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Molecular Chaperones: Biology and Prospects for Pharmacological Intervention

DAVID F. SMITH,[a] LUKE WHITESELL, AND EMMANUEL KATSANIS

Department of Pharmacology (D.F.S.), University of Nebraska Medical Center, Omaha, Nebraska; Department of Pediatrics (L.W., E.K.), Steele Memorial Children's Research Center, University of Arizona, Tucson, Arizona

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[a] Address for correspondence: David F. Smith, Department of Pharmacology, University of Nebraska Medical Center, Omaha, NE 68198-6260. E-mail: dfsmith@mail.unmc.edu.

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I. Protein Folding in the Cell

Successful expression of polypeptide gene products requires transcription machinery to generate messenger ribonucleic acids and translation machinery to synthesize polypeptide chains but the protein product is functional, and thus the gene is effectively expressed, only when the polypeptide chain is folded into its native three-dimensional conformation, appropriately localized within or secreted from the cell, and, in many instances, properly assembled into multicomponent complexes. Although the amino acid sequence alone is sufficient to dictate the native conformation of small proteins in vitro (Anfinsen, 1973), most polypeptides would fail to fold efficiently in the highly concentrated, complex, cellular environment without the assistance of yet another type of machinery. This latter type of machinery involves the so-called molecular chaperones, i.e., proteins adapted to facilitate protein folding. The chaperone concept was first proposed by Ellis (1987), and a large, highly active field of research has since developed to investigate the functions and physiological implications of molecular chaperones. Major aspects of this field have been recently reviewed (Buchner, 1996; Hartl, 1996), and many details have been compiled in a more comprehensive manner (Gething, 1997).

There is an inherent problem in the folding of nascent polypeptide chains. Whether synthesized on free ribosomes in the cytoplasm or on ribosomes associated with endoplasmic reticulum (ER),[b] nascent protein chains emerge in a linear manner. Consequently, hydrophobic stretches and other potentially interactive sites must sometimes wait for downstream sequences to emerge before appropriate folding interactions can be established. Inappropriate interactions can readily lead to poorly reversible conformations and aggregations that reduce the efficiency of native folding reactions (Jaenicke, 1995). Molecular chaperones reversibly interact with nascent chains to minimize off-pathway interactions and increase the yield of native folded protein.

In addition to the folding of nascent polypeptide chains, other cellular processes involve protein folding that requires chaperone participation. One of these is the transport of previously synthesized proteins across cell membranes, as best understood for mitochondrial protein import (Langer *et al.*, 1997). Many mitochondrial proteins are encoded by nuclear genes, synthesized on free ribosomes in the cytoplasm, and subsequently transported across one or both mitochondrial membranes. Proteins are translocated across the mitochondrial membranes in a linear manner as unfolded chains, and there are both cytoplasmic and mitochondrial chaperones that participate in the unfolding and refolding pathways.

II. Molecular and Chemical Chaperones

A. Heat Shock Response and Heat Shock Proteins

Several physical and chemical conditions favor inappropriate folding of proteins and are thus hazardous to cells. Among these proteotoxic conditions are elevated temperature, anoxia, and exposure to ethanol, heavy metals, or other chemical denaturants. It has been recognized for >30 years that mild temperature elevation can induce a so-called heat shock response in all cells (reviewed by Lindquist and Craig, 1988). This response is characterized by a rapid shutdown of the synthesis of most proteins, with a dramatic transient increase in the synthesis of a small set of proteins, accordingly called heat shock proteins (Hsps). A similar response is observed after other proteotoxic insults, so the more general terms stress response and stress proteins are sometimes used. For years the function of Hsps was unknown, but studies in the past 10 years have defined the major Hsps as central components of the molecular chaperone/protein folding machinery. Thus, stress responses are evolutionary adaptations to quickly resolve misfolded proteins and restore the normal protein folding environment of cells. In addition, an initial sublethal exposure to heat or other stresses can condition cells for enhanced survival during exposure to subsequent, even more severe, stresses (Gerner and Schneider, 1975); this is commonly termed thermotolerance and is largely attributable to induction of Hsps (reviewed by Parsell *et al.*, 1993).

Many Hsps and their associated co-chaperones are constitutively expressed in all cells. There are several multigene Hsp families, and individual genes within families differ to varying degrees with respect to sequence and expression patterns, as well as the function

[b] Abbreviations: Aβ, amyloid β protein; 17-AAG, 17-allylaminogeldanamycin; ADP, adenosine diphosphate; APP, amyloid precursor protein; ATP, adenosine triphosphate; CFTR, cystic fibrosis transmembrane conductance regulator; Cyp, cyclophilin; DNA, deoxyribonucleic acid; DSG, deoxyspergualin; ER, endoplasmic reticulum; FKBP, FK506-binding protein; GA, geldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HSF1, heat shock transcription factor 1; Hsp, heat shock protein; Htg, huntingtin; MHC, major histocompatibility complex; NIA, nonimmunosuppressive analog; polyGln, polyglutamine; PPIase, peptidylprolyl isomerase; PR, progesterone receptor; PrP, prion protein; PrP^{C}, cellular form of prion protein; PrP^{Sc}, infectious (scrapie) form of prion protein; TCP, T-complex polypeptide; TPR, tetratricopeptide repeat; TOR, target of rapamycin.

and subcellular localization of the respective gene products (table 1). Major Hsp families, named to reflect the approximate molecular size (in kilodaltons) of family members, are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsp family (typically 20 to 25 kDa). The exact function or range of functions for each of the families and its individual members is being actively investigated in many laboratories. Hsp70, Hsp40, and Hsp60 family members play important roles in nascent chain folding (Hartl, 1996). Hsp70 members are also major components in membrane translocation processes. The small Hsps have important functions in disaggregation or degradation of misfolded complexes (Gething, 1997), but it is not clear how important they are for nascent chain folding. Hsp90 also may have a role in nascent chain folding, but it is most notable for its numerous associations with important regulatory proteins (Pratt and Toft, 1997). Hsp100 family members, such as Hsp104, play an important role in thermotolerance in yeast (Lindquist and Kim, 1996; Sanchez and Lindquist, 1990; Schirmer *et al.*, 1996). An overall increase in Hsp levels correlates with the acquisition of thermotolerance, but increased Hsp104 levels appear to be the critical factor. Importantly, Hsp104 is adept at preventing and reversing protein aggregation (Parsell *et al.*, 1994). In addition to these major Hsp families, there are many additional proteins whose expression is responsive to various stresses, but these are not discussed except as they pertain to the topics presented here.

B. Chaperone-Mediated Nascent Chain Folding

Although much remains to be learned in this relatively new field, some chaperone-mediated processes have been characterized in detail. Illustrative of the coordination among chaperones is the typical pathway for nascent chain folding in *Escherichia coli* (reviewed by Martin and Hartl, 1997). This pathway involves Hsp40, Hsp70, Hsp60, and Hsp10 family members. After approximately 50 residues on the nascent chain emerge from the ribosome, DnaJ (an Hsp40) and DnaK (an Hsp70) have access to the growing polypeptide. As synthesis is completed, the nascent chain, if no more than approximately 50 kDa, is passed from DnaJ/DnaK to the lumen of a double-ring complex formed by 14 subunits of GroEL (an Hsp60). The GroEL ring can be capped by a seven-subunit ring of GroES (an Hsp10), transiently enclosing the nascent polypeptide within the GroEL cavity. The nascent chain undergoes rounds of binding and release from GroEL/GroES complexes until its folding is complete. DnaK and GroEL interactions with the incompletely folded substrate are adenosine triphosphate (ATP)-dependent and regulated by ATP hydrolysis. In addition, at least one other protein, termed GrpE, participates as a partner protein of DnaK.

In eukaryotes, a typical pathway of chaperone interactions with cytoplasmic nascent chains has been more difficult to define, although Hsp40 and Hsp70 homologs are important components (Eggers *et al.*, 1997; Frydman *et al.*, 1994; Hansen *et al.*, 1994). Netzer and Hartl (1997) recently pointed out that nascent chain folding in eukaryotes occurs more at the co-translational level, compared with posttranslational folding in prokaryotes, and co-translational folding correlates with the more common occurrence of multidomain proteins in eukaryotes. These differences may contribute to the dissimilarities of chaperone systems involved in nascent chain folding in prokaryotes versus eukaryotes.

Whereas GroEL is a general nascent chain factor in prokaryotes, the closest Hsp60 homologs in eukaryotes are in mitochondria and chloroplasts (essentially prokaryotic organelles). In the cytoplasmic compartment there is a family of proteins, termed T-complex polypeptides (TCPs), that are loosely homologous with GroEL and form double-ring complexes similar to that of GroEL (Kubota *et al.*, 1995). TCP ring complexes are less abundant in cytoplasm than would appear to be necessary for general protein folding, so they may have a limited range of substrates. Significantly, tubulin and actin subunits are specific substrates that require the chaperoning of TCP ring complexes (Sternlicht *et al.*, 1993; Yaffe *et al.*, 1992).

The bacterial Hsp90 homolog Htpg is expressed at low levels and is nonessential (Bardwell and Craig, 1988). In marked contrast to prokaryotic systems, Hsp90 is typically the most abundant chaperone in eukaryotic cytoplasm, and its expression is essential (Borkovich *et al.*, 1989). The potential role of Hsp90 in nascent chain folding is unresolved (Eggers *et al.*, 1997). In in vitro refolding assays in a minimal buffer, eukaryotic Hsp90 is capable of holding heat-denatured enzymes in a competent state for refolding (Wiech *et al.*, 1992), and Hsp90 has two apparent substrate binding sites (Scheibel *et al.*, 1998; Young *et al.*, 1997). However, based on a study in *Saccharomyces cerevisiae* that examined the chaperone function of Hsp90 in vivo (Nathan *et al.*, 1997), Hsp90 probably is not required for nascent chain folding of most proteins but is required for a subset of proteins. In addition to nascent chain folding and refolding of denatured proteins, the regulatory interactions of Hsp90

TABLE 1
Functions of Hsp families

Family	Major functions	References
Hsp100	Protein disaggregation, thermotolerance	Schirmer *et al.*, 1996
Hsp90	Regulatory interactions with signaling proteins, stabilization of misfolded proteins	Csermely *et al.*, 1998
Hsp70	Protein folding, membrane transport of proteins	Hartl, 1996
Hsp60	Protein folding (limited substrates in eukaryotic cytoplasm)	Hartl, 1996; Buchner, 1996
Hsp40	Protein folding, co-chaperone for Hsp70	Cyr, 1997
Small Hsp	Stabilization of misfolded proteins, thermotolerance, eye lens structural proteins	Buchner, 1996

with a variety of important signal transduction proteins have been extensively characterized.

C. Cytoplasmic Co-chaperones

There are several eukaryotic chaperone components that have no prokaryotic homologs. Some of these have activities similar to those of Hsp90, as determined in vitro in refolding assays (Freeman *et al.*, 1996), perhaps generating redundancy in this potentially important cellular function. On the other hand, a strong case has been developed for their functioning as partner proteins or co-chaperones that directly interact with Hsp70 and/or Hsp90. Included in a growing list of Hsp70-associated partners are Hip/p48 (Hoehfeld *et al.*, 1995; Prapapanich *et al.*, 1996), Hop/p60 (Honore *et al.*, 1992; Smith *et al.*, 1993), Bag-1 (Hoehfeld and Jentsch, 1997; Takayama *et al.*, 1995, 1997), RAP46 (Gebauer *et al.*, 1997; Zeiner *et al.*, 1997; Zeiner and Gehring, 1995), and p16, a member of the Nm23/nucleotide diphosphate kinase family (Leung and Hightower, 1997). These can have either positive or negative effects on Hsp70 activity, in some cases depending on the assay conditions. There are even more Hsp90-binding proteins. Most of the known Hsp90 partners contain a tetratricopeptide repeat (TPR) domain that is required for Hsp90 binding. One of these is the Hsp70-binding protein Hop, which can bind concomitantly with Hsp90 (Chen *et al.*, 1996b; Lassle *et al.*, 1997). Other Hsp90-associated TPR proteins belong to the immunophilin families of FK506-binding proteins (FKBPs) and cyclosporin-binding proteins, i.e., cyclophilins (Cyps). FKBP52/FKBP59/Hsp56, FKBP51, and Cyp40, each of which is expressed in many cell types, compete for binding to Hsp90 and have been noted in a variety of Hsp90 complexes (Nair *et al.*, 1997, and references cited therein; Owens-Grillo *et al.*, 1995; Radanyi *et al.*, 1994; Ratajczak and Carrello, 1996). As with other immunophilin family members, the Hsp90-associated immunophilins have peptidylprolyl isomerase (PPIase) activity, but the importance of this activity is unknown (Barent *et al.*, 1998). An additional TPR-containing, Hsp90-binding protein is the protein phosphatase PP5 (Chen *et al.*, 1996a; Chinkers, 1994; Silverstein *et al.*, 1997). Lacking any TPR motif is p23, an Hsp90 partner that stabilizes Hsp90 binding to various target proteins (Hutchison *et al.*, 1995; Johnson *et al.*, 1994; Johnson and Toft, 1995). A pathway of interactions involving Hsp70, Hsp90, and many of their partner proteins is presented in Section III.

D. Endoplasmic Reticulum: A Special Folding Environment

The ER contains its own complex chaperone machinery (reviewed by Hammond and Helenius, 1995; Helenius *et al.*, 1992; Ruddon and Bedows, 1997). Two of the major ER chaperones are Bip/Grp78 and Grp94/gp96, members of the Hsp70 and Hsp90 families, respectively. There are also unique chaperone components in the ER chaperone machinery, two of which are the Ca^{2+}-binding proteins calnexin and calreticulin.

Compared with cytoplasm, the lumen of the ER is a distinct folding environment in which the redox potential is oxidizing, and there is a relatively high concentration of Ca^{2+}. Other special aspects of protein synthesis and folding in the ER include the common glycosylation of emerging nascent chains, the frequent occurrence of disulfide bonds that stabilize polypeptide conformation and oligomerization, and the dual folding environment for proteins with transmembrane domains. A final consideration is that the ER is only a temporary location for most proteins, because they are usually destined for transit to the Golgi or beyond.

Nascent chains emerging into the ER lumen often are rapidly glycosylated on asparagine and glutamine side chains. As the nascent chain elongates, it associates with ER chaperones, in particular calnexin or calreticulin (which bind in a glycosylation-dependent manner) and/or Bip, but additional ER chaperones have also been detected in nascent chain complexes; this may reflect multichaperone complexes in the ER (Tatu and Helenius, 1997). After chain elongation is complete, the incompletely folded nascent chain may undergo sequential rounds of chaperone binding. Only fully folded chains and properly assembled oligomeric complexes exit efficiently from the ER and progress to the Golgi. Misfolded or unassembled proteins are retained in the ER by continued chaperone interactions, but the exact nature and combination of chaperone interactions vary with different substrates (Zhang *et al.*, 1997b, and references cited therein). Interestingly, the eventual proteolytic degradation of retained proteins (whether integral membrane or soluble proteins) often occurs on cytoplasmic proteasomes through a poorly defined export mechanism (Kopito, 1997). The ER chaperone machinery has often been characterized as a quality control station, and, as seen in Section IV., this quality control function is relevant to several clinical conditions.

E. Cellular Chemicals That Favor Protein Folding

In addition to the protein components of the chaperone machinery, several low molecular weight osmolytes in cells are known to favor protein folding and stability (reviewed by Burg, 1995; Welch and Brown, 1996; Yancey *et al.*, 1982). Glycerol and trehalose are among the more important protein-stabilizing compounds, but other carbohydrates, amino acids, and methylamines can also contribute. Not coincidentally, glycerol and trehalose are commonly recognized as general stabilizing agents for proteins in solution (reviewed by Schein, 1990). Intracellular concentrations of osmolytes can reach dramatically high levels after hypo-osmotic shock (Burg, 1995); moreover, a similar increase in the levels of some osmolytes has been noted after heat shock (Hottiger *et al.*, 1987). To reflect their potentially important role in maintaining a cellular environment conducive for

protein folding, these compounds have been termed chemical chaperones (Welch and Brown, 1996). The general mechanism for stabilization of protein structures by chemical chaperones probably relates to their ability to stabilize the hydration shell around proteins (Schein, 1990).

III. Chaperone-Mediated Regulation of Signal Transduction Pathways

Apart from their role in protein-folding processes, several components of the chaperone machinery appear to function as regulatory factors for a variety of signaling proteins. Hsp90 has been found to interact with multiple regulatory proteins, including the steroid hormone receptors, several transcription factors unrelated to steroid receptors, and various tyrosine and serine/threonine kinases (reviewed by Pratt and Toft, 1997). The progesterone receptor (PR) and glucocorticoid receptor (GR) are the most well characterized targets for Hsp90 regulation, and the following discussion centers on what has been learned from studying chaperone-steroid receptor interactions. Many of the chaperone components and mechanisms of interaction described for steroid receptors appear to pertain to kinases and other Hsp90 targets (outlined in fig. 1); however, there are some important target-specific differences.

Hsp90 does not bind steroid receptors by itself. As determined largely in cell-free assembly assays with rabbit reticulocyte lysates, the assembly of the PR and GR with Hsp90 requires the participation of multiple chaperone components in an ordered assembly pathway. Three assembly stages for PR complexes have been described (Smith *et al.*, 1995). In the early stage, Hsp70 binds free PR, perhaps with assistance from an Hsp40/DnaJ homolog. One of the Hsp70-binding proteins, Hip, also appears in early complexes. An intermediate complex is formed in which the receptor monomer is associated with Hsp70, the Hsp70-binding protein Hip, the Hsp70-/Hsp90-binding protein Hop, and Hsp90. In the final assembly stage, Hsp70, Hip, and Hop are absent from the PR complex, but Hsp90 remains with the Hsp90-associated protein p23 and one of the TPR-containing immunophilins.

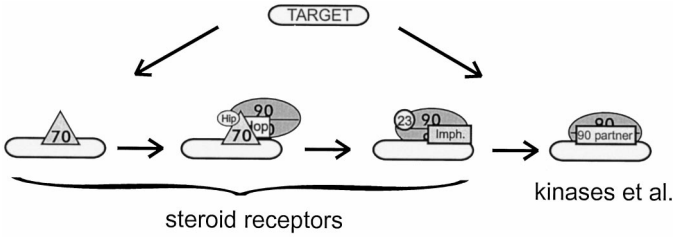


FIG. 1. Chaperone interactions that regulate the activity of target proteins. Steroid receptors undergo an ordered series of chaperone interactions that regulate receptor activity in various ways (see text for details). Other target proteins may interact in a chaperone pathway similar to that for steroid receptors, or there may be alternate chaperone pathways that are target-specific. The assembly pathway has been described in detail only for steroid receptors. However, a common feature in most target pathways is the eventual association with Hsp90 and one or more Hsp90-binding partner proteins. Imph., immunophilin.

Based on a combination of time course studies, chaperone-specific depletions, introduction of mutant chaperones, inhibitor studies, and reconstitution studies, it has been established that the ordered pathway described above is required for the assembly and maintenance of functional receptor complexes (Pratt and Toft, 1997). A key mechanistic element of this pathway involves the apparent roles of Hip and Hop as adaptors that help target Hsp90 to a preexisting Hsp70-receptor complex (Chen *et al.*, 1996b; Prapapanich *et al.*, 1998). Another key element is the p23-dependent stabilization of the interaction of Hsp90 with the receptor (Dittmar *et al.*, 1996; Hutchison *et al.*, 1995; Johnson and Toft, 1995) and the resulting establishment of a high affinity hormone-binding conformation (Scherrer *et al.*, 1990; Smith, 1993). The functions of the Hsp90-associated immunophilins in mature receptor complexes have not yet been established. Furthermore, many of the details relating to transitions from one assembly stage to the next are vaguely defined, and there may be undiscovered components that participate in a highly transient or off-pathway manner. Nevertheless, the basic outline for the pathway of chaperone interactions resulting in functional steroid receptor complexes is well established.

Hormone binding to receptors results in their dissociation from Hsp90 and other chaperones, but hormones are not required to trigger dissociation of receptor complexes. In fact, chaperone interactions with receptors are highly transient at physiological temperatures (Smith, 1993; Smith *et al.*, 1995). In the absence of bound hormone, however, dissociated receptor subunits quickly reassociate with Hsp70 and proceed through the assembly steps, generating a steady state assembly cycle. For the PR, at least, it appears that hormone binding blocks the binding of Hsp70 and re-entry of the PR into the assembly pathway.

Binding of Hsp90 and other chaperone components to steroid receptors is localized to the ligand binding domain (Carson-Jurica *et al.*, 1989; Chambraud *et al.*, 1990; Gehring and Arndt, 1985; Pratt *et al.*, 1988; Schowalter *et al.*, 1991), and chaperone interactions can clearly influence the conformation of this domain, as shown by chaperone-dependent acquisition and stabilization of high affinity hormone binding. But do chaperones complexed with steroid receptors function solely in folding of the ligand binding domain? Several observations argue against this. First, the estrogen receptor is assembled into complexes similar to those of the PR and GR, but the estrogen receptor does not require continued chaperone interactions for hormone binding. Second, steroid receptors lacking chaperones are competent for dimerization and deoxyribonucleic acid (DNA) binding in the absence of hormone. Hsp90 and other chaperone components in mature complexes mask the DNA binding domain of the receptor (Cadepond *et al.*, 1991) and

inhibit dimerization until chaperone interactions are interrupted, typically as a consequence of hormone binding. Third, dissociation of chaperones from receptors correlates with an increased rate of proteolytic degradation of the receptors in intact cells (Segnitz and Gehring, 1997; Whitesell and Cook, 1996). Thus, steroid receptors appear to be adapted for extended chaperone interactions that persist beyond basic folding steps and serve to regulate receptor function at various levels (for further discussions, see Nair *et al.*, 1996; Smith, 1993; Smith *et al.*, 1995). Enhancements of hormone binding and proteolytic half-lives can be considered activating functions, whereas inhibitions of receptor dimerization and DNA binding are repressive functions. Collectively, these activities maintain the receptor in a quiescent state that is competent for binding and responding to hormone.

As mentioned above, many other signaling proteins are targets for Hsp90 and may undergo interactions with multichaperone assemblies similar to those observed with steroid receptors (Nair *et al.*, 1996, and references cited therein). However, several target-specific interactions that relate to Hsp90 partner proteins have been recognized. The three large immunophilins, i.e., FKBP52, FKBP51, and Cyp40, have each been recovered in individual steroid receptor complexes, but there is a clear preference for FKBP51 over the other immunophilins in PR and GR complexes assembled in vitro (Barent *et al.*, 1998). The protein phosphatase PP5 is a TPR-containing protein that competes with immunophilins for Hsp90 binding and may associate preferentially with GR complexes (Silverstein *et al.*, 1997). Another Hsp90-binding TPR protein appears in arylhydrocarbon/dioxin receptor complexes (Ma and Whitlock, 1997), and CDC37/pp50 appears preferentially together with Hsp90 in many kinase complexes (reviewed by Hunter and Poon, 1997), although chaperone interactions with the heme-regulated eIF2α kinase are more similar to those with steroid receptors (Matts *et al.*, 1992; Uma *et al.*, 1997, 1998; Xu *et al.*, 1997). None of the Hsp90 accessory proteins has a clearly defined function in the respective signal protein complexes, but each may modulate the actions of Hsp90 or the target protein in a distinct manner.

IV. Protein Misfolding in Disease

Many heritable and acquired diseases result solely from the loss of wild-type protein activity, because of a mutation that disrupts a critical function of the gene product. On the other hand, some disease states result from production of a mutant protein that misfolds and acquires a novel activity (for example, a tendency to form aggregates) that has pathological consequences. Several human diseases involving protein misfolding have been cataloged in recent reviews (Ruddon *et al.*, 1996; Thomas *et al.*, 1995; Welch and Brown, 1996). A potential confounding factor in these diseases that has been largely overlooked is the possibility that different genetic backgrounds with distinctive patterns of chaperone expression and function might enhance or repress the phenotype of misfolding mutants. Furthermore, mutation of a chaperone component could possibly underlie certain disease states, with the phenotype depending on the range and sensitivity of particular protein substrates for the mutant chaperone. Here we address a few representative disease states, discuss how protein misfolding and chaperones participate in the disease process, and then consider potential treatments that target chaperone activity.

A. Cancer and Inactive or Inappropriately Acting Mutant Proteins

The fact that chaperone interactions play an important role in regulating key components of cellular growth and differentiation pathways suggests that they may be involved in the initiation and progression of human cancers. Dysfunction within the chaperone machinery resulting from mutation or altered expression of specific Hsps may give rise to neoplastic transformation in a unique way by impairing the activity, localization, or stability of multiple signal transduction molecules and/or transcription factors simultaneously. Consistent with this hypothesis, the cytoplasmic sequestration and aggregation of wild-type forms of the tumor suppressor protein p53 have been documented in neuroblastoma (Ostermeyer *et al.*, 1996) and some breast cancers (Moll *et al.*, 1992). A specific defect in chaperone function, however, has yet to be demonstrated in these tumors. The overexpression of certain Hsps in breast (Ciocca *et al.*, 1993) and ovarian (Kimura *et al.*, 1993) cancers has been reported to be associated with poor clinical prognosis, but the reason remains obscure. Given the general role of Hsps in cytoprotection, perhaps overexpression renders cells relatively resistant to conventional chemotherapeutic agents, but this possibility remains to be proven. Finally, our rapidly evolving understanding of the molecular mechanisms of oncogenesis suggests that chaperone interactions may actually provide a useful target for anticancer drug action.

Many of the common mutations in both tumor suppressor genes and dominantly acting oncogenes result in the expression of defective proteins that display unusually stable physical association with normal molecular chaperones. For example, soon after the transforming factor of the Rous sarcoma virus was identified as the constitutively activated tyrosine kinase v-Src, it was noticed that the kinase co-precipitated with several endogenous cellular proteins, including Hsp90 and Hsp70 (Hutchison *et al.*, 1992; Oppermann *et al.*, 1981). Moreover, through mechanisms that remain to be defined, complex formation appears to be required for transforming activity, because drugs that interfere with Hsp90 function revert v-Src-mediated transformation, despite persistently elevated intracellular tyrosine kinase activity (Kwon *et al.*, 1992; Whitesell *et al.*, 1994).

The interaction of Hsp90 and other chaperones with oncogenically mutated kinases appears to differ from that of the normal cellular counterparts primarily in the relative stability of chaperone associations with mutant kinases. As best demonstrated by Matts and colleagues (Hartson *et al.*, 1996, 1998; Hartson and Matts, 1994), c-Src and the cellular Src-related kinase Lck also appear to depend on chaperone interactions for their activity.

Perhaps the most intriguing example of chaperone involvement in malignant transformation can be found in the rapidly evolving description of p53-mediated oncogenesis. Mutations of the *p53* tumor suppressor gene are the most common molecular genetic defects found in human cancers (Harris and Hollstein, 1993). Most *p53* mutations result in the expression of a protein of altered conformation that has lost its cell cycle checkpoint activity. Normal, wild-type p53 is a short-lived protein that is rapidly turned over via selective proteolysis in the ubiquitin-proteasome pathway (Maki *et al.*, 1996). Presumably because of their aberrant conformations, however, many p53 mutants are retained within the chaperone machinery (Davidoff *et al.*, 1992; Selkirk *et al.*, 1994; Sepehrnia *et al.*, 1996) and protected from ubiquitin conjugation and subsequent degradation. As a result, elevated levels of dysfunctional protein accumulate within the tumor cell. A simplified cartoon representation of the mechanisms by which altered chaperone interactions may contribute to p53-mediated oncogenesis is presented in fig. 2. Of note, mutant p53 proteins bound to Hsps no longer function as tumor suppressors, and some mutants may actually interfere with the function of normal p53 (which continues to be expressed from the remaining wild-type allele) by forming heterodimers with it (dominant negative effect). In addition, evidence exists that some mutant p53 proteins can directly activate inappropriate gene expression, contributing to oncogenesis (positive tumor-promoting effect) (Park *et al.*, 1994). Recent work has demonstrated that benzoquinone ansamycin drugs can disrupt the extended chaperone interactions observed with mutant p53 and selectively destabilize mutant proteins without effecting the cellular levels of wild-type protein (Blagosklonny *et al.*, 1995; Whitesell *et al.*, 1997). Drug treatment, however, does not appear to restore wild-type transactivating activity to the mutant protein (Whitesell *et al.*, 1998). Although drug treatment does not appear to restore tumor suppressor activity to mutant p53 protein, it may abrogate the positive transforming activities of mutant p53 within tumor cells. Moreover, in cells that are heterozygous for p53 mutation, destabilization of mutant p53 might restore function to protein expressed from the wild-type allele (Blagosklonny *et al.*, 1995). It remains to be seen whether these interesting cell biological considerations can be translated into clinically useful chemotherapeutic strategies.

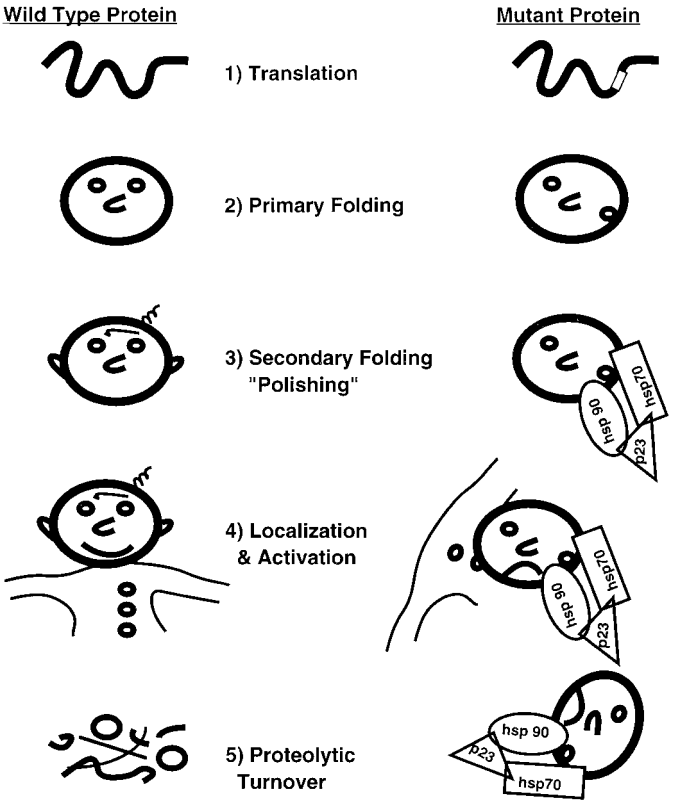


FIG. 2. Simplified cartoon representation of mechanisms by which altered chaperone interactions may contribute to the transforming activity of oncoproteins such as mutant p53. A mutation in the primary amino acid sequence of the target protein leads to an altered conformation (steps 1 and 2). This mutant conformation is recognized by the chaperone machinery as "misfolded," resulting in extended interactions with chaperone complexes (step 3). As a result, the mutant protein is mislocalized within the cell and does not function properly (step 4). In addition, normal proteolytic degradation is impaired, and the mutant protein persists, to accumulate to high levels within the cell (step 5).

B. Cystic Fibrosis and Diversion in Folding Pathways

For several gene mutations that are the bases of human diseases, the mutant membrane or secretory protein product is sufficiently active to prevent the disease state, but the product is captured by the quality control system in the ER and never reaches its site of function. One of the most well characterized examples involves the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel protein whose functional loss underlies cystic fibrosis (reviewed by Welsh, 1994; Welsh and Smith, 1995). The most common disease-related mutation in CFTR is a deletion of Phe508 (ΔF508). Typically, ΔF508 protein never reaches the plasma membrane (Cheng *et al.*, 1990; Lukacs *et al.*, 1994) because it is captured by chaperones in the ER quality control system and is eventually degraded by proteasomes in the cytoplasmic compartment (Ward *et al.*, 1995). Interestingly, a portion of ΔF508 protein can achieve an active conformation and correct localization in the plasma membrane when cells are grown at reduced temperature (Denning *et al.*, 1992) or when ΔF508 protein is overexpressed (Cheng *et al.*, 1995). Expression

at lower temperatures would favor native folding (consider, for example, the correct folding of temperature-sensitive mutants at permissive temperatures), whereas overexpression would be expected to increase the amount of ΔF508 protein that escapes the limiting quality control system. Therefore, a critical problem with ΔF508 (and probably with several other disease-related alleles) is the inability of the protein to move beyond the ER quality control system.

C. Amyloid Diseases and Protein Aggregation

Misfolded proteins can lead to intracellular aggregate formation, disruption of multiple cellular processes, and cell death. However, just as in heat shock and other proteotoxic conditions, the chaperone machinery is designed to prevent aggregation, reverse aggregation that occurs, and/or target misfolded proteins and aggregated complexes for proteolytic degradation. Overall, the chaperone machinery functions efficiently in this regard, but there are some situations in which protein aggregation is not prevented or remedied, resulting in cellular abnormalities. The following neurodegenerative diseases highlight the potential hazards of protein aggregation.

1. Alzheimer's disease. A common marker of Alzheimer's disease, and perhaps the major cause of neurodegeneration associated with this condition, is the formation in brain tissues of amyloid plaques, which are principally composed of the amyloid-β (Aβ) protein (reviewed by Checler, 1995; Dickson, 1997; Selkoe, 1994, 1996, 1997). The amyloid precursor protein (APP), whose normal cellular function is unresolved, is a membrane protein and, distinctively, an intramembranous substrate for proteases that generate a characteristic set of fragments. Aβ is one of these and consists of the amino-terminal 40 to 43 amino acids of APP.

Several factors that predispose individuals to the development of Alzheimer's disease also lead to increased generation of Aβ 42- and 43-amino acid peptides. Mutations in APP associated with Alzheimer's disease all map within or adjacent to the Aβ sequence. However, the most common mutations associated with familial Alzheimer's disease are in the genes for presenilin 1 or 2 (Hutton and Hardy, 1997), proteins that somehow regulate proteolysis of APP (De Strooper *et al.*, 1998).

The carboxyl-terminal region of Aβ exists in a β-sheet conformation, whereas a region toward the amino terminus can exist in either an α-helical or β-sheet conformation (Soto *et al.*, 1995). Various factors, such as Aβ mutations, can shift the conformational equilibrium of the amino-terminal region toward a β-sheet conformation that favors aggregation of Aβ peptides and plaque formation. Once a seed aggregate has formed, equilibrium might be shifted further toward β-sheet conformations as monomers in the transient β-strand conformation are trapped by addition to preexisting aggregates. A conformational shift toward β-sheets is a factor in other diseases involving protein aggregation.

In addition to promoting amyloid plaque formation, accumulation of Aβ may be involved in other neurotoxic mechanisms. In one recent study (Yan *et al.*, 1997), a cellular protein termed endoplasmic reticulum-associated amyloid β-peptide binding protein, which is related to hydroxysteroid dehydrogenases, was shown to bind specifically to Aβ; this protein's abundance and interactions with Aβ correlated positively with Aβ-induced neurotoxicity. In another report (Schulze *et al.*, 1993), recombinant Aβ was found to interact specifically with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis. Conceivably, sequestration of GAPDH and a deficit in GAPDH activity could result in cellular metabolic deficiencies and contribute to neuropathological changes, but this remains to be demonstrated.

2. Huntington's disease. Huntington's disease is one of a class of neurodegenerative conditions caused by aggregation of a poly-glutamine-containing protein (reviewed by Bates *et al.*, 1997; Lunkes and Mandel, 1997). Although different gene products are involved in different disorders, a common genetic feature is that each locus contains CAG tandem repeats that have been expanded by a poorly understood mechanism. Because CAG is a glutamine codon, an in-frame CAG expansion results in a protein product with a corresponding extension of its polyglutamine (polyGln) tract. Beyond a critical length of approximately 40 glutamine residues, protein aggregation occurs that leads to pathological changes.

In Huntington's disease, the responsible gene (Huntington's Disease Collaborative Research Group, 1993) codes for huntingtin (Htg), a 350-kDa protein. Htg appears to be expressed in all tissues, not just the brain, and embryonic lethality is observed in mice nullizygous for the Htg gene (Duyao *et al.*, 1995; Nasir *et al.*, 1995; Zeitlin *et al.*, 1995). In normal individuals, the amino-terminal region of Htg contains a tract of 6 to 39 glutamine residues; in Huntington's disease, the tract is 35 to 180 residues long, but 40 to 55 units are found in most cases. Recent evidence (White *et al.*, 1997) suggests that Htg is required for neurogenesis but that pathological changes associated with expanded polyGln tracts in Htg are the result of a gain of function rather than loss of normal Htg function.

In one recent study (Scherzinger *et al.*, 1997), Htg fragments containing a range of polyGln lengths were generated. Fragments with 20 or 30 glutamines failed to aggregate in an in vitro assay, but fragments with 51 or more glutamines formed fibrillar aggregates. As with Aβ, the aggregating form of Htg is conformationally distinct from nonaggregating protein and appears to be enriched for β-sheet structure. Expression of only the amino-terminal portion of Htg, containing a 100- to 150-glutamine tract, generated Huntington-like pathological markers and symptoms in transgenic mice (Davies *et al.*, 1997). Neuronal intranuclear inclusions were formed that contained the Htg fragment as well as ubiquitin,

suggesting that there might be a defect in ubiquitin-mediated degradation of the fragment. Interestingly, Htg is a substrate for apopain, a protease involved in apoptotic pathways, and cleavage increases with the length of the polyGln tract (Goldberg *et al.*, 1996). Apopain cleavage generates an amino-terminal, polyGln fragment similar to the transgene product observed in the study by Davies *et al.* (1997); therefore, generation of an amino-terminal Htg fragment may be pathogenic in Huntington's disease. Similar to the Aβ interaction with GAPDH noted above, peptides and Htg fragments containing an extended polyGln tract bound specifically to GAPDH (Burke *et al.*, 1996). Again, although it is tempting to speculate that loss of GAPDH activity may be directly related to disease mechanisms, the significance of GAPDH binding has not been resolved.

3. Prion diseases. Sheep scrapie, bovine spongiform encephalopathy, and human Cruetzfeldt-Jakob disease are transmissible neurodegenerative diseases; the infectious agent for each is thought to be a prion vector termed prion protein (PrP) (Prusiner and Scott, 1997). Although findings are still controversial (Caughey and Chesebro, 1997), prion vectors are thought to lack nucleic acids and to consist solely of an alternately folded form of a protein that is normally expressed in the host. Strong genetic evidence for a heritable prion-like protein has come from studies of certain non-Mendelian inheritance patterns in yeast (Lindquist, 1997; Wickner, 1994, 1996).

The mammalian PrP has complex biological characteristics (reviewed by Horwich and Weissman, 1997). The normal function of the noninfectious cellular form of PrP (PrP^C) is poorly defined. PrP^C is a membrane protein found in many tissues, not only in brain. PrP^C has a half-life of 3 to 6 hours in cells and is sensitive to proteases in vitro. The infectious form of PrP (termed PrP^{Sc} to reflect its role in scrapie) undergoes little or no turnover in cells and is resistant to proteolysis in vitro. PrP^{Sc}, but not PrP^C, forms amyloid fibrils associated with scrapie, bovine spongiform encephalopathy, and Cruetzfeldt-Jakob disease. Similar to Aβ and the disease-associated form of Htg, PrP^{Sc} has a much higher content of β-sheet structure than does the normal conformation in PrP^C (Pan *et al.*, 1993), and a recombinant amino-terminal fragment of PrP (142 amino acids) has been shown to switch between α-helical and β-sheet conformations in a pH-dependent manner (Mehlhorn *et al.*, 1996; Zhang *et al.*, 1997a). PrP^{Sc} is thought to propagate itself by inducing PrP^C to change its conformation to the protease-resistant form, which then participates in amyloid formation. PrP^{Sc} appears to be required for infectivity and formation of amyloid fibers, but it may not be required for PrP-dependent neuropathological changes. Several years ago, an observation was made that PrP synthesized in a cell-free system can exist in alternate topological forms in ER membranes (Lopez *et al.*, 1990). Recently, it was shown (Hegde *et al.*, 1998) that transgenic mice expressing a PrP mutant that favors a greater cytoplasmic orientation of PrP domains developed a neurodegenerative phenotype in the absence of protease-resistant PrP^{Sc}.

As recently discussed (Caughey and Chesebro, 1997), several questions remain regarding the mechanism by which PrP^C is converted to PrP^{Sc}, as well as whether PrP^{Sc} is the infectious agent or instead serves as a highly protective reservoir for an unidentified virus. There are some suggestions that conversion may be mediated by an additional cellular protein, perhaps one of the molecular chaperones (Telling *et al.*, 1995). Interestingly, the chaperone Hsp104 has been genetically (Chernoff *et al.*, 1995) and biochemically (Schirmer and Lindquist, 1997) linked to the function of prion-like proteins in yeast. In addition, Hsp104 was recently found to interact in vitro with the β-sheet conformer of a PrP fragment (not the α-helical conformer) and with the Alzheimer's protein Aβ (Schirmer and Lindquist, 1997). Hsp104 and the bacterial chaperone GroEL were found to enhance PrP^{Sc}-dependent conversion of PrP^C in vitro, whereas other protein chaperones had no effect and chemical chaperones inhibited conversion (DebBurman *et al.*, 1997). Still, the biological role of chaperone interactions in the pathogenesis of mammalian prion-based diseases remains to be defined.

V. Natural Products That Bind Chaperone Components

Several natural products synthesized by fungi and bacteria bind with high affinity and specificity to chaperone components. It is likely that these compounds are evolutionarily adapted to deter predators or competitors, with chaperone proteins being effective toxicological targets.

A. Molecules That Bind Immunophilins

The important immunosuppressant drugs cyclosporin A, FK506 (tacrolimus), and rapamycin (sirolimus) function by virtue of their binding to proteins in the two immunophilin families, i.e., the Cyps that bind cyclosporin A and the FKBPs that bind FK506 and rapamycin (for reviews, see Kay, 1996; Marks, 1996). In both immunophilin classes, the drug binds the PPIase active site, inhibiting enzymatic activity, but immunosuppression is not a result of PPIase inhibition. Instead, the drug-immunophilin complex forms a hybrid surface composed partly of protein and partly of a portion of the drug that extends from the binding pocket. This hybrid structure binds avidly to proteins regulating key steps in the activation of immune responses. Both cyclosporin-CypA and FK506-FKBP12 complexes bind to and inhibit calcineurin (Friedman and Weissman, 1991; Liu *et al.*, 1991), a protein phosphatase whose activity is required for T cell activation. On the other hand, the rapamycin-FKBP12 complex, because of structural differences in the exposed regions of rapamycin and FK506 in FKBP12

complexes, fails to bind and inhibit calcineurin; instead, the rapamycin-FKBP12 complex binds a class of proteins termed target of rapamycin (TOR). Mammalian TOR, which was first identified in yeast cells (Heitman *et al.*, 1991) and which has also been termed FKBP-rapamycin-associated protein (Brown *et al.*, 1994) or rapamycin and FKBP12 target 1 (Sabatini *et al.*, 1994), is involved in signaling the G_1 to S phase transition in cells; rapamycin treatment induces G_1 arrest of T cells. TOR proteins share homology with phosphoinositol 3-kinases, but mammalian TOR was recently shown to have protein kinase activity that recognizes a (Ser/Thr)-Pro motif similar to that recognized by mitogen-activated protein kinase (Brunn *et al.*, 1997; Burnett *et al.*, 1998). Therefore, binding of the rapamycin-FKBP12 complex to TOR may alter the ability of TOR to phosphorylate substrates and propagate growth signals.

Many compounds related to FK506 and rapamycin have been generated in hopes of reducing immunosuppressive side effects or discovering novel pharmacological uses. Many nonimmunosuppressive analogs (NIAs) have been identified that bind immunophilins and inhibit PPIase activity without promoting binding to calcineurin or TOR. As demonstrated most clearly by Snyder and colleagues (Steiner *et al.*, 1997a,b), immunosuppressant agents and some NIAs have neurotrophic actions that may be therapeutically useful (reviewed by Sabatini *et al.*, 1997). The major immunophilin targets for the NIAs are probably FKBP12 and CypA, both of which are expressed at high levels in neural tissues (Steiner *et al.*, 1992) and are up-regulated in regenerating nerves (Lyons *et al.*, 1995).

Although immunosuppressive and neurotrophic drug effects are predominantly mediated by FKBP12 and CypA, other immunophilin family members also bind FK506, cyclosporin A, and their respective analogs. The large-molecular size, TPR-containing family members that associate with Hsp90 and steroid receptor complexes bind drugs with lower affinities than the small family members, with no well defined biological consequences. In several reports (reviewed by Pratt and Toft, 1997), the effects of immunosuppressant drugs on steroid receptor assembly and function have been examined. The drugs have little effect on the structure and function of receptor complexes in cell-free studies, although drug-dependent effects (some seemingly contradictory) have been reported using intact cell assays. A problem with some intact cell studies of steroid signaling is that any drug effect is assumed to relate to receptor-associated immunophilins, when drug effects may in fact occur indirectly through other immunophilins (for example, alteration of protein phosphorylation patterns by inhibition of the phosphatase calcineurin) or through immunophilin-independent drug actions (such as drug competition at membrane efflux transporters, for which some steroids are substrates). Additional studies that should better define the potential activities of immunosuppressant drugs that are directly mediated by TPR-containing immunophilins are underway in several laboratories.

B. Molecules That Bind Hsp70

1. Background. Hsp70-class molecular chaperones perform numerous cellular functions, many of which appear to involve the binding and ATP-dependent release of nascent polypeptides or mature cellular proteins. As a result, it has been possible to identify synthetic peptides that bind selectively to Hsp70-class chaperones in the submillimolar concentration range; these reagents have been used in attempts to define the binding specificities of individual Hsp70 family members in vitro (Flynn *et al.*, 1991; Fourie *et al.*, 1994). Unfortunately, because of their inherent limitations in terms of rapid degradation in biological fluids, poor cellular uptake, and relatively low binding affinity, peptides have not proven useful in the study of Hsp70 function in intact cells. Several types of low-molecular weight compounds have been reported to modulate the expression and function of Hsp70-class molecules in whole cells, including drugs such as nonsteroidal anti-inflammatory agents (Morimoto *et al.*, 1994), flavonoid kinase inhibitors (Elia *et al.*, 1996), and serine/threonine phosphatase inhibitors such as okadaic acid (Chang *et al.*, 1993). These drugs, however, are relatively nonspecific and appear to modulate the transcription of Hsp70, rather than interacting directly with the chaperone. In contrast, several derivatives of the natural product spergualin (an antibiotic that was first identified in culture filtrates of the organism *Bacillus laterosporus*) have been shown to interact selectively with an Hsp70 isoform and to alter its function (fig. 3). Spergualin is a peptidomimetic compound that contains the polyamine spermidine within its structure (Umezawa *et al.*, 1981); it was first evaluated as an antitumor agent in murine leukemia models, where it exhibited significant activity (Takeuchi *et al.*, 1981). Subsequently, the semisynthetic analog 15-deoxyspergualin (DSG) was noted to possess potent in vivo immunosuppressive activity, which was distinct from that of the classic immunophilin-binding drugs cyclosporin A and FK506.

2. Hsp70-15-deoxyspergualin interactions. In an attempt to elucidate the mechanism of this novel immu-

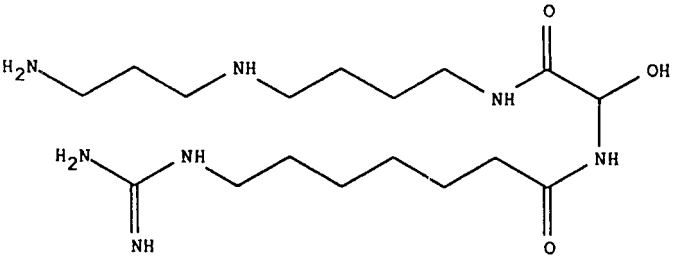


FIG. 3. Structure of DSG [7-[(aminoiminomethyl)amino]-N-[2-[[4-[(3-aminopropyl)amino]butyl]amino]-1-hydroxy-2-oxyethyl]heptanamide]. DSG is typically prepared as the trihydrochloride salt, with a formula weight of 497.

nosuppressive action, Nadler *et al.* (1992) prepared a solid-phase, immobilized, methoxy-DSG derivative to identify the cellular target(s) with which the drug interacted. Surprisingly, they found that a single major protein with an apparent molecular size of 70 kDa was able to bind selectively to DSG in lysates prepared from a variety of cell types. This protein could be eluted with soluble DSG or ATP but not with the polyamines putrescine and spermidine, which show some structural similarity to DSG but are not immunosuppressive. Peptide sequencing data, as well as immunoblotting with a panel of monoclonal antibodies, identified the DSG-interacting protein as Hsc70, the constitutive or cognate member of the Hsp70 class of Hsps. No binding of the inducible isoform of Hsp70 was detected but, because this species is present in much smaller amounts than Hsc70 under nonstress conditions, it is possible that it and other Hsps may also bind DSG. In fact, using affinity capillary electrophoresis and solution-phase DSG, Nadeau *et al.* (1994) found that DSG bound purified Hsc70 (K_d = 4 μM) and Hsp90 (K_d = 5 μM) with comparable affinities in Tris/glycine buffer. Given the typical concentrations of cytosolic Hsc70 (5 μM) and Hsp90 (2 to 10 μM) in cells, these K_d values for DSG interactions with Hsps are consistent with the concentrations required for biological activity in cell cultures and in vivo. This finding and the good correlation between immunosuppressive activity and the ability to bind Hsc70 for a panel of five DSG analogs suggested that Hsp function is actually a primary site of drug action. It is unclear why initial solid-phase affinity precipitation studies did not detect Hsp90 binding, but it is possible that Hsp90 binds to a site on DSG that was attached to the bead matrix or was close enough to result in sufficient steric hindrance to impede binding. DSG does not appear to interfere directly with Hsp90-Hsc70 interactions in vitro, and it does not inhibit the reconstitution of functional steroid receptors in reticulocyte lysates (Smith DF, unpublished observations). DSG binding to Hsc70 at low concentrations was found to stimulate the ATPase activity of Hsc70 2-fold, whereas at high concentrations activity returned to basal levels. Consistent with this finding, it was reported more recently that DSG interacts with Hsc70 in a fashion distinct from that of unfolded peptides (Nadler *et al.*, 1995). Specifically, DSG was ineffective at inhibiting the binding of a cytochrome *c* peptide to purified Hsc70, and this peptide was unable to elute Hsc70 from DSG affinity resin. These observations are reminiscent of a report by Takenaka *et al.* (1995) describing the use of a phage display library to screen for Hsc70-interacting peptides. Those investigators found that Hsc70 recognized two distinct peptide motifs, one thought to be involved in Hsc70 chaperoning of proteins to organelles (NIVRKKK-like sequences) and one involved in Hsc70 facilitation of protein folding (FYQLALT-like sequences). The peptidomimetic structure of DSG may well interact with the hydrophobic-basic peptide binding site of Hsc70, which was shown to bind NIVRKKK sequences, but not the unfolded peptide binding site of Hsc70. Such an interaction could explain the lack of DSG effects on general protein folding while accounting for DSG interference with the chaperoning activity of Hsc70 that is potentially required for antigen processing and elaboration of cellular immune responses (see Section VI.C.).

3. 15-Deoxyspergualin biological activities. DSG was initially identified as a potential antitumor agent because of its activity against several mouse leukemia cell lines in vitro and in syngeneic animal models. It now appears that the cytotoxic activity of DSG in vitro is attributable in large part to its metabolism to aminoaldehydes and hydrogen peroxide by copper amine oxidases, which are present at high levels in fetal bovine serum (Shiro *et al.*, 1992). When cell survival assays are performed in the presence of 1 mM aminoguanidine as an inhibitor of amine oxidase activity or in media containing sera that are low in oxidase activity (horse, mouse, or human serum), DSG appears to exert cytostatic effects that are characterized by G_1 cell cycle arrest (Nishikawa *et al.*, 1991). Despite the demonstrated interaction of the drug with both Hsc70 and Hsp90, as discussed above, DSG does not revert the phenotype of cells transformed by activated tyrosine kinases such as v-Src, which are known to interact with these chaperones (Whitesell L, unpublished observations). Because the antitumor activity of DSG is markedly reduced in immunocompromised mouse models, it has been postulated that this anticancer activity may be the result of alterations in host immune function, but this possibility has yet to be proven. Unlike the immunosuppressants cyclosporin A and FK506, DSG does not interact with P-glycoprotein and does not reverse the multidrug resistance phenotype (Holmes and Twentyman, 1995).

In contrast to its limited antitumor activity, DSG displays potent immunosuppressive activity, prolonging the survival of allografts and xenografts, inducing tolerance, and reversing allograft rejection (Kaufman, 1996). The cellular mechanisms by which DSG achieves these effects are not well understood, but it clearly acts in a manner distinct from that of cyclosporin A and FK506. Unlike those agents, DSG does not inhibit cytokine production. It is a rather poor inhibitor of T cell proliferation but can partially inhibit mixed lymphocyte reactions even when added after the initiation of the culture. DSG strongly inhibits expression of the interleukin 2 receptor on activated T cells (Ramos *et al.*, 1996). DSG has also been reported to exert significant effects on humoral immune function, including inhibition of B cell differentiation and κ light chain expression at the messenger ribonucleic acid level (Tepper *et al.*, 1995). This effect may be mediated via inhibition of the nuclear translocation of transcription factor NF-κB, which is required for light chain expression. Interestingly, in that

model system, DSG also inhibited the nuclear translocation of Hsc70 that is normally observed after heat shock (without producing gross changes in total nuclear protein composition), leading the authors to speculate that DSG may interfere with the postulated role of Hsc70 in chaperoning the energy-dependent translocation of proteins through the nuclear pore (Yang and DeFranco, 1994). Lastly, experiments by Hoeger *et al.* (1994) suggest that DSG may interfere with antigen processing by cells of the monocyte/macrophage or antigen-presenting cell lineage. Pretreatment of antigen-presenting cells but not T cells prevented proliferative responses to conventional antigens such as tetanus toxoid (which require processing and presentation), whereas responses to superantigens were not affected. Such a mechanism for DSG action is attractive in light of the Hsc70-binding activity of the drug, because Hsp70 family members are known to play a role in the binding and intracellular transport of antigenic peptides (Manara *et al.*, 1993; Tamura *et al.*, 1997).

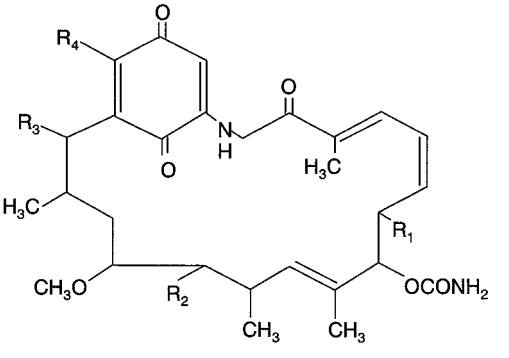
C. Molecules That Bind Hsp90

1. Background. The molecular chaperone Hsp90 plays an essential role in stress tolerance, de novo protein folding, and posttranslational regulation of the stability and function of many important cellular proteins, including steroid hormones, protein kinases, and molecules regulating the cell cycle and programmed cell death. Although Hsp90 knockout is clearly lethal in eukaryotic cells, no well defined biochemical activity has been established for this class of molecular chaperones. Specifically, a role for ATP binding and/or hydrolysis in Hsp90-mediated chaperoning events has remained controversial. Recently, several small-molecule natural products that appear to interact in a selective fashion with Hsp90 have been identified by affinity precipitation techniques. The effects of these compounds on Hsp90 function in a variety of in vitro and cell culture systems are finally beginning to indicate the specific role(s) of Hsp90 in multiprotein chaperone complexes.

2. Hsp90-15-deoxyspergualin interactions. As described in Section V.B.2., Nadeau *et al.* (1994), using affinity capillary electrophoresis techniques, reported that the immunosuppressant DSG can bind purified Hsp90. Using solid-phase immobilized DSG, however, an Hsp90-DSG interaction was not detected in whole-cell lysates (Nadler *et al.*, 1992). This discrepancy may be the result of technical problems associated with the DSG immobilization strategy used or may indicate a lack of physiological significance for the interaction detected by affinity capillary electrophoresis. Because of the lack of detectable effects on well defined Hsp90-dependent processes such as PR assembly and v-Src-mediated transformation, DSG has not proven helpful in defining Hsp90 function.

3. Hsp90-benzoquinone ansamycin interactions. In the course of screening microbial fermentation products for anticancer activity, it was noted that the benzoquinone ansamycins herbimycin A (Omura *et al.*, 1979), geldanamycin (GA) (DeBoer *et al.*, 1970), and macbecin (Muroi *et al.*, 1979) were able to revert the phenotype of cells transformed by the oncogenically activated tyrosine kinase v-Src (Uehara *et al.*, 1986) (fig. 4). Transformation by many other dominantly acting oncogenes, such as *erbB-2, bcr-abl, fps, ros,* and *yes,* have also been reported to be reversed by noncytotoxic concentrations of herbimycin A (Okabe *et al.*, 1992; Uehara *et al.*, 1988). Surprisingly, mechanistic evaluation of this novel biological activity has revealed that the ansamycins possess no intrinsic kinase inhibitory activity; they appear to alter the stability and function of kinases indirectly. Using a solid-phase-immobilized GA derivative, it was found that GA interacts in a highly selective fashion with Hsps of the 90-kDa class and that this interaction disrupts Hsp90 association with mutant kinases such as v-Src, leading to loss of their transforming activity (Whitesell *et al.*, 1994). Similarly, GA causes disruption of Hsp90 interactions with a variety of kinases (Hartson *et al.*, 1996; Nair *et al.*, 1996; Schulte *et al.*, 1995, 1996; Stepanova *et al.*, 1996).

In another approach, a GA derivative incorporating a photoaffinity label was prepared and used to demonstrate specific binding to a 100-kDa cellular protein (Chavany *et al.*, 1996; Miller *et al.*, 1994b), which was subsequently identified as Grp94, an ER homolog of Hsp90. GA binding to Grp94 appears to disrupt association of the receptor tyrosine kinase erbB-2 with this chaperone, leading to destabilization of the kinase and loss of its transforming activity (Miller *et al.*, 1994a). A study of structure-activity relationships has demonstrated a strong correlation between the biological effects of the benzoquinone ansamycins and their ability to bind members of the Hsp90 family (An *et al.*, 1997).



	R_1	R_2	R_3	R_4
Herbimycin A	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H
Macbecin I	$-CH_3$	$-OCH_3$	$-OCH_3$	-H
Geldanamycin	$-OCH_3$	-OH	-H	$-OCH_3$
17-allylamino GA	$-OCH_3$	-OH	-H	$-NHCH_2CH{=}CH_2$

FIG. 4. Structures of the benzoquinone ansamycins under most active evaluation as modulators of Hsp90 function. The molecular weight of herbimycin A is 560.

More recently, two groups have confirmed GA interaction with Hsp90 by establishing the crystal structures of Hsp90 fragments complexed with either GA or ATP. Stebbins *et al.* (1997) used monomeric recombinant human Hsp90 fragments to define a GA binding pocket within the amino terminus of the protein and speculated that this pocket represents a physiological binding site for Hsp90 interactions with partially folded polypeptide substrates. In that model, GA is proposed to act as a competitive inhibitor of target-chaperone interactions. In contrast, another group, using limited proteolysis of the yeast homolog of Hsp90, were able to generate an amino-terminal fragment (residues 1 to 220) that they crystallized as a dimer in the presence of Mg^{2+} and either adenosine diphosphate (ADP), ATP, or adenosine-5′-*O*-(3-thio)triphosphate. That work definitively identified an ADP/ATP binding site within the amino terminus of Hsp90, which shares homology with the ATP binding site of the bacterial type II topoisomerase DNA gyrase B protein (Prodromou *et al.*, 1997). The site also coincides structurally with the GA binding site previously identified by Stebbins *et al.* (1997), which indicates that it is not a peptide binding pocket, as originally proposed. The crystallographic data did not definitively establish an intrinsic ATPase activity for Hsp90, but the conformations of the Hsp90 fragments used in this study did vary substantially in their ATP- versus ADP-bound forms, in agreement with findings reported in a previous biophysical study using native Hsp90 (Csermely *et al.*, 1993). Taken together, the structural findings reported above and recent, well executed, biochemical studies have finally resolved the longstanding controversy of ATP involvement in Hsp90 function (Grenert *et al.*, 1997; Sullivan *et al.*, 1997), and they convincingly demonstrate that GA acts as an Hsp90-specific ATP mimetic that alters the function of the chaperone.

4. Hsp90-radicicol interactions. Radicicol is a macrocyclic antibiotic that was originally isolated from cultures of the fungus *Monosporium bonorden* (Delmotte and Delmotte-Plaquee, 1953). Although radicicol (fig. 5) is structurally distinct from the benzoquinone ansamycins, it too was originally described as a tyrosine kinase inhibitor with the ability to revert the morphological changes of v-Src-transformed fibroblasts (Kwon *et al.*, 1992). In addition to inhibiting transformation by activated tyrosine kinases, radicicol has been reported to revert Ras transformation, presumably through disruption of downstream signaling by Raf kinase, because no alteration in the level of GTP-bound Ras was found in treated cells (Kwon *et al.*, 1995). It was recently demonstrated that radicicol specifically interacts with Hsp90 (Schulte *et al.*, 1998; Sharma *et al.*, 1998) and can compete with GA for binding to the amino-terminal domain of this chaperone (Schulte *et al.*, 1998). Given these biochemical findings, it is not surprising that the drug appears to possess many of the same biological activities as the benzoquinone ansamycins in cell culture. Although radicicol appears to have the same cellular target of action as GA-like compounds, its distinct structure (including the absence of a quinone ring) may result in quite different patterns of toxicity and bioactivity in whole animals because of differences in metabolism and disposition, which remain to be explored.

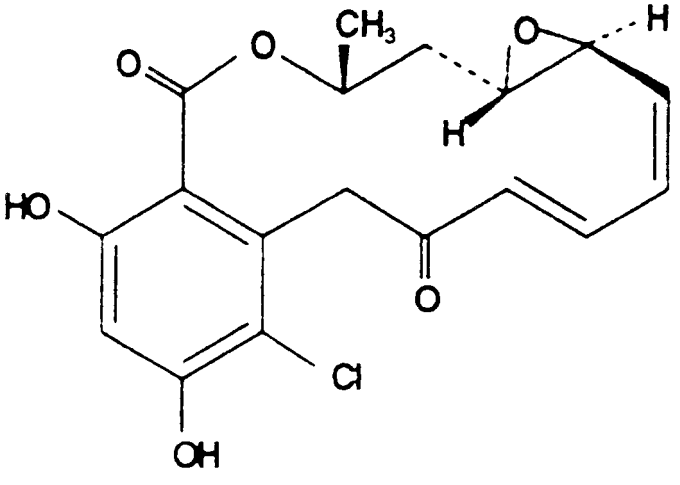


FIG. 5. Structure of radicicol [5-chloro-6-(7,8-epoxy-10-hydroxy-2-oxo-3,5-undecadienyl)-β-resorcylic acid μ-lactone]. The molecular weight is 365.

5. Biological activities. Although the drugs were originally described as selective inhibitors of tyrosine phosphorylation, the biological activities of both radicicol and benzoquinone ansamycins such as GA are best explained in terms of their high affinity interaction with members of the Hsp90 class of molecular chaperones. In the case of Raf-1 (Schulte *et al.*, 1995, 1996) and both receptor-linked (Chavany *et al.*, 1996; Miller *et al.*, 1994a) and Src-family tyrosine kinases (Hartson *et al.*, 1996), it has become clear that the drugs do not directly inhibit kinase activity but, rather, destabilize the kinase proteins and stimulate their degradation. Destabilization occurs via stimulation of the ubiquitin conjugation and proteasome-mediated degradation of the target proteins (Mimnaugh *et al.*, 1996; Sepp-Lorenzo *et al.*, 1995). For many of these drug-sensitive targets, stable association of the kinase with Hsp90 has been demonstrated by co-precipitation, but the way in which drug-induced alterations in Hsp90 complex formation with the target result in its enhanced ubiquitin conjugation remains unclear at this time. GA interaction with Hsp90 also explains some of the more recently reported biochemical activities of GA, such as inhibition of steroid hormone receptor function (Segnitz and Gehring, 1997; Whitesell and Cook, 1996) and alteration of the conformation and stability of mutant p53 species (Blagosklonny *et al.*, 1995, 1996). The consequences of drug interactions with Hsp90 are probably best characterized with respect to the PR, because so much is known about the posttranslational chaperone interactions that take place normally with this steroid receptor (Smith *et al.*, 1995). GA binding of Hsp90 both in vitro and in intact cells does not

inhibit Hsp90 association with the receptor protein but, rather, inhibits the association of Hsp90 with the co-chaperone p23 (Johnson and Toft, 1995). As a result, the formation of a mature receptor heteroprotein complex that is competent to bind hormone in a high affinity manner is blocked. Therefore, these Hsp90-binding agents can function as nonclassical antagonists of hormone action because they do not interact with the receptor protein itself. Such a mechanism of action could prove useful in overcoming the resistance to antiestrogen therapy that often results from acquired mutations of the receptor protein in breast cancers treated with conventional antagonists such as tamoxifen. On the other hand, global disruption of steroid hormone signaling after systemic exposure to these agents could lead to the toxicities characteristic of functional adrenal insufficiency. Although preclinical studies have been reported describing some preliminary toxicological data for GA and derivatives in animals, no data have yet been reported that address these specific issues.

Many other, less well characterized, biological effects have also been reported for the ansamycins and radicicol, such as inhibition of angiogenesis (Oikawa *et al.*, 1989, 1993), induction of Hsp expression (Hegde *et al.*, 1995; Murakami *et al.*, 1991), cytoprotection after hypoxic/ischemic injury (Conde *et al.*, 1997; Ohtsuki *et al.*, 1996), induction of differentiation (Honma *et al.*, 1989; Kondo *et al.*, 1989; Shimada *et al.*, 1995), and inhibition of immune and inflammatory responses (Hwang *et al.*, 1996; June *et al.*, 1990; Nishiya *et al.*, 1995). These diverse effects are consistent with drug-mediated alterations in Hsp90 function rather than simply inhibition of tyrosine kinase activity, but the precise mechanisms of drug action in each system have yet to be defined. In particular, the possibility that some of these effects may be the result of drug-mediated alterations in the expression or function of other Hsps, either alone or in combination with Hsp90, cannot be ruled out.

A concern in the use of GA and similar Hsp90-binding drugs is the potential for pleiotropic adverse effects resulting from disruption of normal cellular folding processes, in both cytoplasm and the ER. GA partially inhibited renaturation of firefly luciferase in a reticulocyte lysate model for refolding of denatured proteins (Thulasiraman and Matts, 1996) and may have similar effects on multiple Hsp90-dependent folding processes in the cytoplasmic compartment. As noted above for erbB2, GA may also alter folding of secretory or membrane proteins whose folding in the ER is dependent on Grp94.

VI. Prospective Therapeutic Rationales That Involve Chaperones

A. Drugs Targeting Specific Chaperone Activities

1. Immunophilins. As discussed above, several natural products that bind specifically to components of the chaperone machinery have been identified. The immunosuppressants cyclosporin A and FK506 are two notable examples that are currently being used in many transplantation procedures, as well as in the treatment of autoimmune and inflammatory diseases. These drugs or their analogs may also prove useful as neurotrophic agents. As noted, the primary protein effectors for immunosuppression and neurotrophic actions are CypA and FKBP12, but other immunophilin family members are attractive pharmacological effectors. The involvement of Cyp40, FKBP52, and FKBP51 in multiple signal transduction pathways compels further research into their potential as therapeutic targets. There is currently a poor understanding of the biological functions of the large immunophilins, but it is reasonable to predict that drugs will be discovered or designed that more effectively, and perhaps specifically, block actions of the large immunophilins in therapeutically important ways.

2. Hsp70. DSG is the only drug currently under clinical evaluation that is known to interact selectively with Hsp70-class chaperones. Although the precise mechanism of the potent immunosuppressive activity of DSG is still unclear, it appears to be unique, because the toxicities of DSG do not overlap with those of standard immunosuppressive agents. Early human trials have demonstrated a favorable safety profile, and it appears that DSG may be able to reverse renal allograft rejection episodes that are refractory to conventional agents. Based on these considerations, the drug may prove quite useful in combination-therapy regimens after solid-organ transplants, but further study is required before its true utility can be defined (Gores, 1996).

3. Hsp90. Drugs specifically binding Hsp90 hold promise as inhibitors of signal transduction pathways influenced by Hsp90-target protein interactions. As discussed above, GA and the benzoquinone ansamycins have received considerable attention as potential anticancer agents (Supko *et al.*, 1995). Preclinical studies have demonstrated significant antitumor effects in several animal models, with structure-activity data indicating much greater in vivo activity for the 17-allylamino derivative of GA (17-AAG) (fig. 4) than for the parent compound (Schnur *et al.*, 1995). To overcome severe solubility problems, 17-AAG has been formulated as a microdispersed suspension in phospholipid. Pharmacokinetic data obtained in rodents using this formulation indicate that plasma concentrations sufficient for good bioactivity in vitro (>1 μM) can be achieved for >3 h after bolus intravenous administration. Microdispersed 17-AAG is now undergoing full Investigational New Drug-directed toxicological evaluation, and it is anticipated that National Cancer Institute-sponsored phase I clinical trials will be initiated with patients with refractory malignancies after regulatory approval (Sausville *et al.*, 1997). Preliminary toxicological studies have shown that 17-AAG is better tolerated than GA in rats and dogs, with the dose-limiting toxicity being hepatic in dogs and renal in rats. Hematopoietic toxicity was ob-

served for 17-AAG but was not dose-limiting (Page *et al.*, 1997). The marked variations in toxicity profiles among animal species and between GA and 17-AAG suggest that drug metabolism, rather than intrinsic limitations associated with drug-Hsp90 interactions, may be a significant component of benzoquinone ansamycin toxicity in vivo. In this regard, several proprietary derivatives of radicicol, a non-quinone-containing structure that appears to interact with Hsp90 in a manner similar to that of GA, are under development by the pharmaceutical company Kyowa Hakko Kogyo. These compounds are said to demonstrate no hepatic or renal toxicity at doses associated with good antitumor activity in several human tumor xenograft models in mice (Akinaga S, personal communication).

In addition to its inhibition of kinase- or steroid receptor-mediated signaling, GA also activates the stress response pathway mediated by heat shock transcription factor 1 (HSF1). Hsp70 and Hsp90 bind to inactive HSF1 (Abravaya *et al.*, 1992; Baler *et al.*, 1992, 1996; Nadeau *et al.*, 1993; Nair *et al.*, 1996) and possibly contribute to repression of HSF1 activity. In a simple model (Morimoto, 1993), the Hsps are competitively depleted from HSF1-repressive interactions when misfolded substrates accumulate after a proteotoxic insult. Activated HSF1 then increases transcription from heat shock genes; as Hsp levels increase, HSF1 again becomes repressed and Hsp production is curtailed. Unfortunately for investigators hoping to understand stress responses, HSF1 regulation is much more complex (Morimoto *et al.*, 1996), but Hsp90 has been clearly implicated as a participant. A common consequence of GA or herbimycin A treatment of cells is the induction of Hsp70 that results from drug-induced activation of HSF1. The exact mechanism by which the Hsp90-binding drugs lead to HSF1 activation has not been determined, but it certainly correlates with a change in chaperone interactions with HSF1. Interestingly, another consequence of GA treatment is activation of the unfolded protein response in ER that leads to increased ER chaperone expression (Lawson *et al.*, 1998). This GA effect is perhaps mediated through Grp94-dependent regulation of this response or accumulation of misfolded proteins in the ER. As discussed in the next section, the ability to elevate chaperone activity in cells may have several therapeutic uses.

B. Induction of Protein and Chemical Chaperones

1. Background. It has been long recognized that heat-treated or chemically stressed cells and tissues gain a tolerance to further stressful insults. This conditioning results in large part from the induction of Hsps and chemical chaperones that enhance the cellular environment for protein folding and stability. A heat shock response is observed in many tissues after an elevation of body temperature of a few degrees, and fever may, in part, be an adaptation to naturally increase intracellular chaperone activity. In a rat model (Blake *et al.*, 1991; Udelsman *et al.*, 1993), behavioral stress induces Hsp70 levels in adrenal and vascular tissues, a biochemical response that is mediated by neurohormonal factors and elevated blood pressure (Xu *et al.*, 1996) and that is attenuated with aging (Blake *et al.*, 1991; Udelsman *et al.*, 1993). Recently, there has been considerable interest in the possibility that prophylactic induction of chaperone activity may be highly beneficial in several clinical situations.

2. Mechanisms for inducing chaperone activity. There are several means by which chaperones may be therapeutically induced. An obvious one is mild heating of tissues, but localizing and controlling temperature elevation within a narrow, therapeutically beneficial range can be difficult. Recently, several pharmacological compounds that can induce or enhance induction of Hsp synthesis were identified. As previously noted, the benzoquinoid ansamycins herbimycin A and GA can induce Hsp70 synthesis and activation of HSF1. These drugs and the unrelated compound radicicol are now known to specifically bind Hsp90, and it appears that their mechanism for activating HSF1 involves disruption of a negative regulatory interaction between Hsp90 and HSF1. These or similar drugs may prove to be effective for acute localized or systemic induction of a heat shock response. Nonsteroidal anti-inflammatory drugs have been found to potentiate the activation of HSF1 and a heat shock response (Amici *et al.*, 1995; Fawcett *et al.*, 1997; Jurivich *et al.*, 1992, 1995; Lee *et al.*, 1995). Perhaps related to this drug action, arachidonic acid can also potentiate HSF1 activation, decreasing the temperature elevation needed for robust Hsp induction (Jurivich *et al.*, 1994). Because arachidonic acid is a key mediator and precursor in inflammatory responses, its potentiation of HSF1 activation may be an adaptation to enhance chaperone activity within cells near the site of injury or infection.

Specific mechanisms for inducing production and accumulation of intracellular chemical chaperones are potentially useful for correcting or preventing protein misfolding, and perhaps drugs will be discovered that serve this purpose. An alternative approach would be to administer, by tissue perfusion, nontoxic chemical chaperones that could be taken up by cells. As discussed in the next section, the feasibility of this approach has been examined in cell culture systems.

3. Injury protection. Currie *et al.* (1988), using rats whose body temperature was raised to 42°C, first demonstrated that heat stress promotes recovery of heart tissues from ischemic injury. This protection correlates with the time course of elevated Hsp70 levels (Karmazyn *et al.*, 1990; Yellon and Latchman, 1992), and transgenic mice overexpressing Hsp70 exhibit reduced postischemic injury (Marber *et al.*, 1995; Plumier *et al.*, 1995). Interestingly, Hsp70-transgenic mice are also protected from ischemic injury in the brain (Plumier *et al.*, 1997).

Induction of a heat shock response might be an effective prophylactic treatment to minimize myocardial injury. Herbimycin A, which can induce activation of HSF1 (Hegde *et al.*, 1995), has been shown to protect cardiomyocytes in culture (Morris *et al.*, 1996). Although it is difficult to predict when natural occlusion of cardiac vessels may occur, induction of Hsps may be beneficial during scheduled surgical procedures that can cause ischemic damage.

A common problem with balloon angioplasty is restenosis, i.e., reclosure of the artery resulting from smooth muscle cell proliferation in response to mechanical injury. In an in vitro model, pretreatment of cells using heat treatment or incubation with chemical inducers of the heat shock response reduced subsequent injury-induced smooth muscle cell proliferation (Slepian *et al.*, 1996). Further investigation may show that chaperone-related pretreatment can reduce postangioplasty restenosis. Similarly, recovery from several stressful therapeutic procedures may be enhanced by induction of chaperone activity that is appropriately timed and localized before the procedure.

4. Enhanced utilization of mutant proteins. As discussed in section IV with respect to the quality control machinery of the ER, disrupted trafficking may be a greater problem than the ultimate ability of a mutant protein to fold into a functional conformation. There are several reports that chemical chaperones can assist mutant proteins in passing ER quality control and continuing on to be secreted or properly localized at membrane sites. The mutant phenotype in cells expressing the ΔF508 form of CFTR could be corrected by treating cells with any of several chemical chaperones (Brown *et al.*, 1996; Sato *et al.*, 1996), including glycerol, trimethylamine N-oxide, and deuterated water. Welch and colleagues have extended their study of chemical chaperones to several additional systems involving misfolded proteins. Chemical chaperones were shown to inhibit formation of PrP^{Sc} (Tatzelt *et al.*, 1996) and were shown to complement, in cells grown at a nonpermissive temperature, temperature-sensitive mutations in the tumor suppressor p53, oncogenic v-Src, or a ubiquitin-activating enzyme (Brown *et al.*, 1997). In the latter study, chemical chaperones were able to promote proper folding of nascent mutant polypeptides; once folded into a native conformation, the mutant proteins were stable at nonpermissive temperatures after removal of chemical chaperones. More than 30 years ago, it was proposed that "osmotic remediation" could correct mutant proteins (Hawthorne and Friss, 1964); possibly in less than another 30 years, this principal will achieve clinical application.

C. Chaperones as Immunological Adjuvants

A basic intracellular function of Hsps is peptide chaperoning within and across cellular compartments. An increasing body of data indicate that Hsp-bound peptides are similar to those that bind major histocompatibility complex (MHC) molecules. Hsp70 members may chaperone potential MHC class I ligand peptides toward the transporter associated with antigen processing. Grp94/Gp96 may help stabilize unfolded class I α chains and shuttle cytosolic peptides to newly synthesized class I molecules in the ER.

The tumor-derived Hsps Hsp70 and Grp94 elicit tumor-specific protective immunity in mice (Suto and Srivastava, 1995; Tamura *et al.*, 1997; Udono and Srivastava, 1993, 1994). These Hsps are not antigenic themselves, but they act as carriers of immunogenic peptides. Hsp70 and Grp94 preparations isolated from unrelated tumors or normal murine tissues were ineffective in generating protection (Udono and Srivastava, 1994). Murine depletion experiments have suggested that Hsp-peptide complexes are taken up by host macrophages, which may direct the associated peptides toward the endogenous (MHC class I) presentation pathway (Suto and Srivastava, 1995; Udono and Srivastava, 1994). Grp94 from T cell lymphoma cells infected with the vesicular stomatitis virus was associated with the immunodominant peptide of vesicular stomatitis virus, which is naturally presented by H-2Kb class I molecules (Nieland *et al.*, 1996). Grp94-associated peptides are not restricted to those that can bind to the particular MHC class I alleles of the host cells but include a wider repertoire of antigenic peptides (Arnold *et al.*, 1995). It appears that Grp94 can bind all peptides that enter the ER by either transporter-associated antigen processing-dependent or -independent mechanisms (Arnold *et al.*, 1997).

There are potential advantages in using Hsp-peptide complexes generated in vivo, rather than purified or synthetic peptides, as the source of tumor antigens. Use of HSP-associated peptides may circumvent the need to identify tumor-specific antigens. Furthermore, individual peptides are restricted to specific MHC molecules, whereas peptides associated with Hsp70 or Grp94 from tumors represent an aggregation of epitopes corresponding to numerous human lymphocyte antigen specificities (Arnold *et al.*, 1995). Professional antigen-presenting cells are capable of processing the appropriate peptides based on the MHC restriction elements and presenting them to the responder human lymphocyte antigen-matched T cells. Immunization of a tumor-bearing host with a specific tumor-associated antigen may induce an antitumor T cell response. However, this may eventually lead to outgrowth of tumor cells not expressing this particular antigen. Hsp-peptide complexes generated in vivo may reduce immunological escape variants by providing the entire antigenic repertoire of that tumor, resulting in the generation of T effector cells directed against different tumor epitopes.

Numerous investigators have recently shown that complexes formed in vitro between peptides and Hsps can also induce specific T cell responses. Complexes of murine liver-derived Grp94 and Hsp70 with a variety of immunogenic peptides induced potent, specific, cytotoxic

T cell responses against these peptides (Blachere *et al.*, 1997). Noncovalent complexes of mycobacterial Hsp70 and influenza A nucleoprotein were effective in eliciting peptide-specific T helper responses in mice (Roman and Moreno, 1996). Moreover, a large fragment of ovalbumin covalently linked to mycobacterial Hsp70 acted as an adjuvant in generating cytotoxic T cells and protected immunized mice against a lethal challenge with transfected melanoma expressing ovalbumin peptides (Suzue *et al.*, 1997). The strategy of using large protein fragments linked to Hsps may offer the advantage of providing numerous peptides that may be processed and presented by the highly diverse MHC molecules.

Transfer of genes encoding mycobacterial Hsps into mammalian tumors can also increase the immunogenicity of the tumors. Transfection of the mycobacterial Hsp65 gene into J774 murine macrophage tumor cells abrogated their tumorigenicity in syngeneic and athymic murine hosts, whereas preimmunization of syngeneic mice with J774-Hsp65 cells conferred protection to challenges with wild-type tumor (Lukacs *et al.*, 1993). Similarly, in vivo mediated gene transfer of mycobacterial Hsp65 into J774 tumors resulted in tumor regression in both immunocompetent syngeneic mice and severe combined immunodeficient mice (Lukacs *et al.*, 1997). However, complete tumor regression was evident only in immunocompetent mice, confirming the role of T cells in tumor rejection.

Taken together, these reports strongly support the potential utility of Hsps as natural immunological adjuvants. Hsp-peptide complexes, Hsp-peptide fusion proteins, or Hsp gene-transfected cells may be effectively used to stimulate potent, durable, and specific T cell responses against tumor cells or virally infected cells.

VII. Summary

In this review, we have presented an overview of protein misfolding as a basis for disease and have provided a prospective look at pharmacological approaches that may ultimately help to prevent or resolve protein-folding problems. Much of this article has focused on the molecular and chemical chaperones that assist in protein-folding processes and may be helpful in alleviating conditions that result from misfolding. A textbook-length treatise would be needed to fully cover these subjects, and we apologize for our oversights and biases in selecting the topics, examples, and citations that appear here. Currently, there are few pharmacological therapies that directly address protein misfolding and chaperone activity, so much of our outlook is necessarily speculative. Although some of our prognostications may prove to be inaccurate or unfeasible, perhaps for reasons we should have recognized, we would be surprised if chaperone-targeting drugs, including ones unforeseen by us, are not added to the clinical arsenal in the near future.

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