DEPLETION OF HSP90β INDUCES MULTIPLE DEFECTS IN B CELL RECEPTOR SIGNALING

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Running Title: Hsp90 Is Required for BCR Signaling

Hsp90 participates in many distinct aspects of cellular functions and accomplishes these roles by interacting with multiple client proteins. To gain insight into the interactions between Hsp90 and its clients, here we have reduced the protein level of Hsp90 in avian cells by gene targeting in an attempt to elicit the otherwise undetectable (owing to the vast amount of cellular Hsp90) Hsp90-interacting proteins. Hsp90-deficient cells can grow, albeit more slowly than wild-type cells. B cell antigen receptor signaling is multiply impaired in these mutant cells; in particular, the amount of immunoglobulin M heavy chain protein is markedly reduced. Furthermore, serum activation does not promote ERK phosphorylation in Hsp90-deficient cells. These multifaceted depressive effects seem to be provoked independently of each other and possibly recapitulate the proteome-wide in vivo functions of Hsp90. Reintroduction of the Hsp90β gene efficiently restores all of the defects. Unexpectedly, however, introducing the Hsp90α gene is also effective in restoration; thus, these defects might be caused by a reduction in the total expression of Hsp90 rather than by loss of Hsp90β-specific function.

INTRODUCTION

Living cells continuously cope with protein folding and assembly. Because cell fate (e.g., proliferation, differentiation and even death) is determined and implemented by a plethora of varied proteins, the folding and assembly of these proteins are crucial to a cell’s life. However, many proteins do not achieve correct folding or proper assembly autonomously in the cell; instead, they require the assistance of molecular chaperones (1-6). The 90-kDa heat shock protein (Hsp90β) is one such molecular chaperone and is also one of the most abundant cytosolic proteins (7-12). The Hsp90 family is highly conserved during evolution and is essential in yeast (13),

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Caenorhabditis elegans (14) and Drosophila melanogaster (15-17). Concordant with these facts, many Hsp90 client proteins (i.e., Hsp90 substrates) are key molecules in signal transduction, including protein kinases and transcription factors (7-12).

Although the proteome-wide function of Hsp90 as a capacitor for morphological evolution that buffers cryptic genetic variation is well known (18-20), elucidating the complete compendium of the Hsp90-interacting proteins remains a challenge (21, 22). Hsp90 is not required for the de novo folding of most proteins in yeast under normal conditions (23) and reduction to 1/20 of its normal level can be tolerated (24). By contrast, mouse mutant embryos lacking Hsp90β die because of defects in the development of the placental labyrinth (25). Higher eukaryotes contain two Hsp90 isoforms that closely resemble each other (Hsp90α and Hsp90β). Although each forms a homodimer (26), no obvious difference in function between the two isoforms has been reported so far. The developmental deficiency observed in the Hsp90β mutant embryos may be attributable to a lack of specific functions that are fulfilled by only Hsp90β (25); alternatively, the reduced expression level of Hsp90 may impinge on a specific aspect of the placental development. These possibilities raise two questions. First, do Hsp90α and Hsp90β have individual functions that cannot be accomplished by one another? Second, to what extent can the expression of Hsp90 be decreased without losing viability in higher eukaryotic cells?

Anti-tumor agents such as geldanamycin (GA) compete with ATP for binding to the specific pocket of Hsp90 and ultimately block its ATPase-driven chaperone cycle (27-29); therefore, they are efficient inhibitors of Hsp90 that can be used to dissect its functions (7-12). Because GA binds to both Hsp90α and Hsp90β (30), this Hsp90-inhibitor is not suitable for discriminating between the functions of the two Hsp90 isoforms. In addition, our attempts to use siRNA-directed gene knockdown of the specific isoforms of Hsp90 have not led to the successful depletion of either Hsp90α or Hsp90β (our unpublished data).

Thus, to address the aforementioned issues, here we have generated cells from the chicken B lymphocyte line DT40 that are deficient in Hsp90β. We show that multiple defects are concurrently elicited in the mutant cells; in particular, components involved in B cell antigen receptor (BCR) signaling are impaired. Most strikingly, the expression level of the immunoglobulin (Ig) M heavy chain is profoundly reduced. These defects can be efficiently corrected not only by reintroducing the Hsp90β gene but also by introducing the Hsp90α gene; therefore, the defects observed in these cells are derived mainly from a reduction in the Hsp90 content and not from a loss of Hsp90β-specific function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs** - Chicken Hsp90β genomic DNA was obtained from DT40-derived genomic DNA by PCR amplification based on previous studies (31, 32) and confirmed by DNA sequencing. Two targeting constructs, Hsp90β-neo and Hsp90β-hisD, were generated by replacing the DNA segment encompassing exons 4 to 6 with drug-resistant cassettes for neomycin (neo) and histidinol (hisD), respectively. Chicken Hsp90α and Hsp90β cDNAs were synthesized by reverse transcription (RT)-PCR using mRNA isolated from DT40 cells, and were cloned either into the pApuro2 vector [made by introducing a multicloning site into the pApuro vector (33)] for their (re)introduction into chicken serum, 50 μ M –mercaptoethanol and 50 μ M –mercaptoethanol and antibiotics (penicillin and streptomycin) at 39.5°C under 5% CO2. Cells were electroporated at 25 μ F and 550 V with a Gene Pulser II apparatus (Bio-Rad); approximately 10^7 cells and 50 μg of...
linearized DNA were used for each transfection (35). Stable transformants were selected with 2 mg/ml of G-418, 1 mg/ml of histidinol and 0.5 μg/ml of puromycin (all from Sigma). Genomic DNAs were isolated using a DNeasy Tissue Kit (Qiagen). The DNAs (5 μg) were digested with PvuII, separated in 0.8% (w/v) agarose gels and transferred onto a Hybond N+ nylon membrane (Amersham Biosciences). Membranes were hybridized with an Hsp90β genomic DNA fragment, as shown in Fig. 1A, which was labeled using a Gene Images random prime labeling and detection system (Amersham Biosciences), as a probe. After incubating cells with tetrazolium salt WST-8 (Cell Counting Kit-8: Dojindo), the probe. After incubating cells with tetrazolium dye WST-8 (Cell Counting Kit-8: Dojindo), the optical density was measured at 450 nm to assess cell viability.

**Northern Blot Analysis and RT-PCR** - Total RNA was isolated by using an RNaseasy Mini Kit (Qiagen). Membranes (5 μg of RNA) were hybridized with the following fluorescently labeled cDNA fragments as probes: the EcoRI-PstI fragment of Hsp90α (0.63 kb); the BglII fragment of Hsp90β (0.8 kb); a PCR fragment of IgM (X01613) synthesized with the two primers 5’-CGGAACAACTGAACGGCAAC-3’ and 5’-GCCCTATCCACCGACTTCTG-3’ (0.3 kb); the PstI-HindIII fragment of Igα (0.16 kb); the BamHI-EcoNI fragment of Igβ (0.27 kb).

Total RNAs (1 μg) were used for the first-strand synthesis with oligo-dT and SUPERSCRIPT II RNaseH Reverse Transcriptase (Invitrogen). For semi-quantitative RT-PCR, total cDNA was subjected to a three-fold dilution. PCR amplification was performed with KOD-Plus DNA polymerase (Toyobo) using the following oligonucleotide primers: μm, 5’-ATCTCTCTCCATTGGCCTCTGGGAG-3’ and 5’-TTTCACCTTGATGAGGTTGAGGACG-3’; μs, 5’-CGGAACAACTGAACGGCAAC-3’ and 5’-CAATCGAGGGCGCTTAAACG-3’; X-box binding protein-1 (XBP-1) (directed against the N-terminal peptide of chicken YB-1) and a Y-box protein-associated acidic protein (YBAP1) (directed against recombinant proteins of chicken YBAP1) will be described in more detail elsewhere (K. Matsumoto, manuscript in preparation). Anti-chicken Hsp90β and anti-Igα antibodies were raised in rabbits using the GST-fusion proteins. Anti-Hsp90α and anti-Hsp90β antisera (10 μl) were mixed with the His-tagged Hsp90β or Hsp90α proteins (100 μg), respectively, and incubated for 2 h at room temperature to eliminate mutual cross-reactivities between them; for anti-Hsp90β antisera, incubation was further continued overnight after the re-addition of Hsp90α protein (100 μg). Anti-chicken IgM...
monoclonal antibody M4 (Southern Biotechnology) and goat anti-chicken IgM antibody (Bethyl Laboratories) were used for BCR stimulation and for immunoprecipitation/immunoblotting, respectively. The following antibodies were purchased: anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology); anti-Raf-1 (C-12 and E-10) and horseradish peroxidase-conjugated anti-goat IgG antibodies (Santa Cruz Biotechnology); anti-ERK, anti-phosphorylated ERK, anti-MEK, anti-phosphorylated MEK, anti-Akt and Phospho-(Ser/Thr) Akt substrate antibodies (Cell Signaling Technology); anti-actin antibody (Sigma); and horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Amersham Biosciences).

Stimulation of DT40 Cells - Cells were washed twice with PBS and resuspended in serum-free RPMI1640 medium. After a 10-min incubation at 39.5 °C, cells were activated by the addition of either 4 μg/ml of anti-IgM antibodies (M4) or 20% (v/v) fetal bovine serum.

Western Blot Analysis and Immunoprecipitation - Where indicated, cells were treated with 2 μM GA (Sigma), 50 μg/ml of cycloheximide (CHX) (Sigma) or 50 nM calyculin A (Cell Signaling Technology) before cell lysis. Cells were lysed in NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10 μM sodium molybdate and 0.2 mM sodium vanadate (33) supplemented with protease inhibitor cocktail (Complete mini: Roche Applied Science). Cell lysates were cleared by centrifugation at 16,000 x g for 15 min and subjected to SDS-PAGE, followed by transfer onto Immobilon-P membranes (Millipore). Blots were incubated with antibodies and subjected to chemiluminescence detection with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Science). Intensity of immunodetected protein bands was quantified using NIH image software after scanning of the blot with a densitometer. For immunoprecipitation, cell lysates were incubated with antibodies for 10 min on ice, either Protein A- or Protein G-Sepharose (Amersham Biosciences) was then added and incubation was continued for a further 3 h. The immunoprecipitates were washed four times with NP40 lysis buffer. To prepare μs proteins for immunoblotting, cells were cultured for 12 h in RPMI1640 with 10% fetal bovine serum, and the secreted μs proteins were immunoprecipitated and immunoblotted with anti-IgM antibodies (Bethyl Laboratories).

Phosphatase treatment - Cell lysates (30 μl) were incubated with or without 60U of lambda protein phosphatase (New England Biolabs) for 30 min at 30 °C.

Flow Cytometric Analysis - Cells were washed with a buffer solution [PBS, 0.3% (w/v) bovine serum albumin, 0.02% (w/v) sodium azide] and incubated with the same solution containing goat anti-chicken IgM antibody for 30 min on ice. After washing twice, cells were incubated with FITC-labeled rabbit anti-goat antibody (Sigma) for 30 min on ice. The stained cells were then resuspended in 1 ml of the above solution and their fluorescent intensity was analyzed by a Beckman Epics XL flow cytometer (Beckman Coulter).

In Vitro Kinase Assay - For the in vitro Raf-1 kinase assay, immunoprecipitates obtained with anti-Raf-1 antibody (E-10) were washed four times with NP40 lysis buffer and then with ice-cold reaction buffer [20 mM Tris-HCl (pH7.5) and 10 mM MgCl2]. The kinase reaction was performed in 30 μl of the reaction buffer containing 250 μM ATP and 1 μg of recombinant MEK-1 substrate (Santa Cruz Biotechnology) at 30 °C for 30 min.

RESULTS

Targeted Gene Disruption of Hsp90β in Chicken DT40 Cells - Two different targeting constructs were designed to disrupt exons encoding the ATP-binding domain of Hsp90β that is essential to its chaperone function (38, 39) (Fig. 1A). We generated Hsp90β-deficient cells by the successive transfection of these two constructs (Fig. 1B). Southern blot analysis indicated that the constructs were properly targeted (Fig. 1B) and this result was corroborated by Northern blotting (Fig. 1C). Expression of Hsp90β protein was not detected in Hsp90β-deficient cells by Western blot analysis (Fig. 1D); albeit a faint band was seen probably due to cross-reactivity of anti-Hsp90β antibody with Hsp90α proteins. Notably, expression of
Hsp90α protein did not increase in the Hsp90β-deficient cells (Fig. 1D); consistent with this, the level of Hsp90α mRNA was comparable to that in wild-type cells (Fig. 1C). The protein levels of Hsp70 and Hsp40 were also unchanged in the Hsp90β-deficient cells (data not shown). Taken together, we conclude that Hsp90β can be successfully depleted by gene disruption and that expression of Hsp90α does not increase to compensate for the lack of Hsp90β protein.

Although the Hsp90β-deficient cells did not lose viability and their saturation densities did not differ from those of wild-type cells (data not shown), they grew more slowly; by contrast, heterozygous mutant cells seemed to grow normally (Fig. 1E). Thus, the gene knockout of Hsp90β apparently affected an aspect of cell proliferation. Initially, we suspected that the heat shock response might be compromised in Hsp90β-deficient cells, because Hsp90 has been suggested to have a role in modulating HSF1 (40). Western blotting showed, however, that the expression levels of the HSFs (avian cells express HSF1-HSF3) were unchanged (Fig. 2A), although HSF2 is not activated by heat stress (36). Within 15 min of sustained exposure to 43 °C, an electrophoretic mobility shift of HSF3 was observed in both the wild-type and Hsp90β-deficient cells (Fig. 2B); by contrast, HSF1 did not show any detectable mobility shift (data not shown). This upshift pattern of HSF3 in the two cell clones was eliminated by phosphatase treatment (Fig. 2C), which reconfirms a previous study showing that the characteristic mobility shift of mammalian HSF1 results from stress-induced phosphorylation (indicating the transient activation) (41): HSF3 is dominant among the three HSFs in regulation of the heat shock response in DT40 cells (42). Most importantly, an equal accumulation of Hsp70 following the upshift of HSF3 bands was detected in the two cell clones (Fig. 2B). We therefore conclude that the heat shock response is intact in Hsp90β-deficient cells.

Expression of IgM Is Markedly Reduced in Hsp90β-deficient Cells - Signaling through the BCR is essential for B cell function (43, 44). BCR cross-linking by anti-IgM antibody induced an immediate increase in tyrosine phosphorylation on several proteins (indicating successful stimulation) and ERK was phosphorylated concurrently in wild-type cells (Fig. 3A). Whereas tyrosine phosphorylation was reduced to some extent, ERK phosphorylation appeared to be almost unaltered in Hsp90β+/− cells (Fig. 3A). By contrast, the amounts of tyrosine-phosphorylated proteins and phosphorylated ERK were markedly decreased in Hsp90β-deficient cells (Fig. 3A). These observations strongly suggest that loss of Hsp90β impairs a factor or factors involved in early phases of BCR signaling.

The BCR consists of the antigen-binding membrane Ig (IgM in DT40 cells) (45) and the signaling heterodimers of Igα and Igβ (43, 44). Western blotting revealed that the amount of the μ chain (i.e., the total amount of both μm and μs in cell lysates) was greatly reduced in Hsp90β-deficient cells, whereas Hsp90β+/− cells showed somewhat decreased but yet significant amounts of μ protein (Fig. 3B). These observations were corroborated by flow cytometric analysis of the expression of μ chains at the cell surface (i.e., μm) in Hsp90β+/− and Hsp90β−/− cells (Fig. 3C). Unexpectedly, μs protein was also drastically decreased in Hsp90β-deficient cells (Fig. 3B). Despite these findings, we observed only a slight, if any, reduction in μ transcripts in these mutant cells, as measured by Northern blotting (Fig. 3D) or by semi-quantitative RT-PCR (Fig. 3E).

These data have a strong resemblance to those of a recent study in which a profound depression in synthesis of IgM protein was detected in mouse B cells deficient in the transcription factor XBP-1, notwithstanding μ transcript levels similar to those of wild-type cells (46). It has been shown that mRNA of XBP-1 must be spliced to promote secretion of IgM and this splicing in turn depends on the production of μm (46, 47). We found, however, that splicing of XBP-1 mRNA in Hsp90β-deficient cells was normal (Fig. 3F). We therefore considered that the function of XBP-1 might be compromised. As EDEM is an XBP-1-dependent target gene (48, 49), we investigated the induction of EDEM transcription to assess the functionality of XBP-1. We found that levels of EDEM mRNA were comparable among the three cell clones (Fig. 3F); thus, we conclude that the function of XBP-1 is intact in the mutant cells.
The Synthetic Pathway of the μ Chain Is Affected by Depletion of Hsp90β – In the absence of any of the four BCR components, partially assembled BCR complexes are presumed to be degraded by the ER-associated degradation (ERAD) pathway (50). Therefore, the marked decrease in μ chains may indicate that assembly of the BCR in the ER is unsuccessful in Hsp90β-deficient cells and, consequently, that the μ chain is proteasomally degraded. To address this issue, DT40 cells were treated with the Hsp90 inhibitor; we found that expression of the μ chain was markedly decreased in wild-type cells treated with GA (Fig. 4A). Since μm has only three residues in the cytoplasm (51), it is more likely that Hsp90β interacts directly with Igα and/or Igβ to assist organization of the BCR (52). Northern blotting showed that the transcript levels of Igα and Igβ were unaltered in the Hsp90β-deficient cells (Fig. 4B). In addition, the expression levels of Igα protein were comparable among the three cell clones (Fig. 4B). Nevertheless, the amount of Igα was reduced in wild-type cells treated with GA (Fig. 4A). This reduction in the amount of Igα is apparently incompatible with the results of Northern and Western blotting (Fig. 4B); however, this observation may be accounted for by the fact that GA affects not only Hsp90β but also Hsp90α (30). Alternatively, because Igα was less sensitive to treatment with GA and reduced after the onset of reduction in μ chains (Fig. 4A), it is plausible that the μ protein is the primary target of GA and the loss of μ chains facilitates reduction in the protein level of Igα by an unknown mechanism. Thus, cells may have to induce ERAD immediately in order to eliminate a partially but unsuccessfully assembled BCR (i.e., the μ chain and Igα), which forms because GA treatment abruptly interferes with ongoing assembly of the BCR within these cells.

When Hsp90β-deficient cells were treated with the proteasome inhibitor MG132, however, we found that the expression levels of the μ chain remained low (data not shown); thus, proteasomal degradation did not explain the reduction in μ chain seen in Hsp90β-deficient cells (Fig. 3B) despite μ transcript level comparable to that of wild-type cells (Fig. 3D and E). Collectively, these observations suggest that de novo synthesis of the μ chain may cease in the mutant cells; therefore, we considered that depletion of Hsp90β might affect synthesis of the μ chain directly.

To investigate this possibility, we determined the turnover rate of the μ chain in wild-type and Hsp90β-deficient cells by treatment with the translation inhibitor CHX to stop the synthesis of μ chains, according to a previously described procedure (53). The half-life of the μ chain in wild-type and Hsp90β-deficient cells was 1.7 and 1.9 h, respectively (Fig. 4C and D); note that prolonged exposure was necessary to visualize μ chains of the mutant cells (Fig. 4C). Upon removal of CHX by washing, translation of the μ chain resumed efficiently in both cell clones and the proteins accumulated with kinetics similar to one another; the amounts of the μ chain in both two cell clones were recovered to 70–80% of the original level after a 4-h incubation following removal of CHX (Fig. 4C), albeit absolute amounts of the μ chain protein remarkably differ from one another (Fig. 4D, right).

Thus, it is clearly shown that the reduced expression level of the μ chain seen in the Hsp90β-deficient cells (Fig. 3B) is not due to lability of the μ protein but due to its low translation level. In addition, as well as the expression level (Fig. 4B), the turnover rate of Igα and its accumulation kinetics after removal of CHX in the mutant cells were comparable to those in wild-type cells (Fig. 4C and D); the half-life of Igα in wild-type and mutant cells was 0.7 and 0.6 h, respectively.

Taking these findings together, we conclude that the reduced level of protein synthesis in the Hsp90β-deficient cells is not a general phenomenon but specific to the μ chain.

Posttranscriptional regulation of the translation-initiation machinery is crucial for protein synthesis (54). YB-1 is involved in this regulatory process as a predominant component of messenger ribonucleoprotein particles, and it has been proposed that mRNA species that are silenced by YB-1 are activated by the specific association of YB-1 with YBAP1 (55) or by Akt-mediated phosphorylation of YB-1 (56). We found that all of these three proteins (YB-1, YBAP1 and Akt) were expressed in
Hsp90β-deficient cells in amounts similar to those observed in wild-type cells (Fig. 4E). Although the expression levels of YB-1 and YBAP1 were unchanged by GA treatment, Akt was significantly reduced in GA-treated wild-type cells (Fig. 4F); this finding is consistent with a previous study showing that Hsp90 plays an important role in regulating Akt kinase activity (57). To examine whether Akt is involved in the marked decrease in μ chains, we monitored its kinase activity by Western blotting with an antibody generated against a phosphorylated peptide corresponding to the consensus Akt target sequence (i.e., anti-phospho-Akt substrate antibody). Against expectation, we found that a 68-kDa protein, which has previously been demonstrated to be predominantly recognized by this antibody in DT40 cells (58), was accumulated by treatment with the phosphatase inhibitor calyculin A in the mutant cells to the level comparable to that in wild-type cells (Fig. 4G); thus, it appears that the kinase activity of Akt is not affected by depletion of Hsp90β. Next, we examined whether GA affects the interaction between YB-1 (which was ectopically expressed as a FLAG-tagged protein) and YBAP1 in wild-type cells, and found no effect on the amounts of YBAP1 that was co-immunoprecipitated with YB-1 (data not shown). Taken together, these results indicate that YB-1 is not involved in the decreased synthesis of μ chain proteins.

**BCR Signaling and Serum Activation Are Concurrently Abrogated in Hsp90β-deficient Cells**

Engagement of the BCR provokes a downstream signaling cascade: the protein tyrosine kinase Syk transfers the BCR signal to downstream components, including the Raf-1-MEK-ERK pathway (59-61). Notably, we found that the level of Syk phosphorylation (reflecting its activation) and its total content were not altered in Hsp90β-deficient cells (Fig. 5A). By contrast, a Syk substrate, BLNK (37, 61), was barely phosphorylated in the mutant cells, whereas its total amount remained substantially unchanged (Fig. 5A). These observations may indicate either that Syk has lost its kinase activity or that BLNK is incorrectly folded such that it is not recognized as a substrate by Syk, although this issue still remained to be resolved.

Next, we analyzed serum activation of a well-characterized Hsp90 client kinase, Raf-1 (16, 63, 64). Strikingly, on addition of serum, phosphorylation of MEK and also of ERK was hardly detected in Hsp90β-deficient cells (Fig. 5B). These data suggest that either Raf-1 or MEK is deteriorated in Hsp90β-deficient cells in terms of an enzyme or substrate, respectively; regardless of which protein is defective, the slower growth of Hsp90β+/− cells (Fig. 1E) may be substantially attributable to a defect in the Raf-1-MEK-ERK pathway, because proliferation can be induced by activation of this signaling pathway (43). To distinguish between the above two possibilities, Raf-1 was isolated from both wild-type and Hsp90β-deficient cells and its in vitro kinase activities were measured. Raf-1 from the mutant cells retained its ability to phosphorylate MEK (recombinant protein) on addition of serum (Fig. 5C); thus, abrogation of MEK but not Raf-1 is the most likely cause of the defect observed in MEK and ERK phosphorylation on serum activation. MEK has been reported to physically interact with Hsp90 (65). Our data reinforce this notion and strictly demonstrate that MEK requires the assistance of Hsp90 to fold correctly; thus, MEK is defined as a genuine Hsp90 client kinase.

Overall, we found that several components of cellular signaling are simultaneously impaired by depletion of Hsp90β. We consider that these multiple defects may faithfully represent a snapshot of the cellular functions of Hsp90.

**Elevation of the Hsp90 Expression Levels Rescues Defects of Hsp90β-deficient Cells**

To establish that the above defects resulted solely from loss of the Hsp90β gene, we reintroduced the Hsp90β gene into the Hsp90β-deficient cells. Stable cell lines were successfully isolated and two of them (β17 and β19) were examined. The expression levels of Hsp90β protein were a few- or several-fold increased in β17 or β19 cells, respectively, as compared with wild-type cells (Fig. 6A). These two Hsp90β-reconstituted cells produced μ chains in amounts comparable to those of wild-type cells (Fig. 6B). Cell surface expression of the μ chain in β17 cells was confirmed (Fig. 3C). Upon BCR stimulation, tyrosine-phosphorylated proteins and phosphorylation of BLNK, MEK and ERK were largely restored in β17 and β19 cells (Fig. 6C);
However, phosphorylation of ERK in the reconstituted cells seems to be recovered less efficiently. Although we do not know the precise reason for this partial recovery, it may be due to an inhibitory effect that is derived from an unidentified protein encoded by a gene neighboring Hsp90β cDNA which is stochastically integrated into chromosomes; alternatively, an excess amount of Hsp90β protein expressed in the reconstituted cells (Fig. 6A) may be harmful for restoring a defect in phosphorylation of ERK. Notwithstanding, it appears that all of the defects observed in Hsp90β-deficient cells may be substantially attributable to the lack of Hsp90β protein.

To verify that this restoration was specific to Hsp90β, the Hsp90α gene was introduced into the Hsp90β-deficient cells in place of the Hsp90β gene. The Hsp90α-introduced cells expressed marginally increased (less than 2-fold) levels of Hsp90α protein as compared with wild-type cells (Fig. 6A and data not shown). Although the precise reason for this small increase is uncertain, it is conceivable that expression of Hsp90α proteins is strictly controlled and that, unlike Hsp90β, its overproduction is prohibited in cells. Unexpectedly, despite the small increase in Hsp90α protein, the amount of μ chain was strikingly restored in two Hsp90α-introduced cell lines, α9 and α11 (Fig. 6B) and its surface expression on α9 cells was clearly observed (Fig. 3C). In addition, tyrosine phosphorylation of cellular proteins and phosphorylation of BLNK, MEK and ERK upon BCR stimulation were substantially restored in α9 and α11 cells (Fig. 6C). Because the surface expression level of μ chains in α9 cells seemed to be somewhat lower than that in wild-type and β17 cells (Fig. 3C), the expression of surface IgM may be facilitated more efficiently by Hsp90β than by Hsp90α; alternatively, the expression level of Hsp90α may be insufficient for this defect to be restored.

In summary we conclude that the defects observed in Hsp90β-deficient cells can be largely ascribed to a reduction in the total amount of Hsp90 (Hsp90α and Hsp90β) protein rather than to an abrogation of the Hsp90β-specific functions.

**DISCUSSION**

Hsp90 is relatively unique among molecular chaperones in that its client proteins seem to be restricted to a select group of proteins such as protein kinases and transcription factors (7-12). Nevertheless, because these client proteins presumably occur across the proteome, Hsp90 must be able to execute multifaceted functions, as indeed has been shown clearly in previous studies (18-20). Because each client exists in much smaller amounts than Hsp90 (i.e., 1-2% for cytosolic proteins), however, the whole spectrum of Hsp90 client proteins has not been fully delineated as yet and remains tantalizing (21, 22).

Against this background, we aimed to elicit the otherwise undetectable Hsp90-interacting proteins by lowering the Hsp90 expression level in cells. Ultimately, we used gene disruption to generate Hsp90β-deficient DT40 cells, which successfully afforded varied defects. These defects appeared concurrently but independent of each other; for example, synthesis of the μ chain and phosphorylation of BLNK (a substrate of Syk) and MEK (a substrate of Raf-1) were abrogated. In addition, these defects were mostly restored by either reintroducing the Hsp90β gene or introducing the Hsp90α gene. On the one hand, these data clearly demonstrate that the defects are caused largely by a reduction in total Hsp90 proteins and not by a lack of the Hsp90β-specific functions. Consequently, we conclude that the cellular amount of chicken Hsp90 can be reduced to a half [Hsp90α and Hsp90β proteins are comparably expressed in DT40 cells (data not shown)] without losing viability; however, the growth rate of Hsp90β-deficient cells slows as compared with normal cells, probably because of certain defects that are already explicit. On the other hand, we have still no evidence that indicates the existence of Hsp90α- and/or Hsp90β-specific client proteins. This fact may be beneficial for us, however, because the Hsp90β-deficient cells can be used as a resource to capture currently unknown client proteins in living cells regardless of the Hsp90 isoforms.

Organization and surface expression of the BCR are crucial for B cell development (51, 52). A partially assembled BCR lacking any of its four constituents is presumably retained in the ER and degraded by ERAD (50, 52, 66).
Although this hypothesis has not been conclusively proved as yet, molecular chaperones within the ER such as BiP and calnexin are involved in formation of the BCR complex (50, 67). Intriguingly, our results show that the level of μ chains in wild-type cells is markedly reduced by treatment with the Hsp90 inhibitor GA, even though Hsp90 is cytosolic. This finding strongly suggests that Hsp90 is critically involved in assembly of the BCR on the cytoplasmic side of the ER. Not only Hsp90 (68) but also cytosolic Hsc70 and Hsp40 (69) reportedly facilitate biogenesis of cystic fibrosis transmembrane conductance regulator, and Hsp90 has also been reported to mediate the maturation of another transmembrane protein, ErbB1, on the ER (70). Consequently, we propose that Hsp90 may contribute more generally than has been predicted so far to the maturation of nascent integral membrane proteins, which in turn may potentially include many but as-yet to be determined client proteins.

Our data demonstrate that a reduction in μ chain proteins in Hsp90β-deficient cells, which occurs posttranscriptionally (because the μ chain transcript level is unchanged), does not seem to be mediated by ERAD; thus, we consider that Hsp90 is involved directly in the synthetic pathway of the μ chain. It is conceivable that Hsp90 fills this role by assisting some as-yet unknown factor that promotes synthesis of μ chains. YB-1 is one such candidate factor that has been examined in this study. YB-1 is known to bind to mRNAs and to repress cap-dependent translation; YB-1 exhibits selectivity towards specific mRNA subsets, including transcripts encoding signal transducers (56). The translational repression activity of YB-1 is alleviated by its interaction with YBAP1 (55) or by its phosphorylation by Akt (56). In this study, however, it was found that the kinase activity of Akt is intact in Hsp90β-deficient cells and that GA treatment does not affect either the expression of YB-1 and YBAP1 or the interaction between these two proteins. Thus, YB-1 does not seem to be involved in the depression of μ chain synthesis.

XBP-1 may be another candidate factor, because it is intricately involved in biosynthesis of IgM, although its function remains obscure (47); furthermore, it has been proposed that XBP-1 may specifically control the efficacy by which nascent μ chains are targeted into the ER (46). Although a clear function of XBP-1 – namely, the induction of EDEM transcription (48, 49) – is not compromised in Hsp90β-deficient cells, this activity is executed by its spliced form but not by its unspliced form. It is therefore possible that unspliced XBP-1 contributes to synthesis of μ chains by an as-yet unknown mechanism, as has been proposed previously (47).

Depletion of Hsp90β impinged on the Raf-1-MEK-ERK pathway, in which MEK was abrogated. Although it has been reported that MEK interacts with Hsp90 (65), our study compellingly demonstrates that MEK requires the Hsp90 assistance for its correct folding; thus, it could be called a genuine Hsp90 client. Even though Akt and Raf-1 are Hsp90 client kinases, these proteins were not compromised in the Hsp90β-deficient cells. Another client IRE1 that is a transmembrane protein kinase/endoribonuclease (71) seemed to be intact in Hsp90β-deficient cells, an observation that is underpinned by the fact that XBP-1 is properly spliced in these cells as XBP-1 splicing is executed by IRE1α (72). Thus, reliance on the Hsp90 chaperoning function might be distinctly graded among Hsp90 client proteins. Although further reduction of the Hsp90 expression level may be too harmful for cells to survive, it will be both tantalizing and challenging to see whether more defects (i.e., client proteins) will be identified by gradually diminishing the Hsp90 content in Hsp90β-deficient cells. Irrespective, it should be emphasized that the Hsp90β-deficient cells on their own are potentially a good resource for finding previously unidentified Hsp90 clients, because in this study we have investigated only a limited number of the phenotypes that are elicited in these mutant cells.

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**FOOTNOTES**

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2Abbreviations used: BCR, B cell antigen receptor; CHX, cycloheximide; EDEM, endoplasmic reticulum degradation-enhancing α-mannosidase-like protein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GA, geldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione-S-transferase; HSF, heat shock factor; Hsp90, 90-kDa heat shock protein; Ig, immunoglobulin; μ, the IgM heavy chain; μm, a membrane-bound form of the μ chain; μs, a secreted form of the μ chains; RT, reverse transcription; XBP-1, X-box binding protein-1; YBAP1, a Y-box protein-associated acidic protein; YB-1, Y-box-binding protein 1.

**FIGURE LEGENDS**

**Fig. 1.** Generation of DT40 cells deficient in Hsp90β.  
A. Schematic showing part of the Hsp90β locus and targeting constructs. Exons (from exon 1 to part of exon 12) are indicated by filled boxes.  
B. Hsp90β-neo and Hsp90β-hisD were transfected in consecutive steps. Genomic DNAs from wild-type (wt), Hsp90β heterozygous mutant (β+) and homozygous mutant (β−) cells were hybridized with the probe indicated in A.  
C. Total RNAs from the indicated cells were subjected to Northern blot analysis. Expression of Hsp90α and Hsp90β mRNAs (upper) and ethidium bromide staining of total RNAs (lower) are shown.  
D. Expression of Hsp90α and Hsp90β proteins in the indicated cell lysates was analyzed by Western blotting.  
E. Growth curves of the indicated cells are shown. Results are the mean ± the standard deviation of five independent experiments.

**Fig. 2.** Heat shock response.  
A. Expression of HSF1-HSF3 proteins in wild-type (wt) and Hsp90β homozygous mutant (β−) cells was analyzed by Western blotting.  
B. Wild-type and Hsp90β-deficient cells were exposed to 43 °C for the indicated times ranging from 15 min to 4 h or were left untreated (C), and then the corresponding cell lysates were subjected to Western blotting with the indicated antisera. Actin serves as a loading control.  
C. Cell lysates (input) prepared from wild-type and Hsp90β-deficient cells exposed to 43 °C for 30 min were either mock treated (PPase -) or treated with lambda phosphatase (PPase +), and subjected to Western blotting with anti-HSF3 antibody.

**Fig. 3.** Cell surface expression of IgM is reduced in Hsp90β-deficient cells.  
A. Wild-type (wt), Hsp90β heterozygous mutant (β+/−) and homozygous mutant (β−) cells were stimulated with anti-IgM antibodies for the indicated times, and then the corresponding cell lysates were subjected to Western blotting with anti-phosphotyrosine (pY), anti-ERK (ERK) and anti-phosphorylated ERK (pERK)
antibodies. Molecular weight markers are shown at the right of the panel. B, The μ chain (μm plus μs) in cell lysates (μ) and the secreted μ chain (μs) of the indicated cells were analyzed by Western blotting. C, Cell-surface expression of IgM on wild-type, Hsp90β−/−, Hsp90β+/−, Hsp90β-reconstituted (β17) and Hsp90β-deficient/Hsp90α-introduced (α9) cells (filled graphs). Cells stained with the second antibody alone were used as negative controls (open graphs). Cells stained with the second antibody were subjected to Western blotting with the indicated antibodies after immunoprecipitation with anti-Syk and anti-BLNK antibodies, respectively. D, Total RNAs from the indicated cells were analyzed by Northern blotting to measure μ (μm plus μs) mRNA levels. E, RT-PCR analysis of cDNA [μm, μs and GAPDH (as a reference)] prepared from RNA obtained from the indicated cells. A three-fold dilution series of the cDNAs was used for semi-quantitative RT-PCR. F, RT-PCR analysis of cDNA [unspliced (U) and spliced (S) XBP-1, EDEM and GAPDH] prepared from RNA obtained from the indicated cells.

**Fig. 4.** Synthesis of μ chains is abrogated in Hsp90β-deficient cells. A, Wild-type cells were mock-treated or treated with GA for the indicated times and the corresponding cell lysates were immunoblotted as indicated. Actin serves as a loading control. B, Total RNAs from wild-type (wt), Hsp90β heterozygous mutant (β+/−) and homozygous mutant (β−/−) cells were subjected to Northern blot analysis. Expression of Igα in the indicated cells was determined by Western blotting. C, Wild-type and Hsp90β homozygous mutant cells were cultured in the presence (CHX) or absence (Mock) of CHX for the indicated times. The cells that had been cultured for 4 h in the presence of CHX were either washed to remove the inhibitor (Wash +) or untreated (Wash -) and the culture was continued for a further 2 or 4 h as indicated. The corresponding cell lysates were immunoblotted as indicated. Note that prolonged exposure was necessary to visualize μ chains in the mutant cells; the lane indicated by an asterisk [i.e., β−/−, Wash (-), 2 h] is obscure for unknown reasons. D, Left, the extent of the μ chain was quantified by scanning the blot (Fig. 4C) with the NIH image program. The original protein level of the μ chain in wild-type (closed circles) or Hsp90β-deficient (open squares) cells is set to 100% and data are expressed as a percentage of the remaining value. Right, the extents of the μ chain and Igα in cells that were cultured for 4 h after removal of CHX (indicated by Wash) were quantified by scanning the blot; the blot for the μ chain in the mutant cells was exposed for the same time as others (Fig. 4C and data not shown). The protein amounts of the μ chain and Igα in wild-type cells (closed circles) are set to 1 and relative amounts of the corresponding proteins in Hsp90β-deficient cells (open squares) are presented. E, Cell lysates from wild-type and Hsp90β-deficient cells were subjected to Western blotting with the indicated antibodies. F, Wild-type cells were mock-treated or treated with GA for the indicated times and the corresponding cell lysates were immunoblotted as indicated. G, Wild-type and homozygous mutant cells were mock treated (-) or treated with the serine/threonine protein phosphatase inhibitor calyculin A (+) for the indicated times to stabilize phosphorylated proteins and the corresponding cell lysates were immunoblotted with the antibody to phospho-Akt substrate. A molecular weight marker is shown at the left of the panel.

**Fig. 5.** BCR signaling and serum activation are abrogated in Hsp90β-deficient cells. A, Wild-type (wt) and Hsp90β homozygous mutant (β−/−) cells were stimulated with anti-IgM antibodies for the indicated times, and then the corresponding cell lysates were subjected to Western blotting with the indicated antibodies. Phosphorylated forms of Syk (pSyk) and BLNK (pBLNK) were detected with anti-phosphotyrosine antibodies after immunoprecipitation with anti-Syk and anti-BLNK antibodies, respectively. B, Wild-type and Hsp90β homozygous mutant cells were stimulated by the addition of serum for the indicated times, and the corresponding cell lysates were subjected to Western blotting with the indicated antibodies. Phosphorylated forms of MEK and ERK are indicated as pMEK and pERK, respectively. C, Raf-1 purified from wild-type and Hsp90β-deficient cells that were either serum-stimulated for 10 min (+) or left untreated (-) was immunoblotted (WB) with anti-Raf-1 antibodies to confirm equal protein immunoprecipitation (IP) (top), and then subjected to an in vitro kinase assay toward recombinant MEK-1 in the absence (-) or presence (+) of ATP, and then...
immunoblotting with anti-phosphorylated MEK antibodies (bottom).

**Fig. 6.** Increase in Hsp90 expression levels.  
**A,** Left, expression of Hsp90β protein in wild-type (wt), Hsp90β-deficient (β−) and Hsp90β-reconstituted (β17 and β19) cells was determined by Western blotting with anti-Hsp90β antibodies.  
**Right,** expression of Hsp90α protein in wild-type, Hsp90β-deficient and Hsp90β-deficient/Hsp90α-introduced (α9 and α11) cells was determined by Western blotting with anti-Hsp90α antibodies. Actin serves as a loading control.  
**B,** Expression of μ chain proteins in the indicated cell lysates was determined by Western blotting with anti-chicken IgM antibodies.  
**C,** The indicated cells were stimulated with anti-IgM antibodies for the indicated times, and the corresponding cell lysates were subjected to Western blotting with the indicated antibodies. Phosphorylated form of BLNK was detected with anti-phosphotyrosine antibodies after immunoprecipitation with anti-BLNK antibody. Tyrosine-phosphorylated proteins, phosphorylated forms of BLNK, MEK and ERK are indicated as pY, pBLNK, pMEK and pERK, respectively. Molecular weight markers are shown at the right of the panel.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Depletion of HSP90β induces multiple defects in B cell receptor signaling
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