Cell, Vol. 79, 1129–1132, December 30, 1994, Copyright © 1994 by Cell Press

Protein Folding and the Regulation of Signaling Pathways

Minireview

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Summary

A growing number of intracellular signaling molecules are found associated with components of the cellular protein folding machinery. In this minireview we suggest that the same ancient cellular process that promotes the folding and assembly of nascent proteins plays a pivotal role in signal transduction by promoting the regulated folding or assembly and disassembly of mature signaling molecules between active and inactive states. Members of the protein folding machinery mediate the activity of various kinases, receptors, and transcription factors. These may be poised in late stages of folding or assembly until upstream signaling events trigger their biogenesis into activated molecules.

Introduction

Most aspects of protein homeostasis, from the folding and assembly of newly synthesized proteins to their repair or degradation, are supported by a sophisticated cellular machinery that is thought to have been present throughout prokaryotic and eukaryotic evolution (Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993). Protein folding in the cell is facilitated by molecular chaperones and enzymes (Gething and Sambrook, 1992). The major chaperone families are Hsp70 (DnaK), Hsp60 (chaparonin/GroEL/TCP1/TRiC), and associated components (e.g., DnaJ, GroES, and GrpE) (Georgopoulos and Welch, 1993). Recently, Hsp90 has also been implicated in protein folding (e.g., Melnick et al., 1994), but the mechanistic details remain obscure (Jakob and Buchner, 1994). The past few years have seen major advances in the detailed understanding of Hsp60 and Hsp70 function. In essence, chaperone activity is based on their selective affinity for nonnative proteins. A characteristic of nonnative proteins is the solvent exposure of interior hydrophobic residues. By binding to proteins prior to their folding or assembly into oligomers, chaperones protect these exposed hydrophobic residues from nonproductive inter- and intramolecular aggregation. Transient binding of nonnative proteins by chaperones destabilizes kinetically trapped intermediates that can then resume potentially productive folding pathways. As native proteins are not generally recognized, such cycles of binding, unfolding, and release favor the accumulation of folded versus nonnative forms.

Different chaperones are clearly distinct from each other in their substrate specificities and cellular function. For instance, Hsp70 preferentially recognizes extended conformations and acts prior to Hsp60 in the folding pathway. Similarly, proteins undergoing folding in the lumen of the endoplasmic reticulum (ER) interact with different ER resident chaperones during successive stages of their biogenesis (e.g., Melnick et al., 1994).

Chaperones also make long-term associations with their targets. Stable associations allow the target proteins to remain competent for assembly with additional subunits as they become available. This may be particularly important in the assembly of large protein complexes. In some circumstances, chaperones recognize mature proteins and promote their unfolding or the disassembly of oligomeric complexes. For example, the phage λ DnaB helicase complex requires disassembly by Hsp70 to release active helicase, and clathrin cages are disassembled by Hsp70 during vesicle budding (Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993).

Chaperones work in concert with each other and with enzymes that catalyze conformational changes that may provide energy barriers to folding. Two rate-limiting events that have been determined for the folding of polypeptides in vitro are the sampling and attainment of the proper configuration of disulfide bonds and the *cis–trans* isomerization of the polypeptide backbone around the peptide bond adjacent to proline residues. These reactions are catalyzed by two enzyme families found in all cell types. Protein disulfide isomerases reside in the reducing environment of the ER and catalyze the making and breaking of disulfide bonds. Peptidylprolyl *cis–trans* isomerases (PPIases) are found in the cytoplasm and in most subcellular compartments, where they may aid the folding of cellular proteins (e.g., Stamnes et al., 1991).

There are two known classes of PPIase making up two structurally distinct families. These are cyclophilins and FK506-binding proteins (FKBPs). The importance of the cyclophilins and FKBPs in protein folding has sometimes been overshadowed by their medical importance in mediating the effects of the immunosuppressant drugs cyclosporin A and FK506. Both drugs in complex with their respective binding proteins block intracellular signaling pathways important for the activation of T lymphocytes by inhibiting calcineurin function. However, this appears to be unrelated to the general function of cyclophilins and FKBPs in protein folding and has been reviewed elsewhere (Schreiber and Crabtree, 1992).

Several unifying features characterize both the molecular chaperones and the PPIases. First and most important, they are both involved in protein folding. Second, they belong to large, highly conserved families, so conserved in fact that they are believed to have existed in the oldest common ancestor of all living things. Third, they are abundant, with constitutively expressed cytoplasmic isoforms individually making up 1%–2% of the total cellular protein (together the chaperones and PPIases may account for 5%–10% of the protein in the cell). Fourth, they have isoforms that are induced by subjecting cells to treatments causing protein damage (heat shock proteins [hsps]). Finally, members of both the chaperone and PPIase families reside in most subcellular compartments in all cells, from bacteria to plants to mammals.

Chaperones and PPIases Mediate Intracellular Signaling

A number of chaperones and PPIases have been found

associated with various signaling molecules. However, because of the abundance of these proteins and their wide substrate specificity, the significance of these biochemical associations has been slow to emerge. Furthermore, many of the protein–protein interactions have been observed in nonphysiological, low salt buffers, raising the question of specificity and relevance in vivo. In light of our growing understanding of the role of molecular chaperones and enzymes in protein folding and maintenance and because of recent evidence for the involvement of chaperones in signaling processes, these associations deserve renewed attention. Given the pervasiveness of members of the folding machinery and their ability to facilitate conformational changes, it is tempting to speculate that these proteins may have allowed the evolution of signaling strategies that rely on structural transitions to switch between "on" and "off" states.

Signaling by Receptor Tyrosine Kinases Requires Hsp90

Recent studies in Drosophila have implicated Hsp90 in signaling by the membrane tyrosine kinase receptors *sevenless* and *Torso* (Cutforth and Rubin, 1994). *hsp90* is one of seven genes originating from the saturating genetic screen pioneered by Simon et al. (1991) that helped identify the GTPase $p21^{Ras1}$, the guanine nucleotide exchange factor Sos, and the SH3–SH2–SH3 domain protein Drk as important components of the *sevenless* signaling pathway. All of the five alleles of *hsp90* obtained in this screen are lethal as homozygotes. As heterozygotes, they decrease the *hsp90* gene product by one half and, when in combination with a weakly mutant *sevenless* receptor, impair the crippled *sevenless* signaling cascade to the point where it becomes nonfunctional. The isolation of *hsp90* in this screen indicates that it acts somewhere in the normal *sevenless* pathway. Importantly, these alleles also interact with signaling by the *Torso* tyrosine kinase receptor (Doyle and Bishop, 1993), establishing Hsp90 as a player in at least two Ras-dependent signaling pathways.

In the simplest interpretation, Hsp90 may be required for the folding or assembly of an important component(s) required in the Ras signaling pathway. However, there is a more intriguing possibility: Hsp90 may be required to facilitate the transition between active and inactive states of a component of this pathway. Indeed, research performed in the past decade has led to the inescapable conclusion that Hsp90 function is intimately tied to the activation of at least two signaling pathways, those mediated by the steroid receptor superfamily of transcription factors and $pp60^{v\text{-}src}$.

Steroid Receptor Function Is Mediated by Hsp90

The steroid receptor superfamily includes several nuclear and cytoplasmic members that, upon ligand binding, are converted into active modulators of transcription. Some of the steroid receptors can be isolated in complex with Hsp90; these include the estrogen, progesterone, androgen, glucocorticoid, mineralocoid, and dioxin receptors. These receptors are comprised of distinct DNA-binding and hormone-binding domain. This latter domain is also considered a signaling domain as it is thought to communicate with the DNA-binding domain (reviewed in Bohen and Yamamoto, 1994). In the inactive form, the receptors are associated with large (9S–10S) particles composed in part of chaperones and PPIases. The hallmark of the steroid receptor–hsp heterocomplex is the association of a dimer of Hsp90 per receptor monomer. There are, however, a variety of other proteins also present (see below).

Upon activation, the steroid receptors dissociate from Hsp90 and become competent to bind DNA. Interestingly, receptors can be activated in the absence of ligand by heat or salt conditions that cause their dissociation from the hsp complex (Willmann and Beato, 1986). The tight correlation between receptor activity and dissociation from Hsp90 led to the initial interpretation that the function of Hsp90 is purely inhibitory, playing a passive role in keeping receptor inactive. However, there are several reasons why this is unlikely, both for steroid hormone receptors and for other signaling molecules that interact with Hsp90 complexes prior to activation.

The clearest evidence that the Hsp90 heterocomplex has an enabling, rather than an inhibitory, role in transcriptional regulation by steroid receptors comes from an important series of experiments carried out in the laboratories of Lindquist and Yamamoto. Yeast can be made steroid responsive by the introduction of steroid receptor/steroid response element reporter constructs. Lindquist, Yamamoto, and coworkers used this yeast system to test the genetic requirement for Hsp90 by steroid receptors. They found that the ability of estrogen and glucocorticoids to induce the expression of a β-galactosidase reporter construct via their respective receptors is strikingly dependent on Hsp90. These results established that an Hsp90-dependent function is essential for the activation of steroid hormone receptors in vivo (Picard et al., 1990; Bohen and Yamamoto, 1994).

Biochemical evidence suggests that Hsp90 has a direct role in modulating steroid receptor structure and activation. Hsp90 specifically binds to the ligand binding/signaling domain of steroid receptors and may be responsible for important conformational states associated with the activities attributed to this domain (Bohen and Yamamoto, 1994). For instance, for the glucocorticoid receptor to be responsive to ligands, the signaling domain must be in the high affinity conformation. Association with the Hsp90 heterocomplex is required to generate and, for most of the steroid receptors, to maintain the high affinity binding state (Bresnick et al., 1989). Moreover, glucocorticoid receptors must interact with Hsp90 to have the potential of being activated by ligand. Thus, Hsp90 may provide important conformational support for the high affinity ligand site and may also help promote the proper transition of this domain into a state that no longer prevents DNA binding.

$pp60^{v\text{-}src}$ and Other Kinases Require Chaperone Function

The transforming tyrosine kinase $pp60^{v\text{-}src}$ associates with an Hsp90 heterocomplex (Brugge, 1986). Interestingly, $pp60^{v\text{-}src}$ activation is correlated with its release from this complex and translocation into the plasma membrane. Indeed, temperature-sensitive $pp60^{v\text{-}src}$ alleles that are inactive at the nonpermissive temperature retain their associa-

tion with Hsp90 and are not targeted to the membrane. However, when shifted to the permissive temperature, they dissociate from Hsp90, are targeted to the plasma membrane, and are active as kinases. Using the yeast expression system, Xu and Lindquist (1993) have recently shown that Hsp90 facilitates $pp60^{v\text{-}src}$ kinase activity. Thus, Hsp90 does not simply inhibit the kinase activity of $pp60^{v\text{-}src}$, and, as in the case of the steroid receptors, the chaperone complex appears to have a highly specific role in maturation and activation of this signaling molecule.

Hsp90 also associates with and may modulate the function of serine/threonine protein kinases. Casein kinase II (CKII) is implicated in cell growth and the cell cycle, and eIF-2a kinase is important in the regulation of protein synthesis. Both CKII and eIF-2a kinase reportedly associate with hsp complexes containing Hsp90 and a number of other chaperones (Matts et al., 1992). Unexpectedly, the activity of both kinases appears to rely on Hsp90 in a dose-dependent manner (Rose et al., 1989; Miyata and Yahara, 1992). When purified CKII is incubated in the presence of recombinant Hsp90, CKII activity is dramatically enhanced. Similar results were obtained with eIF-2a kinase. Although these enhancements may simply reflect a chaperone activity of Hsp90 by protecting the kinases from aggregation, the data are suggestive of a regulatory role for Hsp90.

It is not yet clear which component of the highly conserved Ras signaling pathway relies on Hsp90 function. A direct biochemical association between Hsp90 and *sevenless* or *Torso* has not yet been demonstrated, and in theory, Hsp90 could be acting at any point downstream of activated receptor. Interestingly, mammalian Raf has been shown to associate in a native heterocomplex with a number of chaperones, including Hsp90 and Hsp70 (Wartmann and Davis, 1994). As it turns out, Raf activation requires translocation to the plasma membrane, and recent studies have demonstrated that the role of active $p21^{ras}$ is to recruit Raf to the membrane (Leevers et al., 1994; Stokoe et al., 1994). Therefore, it is possible that Hsp90 may maintain Raf in a state competent for interaction with appropriate targets or for its assembly into a signaling complex.

Do Other Chaperones Associate with Signaling Molecules?

In addition to Hsp90, many chaperones and PPIases have been found in stable association with various receptors, kinases, and transcription factors. These include Hsp70, Hsp56/FKBP59, FKBP54 (Smith et al., 1990), and Cyp40 (Kieffer et al., 1993). Although not much is known about specific modulatory roles for these chaperones, this may simply reflect a lack of suitable assays or the identification of biologically relevant targets. For instance, although FKBP12 has been shown to associate with a variety of intracellular proteins, yeast mutants defective in FKBP12 display no overt phenotype. However, two groups have now demonstrated that FKBP12 is involved in the gating properties of the ryanodine receptor. The ryanodine receptor is the calcium release channel of muscle sarcoplasmic reticulum. FKBP12 both colocalizes and copurifies with the ryanodine receptor (Jayaraman et al., 1992). The stoichiometry of their association is one molecule of FKBP12 per receptor, with a functional channel comprising four identical subunits and one molecule of FKBP12. When the ryanodine receptor is expressed in insect cells, its single channel properties do not match those seen in intact muscles. Remarkably, when the receptor and FKBP12 are coexpressed in the same cells, the functional properties of the channel mimic those observed in vivo (Brillantes et al., 1994). Pharmacological data also suggest that FKBP12 is required for ryanodine receptor function. For example, when FK506 or rapamycin, both of which are inhibitors of the PPIase activity of FKBP12, is added to intact muscle cells, the ryanodine channel properties are similar to those of channels expressed without FKBP12 (Brillantes et al., 1994). Taken together, these results are consistent with a model in which FKBP12 facilitates conformational changes or protein–protein interactions that affect the gating properties of the ryanodine receptor.

Zen and the Art of Oligomerization and Transcriptional Control

It appears that molecular chaperones exert important control over the activity of transcription factors and DNA-binding proteins by regulating their state of oligomerization. Two particularly well-characterized examples are the involvement of chaperones in phage λ DNA replication (repA and λP) and in the induction of the eukaryotic heat shock response (HSF) (Georgopoulos and Welch, 1993; Craig et al., 1994). For instance, repA is only active as a monomer, and the transition from oligomer to monomer is mediated by the bacterial chaperones DnaK and DnaJ. In the case of HSF, its activation involves the formation of a homotrimer in which Hsp70 has been implicated (see Craig et al., 1994).

Given their importance in the synthesis and assembly of intracellular protein complexes, it is interesting that a large number of chaperones and PPIases are found in the nucleus. This is particularly intriguing since transcriptional regulation in eukaryotic cells involves a combinatorial assembly of specific transcription factors. Furthermore, important conformational changes are known to take place upon the assembly of factors with their DNA targets; for example, local protein folding transitions are coupled to DNA binding (Spolar and Record, 1994). We propose chaperones may function as important regulators of gene expression by acting as switches in the formation of the appropriate transcription factor–transcription factor or transcription factor–DNA complexes. Such a mechanism would help ensure that complexes form at the right time and place in response to the correct signals.

Concluding Remarks

A picture is emerging whereby the cellular protein folding machinery plays a pivotal role in regulating signaling pathways. Taken as a whole, the accumulated evidence suggests that chaperones and PPIases promote structural changes required for the activation of signaling molecules. These structural modifications are manifest as changes in protein activities, oligomerization states, binding affinities, and single channel properties. It is perhaps not surprising that cells have taken advantage of a ubiquitous and conserved system, such as the protein folding machinery, to

control the activities of signaling proteins. Interestingly in this regard, it has recently been reported that another highly conserved system for maintaining cellular proteins, the hsp–ubiquitin proteosome degradation pathway, is used to activate NF-κB signaling by the regulated cleavage of the p105 precursor of the p50 (NF-κB1) subunit (Palombella et al., 1994). It could well be that we are just beginning to unravel the many interesting and important uses of these often overlooked "housekeeping" proteins.

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