked tetraubiquitin; (C, D) Lys48-linked tetraubiquitin. (A) and (C) correspond to ^{15}N -labelled (indicated in grey) subunit 1; (B) and (D) to ^{15}N -labelled subunit 3. The weighted chemical shift differences, δ_{wt} , are shown (see Experimental procedures). (E, F) Ribbon representation of ubiquitin (PDB code: 1D3Z), with colour coding indicating the chemical shift perturbations observed for Lys63-linked tetraubiquitin (E) and Lys48-linked tetraubiquitin (F). Residues that exhibited weighted chemical shift differences (δ_{wt}) of more than 0.05 p.p.m. are coloured blue if they showed this difference only in subunit 1, green if only in subunit 3 and red if they showed this difference in both subunits. The side chains of the linkage-site residues of the Lys48- and Lys63-linked polyubiquitin chains and the hydrophobic patch-forming residues are also indicated.

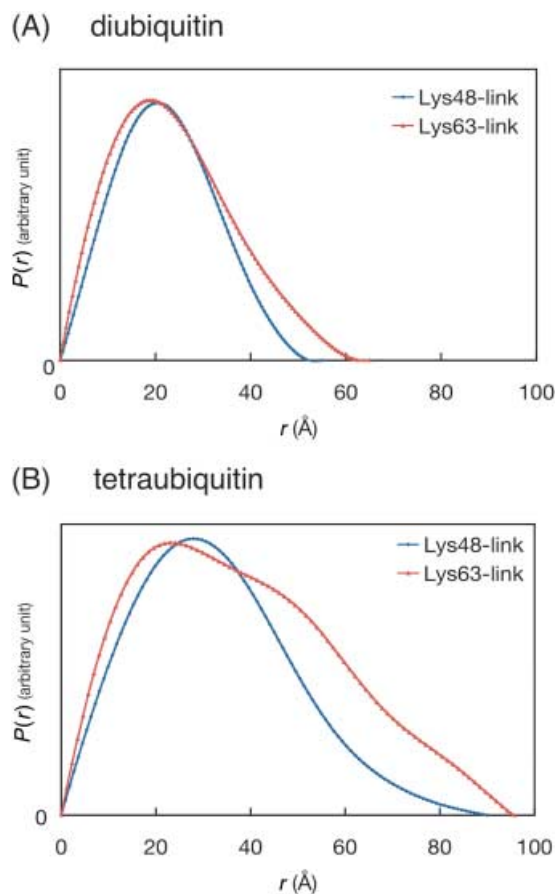


Figure 4 The distance distribution function, $P(r)$, of diubiquitin (A) and tetraubiquitin (B) chains. Those of Lys63-linked chains are shown in red, and those of Lys 48-linked chains in blue. The $P(r)$ functions were calculated by an indirect Fourier transform algorithm using the program GNOM (Semenyuk & Svergun 1991).

results show that Lys48-linked di- and tetraubiquitin and Lys63-linked diubiquitin are globular proteins, whereas Lys63-linked tetraubiquitin has a relatively extended and flexible structure rather than a globular structure.

Discussion

Conformation of Lys63-linked chains

Both the chemical shift perturbation and the cross-saturation experiments indicate the absence of a well-defined non-covalent interface in Lys63-linked di- and tetraubiquitin chains, which is in clear contrast to Lys48-linked chains. This means that the Lys63 chains probably lack well-defined quaternary structure—that is, a stable arrangement of their subunits—and that instead they assume an array like beads on a string, in which the

globular body of each ubiquitin subunit (residues 1–71) is tethered by its C-terminal tail (residues 72–76) to the side-chain terminus of Lys63 in the adjacent ubiquitin subunit.

The variation in the orientation of subunits of Lys63-linked diubiquitin seems to be limited to some extent, as shown by the distance distribution of the Lys63-linked diubiquitin chain (Fig. 4A), which shows a near single Gaussian profile. This observation suggests that the five-residue tail is not totally flexible, or is not long enough to give a large freedom of relative orientations of the two subunits. In contrast, when the number of linkers in the chain increases, it displays an apparent flexible character: The distance distribution of the Lys63-linked tetraubiquitin chain, which has three linkers, is markedly different from those of the Lys48-linked di- and tetraubiquitin chains, but is similar to that of Grb2 (Yuzawa *et al.* 2001) (Fig. 4B). Grb2 is composed of three domains (nSH3, SH2 and cSH3), and its solution structure is well simulated by an ensemble of multiple conformations with relatively open structures. These observations suggest that, in Lys63-linked chains, the C-terminal tails that tether the body of ubiquitin subunits is not rigid but display a certain degree of flexibility.

The chemical shift values of the C-terminal tail residues preceding the linkage site Gly76 (residues 72–75) in subunit 3 of Lys63-linked tetraubiquitin exhibited much less chemical shift perturbation than did the corresponding residues in the Lys48-linked chains (Figs 1 and 3). This observation suggests that the conformations of the tails of the subunits in the Lys63-linked tetraubiquitin chain do not differ radically from that of the C-terminal tail in unlinked ubiquitin, which has been shown to be flexible in solution (Cornilescu *et al.* 1998).

Our present data suggest that the hydrophobic patch comprising the side chains of Leu8, Ile44 and Val70 of ubiquitin is not involved in the intersubunit interface of Lys63-linked chains (Figs 1E, 2F, 3E). This patch has been shown to serve as a major contact surface for numerous proteins, including the proteasome, ubiquitin-interacting motifs (UIMs), CUE domains, and UBA domains (Beal *et al.* 1998; Young *et al.* 1998; Withers-Ward *et al.* 2000; Mueller & Feigon 2002; Shekhtman & Cowburn 2002; Kang *et al.* 2003; Prag *et al.* 2003). It also acts as the major intersubunit interface in Lys48-linked chains [(Cook *et al.* 1992; Phillips *et al.* 2001; Varadan *et al.* 2002) and our present data], which raises the question of why this patch does not interact with its counterpart in the adjacent subunit in Lys63-linked chains. Preliminary modelling of Lys63-linked diubiquitin under the assumption that the C-terminal tails, but

not the structural bodies, of the ubiquitin subunits are flexible suggests that the same intersubunit interaction between the two hydrophobic patches as Lys48-linked diubiquitin in the crystal is not sterically feasible (data not shown). This is because the side chain of Lys63 is located on the opposite side to the hydrophobic patch, and the C-terminal tail is not long enough to establish the homophilic intersubunit contacts between the two patches. Therefore, it seems reasonable to assume that the impossibility of such an interaction between the hydrophobic patches may cause the absence of stable non-covalent intersubunit interactions in Lys63-linked chains.

Conformation of Lys48-linked chains

Our chemical shift perturbation and cross-saturation experiments on Lys48-linked chains have shown that the β -sheet surface centred on the hydrophobic patch acts as the intersubunit interface in all subunits examined, namely, in both subunits of diubiquitin and in subunits 1 and 3 of tetraubiquitin. This result suggests that in solution these polyubiquitin chains possess quaternary structure, in which the subunit arrangement is defined by both covalent intersubunit linkages and non-covalent contacts mediated by the β -sheet interface. Our data are generally consistent with previous chemical shift perturbation data for Lys48-linked di- and tetraubiquitin chains (Varadan *et al.* 2002).

For Lys48-linked diubiquitin, the residues identified at the intersubunit interface through the chemical shift perturbation and cross-saturation experiments are consistent with those seen in its crystal structure (Cook *et al.* 1992), and the solution structure model derived from the chemical shift perturbation, ^{15}N relaxation and residual dipolar coupling data (Varadan *et al.* 2002). All of these studies indicate that the surface of each ubiquitin subunit centring on the hydrophobic patch acts as the intersubunit interface. As this hydrophobic patch has also been shown to function as the binding site for the 26S proteasome and S5a, the lack of binding between diubiquitin and proteasome or S5a (Young *et al.* 1998) can be explained, at least in part, by the sequestration of this surface by intersubunit interaction.

The chemical shift perturbation data revealed that in Lys48-linked tetraubiquitin, similar surfaces of subunits 1 and 3 act as the intersubunit interfaces in a similar manner to that of the subunits of Lys48-linked diubiquitin. The presence of quaternary structure of the tetraubiquitin is also supported by our SAXS data. Currently, the topology of subunit interactions within the Lys48-linked tetraubiquitin chain is unclear. One of the three structural models of Lys48-linked tetraubiquitin

that have been derived from two different crystal forms [(Cook *et al.* 1994; Phillips *et al.* 2001) and PDB code: 1F9J] assumes that subunits 1 and 4, and subunits 2 and 3 within the same chain bind to each other through their hydrophobic patches in a manner similar to that seen in the crystal structure of diubiquitin. Alternatively, it may be possible that subunits 1 and 2 and subunits 3 and 4 contact each other, resulting in a conformation consisting of successive diubiquitin chains. Further structural information is required to differentiate between these possibilities. The ^{15}N longitudinal and transverse relaxation times, T_1 and T_2 , of subunits 3 in Lys48-linked and Lys63-linked tetraubiquitins were determined at the ^{15}N frequency of 50.7 MHz and 303 K. For Lys48-linked tetraubiquitin, the 10% trimmed means and distributions for all the mainchain ^{15}N nuclei are 774 ± 119 ms for T_1 and 64.7 ± 8.0 ms for T_2 . Those for Lys63-linked tetraubiquitin are 638 ± 88.1 ms for T_1 and 64.1 ± 8.0 ms. Therefore, we could find no statistically significant difference between the relaxation parameters of the subunits in the tetraubiquitins under the experimental condition.

While this manuscript was in preparation, a structural study of Lys63-linked diubiquitin was published (Varadan *et al.* 2004). The chemical shift perturbation experiment described in that paper is in good agreement with our data shown in Fig. 1A,B, and indicates that there is no clear interface between ubiquitin subunits. The authors also analysed the relative orientation of subunits of the diubiquitin by using residual dipolar coupling and relaxation data. They found that, although the intersubunit linker is flexible, the two subunits in diubiquitin tumble as one entity. This is also consistent with our SAXS data shown in Fig. 4A.

Conclusion

Our NMR studies of the intersubunit interfaces of di- and tetraubiquitin chains indicate that the interfaces of Lys63-linked chains do not comprise stable non-covalent contacts, unlike those of Lys48-linked chains, which are formed by a hydrophobic patch of Leu8, Ile44 and Val70 residues. Therefore, consistent with our SAXS data, these two forms of polyubiquitin chain adopt very different conformations, which probably accounts for their distinct roles in cell signalling. Our data therefore provide a framework for the specificity of polyubiquitin chains toward their downstream effectors and recently identified deubiquitinating enzymes (Brummelkamp *et al.* 2003; Kovalenko *et al.* 2003; Trompouki *et al.* 2003), both of which mediate a wide array of cellular events.

Experimental procedures

Materials

Ubiquitin and E2–25K were expressed in *Escherichia coli* and purified as described (Piotrowski *et al.* 1997). E1 was expressed in Sf9 insect cells and purified. Yeast ubiquitin hydrolase was a gift from Dr Yutaka Ito. A plasmid for expressing the yeast Mms2-Ubc13 complex, which functions as an E2 enzyme for the synthesis of Lys63-linked polyubiquitin chains, was constructed. cDNAs encoding Mms2 and Ubc13 were obtained from the genomic DNA of *Saccharomyces cerevisiae*. Constructs for the ubiquitin derivatives K48C, D77 and K63C were prepared with a GeneEditor *in vitro* site-directed mutagenesis system (Promega, Madison, WI), according to the manufacturer's instructions. Non-isotopically enriched, ^{15}N - or [^{15}N , ^2H]-labelled ubiquitin derivatives were expressed in *E. coli* strain BL21 (DE3) grown in LB or M9 broth, or in *E. coli* grown in OD 1 DN (^{15}N , ^2H) (Silantes GmbH), respectively, and then purified by chromatography.

Synthesis of Lys48- and Lys63-linked polyubiquitin and subunit-specific isotope labelling

Lys48-linked di- and tetraubiquitin chains were synthesized *in vitro* as described (Piotrowski *et al.* 1997; Thrower *et al.* 2000). For the synthesis of Lys63-linked di- and tetraubiquitin chains, yeast Mms2-Ubc13 complex was used instead of E2–25K as described (Hofmann & Pickart 2001). Subunit-specific isotope labelling of the polyubiquitin chains was achieved by using a ^{15}N - or [^{15}N , ^2H]-labelled ubiquitin derivative (K48C, D77 or K63C) as a template. The subunits of the polyubiquitin chains are numbered according to the convention of Thrower *et al.* (2000). Namely, the subunit at the proximal end of the chain is numbered 1 and the other subunits are numbered sequentially. For Lys48- and Lys63-linked diubiquitins, both subunit 1- and subunit 2-specific ^{15}N - and $^{15}\text{N}/^2\text{H}$ -labelled chains were prepared. For Lys48- and Lys63-linked tetraubiquitin chains, subunit 1- and subunit 3-specific ^{15}N -labelled chains were prepared. The length of these polyubiquitin chains was analysed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and mass spectrometry.

NMR spectroscopy

All NMR studies were performed at 303 K on a Bruker DRX-500 spectrometer equipped with a cryo-probe. [^{15}N , ^1H]-correlation (HSQC) spectra for chemical shift perturbation and cross-saturation experiments were acquired with spectral widths of 1600 Hz and 8000 Hz, and 128 and 1024 complex points, in the ^{15}N and ^1H dimensions, respectively.

For chemical shift perturbation experiments, the [^{15}N , ^1H]-HSQC spectra of Lys48-linked and Lys63-linked di- and tetraubiquitin chains were measured and compared with those of the corresponding unlinked ubiquitin derivatives. For example, the spectra of subunit 1- and subunit 2-labelled diubiquitins linked through Lys63 were compared with those of ubiquitins D77 and K63C, respectively. Samples of diubiquitin comprised 0.1 mM

protein in 20 mM potassium phosphate buffer pH 6.8, 1 mM EDTA and 90% $\text{H}_2\text{O}/10\%$ D_2O . Those of tetraubiquitin comprised 0.1 mM protein in the same buffer. The weighted chemical shift difference of the ^1H and ^{15}N resonances, δ_{wt} , was calculated for each residue as $\{[\delta_{\text{H}} + (\delta_{\text{N}}/5)^2]\}^{1/2}$, where δ_{H} and δ_{N} are the differences in p.p.m. (Garrett *et al.* 1997; Foster *et al.* 1998). The cross-saturation experiments were performed by using the pulse scheme described in Takahashi *et al.* (2000). The WURST-2 decoupling scheme with an adiabatic factor Q_0 of 1 was used to saturate aliphatic protons between 0.9 and 3.5 p.p.m. The spectra with irradiation times of 0.5, 1.0, 1.5, 2.0 and 2.5 s were acquired. The ^{15}N relaxation parameters were measured as described in (Ikegami *et al.* 2000) using samples of subunit 3-specific ^{15}N -labelled Lys48-linked or Lys63-linked tetraubiquitin comprised 0.1 mM protein in 20 mM potassium phosphate buffer pH 6.8, 1 mM EDTA and 90% $\text{H}_2\text{O}/10\%$ D_2O . T_1 relaxation was measured with delays of 10, 100, 200, 400, 600 and 800 ms and T_2 relaxation with delays of 10, 20, 30, 40, 50 and 60 ms. The uncertainties of the relaxation parameters were estimated from the differences of two spectrum sets. All spectra were processed by nmrPipe/nmrWish software (Delaglio *et al.* 1995).

SAXS measurement

All protein solutions were prepared in the same buffer used for NMR measurements. The protein concentration of each solution ranged from 12 to 5 mg/mL. The X-ray source was 0.1×0.1 mm, with a take-off angle of 6° to a 0.1×1 mm spot on the copper anode of a Rigaku ultra-fine focus rotating-anode X-ray generator (FR-D) operated at 50 kV and 60 mA. X-rays focused with an Osmic Confocal Max-FluxTM mirror were used to irradiate the sample solution through two pinhole slits and one scatter suppressor slit. The beam size at the sample position was approximately 0.2×0.2 mm. Each sample solution (15 μL) was introduced into a thin-walled quartz capillary (1 mm ϕ) fixed in the sample holder and maintained at 297 ± 0.5 K. The scattered X-rays were recorded on a Fuji Imaging Plate (IP) with an exposure time of 3 h at 500 mm from the sample position. The size and positional resolution of the IP were 12×12 cm and 50 μm , respectively.

The scattering X-rays recorded on the IP were subjected to a circular average to obtain a one-dimensional intensity profile as a function of q ($q = 4\pi \sin \theta/\lambda$, 2θ : scattering angle, λ : wavelength of X-ray), $I(q)$. The $I(q)$ data were corrected for background scattering from the corresponding buffer solution. Interparticle interference effects were corrected using the program GNOM (Semenyuk & Svergun 1991). Because the intensity profile did not change significantly on de-smearing for the effects of beam divergence and wavelength spread, these corrections were not applied.

The radius of gyration R_g was estimated from both the Guinier plot and the distance distribution function, $P(r)$. All Guinier plots could be approximated by straight lines in a smaller-angle scattering region where $qR_g < 1.2$. The $P(r)$ function was calculated using $I(q)$ data in a q -range from 0.02 to 0.20 \AA^{-1} by an indirect Fourier transform algorithm using the program GNOM (Semenyuk & Svergun 1991). The maximum molecular distance, D_{max} , was estimated from the $P(r)$ function as the distance r where $P(r) = 0$ (Glatter & Kratky 1982).

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