Possible Role for Serine/Threonine Phosphorylation in the Regulation of the Heteroprotein Complex between the hsp90 Stress Protein and the pp60\textsuperscript{v-src} Tyrosine Kinase*

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The abundant, cytoplasmic 90-kDa heat-shock protein associates transiently with the Rous sarcoma virus oncogenic protein tyrosine kinase, pp60\textsuperscript{v-src}, directs its cellular trafficking and negatively regulates its kinase activity. Here we report that the serine/threonine phosphatase inhibitor, okadaic acid, destabilized the heat-shock protein 90-pp60\textsuperscript{v-src} complex in v-src-transfected cells. Concomitant with complex destabilization by okadaic acid, phosphorysorine was doubled and phosphothreonine was increased 20-fold in the heat-shock protein 90. Although phosphorylation of the total pool of immunoprecipitable pp60\textsuperscript{v-src} was unchanged, okadaic acid slightly increased phosphorysorine and phosphothreonine levels specifically in pp60\textsuperscript{v-src} bound to heat-shock protein 90. The low level of tyrosine phosphorylation in the pp60\textsuperscript{v-src} complex with heat-shock protein 90 was further decreased by okadaic acid. Interestingly, okadaic acid-stabilized hyperphosphorylation of the heat-shock protein 90-pp60\textsuperscript{v-src} complex lowered the level of pp60\textsuperscript{v-src} in cell membranes, the functional location for pp60\textsuperscript{v-src}. We suggest that serine/threonine phosphorylation of heat-shock protein 90 and/or pp60\textsuperscript{v-src} functions as a regulatory molecular trigger to release pp60\textsuperscript{v-src} from the chaperone complex at the inner surface of cell membranes.

Neoplastic transformation of avian or mammalian cells by the Rous sarcoma virus (RSV)\textsuperscript{1} results directly from the protein-tyrosine kinase activity of a single phosphoprotein, pp60\textsuperscript{v-src}, that is encoded by the v-src oncogene (1). The phosphorylation cascade initiated by pp60\textsuperscript{v-src} kinase activity at or near the plasma membrane is considered to be critical to the cell transformation process (reviewed in Refs. 2–4).

Although it has been established that nascent pp60\textsuperscript{v-src} protein is synthesized on cytosolic free polyribosomes (5), the mature kinase localizes predominantly to the cytoplasmic surface of the plasma membrane where it is anchored via a myristic acid residue (6). Additionally, some pp60\textsuperscript{v-src} is associated with perinuclear membranous structures (7, 8). During its intracellular trafficking, v-Src oncprotein is transiently associated with a constitutive cytosolic 90-kDa heat-shock or stress protein (hsp90) and a second host cell 50-kDa phosphoprotein of unknown function (9–11). Complexation with hsp90 sharply diminishes the intrinsic kinase activity of pp60\textsuperscript{v-src}, and both autophosphorylation of the v-Src protein at tyrosine 416 and phosphorylation of cellular substrate proteins on tyrosine are minimalized until after pp60\textsuperscript{v-src} is attached to the plasma membrane (12). Therefore, the formation of the hsp90-pp60\textsuperscript{v-src} complex tightly regulates the kinase activity of pp60\textsuperscript{v-src} and plays an obligatory biochemical function in cell transformation by this viral oncoprotein (13).

The aim of the present study was to further characterize the transient interaction of pp60\textsuperscript{v-src} with hsp90. Specifically, we were interested in the role of phosphorylation/dephosphorylation of either hsp90 or pp60\textsuperscript{v-src} in the molecular interactions of these two proteins. Toward this end, we attempted to manipulate the phosphate status of both proteins by treating v-src-transfected cells with okadaic acid (OA), a potent inhibitor of the serine/threonine protein phosphatases PP1 and PP2A (see Ref. 14 for review). OA has been shown to stabilize the hyperphosphorylation of many cellular proteins, notably retinoblastoma protein and p53 (15), and to reverse the neoplastic phenotypic characteristics of v-src-transformed 3T3 cells (16).

MATERIALS AND METHODS

Reagents and Drugs—Antibodies were as follows: mouse anti-hsp90 mAb (SPA-830; StressGen Biotechnologies, Victoria, BC), rabbit anti-hsp86 polyclonal antibody (mouse homolog of human hsp90 α; NeoMarkers, Freemont, CA), rabbit anti-mouse IgG (Cappel, Durham, NC), horseradish peroxidase-conjugated sheep anti-mouse mAb and horseradish peroxidase-conjugated donkey anti-rabbit mAb (Amersham Corp.), mouse anti-Src protein mAb and OA from Upstate Biotechnology Inc. (Lake Placid, NY), and anti-src dose 327 mAb from Oncogene Science (Uniondale, NY). Protein A-Sepharose CL-4B beads were purchased from Pharmacia Biotech Inc.; Affi-Gel 10 beads were from Bio-Rad; Geldanamycin (GA) was obtained gratis from the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD). Other chemicals used in this study were purchased from Sigma.

Tumor Cells—Temperature-sensitive v-src-transfected NIH 3T3 cells (Tsv-src3T3) were generated by subcloning v-src elements from pSR-XD2 into the expression vector ZipNeo followed by transfection into mycoplasma-free NIH 3T3 cells and were grown as described (17). Cells were treated with OA (10–100 nm) dissolved in Me\textsubscript{2}SO for various times (1–24 h); control cells were exposed to the Me\textsubscript{2}SO solvent. Cytosol and membrane fractions were prepared by scraping cells into 50 mm Tris-HCl, pH 7.5, 100 mm NaCl buffer, sonicking on ice, and then centrifuging at 100,000 \times g.

Immunoprecipitations and Immunoblotting—Tsv-src3T3 cells were washed twice in cold phosphate-buffered saline and then lysed on ice with TNEV lysis buffer (50 mm Tris-HCl, pH 7.5, 1% Nonidet P-40, 2 mm EDTA, 100 mm NaCl, 10 mm orthovanadate) with protease inhibi-
tors: 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml of leupeptin, and 20 μg/ml aprotinin. Lysates were scraped, transferred to microtubes, and cleared of debris by centrifugation at 16,000 x g for 20 min at 4°C. Protein concentrations were determined by the biocinchoninic acid method using bovine serum albumin as the standard (18). Aliquots of cell lysates were incubated at 4°C with primary antibodies followed by secondary antibodies conjugated to protein A-Sepharose beads to immunoprecipitate protein complexes. In some cases, hsp90-pp60v-src complexes were precipitated with GA beads prepared exactly as described (19). GA is a benzoxquinone ansamycin that specifically binds to hsp90, and after being coupled covalently to Affi-Gel microbeads, it efficiently precipitates hsp90-pp60v-src complexes from cell lysates (19). Proteins were eluted from the immune complexes by heating in reducing loading buffer (20), and after fractionating by SDS-PAGE, they were electrophoresed into nitrocellulose or to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). After blocking the membranes with 5% fat-free dry milk in 10 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM EDTA buffer, the protein bands were detected by Western immunoblotting followed by chemiluminescence visualization (21), using a commercial kit (Renaissance™, DuPont NEN).

Metabolic Labeling—T5v-src3T3 cells were preincubated with phosphate-free RPMI 1640 medium containing 10% dialyzed fetal bovine serum and 2 mM glutamine for 2 h. They were then incubated with 0.5 mCi of [32P]orthophosphate (specific activity 370 MBq/ml, 10 mCi/ml, Amersham) for 2 h (2 mM/100-mm plate), rinsed twice in 0.9% NaCl solution, and lysed in 2 ml of TNE SV buffer with protease inhibitors. Aliquots of cleared lysates or lysates immunoprecipitated with anti-anti-src protein mAb, anti-hsp90 antibodies, or GA beads were separated by SDS-PAGE, and radioactive phosphoprotein bands were detected by autoradiography of dried gels.

Phosphoamino Acid Analysis—The relative extent of phosphorylation of specific amino acids after OA treatment was determined by labeling cell phosphoproteins with [32P]orthophosphate as described above, followed by immunoprecipitation of pp60v-src and hsp90, separation of coprecipitated proteins by SDS-PAGE and electrophoresis to PVDF membranes. Individual radioactive bands were excised and hydrolyzed with 6 N HCl under nitrogen for 2 h at 110°C. Phosphoamino acids were separated by two-dimensional electrophoresis of gelatin thin-layer plates exactly as described (22, 23). The [32P]phosphate content of the three phosphoamino acids was measured with a Phosphorimage analyzer (Molecular Dynamics), and the intensities of the radioactive spots were measured by integration and corrected for any background signal by computer.

RESULTS

Association of hsp90 with the pp60v-src Oncoprotein—T5v-src3T3 cells express a mutant temperature-sensitive RSV pp60v-src protein that, like certain other temperature-sensitive mutants, associates with hsp90 to a greater degree than does wild-type RSV pp60v-src (9, 24). Immunoprecipitation of this mutant pp60v-src protein from these cells with an antibody to mAb coprecipitated hsp90 (actually hsp84 and hsp86 isoforms in mouse 3T3 fibroblasts), as determined by Western immunoblotting with a monoclonal antibody specific for the hsp90 family of stress proteins (AC88, 830 mAb, StressGen) (Fig. 1, lanes 1–3). The association of hsp90 and pp60v-src was verified when treated with alkaline phosphatase or the serine/threonine phosphatases PP1 and PP2A (data not shown). We then reasoned an alternative approach to destabilize the complex might be to inhibit cellular phosphatases with OA. Phosphatase inhibition would permit hyperphosphorylation of hsp90 or pp60v-src to occur, which could influence the interaction of the two proteins. To test this possibility, we treated T5v-src3T3 cells with OA prior to immunoprecipitating the complex from cell lysates with anti-src mAb (UBI) or with GA beads. Following separation of the coimmunoprecipitated proteins by SDS-PAGE and transfer to nitrocellulose membranes, Western immunoblots for both pp60v-src and hsp90 were performed. OA treatment (2 h) decreased the quantity of hsp90 that coprecipitated with v-src protein (Fig. 2, lanes 1–4), and likewise the amount of v-Src protein associated with hsp90 precipitated by GA beads was diminished (Fig. 2, lanes 5–8). At the same time, there were no major changes in the total cell levels of either hsp90 or pp60v-src within 2 h of OA treatment (compare bands from lysates; Fig. 2, lanes 10–13).

Phosphorylation of hsp90 and v-Src Proteins following OA Treatment—We next examined the effects of OA on the phosphorylation of both pp60v-src and hsp90 proteins. pp60v-src is phosphorylated mainly on serine and tyrosine residues, with a lesser amount of phosphothreonine (25), while hsp90 is constitutively phosphorylated exclusively on serine residues (26, 27). As shown in Fig. 3, there was a severalfold increase in the general [32P]phosphate labeling of phosphoproteins from cells after exposure to OA (lanes 1 and 2). After immunoprecipitation and separation of proteins by SDS-PAGE, we found that OA treatment greatly stimulated the incorporation of [32P]phosphate into hsp90 in the total pool of immunoprecipitable hsp90 (Fig. 3, lanes 5–8) as well as in the hsp90 that coprecipitated with pp60v-src (Fig. 3, lanes 3 and 4). In contrast, OA did not significantly increase the radioactivity associated with the total pool of immunoprecipitable pp60v-src (Fig. 3, lanes 3 and 4). However, phosphorylation of the pool of pp60v-src that was complexed with hsp90 was somewhat increased by OA treatment (compare panels B and C in Fig. 4). When the radioactive phosphate content of the fraction of v-Src protein that coprecipitated with hsp90 was normalized to the

![Fig. 1. Coimmunoprecipitation of hsp90 with pp60v-src. T5v-src3T3 cells were lysed with TNE SV buffer supplemented with protease inhibitors, and pp60v-src was immunoadsorbed from lysates (1 mg of total protein) with anti-src mAb (UBI). hsp90 was immunoprecipitated with SPA-830 anti-hsp90 mAb (StressGen), anti-hsp90 polyclonal antibody (NeoMarkers), or GA beads, with the appropriate secondary antibodies coupled to protein A-Sepharose beads. Proteins were resolved by reducing SDS-PAGE and transferred to nitrocellulose membranes. A, coprecipitated hsp90 was determined by Western immunoblotting using either anti-hsp90 or anti-hsp86 antibodies followed by secondary antibodies conjugated to protein A-Sepharose beads. Proteins were resolved by reducing SDS-PAGE and transferred to nitrocellulose membranes. B, coprecipitated pp60v-src was determined by probing Western blots with the 327 anti-src mAb followed by sheep anti-mouse IgG coupled to horseradish peroxidase with chemiluminescence detection. Lane 1, anti-src immunoprecipitation; lane 2, hsp90 standard, 1 μg; lane 3, SPA-830 anti-hsp90 immunoprecipitation; lane 4, anti-src immunoprecipitation; lane 5, 25 μg of cell lysate. B, coprecipitated pp60v-src was determined by probing Western blots with the 327 anti-src mAb followed by sheep anti-mouse IgG coupled to horseradish peroxidase with chemiluminescence detection. Lane 6, SPA-830 anti-hsp90 immunoprecipitation; lane 7, anti-hsp98 immunoprecipitation; lane 8, GA bead precipitation.]
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**Fig. 2. Effects of OA treatment on the hsp90-pp60\textsuperscript{v-src} heteroprotein complex.** Cells were treated with OA (10–100 nM) for 2 h; control cells were treated with an equivalent concentration of Me\textsubscript{2}SO (0.2%). Cells were lysed in TNE SV buffer with added protease inhibitors, and after protein assay, UBI anti-src mAb was used to immunoprecipitate pp60\textsuperscript{v-src} (lanes 1–4), and GA beads were used to precipitate hsp90 (lanes 5–8). Lane 9 shows 1 μg of hsp90 standard. Aliquots of lysates (25 μg of protein) from control and OA-treated cells are included for comparison (lanes 10–13) and show that both hsp90 and pp60\textsuperscript{v-src} levels were not decreased by OA within 2 h. Following SDS-PAGE separation of proteins, Western immunoblots for hsp90 and pp60\textsuperscript{v-src} were probed on nitrocellulose membranes using the SPA-830 anti-hsp90 mAb and the 327 anti-src mAb, followed by appropriate horseradish peroxidase-coupled secondary antibodies, and detection was by chemiluminescence.

**Fig. 3. OA alters the phosphorylation of both hsp90 and pp60\textsuperscript{v-src}.** Control and OA-treated cells were exposed to [\textsuperscript{32}P]orthophosphate for 2 h in phosphate-free medium containing 10% dialyzed calf serum prior to lysis with TNE SV buffer containing protease inhibitors; hsp90 and pp60\textsuperscript{v-src} were immunoprecipitated using appropriate antibodies as described under "Materials and Methods." Following resolution of proteins by reducing SDS-PAGE, the bands were transferred to PVDF membranes, and phosphoproteins were visualized by autoradiography. OA (100 nM) was added to the cells 30 min prior to [\textsuperscript{32}P]orthophosphate. Shown are cell lysates (10 μg of total protein, lanes 1 and 2), anti-v-src immunoprecipitations (lanes 3 and 4), anti-hsp90 immunoprecipitations (lanes 5 and 6), and GA bead precipitations (lanes 7 and 8) from 1.5 mg of cell lysates. Control samples are indicated by (−) and samples from cells treated with OA by (+). Molecular weight markers are shown on the left side of the lysate lanes.

**Fig. 4. Phosphoamino acid analysis of pp60\textsuperscript{v-src} and hsp90 following OA treatment.** hsp90 and pp60\textsuperscript{v-src} bands were cut from the PVDF membranes and treated as described under "Materials and Methods." The locations of unlabeled marker phosphoamino acids on the thin-layer plates were determined by ninhydrin staining and are indicated by the dotted lines. Radioactivity was measured by direct autoradiography and by scanning the plates with a PhosphorImager analyzer (Molecular Dynamics) capable of integrating radioactive areas and correcting for background values (see also Table I). Shown are phosphoamino acids from control pp60\textsuperscript{v-src} immunoprecipitated using UBI anti-src mAb (A), pp60\textsuperscript{v-src} coprecipitated with hsp90 from control cells using the SPA-830 anti-hsp90 mAb (B), and pp60\textsuperscript{v-src} coprecipitated from OA-treated cells with hsp90 using the SPA-830 anti-hsp90 mAb (C). Note that most of the radioactivity resides on phosphoserine in hsp90-associated pp60\textsuperscript{v-src}, while the general pool of pp60\textsuperscript{v-src} is predominantly labeled on phosphothreonine. D, hsp90 immunoprecipitated from control cells with the SPA-830 mAb; E, hsp90 immunoprecipitated from OA-treated cells with the SPA-830 mAb. It should be noted that the control hsp90 sample (D) includes two bands cut from the PVDF membrane, while the OA-treated hsp90 sample (E) represents only one band. This was done to permit comparison of hsp90 samples with similar radioactivity. The relative positions of the phosphoamino acids from the acid hydrolysates are indicated by PS (phosphoserine), PT (phosphothreonine), and PY (phosphotyrosine), and they are in the same positions in each panel.

amount of v-Src protein detected by Western blot, there was an 8-fold increase in [\textsuperscript{32}P]orthophosphate in the pp60\textsuperscript{v-src} band following OA treatment. This resulted because OA slightly increased the [\textsuperscript{32}P]orthophosphate content of v-Src protein in the heterocomplex as measured by autoradiography and, at the same time, greatly decreased the quantity of pp60\textsuperscript{v-src} that was associated with hsp90 measured by Western immunoblot.

Increased Serine and Threonine Phosphorylation Associated with hsp90-pp60\textsuperscript{v-src} Heterocomplex Instability—We next investigated which amino acids were hyperphosphorylated in the two proteins after OA treatment. Cells were exposed to OA for 30 min prior to and during a 2-h labeling period with [\textsuperscript{32}P]orthophosphate, and then hsp90 and pp60\textsuperscript{v-src} were immunoprecipitated, run on SDS-PAGE, and subjected to phosphoamino acid analysis. OA caused marked changes in the phosphoamino acids in both proteins compared with controls. The phosphoamino acid profile shown in Fig. 4A is representative of the phosphoamino acid analysis of the total pp60\textsuperscript{v-src} pool both before and after OA. Phosphotyrosine content in the general pool of pp60\textsuperscript{v-src} was unchanged by OA, as expected, and this was verified by separate anti-phosphotyrosine immunoblotting of the v-Src protein bands (data not shown). In the specific pool of pp60\textsuperscript{v-src} that co-immunoprecipitated with hsp90, the phosphoserine and especially phosphothreonine levels were much higher than in the total pool of immunoprecipitable pp60\textsuperscript{v-src}, and phosphorylation of these residues was slightly increased by OA treatment (Fig. 4B and C, and Table I). It should be noted that Table I shows the individual phosphoamino acids expressed as percentages of the total phosphoamino acids in each
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**Table I**

Relative phosphoamino acid content in v-Src protein and hsp90 after OA treatment

| Phosphoamino acid | Control pp60\(^v\)-src pool | pp60\(^v\)-src with hsp90 | Total hsp90 pool |
|-------------------|-----------------------------|---------------------------|-----------------|
| Phosphoserine     | 44.6                        | 38.6                      | 74.5            |
| Phosphothreonine  | ND*                         | ND                        | 10.0            |
| Phosphotyrosine   | 55.4                        | 61.4                      | 15.5            |

*ND, nondetectable PT levels were less than background values for an equivalent area on the TLC plate.

**Discussion**

The steady-state level and functional activity of pp60\(^v\)-src are determined in part by its interaction with the cellular chaperone phosphoprotein hsp90. Any compromise of the integrity of the pp60\(^v\)-src-hsp90 complex appears to lead to destabilization and loss of the v-Src protein (2, 3, 5, 13, 19). Because pp60\(^v\)-src coupled to hsp90 is underphosphorylated on tyrosine (5, 12) and has greatly diminished kinase activity (5), a biochemical/physiologic mechanism must exist for the dissociation of pp60\(^v\)-src from hsp90. This concept is reinforced by our observations that treatment of the pp60\(^v\)-src-hsp90 complex with various phosphatases did not dissociate the proteins.

**Table I**

Relative phosphoamino acid content in v-Src protein and hsp90 after OA treatment

- hsp90 and pp60\(^v\)-src were immunoprecipitated with appropriate antibodies from control- and OA-treated (100 nM for 2.5 h), \(\[^{32}\text{P}\]\)orthophosphateabeled cells. Analysis of the radioactive phosphoamino acids was by two-dimensional thin-layer electrophoresis as described under Materials and Methods.* Phosphoamino acid results are expressed here as a percentage of total measured phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) in each protein sample from a representative experiment. hsp90 was also precipitated with GA beads and was found to have a phosphoamino acid profile similar to the anti-hsp90 mAb immune-complexed hsp90 profile in both the control and OA groups.

**Figure 5.** Membrane and cytosol hsp90 and pp60\(^v\)-src levels after OA treatment. After treatment of cells with OA (100 nM) for 2 or 16 h, cell sonicates were prepared and then separated into cytosolic and membrane fractions by ultracentrifugation. Following SDS-PAGE, these fractions were analyzed by Western immunoblotting for both hsp90 and pp60\(^v\)-src. Lanes 1 and 4, control fractions; lanes 2 and 5, OA treatment for 2 h; lanes 3 and 6, OA treatment for 16 h. Different amounts of total protein from the cell fractions were used: hsp90 from cytosol, 50 \(\mu\)g (lanes 1–3); hsp90 from membranes, 100 \(\mu\)g (lanes 4–6); pp60\(^v\)-src from cytosol, 100 \(\mu\)g (lanes 1–3); and pp60\(^v\)-src from membranes, 10 \(\mu\)g (lanes 4–6).

Brief exposure of cells to the serine/threonine phosphatase inhibitor OA resulted in a concentration-dependent dissociation of the pp60\(^v\)-src-hsp90 complex without initially affecting the steady-state constituent level of either protein. However, within 16 h of OA exposure, the steady-state level of membrane-associated, but not the cytoplasmic, v-Src protein was markedly diminished. These data are consistent with destabilization of pp60\(^v\)-src following disruption or prevention of its association with hsp90 (13, 19).

Interestingly, OA did not affect the phosphorylation status of the total pool of pp60\(^v\)-src, but the phosphatase inhibitor did moderately increase serine and threonine phosphorylation of the pp60\(^v\)-src that coprecipitated with hsp90, while decreasing dramatically the amount of v-Src protein that coprecipitated with hsp90. The predominant amino acids that are phosphorylated in pp60\(^v\)-src are the tyrosine 416 autophosphorylation site (2–4, 25) and the serine 17 residue, known to be phosphorylated by cAMP-dependent protein kinase (28). In addition, v-Src protein is also phosphorylated on serine-12 and serine-48 by protein kinase C (29, 30). All of these phosphoserine residues are within the amino-terminal modular domain and close to the membrane-binding domain of pp60\(^v\)-src. It should be pointed out, however, that Resh and Erikson (31) have shown amino-terminal phosphorylation sites in pp60\(^v\)-src to be very labile, presumably because of their susceptibility to phosphoprotein phosphatases. Although the molecular consequences of protein sample. As previously reported by Brugge (12), there was less phosphotyrosine in pp60\(^v\)-src complexed with hsp90 than in the total pool of pp60\(^v\)-src, and phosphotyrosine in hsp90-complexed pp60\(^v\)-src was decreased further by OA (Fig. 4, B and C, and Table I).

In contrast to the rather modest changes in the phosphorylation of amino acids in pp60\(^v\)-src, serine phosphorylation in hsp90 was doubled by OA treatment, and the phosphothreonine content was increased approximately 20-fold (Fig. 4, compare panel D from two hsp90 bands and panel E from one hsp90 band). Expressed as a percentage of the total phosphoamino acid content per sample, phosphothreonine in hsp90 was increased 9-fold by OA (Table I). Although a trace of phosphotyrosine was found in the hsp90 band (0.7% of total phosphoamino acids), this was not appreciably altered by OA (0.5%) and may have been due to a contaminating, unresolved protein.

OA Treatment Decreases pp60\(^v\)-src Levels in the Membrane Fraction of Cells—If the trafficking of newly synthesized pp60\(^v\)-src to its functional location on the plasma membrane specifically depends upon complexation with hsp90, then disruption of the hsp90-pp60\(^v\)-src chaperone complex should ultimately result in a decrease in the membrane content of v-Src protein. We tested this concept by preparing cytosolic and membrane fractions from cells treated with OA at 100 nM for various times. These fractions were then directly examined for both hsp90 and pp60\(^v\)-src levels by Western blotting following separation by SDS-PAGE. Although OA had no measurable effect on pp60\(^v\)-src levels in either fraction after 2 h (Fig. 5, compare lanes 1 and 2 and lanes 4 and 5), OA markedly diminished the amount of membrane-associated pp60\(^v\)-src after 16 h (Fig. 5, lane 6). The decline in membrane-bound pp60\(^v\)-src most likely did not result from a decreased synthesis of the protein because cytosolic pp60\(^v\)-src was unaltered by OA at either 2 or 16 h (Fig. 5, lanes 1–3). hsp90 levels, likewise, were unchanged by OA in either cell fraction (Fig. 5, lanes 1–6).
phosphorylation of pp60src at amino-terminal serine residues is not well understood. Jove and Hanafusa (2) have suggested that transient phosphorylation within its amino-terminal modular domain might regulate pp60src kinase activity. Interestingly, Gottesman et al. (32) have shown that cAMP-dependent phosphorylation of pp60src at serine 17 in RSV-transformed cells stimulates the tyrosine kinase activity of pp60src in vitro and enhances the tumorigenicity of the transformed cells in nude mice.

On the other hand, deletion of serine 17, as well as mutation of serine 12, appears to have little influence on the transforming capability of pp60src (33). Analysis of several pp60src amino-terminal deletion mutants for their ability to stably associate with cellular hsp90 and pp50 suggests that these particular phosphoserine residues are not necessary for the association of pp60src with either hsp90 or pp50, although the mutations may negatively influence the stability of the complex (24). The majority of pp60src mutant proteins containing amino-terminal deletions, however, are more stably associated with hsp90 than is the wild-type V-Src protein (3, 24).

Although phosphoryserine is present in both the total pool of pp60src and in the fraction of pp60src associated with hsp90, phosphothreonine appears only in the hsp90-bound V-Src protein (see Fig. 4 and Table I). These data suggest that threonine phosphorylation in pp60src may be necessary for pp60src to associate with hsp90. OA treatment appears to only moderately increase the level of V-Src protein phosphorythreonine residues (150%). Very little is known about the role of threonine phosphorylation in the regulation of pp60src activity (2, 3), and the significance of this finding remains to be determined.

Both α and β hsp90 isoforms are constitutively phosphorylated by casein kinase II exclusively on serine residues and within a highly charged region of the protein between amino acids 222 and 290 (26, 34). A characterization of avian hsp90 purified in the presence of the serine phosphatase inhibitor, fluoride, revealed 6 mol of phosphate/mol of hsp90 dimer; these investigators also suggested that the phosphorylation state of hsp90 could affect its affinities for other proteins to which it binds (35). Recently, double-stranded DNA-activated protein kinase has been reported to phosphorylate two amino-terminal threonine residues unique to the hsp90 α isoform (36). Since our data demonstrate that both α and β hsp90 isoforms are hyperphosphorylated after OA (see Fig. 3), it is unlikely that this particular kinase is responsible for the increased phosphorylation of hsp90 we have observed. Additionally, since both casein kinase II (37) and the DNA-activated protein kinase (38) are localized within the nucleus, it is unlikely that they could be responsible for our findings, unless the nuclear membrane was damaged. It is more likely that a plasma membrane or cytosolic serine/threonine kinase is responsible for hsp90 hyperphosphorylation following OA treatment. We are presently investigating a number of purified kinases for their ability to phosphorylate hsp90 on threonine and to dissociate preformed hsp90-pp60src complexes. From a functional physiologic standpoint, it would be logical for the particular kinase responsible for hsp90 threonine phosphorylation to be localized at the inner surface of the plasma membrane, where it could promote the release of pp60src to be anchored to the membrane.

The dramatic, 20-fold increase in hsp90 phosphothreonine content following brief OA exposure, compared with the more modest increase in hsp90 phosphoserine, suggests a very robust, specific, and tightly controlled phosphorylation/dephosphorylation cycle for this amino acid in hsp90. Such a rapid and controlled cycle presumably has essential biological significance. We propose that the level of hsp90 threonine phosphorylation regulates the hsp90-pp60src complex stability. This model is similar to one advanced for the regulation of retinoblastoma protein with either the adenovirus E1A protein or the cellular E2F-1 transcription factor whereby hyperphosphorylation of retinoblastoma protein by cyclin-dependent serine/threonine kinases causes E1A or E2F proteins to release from the complex within the nucleus, allowing for transactivation of genes involved in cell proliferation (39, 40).

It will be intriguing to determine whether hsp90 hyperphosphorylation on threonine residues caused by OA treatment disrupts other hsp90-protein complexes, such as those formed with the glucocorticoid receptor or the serine/threonine kinase c-Raf-1 (41, 42). Perhaps not surprisingly, hsp90 association is required for stability and proper intracellular targeting of c-Raf-1 protein (43). Preliminary data in our laboratory also suggest that c-Raf-1 protein is, in fact, rapidly depleted from cells following OA treatment, conceivably as a result of OA-induced c-Raf-1-hsp90 complex instability.2

In summary, we have shown that brief OA treatment of v-src-infected 3T3 cells results in modest increases in the phosphorylation of serine and threonine in pp60src bound to hsp90 and serine residues in hsp90. At the same time, OA stabilizes the hyperphosphorylation of hsp90 threonine residues. Subsequent to these phosphorylation changes, the hsp90-pp60src complex is destabilized, and eventually pp60src is lost from cell membranes. We propose that a phosphorylation/dephosphorylation cycle, likely involving hsp90 threonine residues, functions as a molecular trigger that initiates the assembly and disassembly of the hsp90-pp60src heteroprotein complex, thereby directing the cellular trafficking of pp60src and ultimately regulating cell transformation.

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