MINIREVIEW

Regulation of Signaling Protein Function and Trafficking by the hsp90/hsp70-Based Chaperone Machinery[1]

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Nearly 100 proteins are known to be regulated by hsp90. Most of these substrates or "client proteins" are involved in signal transduction, and they are brought into complex with hsp90 by a multiprotein hsp90/hsp70-based chaperone machinery. In addition to binding substrate proteins at the chaperone site(s), hsp90 binds cofactors at other sites that are part of the heterocomplex assembly machinery as well as immunophilins that connect assembled substrate·hsp90 complexes to protein-trafficking systems. In the 5 years since we last reviewed this subject, much has been learned about hsp90 structure, nucleotide-binding, and cochaperone interactions; the most important concept is that ATP hydrolysis by an intrinsic ATPase activity results in a conformational change in hsp90 that is required to induce conformational change in a substrate protein. The conformational change induced in steroid receptors is an opening of the steroid-binding cleft so that it can be accessed by steroid. We have now developed a minimal system of five purified proteins—hsp90, hsp70, Hop, hsp40, and p23—that assembles stable receptor·hsp90 heterocomplexes. An hsp90·Hop·hsp70·hsp40 complex opens the cleft in an ATP-dependent process to produce a receptor·hsp90 heterocomplex with hsp90 in its ATP-bound conformation, and p23 then interacts with the hsp90 to stabilize the complex. Stepwise assembly experiments have shown that hsp70 and hsp40 first interact with the receptor in an ATP-dependent reaction to produce a receptor·hsp70·hsp40 complex that is "primed" to be activated to the steroid-binding state in a second ATP-dependent step with hsp90, Hop, and p23. Successful use of the five-protein system with other substrates indicates that it can assemble signal protein·hsp90 heterocomplexes whether the substrate is a receptor, a protein kinase, or a transcription factor. This purified system should facilitate understanding of how eukaryotic hsp70 and hsp90 work together as essential components of a process that alters the conformations of substrate proteins to states that respond in signal transduction. Exp Biol Med 228:111–133, 2003

Key words: hsp90; chaperone proteins; signal transduction; steroid receptors; protein trafficking; protein kinases

Hsp90 is a highly conserved and essential stress protein that is expressed in all eukaryotic cells. Despite being a heat-shock protein, hsp90 is one of the most abundant proteins in unstressed cells (1–2% of cytosolic protein), where it performs housekeeping functions controlling the activity, turnover, and trafficking of a variety of proteins. Most of the hsp90-regulated proteins that have been discovered to date are involved in signal transduction. We reviewed the regulation of signal transduction by hsp90 in 1997 (1, 2), and in the intervening 5 years, the number of established substrates or "client proteins" that are regulated by hsp90 has tripled (Fig. 1). In most cases, these proteins have been shown by coimmunoadsorption to exist in cytosolic heterocomplexes with hsp90. In a number of cases, variation in hsp90 expression or hsp90 mutation has been shown to impair signaling via the protein or to impair a specific function of the protein (e.g., steroid binding, kinase activity) *in vivo*. Ansamycin antibiotics, such as geldana-

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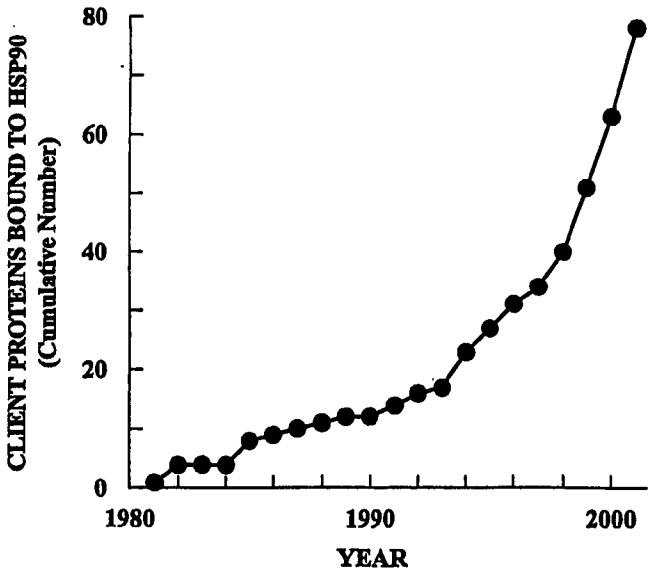

Figure 1. Cumulative number of "substrate" or "client" proteins reported bound to and/or regulated by hsp90. pp60$^{v\text{-}src}$, the first hsp90-bound protein, was reported in 1981, and steroid receptor binding to hsp90 was reported in 1985. Geldanamycin was shown to be an inhibitor of hsp90 in 1994, and it has been in general use for rapid identification of hsp90 substrates since 1997. Proteins bound to Grp94 are not included, and all homologs of a protein are considered as a single protein.

mycin and radicicol, inhibit hsp90 function (3), and treatment of cells with these inhibitors alters the function and/or turnover of hsp90-bound proteins, providing a rapid means of screening for hsp90-regulated targets.

In Tables I–III, we provide a comprehensive listing of the hsp90-bound proteins identified to date. With the exception of actin and tubulin, where cytoskeleton-associated proteins are likely responsible for the linkage, direct interaction of the substrate with the chaperone site(s) in hsp90 is likely and in many cases proven. These substrate·hsp90 heterocomplexes are formed in an ATP-dependent process by a multiprotein hsp90/hsp70-based chaperone machinery that is ubiquitous among cells of the animal and plant kingdoms (1, 2). Tables I–III do not include proteins such as MyoD (4) and calponin (5), for which hsp90 effects have been demonstrated with chaperone fragments in systems without ATP and hsp70. The relevance of *in vitro* passive chaperoning assays, such as the ATP-independent inhibition of enzyme aggregation by hsp90, to the formation of signaling protein·hsp90 complexes *in vivo* is unclear. Thus, the proteins reactivated in these assays are not included in Tables I–III as relevant substrates, and these passive chaperone effects are reviewed elsewhere (6).

As a reflection of the increasing interest in hsp90, several reviews have been published since 1997. A review by Csermely *et al.* (7) is comprehensive, and other reviews focus on hsp90 structure (8–10), studies of chaperone function in yeast (11, 12), hsp90 in plants (13), hsp70/hsp90 in protein folding versus degradation (14), hsp90 chaperoning and signal transduction (15), steroid receptor·hsp90 heterocomplexes (16–18), geldanamycin/radicicol inhibition of hsp90 (19–22), and the role of chaperones in receptor trafficking through the cytoplasm (23, 24) and within the nucleus (25). Despite the many proteins that are known to be regulated by hsp90 *in vivo* (Tables I–III), it is not known how they are regulated, and most of these reviews contain considerable speculation regarding the mechanism of hsp90

Table I. Hsp90-Bound Transcription Factors and Polymerases

Substrate protein	Reference		
	Hsp90 binding[a]	Regulation *in vivo*[b]	Geldanamycin[c]
Glucocorticoid receptor	31	32, 33	34
Progesterone receptor	35, 36		37
Estrogen receptor	38, 39	32	
Androgen receptor	38, 40	41	
Mineralocorticoid receptor	42	32	
Aryl hydrocarbon (Ah) receptor	43, 44	45	46
v-*erb*A	47		
Retinoid receptor		48	
Heat-shock factor	49, 50	49	49, 50
Sim	51		
Tumor promotor-specific binding protein	52		
Hepatitis B virus reverse transcriptase	53		53
p53 mutant	54, 55	55	55
Ecdysone receptor	56		56
Heme activator protein (Hap1)	57	57	
Hypoxia-inducible factor-1α	58		58
Telomerase	59		59
SV40 large T antigen	60		60
MTG8 myeloid leukemia protein	61		

[a] Substrate protein · hsp90 complexes demonstrated directly, usually by coimmunoadsorption.
[b] Variation in hsp90 expression or hsp90 mutation impairs substrate protein function *in vivo*.
[c] Function and/or turnover of the protein is affected by geldanamycin *in vivo*.

Table II. Signaling Protein Kinases Bound to Hsp90

	Reference		
	Hsp90 binding[a]	Regulation *in vivo*[b]	Geldanamycin[c]
v-*Src*, c-*Src*	26, 27, 62	33, 63	64, 65
v-*fps*	66		
v-*yes*	66		
v-*fes*	66		
v-*frg*, c-*frg*	67, 68		
Lymphoid cell kinase p56^{lck}	68		69
Hck	70		71
Wee1	72	72	
Sevenless PTK		73	
Heme-regulated eIF-2α kinase	74, 75		76
Calmodulin-regulated eEF-2 kinase	77		78
Casein kinase II	79		
Raf family kinases: v-*Raf*, c-*Raf*, B-*Raf*	80, 81, 82	84	85, 86
Gag-Mil, Ste11	83, 84		
MEK	86		
Kinase suppressor of Ras	87		87
Phosphatidylinositol 4-kinase	88		
Focal adhesion kinase			89
p210$^{bcr\text{-}abl}$	65		65
Receptor-interacting protein	90		90
Translation initiation factor kinase Gcn2	91	91	91
Insulin receptor	92		93
Insulin-like growth factor receptor			93
Pim-1	94		94
ErbB2	95		95, 96
Epidermal growth factor receptor			93, 97
Platelet-derived growth factor receptor			97
c-*Mos*	98		98
Cdc2		99	
Cdk4	100, 101		101
Cdk6	102		
Cdk9	103		103
Polo mitotic kinase	104		104
Tropomyosin related kinase B (trkB)	105		
Mitogen-activated protein kinase MOK	106		106
Male germ cell-associated kinase MAK	106		
MAK-related kinase	106		
dsRNA-dependent kinase PKR	107	107	107
Akt kinase	108		
Mik1, Swe1	109	109	109
3-Phosphoinositide-dependent kinase-1	231		231

[a] Substrate protein·hsp90 complexes demonstrated directly, usually by coimmunoadsorption.
[b] Variation in hsp90 expression or hsp90 mutation impairs substrate protein function *in vivo*.
[c] Function and/or turnover of the protein is affected by geldanamycin *in vivo*.

action. To limit redundancy, in this review we update our studies of hsp90 and hsp90-bound cofactors (e.g., Hop, p23, immunophilins) as they relate to steroid receptor function and trafficking.

Although the first to be recognized as an hsp90 substrate was pp60$^{v\text{-}src}$ (26, 27), hsp90 has been studied most extensively in relation to steroid receptors. The study of hsp90 effects on steroid receptors has provided a number of unique contributions to our understanding of this important participant in many signal transduction pathways:

1. Several of the steroid receptors (e.g., glucocorticoid receptors) must be bound to hsp90 to have high-affinity steroid-binding activity, and in cell-free systems of receptor·hsp90 heterocomplex assembly, the steroid-binding form is generated, providing an *in vitro* assay of hsp90 action that is clearly relevant to hsp90 action *in vivo*.
2. The study of steroid receptor·hsp90 assembly by reticulocyte lysate led to the discovery of p23 and to the concept that hsp90 and hsp70 work together as multiprotein machinery with the hsp-organizing protein Hop.
3. The receptor·hsp90 assembly system of reticulocyte lysate has now been resolved into a minimal system of five purified proteins—hsp90, hsp70, Hop, hsp40, and p23—that efficiently work together to produce receptor·hsp90 heterocomplexes with steroid-binding activity.

Table III. Other Proteins Bound to Hsp90

	Reference		
	Hsp90 binding[a]	Regulation *in vivo*[b]	Geldanamycin[c]
G protein βγ	110		
$G\alpha_o$	111		111
Gα12	112		112
Nitric oxide synthase (NOS)			
Endothelial NOS	113		113
Neuronal NOS	114		114
Inducible NOS			115
Protease-activated receptor 1 (PAR-1)	116	116	116
Cystic fibrosis transmembrane conductance regulator (CFTR)	117		117
Atrial natriuretic peptide receptor	118		118
Mammalian aminoacyl-tRNA synthetases	119		119
Reovirus cell attachment protein σ1	120		120
HETE binding complex	121		
Apaf-1	122		
Cna2 catalytic unit of calcineurin	123	123	123
Proteasome	124, 125		
Lysosome	126		
Centrin/centrosome	127		128
Actin[d]	129		
Tubulin[d]	130		
Apoprotein B	131		131
β-Galactosidase M15 truncation mutant	132		
Pancreatic bile salt-dependent lipase[e]	133		
Unassembled immunoglobulin chains[e]	134		
Fanconi anemia group C protein[e]	135	135	
Thyroglobulin[e]	136	136	
Macrophage scavenger receptor	232		
Plasmodium falciparum erythrocyte membrane protein	233		

[a] Substrate protein·hsp90 complexes demonstrated directly, usually by coimmunoadsorption.
[b] Variation in hsp90 expression or hsp90 mutation impairs substrate protein function *in vivo*.
[c] Function and/or turnover of the protein is affected by geldanamycin *in vivo*.
[d] Coprecipitation (actin) or coimmunoadsorption (tubulin) of hsp90 probably reflects binding to cytoskeleton-associated proteins and not ATP-dependent and hsp70-dependent formation of heterocomplexes with hsp90.
[e] Bound to Grp94, an hsp90 homolog in the endoplasmic reticulum.

4. The purification of this five-protein system has permitted mechanistic study to define several steps in the assembly of receptor·hsp90·immunophilin complexes, starting with two sequential ATP-dependent events involving first hsp70 and then hsp90.
5. Because the chaperone machinery opens up a hydrophobic steroid-binding cleft to access by ligand, the action of hsp90 as part of the multiprotein machinery is quite different from that of hsp90 acting alone *in vitro* to promote reactivation of partially denatured enzymes.
6. The three hsp90-binding TPR domain immunophilins—FKBP52, FKBP51, and CyP-40—were discovered as components of steroid receptor·hsp90 heterocomplexes.
7. The study of steroid-mediated glucocorticoid receptor translocation from the cytoplasm to the nucleus led to the discovery that the TPR domain immunophilins link hsp90 to a dynein motor protein.

It is clear from these findings that in an approach to hsp90 function from the standpoint of understanding the molecular endocrinology of hormone action, observations made on glucocorticoid receptor (GR) and progesterone receptor (PR) assembly into heterocomplexes with hsp90 have led us to some mechanistic conclusions and speculations that are quite different from those derived from studies on cell stress and protein chaperoning. It seems likely that hsp90 is assembled into complexes with all of the signaling proteins in Tables I–III by the same multiprotein machinery that regulates steroid receptor function (28). Thus, the mechanism of steroid receptor·hsp90 heterocomplex assembly discussed in this review may suggest some broad principles as to how hsp90 affects the function of a wide variety of regulatory proteins regardless of their structure or sequence. It is usually thought that, to be a substrate for hsp90 or hsp70, a protein must be partially unfolded, thus exposing hydrophobic regions that are the sites for chaperone binding. However, there is no indication that steroid receptors that have been stripped of hsp90 by mild salt treatment are in any way denatured before their reactivation by the hsp90/hsp70-based chaperone machinery. When hsp90 and hsp70 are acting together, we find that there is a very focal site of attack on the GR that lies on the surface of the ligand-binding domain at the opening of the hydrophobic steroid-

binding cleft (29, 30). It is not yet clear whether the chaperone machinery has the general ability to recognize cleft openings on other signaling proteins. However, such regions are a general topologic feature of virtually all proteins in native conformation, and one must seriously consider the possibility that the hsp90/hsp70-based chaperone machinery functions on proteins in their native conformations rather than promoting refolding of partially denatured proteins. Whether the example of focal recognition of steroid receptors by the hsp90/hsp70-based machinery will extend to other proteins whose function is regulated by hsp90 is unknown. However, we hope that the following update of how this machinery works on steroid receptors will prove to be heuristic for investigators working with other regulatory systems.

Cofactor Sites on hsp90

The substrate, or client, proteins for hsp90 (Tables I–III) are bound to its chaperone site(s). The location and number of substrate-binding sites on hsp90 are still uncertain. However, hsp90 may be unusual in that it appears to contain two independent chaperone sites that differ in their substrate specificity (137, 138), an observation that may ultimately be important for understanding the mechanism by which the hsp90/hsp70-based chaperone machinery opens the ligand-binding cleft in steroid receptors. As summarized in Table IV, there are a variety of other protein interaction sites on hsp90 that are important for binding components of the heterocomplex assembly machinery or proteins that bind after the heterocomplexes are formed, such as immunophilins in the case of steroid receptor·hsp90 heterocomplexes or $p50^{cdc37}$ in the case of protein kinase·hsp90 heterocomplexes.

Hsp70 is required for the assembly of signaling protein·hsp90 heterocomplexes, and these two chaperones likely interact directly with each other while opening the steroid-binding cleft in the GR (164). Mammalian hsp90s and hsp70s do not form complexes that are stable enough to be detected by the usual biochemical separation techniques (1), but hsp80, the hsp90 homolog of *Neurospora crassa*, binds directly to hsp70 to form a complex that survives routine separation procedures (139), and direct interaction of mammalian hsp70 with hsp90 has been shown by cross-linking (140). It is not known where hsp70 binds to hsp90, but one of the two chaperone sites identified by *in vitro* experiments (137, 138) is a potential hsp70 interaction site.

p23 is a widely distributed acidic 23-kDa protein (141) that binds only to the ATP-dependent conformation of hsp90 (165) to stabilize receptor·hsp90 heterocomplexes once they are formed and the receptor has been converted to the steroid-binding state (166). The p23-binding domain on hsp90 has not been identified, but it is known that p23 binding requires regions outside the 1–221 domain containing the nucleotide/geldanamycin-binding site (167, 205).

The third component of the hsp90/hsp70-based chaperone machinery that directly contacts hsp90 is a 60-kDa protein called Hop (hsp-organizing protein) (144). Hop binds independently via an N-terminal tetratricopeptide repeat (TPR) domain to hsp70 and via a central TPR domain to hsp90 (168, 169) to bring the two chaperones together in an hsp90·Hop·hsp70·hsp40 machinery that opens the GR steroid-binding cleft (170, 171). Hop binds to a TPR acceptor site that is located at the C-terminus of hsp90 (172–175), and Hop and the TPR domain immunophilins compete with each other for binding to this common acceptor site (156, 173, 176–178). For some of the immunophilins, such as CyP-40 and FKBP51, sequences upstream and/or downstream of the TPR domain are also important for stable binding to hsp90 (177, 179), but for FKBP52, the TPR domain alone is sufficient. A mutational study of protein phosphatase 5 binding to hsp90 indicates that interactions between basic residues in a binding groove on one face of the TPR domain and acidic residues in the acceptor site on hsp90 are important for stable binding (175). Hsp90 functions as a dimer (1), and the dimerization domain is located within the TPR acceptor site (172, 174, 204).

The number of TPR acceptor sites per hsp90 dimer is somewhat controversial. Studies of saturation binding of Hop to hsp90 dimer (173) and cross-linking of hsp90·FKBP52 complexes (180) are consistent with one TPR-binding site per hsp90 dimer. However, isothermal titration calorimetry studies of hsp90 binding by yeast Cpr6 and Sti1 (181) and by mammalian CyP-40, FKBP51, and FKBP52 (182) are consistent with binding of two molecules of TPR protein to an hsp90 dimer. Deletion of amino acids 661–677 of chicken hsp90 eliminates dimerization, and the resulting hsp90 monomers bind FKBP52 weakly with respect to the wild-type hsp90 dimer (172). Thus, we favor the model in which each hsp90 monomer contains a half-site that is sufficient for low-affinity TPR binding, and a single higher affinity TPR acceptor site is created by dimerization. In a series of cross-linking studies, Gehring and his colleagues determined a heterotetrameric structure of 1 receptor:2 hsp90:1 immunophilin for glucocorticoid, estrogen, and progesterone receptors (reviewed in Ref. 1). It has also been shown that CyP-40 and FKBP52 exist in separate GR·hsp90 heterocomplexes (176, 183). Thus, the presence of one TPR acceptor site per hsp90 dimer is consistent with all of the observations on native steroid receptor heterocomplexes, and a two-site model is not.

Another TPR protein that interacts with hsp90 is CHIP (carboxy terminus of hsc70-interacting protein). CHIP is a 35-kDa protein that was originally shown in yeast two-hybrid and *in vitro* experiments to bind via its amino-terminal TPR domain to both hsc70 and hsp70 (184). Subsequently, it was shown that when reticulocyte lysate was incubated with CHIP and hsp90 was immunoadsorbed, CHIP was coimmunoadsorbed, Hop binding to hsp90 was decreased, and p23 binding was eliminated (160). This is consistent with CHIP binding to the TPR acceptor site on hsp90 and somehow keeping hsp90 in its ADP-dependent conformation, which does not bind p23. GR translated in the

Table IV. Proteins That Bind Directly to Hsp90 at Sites Other Than the Substrate Site(s)

Protein	Reference	Proposed role in SR or kinase action
hsp70[a]	139, 140	Interacts with hsp90 and is essential for assembly of protein·hsp90 heterocomplexes
p23	141	Binds to ATP-dependent conformation of hsp90, stabilizing protein·hsp90 heterocomplexes; not essential for assembly
Sba1 (yeast p23 homolog)	142, 143	
Proteins binding to the TPR acceptor site		
Hsp organizing proteins		
Hop	144	Bind via independent TPR domains to form the hsp70·Hop·hsp90 machinery for protein·hsp90 heterocomplex assembly; Hop promotes rate of assembly but is not essential
Sti1 (yeast)	145	
Dpit47	146	DNA polymerase interacting TPR containing protein of *Drosophila;* primarily nuclear hsp70·Dpit47·hsp90 complexes are bound to DNA polymerase α
Immunophilins		
FKBP52	147, 148	Found in SR·hsp90 heterocomplexes; proposed to target retrograde SR movement by binding directly via PPIase domain to cytoplasmic dynein
FKBP51	141, 149	Found in SR·hsp90 heterocomplexes
CyP-40	150, 151	Found in SR·hsp90 heterocomplexes and binds to cytoplasmic dynein
Yeast CyP-40 homologs		
Cpr6, Cpr7	152	GR and $pp60^{v\text{-}src}$ signaling adversely affected by *cpr7* null mutation, but Cpr function unknown
Cns1	153, 154	Essential for yeast cell viability; overexpression of CNS1 in *cpr7*Δ cells restores GR activity, but Cns1 function is unknown
Immunophilin homologs		
PP5	155, 156	An okadaic acid-sensitive protein serine phosphatase with a TPR domain and a PPIase homology domain; PP5 is found in SR·hsp90 heterocomplexes, and it binds to cytoplasmic dynein
ARA9 (also called XAP2 and AIP)	157, 158, 159	Found in aryl hydrocarbon receptor (AHR)·hsp90 heterocomplexes; mediates the cytoplasmic localization of the AHR; has a PPIase homology domain that does not interact with cytoplasmic dynein
CHIP (*C*-terminus of *h*sc70 *i*nteracting *p*rotein)	160	Binds via TPR domain to hsc70, hsp70, or hsp90; CHIP interaction with hsc70/hsp70 causes proteasome-dependent degradation of substrate; CHIP binding to hsp90 causes p23 release
UNC-45	234	Binds via N-terminal TPR to hsp90 and via C-terminal regions to myosin
Proteins binding to the p50 acceptor site		
$p50^{cdc37}$	26, 27, 161	Found in a variety of protein kinase·hsp90 heterocomplexes, where it binds directly to both the catalytic domain of the kinase and to a site on hsp90 that is different from the TPR acceptor site; function is unknown
Cdc37 (yeast)	73	
Harc	162	Hsp90-associating relative of Cdc37; a cytoplasmic phosphoprotein with a central hsp90 binding domain homologous with Cdc37; does not bind to Src or Raf-1 kinases, and function is unknown
Calmodulin binding domain		
Ca^{2+}-calmodulin	163	Ca^{2+}-calmodulin binds to hsp90 and inhibits binding of hsp90 to actin filaments; other calmodulin effects have not been reported, and the physiologic significance of calmodulin binding, if any, is unknown

[a] Although biochemically stable binding of hsp90 to hsp70 has been detected in *Neurospora crassa,* interaction of mammalian hsp90 and hsp70 has only been detected by cross-linking a mixture of purified proteins, and it has not been determined if there must be direct interaction between the two chaperones during the assembly of hsp90 heterocomplexes.

presence of CHIP does not achieve the steroid-binding state (160), but that likely reflects CHIP inhibition of hsp70 interaction with the GR before hsp90 can act in the heterocomplex assembly pathway. Thus, it is not totally clear what CHIP's interaction with hsp90 does to the GR.

CHIP possesses a carboxy-terminal U-box that interacts with the ubiquitin-conjugating enzyme family (185), and CHIP promotes GR ubiquitylation (160). But it has not been totally sorted out whether it is only CHIP binding to GR-bound hsp70 that targets the GR for ubiquitylation and subsequent proteasomal degradation or whether CHIP can also bind to GR-bound hsp90 and target the receptor for ubiquitylation. Thus, at this moment, it is clear that CHIP is a very interesting regulator of hsp70 function, targeting hsp70 itself (185) and hsp70 substrates, such as the GR (160), for proteasomal degradation. CHIP has been found in GR·hsp90 heterocomplexes (160), but hsp90 that is bound to steroid-binding receptors is in its ATP-bound conformation, and it seems unlikely that CHIP would bind to this hsp90 and target degradation of this form of the receptor. It should be noted, however, that receptor·hsp90 heterocomplexes are dynamically undergoing disassembly and being reassembled by the hsp90/hsp70-dependent machinery (1, 240); thus, receptor·hsp70 complexes are constantly being formed as targets for CHIP action.

When src·hsp90 heterocomplexes were first identified, they were found to contain a 50-kDa phosphoprotein (26, 27) that was subsequently cloned and shown to be a mammalian homolog of the yeast cell cycle control protein cdc37 (186, 187). p50^{cdc37} is not a TPR protein, and it binds directly to hsp90 (161, 186) at a site that is different from the TPR acceptor site (188). p50^{cdc37} has been recovered with a number of mammalian protein kinase·hsp90 heterocomplexes, and studies in yeast and *Drosophila* have shown that mutations in cdc37 impair signaling via several protein kinases, including v-Src (189), cdc28 (190), MPS1 (191), CDK-Kin28p (192), and the sevenless receptor tyrosine kinase (73). The mechanism by which p50^{cdc37}/cdc37 affects the kinases is not known (23, 193), but it is clearly quite kinase selective. GR·hsp90 heterocomplexes, for example, contain TPR proteins, but they do not contain p50^{cdc37} (80, 161), whereas immune-isolated src·hsp90 (194), cdk4·hsp90 (186), and raf·hsp90 (80) heterocomplexes contain p50^{cdc37} and are essentially devoid of TPR proteins. p50^{cdc37} has been shown to bind directly to cdk4 (186) and to the catalytic domain of raf (188); thus, the kinases can select for its presence in their heterocomplexes.

The specificity of TPR proteins for receptors and of p50^{cdc37} for protein kinases is apparently not absolute. Mutation in *CDC37* in yeast, for example, affects the ligand-binding activity of the androgen receptor (195), and cdc37 interacts with the AR but not the GR ligand-binding domain (196). Also, raf·hsp90 immune pellets can bind a small amount of [^{3}H]FK506, suggesting the existence of a few raf·hsp90·FKBP complexes (197). Nevertheless, a general model in which p50^{cdc37} is predominantly associated with protein kinase substrates of hsp90 in native heterocomplexes stands on a solid body of evidence. In contrast to native complexes, assembly of raf·hsp90 heterocomplexes *in vitro* using reticulocyte lysate yields both raf·hsp90·FKBP52 and raf·hsp90·p50^{cdc37} complexes (80). This may reflect the dynamic nature of the reticulocyte lysate system, resulting in inadequate time for the hsp90-bound kinase to select for the preferred binding partner, p50^{cdc37}.

Although the function of p50^{cdc37} is not known, it has often been proposed that it targets hsp90 to protein kinases. It should be noted that GR, PR, and raf heterocomplexes have been assembled with the purified five-protein system in the absence of immunophilins or p50^{cdc37}; thus, neither protein family is required for formation of heterocomplexes with hsp90.

The number and location of p50^{cdc37} acceptor sites on hsp90 are not resolved. Unpublished observations from cross-linking of hsp90·p50^{cdc37} complexes are consistent with one binding site per hsp90 dimer (A.M. Silverstein, personal communication). *In vitro* binding studies in the Pratt lab showed competition of intact TPR proteins for binding of p50^{cdc37} and competition of p50^{cdc37} for binding of Hop to hsp90 (178, 188). However, a TPR domain fragment that blocked the binding of the TPR proteins did not compete for binding of p50^{cdc37}. This suggests that p50^{cdc37} binds outside the TPR acceptor site on hsp90 but that the p50^{cdc37} acceptor site is close enough to the TPR acceptor site on the surface of hsp90 that p50^{cdc37} and a TPR protein cannot be bound at the same time (188). The question of whether p50^{cdc37} binds to hsp90 in a manner that is exclusive or nonexclusive with respect to TPR proteins is subject to some contention. Silverstein *et al.* (188) reported that immunoadsorption of TPR proteins from reticulocyte lysate did not yield coadsorption of p50^{cdc37}. In contrast, Hartson *et al.* (198) reported that p50^{cdc37} coadsorbed with FKBP52, CyP-40, and Hop and concluded that both are bound simultaneously to the same hsp90 dimer. Obviously, these two models are mutually exclusive, and further work is required to determine which is correct. Ultimately, it will have to be explained why no p50^{cdc37} has been recovered in native glucocorticoid, progesterone, and aryl hydrocarbon receptor·hsp90·immunophilin heterocomplexes and why little or no TPR protein has been recovered in native protein kinase·hsp90·p50^{cdc37} heterocomplexes. At this juncture, the exclusive binding model explains those observations, and the nonexclusive model does not.

Structure/Function Relationships in hsp90 Action

The complex nature of hsp90 action is evident not only from the multitude of cochaperones or partner proteins that participate in its activities but also from the intramolecular interactions and conformational transitions that direct its functioning. Recent studies indicate these complexities, but they are only partially understood. Three aspects of hsp90 function that concern the importance of conformational transitions are briefly reviewed here.

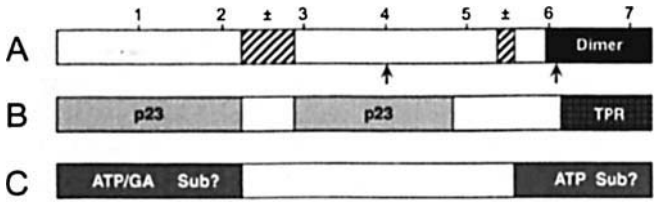

Figure 2. The organization of domains of hsp90. Bar **A** shows two highly charged regions on hsp90 (±) and two hypersensitive sites for trypsin cleavage *(arrows)* (260). The major region for dimer interaction is near the C-terminus that ends in the sequence MEEVD. Shown in Bar **B** are the binding domains for the cochaperone p23 and the TPR-containing proteins (TPR) such as Hop and the immunophilins. Bar **C** shows an N-terminal ATP and geldanamycin (GA)-binding region and a second potential ATP-binding site at the C-terminus. Each of these regions may also participate in binding of substrate (Sub).

Nucleotide Interactions. The most conserved domain of hsp90, and the best understood, is the nucleotide-binding pocket near the N-terminus, approximately residues 1–220 (Fig. 2). Both biochemical and crystallographic studies show this to be the binding site for ATP and ADP and also the site of action for the hsp90 inhibitors geldanamycin and radicicol (167, 199, 200). This ATP-binding site is structurally unique and identifies hsp90 as a member of a small family of proteins, the GHKL family, having related binding pockets for ATP (201). The other members include bacterial DNA gyrase, the DNA repair protein MutL, and several bacterial histidine kinases. These proteins are unrelated in function, but they all require ATP binding and ATPase or phosphotransferase activity in their functions. Although they share little sequence identity in their ATP-binding domains, they contain four common motifs that define a "Bergerat fold" for binding ATP (201, 202). In all cases, ATP binding and hydrolysis are used to regulate the conformational states of the proteins during their activities. With hsp90, the binding of ATP induces dimer interaction near the N-terminus domains of the hsp90 homodimer (203). These interactions are in addition to the constitutive dimer interactions near the C-terminus (204). It has been suggested that the N-terminus domains act as a "molecular clamp" during the chaperoning functions of hsp90 (203). Although conformational transitions in this region of hsp90 clearly occur, it is surprising that the amino termini of hsp90 can be restricted with a minimal effect on its activity. When the N-terminal domains of hsp90 are forced together by fusion with the dimeric protein glutathione-S-transferase, this fusion protein is quite capable of chaperoning the progesterone receptor to its active state (205).

Hsp90 also has a weak ATPase activity that is essential to its biologic functions (206–208). However, this ATPase activity is not exhibited by the nucleotide-binding pocket *per se* (residues 1–220) but requires additional downstream residues that have not yet been clearly defined. A highly charged region directly adjacent to the binding pocket is probably not needed for ATPase activity because this region is absent in hsp90 homologs from bacteria and mitochondria. However, beyond this region, there are residues that are thought to interact with the γ phosphate of ATP during ATP hydrolysis, as has been shown for other members of the GHKL family (201, 209, 210). Downstream residues are also needed for the tight, committed binding of ATP to hsp90 that appears to precede ATP hydrolysis (211). C-terminal truncation mutants of yeast hsp90 show greatly reduced ATPase activity, suggesting that residues near the C-terminus are required either directly or indirectly by promoting dimer formation (203, 211). That dimer interactions are important for ATPase activity is supported by studies with yeast hsp90 mutants and with mixed dimers of wild-type and mutant hsp90 (209). On the other hand, a monomer fragment from chicken hsp90 (residues 1–573) has been shown to be active (212). This fragment actually has a much greater ATPase activity than full-length hsp90, which suggests the presence of a region beyond residue 573 that suppresses ATPase activity. This supports a model in which the ATPase activity is suppressed until hsp90 assumes a conformational state in which ATPase activity is needed for the next transition. Thus, it is likely that the binding of client proteins and cochaperones modulates the ATPase activity of hsp90. In a study by McLaughlin *et al.* (277), the ATPase activity of human hsp90 could be stimulated by the ligand-binding domain of the glucocorticoid receptor. They found that the cochaperones Hop and p23 suppressed this ATPase activity. In the absence of a client protein, the ATPase activity of hsp90 can be inhibited by Hop but not by p23 (181, 213).

Although the binding of ATP and ADP to hsp90 is now well established, this was a controversial issue for several years because this low-affinity nucleotide binding was hard to demonstrate. Surprisingly, recent studies indicate the presence of yet another nucleotide-binding site near the C-terminus of hsp90 (210, 214, 215). This site is not obvious by sequence analysis and is evident only under certain conditions. It appears to be suppressed, but binding at this site can be measured if the N-terminal site is occupied by nucleotide or if that site is inactivated by certain mutations. The C-terminal site can also be activated by deletion of the N-terminal site, but only if the adjacent charged region is also removed (214). On the other hand, binding of the drug novobiocin to the C-terminal site blocks nucleotide binding at the N-terminal site. Thus, these two nucleotide-binding sites appear to interact in a coordinated fashion that is presumably important for regulating the conformational state of hsp90. The purpose of this second site is not known, but it appears to be inhibited by novobiocin and by cisplatin, and these agents should be quite helpful in functional studies (210, 214).

Cochaperone Interactions. Two types of cochaperone interactions have been described for hsp90. Several proteins that contain TPR domains have been shown to bind near the C-terminus of hsp90 (Fig. 2), and it appears that these proteins compete for the same site or overlapping sites on hsp90 (156, 173, 176–178). The binding of Hop to hsp90 occurs transiently within an intermediate complex for chap-

eroning hsp90 targets. Although one purpose of Hop is to physically link the activities of hsp70 with hsp90, Hop also appears to modulate the activities of these chaperones. Hop can enhance the activity of hsp70 in chaperoning protein folding in the absence of hsp90 (216). Hop has been reported to stimulate nucleotide exchange by hsp70 (217); however, this effect was not observed in another study (216), and the mechanism of Hop's influence on hsp70 function remains unknown. Hop binds selectively to hsp70 in its ADP-bound state, suggesting that it may modulate hsp70 when it is bound to a substrate protein (216).

Hop also influences the functioning of hsp90. As noted above, its binding blocks ATP binding and the ATPase activity of hsp90 (181, 277), and this very effectively inhibits the binding of the cochaperone p23 to hsp90, which requires hsp90 in its ATP-bound state (216). Based on these results, one might speculate that Hop, perhaps in complexes with hsp90, would interact specifically with hsp70 that has formed a complex with a target protein or substrate. The hsp90 in this complex would not interact with ATP, and this may be important for inducing hsp90 into a substrate-binding state (see below) and for preventing premature entry of other TPR proteins or p23 into the complex.

The binding of p23 to hsp90 occurs late in the hsp90-chaperoning pathway when hsp90 has apparently dissociated from Hop and can assume an ATP-bound conformation. This binding can be demonstrated in isolation, but it requires elevated temperature and the presence of ATP and molybdate or a nonhydrolyzable analog of ATP (165). Molybdate stabilizes the ATP-bound conformation of hsp90, but the mechanism for this effect is still uncertain (165, 218). The site of p23 binding on hsp90 has been described to some extent. The p23 binding apparently involves the primary ATP-binding domain (residues 1–220), but this alone is not sufficient (167, 205). Because the p23–hsp90 interaction is very conformation dependent, many diverse mutations of hsp90 exhibit a loss of p23-binding activity. Recent studies show that an hsp90 fragment, residues 1–490, is able to bind p23, but only if it is prepared as a fusion protein to form a dimer (205). Thus, p23 binding involves the N-terminal ATP-binding site plus a downstream domain, all in a dimer arrangement. The charged domain within this region can be deleted without loss of p23 binding.

The function of p23 is still unclear. p23 is able to inhibit the aggregation of denatured proteins, and this passive chaperoning activity may be used to chaperone substrates in hsp90 complexes (219, 220). Alternatively, this chaperoning activity might be focused specifically on hsp90, where it may facilitate conformational changes. p23 appears to stabilize hsp90 complexes with steroid receptors and to enhance the proportion of complexes that are in a "mature" state, where the steroid receptor has hormone-binding activity (166). Young and Hartl have recently proposed that p23 may function in the ATP-dependent dissociation of hsp90–substrate complexes (213). Finally, Freeman *et al.* have shown that p23 can influence the biologic activity of steroid receptors after their activation by hormone, suggesting that p23 may affect target proteins after their release from hsp90 (221).

Hsp90–Substrate Interactions. Very little is known regarding the mechanism through which hsp90 binds to substrates or client proteins. How does hsp90 select its substrates? What are the structural and chemical requirements for this interaction? Where is the substrate-binding site on hsp90, and how is the activity of this process regulated? These questions remain unanswered mainly because of the complexities of the process. Hsp90 is unable to bind biologic substrates such as steroid receptors on its own and requires the assistance of several other proteins. With steroid receptors, it is evident that the simple binding of hsp90 is not sufficient to achieve a conformational state able to bind hormone. An additional step requiring ATP and enhanced by p23 is needed, suggesting that there is more than one step in the substrate-binding process. One possibility is that there are multiple sites for substrate binding to hsp90. In this regard, two regions of hsp90, one near the N-terminus and one near the C-terminus, have been shown to prevent the aggregation of denatured proteins (137, 138). It has been suggested from these results that these activities represent domains for substrate binding, and thus, hsp90 may have two substrate-binding sites per monomer. However, in another study, a middle region of hsp90 was also shown to have this antiaggregation activity, and the significance of this activity and the actual location of substrate binding domains on hsp90 remain uncertain (222).

Purified hsp90 and its homolog in the endoplasmic reticulum, GRP94 (gp96), show a weak and inefficient interaction with a variety of peptides (223–226). In this regard, GRP94 has been studied more extensively because of its involvement in antigen processing and its potential use in immune therapy (223, 227, 228). Although GRP94 binds peptides poorly in its purified native state, binding activity can be artificially enhanced by heating the protein at high temperature (e.g., 50°C) that also promotes oligomerization, or by a cycle of denaturation and renaturation using guanidine hydrochloride (224, 225, 229). It is possible that these perturbations simply generate a partially denatured protein that can interact nonspecifically with proteins and peptides, and these treatments may open a substrate-binding site on GRP94 that is normally suppressed. In one study, the peptide-binding site was identified by cross-linking to be near the C-terminus of GRP94 (229). More recently, the binding of the hydrophobic ligand bis-ANS has been shown to enhance peptide binding to GRP94 (230). From these results, it has been suggested that GRP94 can exist in an open and a closed conformation with respect to peptide binding (224). The protein would normally exist in the closed state but could be induced to open by specific events such as contact with a particular type of substrate or interaction with specific cochaperones that help to control or organize the GRP94 (or hsp90) chaperoning process. The mechanisms

that control the opening of GRP94 and hsp90 binding sites remain unknown. In a recent study, Zhao *et al.* (259) presented evidence that the binding of hsp90 to the σ1 protein of reovirus is accompanied by a cycle of phosphorylation and dephosphorylation of hsp90. Greater effort is needed to pursue this and other leads toward understanding the mechanisms for the binding of substrates to hsp90 and the regulation of this key function.

Receptor·hsp90 Heterocomplex Assembly

In our original studies of cell-free assembly of steroid receptor·hsp90 heterocomplexes, immunoadsorbed GR or PR that was salt-stripped of hsp90 was incubated with rabbit reticulocyte lysate, and the receptors became complexed with rabbit hsp90 (235, 236). Because the GR must be bound to hsp90 to have steroid-binding activity, and because steroid-binding activity was generated by reticulocyte lysate in direct proportion to the number of GR·hsp90 heterocomplexes assembled (237), it was clear that the heterocomplexes that were assembled in lysate were functionally the same as native complexes isolated from cytosols. Receptor·hsp90 heterocomplex assembly by reticulocyte lysate was shown to be temperature-dependent and to require ATP, Mg^{2+}, a monovalent cation (e.g., K^+), and hsp70 (237–239). Two other proteins found in PR·hsp90 heterocomplexes assembled in reticulocyte lysate were p23 (235) and the 60-kDa protein Hop (238). In examining the time course of PR·hsp90 heterocomplex assembly by reticulocyte lysate, Smith (240) showed that hsp70 and Hop were present in early complexes, but their levels declined after a few minutes, whereas p23 entered the complex somewhat later, with the amount remaining constant thereafter.

Heterocomplex Assembly Machinery. Since the publication of our original review (1), the heterocomplex assembly system has been reconstituted (241), and it is now possible to assemble receptor·hsp90 heterocomplexes with a minimal system of five purified proteins—hsp90, hsp70, Hop, hsp40, and p23 (171, 242). An overview of the assembly process is modeled in Fig. 3. All of the Hop in reticulocyte lysate is present in an hsp90·Hop·hsp70 complex with an apparent stoichiometry of 2:1:1 (140). About 30% of the hsp90 and 9% of the hsp70 in lysate are in these complexes. Hsp40, a cochaperone that binds to hsp70 and promotes its ATPase activity, is also present in these com-

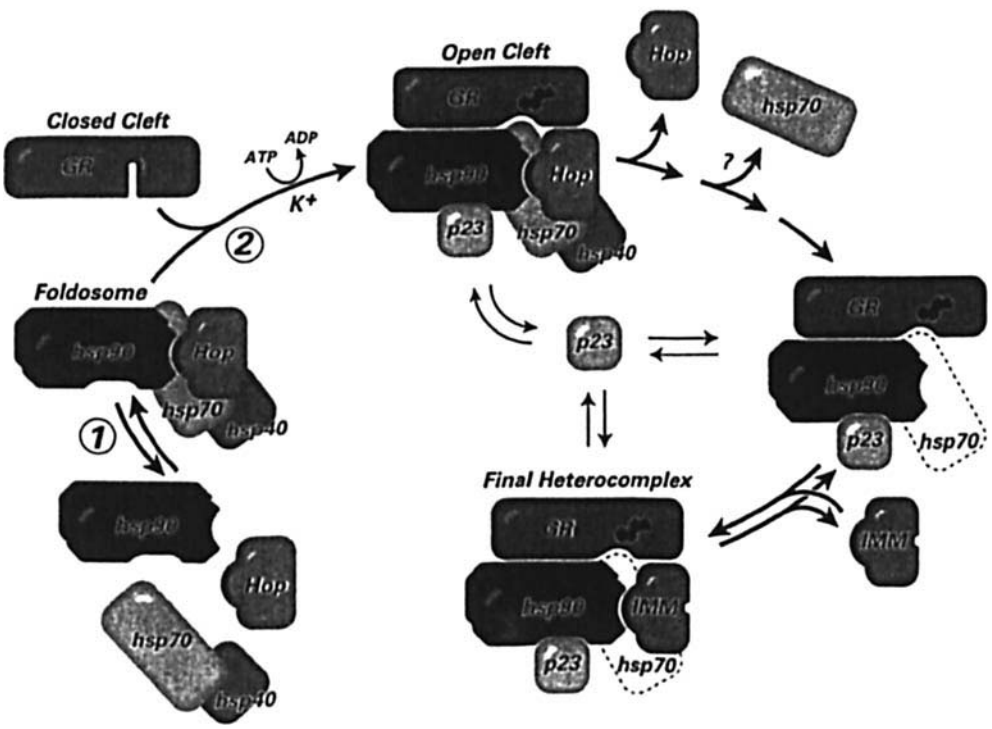

Figure 3. Model of steroid receptor·hsp90 heterocomplex assembly. The hsp90/hsp70-based chaperone machinery, or *foldosome*, converts the glucocorticoid receptor (GR) ligand-binding domain from a folded conformation in which the steroid-binding cleft is closed and not accessible to hormone to an open cleft conformation that can be accessed by steroid. Hop binds via independent tetratricopeptide repeat (TPR) domains to hsp90 and hsp70 to form the foldosome machinery. Hsp90 functions as a dimer, and one Hop is bound to an hsp90 dimer. Hsp40 is an hsp70-bound cochaperone that is also present in the machinery that carries out the ATP/Mg^{2+}-dependent and K^+-dependent opening of the steroid-binding cleft. During cleft opening, hsp90 is converted to its ATP-dependent conformation, which is dynamically stabilized by p23, and Hop is released from hsp90. Thus, the complex shown at the end of Step 2 represents a composite in which several changes are occurring in dynamic fashion. The immunophilins (IMM) also bind to hsp90 via TPR domains (indicated by the *solid black crescents*), and when Hop exits the complex, an immunophilin can bind to the single TPR acceptor site on the receptor-bound hsp90 dimer. Some hsp70 also leaves the intermediate complex, and the *broken line* for hsp70 in the final heterocomplex indicates that it is present at substoichiometric levels with respect to the receptor.

plexes (171), but the stoichiometry of hsp40 binding to other proteins in the complex is unknown. The hsp90 co-chaperone p23 is not present in the hsp90·Hop·Hsp70·Hsp40 complex immunoadsorbed from lysate with an antibody against Hop (144, 166). This complex has been called a *foldosome* (243), but the resistance of reviewers to that term has led us to refer to it as the hsp90/hsp70-based chaperone machinery.

The hsp90·Hop·hsp70·hsp40 complex is formed spontaneously on mixing of the purified proteins, and both the immunoadsorbed purified protein complex and the native complex from reticulocyte lysate convert the GR ligand-binding domain (LBD) to a steroid-binding state (166, 170, 171). In Figure 3, we show the receptor that interacts with this machinery as being in a folded state in which the hydrophobic steroid-binding cleft is closed and not accessible to steroid. It is known that steroid receptors completely envelop the ligand in a hydrophobic space that is large and appears to be altered or collapsed in the absence of ligand. Thus, the receptor must change its conformation to allow entry of the ligand (305). Hsp90 binds directly to the ligand-binding domain of the receptor (1) to promote conformational changes that, in addition to opening the cleft, cause enhanced sensitivity of the GR LBD to attack by thiol derivatizing agents and trypsin (244–246). The site of hsp90 binding to the GR is unknown; however, a short segment of the receptor lying at the opening of the steroid-binding cleft in the N-terminus of the LBD is required for GR·hsp90 heterocomplex assembly (29, 30). When a GR fragment containing this segment is fused to the LBD of the retinoic acid receptor, the chimera is complexed with hsp90 and undergoes cytoplasmic–nuclear translocation in response to retinoic acid (247). This N-terminal region of the LBD is a likely site of interaction between the chaperone machinery and the GR.

To have an open steroid-binding cleft (as indicated in the complex produced by Step 2 in Fig. 3), the receptor-bound hsp90 must assume its ATP-dependent conformation (208). This is the conformation of hsp90 that is bound by p23 (165), and it is at this stage that p23 binds to receptor-bound hsp90 in quite dynamic fashion, stabilizing the GR·hsp90 heterocomplex (166). Although binding of p23 to purified hsp90 is ATP-dependent (165), p23 will stabilize GR·hsp90 heterocomplexes immunoadsorbed from cytosol without a requirement for ATP (166) because these native, final heterocomplexes are in the steroid-binding state and already have their receptor-bound hsp90 in the ATP-dependent conformation. In Figure 3, p23 is shown interacting with a steroid-binding intermediate complex containing Hop as well as with the Hop-free final heterocomplex. It is important for the reader to realize that the intermediate-stage product of Step 2 in the model does not reflect a single static state; rather, a series of events is occurring that depend on the multifunctional properties of the hsp90·Hop·hsp70·hsp40 complex. Binding to the GR is actually initiated by interactions with hsp70 and hsp40 as described below. As the cleft is opened on hsp90 binding, hsp90 achieves its ATP-bound conformation, Hop dissociates from the receptor-bound hsp90, and p23 binds to the ATP-bound hsp90. Hop, however, is still associated with the recep tor-bound hsp70 and must somehow exit the complex.

Exit of Hop and hsp70. The exit of Hop from the intermediate complex formed in Step 2 frees the TPR acceptor site on hsp90 to bind TPR domain immunophilins such as FKBP52, FKBP51, PP5, or CyP-40. It is not clear how Hop exits the intermediate complexes. The affinity of Hop for both hsp70 and hsp90 is reduced when these proteins are in their ATP-bound states (216), and this may favor competing interactions by other TPR domain proteins. When receptor·hsp90 heterocomplexes are assembled by reticulocyte lysate, there are few complexes containing Hop, and these are probably complexes that are in the process of assembly at the time of their isolation. Again, it is important to realize that this whole process is very dynamic (240) and that heterocomplex assembly and disassembly [by a mechanism that is not understood but appears to be promoted by ATP (166)] are occurring simultaneously. In contrast to native receptor·hsp90 heterocomplexes or to those formed by reticulocyte lysate, heterocomplexes formed by the purified five-protein system contain substantial amounts of Hop (170). This suggests that reticulocyte lysate may have a factor (or factors) that promotes Hop release and is not present in the five-protein system, which is a minimal system for assembly of stable receptor·hsp90 heterocomplexes. Reticulocyte lysate, for example, contains the hsp70 cochaperone BAG-1, which binds to the ATPase domain of hsp70 (248, 249) and promotes the release of ADP (250, 251). Hop binds preferentially to the ADP-bound form of hsp70 (216), and addition of physiologic amounts of purified BAG-1 to the five-protein assembly system results in GR·hsp90 heterocomplexes with less Hop (252). It is possible that BAG-1 plays a role in promoting Hop release in assembly systems that are more complex than the minimal five-protein system.

As indicated in Figure 3, some hsp70 is also released during the assembly process (240). Native receptor·hsp90 heterocomplexes isolated from cell lysates may contain no hsp70, as is often the case with the GR, or considerable hsp70, as is always the case with the PR (1). In those cases where it has been measured, the stoichiometry of hsp70 in the final complex was found to be less than 1:1 with respect to the receptor (1). The factors that determine the amount of hsp70 in the final heterocomplex are unknown, but there is always hsp70 in GR·hsp90 and PR·hsp90 heterocomplexes assembled by the five-protein system.

Hip Is Not Required for Assembly. A 48-kDa protein recovered in PR·hsp90 heterocomplexes early in their assembly by reticulocyte lysate (240) was subsequently found (253) to be the hsp70 cochaperone Hip (hsc70-interacting protein) discovered by Höhfeld *et al.* (254). Hip binds to the ATPase domain of hsp70, stabilizing the ADP state, which has a high affinity for nonnative sub-

strate protein (254). Hip and BAG-1 compete with each other in binding to the hsp70 ATPase domain (250, 255). Hip was recovered in PR·hsp90 heterocomplexes assembled in the presence of a limiting amount of ATP or in the presence of geldanamycin (37, 253). This suggested that Hip might be a functional component for intermediate steps in the assembly process. Also, a mutant form of Hip was reported to inhibit PR·hsp90 heterocomplex assembly by reticulocyte lysate, leading to a model of assembly in which Hip was required for progression from early receptor complexes with hsp70 into later complexes containing hsp90 (256). For these reasons, in some reviews of the receptor·hsp90 assembly mechanism (8, 17), Hip is considered to be an important functional component. When this notion was tested using the purified five-protein system, GR·hsp90 heterocomplex assembly was unaffected by the addition of Hip (257). Addition of very large amounts of BAG-1 that are stoichiometric with respect to hsp70 inhibit GR·hsp90 assembly by the purified five-protein system, and overexpression of BAG-1 inhibits assembly *in vivo* (252). Hip counteracts BAG-1 inhibition both in the purified five-protein system and *in vivo* (257). Thus, Hip appears not to be essential for the assembly of receptor heterocomplexes, but it could play a regulatory role in opposition to BAG-1, or it may have another role that is not evident when the purified five-protein assembly system operates.

Essential versus Nonessential Chaperones. The purified, five-protein system has permitted definition of essential versus nonessential chaperones for steroid-binding cleft opening and stable receptor·hsp90 heterocomplex assembly. When either hsp70 or hsp90 is omitted from the purified system, no steroid-binding activity is generated (242, 258). In general, steroid receptor heterocomplexes are assembled at 30°C and then incubated on ice with steroid to determine steroid-binding activity. This procedure assays the formation of stable heterocomplexes that remain intact during subsequent incubation with steroid, and when p23 is omitted from the purified assembly system, no steroid-binding activity is generated in this assay (170, 242, 258). However, opening of steroid-binding clefts in the absence of p23 can be detected by having steroid present during the assembly reaction at 30°C. Under these conditions, as soon as the steroid-binding cleft is opened, the steroid enters, and generation of steroid binding shows that the appropriate conformational change in the receptor has occurred in the absence of p23 (166). In this assay, some steroid binding can be generated by hsp70 and hsp90 in the absence of the other three proteins, although the efficiency of the process is dramatically reduced (258). Neither hsp70 nor hsp90 has any activity alone, and both chaperone proteins must be present to produce steroid-binding activity (258). Thus, hsp70 and hsp90 are essential chaperones, and p23, which is required for production of stable receptor heterocomplexes, is a nonessential cochaperone for steroid-binding cleft opening. This is consistent with yeast studies showing that deletion of *SBA1*, which encodes the yeast p23 ortholog, does not affect dexamethasone-dependent activation of transcription (142, 143). Thus, the receptor can achieve a functional conformation *in vivo* in the absence of p23.

From studies in reticulocyte lysate, Hop was considered essential for formation of functional PR·hsp90 heterocomplexes (168, 240, 256, 261, 262), and immunodepletion of Hop from reticulocyte lysate markedly reduced its PR·hsp90 (262) and GR·hsp90·(241) heterocomplex assembly activity. However, when Hop was eliminated from the purified system, generation of steroid-binding sites for both the PR and GR was decreased ~60% during assembly for 30 min at 30°C (242, 258). It was found that the presence of Hop accelerates the rate at which GR steroid binding is generated, but in the absence of Hop, the other four proteins will continue to activate the receptor to approach the maximum level of steroid binding attained with Hop (258). Thus, the assembly system works faster when the essential chaperones are brought together by Hop, but Hop is a nonessential cochaperone for receptor·hsp90 heterocomplex assembly. Hop is the mammalian ortholog of the nonessential yeast protein Sti1, and the Lindquist laboratory has shown that deletion of *STI1* reduces but does not eliminate GR activity *in vivo* (263).

Instead of mammalian hsp40, we use the purified yeast ortholog YDJ-1 in our five-protein assembly system. The important role of a J protein in this system should not be deemphasized, but when YDJ-1 is omitted, there is still substantial generation of steroid-binding activity for both the PR and GR (242, 258). Thus, YDJ-1/hsp40 is also a nonessential cochaperone for opening of the steroid-binding cleft. Genetic studies in yeast support the notion that YDJ-1 is important but not essential for attaining the hormone-binding state *in vivo*. For example, yeast with a mutation in *YDJ-1* and expressing glucocorticoid receptors still respond to steroid (265). The androgen receptor also requires hsp90 to achieve a high-affinity ligand-binding conformation (41, 264), and androgen receptors have some steroid-binding activity in *YDJ-1* mutant yeast (266) and in yeast deleted for *YDJ-1* (267).

Although hsp90 and hsp70 are together necessary and sufficient for opening the steroid-binding cleft (258, 269), the importance of the cochaperones for efficient receptor hetercomplex assembly should be emphasized. Some investigators have repudiated a model of machinery-mediated GR·hsp90 assembly as well as any role for Hop and YDJ-1/hsp40 in the assembly process (269). In our experience with both GR and PR heterocomplex assembly, addition of Hop and hsp40 has always yielded a substantial increase in the amount of steroid-binding activity that is generated (171, 241, 242, 258), and p23 is required for stable heterocomplexes.

Mechanism of Cleft Opening. Early experiments on the time course of PR heterocomplex assembly indicated an ordered series of steps that are not shown in the model of Figure 3. In the earlier model of heterocomplex assembly, the PR first associated with an hsp90·Hop·hsp70

complex, and these proteins were then replaced by a p23·hsp90·immunophilin unit to yield the final receptor heterocomplex (253, 261, 270). This concept of one hsp90 complex being replaced by another hsp90 complex now seems to have been wrong. Stepwise assembly experiments with purified proteins are beginning to separate the cleft-opening process into an ordered series of events as diagramed in Figure 4. Two ATP-dependent steps have been resolved. In the first step, immunoadsorbed receptor is incubated with purified hsp70 and hsp40 (YDJ-1) in the presence of ATP (268, 271). This produces a receptor·hsp70·hsp40 complex that can be washed free of unbound hsp70 and hsp40 and then incubated with purified hsp90, Hop, and p23. In this first reaction, the receptor is "primed" to be activated by hsp90 during the second incubation. Both the initial priming step with hsp70 and the second activating step with hsp90 are ATP-dependent, and steroid-binding activity is generated only during the second step. Experiments are ongoing in both of our laboratories to understand the mechanisms of the priming and activating steps (164, 271, 272).

In addition to hsp70, the first step requires hsp40 and sustained high levels of ATP for formation of a substantial number of primed GR·hsp70·hsp40 complexes that can be activated in the second step (164). For the GR, it has been found that hsp70 binds to stripped receptors when the chaperone is in its ATP-bound state, even in the absence of hsp40 (272). At the end of the priming incubation, the GR-bound hsp70 is in both ADP-bound and ATP-bound states (164). Consistent with a very limited site of attack in the LBD, the number of molecules of hsp70 in the primed complex is low; one to two hsp70 per receptor (271; P. Murphy, unpublished data). The ATPase activity of hsp70 is K^+-dependent (273, 274), and both the priming step and the subsequent activating step shown in Figure 4 are K^+-dependent (164). The requirement for a high level of ATP and the dependence on hsp40 and K^+ show that both ATP binding and ATPase activity are required to produce primed GR·hsp70 complexes. This has led to the notion that, once hsp70 is bound to the GR, the chaperone may oscillate back and forth between ATP-bound and ADP-bound configurations as it performs an iterative function during the priming step (164).

One observation that seems clear for both GR and PR is that receptor activation in the two-step protocol is more dependent on the presence of hsp40 than is activation by a single incubation with all five proteins at once (164, 271). The reason for this difference is unknown, and an explanation will require more knowledge about the mechanism of hsp40 action in these systems. In some cases, J domain proteins are thought to interact with substrates directly to target the subsequent binding of hsp70 (for review, see Ref. 275). It has been shown that hsp40 binds directly to the PR in the absence of the hsp70 with a stoichiometry of one molecule of hsp40 to one molecule of receptor, and hsp40 remains bound to the PR in a static manner during the assembly process (271). That is, the PR·hsp40 complex can be washed free of unbound hsp40 and then incubated with the other components of the system to yield the steroid-binding state of the receptor (271). Although hsp70 in its

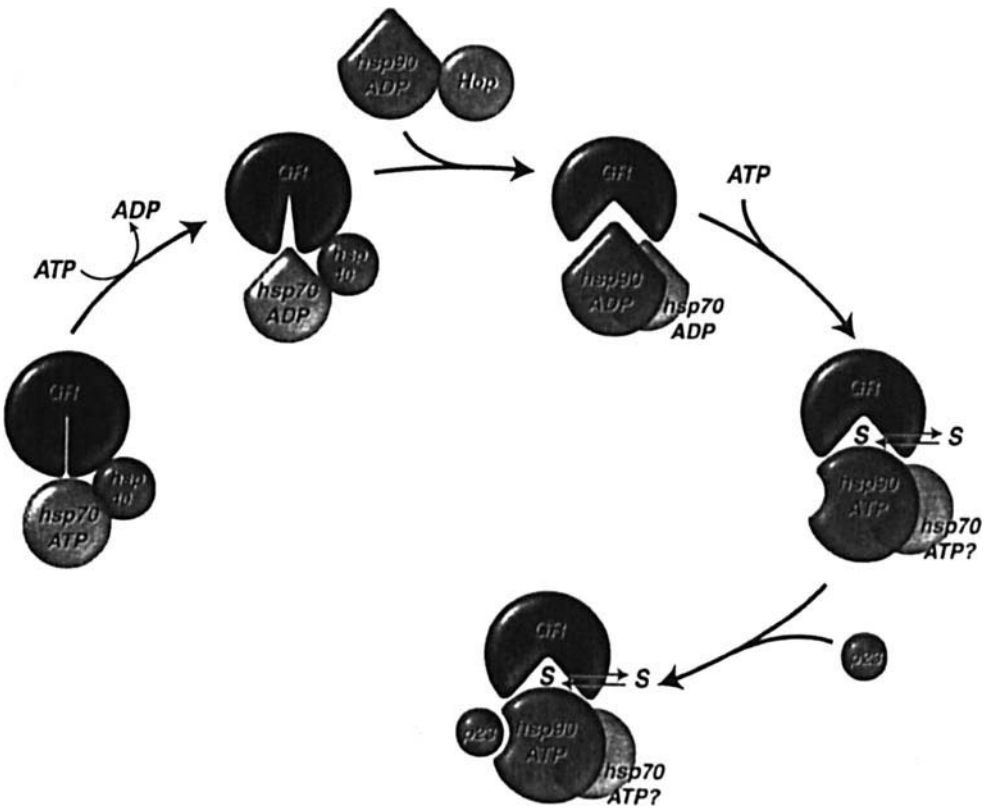

Figure 4. Mechanism by which hsp70 and hsp90 open the steroid-binding cleft. The mechanism is derived from stepwise assembly experiments in which hsp70 binds to the GR in an ATP-dependent and hsp40-dependent step to form a "primed" GR·hsp70 complex that can then bind Hop and hsp90. After hsp90 binding, there is a second ATP-dependent step (or steps) that is rate-limiting and leads to opening of the steroid-binding cleft, which enables access by the steroid. The hsp90 is now in its ATP-dependent conformation and can be bound by p23, which stabilizes the chaperone in that conformation, preventing disassembly of the GR·hsp90 heterocomplex. The hsp40 and Hop components of the five-protein system have been omitted from later steps for simplicity.

ATP-bound form, but not in its ADP-bound form, can bind to the GR in the absence of hsp40 (272), it has not yet been determined whether it is hsp70 or hsp40 that reacts initially with the GR during the priming reaction. Thus, in Figure 4, we show both hsp70 and hsp40 contacting the receptor.

In the mechanism of Figure 4, we suggest that hsp90 binds to the primed GR·hsp70 complex in its ADP-bound state. Actually, the purified hsp90 we use is not bound by nucleotide (164), but the ADP-bound and unbound states may be essentially the same in terms of the overall conformation of hsp90 (165). Scheibel *et al.* (276) have calculated that about 70% of the hsp90 in cells would be in the ATP-bound form. This is quite consistent with ~30% of the hsp90 being bound to Hop (140), which binds preferentially to the ADP-bound form (216). The ADP-bound conformation of hsp90 possesses high affinity for hydrophobic substrate (165, 167), and in the model of Figure 4, we suggest that in the priming step with hsp70, the steroid-binding cleft is partially opened such that hydrophobic residues in the interior are now available for interaction with hsp90. Although this is a reasonable operating model, as of this writing, there is no direct evidence that hydrophobic residues are exposed during priming or that it is the ADP-bound (or unbound) conformation of hsp90 that initially binds to the primed GR·hsp70 complex.

When the GR is incubated with the five-protein system at 30°C, activation of steroid-binding activity achieves a plateau at 10–15 min. The priming step is complete in ~1 min (164), and hsp90 binding to the primed GR·hsp70 complex during the second step achieves a plateau within ~30 sec (272). Thus, it is the ATP-dependent opening of the binding cleft that is rate limiting for the overall process. Although this ATP-dependent opening of the binding cleft to access by steroid is indicated as a single step in Figure 4, it is likely that several changes in chaperone conformation occur during cleft opening. Although it is clear that the receptor-bound hsp90 must be in the ATP-bound conformation that binds p23 when the cleft is open for steroid binding, the conformation of receptor-bound hsp70 at that stage is not known. We have drawn hsp70 in its ATP-bound state but with a question mark to indicate that that is purely speculative.

Immunophilins, hsp90, and Receptor Trafficking

Although the general features of chaperone interaction may be common to all hsp90 client proteins, diversity arises from a number of TPR-domain proteins that associate with the client protein and hsp90. These TPR-domain proteins may provide a variety of functions to hsp90 complexes, but their roles remain unclear. A subset of TPR-domain proteins, the immunophilins and immunophilin-related proteins, have been studied in a number of systems. These enter in the final step of receptor·hsp90 heterocomplex assembly (Fig. 3), and they do not appear to be needed for generation of an active steroid receptor or for the stabilization of receptors against proteasomal degradation. TPR-domain immunophilins are distributed widely (possibly ubiquitously) among animal and plant cells, and TPR-domain binding to hsp90 is conserved (278–280). This suggests that immunophilin binding to hsp90 is essential both for the action of the TPR-domain immunophilins and for a major function(s) of hsp90. In this section, we review the evidence that hsp90 is involved in receptor trafficking and that the hsp90-binding immunophilins engage in protein interactions consistent with a role in targeting this trafficking.

Several of the hsp90-binding immunophilins (FKBP52, FKBP51, and CyP-40) were discovered in characterizing components of steroid receptor·hsp90 heterocomplexes, and we have previously reviewed their discovery, cloning, and structure (1, 2). The common feature of immunophilins is the peptidyprolyl isomerase (PPIase) domain, and their PPIase activity is inhibited by immunosuppressant drugs of the FK506 or cyclosporin A classes. However, inhibition of PPIase activity has not been found to affect any intrinsic function of steroid receptors, including receptor trafficking (1). Recent observations support the notion that the PPIase domains of the hsp90-binding immunophilins function as protein–protein interaction domains that link steroid receptors to a cytoplasmic–nuclear trafficking system.

The steroid receptors move continuously into and out of the nucleus (reviewed in Ref. 281), and when the GR becomes bound by steroid, the receptor shifts from a predominantly cytoplasmic localization to the nucleus. Normally, this steroid-dependent translocation is rapid ($t_{1/2}$ ~ 4.5 min), but treatment of cells with the hsp90 inhibitor geldanamycin slows the rate of translocation by an order of magnitude ($t_{1/2}$ ~ 45 min) (282, 283). The rapid, hsp90-dependent movement occurs along cytoskeletal tracts (283), and a variety of observations (reviewed in Ref. 23) support the notion that the steroid receptors traffic through the cytoplasm to the nucleus while they are in heterocomplex with hsp90. From the study of protein interactions and cellular localizations of hsp90-binding immunophilins, it was proposed that these proteins may serve to target the movement of the steroid receptors (178).

The most studied immunophilin in steroid receptor heterocomplexes is the FK506-binding protein FKBP52 (1). Although the majority of FKBP52 is located in the nucleus, where it colocalizes with nuclear GR (284), a minority of FKBP52 that is cytoplasmic colocalizes with microtubules (284, 285). Microinjection of antibody against FKBP52 was found to impede steroid-induced movement of the GR from the cytoplasm to the nucleus, consistent with a role for the immunophilin in receptor trafficking (286). Also consistent with a role in targeting receptor trafficking is the demonstration that FKBP52 binds directly to the GR and that a 35–amino acid segment of the receptor that spans the protosignals comprising the NL1 nuclear localization signal is sufficient for binding (180).

In addition to the proteins shown in the final heterocomplex in Figure 3, GR·hsp90 heterocomplexes immunoadsorbed from cell lysates contain cytoplasmic dynein (287,

288). Cytoplasmic dynein is a motor protein responsible for retrograde movement of vesicles along microtubular tracks toward the nucleus (reviewed in Ref. 289). Immunoadsorption of FKBP52 from cell lysates is accompanied by coimmunoadsorption of cytoplasmic dynein (284). Coimmunoadsorption of dynein is prevented by competition with an FKBP52 fragment comprising its PPIase domain, but dynein coadsorption is not affected by FK506 (180). This suggests that the PPIase domain functions as a dynein interaction domain independent of its PPIase activity, and it has recently been shown that the purified PPIase domain of FKBP52 binds directly to the purified intermediate chain of cytoplasmic dynein (290).

Two other receptor-associated immunophilins, the cyclophilin CyP-40 and the combined immunophilin homolog and protein phosphatase PP5, also bind directly via their PPIase domains to cytoplasmic dynein (290). The non-hsp90-binding small immunophilin FKBP12 does not compete for dynein binding (180), suggesting that there is a subclass of PPIase domains that bind to dynein. By use of a green fluorescent protein (GFP)–GR chimera to monitor steroid-dependent cytoplasmic–nuclear receptor translocation, it was shown that GFP-GR movement was inhibited by cotransfection with the FKBP52 PPIase domain but not by FKBP12 (287). As with hsp90-dependent GFP-GR movement, immunophilin-dependent (i.e., PPIase domain-dependent) GFP-GR movement requires intact microtubules (287). Finally, it has been shown that the presence of cytoplasmic dynein in GR·hsp90 heterocomplexes assembled by either rabbit reticulocyte lysate (287) or wheat germ lysate (280) depends on the presence of animal or plant immunophilins, respectively. This conservation of PPIase domain–dynein interaction implies a fundamental role for the hsp90-binding immunophilins in protein trafficking in eukaryotic cells.

ARA9 (also called XAP2 and AIP) is an immunophilin homolog that was isolated in yeast two-hybrid screens for proteins interacting with the aryl hydrocarbon receptor (AHR) (157–159). ARA9 contains three TPRs in its C-terminus and a PPIase homology domain (50% similarity and 27% identity with human FKBP52 PPIase domain) in its N-terminus (157–159). ARA9 is recovered in AHR·hsp90 heterocomplexes, where it is bound both to the receptor and, via its TPR domain, to hsp90 (157, 158). Direct binding to receptor appears to account for the fact that ARA9 is specifically found in immunoadsorbed AHR·hsp90 complexes but not in GR·hsp90 complexes (291), whereas the reverse is the case for FKBP52. Although the AHR is structurally very different from the steroid receptors, like steroid receptors, the AHR shuttles into and out of the nucleus, and ligand-induced nuclear accumulation of the receptor is inhibited by geldanamycin (292, 293). Overexpression of ARA9 increases the amount of AHR recovered in the cytosolic fraction (294), and immunofluorescence data show a redistribution of the receptor to the cytoplasmic compartment (294–296). Overexpression of ARA9 also delays ligand-induced nuclear accumulation of the AHR (292).

Thus, ARA9 is thought to mediate cytoplasmic retention of the AHR, although how such retention is mediated is not clear. It is interesting that ARA9 does not bind to cytoplasmic dynein (290), and it is possible that it promotes cytoplasmic localization of the AHR by effectively competing for the association of dynein-binding immunophilins to the TPR acceptor site on hsp90. Despite our lack of understanding of how the hsp90-binding immunophilins and immunophilin homologs work, there is considerable evidence that they are involved in receptor trafficking.

Speculations on Signal Protein·hsp90 Interactions

As one peruses Tables I–IV, the overall impression is that hsp90 is a protein with complex substrate and cofactor interactions that endow it with the capacity for truly pleotropic action in the eukaryotic cell. When nucleated cells evolved, the genes for hsp90 and hsp70 duplicated (297), and hsp90 became an essential protein (298). The essential function of hsp90 in the primitive eukaryote is unknown, but we speculate that it relates to the ability of the eukaryotic hsp70 and hsp90 to work together. We have outlined here how this machinery interacts with a native conformation of a steroid receptor to open a hydrophobic cleft to access by steroid. The ability of the machinery to open clefts may be of fundamental significance. For example, hsp90 is required for the binding of heme by aponeuronal nitric oxide synthase (299), which must bind heme to form the catalytically active homodimer. In eukaryotes there are many enzymes that must bind cofactors, such as heme or flavins, in hydrophobic clefts, and the opening of such clefts to facilitate cofactor entry may be an important and perhaps essential function of the hsp90/hsp70-based chaperone machinery.

Regions where hydrophobic clefts emerge on the protein surface are a normal topologic feature of properly folded proteins regardless of their size or sequence. Thus, the hsp90/hsp70-based chaperone machinery has at least a theoretical potential for interacting with hundreds or even thousands of proteins. The acquisition of a TPR-domain acceptor site on the hsp90 dimer gave the chaperone the additional ability to interact with a variety of proteins (i.e., immunophilins, immunophilin homologs, and UNC-45) that bind to motor proteins, giving hsp90 the potential for facilitating protein movement along cytoskeletal highways. Although this potential for hsp90 to bind soluble proteins and facilitate their trafficking to and from the nucleus and other organelles may not have been essential for protein movement within the body of nucleated cells, it is likely that the possession of a system, or systems, for rapid protein trafficking helped these large cells to function better. Clearly, such protein movement systems would have to evolve before cells could develop axons, where movement by diffusion is not possible and proteins must be moved by a specialized mechanism. Thus, in the integrated, multicellular

organism possessing a nervous system, a function of hsp90 in protein trafficking may have become essential.

If a major function of the TPR-domain immunophilins is to link proteins that are transiently complexed with hsp90 to a motor protein, then redundancy of this immunophilin action would be predicted, and one specific immunophilin might not be essential. Such a trafficking function of a TPR-domain immunophilin could itself be regulated, as suggested by experiments in which phosphorylation of FKBP52 by casein kinase II abrogated its binding to hsp90 (300). Because most client proteins do not form the more persistent complexes with hsp90 that we have described for steroid receptors, the cycle of heterocomplex assembly/disassembly would be more rapid, and their rapid movement would be saltatory as proteins become attached to and detached from protein motors with each assembly cycle.

One can ask why so many of the substrate proteins of hsp90 that are listed in Tables I–III are involved in signal transduction. There may be two reasons for this. First, it is inherent to signal transduction that the signal must move. Thus, before the evolution of the signaling process, many signaling proteins may have been transiently associated with hsp90 as they were moved to their sites of action in the cell. Second, signal transduction involves changes in interactions between proteins. Evolution of persistent binding to hsp90 may have been selected because cleft opening yielded a metastable state in which the proteins were conformationally set up to respond to ligands (as in the case of steroid receptors), to protein modification (e.g., phosphorylation), or to the binding of mediators (e.g., calcium/calmodulin, phosphatidyl inositol), all of which are events that facilitate subsequent protein interactions.

The tumor-suppressor protein p53 may provide us with an example of such a switch from a dynamic to persistent interaction with hsp90. In response to DNA damage, wild-type p53 accumulates in the nucleus, where it activates genes involved in growth arrest and apoptosis (301, 302). Wild-type p53 is transported to the nucleus along microtubules by cytoplasmic dynein (303). Some tumor cells contain p53 mutants that are inactive because they remain in the cytoplasm and are in persistent interaction with hsp90 (54, 55). For example, the temperature-sensitive mutant murine p53 ($TSp53^{Val\text{-}135}$) is cytoplasmic and bound to hsp90 at nonpermissive temperature, whereas at the permissive temperature the mutant p53 translocates to the nucleus and displays little or no hsp90 binding (54). At the permissive temperature, the p53 mutant may be in dynamic assembly/disassembly of complexes with hsp90 and engage with the dynein-mediated movement system proposed here for many soluble (i.e., nonvesicular) proteins. At the nonpermissive temperature, the mutant behaves like the glucocorticoid receptor, which is cytoplasmic and in persistent interaction with hsp90 in hormone-free cells. If the conversion of mutant p53 from its nonpermissive to its permissive state were under hormonal control, the result would be a steroid-type signal transduction system.

A major advance that has been made in the 5 years since our previous reviews of hsp90 regulation of signaling protein function is the definition of the five-protein minimal system for assembly of steroid receptor·hsp90 heterocomplexes. This purified system is already permitting mechanistic understanding of how the hsp90/hsp70-based machinery opens the steroid-binding cleft and forms stable complexes with hsp90 (Fig. 4). The five-protein system also assembles heterocomplexes between Raf-1 and hsp90 (K.D. Dittmar and W.B. Pratt, unpublished data), suggesting that it has the capacity to assemble heterocomplexes with the protein kinases listed in Table II. The nicest example of the broad application of the minimal purified system is the reconstitution of a functional hepadenovirus reverse transcriptase (304). In this case, transcription from the RNA template requires binding of the reverse transcriptase to an RNA signal (the packaging signal, ε) on the template. The reverse transcriptase·ε interaction and self-priming by the transcriptase require hsp90, and Hu *et al.* (304) reconstituted reverse transcriptase activity with the purified five-protein system. Four proteins of the machinery, hsp90, hsp70, Hop, and hsp40, were required for activity, and p23 further enhanced the kinetics of the process. This suggests to us that the purified assembly system may be applicable to a wide variety of biologic processes dependent on hsp90 function.

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