c-myc Gene-induced Alterations in Protein Kinase C Expression: A Possible Mechanism Facilitating myc-ras Gene Complementation

Linda F. Barr,2 Mack Mabry, Barry D. Nelkin, Greg Tyler, W. Stratford May, and Stephen B. Baylin

Departments of Medicine [L. F. B., S. B. B.] and Oncology [M. M., B. D. N., G. T., W. S. M., S. B. B.], The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

The mechanism(s) by which the c-myc nuclear protein and the membrane-associated ras protein interact to mediate phenotypic changes is unknown. We now find that c-myc gene expression is associated with alterations in the principal signal transduction pathway through which the ras protein is thought to function. We studied the transcript and protein expression of protein kinase C (PKC) isofoms in a culture line of human small cell lung cancer cells (NCI H209) in which expression of inserted c-myc and Ha-ras genes together, but not alone, causes a transition to a large cell phenotype. In control H209 cells, at the transcription and cell membrane protein levels, PKC-α is the dominant PKC species. In this cell line, the expression of an exogenous c-myc gene, but not of a viral Ha-ras gene, causes a 5- to 10-fold increase in the PKC-β isoform transcript and protein. The insertion of ras into the exogenous myc-expression 209 cells, in addition to causing phenotypic transition, results in the translocation of the PKC-β protein from the cytosol to the membrane fraction and a decrease in membrane-associated PKC-α. Concomitant with these changes, the increased PKC isoform transcript levels induced by myc alone are completely reversed. These observations suggest that a complex set of PKC transcript and protein alterations, most prominently involving an increased PKC-β protein level in the cell membrane, a decrease in PKC-α protein, and a decrease in all PKC isoform transcripts, may represent a fundamental event(s) for c-myc collaboration with Ha-ras to alter cell phenotype.

INTRODUCTION

The myc family oncogenes complement ras family oncogenes to transform primary cells in culture (1). We have found that the c- or N-myc and the Ha-ras genes can also collaborate to induce tumor progression events, characterized by transition to a large cell phenotype, in a cell culture model of human SCLC (2, 3). The mechanism(s) underlying this gene cooperation are unknown.

The p21 ras protein functions, in part, by coupling signals generated at the plasma membrane with diacylglycerol-induced activation of PKC (4–6); as well, PKC may affect the activation state of p21 ras (7). PKC activity results from a family of genes, and the activities of different isoforms could account for the pleiotropic effects which may follow either physiological or pharmacological activation of PKC (8). Expression of the different PKC isoforms has not been examined in detail in ras-infected cells. Further, the possibility that alterations in PKC gene transcription and/or translation might play a role in the mechanism by which myc genes complement the action of the ras genes has not been directly studied. We now report, in a study of cultured lung cancer cells, that c-myc gene-induced alterations in PKC gene expression, especially that of PKC-β, may play a critical role in the phenotypic changes induced by myc-ras complementation.

MATERIALS AND METHODS

Cells and Transfection. All SCLC cultures studied are well-established lines (9, 10) and were grown as previously described (3). The 209 myc cell line is a gift from Bruce Johnson, National Cancer Institute-Navy Medical Oncology Branch, Naval Hospital, Bethesda, MD. The c-myc construct used for transfection of the 209 cells consists of a normal human c-myc gene behind Simian Virus 40 promoter and contains a neomycin resistance gene in a pBR 327 plasmid (11). Cells containing this construct (209 myc, 209 myc/ras) were grown in the presence of 0.4 µg/ml of G418 (Sigma). Infection with the 1504A pseudotype of Harvey murine sarcoma virus (12) was performed as described previously (13).

DNA Probes. cDNA probes were oligolabeled with [α-32P]dCTP to a specific activity of approximately 108 cpm/µg of DNA (14). Probes were prepared as inserts of human PKC-α, -β, and -γ cDNA restricted from recombinant plasmids obtained from American Type Tissue Culture (15, 16). A recombinant plasmid containing human β-actin sequences was provided by Don Cleveland, Johns Hopkins University School of Medicine.

Northern Hybridization. Cellular RNA extraction and preparation of polyadenylated RNA were carried out exactly as in our previous studies (3) by standard procedures (17, 18). Membranes were hybridized with labeled probes, washed exactly as in previous studies (3), and exposed for autoradiography at ~70°C for various times as described in “Results.” In one PKC-γ hybridization, the denatured probe was preincubated at 42°C for 30 min with an excess of ribosomal RNA prior to hybridization. This resulted in a specific diminution of the signals of two previously seen wide bands at 4.0 and 1.9 kilobases that likely represented nonspecific ribosomal RNA binding of the PKC-γ probe. RNA was quantified by densitometric readings of PKC bands and corrected for loading by dividing these values by the densitometries of β-actin bands in each lane obtained during subsequent hybridization. For sequential hybridization of the same filter, membranes were stripped according to manufacturers’ protocols.

Fractionation and Partial Purification of PKC Proteins from Cell Homogenates. Cells (1 × 10⁸) from each sample were washed 3 times with cold, sterile PBS and hypotonically swelled in STM buffer containing 1% (v/v) 2-mercaptoethanol, 0.1 mM NaVO₃, 20 mM NaF, 10 µg/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 17 µg/ml of Calpain I inhibitor, and 7 µg/ml of Calpain II inhibitor (Boehringer-Ingelheim) (19) for 10 min on ice. Cells disrupted by shearing and sonication were centrifuged for 30 min at 200,000 × g yielding the cytosolic fraction. The pellet was resuspended in STM buffer with 0.02% (w/v) Nonidet P-40, agitated at 4°C for 25 min, and spun at 3,000 rpm for 15 min at 4°C to isolate the membrane fraction in the supernatant. The crude cytosolic and membrane extracts were passed over DEAE-cellulose columns which had been equilibrated with 25 mM Tris (pH 7.4), 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N',N",N"-tetra-acetic acid, 10 µg/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride and batch eluted with buffer containing 400 mM NaCl. These eluants were adjusted to 1.5 mM NaCl and applied to phenyl-Sepharose columns that had been equilibrated with 20 mM Tris, 0.1 M EDTA, 0.1 M.
contain isoform-specific sequences, bind total PKC isolated from rat brain in a quantitative, isoform-specific manner and are selectively blocked when preincubated with the peptides against which they were raised.

Western Hybridization. Equal protein amounts (approximately 100 μg/lane) of purified cell fractions were solubilized in sodium dodecyl sulfate sample buffer by boiling for 2 min, resolved on a 1% sodium dodecyl sulfate/10% polyacrylamide gel (23), and transferred to nitrocellulose at a constant 40 V for 12 to 16 h (24). Following a 12-h, room temperature incubation in 3% (w/v) BSA and 1% (w/v) azide in PBS, the blocked filters were incubated with PKC antibodies specific for PKC-β(II), PKC-α, and PKC-γ for 60 min at room temperature, asculted with 0.1% (v/v) Tween 20 in PBS, incubated with 2.5 μCi of 125I-Protein A in 3% (w/v) BSA and 0.1% (v/v) Tween 20 in PBS, washed as before, and autoradiographed using Kodak XAR-5 film at -70°C for 1 to 8 days.

RESULTS

Phenotypic Response of NCI-H209 Cells to c-myc and Ha-ras Gene Insertion. The cell line NCI H209 (209) is a small cell lung cancer line which responds, as described in detail elsewhere (25), to coinsertion of a human c-myc gene and a viral Harvey ras (Ha-ras) gene by taking on properties of large cell lung carcinoma. These changes are identical to those produced by insertion of the Ha-ras gene into SCLC cells containing endogenously amplified c- or N-myc genes (2, 3). In brief, insertion of a constitutively expressed human c-myc gene, providing a 3- to 4-fold increase in the c-myc message, results in decreased doubling time, increased cloning efficiency, and looser cell aggregates (11). However, these myc-containing cells retain neuroendocrine characteristics of the parent 209 SCLC line and continue to grow in suspension, with the morphological characteristics of “variant” SCLC (11). 209 cells infected with and highly expressing the Ha-ras alone (209 ras) are phenotypically unchanged from 209 control cells. In contrast, 209 cells that have been transfected with both c-myc and Ha-ras (209 myc/ras) undergo the transition to a large cell carcinoma phenotype, with characteristics including decreased expression of neuroendocrine markers, growth as monolayers rather than as floating aggregates, and assumption of the electron microscopic features of the large cell phenotype (25).

Changes in PKC Isoform Gene Expression in 209 Cells with Inserted myc and ras Genes. Using the above-described cells, we first found that the basal transcript levels for the PKC isoforms differed markedly from one another in the parent 209 cells. Representative Northern hybridizations from one of five separate experiments are shown in Fig. 1. Thus, PKC-α mRNA can be visualized at 1-day exposure time, whereas PKC-β is barely visible even after 7 days of exposure. PKC-γ, requiring 14 days of exposure in the blot shown, was visualized in only 2 of these experiments. This suggests that the major fraction of PKC mRNA in these cells is PKC-α, with lesser amounts of PKC-β.

The most striking changes with insertion of the individual oncogenes were an increase in PKC-β gene expression in 209 cells containing the exogenous c-myc gene. In this setting, as compared to β-actin transcripts (Fig. 1), there was an increase in the steady-state levels of PKC-β transcripts (Fig. 1b and e), a much smaller increase in PKC-α transcripts (less than 2-fold in the blot shown and not significant over multiple experiments), and little or no change in the PKC-γ isoform transcripts. This myc-induced change in PKC-β and -α transcript expression was not due to amplification or structural alteration of the PKC-β or -α genes in these cells, as shown by analyses of DNA with multiple restriction enzymes (data not shown). By contrast, expression of the exogenous Ha-ras gene alone, which has no phenotypic consequences in the 209 cells, had no consistent effect on the steady-state level of any of the PKC isoform transcripts (Fig. 1).

Very different PKC transcript changes occur in 209 cells expressing the exogenous ras and myc genes together, and which assume the phenotypic characteristics of large cell lung cancer. As compared to the 209 myc cells, these cells display a profound decrease in the steady-state transcript levels of the
PKC-α and -β genes, including a complete reversal of the increased level of PKC-β which was induced by expression of the c-myc gene alone (Fig. 1). This transcript decrease was not due to an alteration in expression of the exogenous c-myc gene in the 209 myc/ras cells as verified by probing the same Northern blot with myc exon 2 (data not shown). This demonstrated that the changes in PKC isoform expression associated with ras infection of 209 myc cells were not due to selection of a clone which had lost expression of the transfected myc.

Changes in PKC Isoform Protein Levels in SCLC Cells with Inserted ras and myc Genes. To evaluate the potential functional consequences of the transcript alterations described above, we analyzed the protein levels for each of the PKC species present in the 209-derived cell lines. Representative Western hybridization studies with specific rabbit polyclonal antibodies raised to the PKC-α, -β(II), and -γ isoforms are shown in Fig. 2. A series of PKC protein changes were seen, some of which paralleled the above RNA findings, and some of which did not. For control 209 cells, as was seen for transcripts of PKC isoforms, the PKC-α isoform comprises the majority of the PKC protein in the cell cytosol and membrane (Fig. 2). Comparable relative PKC-α protein expression has been observed in other lung cancer cells (26). Consistent with the findings of a low PKC-β mRNA level in control H209 cells, in four separate experiments, PKC-β(II) protein cannot be detected in the cytosol or membrane. By contrast, PKC-β(II) is easily detected in another SCLC line, NCI-H69 (Fig. 2c). This cell line, which has been previously demonstrated to have very high levels of total PKC enzyme activity (26), contains an amplified N-myc gene (27) and, like H209 cells, responds to Ha-ras by transition to the large cell phenotype (3). Finally, consistent with the mRNA data, PKC-γ protein levels were low and found only in the cytosolic fraction (Fig. 2b).

For the oncogene insertion studies, a series of PKC isoform protein changes were noted, not all of which correlated with the mRNA changes. First, insertion of the c-myc gene alone caused an increase of PKC-β protein, as would be predicted from the mRNA findings. In two experiments, this increase was light and could be detected as an intact M, 66,000 PKC-β species in the membrane and cytosolic fractions of 209 cells expressing exogenous c-myc. In two other experiments, one of which is shown in Fig. 2c, the level of a M, 66,000 PKC-β(II) species is markedly increased in the cytosolic fraction, and a much smaller quantity of a M, 60,000 species appears in the membrane fraction of these 209 myc cells (Fig. 2c). The identity of the M, 66,000 band is not certain, but likely represents a metabolic product of PKC-β which is also seen in the cytosolic fraction of the H69 cells on this blot, and which has been reported in other cell types (28, 29). Also, the difference in size in the higher molecular weight PKC-β species in the H69 cell line may be due to differences in phosphorylation of this protein (30). Importantly, neither the M, 66,000 nor the M, 60,000 PKC-β protein species is increased in the 209 ras cells.

One prominent change in PKC proteins in 209 cells expressing either the exogenous c-myc or the Ha-ras gene alone, which did not parallel the transcript changes, was a prominent increase in membrane-associated M, 60,000 PKC-α species (Fig. 2). This increase presumably reflects posttranslational alterations in this PKC isoform species.

Unlike for PKC-β and -α proteins, and as predicted from the mRNA data, the PKC-γ protein level does not change with insertion of c-myc on Ha-ras alone and is detected only in the cytosolic fraction of all of these cells.

A series of distinct changes in PKC-α and -β proteins accompanied the simultaneous expression of the exogenous c-myc and Ha-ras genes, some of which paralleled the transcript findings. As for the mRNA data, PKC-α protein in cytosolic and the membrane fraction was reduced compared to levels in cells expressing each exogenous oncogene alone. Further, no changes were seen for PKC-γ protein. Alterations in PKC-β protein expression were more complex, and, perhaps, the most significant. Despite the marked fall in PKC-β transcript levels in 209 myc/ras cells as compared to 209 myc cells (Fig. 1), total PKC-β protein levels remained increased, and most importantly, showed a marked shift to presumably activated forms in the cell membrane (Fig. 2c).

To further assess the functional significance of the PKC protein changes, we measured total PKC activity in the 209 cell lines (Fig. 3). The changes correlated most closely with the amplitude and pattern of immunoreactive PKC forms in the cells membrane fraction (Fig. 2) and particularly with PKC-α protein. In 209 ras and 209 myc cells, cytosolic and especially membrane PKC activity was increased (Fig. 3), presumably due to the increased PKC-α protein levels (Fig. 2). Also, in 209 myc/ras cells, overall PKC activity levels returned to that seen in control 209 cells, again paralleling the changes seen in PKC-α transcripts (Fig. 1a) and protein (Fig. 2a).

For the membrane fraction, the only difference between the total PKC activity and the quantity of isoform protein seen on the immunoblots is that the total kinase activity in the 209 myc/ras cells does not reflect the increased PKC-β isoform seen by Western analysis. This is likely because the pattern of total PKC-β expression is markedly increased in the cytosolic fraction, and a much smaller quantity of a M, 66,000 species appears in the membrane fraction (Fig. 2c).
PKC activity reflects the larger contribution of the PKC-α isoform protein more than the less populous PKC-β isoform protein. However, this in no way negates the potential importance of the PKC-β changes for the phenotypic changes we are tracking. The differences between total PKC activity and immunoblot quantitation probably reflect the differential activation states of the PKC isoforms which may result from subcellular location, substrate specificity, and differences in regulation of each PKC isoform as have been observed in other cell types (8, 22, 31-33). In addition, we cannot eliminate a possible contribution to kinase activity by a PKC isoform that was not evaluated by immunoblotting.

DISCUSSION

Our observations in human SCLC cells are summarized in Table 1 and suggest the possible association between changes in PKC isoform gene expression and the action(s) of the myc gene to complement the ras gene to induce the biological changes we are studying in SCLC cells. Since ras proteins are known to interact with PKC (4-7), it was not unexpected that insertion of Ha-ras into 209 cells causes a series of posttranslational changes in PKC including an increase in overall PKC activity, due, apparently, to increased levels of PKC-α protein in the cell membrane fraction. However, in this cell type, no phenotypic changes ensue with this apparent activation.

Our surprising finding is that the expression of the c-myc gene, which is required in SCLC cells for ras to induce a phenotypic effect, causes a series of alterations in PKC isoforms, which involved both changes in steady-state transcript levels and changes in protein amounts. The most striking of these is an increase in transcripts and protein for PKC-β. In addition, there are an increase in protein and a small increase in transcripts for PKC-α.

Furthermore, a series of changes occurred with the insertion of ras into exogenous myc-expressing 209 cells, which could be critical for the transition to a large cell phenotype induced by expression of these two genes. (a) The large cell phenotype is associated with an increased PKC-β protein level in the cell membrane (presumably activated) compartment and with a decrease in PKC-α transcript and protein levels. (b) There was a striking abolition of the increased PKC-β transcript expression that is seen when the exogenous c-myc gene is expressed alone and also a reduction of the PKC-α transcript to levels well below those for control 209 cells (Fig. 1a).

Our findings lead us to hypothesize that complex changes in PKC isoform gene expression may play a role in the myc-ras gene complementation events which alter cell phenotypes. Most notably, we find increased levels of PKC-β mRNA and protein in cells transfected with c-myc gene alone. These data, suggesting a role for PKC in mediating cellular effects of c-myc, are complemented by several previous reports. Increased PKC activity can mimic cell responses to increased myc expression, including increased cell growth, and, often, "immortalization" of cells (34-36). Also, studies of other investigators indirectly suggest that PKC activity may be important for complementation events between myc and ras genes. Thus, 12-O-tetradecanoylphorbol 13-acetate, which activates one or more PKC isoforms, collaborates with Ha-ras transfection, but not with v-myc transfection, to transform primary rat embryo fibroblasts (37).

In the cell system that we are studying, we observed increased expression of a membrane-associated PKC-β protein and decreased expression of the PKC-α protein in the cells expressing both myc and ras compared with cells expressing myc alone. It is possible that alterations in the isoform balance in a specific cell may be as critical in affecting cell dynamics as an alteration in contribution of any specific isoform alone. In support of this hypothesis, alteration in PKC isoform expression (38) and in total PKC activity (39) accompanies differentiation changes in other cell types. Further, a recent study suggests that the relative enrichment of PKC-β and -α isoforms in the dorsal ectoderm of Xenopus underlies the susceptibility of this region to neurodifferentiating stimuli and contrasts with the insensitivity of the relatively PKC-γ-enriched ventral region to these same stimuli (40).

There are also data to support our findings that the PKC-β isoform may play a central role in myc-ras complementation events. Transfection of a PKC-β gene renders rat embryo fibroblast cells sensitive to the transforming activity of a mutated ras oncogene (41). Other studies have highlighted the importance of the PKC-β isoform expression in cell differentiation events. The level of PKC-β expression, and not of PKC-α expression, correlated with the responsiveness of KG-1 myeloid leukemia cells to the differentiating effects of phorbol ester (42). Further, 1,25-dihydroxyvitamin D₃-induced differentiation of HL-60 cells resulted in an increase in PKC transcripts which were predominantly of the β species (43).

Another new observation in our work which implicates the PKC pathway in myc-ras complementation is the profound steady-state decrease of PKC isoform transcript levels in those SCLC cells which are phenotypically responsive to Ha-ras gene insertion. We speculate that ras gene-induced activation of PKC protein may mediate down-regulation of the PKC system at the steady-state transcript level. There are several precedents for this type of down-regulation. (a) ras transformation of NIH 3T3 fibroblasts results in a partial activation and down-regulation of PKC protein (44). (b) The down-regulation of the PKC...
response may be necessary for phorbol ester-induced differentiation of HL60 cells (45). (c) At the transcript level, chronic exposure of KG-1 human myeloid leukemia cells to 12-O-tetradecanoylphorbol 13-acetate results in a selective decrease in PKC-β (and not PKC-α) RNA (42).

Our data imply a complex hierarchy of molecular events which regulate PKC expression in lung cancer cells. Classically, and including findings for expression of the ras oncogene, regulation of PKC activity has been shown to be by posttranslational mechanisms involving increasing cellular diacylglycerol levels (46) and by proteolysis (29, 47) and phosphorylation (30) of the PKC protein. In contrast, the increase in the PKC-β transcript and the smaller increase in the PKC-α transcript observed in vitamin D3-induced differentiation of HL60 cells (changes of magnitudes similar to those we observed in our cells) are accounted for by increased transcription of these genes (43). Transcript expression may also be regulated by a posttranscriptional process such as by altering message half-life. Indeed, c-myc has been shown to regulate the expression of two genes important in rodent fibroblast proliferation, mr1 and mr2, by a posttranscriptional process that does not affect message half-life, likely by modulating RNA export from the nucleus, RNA splicing, or nuclear RNA turnover (48). Such studies will be of interest in our cell system.

In summary, we present a study of cultured human small cell lung cancer cells in which individual and dual expressions of exogenous myc and ras genes are associated with changes in expression of two major PKC species at both the transcript and protein levels. These PKC responses may directly follow the action of the oncogenic products or may be a consequence of some other aspect of the phenotype acquired by the 209 cells following expression of the c-myc plus Ha-ras genes. Our model, using controlled gene manipulation, should allow for further dissection of these complex interactions, including the time frames involved and the specific molecular steps underlying linkage of myc, ras, and PKC gene expression events. Our findings should encourage work to determine whether, in other cell types, the actions of the c-myc protein might complement those of ras proteins by affecting intracellular signal transduction through modulation of expression of the PKC isoforms.

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REFERENCES

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