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The mechanical stability of ubiquitin is linkage dependent

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Ubiquitin chains are formed through the action of a set of enzymes that covalently link ubiquitin either through peptide bonds or through isopeptide bonds between their C terminus and any of four lysine residues. These naturally occurring polyproteins allow one to study the mechanical stability of a protein, when force is applied through different linkages. Here we used single-molecule force spectroscopy techniques to examine the mechanical stability of N-C–linked and Lys48-C–linked ubiquitin chains. We combined these experiments with steered molecular dynamics (SMD) simulations and found that the mechanical stability and unfolding pathway of ubiquitin strongly depend on the linkage through which the mechanical force is applied to the protein. Hence, a protein that is otherwise very stable may be easily unfolded by a relatively weak mechanical force applied through the right linkage. This may be a widespread mechanism in biological systems.

Naturally occurring ubiquitin chains are found to be either N-C–linked or linked between their C terminus and an exposed lysine residue that can be Lys63, Lys48, Lys29 or Lys11, wherein each ubiquitin chain is assembled with a single type of linkage between the ubiquitin monomers[1–4]. These ubiquitin chains offer a new opportunity for studying the mechanical stability of a protein under conditions in which the mechanical force is applied to the protein through different linkages. Our studies made use of single-molecule force spectroscopy[5,6] and SMD techniques[7] to examine how the type of linkage affects the mechanical stability of native ubiquitin chains. Single-molecule techniques have been used to examine the mechanical stability of native proteins such as titin[5] and fibronectin[6]. However, the heterogeneous mixture of modules found in these proteins made it difficult to assign mechanical properties to individual modules. The engineering of proteins made of tandem repeats of an identical module, called polyproteins, has permitted a module-by-module dissection of the mechanical properties of native proteins[8–15]. However, polyproteins are rare in nature, with the exception of ubiquitin. The different polyubiquitin linkages have important functions in cell signaling and target proteins to different pathways. For example, protein substrates tethered by a Lys48-linked polyubiquitin are specifically tagged for degradation by the proteasome[16]. Here we use single-molecule atomic force microscopy (AFM) techniques[10,11] to study the effect of a mechanical force on polyubiquitin chains. We study the mechanical properties of two types of ubiquitin polyproteins, an N-C–linked polyprotein composed of nine repeats (N-Ub_9; **Fig. 1a**) and a Lys48-C–linked polyprotein ranging from two to seven repeats (Lys48-$Ub_{2–7}$).

RESULTS

The mechanical stability of N-C–linked ubiquitin chains

Stretching single N-C–linked polyproteins generates force-extension curves with a characteristic sawtooth pattern fingerprint with equally spaced unfolding force peaks (**Fig. 1b**). The average force required to unfold N-C–linked ubiquitin at a pulling speed of 400 nm s^{-1} is 203 ± 35 pN (**Fig. 1c**). A Monte Carlo simulation of forced unfolding[9] can qualitatively reproduce the magnitude and the distribution of the force required to unfold ubiquitin (blue trace in **Fig. 1c**). This simulation was made assuming two-state unfolding for ubiquitin[17], using reported values for the spontaneous unfolding rate measured with guanidinium chloride and extrapolated to zero denaturant concentration (4×10^{-4} s^{-1})[17]. The distance from the native state to the transition state was assumed to be Δx = 0.25 nm, which is similar to that found for the mechanical unfolding of titin immunoglobulin domains[9].

The force-extension relationship leading up to each unfolding peak was fitted with the worm-like chain (WLC) model of polymer elasticity[18] (for example, blue lines in **Fig. 1b**). Fits with the WLC measure the persistence length p and the contour length L_c. Fits to two consecutive force peaks measure the increase in contour length caused by the unfolding of a single ubiquitin molecule (ΔL_c, **Fig. 1b**). We measure an average of ΔL_c = 24 ± 5 nm (**Fig. 1d**). A folded ubiquitin measures 3.8 nm from its termini. An unfolded ubiquitin should be ~27.4 nm long (76 residues × 0.36)[19] when fully extended. Hence, unfolding should cause an increase in contour length of ΔL_c = 23.6 nm, in close agreement with our experimental results.

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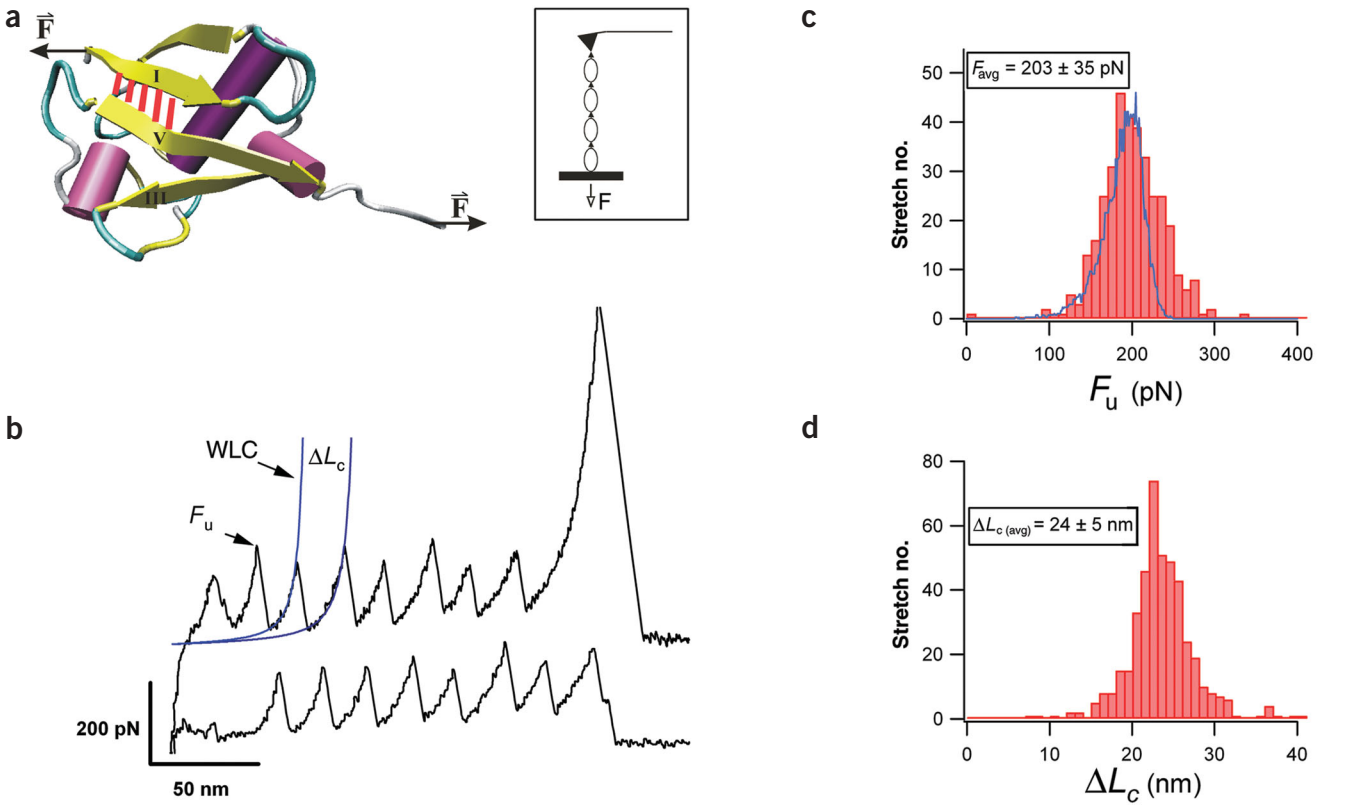

Figure 1 Mechanical properties of N-C polyubiquitin. (**a**) Ribbon diagram showing the α/β structure of ubiquitin. In N-C polyubiquitin the monomers are covalently linked to each other through the N and C termini. The backbone hydrogen bonds between the I and V β-strands (orange bars) may form the first point of mechanical resistance when exposed to a stretching force (arrows). (**b**) Stretching N-C polyubiquitin results in a characteristic sawtooth-like force-extension relationship, as shown by two typical recordings. The peak value of each force peak, F_u, corresponds to the force needed to unfold a ubiquitin domain. The force-extension relationships can be well described by the WLC model for polymer elasticity (blue lines). ΔL_c is the increase in contour length of the protein upon domain unfolding. (**c**) Unfolding force frequency histogram obtained at an average pulling speed of 0.3 nm ms^{-1} (range 0.25–0.41 nm ms^{-1}). The mean unfolding force is 203 ± 35 pN (n = 549). Blue line: a Monte Carlo simulation of the unfolding forces (1,000 trials) of eight ubiquitin domains placed in series, with parameters Δx_u = 0.25 nm and $\alpha_0 = 4 \times 10^{-4}$ s^{-1}. The unfolding rate at zero force, α_0, is identical to the chemical unfolding rate at zero denaturant. (**d**) A contour length increment frequency histogram for N-C polyubiquitin shows a mean value of ΔL_c = 23.6 ± 4 .5 nm (n = 441).

The mechanical stability of Lys48-C–linked ubiquitin chains

In contrast to N-C–linked polyubiquitin, Lys48-C–linked polyubiquitin showed a much lower mechanical stability (**Fig. 2**). In this case, the sawtooth patterns that we observed in the force-extension curves obtained for Lys48-C–linked polyubiquitin were smaller and had much shorter spacing. The average unfolding force was only 85 ± 20 pN at a pulling rate of ~300 nm s^{-1} (**Fig. 2b**), and the increment in contour length was only ΔL_c = 7.8 ± 2.8 nm. The smaller increase in ΔL_c clearly fingerprints a Lys48-C–linked polyubiquitin. There are only 29 amino acids between Lys48 and C-terminal Gly76. The distance between these two residues (carbonyl carbon of Gly76 and ε-amino nitrogen of Lys48) in the folded structure is ~3.1 nm (ref. 20). The fully extended contour length of the 29 amino acids is calculated to be L_c = 10.4 nm. Hence, we estimate that unfolding of a Lys48-C–linked polyubiquitin extends the contour length by ΔL_c ~7.3 nm, in agreement with our observations.

It is improbable that the large difference in mechanical stability between N-C–linked and Lys48-C–linked ubiquitin chains could have been anticipated from the study of thermodynamic stability by chemical or thermal denaturation, because thermodynamic stability is a poor predictor of mechanical stability[21] and the ubiquitin fold is unchanged by the type of linkage[22]. However, given the lack of measurements of thermodynamic stability for polyubiquitins, it is still possible that the observed changes may partially result from a destabilizing effect of the type of linkage. Another caveat arises from the possibility that the different linkages are associated with specific tertiary and/or quaternary interactions that could affect the mechanical properties of the polyubiquitin protein[23].

The kinetics of forced unfolding of ubiquitin chains

The average force required to trigger the mechanical unfolding of a protein has been shown to be dependent on the rate at which the force is applied[6,9]. These measurements are useful because, assuming a simple two-state model for unfolding, we can use Monte Carlo simulations to estimate the unfolding rate at zero force, α_0, and the position of the transition state, Δx (refs. 6,9). We have found that the force required to unfold Lys48-C–linked polyubiquitin is weakly dependent on the pulling rate, in contrast to the situation with N-C–linked polyubiquitin chains, which have a strong rate dependency (**Fig. 3**).

We simulated the mechanical unfolding of these proteins using the Monte Carlo technique (solid lines in **Fig. 3b**), using a fixed value for $\alpha_0 = 4 \times 10^{-4}$ s^{-1} in both cases but adjusting the value of Δx for best fit. The value of α_0 that we used in the simulations is the same rate of unfolding that had been determined using chemical denaturants[17]. The values of Δx that best described our data were Δx = 0.25 nm for N-C–linked polyubiquitin and Δx = 0.63 nm for Lys48-C–linked

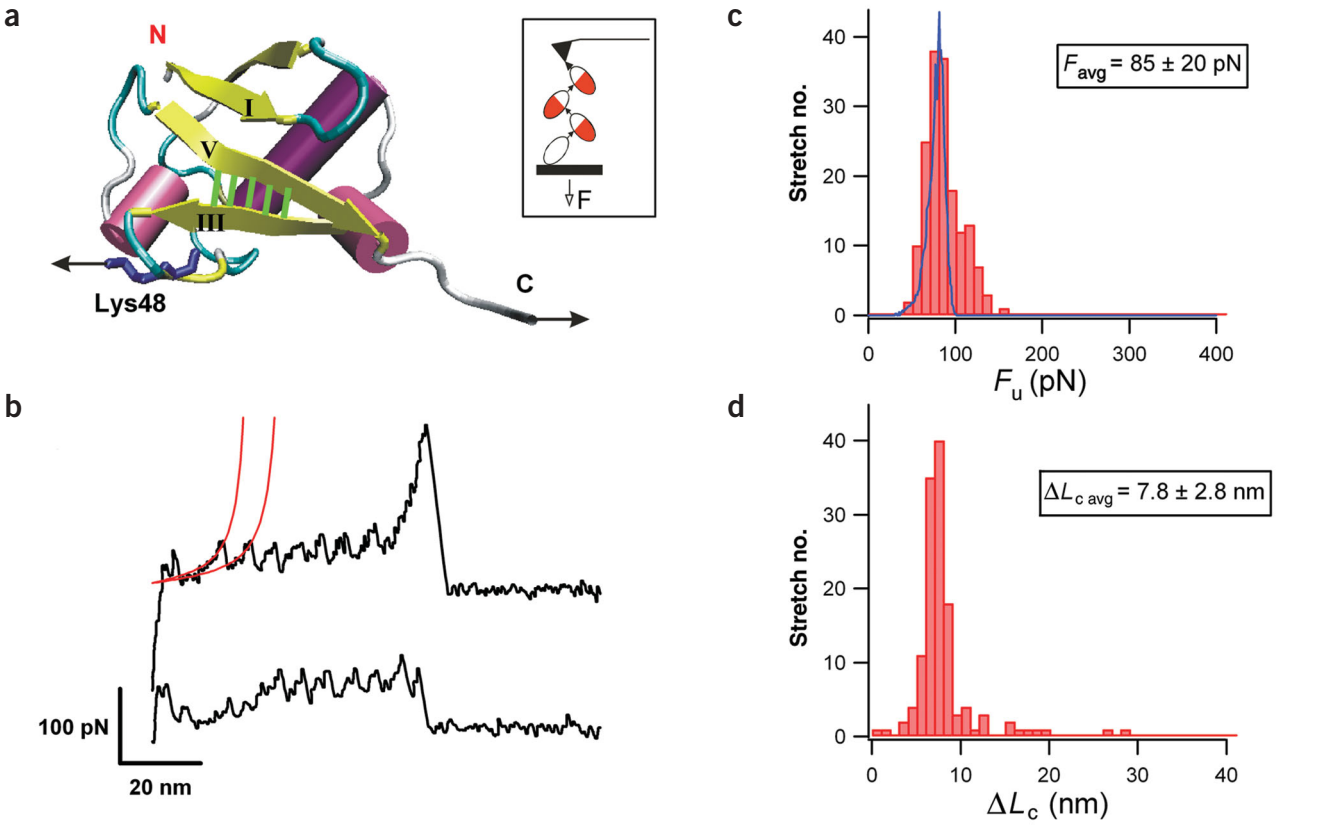

Figure 2 The linkage between domains markedly affects the mechanical properties of ubiquitin. (**a**) In the Lys48-C polyubiquitin the monomers are linked between Lys48 of one domain and the C terminus of the next domain. The green bars represent backbone hydrogen bonds between the III and V β-strands. This patch of hydrogen bonds is probably the first point of mechanical resistance when the domain is exposed to a mechanical force (represented by the arrows). (**b**) Two examples of force-extension relationships obtained for Lys48-C polyubiquitin proteins. The unfolding forces and contour length increments are both substantially smaller than those observed for N-C polyubiquitin. The red lines correspond to fits of the WLC equation to the force extension. (**c**) Unfolding force frequency histogram for the Lys48-C polyubiquitin obtained at an average pulling speed of 0.3 nm ms^{-1} (range 0.28–0.31 nm ms^{-1}). The mean unfolding force is 85 ± 20 pN (n = 166). The blue line corresponds to a Monte Carlo simulation of the unfolding forces (1,000 trials) of eight domains placed in series using Δx_u = 0.63 nm and $\alpha_0 = 4 \times 10^{-4}$ s^{-1}. (**d**) Contour length increment frequency histogram for Lys48-C polyubiquitin shows a mean value of ΔL_c = 7.8 ± 2.8 nm (n = 127).

polyubiquitin. The large increase in the position of the transition state explains the decrease in mechanical stability[24] observed for the Lys48-C–linked polyproteins, because the effect of an applied force on the unfolding rate is exponentially dependent on the value of Δx (ref. 25). Although we can explain the speed dependency of the unfolding forces of the different linkages by assuming a single value of α_0, the use of Monte Carlo simulations to estimate these parameters has a low sensitivity[9,26]; therefore, the values of α_0 and Δx used in these Monte Carlo simulations can only be taken as rough estimates. A more precise determination of the ubiquitin unfolding rate constants may be obtained in the future from force-clamp experiments that resolve many of the uncertainties inherent to sawtooth pattern data[27]. Nonetheless, it is notable that, given these Monte Carlo estimates, the amount of work necessary to mechanically unfold ubiquitin, $W_{unfold} = F\Delta x$, is independent of the linkage. For example, for a Lys48-C linkage we measure ~W_{unfold} = 54 pN nm, whereas for an N-C linkage we measure W_{unfold} = 51 pN nm. This similarity remains true for all speeds measured.

Simulations of the mechanical unfolding of ubiquitin

The process of unfolding ubiquitin by an external force was simulated by SMD[7,28,29], a technique that allows an atomic description of the crucial events involved during the mechanical stretching process. We have simulated the stretching of ubiquitin molecules between their C-terminal end and Lys63, Lys48 and the N terminus. As the force-extension curves show (**Fig. 4a**), after unfolding, ubiquitin becomes a linear polypeptide that is stretched at increasingly high forces, as evidenced by the large forces developed toward the end of the simulated extensions. The WLC model of polymer elasticity predicts that, after ubiquitins unfold and the force reaches 2 nN, these polypeptides would have extended to 97% of their contour length (using a persistence length of 0.4 nm). Beyond 2 nN, the observed linear elongation results from bond stretching. We fitted this linear region and extrapolated to a force of 2 nN to estimate the contour length increment caused by unfolding for each form of ubiquitin (**Fig. 4a**, dashed lines and arrows). The SMD simulations predict that upon unfolding there is an increase in contour of ΔL_c = 24.4 nm for N-C–linked, ΔL_c = 7.8 nm for Lys48-C–linked and ΔL_c = 1.5 nm for Lys63-C–linked ubiquitin. These predictions are in excellent agreement with our experimental data (**Figs. 1d** and **2d**). The simulation with Lys63-C–linked ubiquitin demonstrated the futility of attempting to stretch this polyprotein given that it showed a monotonic increase in force due to the absence of unfolding, and it would cause an increase in contour length of only ~1.5 nm per ubiquitin, which is our current resolu-

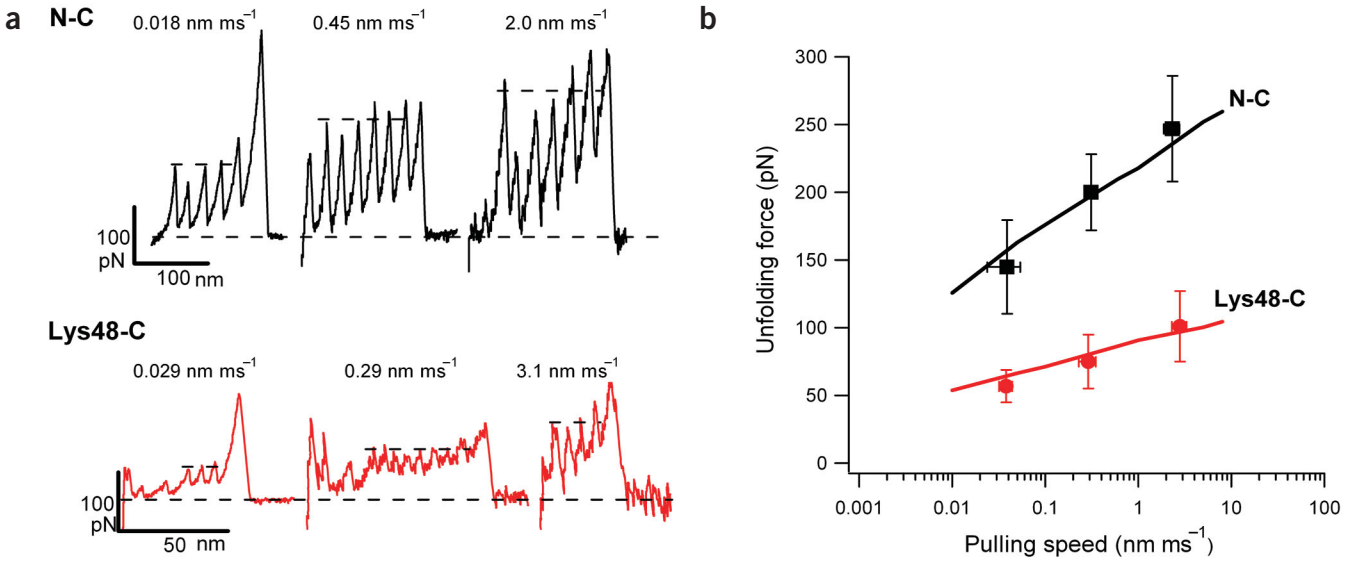

Figure 3 The mechanical unfolding forces of ubiquitin chains depends on the rate at which they are pulled. (**a**) Force-extension curves of N-C–linked (black traces) and Lys48-C–linked (red traces) polyubiquitin at different pulling speeds. (**b**) Plot of the average unfolding force as a function of the pulling speed for N-C–linked (black squares) and Lys48-C–linked (red squares) polyubiquitin. The solid lines correspond to Monte Carlo simulations with $\Delta x_u = 0.25$ nm and $\alpha_0 = 4 \times 10^{-4}$ s^{-1} for N-C–linked (black line) and $\Delta x_u = 0.63$ nm and $\alpha_0 = 4 \times 10^{-4}$ s^{-1} for Lys48-C–linked (red line) polyubiquitin. We used the same value of α_0 for both N-C–linked and Lys48-C–linked polyubiquitin to show that it is possible to explain the changes in mechanical stability using the same values of spontaneous unfolding rates for all linkages.

tion. Hence, the SMD simulations predict that mechanical stretching of Lys63-linked ubiquitin chains do not trigger ubiquitin unfolding and would cause the force to rise rapidly on extension of the protein. These simulations show major differences between the differently linked ubiquitin chains.

Figure 4b shows an amplified detail of the main energy barrier to unfolding predicted by SMD for ubiquitin stretched between their C-terminal end and either Lys48 or the N terminus. A direct comparison of the resulting forces is not possible because SMD simulates the unfolding process over a very short period of time (~2.5 ns), whereas our experimental data are obtained over a much longer period of time (~1 s), and the unfolding forces are known to depend strongly on the pulling rate. However, a force barrier was found for N-C–linked ubiquitin that was higher and appeared at a shorter distance than the force barrier for Lys48-C–linked ubiquitin, in good agreement with our experimental observations.

From the force peak region in the simulated force-extension curve (**Fig. 4b**), we identified the location of the transition state and the rupture distance for both the N-C–linked and the Lys48-C–linked ubiquitin chains. The simulations ($n = 5$) indicated that the major unfolding barrier for N-C–linked ubiquitin was formed by the rupture of five hydrogen bonds between two parallel β-strands, I and V (**Fig. 1a**), during the extension from 10 to 13 Å (**Fig. 4b**). By contrast, the mechanical barrier in Lys48-C–linked ubiquitin was due to the rupture of five hydrogen bonds between a different pair of β-strands, antiparallel strands III and V, during the extension from 14 to 21 Å ($n = 5$; **Fig. 4b**). The lower force required to unfold Lys48-C ubiquitin results from the much larger distance required to break the crucial hydrogen bonds as compared with that for N-C–linked ubiquitin, in agreement with our experimental observations. These predictions can be directly tested by combining site-directed mutagenesis with single-molecule force spectroscopy, as has been done for the immunoglobulin modules of the giant protein titin[24].

An important feature of the ubiquitin fold is the location of three exposed hydrophobic residues, Leu8, Ile44 and Val70, which participate in the binding of Lys48-C–linked polyubiquitin to the proteasome[30]. These hydrophobic residues are positioned, in the folded protein, close to the C-terminal end, forming a multivalent hydrophobic patch[30]. Upon the application of force along the linkage direction, the spatial distribution of the three residues of the hydrophobic patch changes in different ways depending on the linkage (**Fig. 4**). A bound ubiquitin may break away from certain sites but not from others depending on the way that these hydrophobic residues respond to a stretching force (**Fig. 4**). This feature may be important when targeting differently linked ubiquitin chains to various intracellular pathways.

DISCUSSION

The biological significance of the finding that Lys48-C–linked ubiquitin can withstand a stretching force is best illustrated when considering the role of ubiquitin in proteasomal degradation. Recognition of a degradation signal involves binding of a Lys48-linked polyubiquitin chain to the proteasome[31]. After binding, unfolding of the targeted protein is known to occur[31]. It has been proposed that a mechanical force generated by the proteasomal ATPase motor triggers this unfolding[3,4]. The proteasomal motor is an AAA ATPase. AAA ATPases are ringlike hexameric motor proteins that are thought to convert conformational changes of the ring into a pulling force along a linear processive motion[32]. Like a medieval rack, a ringlike ATPase motor is thought to pull a targeted protein against a polyubiquitin chain bound to the proteasome[32]. However, for this mechanism to work, it is essential that the polyubiquitin chain remain folded and bonded to the proteasome long enough for the targeted protein to unfold. These requirements are evident when we consider that mechanical unfolding of a protein is exponentially accelerated by an applied force[9], but so is the unfolding rate of ubiquitin, which in turn limits the lifetime of the ubiquitin-proteasome bond. Hence, the system must be designed so that the target protein unfolds before the polyubiquitin-proteasome bond fails.

Force spectroscopy of proteins has the advantage of defining the reaction coordinate as the end-to-end distance[5,9]. By contrast, the

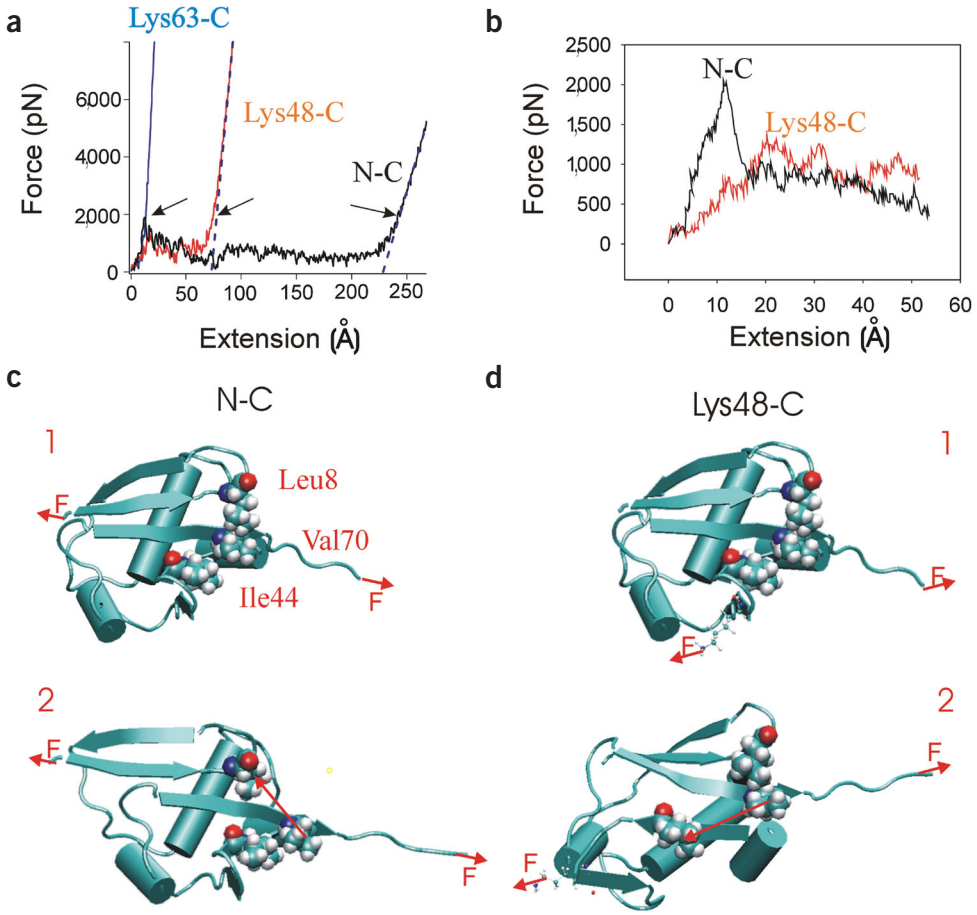

Figure 4 The linkage-dependent properties of ubiquitin reproduced *in silico* using steered molecular dynamics. (**a**) Long-range force-extension curves obtained from SMD simulations by stretching ubiquitin between its C terminus and Lys63, Lys48 and its N terminus, respectively. The steep slope at the end of the extensions corresponds to bond stretching after fully extending the polypeptide (the dashed lines are linear fits to this region). The arrows mark the 2-nN point at which the contour length is measured. (**b**) Expansion of the detail around the region in which the main unfolding barriers for unfolding are found. The black line is the average of three simulations that stretched the molecule by its N and C termini. A peak force of ~2,000 pN occurred at ~11.5 Å of the extension. The red line is the average of three simulations that stretched the molecule by Lys48 and the C terminus. These simulations generated lower force maxima (~1,200 pN) that occurred at considerably longer extensions (>20 Å). (**c**) Two snapshots of ubiquitin stretched from its termini line taken at rest (1) and after 84 ps of simulation (2). Three exposed hydrophobic residues, Leu8, Val70 and Ile44, are highlighted. These residues form a hydrophobic patch that is important for ubiquitin binding. The spacing between Leu8 and Val70 increased substantially during the extension (yellow arrow), whereas the spacing between Val70 and Ile44 remained almost unchanged. (**d**) Snapshots of ubiquitin, stretched between its C terminus and Lys48 taken at rest (1), and 122 ps of the simulation (2). Here, Ile44 was pulled away from Val70, whereas the distance between Val70 and Leu8 remained nearly unchanged. The pulling speed in these simulations was 0.1 Å ps^{-1}. The figure was generated using VMD[43].

reaction coordinate in temperature or chemical denaturation experiments (which act globally on the protein) is less well defined. This property of force spectroscopy allowed us, for the first time, to study the denaturation of a protein along two distinct pathways. If the existence of several distinct unfolding pathways is a common feature of proteins, this will raise the question of which pathways are relevant *in vivo* and what their possible functional and evolutionary significance is. Indeed, there is already evidence that the existence of several unfolding pathways is not unique to ubiquitin. In a separate study reported in this issue, Radford and colleagues conclude that, much like with ubiquitin, the mechanical stability of the Eclip3 protein also depends on the pulling geometry[33].

Forceful unfolding of a wide variety of proteins can be achieved by the application of a nonspecific mechanical stretching force. In contrast to other means of unfolding proteins such as temperature jumps or large concentrations of chemical agents such as urea, mechanical forces occur naturally in a wide variety of biological settings. It is probable that forced unfolding of proteins does occur *in vivo*, for example during the extension of titin[10,34], during protein import into mitochondria[35], during the action of chaperones[36] or to trigger the unfolding of proteins targeted for proteasome degradation[3,4,37,38]. Our work on ubiquitin adds a new dimension to this picture by demonstrating that the mechanical stability of a protein depends on the direction in which the force is applied. Thus, a stable protein may be more readily unfolded by applying force to a 'weak spot'. We predict that this feature may determine the precise location of the exposed lysine residues that are polyubiquitinated when proteins are targeted for proteasomal degradation.

METHODS

Protein engineering. N-C–linked polyubiquitin was cloned by PCR using mutagenic primers carrying *Bam*HI (upstream) and *Kpn*I (downstream) restriction sites, plus two C-terminus cysteine codons, Vent DNA polymerase, 1.5 M betaine (Sigma) and a cDNA clone from a human precursor carrying nine repeats of the ubiquitin sequence (provided by Jens Vuust, Statens Serum Institut, Denmark)[39]. The PCR product was purified, cloned into a pCRBlunt TOPO II vector (Invitrogen), and then digested with *Bam*HI and *Kpn*I and subcloned into a pQE80L expression vector using Sure-2 *Escherichia coli* cells (Stratagene). The polyproteins were then transformed into and expressed in the *E. coli* recombination-defective strain BLR(DE3) (Novagene). Bacterial cells were lysed by treatment with 1 mg ml^{-1} lysozyme and 1% Triton X-100, according to Sambrook and Russell[40]. The polyprotein Ubi_9 was purified by Co^{2+} affinity chromatography using a Talon resin (Clontech) and then kept at 4 °C in a solution containing 50 mM sodium phosphate buffer at pH 7.0, 300 mM NaCl and 150 mM imidazole.

Lys48-C–linked polyubiquitin was purchased from Affinity Research Products Ltd and redissolved, according to the manufacturer instructions, in 50 mM Tris, pH 7.6, to a final concentration of 2 mg ml^{-1}. These polyubiquitin chains ranged in length from two repeats up to seven. However, higher order polyproteins are also found, albeit with less frequency.

Single-molecule atomic force microscopy. Ubiquitin polyproteins were applied to freshly evaporated gold-coated coverslips and allowed to adsorb for ~10 min, then rinsed with PBS and mounted into an AFM chamber. Our custom-made single-molecule atomic force microscope and its mode of operation are identical to those earlier described[6,27]. The spring constant of each individual AFM cantilever (Si_3N_4 tips, ThermoMicroscope), ~40 pN nm^{-1}, was calibrated using the equipartition theorem.

Steered molecular dynamics. The structure of human ubiquitin[20] was solvated and equilibrated with CHARMM22 (ref. 41) force field for 1 ns following the procedure described by Lu *et al*[7]. The stretching forces were applied along the line connecting the Cα of Gly76 at the C terminus and one of the following atoms: Cα of the Met1 at the N terminus for linear ubiquitin; or nitrogen atoms of Nε groups of residues Lys48 and Lys63, for Lys48-Gly76 and Lys63-Gly76 branched ubiquitin. Simulations were done with the molecular dynamics program NAMD[42] on a parallel LINUX cluster with 18 nodes. Proteins were stretched at a constant speed of 0.1 Å ps^{-1} until their extension exceeded 99% of the contour length. The trajectories were recorded every 1 ps and analyzed with VMD[43]. We have run five simulations of the extension of N-C and Lys48-C ubiquitins with similar results.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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