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EFFECT OF SERUM STARVATION ON EXPRESSION AND PHOSPHORYLATION OF PKC-α AND p53 IN V79 CELLS: IMPLICATIONS FOR CELL DEATH

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The effect of serum starvation on the expression and phosphorylation of PKC-α and p53 in Chinese hamster V79 cells was investigated. Serum starvation led to growth arrest, rounding up of cells and the appearance of new PKC-α and p53 bands on Western blots. Prolonged incubation (≥48 hr) in serum-deprived medium led to cell detachment and death. Moving cells to fresh medium containing 10% serum before, but not after, cell detachment reversed the changes observed in PKC-α and p53, and also prevented later cell detachment. Radiolabelling studies showed that the higher-molecular-weight PKC-α and p53 bands result from increased phosphorylation, while a lower-molecular-weight PKC-α band reflects newly synthesized protein. Immunocomplex kinase assays have shown that the increased phosphorylation of PKC-α is associated with its increased activity. To study the relationship between PKC-α, p53 and cell death, cells were treated either with TPA, to down-regulate PKC or with staurosporine, to inhibit PKC activity. Staurosporine, a potent PKC inhibitor and inducer of programmed cell death, caused the appearance of new PKC-α and p53 bands similar to those induced by serum starvation. If serum starvation was preceded by prolonged (48 hr) TPA treatment to down-regulate PKC-α, cell detachment and death did not take place within the same time frame. Intracellular fractionation of cells demonstrated that increased expression of PKC-α and the appearance of the associated higher and lower molecular-weight bands occurred in the nucleus. These data highlight the association of PKC-α and p53 with cellular events leading to cell death. *Int. J. Cancer* 80:400–405, 1999.
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Protein kinase C (PKC) is a protein serine/threonine kinase involved in various signal-transduction pathways which are important regulators of many cellular functions including proliferation, death and differentiation. Mitogenic stimulation of cells by agents such as the tumour promoter TPA, causes activation and translocation of PKC-α (among other PKC isoforms) from the cytosol to the membrane (Hasan *et al.,* 1996). PKC can also translocate to the nucleus after stimulation of cells and can independently cause the activation of transcription factors (Buchner, 1995). Activation of PKC by TPA is time-dependent, and prolonged treatment leads to down-regulation of PKC (Mitchell *et al.,* 1989). PKC phosphorylation/activation, and PKC-α in particular, have been shown to cause direct activation of Raf-1 kinase. Activated Raf-1 triggers a protein-kinase cascade by direct phosphorylation of Map-kinase, which in turn translocates to the cell nucleus and causes activation of transcription factors (Kolch *et al.,* 1993).

The tumour suppressor p53 is a phosphoprotein involved in the positive and negative regulation of the cell cycle. It can cause cell-cycle delay to allow for DNA repair after cellular damage, or it can promote cell death via apoptosis. Analysis of p53 structure has revealed that the DNA-binding and oligomerization-promoting domain shows close similarity with PKC phosphorylation sites, indicating that p53 may be a PKC substrate. Indeed, it has been shown that PKC activation causes p53 phosphorylation both *in vitro* and *in vivo* (Baudier *et al.,* 1992). PKC-α has also been shown to directly phosphorylate p53 in cell extracts (Price and Youmell, 1997). However, Milne *et al.* (1996) have cast some doubt on the ability of PKC to interact directly with p53 in cultured cells. Known PKC substrates include DNA-regulatory proteins such as lamin B, RNA and DNA polymerases, Poly ADP-ribose polymerase, CREB and others (Buchner, 1995). Phosphorylation of these proteins changes their DNA-binding or regulatory properties, which points to a link between activation of PKC and regulation of transcription.

The role of PKC in cell proliferation and apoptosis is complex and dependent on cell type, since different cell lines express different PKC isoforms. For example, over-expression of PKC-β1 in HT29 cells has been found to cause growth inhibition and tumour suppression (Choi *et al.,* 1990), and it was suggested that PKC might act as a ''growth-suppressor'' gene. On the other hand, growth arrest has also been found to be caused by inhibition of PKC, and it has been suggested that translocation of certain PKC isoforms is not only a consequence of PKC activation but, in the case of staurosporine, also of PKC inhibition (Courage *et al.,* 1995). These apparent contradictions might arise because different PKC isoforms have different effects on the same system. For example, it has been reported that the 2 closely related PKC isoforms, PKC-α and -β1, have divergent effects on the growth and transformation of the same parental R6 rat-embryo fibroblast cell line (Borner *et al.,* 1995; Housey *et al.,* 1988). It is therefore important to detail isoform information in studies with PKC.

In this study, the effect of serum starvation on the expression and phosphorylation of PKC-α and p53 has been investigated, and the association of PKC-α with cell death is discussed.

MATERIAL AND METHODS

Cell culture

V79 Chinese hamster lung fibroblasts were grown as monolayers to mid-exponential phase (usually 3 days from the plating of cells) in RPMI-1640, supplemented with 10% FCS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. These cells were spontaneously transformed more than 30 years ago, and have been sub-cultured many times since. They have rapid growth, high plating efficiency and a stable karyotype. Serum starvation was carried out by changing the culture medium to fresh RPMI-1640 supplemented with only 0.1% FCS.

Immunoblot analysis

For measurement of PKC and p53, cells were first washed and then scraped off in ice-cold PBS, then pelleted by centrifugation. Pelleted cells were frozen at −70°C until ready for analysis. Cells were lysed directly in sample buffer containing SDS and dithiothreitol, then heated for 5 min at 95°C. Aliquots containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Nycomed Amersham, Aylesbury, UK) and probed with polyclonal antibodies (kindly supplied by Dr. P. Parker, ICRF, London) selective to different PKC isoforms (Marais and Parker, 1989). For the detection of p53, monoclonal antibody DO-7

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(DAKO, Glostrup, Denmark), which recognises both wild- and mutant-type p53, was used.

Samples of mouse brain, which is rich in PKC, were used as positive controls to indicate the bands of interest in case of the presence of non-specific binding of the polyclonal antibodies. Rainbow molecular-weight markers (Nycomed Amersham) were also used to estimate the M_R of bands of interest. An ECL-chemiluminescence Western-blot detection system (RPN-2106, Nycomed Amersham) was used to visualize immunoreactions on immunoblots. Several exposure times were used to observe high- and low-intensity bands clearly.

Cellular fractionation

Separation of intracellular contents into cytosolic and nuclear fractions was carried out according to Greif *et al.* (1992). In brief, cells were pelleted as described, then lysed in 0.75 ml of homogenization buffer (20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 5 mM EGTA, 10 mM benzamidine, 0.3% mercaptoethanol, 50 µg ml^{-1} leupeptin and 50 µg ml^{-1} PMSF) and left to swell for 20 min on ice. Samples were dounce-homogenized until microscopic examination indicated that the cells were ruptured. The cell homogenate was then overlaid on 0.5 ml of homogenization buffer containing 25% glycerol and centrifuged at 400 *g* at 4°C for 5 min. The supernatant was taken as the cytosolic fraction (containing the plasma membrane) and the nuclear pellet was washed and re-suspended in 0.5 ml homogenization buffer containing 25% glycerol and 0.1% Triton-X 100, then disrupted by brief sonication.

Characterization of cell death

Acridine orange (AO) staining. Cells were grown on glass coverslips, washed in PBS, fixed in methanol/acetic acid (9:1), then stained in AO (10 µg ml^{-1}) in PBS for 2 to 3 min and viewed by microscopy.

Terminal deoxynucleotidyl transferase (TdT) labelling. Cells were washed in PBS and sodium cacodylate, then labelled with fluoresceinated avidin as described by Gorczyca *et al.* (1993). Cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) using the Lysis II program.

PKC-α assay

Detection of MBP4-14 phosphorylation. V79 cells were homogenized in cell-lysis buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.15 units ml^{-1} aprotinin, 1 mM Na_3VO_4, 50 µg ml^{-1} PMSF, 25 µg ml^{-1} leupeptin and 10 mM benzamidine. PKC-α was precipitated by incubation with PKC-α antibodies for 4 hr at 4°C, then the immunocomplexes were collected by the addition of 20 µl protein A-Sepharose 4B beads (Pharmacia, Milton Keynes, UK) for 1 hr at 4°C. Immunocomplexes were mixed with 50 µl of reaction buffer containing 5 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 5 µM ATP, 40 µg ml^{-1} phosphatidylserine, 3.3 µM dioleoylglycerol, 1.2 mM $CaCl_2$, 25 µM MBP4-14, 2 µCi [γ-^{33}P]ATP. After incubation for 10 min at 30°C, the transfer of ^{33}P phosphate to MBP was quantified by transferring 30 µl of the reaction mixture to a P-81 ion-exchange chromatography paper (Whatman, Maidstone, UK) which was immediately washed 4 times in 75 mM H_3PO_4 for 5 min each time. The amount of radioactivity was then quantified by liquid scintillation.

PKC-α and p53 phosphorylation in cells

Cells were washed in phosphate-free DMEM (Sigma, St. Louis, MO) for 2 hr. Then 20 µCi ml^{-1} [^{33}P] orthophosphate (Nycomed Amersham) were added to the cell culture. After the appropriate period, cells were washed and lysed in cell-lysis buffer (see above), and PKC-α or p53 was immunoprecipitated with the corresponding antibodies. Immunocomplexes were then collected by addition of protein A-Sepharose 4B beads for 1 hr at 4°C, then spun and boiled in sample buffer. Samples were analyzed by electrophoresis on 10% SDS-PAGE. The gels were dried and autoradiographed at −70°C.

Protein synthesis

^{35}S labelling. Cells were grown to mid-late exponential phase, then washed and incubated in methionine/cysteine-free medium (Sigma) for 2 hr after which 20 µl ml^{-1} PRO-MIX L-[^{35}S] *in vitro* cell-labelling mix (Nycomed Amersham) was added. After the appropriate times, cells were collected and PKC-α was immunoprecipitated with antibodies. Immunocomplexes were collected by protein A and analyzed by electrophoresis as described above.

RESULTS

Cells grown in normal conditions were tested for PKC expression by several polyclonal and monoclonal antibodies specific to different isoforms. Western blotting showed the presence of one band corresponding to each of PKC-α, β1 and ζ but not ϵ, δ or γ. Cells also showed one band corresponding to the p53 protein when anti-p53 (Do-7) monoclonal antibodies were used.

Serum starvation

Serum starvation inhibited cell growth and caused cellular rounding up. This morphological change started to appear about 10 hr after initiation of serum starvation, and cells were fully rounded up by 24 hr. Cells returned to normal morphology only if supplied with fresh medium containing 10% FCS before subsequent detachment had occurred. Prolonged exposure to low serum caused the appearance of new PKC bands specific to the α isoform; one was higher and the other was lower on the blot than the original PKC band (Fig. 1). Figure 1 also shows that over-exposure to ECL detection shows up a non-specific band at around 50 kDa in all samples, confirming that this change in the PKC-α pattern and expression is real, since the 50-kDa band showed no differences in mobility between samples from control and from starved cells. This band also indicates that gel loading was roughly equal in the 3 lanes. As shown in Figure 1, expression of PKC-α in serum-starved cells is at least twice that in control cells. The use of antibodies specific to other PKC isoforms expressed in V79 cells, such as β1 and ζ showed no similar effects (data not shown).

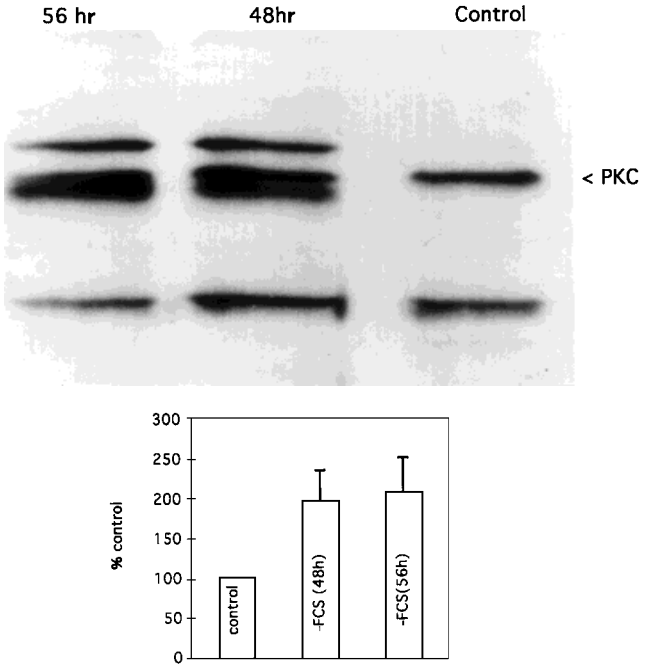

FIGURE 1 – Western blot showing the effect of serum starvation on the expression of PKC-α (top). Densitometric quantitation of PKC expression in 3 independent experiments (bottom). Error bars represent SD of percentage increase in expression.

Characterization of apoptosis

Serum starvation inhibited cell growth and caused rounding up of cells and the appearance of new PKC-α bands on Western blots. It finally led to cell detachment and subsequent death by apoptosis. Staining of serum-starved cells with acridine orange demonstrated the presence of more condensed and fragmented chromatin in the cell nucleus and the appearance of apoptotic bodies (data not shown). Apoptosis was confirmed by TdT labelling; serum starvation for 48 hr resulted in more than 50% of cells labelled (Fig. 2).

Effect of serum starvation on p53

The changes in PKC-α were accompanied by similar changes in p53. To demonstrate that the changes in PKC-α and p53 occurred in the same sample in identical electrophoresis conditions, the nitrocellulose membrane was cut just below the 67-kDa marker, after protein transfer from the gel, and the upper half of the membrane was probed for PKC-α while the lower half was probed for p53. Figure 3 shows that serum starvation of cells led to the formation of double bands both of PKC-α and of p53, as compared with only one band in untreated cells. Lane 4 also shows that the changes observed in PKC and p53 were reversible if cells were returned to medium containing 10% serum after 36 hr of serum starvation. Figure 3 also shows that these effects were not observed when cycloheximide but not actinomycin D was added to cells subjected to serum deprivation (see below).

Staurosporine or TPA treatment

Exposure of cells to the potent PKC inhibitor staurosporine (100 nM) for 16 hr caused cell detachment and the appearance of new PKC-α bands similar to those observed after serum starvation (Fig. 4). The change in PKC-α was again accompanied by a similar change in p53, suggesting a possible coupling between PKC-α and p53. To investigate this relationship further, cells were incubated with TPA for 48 hr to down-regulate PKC-α. Subsequent serum starvation of these PKC-down-regulated cells inhibited the formation of new p53 bands and prevented cell detachment and death. Interestingly, some cells (<10%) detached after prolonged TPA treatment alone; these cells expressed only the higher PKC-α band and showed a p53 double band, indicating that the activation of p53 may relate to the higher, presumably phosphorylated, PKC-α band. These cells were able to survive and proliferate if re-plated in fresh medium containing 10% FCS. These data suggest that the phosphorylation of p53 may be dependent on the presence of functionally active PKC-α. Prolonged TPA treatment also caused down-regulation of PKC-β1 but not PKC-ζ.

Mechanism of the appearance of new bands

The development of the new PKC-α bands, *i.e.*, the appearance of higher and lower bands following serum starvation, occurred in 3 stages, as shown in Figure 5. First, the formation of only the higher band which was seen at 30 to 40 hr followed by the appearance of a lower band at 40 to 48 hr and, finally, the disappearance of the original ''middle'' band leading to the formation of 2 clear split bands (>48 hr), one higher and one lower than the original band.

The early change in PKC (formation of the higher band) was reversible if cells were returned to medium containing 10% FCS. The final phase, with 2 clear split bands, was irreversible even if cells were put back in medium containing 10% FCS. At this stage, cells were very loose and detaching. The change in p53 was reversible/irreversible in the same manner: cells resumed growth if replenished with medium containing serum during the first and the second stages, but did not re-attach and resume growth after the final stage. This demonstrates an association between PKC-α, p53 and cell death.

Intracellular fractionation

Separation of intracellular contents into cytosolic and nuclear fractions showed that serum starvation led to increased protein expression of PKC-α in the doublet pattern (widely separated) in the nucleus (Fig. 6): the appearance of the lower band is clearly associated with the nuclear fraction. We showed earlier (Hasan *et al.*, 1996) that when V79 cells were fractionated into cytosol, nuclear and membrane fractions, the doublet band was still seen only in the nuclear and not in the cytosolic or membrane fractions. Close but separate double bands can be seen in cytosolic fractions. Thus the appearance of 2 clearly separated PKC-α bands is unique to the nucleus. We also showed (Hasan *et al.*, 1996) that TPA treatment results in translocation of PKC-α from the cytosol to the membrane as a single band and that no doublet is observed in this situation.

Effect of cycloheximide and actinomycin D

Cells treated with the protein-synthesis inhibitor cycloheximide (1 μg ml^{-1}) and then starved did not show the morphological changes associated with serum starvation. Moreover, changes in PKC-α and p53 were not observed if cycloheximide was added at the time when serum starvation was initiated, but not if it was added at 8 or more hours after initiation. This indicates the involvement of protein synthesis in the early events induced by serum starvation. In contrast, the RNA-synthesis inhibitor actinomycin D (0.2 μg ml^{-1}) caused morphological changes and the

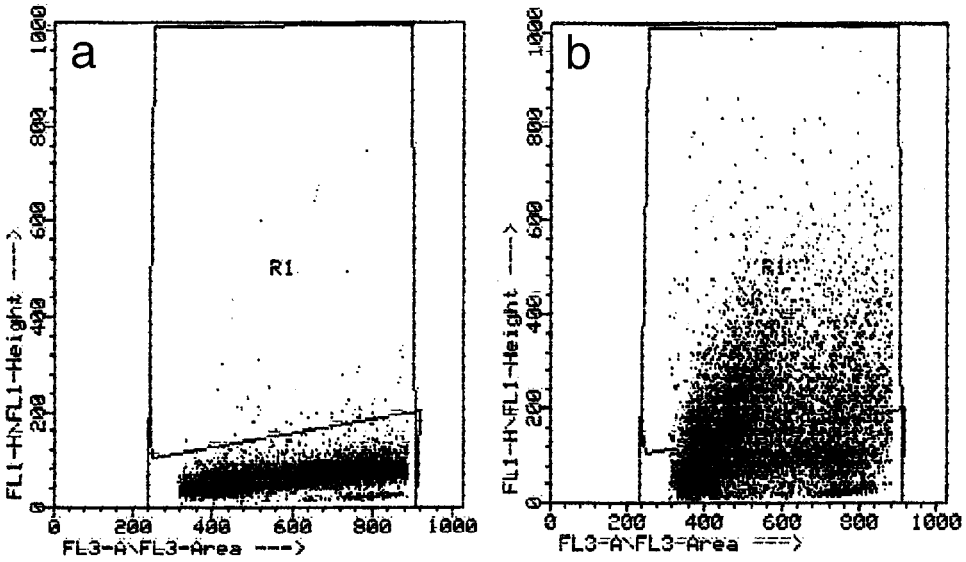

FIGURE 2 – Dot plot showing TdT labelling of cells serum-starved for 48 hr *(b)* compared with untreated cells *(a)*. FL1, FITC fluorescence; FL3, propidium iodide (PI) labelling. Increased fluorescence in R1 is due to TdT labelling of degraded DNA.

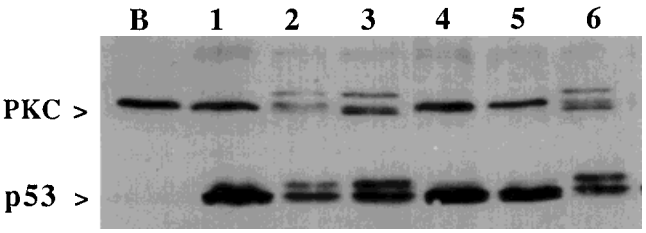

FIGURE 3 – Effects of serum starvation on expression of PKC-α and p53: changes due to serum starvation in PKC-α are accompanied by similar changes in p53. Both effects are reversible when cells are returned to full medium. Lane B, mouse brain (PKC positive control); lane 1, untreated cells. Serum starvation for 36 hr (lane 2), 48 hr (lane 3) and serum starvation for 36 hr followed by full medium for 4 hr (lane 4). Cycloheximide (1 μg ml^{-1}) plus 48 hr serum starvation (lane 5); actinomycin D (0.1 μg ml^{-1}) for 10 hr (lane 6).

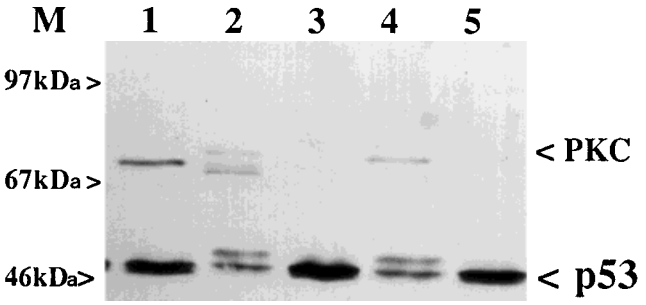

FIGURE 4 – Effect of staurosporine and TPA on expression of PKC-α and p53. M, marker. Lane 1, untreated cells; lane 2, cells incubated with staurosporine (100 nM) for 16 hr; lane 3, cells incubated with TPA (100 nM) for 48 hr and starved for 48 hr; lane 4, cells that detached after TPA treatment; lane 5, p53 positive control.

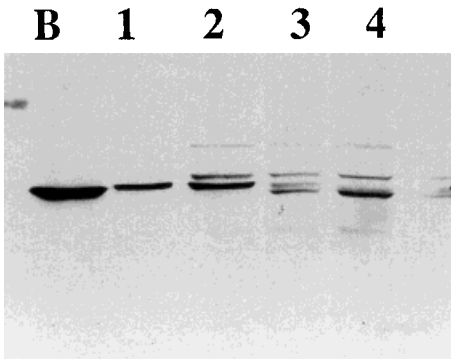

FIGURE 5 – Blot showing the development of PKC-α bands. A single PKC band (untreated cells) splits into 2 bands, one higher and one lower than the original band, which finally disappears. Lane B, mouse brain (PKC positive control). Cells not incubated (lane 1) or incubated in low serum for 36 hr (lane 2), 48 hr (lane 3) and 56 hr (lane 4).

induction of PKC-α and p53 in a similar manner to serum starvation and staurosporine, as shown in Figure 3.

Higher bands in PKC-α and p53 are due to phosphorylation

Immunoprecipitation of PKC-α and p53 by antibodies from cell extracts radiolabelled with radioactive phosphate during serum starvation shows that the upper but not the lower PKC-α and p53 bands are associated with increased phosphorylation (Fig. 7*a*). TPA-detached cells were selected in lane 3 because they show only the upper band.

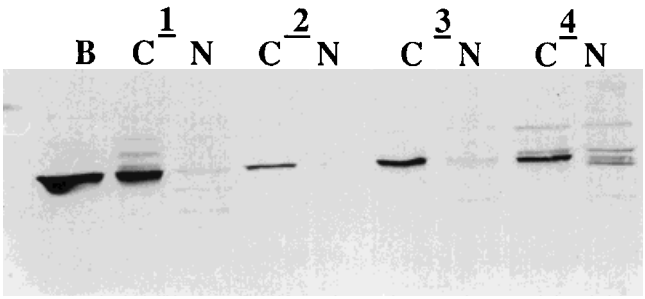

FIGURE 6 – PKC-α expression in cytosolic (C) and nuclear (N) fractions. Lane B, mouse brain (PKC positive control); lane 1, cells growing in 10% FCS. Cells incubated in low serum for 12 hr (lane 2), 24 hr (lane 3) and 48 hr (lane 4).

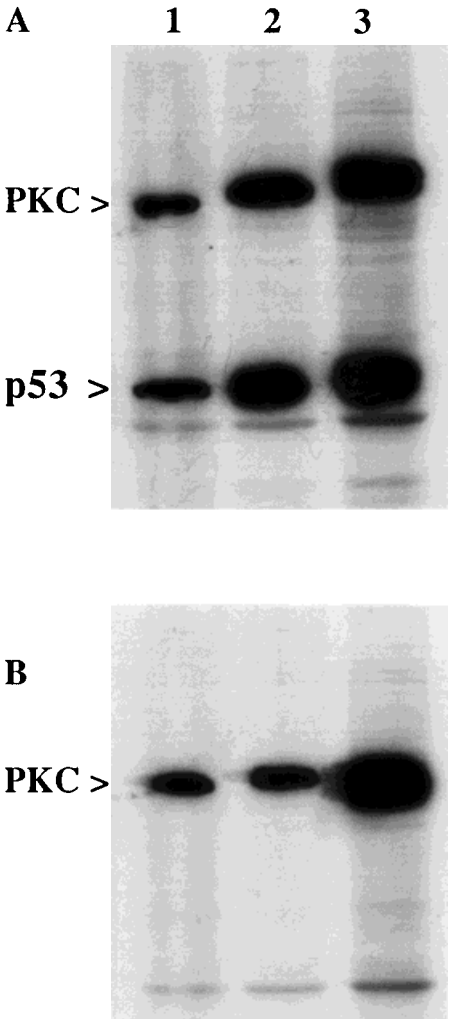

FIGURE 7 – *(a)* Radiograph of immunoprecipitated PKC-α shows that the appearance of the upper band is associated with increased phosphorylation. Lane 1, control cells; lane 2, serum-starved cells, 48 hr; lane 3, TPA-detached cells, 48 hr. *(b)* The lower PKC-α band is associated with increased protein synthesis. Lane 1, control cells; lane 2, serum starvation, 32 hr; lane 3, serum starvation, 48 hr.

Lower PKC-α band is due to protein synthesis

Labelling of cells with ^{35}S during serum starvation followed by immunoprecipitation shows that the lower PKC-α band results

from new protein synthesis. Figure 7*b* shows that serum starvation causes induction of newly synthesized, presumably dephosphorylated PKC-α.

Upper PKC-α band is associated with increased activity

Figure 8 shows that the upper PKC band is also associated with increased activity. Phosphorylation of the PKC-specific and -sensitive substrate (myelin basic protein) MBP4-14 (Shao *et al.,* 1997) by immunoprecipitated PKC-α from TPA-treated cells, which show only the upper PKC band, increased to more than 2-fold that in untreated cells, and almost 2-fold that of the serum-starved cells showing the doublet band. This also suggests that the lower PKC band is not associated with increased PKC activity.

DISCUSSION

The changes we found in PKC expression appear to be specific to PKC-α, indicating that this isoform plays a selective role in this system. Serum starvation caused increased expression of PKC-α in the nucleus without clear changes in its localization. This response to serum starvation is unlike some mitogenic responses in which cells respond to agents such as TPA treatment by completely translocating PKC to the membrane. For example, we have shown (Hasan *et al.,* 1996) that TPA treatment of V79 cells caused complete translocation of cytosolic PKC-α to the membrane, while exposure of cells to hypoxia or to ionizing radiation caused the increased expression, but not complete translocation, of PKC-α in/to the nucleus in a doublet form. Here, we have shown that the appearance of clearly separated double bands (''higher'' and ''lower'' bands) is unique to the nucleus, suggesting that the lower band could be due to newly synthesized dephosphorylated PKC-α and not due to degradation or translocation from cytosol. Proteolysis of PKC-α has been specifically shown to result in PKC-α bands around 45 to 46 kDa (Kikuchi and Imajoh Ohmi, 1995) which is clearly not the case in this study. Considering all the data together, the conclusion is that there is increased expression and phosphorylation of PKC-α following serum starvation, which appears to be associated with the cell nucleus. This would appear to be a novel aspect of PKC regulation requiring further investigation.

Changes in electrophoretic mobility of PKC-α have been attributed to changes in phosphorylation (Hasan *et al.,* 1996); similar electrophoretic shifts are observed upon phosphorylation/dephosphorylation of many protein kinases and have been widely reported. Closely-spaced double bands (''higher'' and ''middle'') can be observed in the cytosol of serum-starved cells (Fig. 6). The doublet (higher and lower) PKC-α pattern in the nucleus can therefore be explained by proposing that nuclear PKC-α exists in 2 forms, one hyperphosphorylated (the higher band) and the other dephosphorylated (the lower band). According to this explanation, cytosolic PKC would be of a hypophosphorylated type, hence the original band in the middle. This model would explain the appearance of 3 bands in whole-cell homogenates of cells exposed to serum starvation, as seen in Figure 5. It is obvious from Western blots that PKC-α is over-expressed in cells undergoing apoptosis by serum starvation or other agents when compared to untreated control cells. This suggests that the over-expression could not be due to mere changes in phosphorylation and dephosphorylation or degradation of the original PKC.

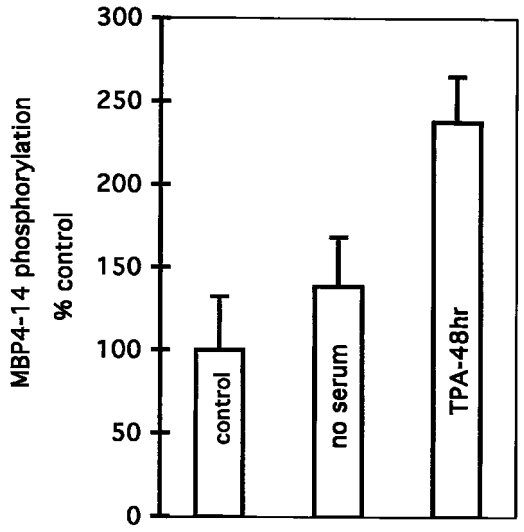

FIGURE 8 – Immunocomplex kinase assay showing that the upper PKC-α band is associated with increased activity. Error bars represent SD of quadruple samples.

The increased expression/synthesis of PKC-α in the nuclear fraction has many implications regarding gene activation which could alter the fine balance between cell survival and death. It has been found that the expression of PKC-α and -β was most intense in cells undergoing apoptosis and that differential sub-cellular localization of PKC isoforms is associated with apoptosis in epithelial cells (Knox *et al.,* 1993). Lee *et al.* (1996) have also shown that ceramides cause apoptosis in Molt-4 cells via PKC-α inactivation. These paradoxical observations are seen in V79 cells undergoing apoptosis, they show both forms of PKC-α, the upper phosphorylated (active) and the lower dephosphorylated (inactive) form. The dephosphorylated PKC-α in the nucleus may be associated with of cell death but is not the cause. This suggests that prolonged PKC-α activation is the factor behind cell death. Shao *et al.* (1997) demonstrated that PKC-α is activated by increased phosphorylation in HL60 cells undergoing apoptosis by exposure to staurosporine derivatives.

Our observation of cell death induced by prolonged serum starvation or by exposure to staurosporine or actinomycin D is in agreement with other studies. Serum starvation (Chou and Yung, 1997), staurosporine (Couldwell *et al.,* 1994) and actinomycin D (Chou and Yung, 1997) have all been shown to induce cell death via apoptosis. In the present study, we have shown that these agents induce similar changes in the expression both of PKC-α and of p53, suggesting that these proteins share a similar response mechanism in this system. The change in p53 appears to depend on the presence of functionally active (phosphorylated) PKC-α as shown with TPA-treated cells. This is supported by the observation that appearance of new p53 bands was inhibited in PKC-α down-regulated serum-starved cells and that a p53 doublet band could be seen only in cells where the higher (phosphorylated) PKC band was present. This indicates that PKC-α might be linked to the activation of p53, which would contradict the report by Milne *et al.* (1996) that PKC cannot interact with p53 in cells, but would agree with earlier observations by Baudier *et al.* (1992), who showed that p53 can be a substrate for PKC both in cells and in cellular extracts. The reason for these discrepancies could be the use of different cell lines and different methodologies or to non-specificity of PKC isoform identification. We here report that changes only in the α isoform of PKC were observed and that changes in p53 were associated with this isoform. Other cellular studies have not looked directly at specific PKC isoforms and have shown only indirect evidence of PKC involvement in p53 activation.

The reversibility/irreversibility of changes in PKC-α and p53 and the inhibition of cell detachment in PKC-down-regulated cells indicate that PKC-α over-expression is associated with events leading to cell death. Cells can survive only when these PKC-α changes are reversible. It has been reported that PKC regulates not only p53 but also other proteins, including Bcl2 in serum-depletion-induced apoptosis (Itano *et al.,* 1996). Furthermore, PKC-α has been shown to directly activate Raf-kinase, which in turn activates Map-kinase (Kolch *et al.,* 1993). Activation of Map-kinase causes its translocation to the nucleus and activation of genes involved in cell proliferation or death pathways. The fine balance between

proliferation and apoptosis (Kyprianou *et al.,* 1996) is reflected in the reversible or irreversible changes we have seen in PKC-α and p53 and in the presence of both forms of PKC-α. Activation of PKC has been shown to regulate either cell proliferation or cell death, depending on cell type and PKC isoform involved (Borner *et al.,* 1995; Housey *et al.,* 1988; Leszczynski *et al.,* 1996). Housey *et al.* (1988) and Borner *et al.* (1995) showed that increased expression of 2 closely related PKC isoforms, α and β, has divergent effects on growth and transformation of the same cell line. Leszczynski *et al.* (1996) found that induction of apoptosis via PKC dependent pathways was prevented by supplementing the culture medium with serum (a finding similar to our own), and have even suggested that PKC-α should be added to the list of proteins such as c-myc and cdc2 kinase which could be therapeutically targeted to prevent tumour progression.

p53 activation is known to play an important role in the G_1 checkpoint after ionizing radiation where it facilitates DNA repair or promotes cell death via apoptosis (Joypaul *et al.,* 1994). Our data suggest that p53 is a substrate for PKC-α and that p53 is involved in apoptosis induced by serum starvation. p53 phosphorylation is a clear sign of upstream signalling (Hao *et al.,* 1996). Hence we suggest that p53 is activated upon serum starvation of V79 cells via a PKC-α-dependent, DNA damage-independent pathway and may be involved directly in apoptosis induced by serum starvation. However, it has been shown that serum starvation resulted in apoptosis both in wild-type p53 parental 32D haematopoietic cells and in the same cells transfected with mutant p53 (Blandino *et al.,* 1995), suggesting the involvement of signalling pathways upstream of p53. Serum starvation also resulted in G_1/S arrest and cell death in a NIH 3T3 sub-clone independent of the presence of wild-type p53, and Huang *et al.* (1996) reported that DNA-damaging agents resulted in distinct responses in 2 sub-clones of NIH 3T3 which possess the same wild-type p53 DNA sequence. These studies show that the role of p53 in serum-starvation-induced apoptosis is complex, and could differ from one cell line to another. However, mutant p53 does appear to be involved in serum-induced apoptosis in V79 cells.

In conclusion, our data show that serum starvation, a non-genomic stress, causes growth arrest and changes in the expression and phosphorylation of PKC-α and p53. These events finally lead to cell detachment and death via apoptosis.

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