

from the cytosol markedly stimulated MVL formation, whereas control antibodies had no effect (Fig. 2, C and E).

When Alix expression was silenced by small interfering RNAs (siRNAs) in HeLa cells (Fig. 3A), the number of acidic, late endocytic compartments appeared to be reduced (based on a probe selective for pH values between 5 and 5.5; Fig. 3B), whereas the acidification properties of other organelles (such as early endosomes and the trans-Golgi network) seemed to be unaffected (based on a probe detecting less acidic pH; Fig. 3C). By EM, LBPA (Fig. 3, D and E, open arrows) was found in HeLa cells both in multilamellar regions (Fig. 3D, black triple arrow) and multivesicular regions (Fig. 3D, star and inset), frequently within the same late endosome. Consistently, Alix siRNAs reduced the number of late endosomes containing multilamellar regions (which could be unambiguously identified on sections) (Fig. 3B), whereas other organelles, including the Golgi complex, seemed to be unaffected (Fig. 3G).

A biochemical analysis showed that Alix downexpression reduced LBPA to $\approx 50\%$ of that of the control. Consistently, the LBPA staining of Lamp1-positive late endosomes was decreased by immunofluorescence (Fig. 3J), as were the total number of anti-LBPA gold particles both per cell profile (Fig. 3H) and per late endosomes (Fig. 3, E and F, quantification in I) by EM. Vesicular stomatitis virus (VSV) infects cells from the endocytic pathway, but beyond early endosomes (26, 27). The acidic endosomal pH triggers fusion of the VSV envelope with endosomal membranes, allowing nucleocapsid release into the cytoplasm. Viral infection (Fig. 3K, quantification in L) was inhibited by Alix downexpression, presumably because the number of acidic late endosomes was decreased. Alternatively, Alix-dependent dynamics of late endosomal membranes may be required for efficient nucleocapsid release.

We found that LBPA possesses the capacity to drive the formation of membrane invaginations within acidic liposomes. We also found that Alix controls this invagination process in vitro and the organization of LBPA-containing endosomes in vivo. We postulate that internal vesicles and the limiting membrane interact dynamically by means of fission and fusion events, and that the inward fission process is controlled, at least in part, by transient interactions between LBPA membranes and Alix (28), presumably together with other factors, including perhaps ESCRT proteins (22, 23).

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Snapshots of DsbA in Action: Detection of Proteins in the Process of Oxidative Folding

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DsbA, a thioredoxin superfamily member, introduces disulfide bonds into newly translocated proteins. This process is thought to occur via formation of mixed disulfide complexes between DsbA and its substrates. However, these complexes are difficult to detect, probably because of their short-lived nature. Here we show that it is possible to detect such covalent intermediates in vivo by a mutation in DsbA that alters *cis* proline-151. Further, this mutant allowed us to identify substrates of DsbA. Alteration of the *cis* proline, highly conserved among thioredoxin superfamily members, may be useful for the detection of substrates and intermediate complexes in other systems.

The formation of disulfide bonds is essential for the folding, activity, and stability of many proteins exported from the cytoplasm. In both prokaryotes and eukaryotes, proteins with the thioredoxin fold are responsible for introducing disulfide bonds into substrate proteins (1–3). When a substrate protein appears in the periplasm of *Escherichia coli*, DsbA rapidly oxidizes it by donating the disulfide bond from its active site, Cys³⁰-Pro³¹-His³²-Cys³³,

to a pair of cysteines in the substrate (4–7). DsbA, in turn, is maintained in its oxidized form (8) by a membrane protein DsbB (9), which transfers electrons from DsbA to quinones in the respiratory chain using its four essential cysteines (10–12).

Substrate oxidation by DsbA likely begins with a deprotonated cysteine in the substrate attacking the disulfide bond in the active site of DsbA (2, 3, 6, 13) (Fig. 1A). This reaction leads to the formation of a complex in which DsbA and the substrate are linked together by an intermolecular disulfide bond. In the next step, the mixed disulfide bond is attacked by a second deprotonated cysteine of the substrate to resolve the complex and release the oxidized substrate and the reduced DsbA. Although in vitro data are consistent with this model (6, 13, 14), the covalent reaction intermediate between DsbA and substrate has not

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been detected during oxidative folding of protein under physiological growth conditions (15). Nor is it known at which point in the protein translocation process disulfide bond formation takes place, or how DsbA recognizes substrates. Here we describe the properties of a mutant DsbA protein that appears to slow down step 2 of the DsbA reaction (Fig. 1A), thus accumulating DsbA-substrate complexes. Mutants of this type should be generally useful for studying the mechanism of thioredoxin superfamily members.

To find new mutants of the DsbA-DsbB pathway, we conducted a genetic screen using a chromosomally encoded *malF-lacZ* fusion that expresses β -galactosidase activity only when there is a defect in disulfide bond formation (16, 17). From approximately one million ultraviolet (UV)-mutagenized colonies (18), we obtained three missense mutations in *dsbA* that altered amino acids other than cysteines: V150G (Val¹⁵⁰ \rightarrow Gly¹⁵⁰) (two isolates), P151T (Pro¹⁵¹ \rightarrow Thr¹⁵¹) (two isolates), and P151S (Pro¹⁵¹ \rightarrow Ser¹⁵¹) (two isolates). The Val¹⁵⁰ and Pro¹⁵¹ residues are both located at the N-terminal end of a β α motif of DsbA and touch the active site cysteines in the structural model of DsbA (Fig. 1B) (1, 19, 20). The Pro¹⁵¹ residue, which is conserved in most thioredoxin superfamily members, assumes a *cis* configuration (1, 19).

We transferred these mutations to other strains by P1 transduction and examined the ability of the mutants to promote disulfide bond formation in β -lactamase, a periplasmic protein with one disulfide bond. To determine the oxidation status of proteins including β -lactamase, we used an "acid trap" technique to inhibit thiol-disulfide reactivity and then alkylated free cysteines with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) (18). This modification retards the mobility of the reduced form of proteins on gels. All of the mutants were weak. They were partially defective in disulfide bond formation, oxidizing 40 to 50% of β -lactamase (Fig. 1C).

To further characterize the mutants, we assessed the redox state of DsbA by probing AMS-alkylated cell lysates with antibody to DsbA. The three mutants exhibited different phenotypes. In normal cells, DsbA exists mainly as the oxidized monomer (Fig. 2A). However, the P151S mutant, while accumulating both oxidized and reduced DsbA, also accumulated a major band with an apparent molecular mass of 36 kD (Fig. 2A). This band, which disappeared upon sample treatment with reducing agent, was also recognized by antibody to DsbB (21). Thus, it represents a presumed intermediate in DsbA reoxidation, the mixed disulfide complex between DsbA and DsbB (15, 22, 23). The DsbA-DsbB

complex accumulates in the mutant only when the mutant DsbA is actively oxidizing substrate proteins; coexpression of wild-type DsbA resulted in disappearance of the DsbA-DsbB complex (21). Thus, the DsbA P151S-DsbB complex was formed in the process of oxidation of the P151S protein by DsbB. The accumulation of substantial amounts of the complex suggests that the P151S mutant has a defect in a step required for the resolution of the mixed disulfide intermediate. By contrast, in the V150G mutant, more than half of the DsbA

remained reduced with accumulation of less DsbA-DsbB complex than wild-type (Fig. 2A), suggesting that a step in the complex formation is defective.

Remarkably, the P151T mutant accumulated a large number of bands of different apparent molecular masses (Fig. 2A, solid arrowheads) that reacted with antibody to DsbA. We suggest that these bands represent DsbA-substrate complexes for the following reasons: First, these bands disappeared when samples were treated with reducing agent before electrophoresis (21) (Fig. 2C). Sec-

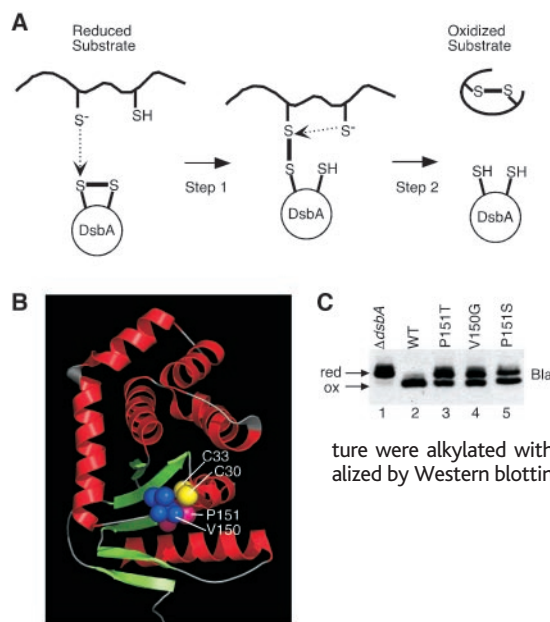
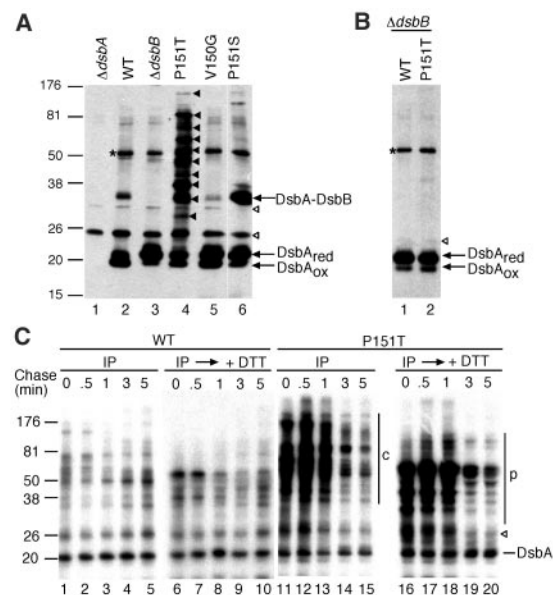


Fig. 1. New DsbA mutants. (A) Oxidation of a substrate protein by DsbA. (B) Ribbon representation of DsbA structure (PDB accession number, 1FVK) generated by PyMol. Yellow spheres represent sulfurs of active site cysteines. Pro¹⁵¹ and Val¹⁵⁰ residues are also indicated by red and blue spheres, respectively. (C) Disulfide bond formation is partially defective in the *dsbA* mutants. Strains HK354 ($\Delta dsbA$, lane 1), HK353 [wild-type (WT), lane 2], HK348 (*dsbA* P151T, lane 3), HK349 (*dsbA* V150G, lane 4), and HK351 (*dsbA* P151S, lane 5) were transformed with pUC18 carrying *bla* and were grown at 30°C. Proteins of culture were alkylated with AMS, separated by SDS-PAGE, and visualized by Western blotting with antibody to β -lactamase (Bla) (18).

Fig. 2. Characteristics of the *dsbA* mutants. (A) The redox state of DsbA in the mutants. AMS-alkylated lysates of HK354 (lane 1), HK353 (lane 2), HK355 ($\Delta dsbB$, lane 3), HK348 (lane 4), HK349 (lane 5), and HK351 (lane 6) were subjected to SDS-PAGE and Western blotting with antibody to DsbA. Open arrowheads, nonspecific bands; asterisk, unknown complex. The positions of marker proteins are indicated on the left in kD. (B) Formation of the mixed disulfide complexes in the P151T mutant depends on DsbB. AMS-alkylated lysates of HK355 ($\Delta dsbB$, lane 1) and HK357 ($\Delta dsbB$, *dsbA* P151T, lane 2) were analyzed with antibody to DsbA. (C) Formation of mixed disulfide complexes in the P151T mutant occurs only transiently after the synthesis of the substrates. HK353 (lanes 1 to 10) and HK348 (lanes 11 to 20) were pulse-labeled with [³⁵S]-methionine for 30 s and chased with excess cold methionine for the indicated times. After alkylation of the pulse-chased samples with AMS, the DsbA and DsbA-substrate complexes were immunoprecipitated from the lysates with antibody to DsbA and analyzed by SDS-PAGE and fluorography. Samples in lanes 6 to 10 and 16 to 20 were incubated with 50 mM dithiothreitol before electrophoresis. DTT, dithiothreitol; c, DsbA P151T-substrate complexes; p, partners of DsbA P151T (substrates).



ond, the formation of the complexes depended on DsbB (Fig. 2B), indicating that their presence requires that DsbA be in the oxidized state. Finally, coexpression of wild-type DsbA from a plasmid to oxidize substrate proteins resulted in decreased amounts of the complexes (21), indicating that these complexes were formed during the oxidation of the substrate proteins by the P151T mutant and not, for example, by reaction of reduced DsbA with oxidized substrates.

We followed the formation of the mixed disulfide complexes in the P151T mutant by pulse-chase experiments. Cellular proteins were labeled, and DsbA complexes immunoprecipitated with antibody to DsbA. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and reducing conditions (Fig. 2C). Complex formation reached a maximum within 1 min after the synthesis of substrates, indicating that these complexes were formed between DsbA P151T and newly synthesized substrate proteins.

To identify the complexed substrates, we purified the complexes using a deca-histidine tag fused to the C-terminus of the P151T mutant protein. The purified complexes were separated by two-dimensional (2D) gel electrophoresis in which the first dimension was nonreducing and the second was reducing (18). A number of spots migrated at 21 kD in the second dimension (Fig. 3). Because antibody to DsbA reacts specifically with these spots (21), they are DsbA P151T. Proteins in each spot along the off-diagonal line (flanked by two open arrows in Fig. 3) represent the partners of DsbA P151T. For example, a 53 kD complex in the first dimension was resolved into a 32 kD protein and DsbA P151T (21 kD) in the second dimension. Similarly, every other spot between the two open arrows

has a corresponding DsbA spot; the sum of the molecular masses of the two spots in each pair agrees well with the apparent molecular mass of the complex in the first dimension.

More than 10 spots are clearly recognizable between the two open arrows on the Coomassie brilliant blue-stained gel (Fig. 3). Proteins in each of these spots were analyzed by mass spectrometry. Fifteen different proteins were identified in this way, including well-characterized proteins and proteins identified as open reading frames (table S1). Among these, OmpA is the only well-studied substrate of DsbA. Most of the other proteins are known (eight proteins) or predicted (five proteins) to be exported and each of them has at least one cysteine residue in its sequence. Thus they are potential substrates of DsbA. (A protein with only one cysteine may form an intermolecular disulfide bridge). We also identified Ef-Tu (elongation factor Tu) as a minor component of one of the spots. This protein apparently represents a false positive because it is located in the cytoplasm. Ef-Tu has also appeared as the major false positive mixed disulfide complex with other redox active proteins studied in our laboratory (24), probably because of its extremely high abundance and the high reactivity of its cysteines (Cys⁸¹ and Cys¹³⁷) (25).

To confirm that the identified proteins are substrates of DsbA, we cloned some of the genes and fused a c-Myc tag at the C-terminus of the proteins for their detection (Fig. 4) (18). Proteins tested were OmpA (positive control), LivK, YodA, RcsF, and YbjP. In the wild-type strain, the cysteines of these proteins were completely oxidized. In a $\Delta dsbA$ strain, however, they were mostly or partially reduced. All of the tested proteins seemed to have one or more disulfide bonds in vivo and require DsbA for their efficient disulfide

bond formation, showing that they are substrates of DsbA.

The accumulation of DsbA-substrate complexes in the P151T mutant indicates that resolution of these complexes is retarded. We considered the possibility that alteration of the redox potential of the protein was responsible for the defect. We measured the standard redox potentials of the mutant proteins (18) and found them to be equal to or slightly lower than that of wild-type enzyme, which is -127 mV (P151T, P151S, and V150G mutant proteins were -152 mV, -151 mV, and -128 mV, respectively). Because the P151T and P151S proteins had almost the same redox potential but showed different phenotypes, redox potential alone cannot explain why the P151T mutant accumulates the DsbA-substrate complexes.

We suspect that specific structural features of the P151T mutant prevent rapid resolution of DsbA-substrate complexes. The published crystal structure of human thioredoxin complexed with the Ref-1 peptide (in which one cysteine in Ref-1 and one in thioredoxin are mutated to stabilize the complex) (26) provides a possible explanation for our findings. In the structure, the ring of this *cis* proline residue abuts the sulfur of the substrate cysteine that is involved in the mixed disulfide. This interaction may be important for maintaining the correct positioning of the mixed disulfide bond (26). The P151T alteration in DsbA may change this positioning, leading to slower resolution of complexes.

On the basis of sequence analysis, more than 1000 proteins have been proposed to contain a redox-active Cys-X-X-Cys motif in a thioredoxin-like fold (27). Most of these proteins are believed to carry out the oxidation, reduction, or isomerization of substrate proteins via formation of mixed disulfide complexes (2, 3). A second reaction of these

Fig. 3. Purification and identification of the partners of DsbA P151T. Culture (4 l) of HK354 carrying a DsbA-His₁₀ P151T expression plasmid pHK630 was alkylated with iodoacetamide (18). DsbA P151T-containing mixed disulfides were purified from the alkylated lysate using a deca-histidine tag fused to the C-terminus of DsbA P151T, separated on a nonreducing-reducing 2D gel, and visualized by Coomassie staining (18). Proteins in spots along the off-diagonal line flanked by two open arrows represent partners of DsbA P151T. They were identified by mass spectrometry and are indicated. DsbA P151T and DsbB, which co-purified with DsbA P151T, are also indicated. Note that Imp was found at two positions. SlyD, on the diagonal line, is a histidine-rich protein that binds to the Ni column. Some spots running below the off-diagonal line could originate from oligomeric disulfide-bonded complexes.

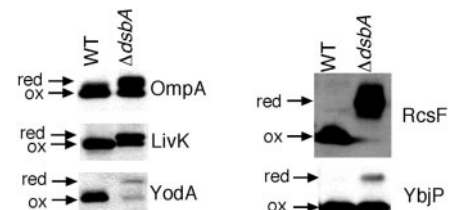
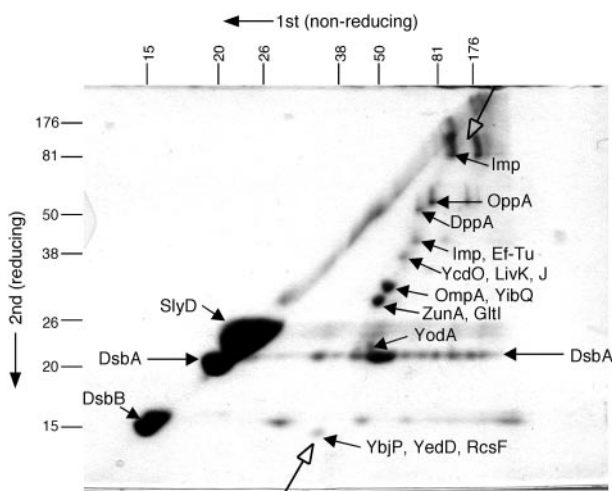


Fig. 4. The partners of DsbA P151T have one or more disulfide bonds in vivo and require DsbA for their efficient oxidative folding. AMS-alkylated lysates of HK353 (WT) or HK354 ($\Delta dsbA$) carrying a plasmid, pHK643 (OmpA-c-Myc), pHK641 (LivK-c-Myc), pHK642 (YodA-c-Myc), pHK646 (RcsF-c-Myc), or pHK648 (YbjP-c-Myc) were separated by SDS-PAGE and visualized by Western analysis with antibody to c-Myc. The positions of the oxidized (ox) and reduced (red) forms of each protein were determined using the samples without AMS alkylation (for oxidized position) and the samples that were first reduced with 50 mM DTT and then alkylated with AMS (for reduced position) (27).

proteins is with enzymes that are responsible for maintaining them in the appropriate redox state for their activities, analogous to the DsbB-DsbA interaction. These reactions also appear to involve mixed disulfide intermediates. However the mixed disulfide bonded reaction intermediates in either direction are, in most cases (28), difficult to detect probably because such complexes resolve very quickly (29). Here, in the case of DsbA, we show that it is possible to detect such intermediates by mutations that alter a *cis* proline residue highly conserved in the structures of proteins with a thioredoxin-like fold. A striking finding is that one of the mutations (P151T) results in accumulation of complexes between DsbA and its substrates, whereas the other (P151S) accumulates complexes between DsbA and the enzyme that oxidizes it, DsbB. Because this proline residue is conserved in most thioredoxin family members, it seems possible that mutational alteration of this residue in other family members will also allow detection of reaction intermediates. However, success in such efforts may depend on the precise geometry and distance of the proline relative to the active site and the nature of the amino acid substituted.

Recently, mutants lacking the C-terminal cysteine of the Cys-X-X-Cys motif of a plant thioredoxin were used to trap putative enzyme-substrate complexes (30, 31). This approach may not be as informative for proteins that act as oxidants such as DsbA, where the formation of such complexes would represent the reverse reaction from that normally carried out by the protein.

Further studies with the DsbA P151T mutant should allow identification of a larger number of DsbA substrates. Analysis of these complexes should also yield a more detailed understanding of the mechanism of DsbA action, including (i) which cysteines in substrate proteins are recognized during its action, (ii) at what point during protein translocation these reactions take place, and (iii) the role of the *cis* proline in this process.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/303/5657/534/DC1

Materials and Methods

Tables S1 to S3

References

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Extensive Gene Traffic on the Mammalian X Chromosome

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Mammalian sex chromosomes have undergone profound changes since evolving from ancestral autosomes. By examining retroposed genes in the human and mouse genomes, we demonstrate that, during evolution, the mammalian X chromosome has generated and recruited a disproportionately high number of functional retroposed genes, whereas the autosomes experienced lower gene turnover. Most autosomal copies originating from X-linked genes exhibited testis-biased expression. Such export is incompatible with mutational bias and is likely driven by natural selection to attain male germline function. However, the excess recruitment is consistent with a combination of both natural selection and mutational bias.

The mammalian X and Y chromosomes changed profoundly in their differentiation from ancestral autosomes (1–3). Throughout this process, the selective placement of new genes can be driven by gene duplication (1, 4). The Y chromosome has been shown to recruit male-specific genes (1, 2), whereas a few individual X-linked genes have male-specific duplicate counterparts on autosomes (4). Furthermore, some male-specific genes appear to be enriched on the X chromosome (5, 6). However, analysis of human genome project data indicated that no pattern exists for gene movements involving the X chromosome in humans (7). To elucidate gene movements in the human and mouse genomes, we

analyzed duplicate genes produced by retroposition, whereby a mature messenger RNA (mRNA) is reverse-transcribed and re-integrated into the genome (8).

Retroposition is an important mechanism of gene copying (9) and produces a large number of functional genes in mammalian genomes. It accounts for approximately 10,000 duplication events in the human genome (10), of which approximately 10% are functional (11). The direction of copying can be inferred from sequence features of each member of the duplicate pair (12): the processed retrocopy is intronless, whereas its parental gene usually contains introns (13). Retrocopies that recently integrated into the genome may also display a 3' polyadenylate [poly(A)] tract and flanking direct repeats. We screened annotated genes in the human genome (7, 14, 15) for functional retropositions (16), identifying 655 retroposition events, of which 366 involved interchromosomal movements. Furthermore, with the use of stringent functionality criteria based on both selective constraint, a nonsynonymous to synonymous substitution rate ratio (K_A/K_S) less

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