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# Split ubiquitin as a sensor of protein interactions *in vivo*

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**ABSTRACT** **We describe an assay for *in vivo* protein interactions. Protein fusions containing ubiquitin, a 76-residue, single-domain protein, are rapidly cleaved *in vivo* by ubiquitin-specific proteases, which recognize the folded conformation of ubiquitin. When a C-terminal fragment of ubiquitin ($C_{ub}$) is expressed as a fusion to a reporter protein, the fusion is cleaved only if an N-terminal fragment of ubiquitin ($N_{ub}$) is also expressed in the same cell. This reconstitution of native ubiquitin from its fragments, detectable by the *in vivo* cleavage assay, is not observed with a mutationally altered $N_{ub}$. However, if $C_{ub}$ and the altered $N_{ub}$ are each linked to polypeptides that interact *in vivo*, the cleavage of the fusion containing $C_{ub}$ is restored, yielding a generally applicable assay for kinetic and equilibrium aspects of *in vivo* protein interactions. This method, termed USPS (ubiquitin-based split-protein sensor), makes it possible to monitor a protein–protein interaction as a function of time, at the natural sites of this interaction in a living cell.**

Multiprotein complexes mediate the bulk of biological processes (1, 2). The knowledge of these complexes is extensive for oligomeric proteins whose subunit interactions are strong enough to withstand *in vitro* conditions. However, many oligomeric assemblies, while relevant physiologically, are transient *in vivo* or unstable *in vitro*. The understanding of *in vivo* protein interactions (and especially of their temporal aspects) is still fragmentary and largely qualitative, the limitations of existing *in vivo* methods being a major reason. Assays for *in vivo* protein interactions include crosslinking of proteins with cell-penetrating reagents (2) and use of resonance energy transfer between dye-coupled proteins microinjected into cells (3). Genetic analyses of protein interactions include searches for synthetic lethal or extragenic suppressor mutations (4) which occur in genes whose products are at least functionally (and often physically) associated with a protein of interest. Another approach, the two-hybrid technique (5–8), is based on expressing one protein as a fusion to a DNA-binding domain of a transcriptional activator and expressing another protein as a fusion to a transcriptional activation domain. If the test proteins interact *in vivo*, a transcriptional activator is reconstituted, resulting in the induction of a reporter gene. This otherwise powerful method cannot address temporal aspects of a protein–protein interaction. In addition, the two-hybrid technique limits the set of detectable protein interactions to those that occur (or can be "reproduced") in the nucleus, in proximity to the reporter gene (5–8).

We describe a ubiquitin (Ub)-based split-protein sensor (USPS; Fig. 1) that makes it possible to examine kinetic and equilibrium aspects of a protein–protein interaction at its natural sites in a living cell.

## MATERIALS AND METHODS

**Strains, Media, Pulse–Chase, and Immunoblotting.** All experiments used the YPH500 strain of the yeast *Saccharomyces cerevisiae* (*MATα ura3 lys2 trp1 ade2 his3 leu2*) (11, 12) grown at 30°C to an $OD_{600}$ of ≈1 in a synthetic (SD) medium (13, 14) containing 0.1 mM $CuSO_4$. Pulse–chase experiments, including the preparation of cell extracts in the presence of N-ethylmaleimide (to inhibit UBPs), immunoprecipitation with a monoclonal antibody to the "ha" epitope (14), SDS/12% PAGE, and fluorography, were carried out as described (11, 14), except that zero-time samples were withdrawn and processed 1 min after the addition of a chase medium (11). Immunoblotting with anti-ha antibody (11) was performed with the ECL detection system (Amersham).

**Test Proteins.** Detailed protocols are available upon request. The final constructs [verified by sequencing (15)] resided in plasmid pRS314 or pRS316 (12) and were expressed from the induced $P_{CUP1}$ promoter. The *S. cerevisiae* Ub gene was amplified by PCR (15) from the previously engineered *Sal* I site immediately upstream of the Ub start codon (11) to the first cytosine of codon 37, and from codon 35 to codon 76. The primers were constructed in a way that yielded, after ligation of the two amplified fragments, a *Bam*HI site between codons 35 and 37 in the Ub open reading frame (ORF). Ligation of this ORF to a fragment encoding mouse dihydrofolate reductase with an ha epitope tag (DHFR-ha, dha) (11) yielded an ORF encoding Ub-dha (Fig. 2, construct I), which contained the sequence Met-His-Arg-Ser-Gly-Ile-Met between Gly-76 of Ub and Val-1 of DHFR. Constructs II–IV (Fig. 2) were produced by replacing the *Sal* I–*Bst*XI fragment in construct I with appropriately designed double-stranded oligonucleotides. Construct V (Fig. 2) was produced by PCR using *S. cerevisiae* genomic DNA and primers designed to amplify the region of *STE6* (16, 17) from codon 196 to codon 262. The resulting fragment was inserted into the *Bam*HI site between the Ub codons 35 and 37 in constructs I–IV, yielding constructs V–VIII. In construct IX (Fig. 2), residue 35 of Ub was preceded by a 32-residue linker all of whose residues except the N-terminal Met-Gly-Gly were specified by codons 234 to 262 of *STE6*. The $z_1$-$C_{ub}$ portion of construct XIV encoded the above Ste6-derived sequence preceding the $C_{ub}$ moiety, the leucine zipper region of *S. cerevisiae* Gcn4 (residues 235–281, denoted as $z_1$) (18–21), the construction-generated N-terminal Met, and the sequence Gly-Glu-Ile-Ser-Thr. Constructs X–XIII (Fig. 2), derived from construct XIV and constructs V–VIII, encoded Gly-Glu-Ile-Ser-Thr-Leu-Glu C-terminally to $z_1$, with Gly-Gly-Ser-Thr-Met between $z_1$ and $N_{ub}$. The $z_1$ motif in $N^{wt}_{ub}$-$z_1$ and its derivatives but not in $z_1$ $C_{ub}$-dha bore a Met-250 → Thr-250 replacement (residue numbers of Gcn4), which occurred during construction; this replacement would be expected to weaken the interaction between $z_1$ domains (18–21).

## RESULTS

***In Vivo* Folding of Ub Containing an Insertion and/or a Single-Residue Replacement.** Ub is a 76-residue, single-

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Abbreviations: Ub, ubiquitin; USPS, Ub-based split-protein sensor; UBP, Ub-specific protease; $N_{ub}$, N-terminal fragment of Ub; $C_{ub}$, C-terminal fragment of Ub; DHFR, dihydrofolate reductase.

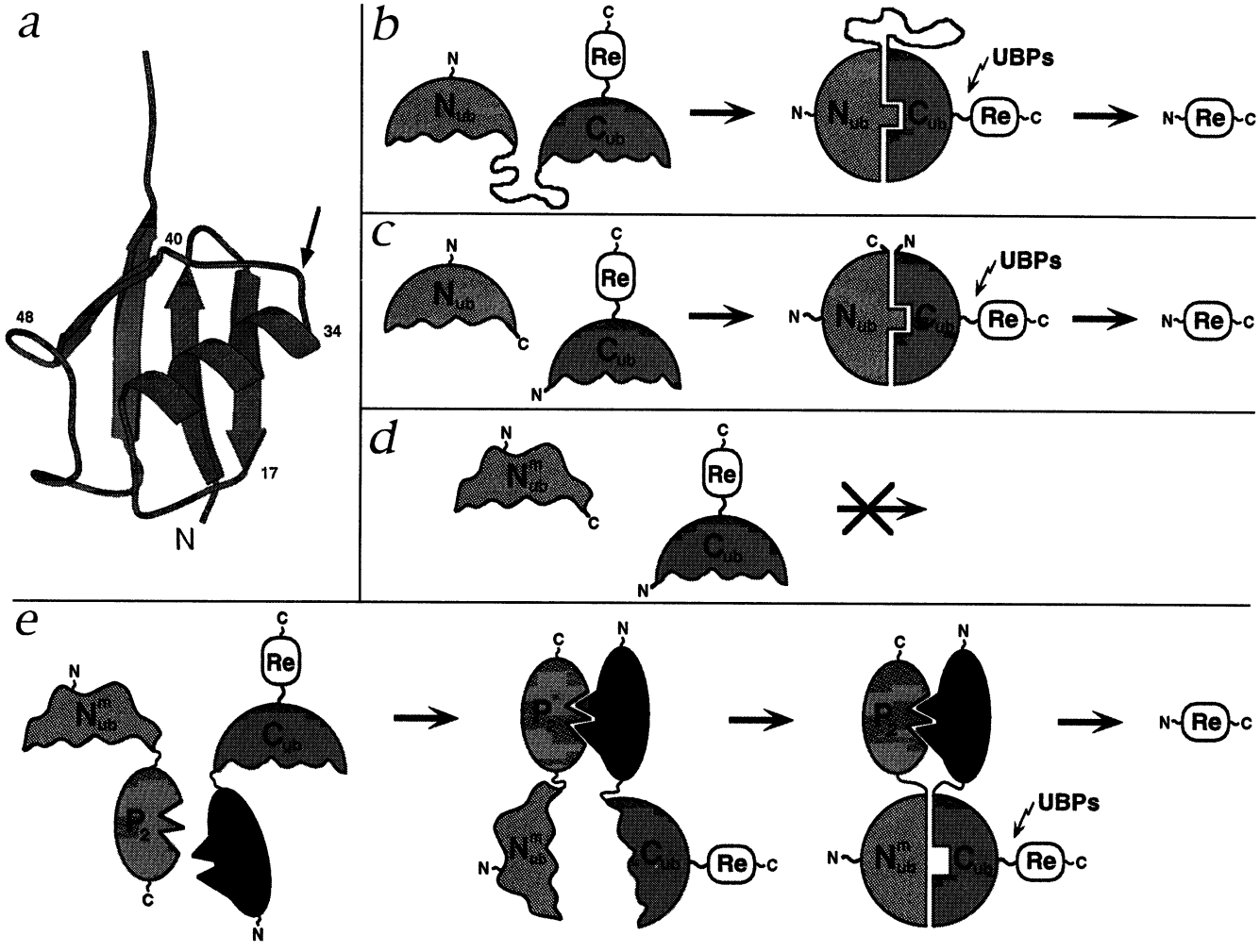


FIG. 1. Split ubiquitin as a proximity sensor *in vivo*. (*a*) A ribbon diagram of Ub (9, 10), with its two subdomains colored pink and green (as in *b–e*). Arrow denotes the site of either a 68-residue insertion or a cut between the subdomains. Some of the residue numbers are indicated. Ile-13, the site of mutations analyzed in this work, is in the second strand of the β-sheet, where it interacts with the hydrophobic face of the α-helix (9). (*b*) A newly formed Ub moiety bearing an insertion (wavy red line) between its N-terminal ($N_{ub}$; pink) and C-terminal ($C_{ub}$; green) subdomains and linked to a reporter protein (Re; yellow). The insertion did not detectably interfere with the Ub folding, which was required for the *in vivo* cleavage of the fusion by Ub-specific proteases (UBPs; red lightning arrow), yielding the free reporter. (*c*) When $N_{ub}$ and $C_{ub}$ were coexpressed as separate fragments, with $C_{ub}$ still linked to the reporter, significant *in vivo* reconstitution of a quasi-native (recognizable by UBPs) Ub moiety was observed. (*d*) *In vivo* reconstitution of Ub from its separate, coexpressed fragments did not occur with a mutant $N_{ub}$ fragment, denoted as $N^{m}_{ub}$, that bore a single-residue replacement at position 13. Conformational destabilization of $N^{m}_{ub}$ relative to its wild-type counterpart $N_{ub}$ is indicated by the altered shape of the $N^{m}_{ub}$ subdomain. (*e*) USPS. $N^{m}_{ub}$, an altered Ub fragment that failed to reconstitute Ub in the presence of $C_{ub}$, did support reconstitution if the two Ub fragments were linked to polypeptides $P_1$ and $P_2$ (red and blue, respectively) that interacted *in vivo*. Reduced conformational stability of Ub that has been reconstituted with $N^{m}_{ub}$ instead of $N_{ub}$ is denoted by a gap between the Ub subdomains.

domain protein (Fig. 1*a*) that is present in cells either free or covalently linked to other proteins. Ub plays a role in a number of processes, primarily through routes that involve protein degradation (22–27). In eukaryotes, newly formed Ub fusions are rapidly cleaved by UBPs after the last residue of Ub at the Ub-polypeptide junction (13, 28–30). The cleavage of a Ub fusion by UBPs requires the folded conformation of Ub (11).

In the constructs of this work, Ub was joined to the N terminus of mouse DHFR, whose C terminus was extended with the ha epitope tag (14), yielding a Ub-dha test protein of 22 kDa. The Ub moiety whose Ile-3 and Ile-13 residues [which are buried in the hydrophobic core of Ub (9)] have been replaced by Gly is a poor UBP substrate (11). To make similar but less destabilizing alterations of Ub, only Ile-13 was replaced with either Val, Ala, or Gly. The resulting Ub fusions (Fig. 2, constructs II–IV) were completely cleaved *in vivo* by the end of either a 5-min or a 2-min labeling with [$^{35}$S]methionine, as was Ub-dha, bearing wild-type Ub (Fig. 3*A*, lanes a–d, data not shown). We then asked whether a 68-residue insertion (i68) within a loop (residues 34–40) connecting the only α-helix of Ub to a β-strand (Fig. 1 *a* and *b*) and a substitution at position 13 of Ub, if present together, result in a less efficient cleavage of a fusion by UBPs. The i68 insertion was derived from the cytosolic region of the *S. cerevisiae* Ste6 protein between its transmembrane segments 4 and 5 (16, 17). This region was chosen because it was expected to be either flexible or folded in a way that positions its ends in proximity to each other. By the end of a 2-min pulse, no uncleaved $Ub^{i68}$-dha, and at most traces of $Ub^{V13,i68}$-dha, could be detected (Fig. 2, constructs V and VI; 3*B*, lanes a and b). However, the cleavage of $Ub^{A13,i68}$-dha and especially $Ub^{G13,i68}$-dha was much slower, in that significant amounts of the uncleaved fusions were observed by the end of a 2-min pulse (Fig. 2, constructs VII and VIII; Fig. 3*B*, lanes c and d).

The i68 insertion places the two "halves" of a nascent Ub farther apart (Fig. 1 *a* and *b*) and therefore is expected to

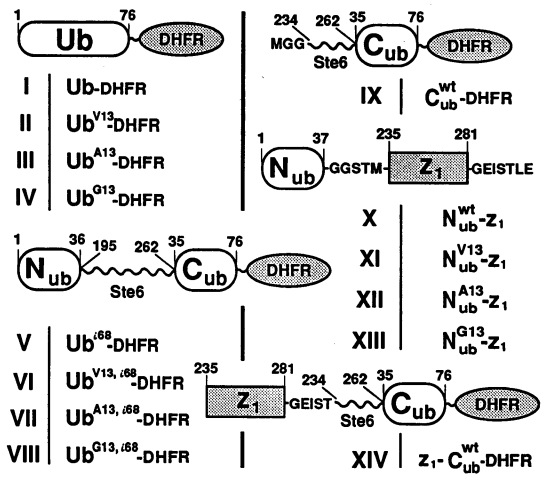


FIG. 2. Fusion constructs. Fusions used in this work contained some of the following elements: (*i*) a Ub moiety, either wild-type (construct I) or bearing single-residue replacements at position 13 (constructs II–IV). (*ii*) A Ub moiety containing the 68-residue insertion (denoted as Ste6) derived from the cytosolic region of *S. cerevisiae* Ste6 between its transmembrane segments 4 and 5 (16, 17). The insertion was positioned after residue 36 of Ub (construct V). (*iii*) A Ub moiety bearing both the above insertion and a single-residue replacement at position 13 (constructs VI–VIII). (*iv*) A C-terminal fragment of Ub ($C_{ub}$, residues 35–76) bearing the 32-residue, Ste6-derived sequence at its N terminus (construct IX). (*v*) The same fusion whose N terminus was extended, via the linker sequence Gly-Glu-Ile-Ser-Thr, with the 47-residue homodimerization motif ("leucine zipper", or $z_1$) of *S. cerevisiae* Gcn4 (18–21) (residues 235–281 of Gcn4) (construct XIV). (*vi*) An N-terminal fragment of Ub ($N_{ub}$, residues 1–37) bearing the wild-type Ub sequence or a single-residue replacement at position 13 and a C-terminal extension containing the linker sequence Gly-Gly-Ser-Thr-Met followed by the $z_1$ leucine zipper of Gcn4 (constructs X–XIII). (*vii*) Mouse DHFR bearing a C-terminal ha epitope (14) (denoted as DHFR in this diagram and as dha in the text and in Figs. 3 and 4).

retard the folding of Ub; this effect could be detected by the 2-min pulse–cleavage assay if another destabilizing Ub alteration such as Ile-13 → Gly-13 (which by itself is insufficient to cause a detectable retardation of Ub folding) was present as well (Fig. 3 *A* and *B*). These results can be interpreted within the diffusion–collision model of protein folding (2, 31, 32), in which marginally stable units of isolated secondary structure form early and then coalesce into the native conformation. Indeed, in native Ub, its first 34 residues are folded into an α-helix interacting with a double-stranded antiparallel β-sheet (Fig. 1*a*) (9). Thus, the i68 insertion retards Ub folding primarily through a reduction in the frequency of collisions between the N-terminal and C-terminal subdomains of Ub, whereas the effect of substitutions at position 13 of Ub is a decreased conformational stability of its N-terminal subdomain (Fig. 1*a*). This *in vivo* evidence for Ub subdomains (see also below) is in agreement with the results of circular dichroism and NMR analyses of Ub and its fragments in a methanol/water solvent at low pH (33, 34).

***In Vivo* Reconstitution of Ub from Its Fragments.** The relative insensitivity of Ub folding to a large insertion within the 34–40 loop (Fig. 1 *a* and *b*) suggested that separate, coexpressed fragments of Ub (produced by a cut within the 34–40 loop) may be able to reconstitute native Ub. In a test of this conjecture, a C-terminal fragment of wild-type Ub (residues 35–76, denoted as $C_{ub}$) was expressed as a fusion to the dha reporter ($C_{ub}$-dha), while an N-terminal fragment of wild-type Ub (residues 1–37, denoted as $N_{ub}^{wt}$) was expressed as a fusion to the "leucine zipper" homodimerization domain of the yeast Gcn4 protein (denoted as $z_1$) (Fig. 2, constructs IX and X; refs. 18–21). (The reason for linking $N_{ub}^{wt}$ to $z_1$ will become clear later.) When expressed by itself, $C_{ub}$-dha remained largely uncleaved (Fig. 4*A*, lanes a–c). However, coexpression of $C_{ub}$-dha and $N_{ub}^{wt}$-$z_1$ resulted in the cleavage of $C_{ub}$-dha, yielding dha, which accumulated during a 30-min chase (Fig. 4*A*, lanes d–f). This cleavage was slow in comparison to the cleavage of a fusion containing wild-type Ub (Fig. 3*A*, lane a). We conclude that the cleavage of $C_{ub}$-dha requires the presence of $N_{ub}$ and is the consequence of an *in vivo* association between the $C_{ub}$ and $N_{ub}$ fragments. This association results in at least transient formation of a Ub moiety which is similar enough to native Ub to be a substrate of UBPs. That fragments of a protein can associate to form a functional, quasi-native species has been demonstrated

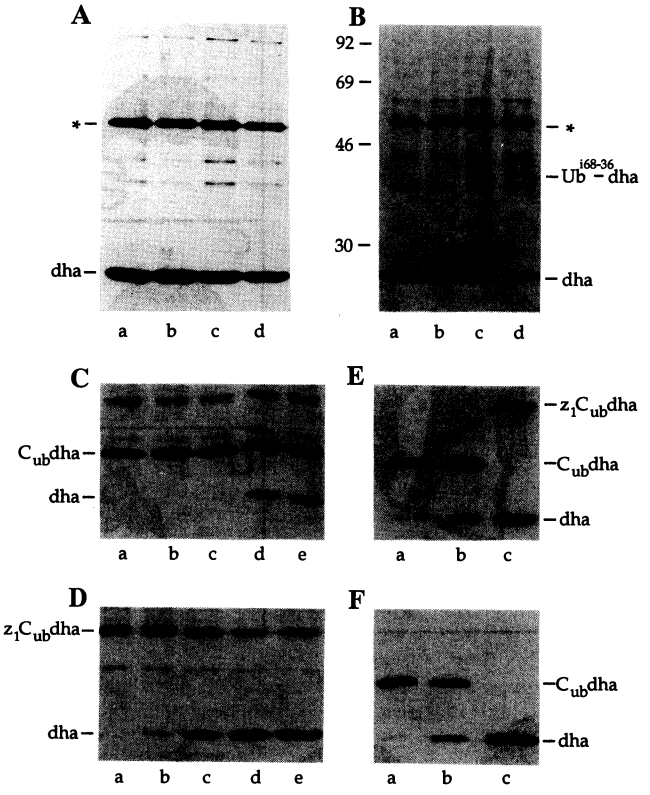


FIG. 3. Kinetic and equilibrium aspects of ubiquitin reconstitution *in vivo*. (*A*) Lane a, *S. cerevisiae* cells expressing Ub-dha (Fig. 2, construct I) were labeled for 5 min with [$^{35}$S]methionine, followed by extraction of proteins, immunoprecipitation with anti-ha antibody, and SDS/PAGE. Lanes b–d, same as lane a but with the Ub moiety containing, respectively, Val, Ala, or Gly instead of wild-type Ile at position 13 (Fig. 2, constructs II–IV). (*B*) Lane a, same as lane a in *A* but with a labeling time of 2 min and with *S. cerevisiae* expressing $Ub^{i68}$-dha (Fig. 2, construct V). Lanes b–d, same as lanes b–d in *A* but with the single-residue replacements at position 13 in the $Ub^{i68}$ moiety (constructs VI–VIII). (*C*) Lane a, same as lane a in *B* but with *S. cerevisiae* expressing $C_{ub}$-dha (construct IX). Lane b, $C_{ub}$-dha was coexpressed with $N_{ub}^{G13}$-$z_1$ (construct XIII). Lane c, $C_{ub}$-dha was coexpressed with $N_{ub}^{A13}$-$z_1$ (construct XII). Lane d, $C_{ub}$-dha was coexpressed with $N_{ub}^{V13}$-$z_1$ (construct XI). Lane e, $C_{ub}$-dha was coexpressed with $N_{ub}^{wt}$-$z_1$ (construct X). (*D*) Same as lane a in *C* but with *S. cerevisiae* expressing $z_1C_{ub}$-dha (construct XIV). Lane b, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{G13}$-$z_1$. Lane c, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{A13}$-$z_1$. Lane d, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{V13}$-$z_1$. Lane e, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{wt}$-$z_1$. (*E*) Whole-cell extracts of *S. cerevisiae* expressing $C_{ub}$-dha (lane a), or $C_{ub}$-dha and $N_{ub}^{G13}$-$z_1$ (lane b), or $z_1C_{ub}$-dha and $N_{ub}^{G13}$-$z_1$ (lane c) were fractionated by SDS/PAGE and analyzed by immunoblotting with anti-ha antibody. (*F*) Same as *E* but with *S. cerevisiae* expressing $C_{ub}$-dha (lane a), or $C_{ub}$-dha and $N_{ub}^{A13}$-$z_1$ (lane b), or $z_1C_{ub}$-dha and $N_{ub}^{A13}$-$z_1$ (lane c). $Ub^{i68}$-dha, free reporter (dha), $C_{ub}$-dha, and $z_1C_{ub}$-dha are indicated. The asterisk in *B* denotes an unrelated *S. cerevisiae* protein that crossreacted with anti-ha antibody (11, 14).

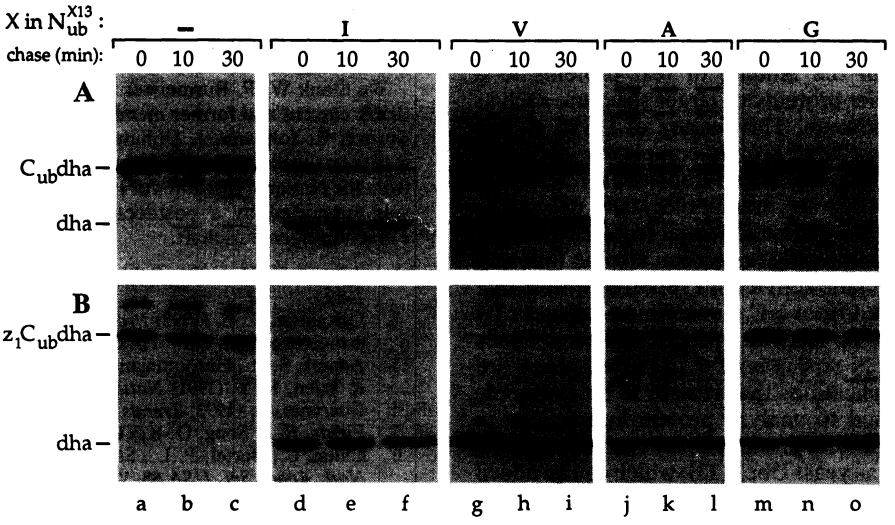


FIG. 4. Reconstitution of the folded ubiquitin from its coexpressed fragments: enhancement by cis-linked interacting polypeptides. (*A*) Lanes a–c, *S. cerevisiae* cells expressing $C_{ub}$-dha (Fig. 2, construct IX) were subjected to incubation for 5 min with [$^{35}$S]methionine, followed by a chase for 0, 10, and 30 min, extraction of proteins, immunoprecipitation with anti-ha antibody, and SDS/PAGE. Note a slow degradation of $C_{ub}$-dha and the absence of the cleavage that yields dha. Lanes d–f, same as lanes a–c, but $C_{ub}$-dha was coexpressed with $N_{ub}^{wt}$-$z_1$ (construct X). Lanes g–i, $C_{ub}$-dha was coexpressed with $N_{ub}^{V13}$-$z_1$ (construct XI), which bore Val instead of wild-type Ile at position 13 of Ub. Lanes j–l, $C_{ub}$-dha was coexpressed with $N_{ub}^{A13}$-$z_1$ (construct XII). Lanes m–o, $C_{ub}$-dha was coexpressed with $N_{ub}^{G13}$-$z_1$ (construct XIII). (*B*) Lanes a–c, same as lanes a–c in *A* but with $z_1C_{ub}$-dha (construct XIV) instead of $C_{ub}$-dha. Lanes d–f, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{wt}$-$z_1$. Lanes g–i, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{V13}$-$z_1$. Lanes j–l, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{A13}$-$z_1$. Lanes m–o, $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{G13}$-$z_1$.

both *in vitro* and *in vivo* for a variety of proteins other than Ub (35–41).

The efficiency of Ub reconstitution depended on the conformational stability of its N-terminal subdomain: coexpression of $C_{ub}$-dha with either $N_{ub}^{G13}$-$z_1$ or $N_{ub}^{A13}$-$z_1$ (Fig. 2, constructs IX, XII, and XIII), bearing Gly or Ala instead of Ile at position 13, resulted in virtually no cleavage of $C_{ub}$-dha, in contrast to the results with either $N_{ub}^{wt}$-$z_1$ or $N_{ub}^{V13}$-$z_1$ (Fig. 2, constructs X and XI), which bore either wild-type Ile or Val, a hydrophobic residue larger than Ala and Gly, at position 13 (Fig. 1 *c* and *d* and Fig. 4*A*, lanes j–o; compare with lanes d–i). These results, together with the discovery that the UBP-mediated cleavage of a Ub fusion requires folded Ub (11), led to development of an assay for *in vivo* protein interactions, as shown below.

**Split Ubiquitin as a Proximity Sensor.** We asked whether the linking of two polypeptides that interact *in vivo* to $N_{ub}$ and $C_{ub}$ facilitates reconstitution of Ub. $C_{ub}$-dha was linked to a region of *S. cerevisiae* Gcn4 (residues 235–281) that contained the leucine zipper homodimerization domain (denoted as $z_1$) (18–21). In the resulting $z_1C_{ub}$-dha, a 32-residue linker was inserted between $z_1$ and $C_{ub}$ (Fig. 2, construct XIV) to ensure that $N_{ub}$ and $C_{ub}$ subdomains could be spatially proximal within a $z_1$-mediated complex between $z_1C_{ub}$-dha and $N_{ub}$-$z_1$. When expressed by itself, $z_1C_{ub}$-dha remained uncleaved and was slowly degraded during the chase (Fig. 4*B*, lanes a–c). However, coexpression of $z_1C_{ub}$-dha and $N_{ub}^{G13}$-$z_1$, bearing Gly instead of Ile at position 13 of Ub, resulted in a significant cleavage of $z_1C_{ub}$-dha (yielding dha) in the course of a 30-min chase (Fig. 2, constructs XIII and XIV; Fig. 4*B*, lanes m–o). By contrast, no such cleavage was observed when $N_{ub}^{G13}$-$z_1$ was coexpressed with $C_{ub}$-dha, which lacked $z_1$ (Fig. 4*A*, lanes m–o). Similar results (but with faster cleavage of $z_1C_{ub}$-dha) were obtained upon coexpression of $z_1C_{ub}$-dha and $N_{ub}^{A13}$-$z_1$ (Fig. 4, lanes j–l). Moreover, the enhancement of Ub reconstitution by $z_1$–$z_1$ interactions was observed even with pairs of Ub fragments that could yield Ub by themselves (in the absence of linked $z_1$). Specifically, whereas the coexpression of $C_{ub}$-dha and $N_{ub}^{wt}$-$z_1$ or $N_{ub}^{V13}$-$z_1$ resulted in detectable but slow cleavage of $C_{ub}$-dha that was still incomplete after 30 min of chase, coexpression of $z_1C_{ub}$-dha and $N_{ub}^{wt}$-$z_1$ or $N_{ub}^{V13}$-$z_1$ resulted in the nearly complete cleavage of $z_1C_{ub}$-dha (yielding dha) by the end of a 5-min pulse (Fig. 4, lanes d–i). The temporal resolution of this assay could be increased by shortening the labeling time from 5 to 2 min. For example, the fraction of $z_1C_{ub}$-dha cleaved by the end of a 2-min pulse progressively increased when $z_1C_{ub}$-dha was coexpressed with $N_{ub}^{G13}$-$z_1$, $N_{ub}^{A13}$-$z_1$, $N_{ub}^{V13}$-$z_1$, or $N_{ub}^{wt}$-$z_1$ (Fig. 3*D*, lanes b–e). By contrast, no cleavage of $z_1C_{ub}$-dha was observed when it was expressed by itself (Fig. 3*D*, lane a), or when $N_{ub}^{A13}$-$z_1$ or $N_{ub}^{G13}$-$z_1$ were coexpressed with $C_{ub}$-dha, which lacked the $z_1$ zipper (Fig. 3*C*, lanes b and c).

To determine steady-state levels of test proteins, cell extracts were analyzed by immunoblotting with anti-ha antibody (Fig. 3 *E* and *F*). When $C_{ub}$-dha was expressed by itself, it remained largely uncleaved (Fig. 3 *E* and *F*, lanes a). When $N_{ub}^{A13}$-$z_1$ was coexpressed with $C_{ub}$-dha, a fraction of $C_{ub}$-dha was cleaved to yield dha (Fig. 3*F*, lane b). However, when $N_{ub}^{A13}$-$z_1$ was coexpressed with $z_1C_{ub}$-dha, virtually all of $z_1C_{ub}$-dha was cleaved to yield dha (Fig. 3*F*, lane c). Similar results were obtained with $N_{ub}^{G13}$-$z_1$, except that a significant fraction of $z_1C_{ub}$-dha remained uncleaved in the presence of $N_{ub}^{G13}$-$z_1$ (Fig. 3*E*, lanes b and c).

## DISCUSSION

Selecting appropriate Ub fragments, altering one of them to reduce the rate of Ub reconstitution by fragments alone, and linking these fragments to a pair of test polypeptides yielded USPS, an assay for kinetic and equilibrium aspects of *in vivo* protein interactions. Features of USPS that distinguish it from the two-hybrid technique (5–8) include the possibility of monitoring a protein–protein interaction as a function of time, at the natural sites of this interaction in a living cell. Enhancement of Ub reconstitution by interacting polypeptides linked to fragments of Ub stems from a local increase in concentration of one Ub fragment in the vicinity of the other. This increase results in a higher probability of the two Ub fragments associating to form a quasi-native Ub moiety, an event detected through the irreversible cleavage of the fusion by UBPs (Fig. 1*e*).

USPS detects a spatial proximity of proteins but not necessarily their direct interaction. Therefore, a USPS signal might also result from the binding of test proteins to a common ligand—another protein or a larger structure such as a microtubule or a ribosome. This feature of USPS should allow *in vivo* analyses of large protein complexes, including those that may be too unstable or too transient for detection by other methods. A separate, immunologically detectable reporter domain (Fig. 1) is not an essential feature of USPS: the cleavage of a $C_{ub}$-containing fusion can also be followed by measuring the enzymatic activity of a reporter protein that is inactive until released from a $C_{ub}$-containing fusion or by using an antibody specific for a test protein linked to either the C terminus or the N terminus of $C_{ub}$. Listed below are some of the USPS applications that remain to be explored.

(*i*) USPS can be used to analyze protein interactions *in vitro*—in cell extracts or with purified fusions in the presence of a purified UBP such as yeast Ubp1 (13), which can be added at a desired time after the mixing of interacting species. The kinetics of Ub reconstitution from its fragments *in vitro* can be compared with analogous reconstitutions in living cells to address the influences of translation, chaperonins, macromolecular crowding, and other aspects of the *in vivo* condition. *In vitro* USPS can also be used to screen for peptide or nonpeptide ligands that interact with a ligand of interest.

(*ii*) Many proteins have more than one protein ligand. A version of USPS for a "many-body" interaction could be as follows. $N_{ub}$-$P_1$, a fusion between $N_{ub}$ and a protein of interest, $P_1$, is coexpressed with two other proteins: $P_2C_{ub}$-$R_1$, a fusion of $C_{ub}$, $P_2$ (a putative ligand of $P_1$), and a reporter $R_1$, and a similarly designed $P_3C_{ub}$-$R_2$ fusion containing a different reporter, $R_2$, and $P_3$, another putative ligand of $P_1$. Comparing the kinetics of *in vivo* cleavage of $P_2C_{ub}$-$R_1$ and $P_3C_{ub}$-$R_2$ in the presence of $N_{ub}$-$P_1$ should provide information about relative affinities of $P_1$ for $P_2$ and $P_3$ and about kinetic aspects of these interactions as well.

(*iii*) Lateral diffusion and interactions of membrane proteins underlie membrane-based reactions such as activation of receptors by hormones and adhesion between cells. In this version of USPS, the two Ub subdomains can be positioned within cytosol-exposed regions of two membrane proteins.

(*iv*) A USPS-based screen for genes whose products interact with a protein of interest $P_1$ can employ a reporter R whose function is incompatible with an N-terminal extension such as $P_1C_{ub}$. The reporter would be activated upon Ub reconstitution (Fig. 1*e*) and the cleavage of $P_1C_{ub}$-R that yields R. In this approach, cells expressing $P_1C_{ub}$-R are transformed with an expression library encoding random translational fusions to a mutant $N_{ub}$ moiety and screened for cells expressing active reporter.

USPS was demonstrated here with homodimerizing polypeptides of the leucine zipper type (Figs. 2 and 4). In addition, we recently used USPS to detect and analyze *in vivo* interactions between *S. cerevisiae* Sec62 protein and the signal sequences of either the *SUC2*-encoded invertase or the *MFα1*-encoded precursor of α-factor, a mating pheromone. Sec62 is an integral membrane protein and essential component of the translocation complex which mediates the transport of proteins bearing signal sequences across the endoplasmic reticulum membrane (42). The USPS assay detected specific, transient interactions between Sec62 and the signal sequences; it also made possible a kinetic analysis of these *in vivo* interactions, which have previously been demonstrated using photocrosslinking in a cell-free system (43). The transient proximity between a signal sequence and the membrane-embedded Sec62 could be detected by using several $N_{ub}$ and $C_{ub}$ arrangements in Sec62 and signal sequence-bearing test proteins. Together with the results of the present work, these findings (unpublished data) illustrate the versatility of USPS, its sensitivity to relatively weak and transient protein interactions, and the remarkable flexibility of "allowed" $N_{ub}$ and $C_{ub}$ configurations within test proteins.

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