The 28-kDa Protein Whose Phosphorylation Is Induced by Protein Kinase C Activators in MCF-7 Cells Belongs to the Family of Low Molecular Mass Heat Shock Proteins and Is the Estrogen-regulated 24-kDa Protein*

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We have previously reported the presence of a 28-kDa protein in human mammary adenocarcinoma MCF-7 cells, whose phosphorylation by phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and permeant diacylglycerol 1,2-dioctanoyl-sn-glycerol was correlated to growth arrest induced by the protein kinase C (PKC) activators. We now investigate the possible identity of this protein with the estrogen-regulated "24-kDa" protein shown as related to the mammalian heat shock protein 27 (Fuqua, S. A. W., Blum-Salingaros, M., and McGuire, W. L. (1989) Cancer Res 49, 4126-4129). 32P-Labeled 28-kDa protein from TPA-treated MCF-7 cells was immunoprecipitated with a 24-kDa-specific monoclonal antibody. Immunoblots from cell extracts fractionated by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis demonstrated that TPA induced the conversion of a 28-kDa isoform "a" (pl 6.7) to a more acidic isoform "b" (pl 6.2). Two-dimensional gel analysis of 32P-labeled 28-kDa protein revealed that phosphorylation of 28-kDa protein, heat shock induced both synthesis (increase of isoform a) and phosphorylation (conversion of isoforms a to b) of the protein. 32P labeling of MCF-7 cells allowed demonstration of the presence of an extra phosphoisoform "c" (pl 5.9) upon TPA as well as heat shock treatment. When cells were pretreated with the bisindolylmaleimide GF109203X, a selective inhibitor of PKC, the heat shock-induced phosphorylation was unchanged, while the TPA effect was almost abolished, suggesting that the heat shock-activated protein kinase was very likely different from PKC. However, peptide mapping of the 28-kDa phosphoprotein suggested identical sites of phosphorylation upon TPA and heat shock stimulation. Partial amino acid sequencing of the 28-kDa protein revealed identity with both the 24-kDa protein and the mammalian HSP27. The fact that estrogens and PKC, respectively, regulate expression and phosphorylation of this 24/28-kDa protein strongly argues for its key role in MCF-7 cell proliferation and differentiation.

Heat shock proteins (HSPs) consist of a number of highly conserved proteins that are synthesized by all pro- and eukaryotic organisms in response to environmental stress including hyperthermia (1-3). Although these proteins are thought to play primarily a protective role in cells subjected to high temperature and other stresses, several lines of evidence suggest that they are involved in a number of other cell functions (2, 3). High molecular weight HSPs including HSP90, HSP70, and HSP60 have been studied with the greatest details and demonstrated as molecular "chaperones" in protein-protein interactions (1, 3). For example, HSP90 has been shown to be associated with steroid receptor (4) or with tyrosine kinases encoded by oncogenes (5). HSP70 and HSP60 have been implicated in protein folding, unfolding, oligomerization, and translocation (2, 3). The low molecular weight HSPs are much less understood. Like the other families of heat shock proteins, they are involved in thermotolerance (6) and very likely in other cell functions, including cell growth and differentiation, for the following reasons. (i) Although their synthesis is stress-induced, they have been shown as constitutive proteins that are expressed at specific stages of development at normal temperatures (1). (ii) They are phosphorylated in response to a wide variety of stimuli including growth factors (7, 8).

Protein kinase C (PKC) is believed to play a key role in transmembrane signaling leading to cell differentiation and proliferation (9). Characterization and identification of endogenous proteins phosphorylated by PKC activators has received particular attention. We have previously demonstrated the presence of a 28-kDa protein in human mammary adenocarcinoma cell line MCF-7 (10), whose phosphorylation by TPA and Dic was closely correlated to growth arrest

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank**‡EMBL Data Bank with accession number(s) X84079.

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1 The abbreviations used are: HSP, heat shock protein; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; Dic, 1,2-dioctanoyl-sn-glycerol; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.
Identification of 28-kDa Phosphoprotein in MCF-7 Cells

Materials and Methods

Reagents

TPA, protease V8 (from Staphylococcus aureus), diphenyldimethylchloride-treated trypsin, keyhole limpet hemocyanin, and Tween 20 were purchased from Sigma. Staurosporine was from Calbiochem. Acrylamide and bisacrylamide were from Bio-Rad. Ammonium and protein A-Sepharose CL-4B were obtained from Pharmacia LKB Biotechnology Inc. Nitrocellulose (0.45 pm) was purchased from Schleicher & Schuell. [32P]Phosphoric acid, [3H]leucine, [14C]glucose, and [15N]arginine were purchased from Schuel. [32P]Phosphoric acid, [3H]leucine (146 Ci/mmol), [14C]glucose, [15N]arginine, and [15N]lysine were purchased from Amersham Corp. Thin-layer cellulose plates were obtained from Merck. Normal mouse serum and rabbit anti-mouse IgG were purchased from Nordic Immunological Laboratories. All other reagents were of reagent grade.

Cell Culture—MCF-7 cells were adapted to grow in the absence of serum (17). Cultures were maintained at 37 °C in RPMI 1640 medium, supplemented with 2 g/liter sodium bicarbonate, pH 7.3, 2 mM l-glutamine, 1 mM insulin, and 0.1 mM transferrin.

For protein phosphorylation studies, subconfluent cultures (0.5-1×10^6 cells/35-mm dish) were washed in PBS, incubated with 0.1 M Tris-HCl, pH 6.7, and 0.5% (v/v) glycerol containing 0.5% (v/v) B-mercaptoethanol, and then boiled for 3 min at 100 °C.

One-dimensional Gel Electrophoresis—Following [32P]leucine or [3H]leucine labeling, cells were rapidly washed with 1 ml of cold phosphate-buffered saline (PBS), then homogenized in 0.1 ml of electrophoresis sample buffer containing 60 mM Tris-HCl, pH 6.7, 2% (w/v) SDS, 8% (v/v) glycerol, 2% (v/v) B-mercaptoethanol, and 0.005% bromphenol blue, then boiled at 100 °C for 5 min. Proteins were fractionated by electrophoresis on 4.5-12% (w/v) discontinuous SDS-PAGE.

Two-dimensional Gel Electrophoresis—Alternatively, samples were analyzed by two-dimensional IEF/SDS-PAGE. After washing as indicated above, cells were solubilized in 0.1 ml of electrophoresis sample buffer containing 30 mM Tris-HCl, pH 6.7, 1% (w/v) SDS, 5% (v/v) glycerol, 1% (v/v) B-mercaptoethanol, and 0.005% bromphenol blue. After heating at 100 °C for 5 min, the samples were cooled and then adjusted to contain 9.5 M urea, 3.7% (v/v) Nonidet P-40, and 2% (w/v) Amphotolines in a final volume of 0.3 ml. Two-dimensional IEF/SDS-PAGE was carried out as described by O'Farrell (18). The proteins were visualized using 1 ml of 3.5-4.5% Ampholines in the IEF dimension and 4.5-10% (w/v) SDS-PAGE in the second dimension.

Immunoblotting—For immunological studies, one- or two-dimensional gels from unlabeled MCF-7 cells were electrophoresed to nitrocellulose membranes for 90 min at 25 V. Western blots were blocked for 1 h in PBS, pH 7.3, containing 5% (w/v) casein and 0.5% (v/v) Tween 20. Blots were then subsequently incubated with (i) 24-kDa-specific monoclonal antibody C11 (dilution 1/500), (ii) rabbit anti-mouse IgG (dilution 1/100) in PBS containing 0.5% gelatin (w/v), 0.05% (w/v) bovine serum albumin, and 0.5% (v/v) Tween 20, and (iii) 0.1 μl/ml 125I-protein A in PBS containing 0.1% (v/v) Tween 20. Alternatively, blots were incubated with the polyclonal anti-HPSE-27 peptide antibody (dilution 1/200). In this case, the rabbit anti-mouse IgG step was omitted.

Immunoprecipitation—32P-Labeled MCF-7 cells were incubated for 2 h in the absence or the presence of 100 ng/ml TPA. After cell washing in cold PBS, cells were rapidly harvested in PBS, and cell pellets were homogenized with Dounce in 0.1 ml of 20 mM Tris-HCl, pH 7.4, 10% (v/v) glycerol, 1 mM EDTA, 10 μg/ml leupetin, and 5 mM β-mercaptoethanol. Homogenates were centrifuged for 1 h at 105,000×g and cytosols incubated for 2 h at 20 °C with protein A-Sepharose CL-4B previously coupled to rabbit anti-mouse IgG (incubation for 2 h at 20 °C; antibody dilution, 1/10) and 24-kDa-specific monoclonal antibody (incubation for 2 h at 20 °C; dilution, 1/50). The antigen-antibody complexes were then extracted using 0.1 ml of the electrophoresis sample buffer, heated at 60 °C for 10 min, and analyzed by multidimensional SDS-PAGE.

Peptide Mapping of 28-kDa Protein Isolomers—One-dimensional peptide mapping was carried out according to Cleveland et al. (19) using protease V8 from S. aureus. After 32P-labeled MCF-7 cells were exposed to TPA or heat shock and fractionated on two-dimensional IEF/SDS-PAGE, the respective phosphoforms b and c of 28-kDa protein were excised from the gels and directly loaded on 4.5-15% SDS-PAGE and then overlaid with protease V8 (200 ng). Digestion proceeded in the stacking gel during the subsequent electrophoresis.

For two-dimensional peptide analysis, pieces of gel containing the required b and c phosphoisoforms were excised, mixed, and incubated overnight with 12.5 μg of diphenylcarbamyl chloride-treated trypsin in 0.5 ml of 50 mM ammonium bicarbonate, pH 8. Lyophilized phosphopeptides were dissolved in 5% acetic acid and then applied to thin-layer cellulose plates and electrophoresed (590 V for 25 min) using a pH 4.4 buffer containing 15% aqueous, 2% pyridine, 4% acetic acid, and 73% water and 30% acetic acid. Following chromatography, a piece of gel was excised in 37.5% butanone, 7.5% acetic acid, 25% pyridine, and 30% water.

Autoradiography—Dried 3H- and 32P-labeled gels, thin-layer cellulose plates, or 32P-labeled blots were exposed to Hyperfilms-MP for 48-72 h in Kodak X-Omat AR cassettes equipped with intensifying screens. Before drying, 3H-labeled gels were treated with Amplify.

Polyclonal Anti-HPS27 Peptide Antibody—An oligopeptide corresponding to the carboxyl-terminal end of human HSP27 (residues 184-193: TFESQALGG) was synthesized by the standard solid phase method using an Applied Biosystems 430A peptide synthesizer. The following side chain protecting groups were used on the t-Butoxycarbonyl amino acids: Tosyl (Tos), benzyl ester (Bz), and benzyl ester (Glu). Cleavage of the peptide from the resin and removal of side chain protecting groups were performed using 7 M hydrochloric acid (93% recovery).

Peptide purity was checked by reverse phase HPLC analysis using an RF900 C8 column with a linear acetonitrile-0.1% trifluoroacetic acid gradient. Molecules were identified using electrospray ionization mass spectrometry (M+H, 1065.4) using a ZAB-HS double focusing spectrometer (VG analytical, Manchester, UK). The peptide (5 mg) was coupled to keyhole limpet hemocyanin (5 mg) in 5 ml of 0.1 M NaHCO3, pH 8.6, and 0.005% glutaraldehyde, and the mixture was incubated for 24 h at 4 °C with 10% Freund's adjuvant. The conjugate was then injected subcutaneously into rabbits (0.25 mg of peptide). After 4 weeks, animals were boosted every 2 weeks for 6 weeks with the same amount of peptide in.
Identification of 28-kDa Phosphoprotein in MCF-7 Cells

RESULTS

Immunodetection of the 28-kDa Phosphoprotein in MCF-7 Cells—Previous data indicated that the 28-kDa protein phosphorylated in MCF-7 cells under TPA or DiC8 stimulation was very likely a member of the low molecular weight HSP family (12). We wondered whether this protein could be related to the estrogen-regulated 24-kDa protein reported by McGuire and co-workers (14) and further demonstrated as homologous to the mammalian HSP27. To assess the possible identity of the respective 28- and 24-kDa proteins, we performed immunoprecipitation studies with a 24-kDa-specific monoclonal antibody (C11) following stimulation of 32P-labeled MCF-7 cells with the PKC activator TPA. Immunoprecipitates were fractionated on SDS-12% PAGE, and the gels were submitted to autoradiography. Fig. 1A shows that the C11 antibody immunoprecipitated the 28-kDa protein phosphorylated upon TPA stimulation of cells. The specificity of this immunoprecipitation was assessed by using a normal mouse serum instead of the 24-kDa-specific antibody. Immunodetection of the 28-kDa protein was also performed after Western blotting of unlabeled MCF-7 cell extracts fractionated by SDS-PAGE (Fig. 1B) or by two-dimensional IEF/SDS-PAGE (Fig. 1C). While TPA stimulation of cells did not increase the amount of the specifically recognized 28-kDa protein (Fig. 1B), it clearly induced its phosphorylation, leading to the conversion of the isoform a (pI = 6.7) to the more acidic isoform b (pI = 6.2).

Two-dimensional IEF/SDS-PAGE Analysis of [3H]Leucine- and 32P-Labeled 28-kDa Protein Upon TPA or Heat Shock Treatment of MCF-7 Cells—Previous data suggested that heat shock treatment of MCF-7 cells could induce both synthesis and phosphorylation of the 28-kDa protein (12). To further characterize this phenomenon and to compare it to the TPA effect, we performed two-dimensional IEF/SDS-PAGE analysis of [3H]leucine- and 32P-labeled MCF-7 cells following TPA and heat shock treatment (Fig. 2). [3H]Leucine labeling showed that TPA induced phosphorylation, i.e. conversion of isoform a (pI = 6.7) to isoform b (pI = 6.2), but not synthesis of the 28-kDa protein (no increase of isoforms a + b from TPA-treated cells versus isoform a from control cells) while heat shock induced both phosphorylation (appearance of isoform b) and synthesis (increase of isoform a) of the 28-kDa protein. The sensitivity of 32P labeling was allowed to demonstrate the presence of two phosphoisoforms, b (pI = 6.2) and c (pI = 5.9), upon TPA as well as heat shock treatment. A small amount of phosphoprotein b was visible in the control confirming the two-dimensional pattern observed in Fig. 1C where isoform b was weakly present in the control.

To investigate the nature of the protein kinase involved in the heat shock-induced phosphorylation of the 28-kDa protein, we studied the protein phosphorylation pattern observed when cells were pretreated with staurosporine, a compound that is believed to be a potent PKC inhibitor. As shown in Fig. 3, in such staurosporine-treated cells, the TPA-induced 28-kDa protein phosphorylation was markedly inhibited with a total disappearance of the phosphoisoform c and a marked reduction of the 32P labeling of isoform b. Staurosporine also decreased, although at a lesser extent, the heat shock-induced 28-kDa protein phosphorylation. As staurosporine has been recently reported to inhibit other protein kinases than PKC (20, 21), we performed identical studies in the presence of the bisindolylmaleimide GF109203X, a potent and more selective inhibitor of PKC (22). Fig. 4 shows that, in GF109203X-treated cells, the effect of TPA on 28-kDa protein phosphorylation was almost abolished while heat shock-induced phosphorylation was unchanged, suggesting that the heat shock-activated protein kinase is very likely different from PKC. Such a hypothesis is further reinforced by the fact that TPA but not heat shock induced the phosphorylation of a 80-kDa/PI 4.5 protein, the selectivity of GF109203X being demonstrated by the disappearance of this PKC-specific protein phosphorylation in cells treated with this compound.

To investigate whether TPA and heat shock induced phosphorylation of the 28-kDa protein at the same sites, protease V8 peptide maps were performed from the individual b and c isoforms.
Identification of 28-kDa Phosphoprotein in MCF-7 Cells

Fig. 3. Effect of MCF-7 pretreatment with staurosporine on 28-kDa protein phosphorylation. 32P-Labeled MCF-7 cells were incubated in the absence (Normal cells) or in the presence (Staurosporine-treated cells) of 300 nM staurosporine and submitted to TPA or heat shock (HS) treatment. Cell lysates were subjected to two-dimensional electrophoresis. Only portions of the respective autoradiographs corresponding to the 28-kDa isoforms a, b, and c are represented. Cont., control.

Fig. 4. Effect of the selective PKC inhibitor GF109203X on protein phosphorylation pattern. 32P-Labeled MCF-7 cells were incubated in the absence (Normal cells) or in the presence (GFX-treated cells) of 5 μM GF109203X and submitted to TPA or heat shock (HS) treatment. Cell lysates were subjected to two-dimensional electrophoresis. Portions of autoradiographs corresponding to 28-kDa phosphoisoforms b and c as well as the 80-kDa protein are shown. Cont., control.

phosphoisoforms obtained from two-dimensional IEF/SDS-PAGE. Fig. 5A shows identical phosphopeptide maps for both isoforms b and c from the 28-kDa protein phosphorylated upon TPA as well as heat shock treatment of MCF-7 cells. To confirm this finding, we performed two-dimensional peptide analysis following trypsin digestion of b and c 28-kDa isoforms upon phosphorylation by TPA and heat shock. Fig. 5B again shows similar patterns with two major spots observed in each case.

Subcellular Localization of the 28-kDa Protein upon TPA and Heat Shock Treatment of MCF-7 Cells—Low molecular weight HSPs have been previously shown to translocate from cytosol to nuclear compartment during heat shock exposure of cells (23–25). To further compare the effects of TPA and heat shock on 28-kDa protein, we investigated the cellular localization of the protein upon the two distinct treatments. Fig. 6 illustrates an immunodetection of the 28-kDa protein in the respective cytosolic and nuclear fractions using a polyclonal antibody raised against a synthetic peptide derived from the HSP27 amino acid sequence. While heat shock induced the expected redistribution of 28-kDa protein from cytosol to nuclear pellet, TPA did not change the initial cytoplasmic localization of the protein. The heat shock-induced translocation of the 28-kDa protein concerned, at least in part, the phosphorylated protein as the 32P labeling of 28-kDa protein followed the redistribution pattern observed when the total amount of the protein was measured (not shown).

Partial Sequencing of the 28-kDa Protein—Attempts to obtain protein sequence information of the 28-kDa isoform a from two-dimensional gel transferred to polyvinylidene difluoride Immobilon membranes were unsuccessful, very probably because the amino terminus of the protein was blocked. Thus, to obtain internal sequence information, we digested the protein in the gel matrix after isolation on two-dimensional gels. The resulting peptides were separated on HPLC and three peaks were selected for sequencing. The material eluting in two of those peaks gave unique sequences corresponding to the peptides 13–20 and 38–46 of the human HSP27 (16). The material eluting in the third peak showed that it was a mixture of three peptides. The deduced sequences could be assigned to peptides 97–110, 141–154, and 172–186 of HSP27. In other words, the peptide sequences obtained were found to be identical to the amino acid sequences of 24-kDa protein (stress-responsive protein 27) and human HSP27 (Fig. 7).
Identification of 28-kDa Phosphoprotein in MCF-7 Cells

FIG. 6. Subcellular localization of 28-kDa protein upon TPA and heat shock treatment of cells. Unlabeled MCF-7 cells were incubated in the absence (Cont) and in the presence of 100 ng/ml TPA (top) or submitted to heat shock (HS) (bottom). Cells were homogenized with Dounce in 20 mM Tris-HCl, pH 7.4, 10% glycerol, 1 mM EDTA, 10 μg/ml leupeptin, and 5 mM β-mercaptoethanol. 2,000 χ g pellets (P) and 105,000 χ g supernatants (S) were subjected to SDS-PAGE fractionation. Western blots were probed with the polyclonal antibody raised against the HSP27 peptide.

FIG. 5. Peptide maps of phosphoisoforms b and c of the 28-kDa protein. 32P-Labeled isoforms b and c from TPA- and heat shock (HS)-treated cells were submitted to limited proteolysis with 200 ng of protease V8 (A) or extensively digested with 12.5 μg of diphenylcarbamyl chloride-treated trypsin (B). Shown are the peptide maps obtained after one-dimensional SDS-PAGE (A) or two-dimensional electrophoresis/chromatography (B) fractionation and autoradiography. In B, the sample application point is indicated by the x. The horizontal arrow indicates the direction of electrophoresis to the cathode, while the vertical arrow indicates the ascending chromatography.

DISCUSSION

Growth arrest of MCF-7 cells by PKC activators TPA and DiC8 has been correlated previously to the phosphorylation of a 28-kDa endogenous protein (11). In the present study, we have identified definitely this protein as a member of the low molecular weight HSP family. [3H]Leucine labeling of MCF-7 cells (Fig. 2) demonstrated that heat shock induced both synthesis (increase of isoform a) and phosphorylation (appearance of isoform b) of 28-kDa protein while TPA caused only its phosphorylation (conversion of isoform a to isoform b). 32P labeling of MCF-7 cells was allowed to detect a second phosphoisoform of the 28-kDa protein, isoform c in addition to isoform b, confirming the capability of both TPA and heat shock to induce the phosphorylation of 28-kDa protein. In several but not all experiments, the labeling of isoform c was more pronounced after heat shock than upon TPA exposure (Figs. 3 and 4). However, it is difficult to make conclusions about this phenomenon because of the distinct procedures used. 32P labeling of cells was either performed for 1 h at 37 °C followed by 1 h at 42 °C (heat shock-induced phosphorylation) or 2 h at 37 °C, TPA being added the last 20 min (TPA-induced phosphorylation). When 32P labeling of cells was carried out for 2 h at 37 °C following 1 h of heat shock at 42 °C, isoform c was much less evident (not shown), suggesting that this isoform is likely subject to rapid dephosphorylation or degradation. Whether the poor labeling of isoform c following TPA treatment of cells reflects the stimulation by the phorbol ester of specific phosphatase or protease for 28-kDa c remains to be established. Alternatively, the heat shock-dependent phosphorylation of the 28-kDa protein might involve a protein kinase distinct from PKC, inducing a more pronounced degree of phosphorylation of 28-kDa protein with the appearance of isoform c. In any case, the stoichiometry of phosphorylation of the 28-kDa protein was difficult to assess pronounced degree of phosphorylation of 28-kDa protein with the appearance of isoform c. In any case, the stoichiometry of phosphorylation of the 28-kDa protein was difficult to assess phosphorylation at similar sites, as the phosphopeptide maps of the 28-kDa isoforms b and c obtained upon TPA as well as heat shock treatment were identical (Fig. 5, A and B). As two-dimensional peptide analysis following trypsin diges-
tion of b showed two major spots, it is tempting to postulate the presence of two phosphorylation sites in this isoform. The identity of b and c peptide maps is rather intriguing as c is supposed to contain an additional phosphate with regard to b. Such a result could suggest that the putative extra phosphorylation site in c is very close to one of the sites phosphorylated in b. This point needs further investigation.

The other important feature of our study is the demonstration that the 28-kDa phosphoprotein was the estrogen sequen-
ted 24-kDa peptide (b). First, the specific monoclonal antibody C11 raised against this 24-kDa protein recognized the 28-kDa protein phosphorylated upon MCF-7 cell stimulation by TPA (Fig. 1). There was a striking coincidence in the respective PI values of the phosphorylated a and phosphorylated b isoforms of the immunodetected 28-kDa protein when compared with those found for the 32P- and [3H]leucine-labeled protein (Figs. 2-4). Second, the 28-kDa protein was also recognized by a specific polyclonal antibody raised against a synthetic peptide derived from the carboxy-terminal amino acid sequence of HSP27. McGuire and co-workers have reported previously that the carboxy-terminal amino acids deduced from the partial cDNA encoding the 24-kDa protein showed total homology with HSP27 previously cloned from a human genomic library (16).

Finally, our partial sequencing of the 28-kDa protein demonstrated identity between the peptides sequenced and the corresponding sequences of both HSP27 and 24-kDa protein. Although discrete differences in the whole amino acid sequence of 28- and 24-kDa proteins cannot be totally ruled out, it is more probable that both proteins are in fact the same molecule.

Our study also demonstrates that the 28-kDa protein is susceptible to nuclear targeting under heat shock treatment of MCF-7 cells, this subcellular redistribution concerning at least in part the phosphorylated form of the protein. This finding confirms similar results obtained in other cell systems showing translocation of low molecular weight HSPs from cytosol to nuclear compartment upon heat shock (23-25). Whether this phenomenon has a physiological significance remains to be established. However, the fact that TPA did not induce a similar targeting of the 28-kDa protein might suggest different cellular functions of the protein depending on the stimuli. Such a multifunctionality of this HSP is further indicated by the fact that the 24/28-kDa protein is induced both by heat shock (this paper and Ref. 14) and estrogen (14). Moreover, recent immunoevidential evidence (29) suggests that the 24/28-kDa protein from breast cancer might be related to an estrogen-associated protein previously reported in human myometrium (30) and so far not identified. Thus, the function of the 24/28-kDa protein might depend not only on its cellular expression and subcellular localization but also on its possible implication in estrogen receptor machinery. Finally, one can postulate reasonably that the state of phosphorylation of the protein might account for its cellular function. Indeed, it is tempting to hypothesize that the unphosphorylated form of the protein may be related to the stimulation of cell proliferation, while its phosphorylated forms might, on the contrary, refer to cell growth arrest. In any case, the different levels of regulation of this 24/28-kDa protein might be associated with other proteins, subcellular localization, and possible association with estrogen receptor, strongly argue for its key role in MCF-7 cell function.

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