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The expression of PDGF-α but not PDGF-β receptors is suppressed in Swiss/3T3 fibroblasts over-expressing protein kinase C-α

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Abstract

The generation and characterization of Swiss/3T3 cells which stably over-express protein kinase C (PKC)-α were previously described by us. In these cells over-expression of PKC-α reduced the expression of epidermal growth factor (EGF) receptor molecules [(1990) J. Biol. Chem. 265, 13290–13296]. Here we show that the expression of PDGF-α receptors, but not PDGF-β receptors, was specifically decreased in these cells. Not only were the levels of PDGF-α receptor mRNA transcript and protein significantly diminished in the PKC-α over-producing cells, but their ability to respond to short- and long-term growth factor signals was appropriately compromised. This was reflected in a reduced tyrosine autophosphorylation signal in response to PDGF-AA, as well as in decreased growth rates of PKC-α over-expressing cells when supplied with external PDGF-AA. A similar decrease in PDGF-α receptors was also demonstrated in parental Swiss/3T3 cells treated with phorbol esters. Our studies imply that PKC-α is involved in a cellular mechanism suppressing the expression of PDGF-α receptors in Swiss/3T3 cells. Hence, activation of PKC-α or alterations in its cellular levels may affect, in turn, the expression of a specific set of cell surface receptors and their responses to external growth factors.

Key words: PKC-α; PDGF-α receptor; Receptor expression

1. Introduction

The Ca^{2+} and phospholipid-dependent protein kinase C (PKC) is thought to be an important regulator of various cellular processes including exocytosis, metabolism, cell proliferation and gene expression [1,2]. The role of PKC in cellular proliferation and transformation is supported by the fact that phorbol ester tumor promoters are potent and selective activators of this enzyme [1]. In spite of extensive research in the last years, it still remains unclear how exactly the signaling pathways, which lead to the above events, are activated or suppressed by PKC. The picture is further complicated by the fact that PKC exists as a gene family of classical [3–7] and PKC-related [8–11] isoenzymes with variations in properties such as kinetics, substrate specificities, degradation rates, patterns of translocation, and tissue/cell distribution [12]. Moreover, the fact that more than one isoform is usually expressed within a particular cell type has suggested that these are not mere isoenzymes of identical function but rather that they are different enzymes executing distinct cellular functions [13].

In order to examine the role of PKC isoforms in the regulation of cellular functions, specific isoforms were stably expressed in cultured cells. Phorbol ester-induced morphological changes, enhanced growth in soft agar, and tumorogenicity in nude mice resulted from the over-production of PKC-β1 and -γ isoforms in rat6 and NIH/3T3 cells, respectively [14–16]. In contrast to these reports, over-expression of PKC-α in Swiss/3T3 [17] was not able to confer a transformed phenotype. Similar results were reported for the over-expression of PKC-α in Balb/c and rat6 cells [18]. Likewise, the expression of the PKC-β1 isoform in C3H 10T1/2 or rat liver epithelial cells was unable to induce changes characteristic of transformed cells [19,20]. Moreover, its over-production in HT29 colon cancer cells suppressed cellular growth events and reduced their tumorogenicity in nude mice [21]. The discrepancies seen in these different studies may be attributed to intrinsic differences between the PKC isoenzymes themselves as well as to the specific cell context into which they were introduced. It appears, however, that although PKC by itself may not be sufficient to induce complete cell transformation, it can alter specific cellular growth characteristics.

In a previous study, we showed that over-expression of PKC-α in Swiss/3T3 cells resulted in the reduced expression of epidermal growth factor (EGF) receptor molecules at the cell surface, correlating with lower levels of their mRNA transcripts [17]. In the work described herein, we examined the effect of PKC-α on the receptors for PDGF. PDGF is a growth factor originally discovered in serum platelets, which produces a wide range of

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effects on cells, including motility and growth responses [22]. Its action is mainly exerted on cells of mesenchymal origin due to the presence of two receptor subtypes, α and β [23]. Both homodimers PDGF-AA and -BB, as well as the heterodimer, AB, have been isolated from normal and transformed cells [24,25]. The response of different cell types to the three dimeric forms appears to be related to the specific receptor subtype expressed on the cell's surface [26–28]. The regulation of these receptors will therefore determine the ability of a cell to respond physiologically to growth factors in its microenvironment. Here we show a specific effect of PKC-α on the expression of PDGF-α receptors but not on PDGF-β receptors.

2. Materials and methods

2.1. Cells and transfections

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, and 1% combined antibiotics. The generation of geneticin-resistant, PKC-α over-expressing clones was described elsewhere [17].

2.2. Northern blot analysis

Total cellular RNA was extracted using a modification of the guanidine thiocyanate method [49]. Equal amounts of denatured RNA samples were fractionated in 1% agarose gels containing formaldehyde and transferred to nitrocellulose filters. Blots were prehybridized for 4 h and then hybridized for at least 18 h at 42°C in a solution containing 50% formamide, 5 × SSC, 50 mM sodium phosphate, pH 6.5, 0.1% bovine serum albumin, 0.1% Ficoll-400, 0.1% polyvinyl pyrolidine, 0.1% SDS, 20–25 μg/ml denatured salmon sperm DNA and 10% dextran sulfate. Probes were labeled with [α-^{32}P]dATP by random priming or by nick translation and added at 10^6 cpm/ml. Filters were subsequently washed three times in 3 × SSC, 0.1% SDS at 42°C, and three times in 0.5 × SSC, 0.1% SDS at 65°C. The PDGF-α receptor full-length cDNA was recently isolated by Do et al. [50] and the PDGF-β receptor cDNA was kindly provided by Y. Yarden (Weizmann Institute, Israel).

2.3. [^{35}S]Methionine labeling and cell lysis

Cells were grown to subconfluence in 10 cm plates, incubated for 1–2 h in methionine-free DMEM supplemented with 5% dialyzed FCS and then labeled for 2–3 h in the same medium containing 100 μCi per ml [^{35}S]methionine (New England Nuclear). After washing twice with PBS, the labeled cells were scraped into 1 ml of cold solubilization buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 1 mM NaF, 1 mM PMSF, 0.4% aprotinin (24.4 TIU/ml), 5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. Cells were solubilized on ice for 20 min with frequent vortexing and then centrifuged at 17,000 × g for 15 min at 4°C. The collected supernatant was subsequently precleared with 1/10 of its volume of 50% protein A–Sepharose (all protein A–Sepharose beads used were first blocked with 10 mg BSA/ml) and rotated end-over-end for 45 min at 4°C. The solubilized fractions were monitored for total radiolabeled proteins by precipitating a small aliquot with 10% TCA. Equivalent amounts of labeled protein were used in subsequent immunoprecipitations. In experiments requiring PDGF stimulation, cells were incubated overnight in defined media DCCM (Biological Industries, Beit HaEmek, Israel) and labeled as above, except that methionine-free DMEM medium supplemented with 0.3% dialyzed FCS was used. At the end of the labeling period PDGF-AA, at 200 ng/ml, was added directly to the labeling medium. Cells were washed before solubilization with 10 mM Tris-HCl, pH 7.4, 140 mM NaCl and 2 mM Na_3VO_4.

In several experiments, unlabeled cells were treated at different time points with 200 ng/ml TPA in 0.01% dimethyl sulfoxide and 10% calf serum, or with the same medium lacking TPA. Cells were washed and lysed as above, and equivalent amounts of protein lysates were subjected to immunoprecipitation and immunoblotting as described below.

2.4 Immunoprecipitation experiments

Anti-phosphotyrosine, anti-PDGF-α receptor (R5) or non-immune serum (NS) coupled to protein A–Sepharose, were added to the lysates at a ratio of 1:100 to 1:50 and rotated end-over-end for 2 h. Cell lysates were subjected to sequential immunoprecipitations: first cleared with NS and then reacted with immune-specific serum. Both non-immune and immune-specific immunoprecipitates were washed three times with 1 ml buffer containing 10 mM Tris, pH 7.5, 140 mM NaCl, 0.1% SDS, 1% NP-40, 10 mM EDTA, 2 mM EGTA, aprotinin, leupeptin, soybean trypsin inhibitor and PMSF (in concentrations as above) followed by two washes in low-salt buffer containing 10 mM Tris, pH 8.0, 0.1% NP-40, 10 mM EDTA, 2 mM EGTA, aprotonin, leupeptin, STI and PMSF. Sample buffer was added to the washed beads and they were boiled for 5 min. The samples were analyzed by gel electrophoresis on 7.5% SDS-polyacrylamide gels. The fixed and stained gels were treated with 2 changes of DMSO and then with 22.5% PPO (2,5 diphenyl oxazole) for 2 h. Dried gels were autoradiographed on Agfa X-ray film at −70°C.

2.5. Immunoblotting analysis

Subconfluent cells in 10-cm dishes were washed with PBS and solubilized in 0.5 ml of solubilization buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptin, 1% aprotinin, and 1 mM PMSF. Solubilized cells were centrifuged for 10 min at 4°C at 17,000 × g, and the protein concentrations of the resulting supernatants were determined. Equivalent amounts of total lysate protein or washed immunoprecipitates were separated on 7.5% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and reacted with PDGFa [30] or PDGF-β (a kind gift of C.-H. Heldin) specific antibodies. Detection of primary antibodies was performed with ^{125}I-labeled protein A, ^{125}I-labeled goat anti-rabbit IgG or with the ECL Western blotting detection system (Amersham).

2.6. Cell growth assays

Cultured cells were set up in 24-well plates at a density of 1×10^4 cells/well. Initial plating was done in DMEM containing 1% CS. Approximately 18 h post-plating the medium was replaced with 1 ml of fresh DMEM containing 1% CS and the appropriate concentration of PDGF-AA (a kind gift of Imclone Inc., NY). After a 4 day growth period, the cells were quantitated using a modified Crystal violet staining procedure [51]. Briefly, cells were first fixed by the addition of 12.5% gluteraldehyde directly to the wells. After 30 min, the wells were washed 3 × with distilled water and 250 μl 0.1% Crystal violet was added. The plates were rocked for 30 min, washed three times with distilled water, and allowed to air dry. Once dry, 10% acetic acid was added to dissolve absorbed dye. After 15 min on a shaker, 100 μl was transferred in duplicate from each well to a corresponding well in a 96-well plate. The latter plates were read on a micro-ELISA reader, using a test wavelength of 570 nm to measure the optical density of dye extracts.

3. Results and discussion

3.1. PDGF receptors in PKC-α over-expressing cells

The Swiss/3T3-derived cell lines denoted SF1.4 and SF3.2 are characterized by stable over-expression of PKC-α with at least 10-fold higher PKC activity and 40-fold higher immunoreactivity than SC1 control lines [17]. In the study presented here we have analyzed the receptors for PDGF in these cells since this factor was shown to be a potent mitogen for Swiss/3T3 cells [29]. We first examined the levels of steady-state RNA found in these cells coding for both PDGF-α and -β type receptors. RNA isolated from PKC-α over-expressing (SF1.4 and SF3.2) and control (SC1) cells, was probed with ^{32}P-labeled full-length cDNAs of PDGF-α and PDGF-β receptor types. Fig. 1A depicts a drastic reduction in

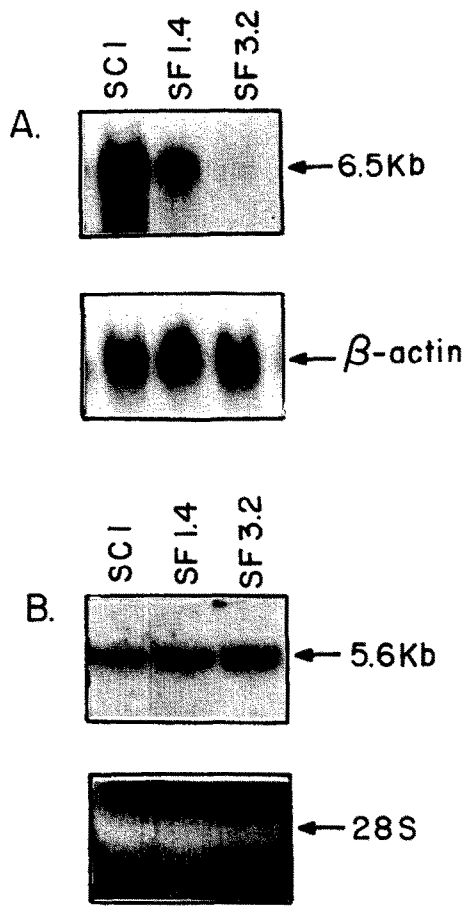

Fig. 1. PDGF-α and -β receptors RNA levels in PKC-α over-expressing cells. Total RNA from PKC-α over-expressing (SF1.4 and SF3.2) and control (SC1) cells were isolated and probed with ^{32}P-labeled full-length cDNAs of the PDGF-α (A) and PDGF-β (B) receptors as described in section 2. The amounts of RNA loaded on the gels were similar, as seen by the expression of β-actin (A) and ethidum bromide staining of 28 S RNA (B) in the α- and β-receptor probed blots.

PDGF-α receptor transcripts in PKC-α over-expressing lines SF1.4 and SF3.2 when compared to control SC1 cells. Interestingly, this alteration in RNA expression was confined to the α-receptor only, since the β-receptor probe revealed equivalent amounts of RNA transcripts in all the cell lines examined (Fig. 1B).

In order to confirm that this differential RNA expression is also reflected at the protein level, PDGF receptors were examined in SF1.4 PKC-α over-expressing cells, which displayed an intermediate level of RNA reduction when compared to control SC1 cells. Both cell types were radiolabeled with [^{35}S]methionine and cell lysates were immunoprecipitated with a PDGF-α receptor-specific polyclonal antibody [30]. Fig. 2A shows significant reduction in the incorporation of [^{35}S]methionine into this receptor in PKC-α over-expressing cells. Thus, consistent with the RNA findings, fewer PDGF-α receptor polypepetide molecules were synthesized in these cells. Furthermore, immunoblot analysis of the same cell lysates, probed with PDGF-β receptor-specific antibodies, confirmed also that the latter receptor is expressed at similar levels (Fig. 2B), as was expected from the Northern blot analysis (Fig. 1B).

Direct activation of PKC by TPA (12-*O*-tetradecanoylphorbol-13-acetate) in parental Swiss/3T3 cells (non-transfected) also caused a specific decrease of PDGF-α receptors (Fig. 3). A time-dependent reduction in PDGF-α receptors was shown by immunoprecipitations and Western blot analysis of TPA-treated Swiss/3T3 cells over a 24-h period (Fig. 3). Thus, TPA treatment of parental Swiss/3T3 cells caused a similar effect on PDGF-α receptors to that observed by the over-expression of PKC-α in the same cells, suggesting that PDGF-α receptor regulation is not merely a result of PKC-α over-expression. TPA treatment of Swiss/3T3 cells was previously reported to induce a small but repro-

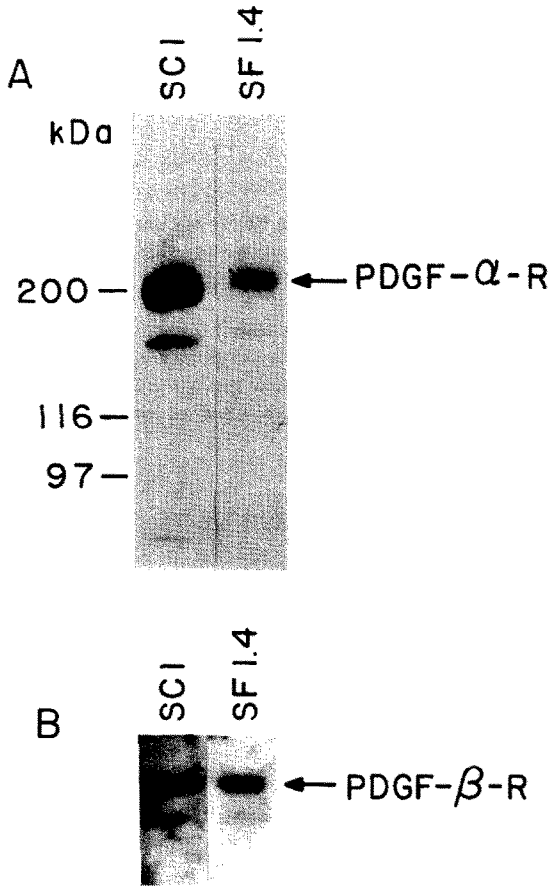

Fig. 2. Immunoprecipitation and immunoblotting of PDGF-α and -β receptors in extracts from PKC-α over-expressing cells. (A) Subconfluent cells were labeled with [^{35}S]methionine in methionine-free DMEM. Labeled cells were solubilized, cell lysates normalized for equal amounts of trichloroacetic acid-precipitable radioactivity, and immunoprecipitated with anti-PDGF-α receptor antibodies coupled to protein A–Sepharose. Immunoabsorbed material was fractionated on 7.5% SDS-polyacrylamide gels and proteins visualized by fluorography. Immunoprecipitation of PDGF-α receptors from control cells (SC1) and from PKC-α expressing cells (SF1.4) is presented. (B) Cell extracts prepared from indicated cell lines were subjected to SDS-PAGE, followed by immunoblot analysis using anti-PDGF-β receptor antibodies as described in section 2.

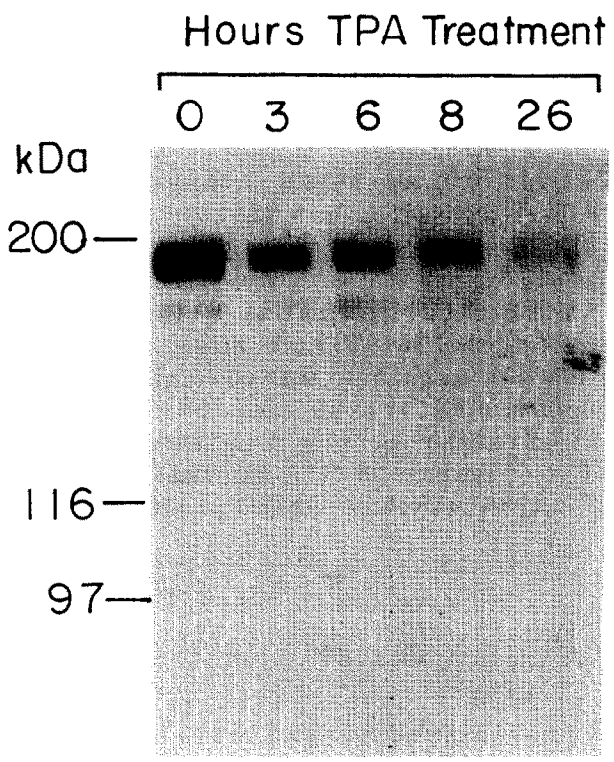

Fig. 3. Effect of TPA treatment on PDGF-α receptors. Parental non-infected Swiss/3T3 cells were treated (or not) with TPA (200 nM) over a 24 h period. Immunoprecipitations of equivalent amounts of solubilized protein cell extracts with anti-PDGF-α receptor antibody R5 [30] were performed as described in section 2. Immunoabsorbed material was separated on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and the blot reacted with the same R5 antibody. Detection of primary antibody was done with the ECL Western blotting detection system.

ducible decrease in [^{125}I]PDGF binding, apparently mediated by a decrease in the number of available cell surface PDGF receptors [31]. The relatively small reduction in PDGF binding seen in these earlier experiments may be explained by the mixture of human platelet-derived PDGF isoforms used; a larger specific decrease in PDGF-α receptor content, following TPA treatment, was most likely masked by the presence of unaffected PDGF-β receptors in these cells which were able to bind the BB and AA isoforms of human platelet-extracted material [23]. Furthermore, the reduction in PDGF-α receptors obtained in our experiments is most probably not due to their internalization and degradation since TPA was shown to have no effect on the kinetics of internalization and degradation of PDGF receptors in Swiss/3T3 cells [31]. It is important to note that it was not necessary to treat our PKC-α over-expressing clones with phorbol esters in order to induce suppression of PDGF-α receptor expression. This might be explained by the observation that a large proportion of PKC-α molecules is already associated with cell membranes in these non-stimulated cells [32] and thus, perhaps, partially activated.

3.2. *Reduced phosphorylation and growth responses to PDGF-AA in PKC-α over-expressing cells*

It is well established that PDGF dimers activate the intrinsic protein tyrosine kinase activity of its receptors, which was found to be essential for the mediation of cell growth [23]. We have therefore examined whether the specific reduction of PDGF-α receptors in SF1.4 cells also affected their response to external growth factor stimulation. The α-receptor is the 'universal' receptor, able to bind all three dimers of PDGF (AA, BB, AB), whereas the β-receptor was shown to bind only PDGF-BB and -AB (with less affinity) [33]. Therefore, by stimulating cells with PDGF-AA, only the α-subtype of PDGF receptors is activated. The tyrosine kinase activity in response to PDGF-AA was examined in PKC-α over-expressing SF1.4 cells and SC1 control cells, using anti-phosphotyrosine antibody. As shown in Fig. 4, the antibody's immunoreactivity with the self-phosphorylated 185 kDa PDGF-α receptor in SC1 cells was markedly higher than in SF1.4 cells. This is most likely due to the decreased expression of the receptor protein itself in PKC-α over-expressing cells.

A variety of mutations of the receptors for EGF, insulin, PDGF and others, resulting in the abolishion of tyrosine kinase activity, produced a concomitant loss of growth factor-stimulated DNA synthesis [34]. The lower PDGF-AA-stimulated receptor tyrosine kinase activity in PKC-α over-expressing lines implied decreased biological responses to this growth factor in intact cells. To

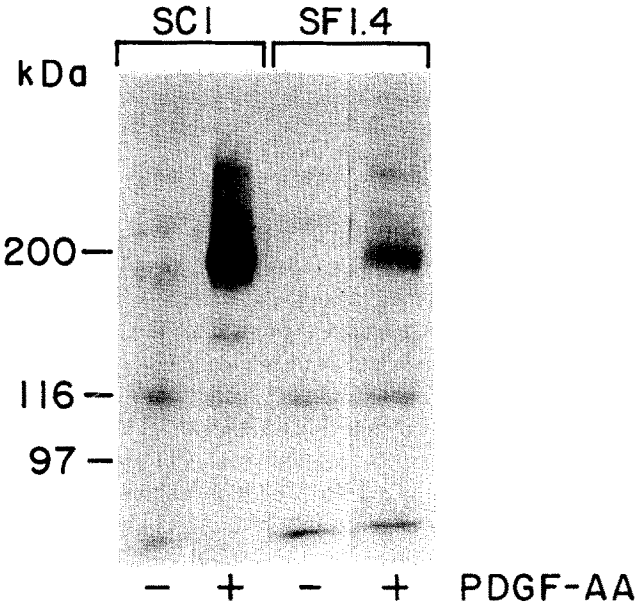

Fig. 4. Stimulation of PDGF-α receptor tyrosine kinase by PDGF-AA. PKC-α over-expressing (SF1.4) and control (SC1) cells were incubated overnight in defined media, labeled with [^{35}S]methionine under low serum conditions, and then stimulated for 10 min with 200 ng/ml PDGF-AA before solubilization. Equivalent amounts of trichloroacetic acid-precipitable radioactive lysates were immunoprecipitated with anti-phosphotyrosine antibodies as described in section 2. Immunoabsorbed material was fractionated on a 7.5% polyacrylamide gel and proteins visualized by fluorography.

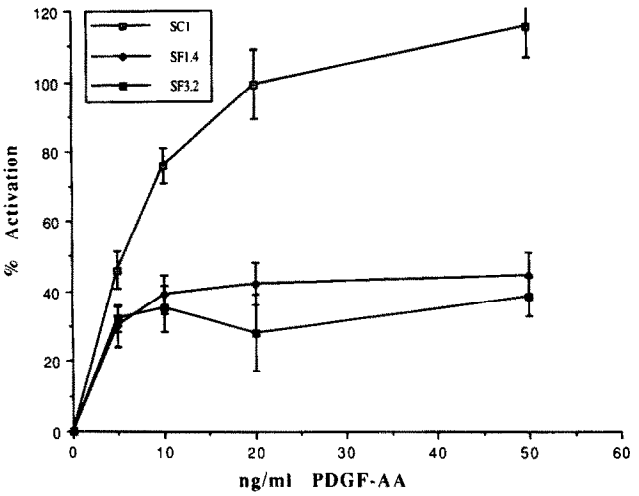

Fig. 5. Effect of PDGF-AA on the growth rates of PKC-α infected cell lines. PKC-α over-expressing cells (SF1.4 and SF3.2) and control (SC1) cells were grown over a 4 day period in 1% calf serum in the absence or presence of increasing (5–50 ng/ml) amounts of PDGF-AA. Cells were quantitated using a Crystal violet staining assay and results are expressed as percent activation above the OD_{570} of cells grown in the absence of growth factor.

test this we examined the growth response of PKC-α over-expressing cells, SF1.4 and SF3.2, and control SC1 cells to increasing amounts of PDGF-AA. As shown in Fig. 5, the growth rates of SC1 cells were greatly induced with PDGF-AA in a dose-dependent manner. The percent activation of these cells in media containing 50 ng/ml PDGF-AA reached 120%. However, both cell lines over-expressing PKC-α showed a significantly lower response to the externally applied growth factor signal, reaching only 44 and 38 percent activation with 50 ng/ml PDGF-AA in SF1.4 and SF3.2 cells, respectively.

3.3. Role of PKC-α in regulation of PDGF receptors

Swiss/3T3 cells normally express approximately equal numbers of α- and β-PDGF receptor subtypes [33, 35]. In this report we have shown that increased expression or activation of PKC-α in these cells resulted in the reduced receptor content of only PDGF-α receptors, but not of PDGF-β receptors. Our previous studies revealed a correlation between the high levels of PKC-α over-expressed in Swiss/3T3 cells and the reduced expression of EGF receptor molecules [17]. FGF receptors were, however, unaffected by the over-expression of PKC-α (Eldar, H. and Livneh, E., unpublished data). Thus, our studies suggest that PKC-α plays a role in regulating the expression of a specific set of cell surface membrane receptors. This selective regulation could play a role in determining the cells' sensitivity to external stimuli, of particular importance during neoplastic development. Indeed, malignancy-dependent changes in the expression of PDGF and PDGF receptors were detected in tumors of fibrosarcomas or glioblastomas (reviewed in [36]). Among other growth factor receptors, only the PDGF receptor appears to be capable of mediating ligand-induced cell division in normal tissues in the absence of other growth factors [29]. However, as noted before, signaling by PDGF is complicated by the existence of three forms of PDGF ligands (BB, AA, and AB) and three types of PDGF receptor dimers (αα, ββ, and αβ) [36]. The cellular significance of this complexity is not yet clear, but a detailed comparison of the functional activities of the α- and β-receptors revealed similarities as well as differences. Whereas both receptors transduce a mitogenic response, only the β-receptor mediated chemotaxis and actin reorganization in the form of circular membrane ruffles [37]. Moreover, in cells having both α- and β-receptors, activation of the α-receptor inhibited β-receptor induced chemotaxis [38,39]. Thus, regulation of PDGF-α expression by PKC-α, revealed in our experiments, may determine at least in part the relative abundance of PDGF-α and -β receptors within a particular cell and the cellular responsiveness to the different isoforms of PDGF.

Binding of PDGF to its receptors was shown to induce association with and phosphorylation of phospholipase C-γ [40,41], which catalyzes the degradation of phosphatidylinositol bisphosphate to diacylglycerol and inositol triphosphate. The latter, by releasing Ca^{2+} from internal stores acts together with diacylglycerol to activate PKC [2]. Since our studies show a role for PKC-α in the suppression of PDGF-α receptors expression, PDGF binding may actually initiate a negative feedback loop, regulating the expression of its α-type receptors only.

A role for PKC was previously proposed in the down-regulation of cell surface receptors via a cellular mechanism that induces their phosphorylation [1]. In fact, phosphorylation of EGF receptor in response to PKC activation was shown to decrease receptor affinity for its ligand [42], reduce its tyrosine kinase activity and the mitogenic effects of EGF [43,44]. Treatment of human T-lymphocytes with PKC activators also resulted in phosphorylation and down-regulation of the T3/T-cell antigen receptor complex molecules by increasing their internalization rates [45,46]. Thus, phosphorylation of certain cell surface receptors by PKC may provide the negative signal for their shut-off and their rapid loss from the cell surface. To our knowledge, no report in the literature has described direct phosphorylation of PDGF receptors by PKC, and no significant changes in their phosphorylation state was observed in cells made PKC deficient [35]. On the other hand, our previous [17] and present studies indicate that PKC-α decreases the steady-state mRNA and protein levels of EGF and PDGF-α cell surface receptors. This could imply the involvement of PKC-α in transcriptional or post-transcriptional processes modulating either the formation or the stability of these mRNAs. Our recent studies suggest potential relevance of the latter possibility. We have shown that the

100 kDa 2-5A synthetase, the enzymatic products of which have the capacity to inhibit protein synthesis and the stability of specific mRNAs, is up-regulated by PKC-α at a post-transcriptional level [47]. In another study, Brooks et al. [48] reported that activation of PKC in Swiss/3T3 cells by phorbol esters reduced the expression of its major phosphorylation substrate, p80, apparently also by a post-transcriptional mechanism affecting the stability of its mRNA. It is therefore possible that similar molecular mechanisms might be active in regulating the expression of EGF, PDGF-α and other receptor molecules.

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