Role of p50/CDC37 in Hepadavirus Assembly and Replication*

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The cellular chaperone Hsp90 has been shown to associate with the reverse transcriptase (RT) of the duck hepatitis B virus and is required for RT functions. However, the molecular basis for the specific interaction between the RT and Hsp90 remains unknown. Comparison of protein compositional properties suggests that the RT is highly related to the protein kinase c-Raf, which interacts with Hsp90 via the cochaperone p50 (CDC37). We tested whether the RT, like c-Raf, is specifically recognized by p50. Immunoprecipitation and pull-down assays showed that p50 or p50ΔC, a p50 mutant defective in Hsp90 binding, could interact specifically with the RT both in vitro and in vivo, indicating that p50 can bind the RT independently of Hsp90. Furthermore, purified p50 and p50ΔC interacted directly with purified RT. The importance of p50-RT interaction for RT functions was underscored by 1) inhibition of protein-primed initiation of reverse transcription by p50ΔC in vitro and 2) stimulation of viral DNA replication and RNA packaging by p50 and their inhibition by p50ΔC in transfected cells. These results suggest that p50 can function as a cellular cofactor for the hepadnavirus RT by mediating the interaction between the RT and Hsp90.

Reverse transcription in hepadnaviruses (hepatitis B viruses) is carried out by a novel virally encoded reverse transcriptase (RT) (1, 2). The RT is able to initiate DNA synthesis in vitro using a specific tyrosine residue located within its N-terminal domain (the terminal protein (TP)) as a protein primer (Refs. 3–6; for a review, see Ref. 7). This protein priming reaction requires the interaction between the RT and a specific RNA signal (termed ε) located on the viral pregenomic RNA (pgRNA; the template for reverse transcription) (8, 9). The ε RNA is used as a specific template for protein priming (and thus, the origin of reverse transcription) (Refs. 9–11; for a recent review, see Ref. 12). In addition, ε serves as the RNA packaging signal (13, 14) and directs, through its interaction with the RT, the selective encapsidation of both the pgRNA and the RT into viral nucleocapsids (8, 15). Therefore, the specific interaction between the RT and ε triggers two essential early steps in hepadnavirus assembly and replication, i.e. the protein-primed initiation of reverse transcription and the assembly of replication-competent nucleocapsids.

Using the duck hepatitis B virus (DHBV) as a model system, we have recently found that the RT requires the assistance of host cell factors to carry out specific ε binding and protein priming functions (16, 17). One such cellular factor is the 90-kDa heat shock protein (Hsp90). Hsp90 associates with the DHBV RT and is required for RT-ε interaction and protein priming in vitro and for pgRNA packaging and DNA synthesis in vivo. Hsp90 is thought to facilitate the functions of a specific subset of cellular proteins (the target or substrate proteins) by helping to establish and maintain certain poised but labile conformations of these target proteins through a dynamic multistage process (18–22). In so doing, Hsp90 invariably collaborates with other factors (the so-called cochaperones or cofactors) and forms various multicomponent chaperone complexes. The precise composition of the Hsp90 chaperone complexes seems to vary depending upon the nature of the target proteins and the stage of the chaperoning process.

Hsp90 target proteins range from steroid receptors and protein kinases to reverse transcriptase (19, 20, 22). These proteins do not share any obvious structural or functional similarities. Hence, one of the most enigmatic, unresolved questions about Hsp90 is how the chaperone can recognize specifically such a diverse group of substrates. In our efforts to understand the molecular basis for the interaction between the Hsp90 complex and the viral RT, we searched the data base for Hsp90 target proteins that might share sequence and/or structural properties with the RT. For this purpose, we used a recently described sequence analysis program called protein property search or PropSearch (23), which was designed to detect weak structural and/or functional protein homologs that cannot be detected using conventional sequence alignment tools. PropSearch defines protein similarities not through sequence alignment, but as a weighted sum of compositional properties such as single and double amino acid compositions. By these criteria, we found that the RT is highly related to the serine/threonine protein kinase c-Raf.

The protein kinase c-Raf and a selected group of several other serine/threonine and tyrosine-protein kinases (e.g. CDK4 and v-Src) are known to be Hsp90 target proteins, requiring the chaperone for their intracellular trafficking, assembly, and maturation (24, 25). An Hsp90 cofactor called p50 (or CDC37) binds specifically to these kinases and to Hsp90 and is thought to be a “kinase-specific subunit” of a particular subset of Hsp90 complexes that targets Hsp90 to the kinase substrates by acting as a bridge between the kinases and Hsp90 (26–28). In light of these results, we decided to examine whether p50/CDC37 plays a role in the functions of the hepadnavirus RT, as suggested by the similarity between the RT and the protein kinases. Herein, we report that p50/CDC37 could indeed spe-
cifically interact with the RT in vitro and in vivo. The functional significance of this interaction was underscored by the fact that p50/CDC37 was able to modulate RT functions, as determined by measuring its protein priming activity in vitro and RNA packaging and DNA synthesis activities in transfected cells. These results thus indicate that p50/CDC37 is a host cell cofactor for hepatovirus replication and may play a more general role in Hep90 chaperone function than originally proposed.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmid pHTP was used for *in vitro* expression of the full-length DHBV RT (6). Plasmids expressing the DHBV mini-RT proteins, pcDNA-MiniRT1 and pcDNA-MiniRT2 (for *in vitro* and mammalian cell expression) and pGST-MiniRT1 and pGST-MiniRT2 (for bacterial expression of glutathione S-transferase (GST) fusion proteins) have been described before (29). pSP-c-Raf directs the expression of the human c-raf gene under the control of the SP6 promoter in *E. coli* and mammalian cell expression and pGST-MiniRT1 and pGST-MiniRT2, respectively, for the GST-p50 subunit of the kinase/RT fusion proteins. pcDNA-MiniRT1 and pcDNA-MiniRT2 were constructed by substituting the GST-MiniRT1 and GST-MiniRT2 cassettes from pGST-MiniRT1 and pGST-MiniRT2, respectively, for the GST-p50 sequences in pGST-p50 and were used to express the GST-MiniRT fusion proteins in mammalian cells. All RT and mini-RT proteins were tagged with a synthetic hemagglutinin (HA) epitope inserted into the spacer domain. p50-FLAG, which directs the expression of FLAG epitope-tagged p50 under the control of the cytomaglovirus immediate-early promoter/enhancer, was kindly provided by Gary Perdew (Pennsylvania State University) (30), pCMV-DHBV, which directs the expression of the DHBV pgRNA under the control of the cytomaglovirus promoter, and its derivative pCMV-YMHA, which harbors two amino acid substitutions in the RT active site abolishing polymerase activity, have been described (25, 26). pEBG-RT1 and pEBG-RT2 were constructed by replacing the c-Myc epitope in the pEBG-MiniRT1 and pEBG-MiniRT2 vectors by a FLAG epitope using the QuickChange site-directed mutagenesis kit (Stratagene). pEBG-MiniRT1 and pEBG-MiniRT2 were then cotransfected with pCMV-yMHA, which harbors two amino acid substitutions in the RT active site abolishing polymerase activity, have been described (25, 26).

**Antibodies**—The monoclonal antibody (mAb) against p23 (clone J33) was generously provided by David Toft (Mayo Clinic) (34), and the anti-p50 mAb (clone C1) by Gary Perdew (30). The anti-Hsp90 mAb (clone 1G10) was purchased from Stressgen Biotech Corp., and the anti-FLAG mAb (clone M2) from Sigma. Anti-HA mAb HA.11 (clone 16B12) was purchased from BAbCO (Berkeley Antibody). The polyclonal rabbit antibody against the DHBV core antigen was kindly provided by William Mason (Fox Chase Cancer Center) (35). The anti-DHBV RT TP domain antibodies were kindly provided by John Tavis (St. Louis University) (36).

**In Vitro Transcription and Translation**—RNAs used for *in vitro* translation were transcribed from linearized plasmids using an *in vitro* transcription kit (MEGAscript, Ambion) and were purified as described previously (16, 29). Purified RNAs were then translated using the rabbit reticulocyte lysate system (Promega). Protein expression in the coupled transcription and translation reaction mixture using the TransTACT rabbit reticulocyte lysate system (Promega) was carried out according to the manufacturer’s instructions.

**In Vitro Protein Priming**—Approximately 10 ng of purified GST-MiniRT proteins were used in an *in vitro* protein priming reaction in a total volume of 10 μl as described previously (29). Rabbit reticulocyte lysate (nuclease-treated; Promega) supplemented with an ATP regenerating system (5 mM ATP, 10 mM creatine phosphate, and 50 μM creatine phosphokinase) was used to reconstitute the RT protein priming activity as described (29). In all reactions, [α-32P]ATP was used as the labeled nucleotide.

**Protein Expression and Purification**—Two truncated minimal DHBV RT fusion proteins (GST-MiniRT1 and GST-MiniRT2) were expressed in *Escherichia coli* and purified as described (29). FLAG epitope-tagged p50 and p50/C were purified from recombinant baculovirus-infected insect cells by immobilized metal affinity chromatography using agarose-cross-linked mAb M2 (Eastman Kodak Co.) as described (27).

**Protein-Protein Interactions**—Full-length DHBV RT and truncation mutants (labeled by *in vitro* translation using the reticulocyte lysate in the presence of [35S]methionine) were diluted with lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 5% Nonidet P-40) and incubated with p50 or p50/C pre-bound to the mAb M2-agarose beads. The unbound material was removed by extensive washing with lysis buffer, and the immunoprecipitates were then resolved by SDS-PAGE. Total proteins were detected by Coomassie Blue staining, and the labeled proteins by autoradiography. To detect direct binding between the RT and p50, purified GST-MiniRT proteins from bacteria were incubated with p50/C-bound mAb M2 under immunofinity beads in lysis buffer, and unbound proteins were removed after extensive washing with lysis buffer. The RT proteins bound to the beads were detected by Western blot analysis using the anti-HA mAb following resolution of the immunoprecipitates by SDS-PAGE.

To assess p50-RT interaction in cultured cells, pcDNA-MiniRT1 and pcDNA-MiniRT2 were cotransfected with pCMV-p50 or pCMV-p50/C into COS-1 cells. The transfected cells were lysed in lysis buffer 3 days after transfection, and the p50 proteins were purified by GSH-agarose beads. The mini-RT proteins bound to the p50 proteins were eluted by GSH and detected by resolving the purified proteins by SDS-PAGE and Western blot analysis using either the anti-HA mAb or mAbs against the TP domains. In addition, the GST-MiniRT proteins were coexpressed using pEBG-MiniRT1 and pEBG-MiniRT2 in 293T or COS-1 cells together with FLAG-tagged p50. The GST-MiniRT fusion proteins were then purified by GSH beads and eluted by GSH. Proteins associated with the RT were detected by SDS-PAGE and Western blot analysis.

**Analysis of Viral DNA, RNA, and Proteins**—The human embryonic kidney cell line 293T and the monkey kidney cell line COS-1 were transfected with pCMV-DHBV, pCMV-yXM(core), or pCMV-YMHA, together with a p50 expression construct (pGST-p50 or pGST-p50/C) or the vector alone (pEBG or GST) using the calcium phosphate (5 Prime → 3 Prime, Inc.) procedure (16). Transfected cells were harvested 3 days after transfection. Replicative viral DNA intermediates were purified from cytoplasmic core particles and analyzed by Southern blot analysis as described (16, 37).

Encapsidated pgRNA in cytoplasmic core particles obtained by polyelectrolyte glycol precipitation (38) was detected by resolving the capsid proteins on agarose gels followed by Southern blot analysis using a 32P-labeled antisense riboprobe (spanning nucleotides 143–391 on the DHBV genome) (16) as described (39). The amount of assembled capsid particles was determined by subsequent reprobing of the same membrane using the anti-DHBV core antibody. To measure the steady-state levels of total DHBV core protein in the transfected cells, a portion of the cytoplasmic extract used for core DNA isolation was analyzed by SDS-PAGE and Western blotting using the anti-DHBV core antibody.

**RESULTS**

**Similarity between the DHBV RT and c-Raf in Compositional Properties as Detected by PropSearch**—Because conventional sequence alignment methods have not revealed any similarity between the hepatitis virus RT and other Hep90 target proteins, we attempted to determine whether the RT is related to any other known Hep90 target proteins using an alternative sequence comparison method called PropSearch (23). PropSearch defines protein similarities not through sequence alignment, but as a weighted sum of compositional properties (a total of 144 parameters), including singlet and doublet amino acid composition and isoelectric point, and has been shown to detect weak structural and/or functional protein homologs that escaped detection by conventional sequence alignment tools.

A strong similarity in amino acid composition between the RT and c-Raf (kraf), a protein kinase target of Hsp90, was detected by PropSearch (Table I and additional search results not shown). In fact, the similarity between the RT and c-Raf proteins (Table I, ranks 7, 8, and 11) was judged to be stronger than that between the RT and other reverse transcriptases from other retroelements (rank 14) and just below that between the DHBV RT and other hepatitis virus RTs (ranks 1–5). It is not yet known whether the other proteins listed in Table I are Hep90 targets or not. However, the strong similarity between the RT and c-Raf raised the intriguing possibility that the RT, like c-Raf, may be targeted to the Hep90 chaperone via p50/CDC37, an Hep90 cofactor known to be responsible for targeting the kinase substrates to the chaperone.

Association between the RT and p50 in Vitro—To determine whether the RT could associate with p50 specifically, we ex-
pressed the $^{35}$S-labeled DHBV RT by in vitro translation in the rabbit reticulocyte lysate, in which an active DHBV RT can be expressed (4) and is associated with Hsp90 and one of the cochaperones, p23 (16, 17). The labeled RT proteins were then co-immunoprecipitated with purified FLAG-tagged p50 pre-bound to antibody affinity beads. Bound proteins were detected by SDS-PAGE and autoradiography. As shown in Fig. 1A, the RT could be co-immunoprecipitated by the p50 beads (lane 1), similar to c-Raf (lane 4), which was used as a positive control. The negative control (in vitro translated luciferase) was not precipitated by the p50 beads (Fig. 1A, lane 7). As p50 is known to bind to Hsp90 (Fig. 1B, lanes 1, 4, and 7) (27, 40), as does the RT (16), it was possible that the RT was associated with p50 via Hsp90. To test whether p50 could associate with the RT independently of Hsp90, a p50 mutant (p50C) with its C-terminal Hsp90-binding domain deleted, and thus unable to bind Hsp90 (Fig. 1B, lanes 2, 5, and 8) (27), was used to precipitate the RT. We found that p50C could also associate with the RT (Fig. 1A, lane 2) as well as c-Raf (lane 5), indicating that the interaction between p50 and the RT (like c-Raf) (27) is independent of its association with Hsp90. Interestingly, p50C seemed to bind even more in vitro translated RT proteins than wild-type p50, which was also the case when they were coexpressed in cultured cells (see Fig. 3 below).

To determine whether the RT could bind directly to p50, as c-Raf does (27, 41), purified GST-MiniRT proteins expressed in bacteria were incubated with purified p50 proteins pre-bound to mAb M2 beads. We found that both GST-MiniRT1 and GST-MiniRT2 (but not GST alone) could bind directly to purified p50 and p50C (Fig. 2) (data not shown). Therefore, these results showed that the DHBV RT could directly associate with p50 in vitro, independently of Hsp90 or any other cellular factors.

Association between the RT and p50 in Vivo—To determine whether the RT could associate with p50 in the cell, we coexpressed functional mini-RT proteins (29) together with GST-tagged p50 in 293T cells, which can be transfected efficiently. As shown in Fig. 3 (lanes 13–18), GST-tagged p50C proteins specifically pulled down the mini-RT proteins expressed in 293T cells. (Similar results were also obtained using COS cells (data not shown).) In addition to the intact mini-RT proteins, we noticed that a major degradation product derived from both mini-RT proteins was also pulled down by p50C. This degradation product reacted with both the anti-HA mAb (Fig. 3A) and several antibodies (both monoclonal and polyclonal) against the TP domain (data not shown), indicating that it represented the TP fragment with some residual sequences from the spacer region where the HA epitope was inserted. On the other hand, the binding of the RT to wild-type p50 seemed to be weaker and more variable (Fig. 3) (data not shown). In addition, we noticed that p50C (but not p50) was predominantly localized to the detergent-insoluble fraction and shifted a significant amount of the mini-RT proteins and the TP fragment into this fraction (Fig. 3, lanes 7–12). As p50C (unlike p50) could not associate with Hsp90 (Fig. 3B, lanes 13–18), these results indicated that the mini-RT proteins and the TP domain could associate with p50 in vivo independently of Hsp90.

To further assess the association of the RT with p50 (and other cellular proteins) in the cell, we expressed the GST-MiniRT proteins in 293T cells. The tagged mini-RT proteins were purified by GSH beads, and RT-associated proteins in the GSH eluate were detected by Western blot analysis using specific antibodies against various cellular proteins. As GST-MiniRT1 was rapidly degraded in the transfected cells, it was difficult to purify significant amounts of MiniRT1 for this purpose. However, we found that GST-MiniRT2 was significantly more stable, and substantial amounts of GST-MiniRT2 could be purified (Fig. 4, fourth panel). When a FLAG-tagged p50 protein was coexpressed with GST-MiniRT2 in 293T cells, the overexpressed exogenous p50-FLAG protein was associated with the MiniRT2 protein (Fig. 4, second panel). The association between the mini-RT protein and endogenous p50 was also detectable in some experiments (data not shown). In addition, we could consistently detect the association between GST-MiniRT1 and endogenous Hsp90 and another cochaperone, p23 (Fig. 4, first and third panels). We noticed that in some experiments, the amount of p23 in association with the RT seemed to be decreased when p50-FLAG was overexpressed (Fig. 4, third panel, lane 1).

In summary, we demonstrated that the RT could associate with several components of the Hsp90 complex, including...
addition of wild-type p50 to the priming reaction had little or no effect (Fig. 5, lanes 5 and 6) (data not shown). As p50C is known to be a dominant-negative inhibitor of p50 function (27), these results suggested that p50 function was important for protein priming, but was not limiting under these in vitro conditions. On the other hand, they could not exclude the possibility that the inhibitory effect of p50C on RT activation was simply due to the sequestration of the RT away from the Hsp90 chaperone by p50C.

**Increase in Viral Replication by p50 and Decrease by p50C in Vivo**—To obtain evidence for a role of p50 in RT function and in viral replication in vivo, we transfected replication-competent DHBV DNA into 293T cells together with p50 expression plasmids. As shown in Fig. 6, the overexpression of p50 led to a significant increase in viral DNA synthesis, whereas the dominant-negative mutant (p50C) showed just the opposite effect and decreased viral replication. The stimulatory and inhibitory effects of p50 and p50C, respectively, were particularly evident on the replicative viral single-stranded DNA intermediates (Fig. 6A). Although the effects of p50 and p50C on viral DNA synthesis were modest (~3-fold increase by p50 and 3-fold decrease by p50C), the opposing effects of p50 and its dominant-negative inhibitor (p50C) clearly demonstrated that p50 plays a role in viral DNA replication.

To understand the mechanism of p50 effect on viral DNA synthesis in the cell, we determined the levels of viral pgRNA...
packaged into the nucleocapsids. Because pgRNA packaging in hepadnaviruses depends on RT function, we predicted that p50, through its effect on the RT, may be required for efficient pgRNA packaging. The preferential effect of p50 and p50/H9254 on the single-stranded DNA intermediate (rather than the mature relaxed circular DNA) (Fig. 6A) also suggested that they might have affected pgRNA packaging. Using a native agarose gel assay of nucleocapsid assembly and RNA packaging (39), we could indeed show that the overexpression of p50 led to a stimulation of pgRNA packaging, whereas the expression of the dominant-negative mutant (p50/H9254C) had just the opposite effect and inhibited pgRNA packaging (Fig. 7).

DISCUSSION

In our attempt to understand the molecular basis for the interaction between the hepadnavirus RT and the host cell Hsp90 chaperone complex, we have now obtained in vitro and in vivo evidence for a specific interaction between the RT and one of the known Hsp90 cofactors, p50/CDC37. This study was initially inspired by the intriguing data base search result suggesting a close relationship between the RT and the protein kinase c-Raf, as judged by their amino acid compositional properties (PropSearch) (23). As p50 is thought to bind specifically to both Hsp90 and c-Raf (and several other Hsp90 kinase substrates) and thus target the chaperone to the kinase substrates, this result prompted us to test whether p50 might also help to target the RT to Hsp90. We have shown here that p50 could bind to the RT in vitro and in vivo independently of Hsp90. Indeed, the RT and p50 could interact directly, at least in vitro. In addition, the in vitro protein priming activity of the

FIG. 3. Association of mini-RT proteins with p50 and p50/CDC37 in cells. pcDNA-MiniRT1 and pcDNA-MiniRT2 were transfected into 293T cells together with pGST-p50, pGST-p50/CDC37, or pGST (as a vector control). Transfected cells were then lysed in lysis buffer. Insoluble materials were removed by pelleting in a microcentrifuge. The supernatant (soluble fraction) was incubated with GSH beads to pull down GST-p50, GST-p50/CDC37, or GST alone. Bound proteins were eluted with GSH (eluate). The soluble (lanes 1–6), insoluble (lanes 7–12), and eluate (lanes 13–18) materials were resolved by SDS-PAGE and detected by Western blot analysis using the anti-HA mAb against the HA epitope inserted into the mini-RT proteins (A) or by Coomassie blue staining (B). The intact MiniRT1 and MiniRT2 proteins and the degradation product containing the TP domain are indicated in A. In B, the arrowheads denote GST-p50 (lanes 3, 6, 9, 12, 15, and 18), GST-p50/CDC37 (lanes 2, 5, 8, 11, 14, and 17), and GST (lanes 1, 4, 13, and 16). p50-associated Hsp90 evident in lanes 15 and 18 is also indicated.

FIG. 4. Association of Hsp90, p23, and p50 with the mini-RT protein. Plasmid DNA expressing GST-MiniRT2 or GST alone was transfected into 293T cells together with p50-FLAG or a vector control (pcDNA3). Transfected cells were lysed in lysis buffer, and soluble materials were incubated with GSH beads. Bound materials were eluted with GSH, resolved by SDS-PAGE, and detected by Western blot analysis using anti-Hsp90 (first panel), anti-p50 (second panel), and p23 (third panel) mAbs or by Coomassie blue staining (fourth panel). Hsp90, p50-FLAG, p23, GST-MiniRT2, and GST are indicated. The asterisk denotes a nonspecific band associated with the GSH beads.

FIG. 5. Inhibition of the protein priming activity of the RT by p50/CDC37. GST-MiniRT1 purified from bacteria was tested for in vitro protein priming activity in presence of purified p50 (200 and 400 ng) (lanes 5 and 6), p50/CDC37 (200 and 400 ng) (lanes 3 and 4), or buffer alone (lanes 1 and 2). All reactions were supplemented with reticulocyte lysate (1 μl), which is required to activate the RT. The 32P-labeled mini-RT protein was then resolved by SDS-PAGE and detected by autoradiography.
RT was inhibited by a dominant-negative inhibitor of p50, p50/CDC37. Furthermore, viral DNA synthesis and RNA packaging in vivo were stimulated by p50 and inhibited by p50/CDC37.

It is not yet known how p50 recognizes the different protein kinases or the viral RT. As the PropSearch program indicated that the RT and c-Raf are highly related in their amino acid composition (but apparently not by sequence alignment), it is possible that this similarity in composition may in turn dictate certain common folding characteristics (pathways) or folding intermediates that are recognized by p50. Interestingly, the same program also identified other classes of Hsp90 substrates, including certain steroid hormone receptors, as related to the RT and c-Raf. Although p50 was originally thought to be a kinase-specific subunit of the Hsp90 complex (26), more recent results suggest that this may not be entirely the case, as other classes of Hsp90 target proteins, including some steroid receptors, may also interact with p50 (42–44). Furthermore, p50 has been found to be present in Hsp90 complexes together with other cochaperones which had been previously thought to be irrelevant for kinase functions (45, 46) but have since been found to associate with at least some kinase substrates and to be required for their functions (47, 48). Our results are therefore consistent with the emerging notion that p50 may not be a dedicated Hsp90 cofactor specific for the kinase substrates, as originally envisioned, but rather functions as a more general cochaperone interacting with a variety of different Hsp90 target proteins. This concept is also supported by the failure, so far, to identify any "dedicated" cochaperones that would target the Hsp90 complex to its increasingly wide array of substrates (19, 20), except for the kinases. Furthermore, genetic experiments in yeast have suggested that Hsp90 folds structurally very different target proteins (e.g. glucocorticoid receptor and v-Src) by a similar mechanism and uses a comparable set of cochaperones (49, 50). If, indeed, no specific targeting factors are responsible for the interaction between the Hsp90 chaper-
one and its diverse substrates, the challenge now will be to identify the common properties that have to be shared among these different substrates and that are recognized by the chaperone. Whatever these properties turn out to be, it appears that both the TP and RT domains of the hepadnavirus RT may share some of these properties, as each of them could independently associate with p50 (Fig. 3) (data not shown) and Hsp90 (29).

The functional importance of p50-RT association in RT activity and viral replication was supported by both in vitro and in vivo experiments. In vitro, a dominant-negative inhibitor of p50 (p50C) decreased reconstitution of protein priming activity by the reticulocyte lysate, suggesting that endogenous p50 present in the reticulocyte lysate played a positive role in RT reconstitution. Furthermore, the overexpression of p50 stimulated viral RNA packaging and DNA synthesis in transfected cells, whereas p50C, a dominant-negative inhibitor of p50 function, inhibited viral RNA packaging and DNA replication. These results thus strongly support a role for p50 in viral replication in vivo. Recently, we have developed a defined reconstitution system whereby five purified components of the Hsp90 complex, i.e. Hsp90, Hsp70, p540, p60/Hop, and p23, are sufficient to partially reconstitute a functional mini-RT (51). Preliminary results so far suggest that p50 does not appear to further enhance the activity of the RT in this defined in vitro reconstitution system. These results may suggest that p50 is required only for RT functions under the more physiological conditions either in the complex mixture of the cell lysate or in the living cell, e.g. by helping to target the RT to the Hsp90 complex. In the defined reconstitution system using purified factors, the requirement for p50 may be bypassed when the RT and the Hsp90 chaperone system are brought together under the selected in vitro conditions. However, other interpretations are possible, and we are currently investigating conditions under which p50 may play a role in RT functions also in this defined system.

Because p50C could still bind the RT, but not Hsp90, it probably inhibited RT activation by sequestering the RT away from the Hsp90 complex. In fact, we found that p50C could bind to the RT more strongly compared with p50 and shifted most of the RT into a detergent-insoluble fraction in the cell. Although the nature of the detergent-insoluble compartment in the cell to which the RT was shifted remains to be characterized, other Hsp90 target proteins have also been shown to partition to detergent-insoluble fractions when the chaperone function is impaired (52). The finding that p50C associated with the RT more strongly compared with p50 also suggests that p50 may only transiently associate with the RT: it helps to bring the RT to Hsp90 and then dissociates from the RT. In this scenario, p50C would remain bound to the RT because it cannot “transfer” the RT to Hsp90. The dynamics of chaperone-RT association in the cell are still unknown. Steroid receptors have been shown to associate with several different Hsp90 subcomplexes (each with a different chaperone complement) along the maturation pathway (53, 54). It is possible that the RT may undergo a similar pathway of conformational maturation/activation. Preliminary results presented here showing that the overexpression of p50 appears to decrease the association of another chaperone (p23) with the RT are consistent with this suggestion. Additional work will be necessary to elucidate the intricate and dynamic pathway of RT activation by the Hsp90 chaperone complex.

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REFERENCES

1. Seeger, C., and Mason, W. S. (2000) Microbiol. Mol. Biol. Rev. 64, 51–68
2. Summers, J., and Mason, W. S. (1982) Cell 30, 403–415
3. Lanford, R. E., Notvall, L., and Beames, B. (1995) J. Virol. 69, 4431–4439
4. Wang, G. H., and Seeger, C. (1992) Cell 71, 663–670
5. Weber, M., Brunzena, V., Bartos, H., Bosscherhoff, A., Bartenschlager, R., and Schaller, H. (1994) J. Virol. 68, 2994–2999
6. Zoulim, F., and Seeger, C. (1994) J. Virol. 68, 6–13
7. Hu, J., and Seeger, C. (1996) Methods Enzymol. 275, 195–208
8. Pollack, J. R., and Ganem, D. (1994) J. Virol. 68, 5579–5587
9. Wang, G. H., Zoulim, F., Leber, E. H., Kitson, J., and Seeger, C. (1994) J. Virol. 68, 8437–8442
10. Wang, G. H., and Seeger, C. (1993) J. Virol. 67, 6507–6512
11. Nassal, M., and Rieger, A. (1996) J. Virol. 70, 2764–2773
12. Hu, J., and Seeger, C. (1997) Semin. Virol. 8, 205–211
13. Hirsch, R. C., Loeb, D. D., Pollack, J. R., and Ganem, D. (1991) J. Virol. 65, 3509–3516
14. Junker-Niempmann, M., Bartenschlager, R., and Schaller, H. (1990) EMBO J. 9, 3389–3396
15. Bartenschlager, R., and Schaller, H. (1992) EMBO J. 11, 3413–3429
16. Hu, J., and Seeger, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1060–1064
17. H. C., Tofit, D. O. (1997) EMBO J. 16, 59–68
18. Bohan, S., Krali, A., and Yamamoto, K. (1995) Science 268, 1303–1304
19. Buchner, J. (1999) Trends Biochem. Sci. 24, 136–141
20. Pearl, L. H., and Prodromou, C. (2000) Curr. Opin. Struct. Biol. 10, 46–51
21. Toft, D. (1998) Trends Endocrinol. Metab. 9, 238–243
22. Young, J. C., Moarefi, I., and Hartl, F. U. (2001) J. Cell Biol. 154, 267–274
23. Holohov, U., and Sander, C. (1985) J. Mol. Biol. 191, 390–399
24. Wartmann, M., and Davis, R. (1994) J. Biol. Chem. 269, 6695–6701
25. van der Straaten, A., Rommel, C., Dickson, B., and Hafem, E. (1997) EMBO J. 16, 1961–1969

[References cited in the text are numbered for easy identification.]
