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THE 27,000 DALTONS STRESS PROTEINS ARE PHOSPHORYLATED BY PROTEIN KINASE C DURING THE TUMOR PROMOTER-MEDIATED GROWTH INHIBITION OF HUMAN MAMMARY CARCINOMA CELLS

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SUMMARY: Phorbol-12-myristate-13-acetate (PMA) inhibited growth of human mammary carcinoma cell lines and increased mainly the phosphorylation of two cytosolic phosphoproteins (pp) of 27 kD with isoelectric points of 5.5 (pp27a) and 5.0 (pp27b). The time course of pp27 phosphorylation closely paralleled the rapid PMA-induced subcellular redistribution of protein kinase C (PKC) activity and its subsequent down regulation. Addition of phospholipase C and fetal calf serum to intact cells or purified PKC to a cell free system enhanced the phosphorylation of both pp27 suggesting that the two polypeptides are specific substrates for PKC. Exposure of human mammary carcinoma cells to stress inducers such as arsenite or cadmium increased the ^{32}P incorporation of both pp27 to an extent comparable to PMA. The increased phosphorus content following stress was rather due to a higher rate of synthesis of both pp27 than to a higher phosphorylation state of these polypeptides as determined by [^{3}H]-leucine labeling. These results indicate that the major substrates of PKC, phosphorylated during the PMA-induced growth inhibition of human mammary carcinoma cells, are members of the stress protein family, suggesting a new possible function for these proteins.

Tumor-promoting phorbol esters elicit a wide array of biological responses in cultured cells including growth regulation and differentiation (1,2). These agents bypass the agonist induced diacylglycerol formation by directly activating protein kinase C (PKC)(3), the major phorbol ester receptor (4). Exposure of human mammary tumor cells to phorbol-12-myristate-13-acetate (PMA) resulted in a reversible, non toxic inhibition of cell growth, indicating an involvement of PKC in the growth regulation of human mammary carcinoma cells (5,6,7). To study the events ensuing the activation of PKC, specific substrates for this enzyme were investigated. This study demonstrate that the initial response of human mammary carcinoma cells to PMA as well as other PKC activators involves the rapid phosphorylation of two 27 kD cytosolic phosphoproteins (pp27) that appear to belong to the family of the stress proteins.

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MATERIAL AND METHODS

Chemicals
Lysine rich histone H1 (calf thymus type V-S), phosphatidylserine, diolein, leupeptin, aprotinin, phorbol-12-myristate-13-acetate (PMA), $NaAsO_2$, $CdCl_2$ and phospholipase C (PLC) were obtained from Sigma. [^{32}P]-orthophosphoric acid (5 mCi/ml), y-[^{32}P]-ATP (S.A. 10 Ci/mM) and [^{3}H]-leucine (S.A. 140 Ci/mM) were from NEN.

In vivo phosphorylation
The human mammary carcinoma cell lines MDA-MB-231, HBL-100, MCF-7 and ZR-75 were routinely grown in IMEM-ZO medium supplemented with 5% fetal calf serum (FCS) as described (5). Cells were grown in 16 mm multiwell dishes to 90% confluency, starved for 24 hours in IMEM-ZO supplemented with 1 mg/ml bovine serum albumin (BSA) and labeled with 100 µCi [^{32}P]-H_3PO_4 (200 µCi/ml) for 4 hours in phosphate-free IMEM-ZO. PMA was added either before or during the last hour of the labeling period. At the end of incubation cells were rapidly washed 3 times with ice cold WB buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM glucose, 50 mM NaF and 100 µM Na_3VO_4), and lysed for 5 min in 0.5 ml SDS buffer (5 mM Tris-HCl, pH 7.9, 3.5 mg/ml dithiothreitol, 0.5 mM EDTA, 1.5% SDS and 10% glycerol) at 95°C.

Synthesis of pp27 under stress conditions
Monolayers (60 mm dishes) were exposed to stress with 100 µM $NaAsO_2$ for 4 hours, followed by labeling in 2 ml of leucine-free IMEM-ZO medium containing 5% FCS and 250 µCi [^{3}H]-leucine for 1 hour in the absence of stress inducers. At the end of the labeling period the cells were washed 3 times with 5 ml ice cold WB, harvested and lysed in 0.6 ml SDS buffer.

Phosphorylation of pp27 by purified PKC
75 µg of cytosolic proteins (100 µl) were incubated at 32°C for 5 min in 40 mM Na-glycerophosphate, 10 mM $MgCl_2$, 10 mM NaF, 0.1 M EGTA, 10 µM ATP (10 µCi y-[^{32}P]-ATP) with 0.2 µg of partially purified PKC in the presence or absence of 100 µg phosphatidylserine (PS) and 1 µM PMA. The reaction was stopped by adjusting the concentrations of Nonidet P40 to 6%, ampholines (pH 3.5-10) to 3%, dithiothreitol to 0.2 M, glycerol to 10% and urea to 9.5 M.

One- and two-dimensional polyacrylamide gel electrophoresis
SDS gel electrophoresis, staining of gels and autoradiography were performed exactly as described (8). For quantitation of $^{32}PO_4$ incorporated into pp27 the protein band was excised and counted for radioactivity. Samples containing equivalent amounts of TCA-precipitable radioactivity (^{32}P: $5x10^5$ cpm, ^{3}H: $1x10^6$ cpm) were analyzed by two-dimensional gel electrophoresis (9) after adjusting the final concentrations of Nonidet P40 to 6%, ampholines (pH 3.5-10) to 3%, dithiothreitol to 0.2 M, glycerol to 10% and urea to 9.5 M using 12% acrylamide gels for the second dimension. Phosphoproteins and [^{3}H]-labeled proteins were detected by autoradiography and fluorography (10) respectively.

Other procedures
Cytosolic and membrane-bound PKC activities were analyzed and quantitated by non-denaturating polyacrylamide gel electrophoresis as described (11). PKC was purified as described (12). Protein was determined by the method of Bradford (13) using the BIO-RAD reagents and BSA as standard.

RESULTS

In all cell lines tested, the phosphorylation of a 27 kD protein (pp27) was the most prominent event in response to PMA (Fig.1). In contrast to other cell types such as

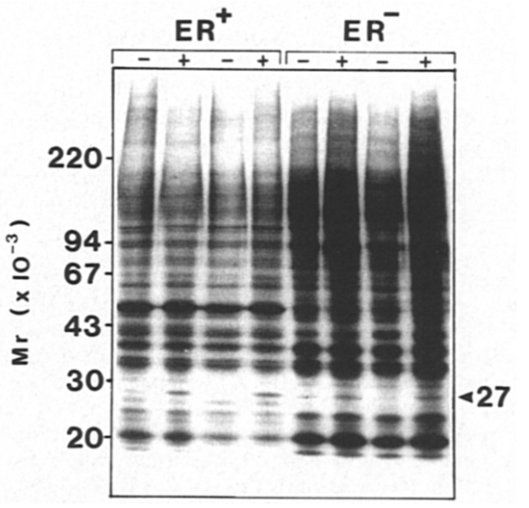

Figure 1. Phosphoprotein pattern of human mammary carcinoma cell lines in response to PMA.
Cells labeled for 4 hours with 100 μCi [^{32}P]-orthophosphoric acid were incubated with 10 nM PMA for 30 min. Phosphoproteins of untreated (-) or treated (+) cells were separated by a linear 5-15% polyacrylamide gradient and autoradiographed. From left to the right: ZR-75, MCF-7, MDA-MB-231, HBL-100. pp27 is indicated by an arrow.
ER+: estrogen receptor positive cell lines.
ER-: estrogen receptor negative cell lines.

fibroblasts and myocytes (14,15), no significant PMA-induced phosphorylation of the 80 kD PKC substrate was observed in the human mammary carcinoma cells (Fig.1). Maximal ^{32}P incorporation into pp27 (3 to 4-fold) was obtained 15 to 30 min after addition of PMA to cells (Fig.2). Prolonged PMA treatment resulted in the gradual

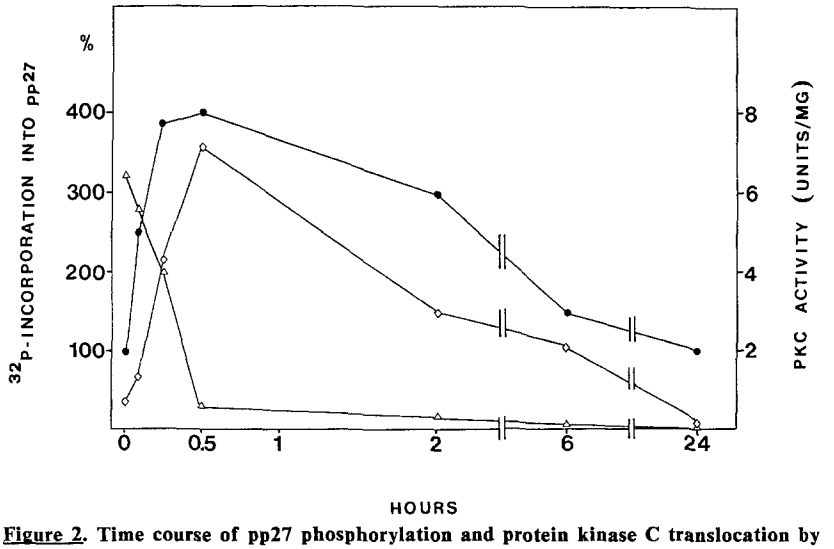

Figure 2. Time course of pp27 phosphorylation and protein kinase C translocation by PMA (10 nM).
The amount of ^{32}P incorporation into pp27 of HBL-100 cells treated for indicated times with 10 nM PMA (●) was quantitated as described in methods. Results are expressed as percent of control. PKC activity of cytosols (△) and membranes (◇).

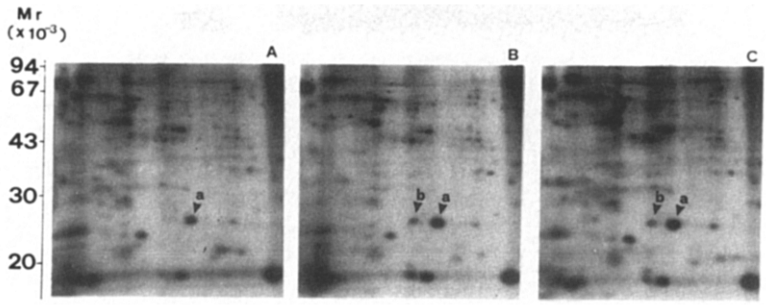

Figure 3. Two-dimensional gel electrophoresis of HBL-100 cells treated with PMA or PLC.
HBL-100 were labeled with $^{32}P_i$ and exposed to PMA (10 nM, 30 min) or PLC (0.25 units, 30 min). Phosphoproteins of control (A), PMA (B) or PLC (C) treated cells were resolved by two-dimensional gel electrophoresis. pp27a and pp27b are indicated by arrows.

loss of ^{32}P from pp27 reaching control levels within 24 hours. The transient phosphorylation of pp27 by PMA closely matched the time course of the PMA-dependent translocation of PKC as well as its subsequent down regulation (Fig.2). Subcellular fractionation of ^{32}P-labeled cells demonstrated that pp27 is predominantly located in the cytosols of both control and PMA treated cells (data not shown). Two-dimensional gel electrophoresis of untreated human mammary tumor cells demonstrated an isoelectric point (pI) of 5.5 for the pp27 (pp27a) (Fig.3A) whereas in PMA-treated cells an additional pp27 exhibiting a pI of 5.0 was obsereved (Fig.3b). This result indicates that the more acidic form of pp27 (pp27b) may be generated from pp27a by a PMA-dependent phosphorylation. Addition of phospholipase C (PLC) (Fig.3c) or 10% FCS (data not shown) to intact cells stimulated the phosphorylation of pp27a and pp27b. In contrast, no phosphorylation of pp27 was observed with insulin, epidermal growth factor (EGF), or dibutyryl-cAMP. Pretreatment of the cells for 24 hours with PMA which results in the complete loss of PKC activity (Fig.2) prevented the PLC-dependent phosphorylations of pp27 (data not shown). Incubation of human mammary cytosols in the presence of purified PKC resulted also,among several other proteins, in a phospholipid-dependent phosphorylation of both pp27 (Fig.4). These data indicate that pp27 is phosphorylated by PKC. Mammalian cells exposed to stress conditions ($NaAsO_2$ or $CdCl_2$) synthesize a family of proteins with properties similar to pp27 (16-19) that are phosphorylated in response to various agents (17). Incubation of human mammary tumor cells with $NaAsO_2$ incorporated ^{32}P into pp27a and pp27b to an extent comparable to the

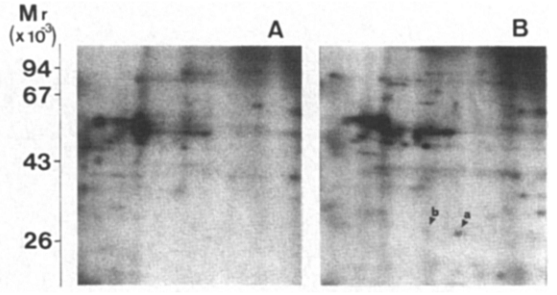

Figure 4. In vitro phosphorylation of pp27 by purified PKC.
Aliquots of cytosol (75 µg of protein) were incubated at 32°C for 5 min with 0.2 µg of purified PKC in the absence (A) or in the presence of phosphatidylserine (100 µg) and PMA (1 µM) (B). Phosphoproteins were analyzed by two-dimensional gel electrophoresis.

PMA-treatment (Fig.5). However, in contrast to PKC activators, the $^{32}PO_4$ incorporation into pp27 during stress treatment was due to increased synthesis of the pp27 as shown by [^{3}H]-leucine labeling (Fig.6). Enhanced synthesis of pp27a and pp27b were detectable only in cells exposed to arsenite (Fig.6b) whereas control cells did not incorporate significant amounts of [^{3}H]-leucine in both pp27 (Fig.6b). Arsenite treatment resulted in the phosphorylation of an additional more acidic pp27 (pp27c) (Fig.5b) whereas a fourth phosphoprotein (pp27d) was only observed in cells exposed to Cd^{2+} (Fig.5c). In cells treated with PMA the stress induced pp27c and pp27d were not detectable. Synthesis of pp27c was also detectable in the arsenite treated cells although after a much longer exposure of the films (data not shown). In contrast synthesis of neither pp27 form was affected by exposure of cells to PMA or to FCS. If [^{3}H]-leucine labeled cells were chased for 12 hours in the presence of unlabeled leucine after the stress treatment, both pp27a and pp27b were found to be

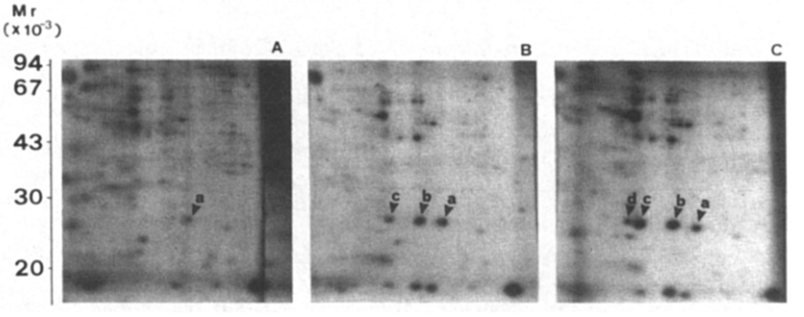

Figure 5. Phosphoprotein pattern of normal and stressed cells.
^{32}P-labeled proteins from control cells (A) and from cells treated for 4 hours with $NaAsO_2$ (100 µM) (B) or $CdCl_2$ (300 µM) (C) were resolved by two-dimensional gel electrophoresis.

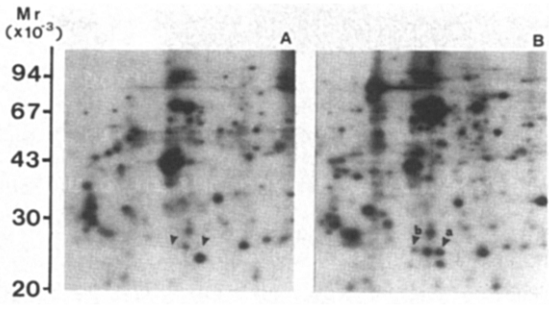

Figure 6. Synthesis of pp27 in arsenite treated cells.
HBL-100 cells were incubated in the presence (B) or in the absence (A) of 100 μM sodium arsenite for 4 hours. The cells were then labeled for 1 hour with [^{3}H]-leucine and analyzed by two-dimensional gel electrophoresis. pp27a and pp27b are indicated by arrows.

partially translocated to the nuclear fraction (data not shown). These results are consistent with reports on the nuclear translocation of stress proteins in rat cells (19).

DISCUSSION

Activation of PKC is thought to be responsible for the PMA-dependent growth inhibition of human mammary carcinoma cells (5,6,7). The PMA treatment predominantly enhances the phosphorylation of two 27 kD cytosolic proteins in all human mammary carcinoma cell lines, independently of their steroid receptor status. Proteins with similar properties have been shown to be rapidly phosphorylated in response to PMA also in other cells (17,21-23). By several criteria we have shown that the pp27 of human mammary carcinoma cells are specific substrates for PKC.

Exposure of cells from a wide variety of species to increased temperature or to other stress inducers leads to a rapid synthesis of a set of highly conserved proteins which are referred to as heat shock or stress proteins (24). Among this group of proteins there is a family of small polypeptides which display properties (MW's, subcellular localization and pI) similar to pp27. These heat shock proteins lack methionine in mammalian cells and are known to be phosphorylated on serine residues (16-19). Although considerable work has provided a large number of details regarding various post-translational modifications of these small heat shock proteins during stress conditions, their physiological function remain unclear. Our data suggest that the two PKC substrates pp27a and pp27b found in the human mammary carcinoma cells belong to the family of the small stress proteins. In fact two different stress treatments (arsenite and cadmium) enhanced the ^{32}P content of both pp27a and b. The phosphorus content in arsenite treated cells reflected a higher rate of synthesis

of pp27a and b rather than a higher phosphorylation of these proteins. The additional forms of pp27, pp27c and d, were only detected under stress conditions suggesting that under normal growth conditions these forms are not expressed or synthesized in undetectable amount. Although the various forms of pp27 are localized in the cytoplasm they seem to partially translocate to the nucleus during the stress treatment (19). PMA and serum factors increase only the phosphate content but neither the synthesis nor the redistribution of these proteins into the nucleus. Thus, the family of pp27 may be involved, albeit by different mechanisms, in the stress response as well as in those processes regulating normal growth. Our results indicate that the two pp27 polypeptides are the major substrates for PKC and may play an important role during the phorbol ester-mediated growth inhibition of human mammary carcinoma cells.

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