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Tumor Cell-mediated Induction of the Stromal Factor Stromelysin-3 Requires Heterotypic Cell Contact-dependent Activation of Specific Protein Kinase C Isoforms*

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Stromelysin-3 (ST3, MMP-11) has been shown to be strongly overexpressed in stromal fibroblasts of most invasive human carcinomas. However, the molecular mechanisms leading to ST3 expression in nonmalignant fibroblasts remain unknown. The aim of the present study was to analyze the signaling pathways activated in normal pulmonary fibroblasts after their interaction with non-small cell lung cancer (NSCLC) cells and leading to ST3 expression. The use of selective signaling pathway inhibitors showed that conventional and novel protein kinase Cs (PKC) were required for ST3 induction, whereas Src kinases exerted a negative control. We observed by both conventional and real time confocal microscopy that green fluorescent protein-tagged PKC α and PKC ϵ , but not PKC δ , transfected in fibroblasts, accumulate selectively at the cell-cell contacts between fibroblasts and tumor cells. In agreement, RNAi-mediated depletion of PKC α and PKC ϵ , but not PKC δ significantly decreased co-culture-dependent ST3 production. Finally, a tetracycline-inducible expression model allowed us to confirm the central role of these PKC isoforms and the negative regulatory function of c-Src in the control of ST3 expression. Altogether, our data emphasize signaling changes occurring in the tumor microenvironment that may define new stromal targets for therapeutic intervention.

Matrix metalloproteinases (MMPs)¹ are zinc-dependent endopeptidases primarily involved in extracellular matrix degra-

tion and tissue remodeling (1, 2). The expression and activity of these extracellular enzymes are controlled at different levels, including transcription, secretion, zymogen activation, and inhibition of their active forms by a family of natural tissue inhibitors of metalloproteinases (TIMPs). Imbalance between MMPs and TIMPs has been implicated in various physiological but also in pathological tissue remodeling processes, notably in multiple steps of tumorigenesis (3). MMPs represent promising therapeutic targets for cancer therapy but additional studies are required to identify the regulatory mechanisms that control MMPs synthesis and activity in the tumor microenvironment (4, 5).

Most MMPs that have been identified in human carcinomas are expressed by the stroma, including fibroblasts, vascular, and inflammatory cells, rather than by tumor cells (6). Among these MMPs, stromelysin-3 (ST3, MMP-11) has received much attention as its expression is elevated at early stages in virtually all invasive human primary carcinomas and in a large part of their associated metastases. Moreover, high ST3 levels have been shown to be associated with poor clinical outcome in various human carcinomas (7–9). ST3 has therefore been proposed as an attractive target for therapeutic approaches directed against the stromal compartment of human carcinomas (8, 10). However, this enzyme exhibits specific properties and both its regulation and its specific function at the tumor-stroma interface remain largely unknown.

Although ST3 possesses the characteristic structure of MMPs, it does not degrade classic ECM components (11) and its only known substrates are serine protease inhibitors (12) and the insulin-like growth factor-binding protein-1 (13). Moreover, unlike most of others MMPs that are secreted as inactive zymogens, the ST3 prodomain contains a recognition site for furin convertase, resulting in the secretion of a 45-kDa active enzyme (14, 15). The near uniform expression of ST3 in early stage tumors strongly suggested that it might participate in the initial development of carcinomas. In agreement with this hypothesis, ST3 expression has been associated with increased tumor take and incidence (16–18) and a diminution of tumor cell apoptosis (19, 20) in various experimental models of tumorigenesis. Our recent data have further confirmed and extended this new function by showing that active ST3 increases tumor cell survival via activation of the p42/p44 MAPK pathway (21). In addition, other studies have recently shown that while ST3

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¹ The abbreviations used are: MMP, matrix metalloproteinases; CM, conditioned medium; EGF, epithelial growth factor; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ST3, stromelysin-3; Tet, tetracycline; TIMP, tissue inhibitor of matrix metalloproteinases; C/EBP, CCAAT/enhancer-binding protein; Ab, antibody; siRNA, small interfering RNA; cPKC,

conventional PKC; nPKC, novel PKC; JNK, c-Jun N-terminal kinase; CA, constitutively active; DN, dominant negative; GFP green fluorescent protein.

promotes cancer cell implantation in connective tissue, its expression is also associated with a decrease in metastatic incidence, illustrating a dual role of this paracrine factor (22).

At a molecular level, ST3 is induced by phorbol esters (23), basic fibroblast growth factor, EGF and platelet-derived growth factor (24, 25), thyroid hormone (26), transforming growth factor- β (27), and retinoic acid (28), a compound that usually represses the expression of other MMPs. The ST3 promoter strongly differs from that of other MMPs and contains three conserved regulatory elements including a C/EBP binding site (23), several retinoic acid responsive elements, and a thyroid responsive element (29). However, aside from thyroid and retinoic acid receptors that appear to control the expression of ST3 during the developmental processes associated with apoptosis (26, 30), the factors regulating its expression in other physiological and pathological processes have not been identified.

Tumor-stroma co-culture assays allow analysis of such a complex regulation in a model that resembles the *in vivo* situation observed in human carcinoma. Using such assays, we and others have demonstrated that the fibroblastic expression of ST3 required a direct contact between fibroblasts and tumor epithelial cells. In addition, its expression was not affected by neutralizing antibodies (Ab) directed against several growth factors including basic fibroblast growth factor, platelet-derived growth factor, EGF, and transforming growth factor- β (31, 32), indicating that these growth factors are not involved. The nature of the tumor-associated factors initiating the stromal response, as well as the signaling pathways activated in fibroblasts and implicated in the induction of ST3 are still unknown. In the present study, we have therefore analyzed the signaling pathways activated in human fibroblasts following their interaction with cancer epithelial cells and we have shown that both classical and novel PKCs are central regulators of ST3 expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The human fibroblast-like cells CCD-19Lu (CCL-210), derived from normal lung tissue, the human NSCLC cell line A549 and the rhabdomyosarcoma tumor cell line RD (CCL 136) were obtained from the American Type Culture Collection (Manassas, VA) and routinely cultured as previously described (23). Direct co-culture was carried out as previously described (31). PMA (Sigma) was used at 20 ng/ml. For inhibitory studies, drugs were added 15 min before treatments at the following concentrations: cycloheximide (Sigma), 10 μ M; GF109203X (Calbiochem), 5 μ M; G66976 (Calbiochem), 2–5 μ M; PD98059 (Calbiochem), 15 μ M; PP2 (Calbiochem), 10 μ M; SB202190 (Calbiochem), 30 μ M; SB203580 (Calbiochem), 15 μ M; LY294002 (Calbiochem), 10 μ M; U0126 (Promega), 10 μ M.

Northern Blot Analysis—Total RNAs were extracted by phenol/chloroform, separated on a 1% agarose/formaldehyde gel, transferred, and hybridized with [α - 32 P]dATP probes as previously described (33). Following exposure of the membranes to storage phosphorscreen, images were quantified using ImageQuantTM software (Amersham Biosciences).

Western Blot Analysis—Conditioned media (CM) were concentrated 20-fold by ultrafiltration (Ultrafree 5K, Millipore Corp.). Cell monolayers were lysed in 50 mM HEPES, pH 7.4, 150 mM NaCl, 20 mM EDTA, 10 mM sodium orthovanadate, 100 mM NaF, 1% Triton X-100 for 30 min under agitation, and centrifuged for 10 min at 12,000 \times *g* at 4 $^{\circ}$ C. Western blot analysis was performed as previously described (21). Anti-ST3 mAb was described elsewhere (clone 1G4) (20). Isozyme-specific anti-PKC Ab were from Transduction Laboratories. Ab raised against phospho-p42/p44 came from New England Biolabs. Anti-c-Src Ab was from Santa Cruz Biotechnology. Anti-Myc mAb (clone 9E10) was provided by UBI. Other Abs were purchased from Cell Signaling.

Transient Transfection and Cell Fractionation—Transient transfection of CCL-210 by constructs coding for hPKC α -GFP, hPKC ϵ -GFP, and hPKC δ -GFP was performed with Exgen 500 (Euromedex, France) in 6-well plates as previously described (34) (2 μ g of ADN and 10 μ l of Exgen/well). Twenty-four hours after transfection, 3 \times 10⁵ A549 tumor cells or PMA were added for different incubation times. Cells were washed with cold phosphate-buffered saline followed by scraping into homogenization buffer (10 mM Tris, pH 7.4, 2 mM EDTA, 1 mM phen-

ylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 1 μ g/ml pepstatin). Cells were then sonicated at 4 $^{\circ}$ C (10 s, 3 times) and centrifuged for 30 min at 13,000 rpm. Supernatants were collected and correspond to the soluble fractions. Pellets, corresponding to the membrane fraction, were resuspended in homogenization buffer supplemented with 1% Nonidet P-40 and incubated for 45 min on ice. Both fractions were subjected to SDS-PAGE and Western blotting using an anti-GFP mAb (Roche Molecular Biochemicals).

Transient Transfection and Observation of Fusion Protein Localization in Living Cells—CCL-210 cells were seeded on 12-mm round coverslips in 24-well plates and transfected with hPKC-GFP constructs as described elsewhere (0.5 μ g of DNA and 2.5 μ l of Exgen-500/well). Twenty-four hours later, cells were stimulated with either PMA or addition of 10⁶ A549 tumor cells. The localization of fusion proteins in living cells was examined by conventional or confocal fluorescence microscopy at different times following stimulation as previously described (35).

siRNA Transfections—Cells were transfected with siRNAs duplexes at a final concentration of 100 nM in 6-well plates using the siImporter reagent (Upstate) 48 h before treatment, according to the manufacturer recommendations. PKC α and PKC ϵ siRNAs were purchased from Upstate. PKC δ siRNA duplex (5'-CGACAAGAUAUCGGCAGATT-3') (36) was synthesized and purified by Eurogentec.

Construction of Plasmids Encoding Constitutively Active (CA)/Dominant Negative (DN) PKC Isoforms and CA c-Src—cDNAs coding for rat CA PKC ϵ A159E and DN PKC ϵ K436R (37) (kindly provided by Dr. Gottfried Baier) were amplified with the following synthetic oligonucleotide primers (sense: 5'-ACCATGGTAGTGTTCATGGCCTT-3'; antisense: GGGCATCAGGTCTTACACAAA), subcloned into PCRscript, digested by KpnI/NotI, and finally subcloned into the Tet-inducible vector pCDNA4/TO (Invitrogen).

Plasmid containing human PKC α -EGFP cDNA (PKC α -EGFP MercuryTM probe, Clontech) was digested with SacII/XhoI to extract PKC α and the resulting insert was cloned into the pCDNA4/TO vector. Single mutations were introduced to generate CA PKC α A25E and DN PKC α K368R with the QuikChangeTM site-directed mutagenesis kit (Stratagene) using the following mutation oligonucleotide primers: CA PKC α , sense: 5'-CCCAGCAAAGGGAGCTGAGGCAGAAG-3'; antisense: 5'-CTTCTGCCTCAGCTCCCTTTGCGGG-3'; DN PKC α , sense: 5'-GAACTGTATGCAATCAGAATCCTGAAGAAGGATGTGG-3'; antisense: 5'-CCACATCCTTCTTCAGGATTCTGATTGCATACAGTTC-3'.

cDNA coding for PKC δ was amplified by reverse transcriptase-PCR from RD cells mRNA using the following primers (sense: 5'-ACCATGGCGGTTCTGCGCATC-3'; antisense: 5'-ATCTTCCAGGAGGTGCTCGAATTT-3'), ligated into the PCRscript plasmid, digested with EcoRV/NotI, and finally subcloned into pCDNA4/TO plasmid. Single mutations were introduced to generate CA PKC δ A148E and DN PKC δ K379R as described above using the following mutation oligonucleotide primers: CA PKC δ , sense, 5'-GAACCGCCGCGGAGAAATCAAAACGGCCAGATCC-3'; antisense, 5'-GGATTTTGCCCTGTTGATTTCTCCGCGCGGTTCTGCGCATC-3'; DN PKC δ , sense, 5'-GTACTTTGCCATCAGGGCCCTCAAGAAGG-3'; antisense, 5'-CCTTCTTGAGGGCCCTGATGGCAAAGTAC-3'. All constructions were entirely sequenced. c-Src cDNA was extracted with XbaI from pSG5 plasmid containing chicken CA c-Src Y527F cDNA (kindly provided by Sarah Courtneidge, San Francisco, CA), ligated into PCRscript plasmid, digested by EcoRI and finally subcloned into pCDNA4/TO plasmid.

Generation of Stable RD Transfectants Expressing Tetracycline-inducible CA/DN Forms of PKCs and c-Src—T-REXTM system (Invitrogen Corp.) was used to obtain a Tet-induced expression system in RD cells. We first established a stable cell line that constitutively expressed the Tet repressor by RD cells electroporation (400 V, 125 μ F) with the pCDNA6/TR plasmid followed by selection with 10 μ g/ml blasticidin. Twenty independent subclones were expanded and tested for Tet-inducible gene expression by transient transfection with a positive control plasmid expressing β -galactosidase. The clone with the lowest level of basal transcription and the highest level of β -galactosidase expression after addition of Tet was selected for subsequent transfection with the different expression plasmids (RD-TR cells). RD-TR cells were electroporated with the different kinase constructs described above and a second selection was performed using 5 μ g/ml blasticidin and 200 μ g/ml Zeocin. Following selection, positive clones were routinely cultured in Dulbecco's modified Eagle's medium, 10% fetal calf serum supplemented with 2.5 μ g/ml blasticidin and 200 μ g/ml Zeocin and expanded. In all experiments, at least two independent clones were analyzed for each construct.

Stimulation of Stable RD Transfectants Expressing Tetracycline-inducible Kinases—RD-TR clones expressing the CA or DN forms of

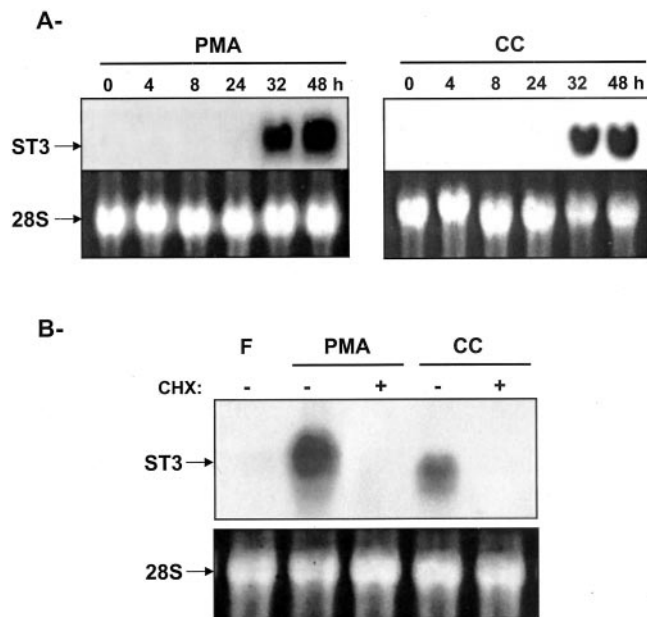


FIG. 1. Comparison of ST3 induction in fibroblasts exposed to PMA or co-cultured with A549 tumor cells. PMA (20 ng/ml) or A549 tumor cells (10^6 cells) were added on a confluent monolayer of human pulmonary fibroblasts in 35-mm dishes in serum-free medium. *A*, time course of ST3 mRNA expression: after the indicated times of stimulation, RNAs were extracted and analyzed by Northern blot performed with 10 (for PMA) or 20 μg (for co-culture, CC) of total RNA. *B*, protein synthesis requirement. PMA-stimulated fibroblasts or co-cultures were incubated in the absence or presence of 10 μM cycloheximide (CHX) for 36 h and ST3 mRNA expression was performed by Northern blot (F, fibroblasts; CC, co-culture).

kinases were stimulated by either 20 ng/ml PMA, 4 μg/ml Tet, or a combination of the 2 drugs in the absence of serum. At different times following stimulation, CM were harvested and cells were lysed as described elsewhere.

Transient Transfection of RD-TR Cells and Luciferase Assay—RD-TR cells at 80% confluence in 6-well dishes were transiently transfected with Exgen-500 using luciferase reporter plasmid 2.5-ST3-LUC (−2447 to +15) (23) or a control pGL3-Basic reporter plasmid. Eighteen hours after transfection, cells were washed twice with phosphate-buffered saline and stimulated by either 20 ng/ml PMA, 4 μg/ml Tet, or a combination of the 2 drugs in the absence of serum for 32 h before determination of luciferase activity, as previously described (21).

Statistical Analysis—Results are expressed as mean ± S.D. and statistical analysis was performed using the Student's *t* test with a statistical significance of at least $p < 0.05$.

RESULTS

Time Course of ST3 Induction in Normal Human Pulmonary Fibroblasts following Co-culture with NSCLC A549 Cells—Using a co-culture assay in which A549 cells are grown on a monolayer of normal pulmonary fibroblasts, we had previously shown that direct contact between the two cell types specifically induces ST3 mRNA in fibroblasts (31). To determine the mechanism leading to ST3 expression in these conditions, the induction kinetic of ST3 mRNA in co-culture was compared with that of fibroblasts treated with the phorbol ester (PMA), whose AP1-independent transcriptional activation has been demonstrated (23). As shown in Fig. 1A, the induction pattern of ST3 was similar in both conditions, with no detectable levels of mRNA before 24 h and a stable expression of the transcript after 32 h. Because PMA-mediated induction of ST3 is dependent on *de novo* protein synthesis (23), we examined the effect of cycloheximide on ST3 induction in the tumor-stroma co-culture. As shown in Fig. 1B, cycloheximide totally blocked ST3 transcript induction in both co-culture and PMA conditions, indicating that the fibroblastic induction of ST3 by cancer cells also requires protein neosynthesis.

Effect of Major Signaling Pathways Inhibitors on PMA- and Co-culture-mediated ST3 Induction—To determine the potential role of specific signaling pathways in PMA or co-culture-mediated ST3 induction, we tested known inhibitors of kinases for their ability to affect ST3 expression (Fig. 2). TIMP-1, a gene constitutively expressed in both fibroblasts and A549, was used as an internal control and its expression was evaluated by Northern blot analysis together with that of ST3 (Fig. 2A). The induction of ST3, observed after 32 h stimulation by PMA or co-culture, was totally abolished by GF109203X, an inhibitor of both conventional (cPKC) and novel PKCs (nPKC) but only partially by the cPKC inhibitor G66976 (Fig. 2, A and B). SB203580, a selective inhibitor of p38 MAPK had a low but significant inhibitory effect on ST3 induction, an effect that was more pronounced with SB202190, a dual inhibitor of p38 and JNK kinases. No alteration of ST3 expression was observed in the presence of the MEK inhibitor PD98059, indicating that p42/p44 MAPK was not involved in this process. Similar findings were obtained in the presence of the selective phosphoinositide 3-kinase inhibitor LY294002. Interestingly, treatment with the Src kinase inhibitor PP2 led to a significant increase in ST3 transcript in co-culture (+80%, Fig. 2B). Concerning TIMP-1 transcript level, GF109203X abrogated PMA-mediated induction, whereas other inhibitors had no significant effect (Fig. 2A, left panel). As previously described, the level of the TIMP-1 transcript did not vary in co-culture conditions (33) and none of these inhibitors significantly affected its level, thereby indicating that these drugs had no toxic effect under these conditions (Fig. 2A, right panel).

To test the effect of these various kinase inhibitors at the protein level, analysis of conditioned media from 48-h stimulated fibroblasts was performed by Western blot in the same conditions (Fig. 2C). No ST3 secretion was detected in the medium of the NSCLC cell line cultured alone (data not shown). Consistent with the transcriptional activation of the ST3 gene observed in Fig. 2A, a major secreted ST3 species of 45 kDa was detected in fibroblasts exposed to PMA or to the NSCLC cancer epithelial cells. This 45-kDa secreted ST3 results from the intracellular cleavage of the inactive ST3 precursor pro-enzyme by furin (14). Its activity was previously demonstrated for the purified recombinant enzyme (11–13), as well as for the enzyme secreted in the same co-cultures as those used in the present study (31). The secretion of this active 45-kDa ST3 enzyme (E) was significantly inhibited by PKC inhibitors and to a lesser extent by stress kinase inhibitors in co-culture conditions, whereas it was induced by the Src kinase inhibitor PP2 (Fig. 2C), demonstrating that alteration of the ST3 transcript level also results in a similar modification of the amount of active enzyme in conditioned media.

Altogether, these data indicate that PMA and co-culture-dependent ST3 induction require common signaling pathways, involving essentially c- and nPKCs and stress-activated kinases, whereas Src kinases exerted an inhibitory action. Finally, similar results were obtained by using other tumor cell types (breast tumor cell line MCF-7, squamous tumor of the tongue CAL-33) or other fibroblasts (fetal human fibroblasts CCL-153, infiltrated fibroblasts from the CAL 33 carcinoma) (data not shown), indicating that this mechanism is not restricted to a specific type of cancer cells or fibroblasts.

Specific Accumulation of Fibroblast PKC α - and PKC ϵ -GFP at the Cell-Cell Contact with Tumor Cells—Because activation of c- and nPKCs isoforms is likely to represent an early and central event in the control of ST3 expression, we analyzed the expression and the potential relocalization of several PKC isoforms in normal fibroblasts cultured alone or co-cultured with

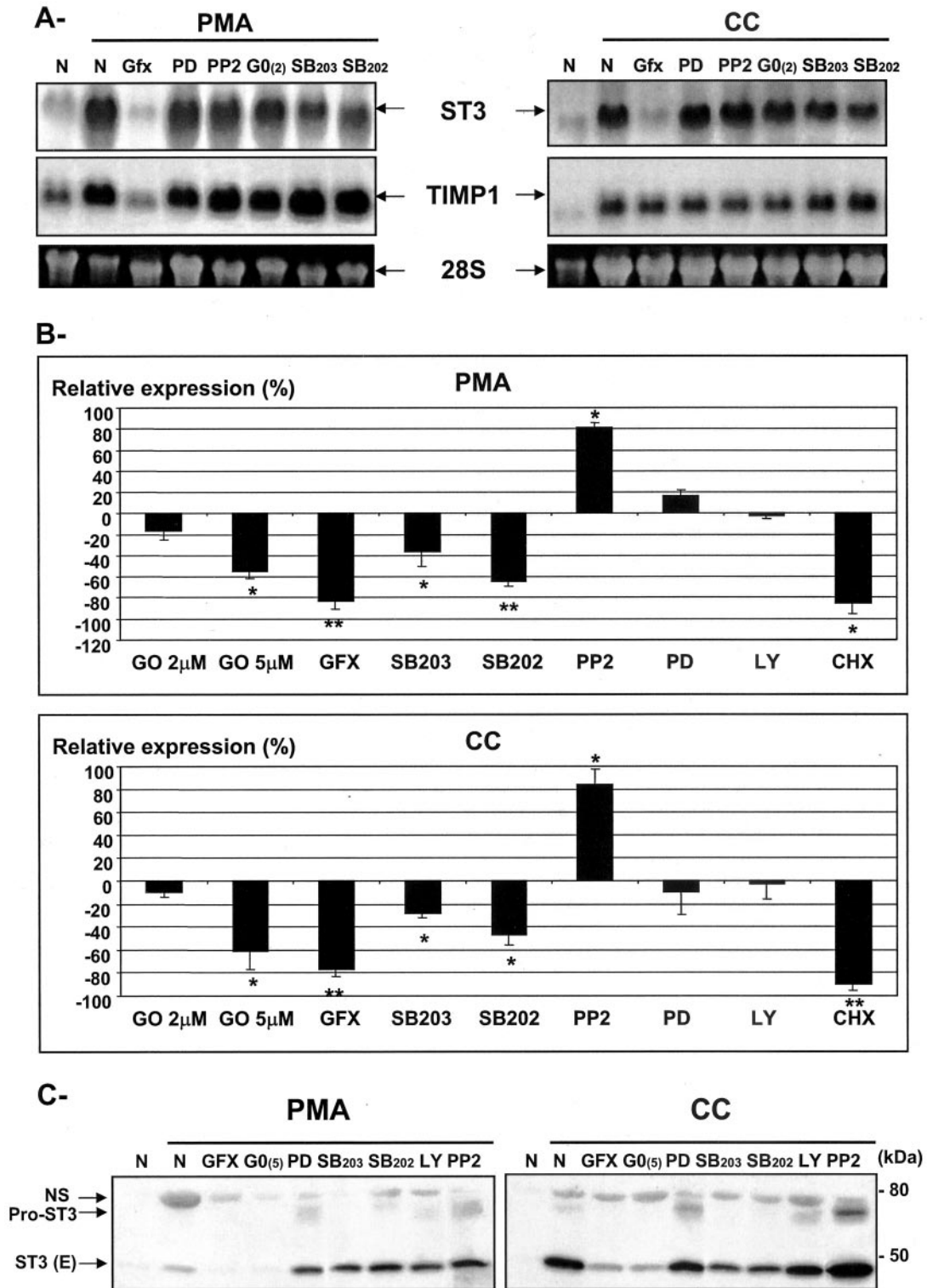


FIG. 2. Effect of the major signaling pathway inhibitors on ST3 induction in fibroblasts exposed to PMA or co-cultured with A549 tumor cells. Control, PMA-stimulated fibroblasts, or co-cultures were incubated in the absence (N) or presence of different pharmacological inhibitors in serum-free medium for 2 days. A, ST3 and TIMP-1 transcripts were analyzed by Northern blot using 10 or 20 µg of total RNA. B, experiments were repeated three times and Northern blot results were quantified with a phosphorimager. The average effect ± S.D. obtained for each inhibitor compared with the untreated PMA- or tumor cell-stimulated condition is represented. C, ST3 protein expression was analyzed in 10 times concentrated conditioned media by Western blot. GFX, GF109203X; G0, G6976; PD, PD98059; SB202, SB202190; SB203, SB203580; LY, LY294002; CHX, cycloheximide. *, $p < 0.05$; **, $p < 0.005$ versus control.

NSCLC cells. Western blot analysis using specific Abs directed against the main isoforms of c- and nPKCs indicated that PKCα, PKCε, and PKCδ are constitutively expressed in fibroblasts (Fig. 3A). These 3 isoforms are also present in the epithelial tumor cells A549, with PKCδ being mainly produced as

a 42-kDa fragment that is likely to correspond to its C-terminal catalytic fragment (38, 39). We then analyzed the potential relocalization of exogenous hPKC-GFP chimeric proteins in fibroblasts. Fibroblasts were transiently transfected with constructs encoding hPKCα, hPKCε, or hPKCδ fused to GFP (34,

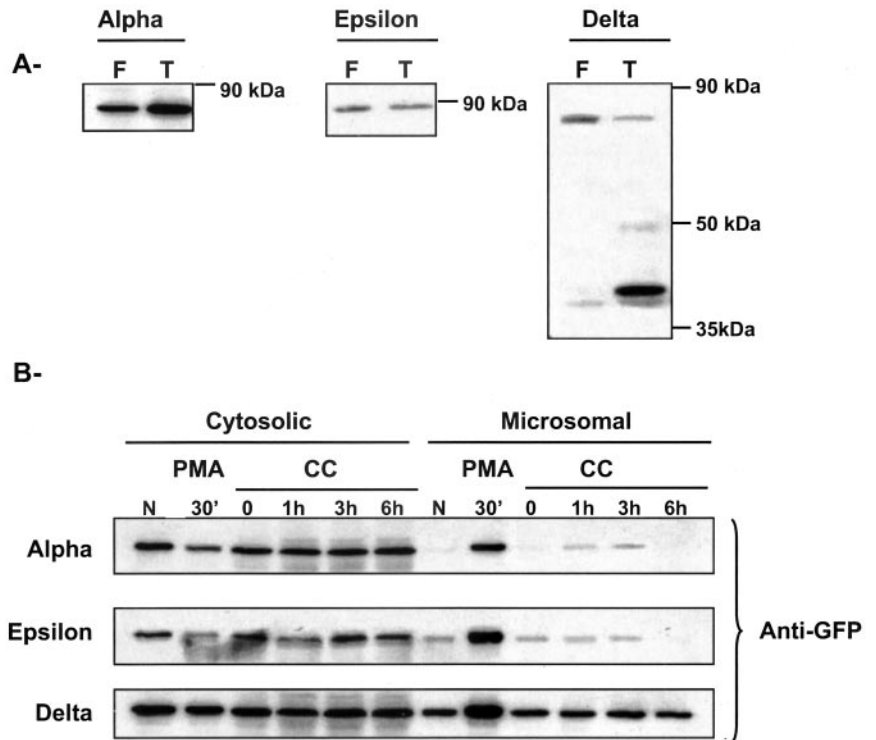


FIG. 3. Analysis of expression and plasma membrane translocation of PKC α , PKC ϵ , and PKC δ in fibroblasts exposed to PMA or co-cultured (CC) with A549 tumor cells. A, expression of the different PKC isoforms in total lysates of normal fibroblasts (F) and A549 tumor cells (T) was determined by Western blot using isoform-specific anti-PKC Abs. B, human fibroblasts transiently transfected with hPKC α -GFP, hPKC ϵ -GFP, or hPKC δ -GFP were treated with PMA or co-cultured with A549 tumor cells for the indicated times. Cells were lysed, fractionated as described under "Experimental Procedures," and localization of PKC isoforms was analyzed by Western blot with an anti-GFP Ab.

39, 40) and subsequently stimulated by PMA or co-cultured with tumor cells. Analysis of PKCs-GFP subcellular localization was first performed by Western blot in cytosolic and microsomal fractions of cell lysates (Fig. 3B). As expected, PMA induced a significant relocalization of the three PKC isoforms from the cytosolic to the microsomal fraction of fibroblasts. Interestingly, a low but significant translocation of PKC α is also detected after 1 and 3 h of co-culture. In contrast, no modulation in PKC ϵ and PKC δ localization could be observed but the presence of both isoforms in the microsomal fraction of control cells may mask a modest relocalization. We therefore analyzed the localization of these hPKC-GFP isoforms in live fibroblasts by conventional fluorescent microscopy (Fig. 4). As expected, PKC α and PKC ϵ showed a typical cytoplasmic localization in control fibroblasts (Fig. 4, A and D). Interestingly, a strong accumulation of these 2 isoforms at some cell-cell contacts between fibroblasts and tumor cells was reproducibly observed (Fig. 4, B and C and E and F) and remained stable for at least 3 h. The PKC α and PKC ϵ accumulation was specific to the contacts between fibroblasts and tumor cells as it was not observed at cell-cell contacts between fibroblasts themselves (data not shown). In contrast with PKC α and PKC ϵ , PKC δ appeared homogeneously distributed, with no clear compartmentalization between the cytoplasm and the nucleus in control cells (Fig. 4G). In the presence of tumor cells, there was no significant relocalization of PKC δ during at least the first 3 h of co-culture.

To better define kinetics of relocalization for PKC α and PKC ϵ , we then recorded by real time confocal microscopy the subcellular distribution of each fusion protein in live fibroblasts during a PMA stimulation or immediately after addition of tumor cells (Figs. 5 and 6). As expected, PMA induced profound changes in subcellular distribution of all 3 PKCs (41, 42). Of note, some differences between the kinetics of redistribution of the three isoforms could be observed (Fig. 5), such as novel PKC ϵ and PKC δ relocalizing more rapidly to the plasma membrane than the classical PKC α (3–5 versus 10–15 min). Interestingly, whereas PKC α and - ϵ relocalized only to the plasma

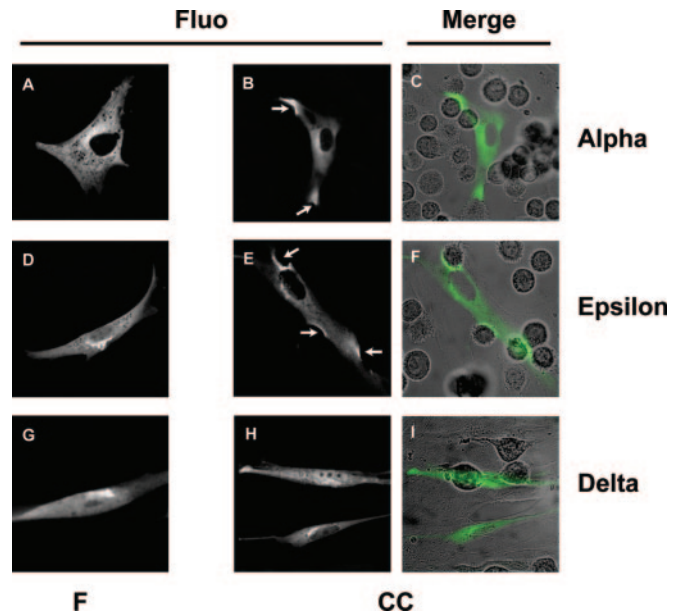


FIG. 4. Targeting of PKC α and PKC ϵ at heterotypic cell contacts in fibroblasts co-cultured (CC) with A549 tumor cells. Fibroblasts (F) expressing hPKC α -GFP, hPKC ϵ -GFP, or hPKC δ -GFP were imaged using conventional fluorescence microscopy from 30 min to 3 h after the addition of fresh medium containing or not A549 tumor cells. In control conditions, hPKC α -GFP (A) and hPKC ϵ -GFP (D) were mainly localized in the cytoplasm. After A549 tumor cell addition, hPKC α -GFP (B and C) and hPKC ϵ -GFP (E and F) were targeted at the interface with tumor cells (see arrows). Such a relocalization was not observed in isolated fibroblasts in co-culture conditions. The subcellular localization of hPKC δ -GFP remained cytosolic and nuclear in basal (G) or co-culture (H and I) conditions.

membrane, PKC δ initially relocalized to the plasma membrane and subsequently to the nuclear membrane. PKC δ that accumulated at the plasma membrane originated from the PKC δ cytoplasmic pool, whereas PKC δ that accumulated at the nuclear membrane originated from the PKC δ nuclear pool. Data

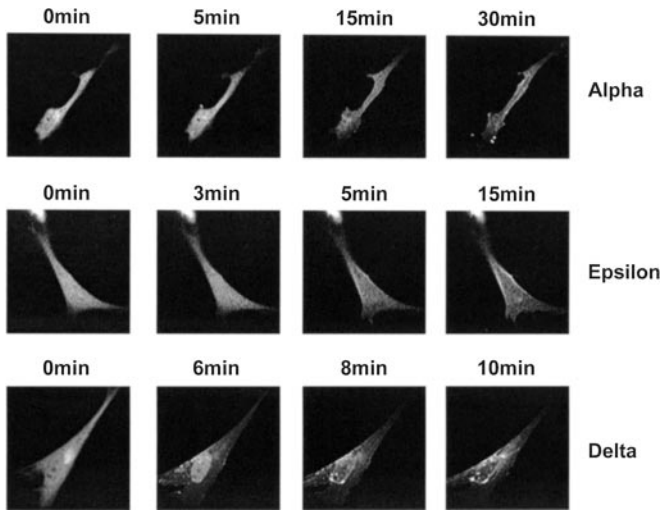


FIG. 5. Time course of plasma membrane translocation of hPKC α -GFP, hPKC ϵ -GFP, and hPKC δ -GFP in response to PMA stimulation. Fibroblasts expressing hPKC α -GFP, hPKC ϵ -GFP, or hPKC δ -GFP were observed with a confocal microscope immediately before and during stimulation with 20 ng/ml PMA. Images were recorded every 15 s for 30 min.

obtained in co-culture conditions confirmed that PKC α and PKC ϵ were selectively targeted to a significant number of tumor cell/fibroblast contacts. This relocalization occurred with a similar kinetic for both isoforms within 15–20 min after addition of tumor cells and then remained stable (Fig. 6, A and B). Fig. 6A shows two examples of confocal recordings where PKC α accumulated at the exact location of a tumor cell. In Fig. 6B, PKC ϵ accumulated at the plasma membrane where two tumor cells are at close vicinity of a fibroblast pseudopod. Altogether, these data demonstrate that the PKC activation is clearly observed by the translocation of two isoforms that represent an early event preceding the induction of ST3. More importantly, the fact that this co-culture-mediated ST3 induction in fibroblasts is contact-dependent is also visualized by the selective accumulation of these PKC isoforms at the cell-cell contacts between fibroblasts and tumor cells.

PKC α and PKC ϵ Are Required for Co-culture-mediated ST3 Induction—To directly assess the role of these PKC isoforms on ST3 production by normal pulmonary fibroblasts we used an siRNA approach. As shown in Fig. 7A, PKC α , δ , and ϵ siRNA transient transfection led to a significant and specific decrease in the expression of the 3 PKC isoforms. Consistent with the relocalization data, a decrease in PKC α and PKC ϵ expression led to a 60 and 90% reduction of the 45-kDa active ST3 expression level in co-culture-stimulated fibroblasts, respectively, whereas a reduction of PKC δ expression had no significant effect (Fig. 7B). Altogether, these data strongly support a specific involvement of these 2 PKC isoforms in the signal transduction pathways leading to ST3 expression.

A Tetracyclin-inducible Model to Evaluate the Role of Specific PKC Isoforms on ST3 Expression—To better define the effect of the selective activation or inhibition of these PKC isoforms on ST3 expression, we generated various constructs coding for CA and DN forms of PKC α , PKC ϵ , and PKC δ . However, we could not use primary pulmonary fibroblasts to establish a stable cell line expressing recombinant PKC isoforms. We therefore used the mesenchymal-derived rhabdomyosarcoma RD cell line that was shown to have a similar ST3 expression pattern in response to various agents (23, 28). RD subclones that could be induced to express the different forms of kinases by tetracyclin (Tet-on system) were established.

We first isolated a RD subclone expressing a high level of Tet

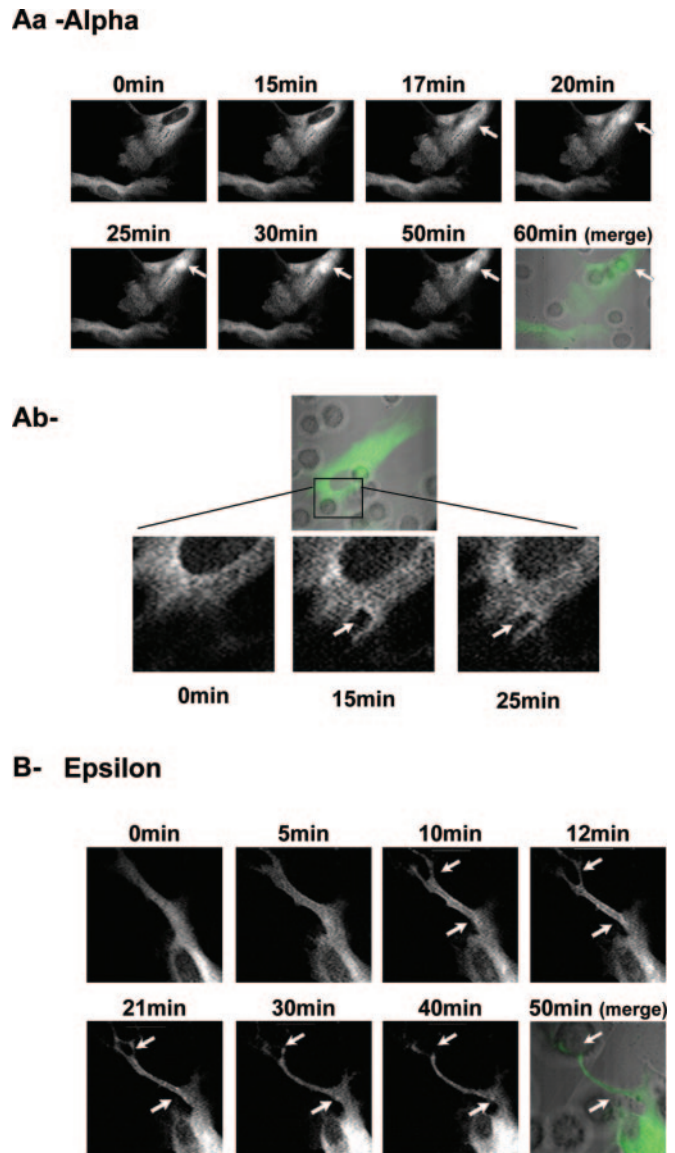


FIG. 6. Time course of plasma membrane accumulation of hPKC α -GFP and hPKC ϵ -GFP in primary fibroblasts in response to A549 tumor cell additions. Fibroblasts expressing hPKC α -GFP or hPKC ϵ -GFP were imaged by real time confocal microscopy immediately before and after addition of A549 tumor cells. Images were acquired every 1 min for 50 min. A, two examples of selective translocation of hPKC α -GFP to heterotypic cell-cell contacts. Aa, translocation is observed 17 min after addition of tumor cells. Ab, zoom of a fibroblast-tumor cell contact. Targeting of hPKC α -GFP at cell-cell contact occurred 15 min after addition of tumor cells. B, selective translocation of hPKC ϵ -GFP in a fibroblast in direct contact with a tumor cell 12 min after addition of A549 cells.

repressor (RD-TR). As illustrated in Fig. 8A, this subclone could express a high level of ST3 when exposed to PMA, as previously described for the original RD cell line (23). Moreover, the expression of PKC α , PKC ϵ , and PKC δ isoforms was verified by Western blot analysis (Fig. 8B). We next tested the effect of main signaling pathway inhibitors on PMA-dependent ST3 induction (Fig. 8C). The overall inhibition profile was similar to that observed in PMA- or tumor cell-stimulated normal fibroblasts, with a complete inhibition of ST3 expression by the c/nPKC inhibitor GF109203X, a partial effect of the cPKC inhibitor Gö6976, and a more efficient inhibitory effect of the dual JNK/p38 inhibitor SB202190 than the selective inhibitor of p38 MAPK SB203580. Whereas no significant effect was observed in the presence of a MEK inhibitor in pulmonary

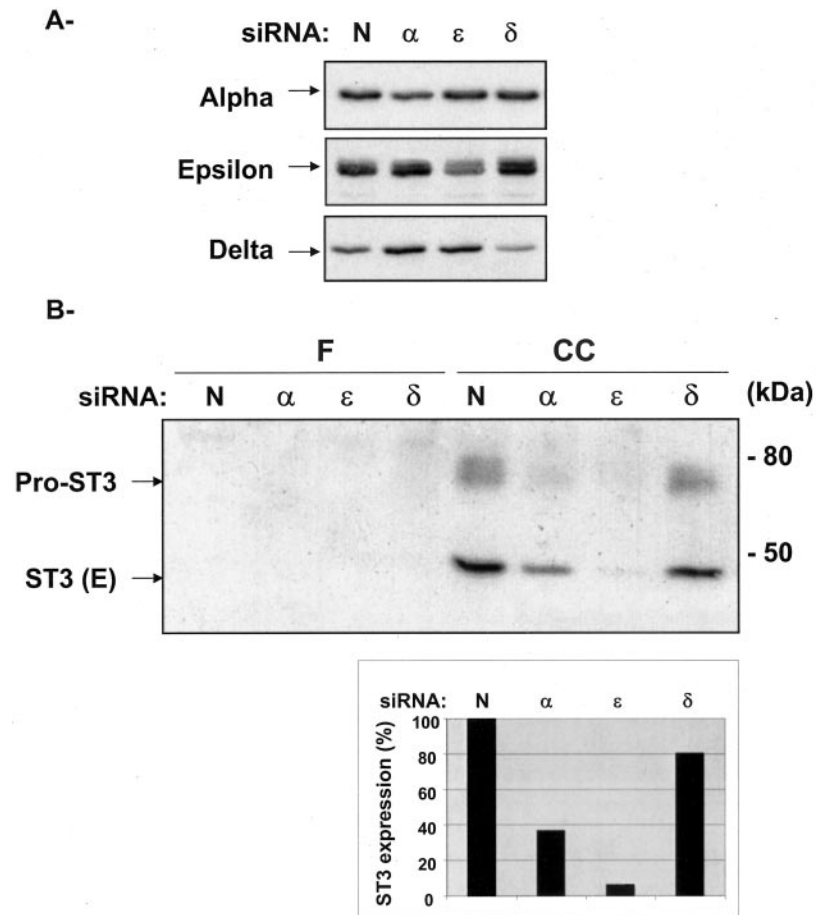


FIG. 7. PKC α and PKC ϵ mediate co-culture-induced ST3 expression. Fibroblasts were transiently transfected with PKC α , PKC δ , and PKC ϵ siRNA as described under "Experimental Procedures." 48 h later, fresh medium containing or not 2×10^5 A549 tumor cells was added for 48 h. **A**, expression of the different PKC isoforms in total lysates of fibroblasts was determined by Western blot using isoform-specific anti-PKC Abs. **B**, ST3 protein expression was analyzed in CM of fibroblasts (**F**) and co-culture (**CC**) by Western blot. Quantification was performed using NIH Image 1.62 software. This experiment is representative of three independent experiments.

fibroblasts, blockade of this pathway by U0126 strongly affected the level of ST3 expression in RD cells stimulated by PMA. However, previous data indicated that in this cell line, MEK1/2 are upstream activator kinases of JNK (43), suggesting that the effect of U0126 could be mediated by the inhibition of the JNK pathway. Finally, conversely to pulmonary fibroblasts, treatment with the Src kinase inhibitor PP2 did not significantly potentiate PMA-mediated ST3 induction.

From the RD-TR subclone, additional clones were generated by stable transfection with c-Myc-tagged constructs coding for CA and DN forms of PKC α , PKC ϵ , and PKC δ and a CA form of c-Src. Zeocin-resistant clones were isolated and construct expression was checked by Western blot in the presence or absence of Tet. Clones with an undetectable or low basal expression level and a strong induction of the different transgenes in the presence of Tet were selected for further experiments. We first analyzed the kinetics of induction of the different CA PKCs following the addition of Tet and their subsequent effect on MAPK pathway activation compared with a 30-min PMA treatment (Fig. 8D). Expression of PKC transfectants, analyzed by immunoblotting with an anti-Myc and a pan-phospho-PKC Ab, indicated that all three PKCs were detected as soon as 4–8 h after addition of Tet and corresponded to phosphorylated proteins. No cross-phosphorylation between the different isoforms was observed. A 30-min stimulation with PMA resulted in an increased phosphorylation of ERKs and JNK and to a lesser extent of p38 MAPK in all three transfectants. Induction of CA PKC ϵ also correlated with a rapid and sustained activation of p42/p44 and JNK (mainly p46) and a transient activation of p38. By contrast, CA PKC α expression resulted in a moderate activation of ERK and a slight and late activation of p38. Finally, no significant modulation of ERK and a slight and

transient phosphorylation of JNK and p38 was observed when CA PKC δ was overexpressed. Altogether, these data demonstrate that the present Tet-on inducible system is an appropriate tool to control the expression level of recombinant PKCs and suggest a differential activation of MAPK pathways by specific PKC isoforms.

Effect of CA PKC Constructs Induction on ST3 Expression—We next addressed the question whether the selective expression of CA PKC isoforms could modulate ST3 expression (Fig. 9). Stable cell lines expressing inducible levels of each PKC isoform (Fig. 9A) were stimulated 48 h by PMA or Tet and ST3 expression was evaluated in conditioned media by immunoblotting (Fig. 9B). As expected, PMA induced 45-kDa active ST3 in the 3 types of transfectants. Interestingly, addition of Tet also resulted in increased ST3 protein for all CA PKC isoforms expressing clones. To find out whether this induction was resulting from a transcriptional activation, the three types of PKC-inducible RD subclones were transiently transfected with the 2.5-ST3-LUC promoter construct (23), and luciferase activity was evaluated after a 32-h PMA or Tet stimulation. As shown in Fig. 9C, overexpression of each CA PKC isoform strongly activated the ST3 promoter, indicating that the induction of the ST3 protein observed in response to each isoform is mediated by a transcriptional mechanism.

Effect of DN PKC Construct Induction on PMA-mediated ST3 Expression—To further define the relative contribution of specific PKC isoforms in PMA-mediated ST3 induction, we analyzed the effect of DN PKC isoforms (Fig. 10). Stable cell lines expressing inducible levels of each PKC (Fig. 10A) were pre-treated or not with Tet for 18 h before a 48-h PMA stimulation and ST3 secretion was evaluated in culture media (Fig. 10B). Overexpression of DN PKC α reproducibly decreased both basal

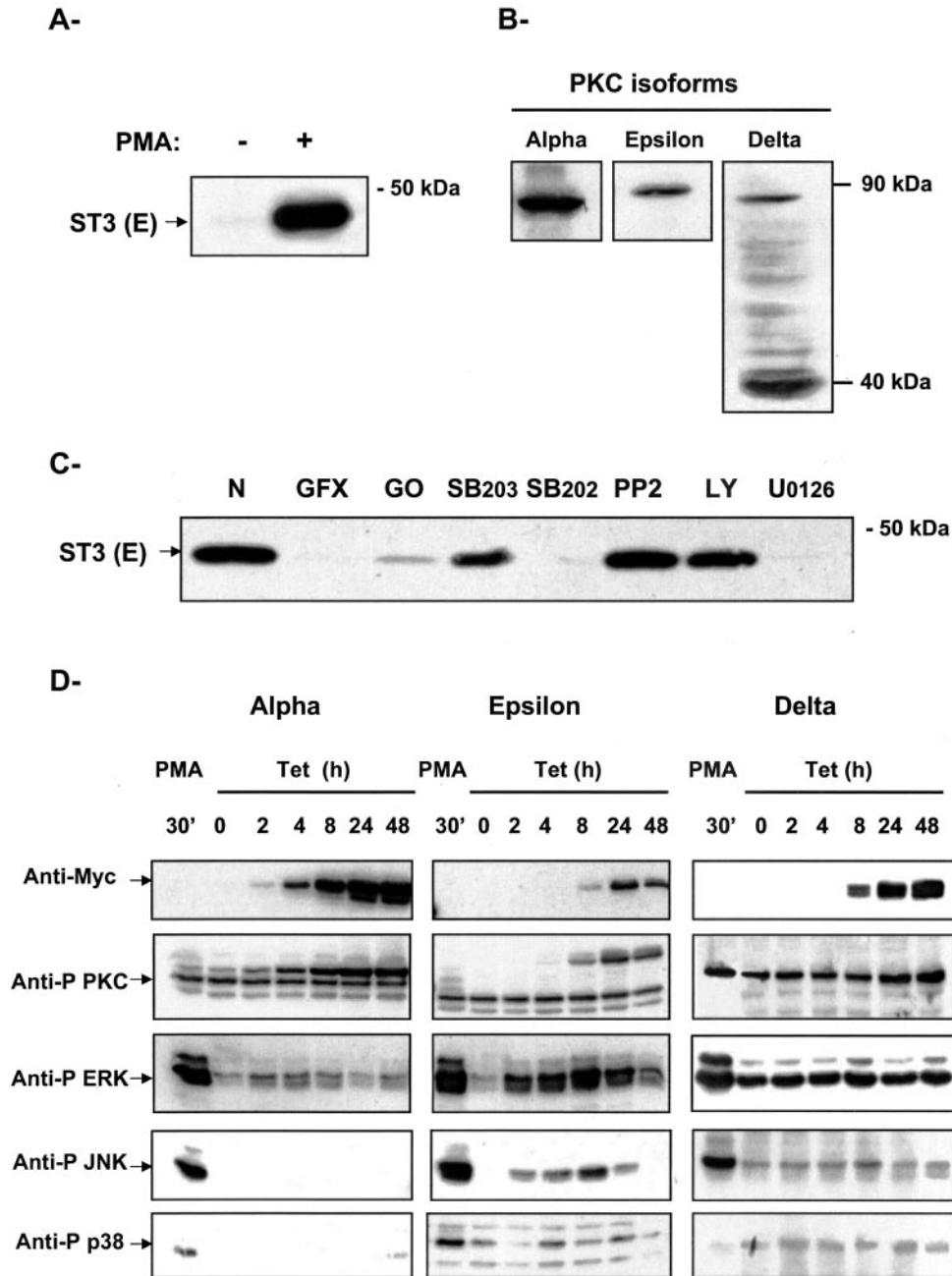


FIG. 8. RD cells, a suitable model to study PKC-mediated ST3 expression. A, ST3 induction in RD-TR cells (RD containing Tet repressor) exposed to PMA. Cells were activated with 20 ng/ml PMA in serum-free medium for 2 days. ST3 expression was analyzed in CM by Western blot. B, expression of the different PKC isoforms in total lysates of RD-TR cells was determined by Western blot using isoform-specific anti-PKC Abs. C, effect of the major signaling pathway inhibitors on ST3 induction in RD-TR cells exposed to PMA. PMA-stimulated RD-TR cells were incubated in the absence (N) or the presence of different pharmacological inhibitors in serum-free medium for 2 days. ST3 protein expression was analyzed in CM by Western blot. *GFX*, GF109203X; *GO*, G66976; *SB202*, SB202190; *SB203*, SB203580; *LY*, LY294002. D, time course of CA PKC isoform induction and MAPK pathway activation in RD-TR cells after treatment with Tet or PMA. RD-TR cells, stably transfected with CA PKC constructs were treated with PMA or 4 μ M Tet and lysed at different times following activation. Immunoblots of total lysates were performed using Abs against c-Myc and phospho-active forms of PKCs and MAPKs. Results shown are representative of two experiments performed with two independent clones for each construct.

level and PMA-mediated level of ST3. In contrast, the induction of DN PKC ϵ and DN PKC δ had no significant effect on PMA-mediated ST3 induction. Evaluation of ST3 promoter activity was performed in similar conditions of activation and showed that DN PKC α and PKC ϵ overexpression reproducibly decreased PMA-mediated luciferase activity, whereas DN PKC δ had no effect (Fig. 10C). The apparent discrepancy between the results obtained for PKC ϵ in terms of ST3 protein level and promoter activity is puzzling but may reflect different levels of regulation for ST3 transcript and protein.

Induction of a CA Form of c-Src Abolishes PMA-mediated ST3 Induction—Finally, to further investigate the negative regulation of PMA-mediated ST3 induction by Src kinases, we looked at the effect of a CA mutant of c-Src on PMA-mediated ST3 induction. Interestingly, this mutant inhibited both basal and PMA-mediated ST3 expression in conditioned media (Fig. 11B) as well as the ST3 promoter activity (Fig. 11C). Therefore, these observations further support the negative role of c-Src activation in the regulation of the signaling pathways leading to ST3 expression.

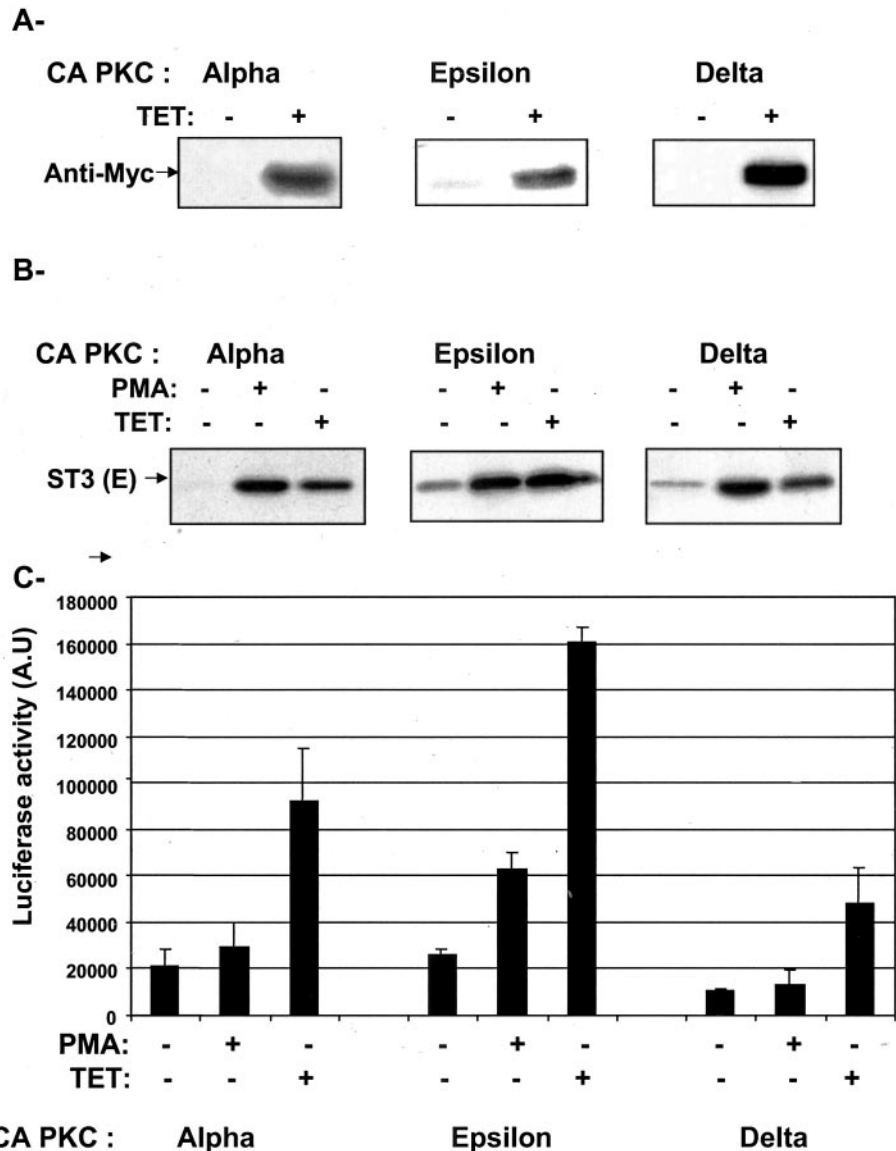


FIG. 9. Effect of CA PKC isoforms induction on ST3 expression. *A*, selective induction of CA PKC isoforms after a 24-h Tet treatment (immunoblot with anti-Myc Ab). *B*, RD-TR cells, stably transfected with CA PKC constructs were treated with 20 ng/ml PMA or 4 μ M Tet for 48 h. Analysis of ST3 protein in CM was performed by Western blot. *C*, RD-TR cells were transiently transfected with 2.5-*ST3*-LUC luciferase reporter plasmid and treated with PMA or Tet for 32 h. Cells were then harvested and assayed for luciferase activity. The values are representative of three independent experiments performed in triplicates on two clones for each construct.

DISCUSSION

In this study, we provide new insights into the regulation of ST3 expression at the tumor-stroma interface and point out early signaling events in fibroblasts following heterotypic interaction with epithelial tumor cells.

Using a pharmacological approach, we first established a global pattern of drug inhibition on co-culture-mediated ST3 induction. We found that inhibitors directed against cPKCs and nPKCs and to a lesser extent, stress kinase inhibitors, down-regulated ST3 expression at both the RNA and protein levels, whereas the Src kinase inhibitor potentiated this response. These data strongly supported a critical role for PKCs in this process, an hypothesis that was reinforced by the close similarities observed between co-culture and PMA-mediated ST3 induction in terms of time course of induction and protein synthesis requirement. The PKC family comprises nine members divided in three subgroups that are structurally and functionally distinguished (44, 45). We focused our study on the 3 main cPKCs and nPKCs expressed in fibroblasts, PKC α , PKC ϵ , and PKC δ . Analysis of endogenous PKCs relocalization was particularly complex in such a co-culture model and required transfection of fibroblasts with chimeric PKC-GFP constructs. Using live fluorescent microscopy, we showed evidence of an intense isozyme-selective relocalization of PKCs following het-

erotypic cell contact with epithelial tumor cells. We demonstrated that both PKC α and PKC ϵ targeted plasma membrane spots in direct contact with tumor cells, whereas no significant relocalization could be observed for PKC δ . Targeting of PKC α and PKC ϵ was detected about 15 min after addition of tumor cells and then remained stable for at least 3 h. Altogether, our data demonstrate that heterotypic cell-cell contacts induce a rapid and selective activation/relocalization of PKC α and PKC ϵ in fibroblasts that ultimately leads to ST3 expression.

To the best of our knowledge, our study provides the first evidence for the spatiotemporal localization of several PKC isoforms in fibroblasts following their heterotypic interactions with cancer cells. However, it seems noteworthy that a similar relocalization has been previously observed in the context of homotypic cell adhesion occurring in TRH- or PMA-stimulated pituitary cells (34). Interestingly, the selectivity of targeting to cell-cell contacts in these models is also restricted to PKC α and PKC ϵ , suggesting a common mechanism of compartmentalization via association to anchoring proteins. The nature of these interactions is not yet known but this specific relocalization requires a restricted amino acid sequence located in the V3 hinge region of PKC α and PKC ϵ (35, 40). The possible role of this domain in the context of our experimental model is an important issue to address and will require further investiga-

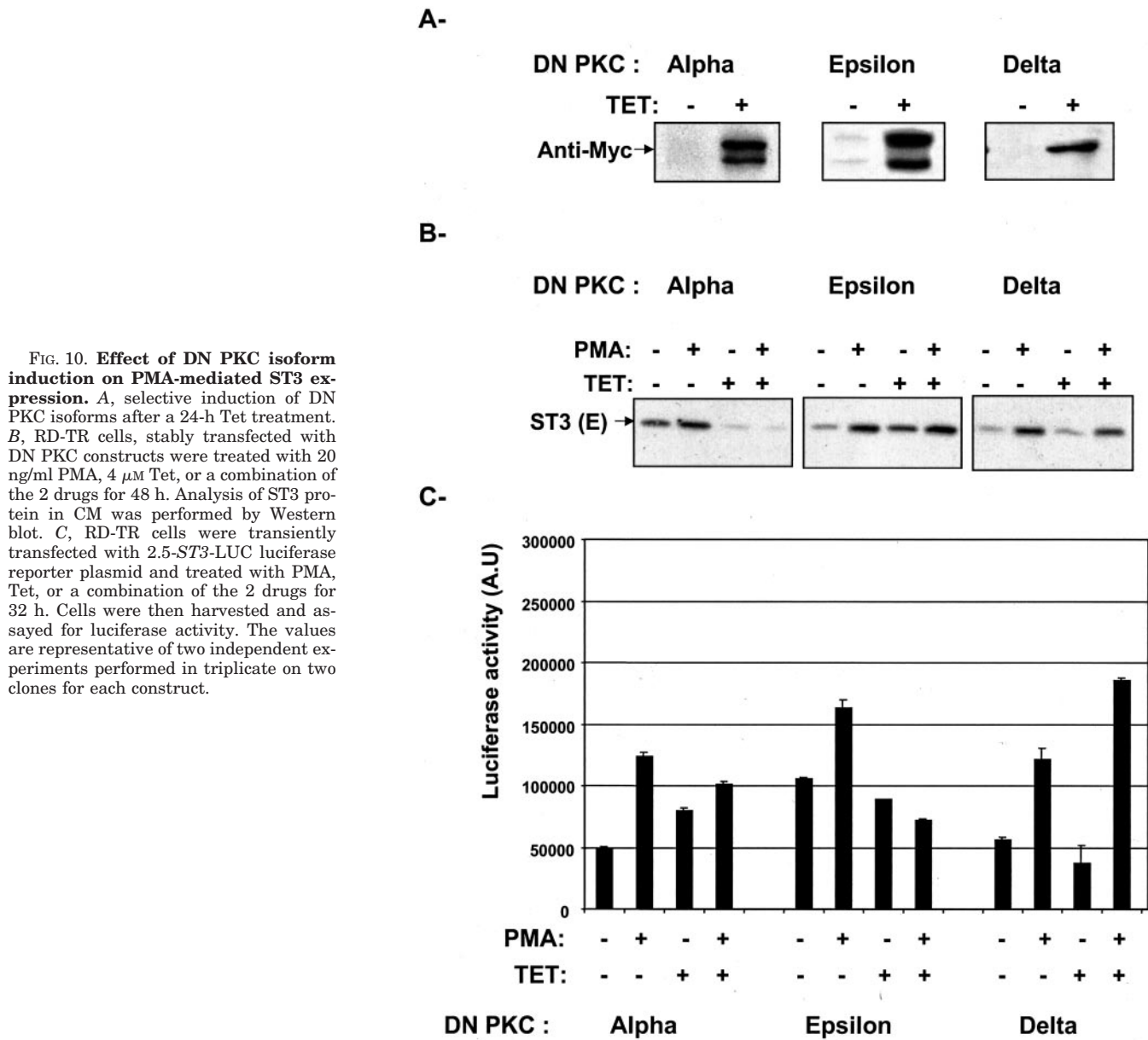


FIG. 10. Effect of DN PKC isoform induction on PMA-mediated ST3 expression. *A*, selective induction of DN PKC isoforms after a 24-h Tet treatment. *B*, RD-TR cells, stably transfected with DN PKC constructs were treated with 20 ng/ml PMA, 4 μ M Tet, or a combination of the 2 drugs for 48 h. Analysis of ST3 protein in CM was performed by Western blot. *C*, RD-TR cells were transiently transfected with 2.5-*ST3*-LUC luciferase reporter plasmid and treated with PMA, Tet, or a combination of the 2 drugs for 32 h. Cells were then harvested and assayed for luciferase activity. The values are representative of two independent experiments performed in triplicate on two clones for each construct.

tion. In respect to heterotypic cell-cell contacts, PKC θ appears to be the only PKC isoform for which a similar translocation has been reported and that involves its highly selective recruitment to the central supramolecular activation complex region of the immunological synapse in antigen-stimulated T cells (46).

The cell-cell contact-dependent activation of PKCs observed in our co-culture model appears to be consistent with our previous data indicating that ST3 induction required direct cell-cell contact and was not influenced by neutralizing Abs directed against several growth factors that have been involved in the regulation of the ST3 transcript *in vitro* (31). Very little information is known concerning the membrane receptors that are potentially involved in epithelial-mesenchymal interactions. Among these receptors, EMMPRIN (basigin/CD147), a glycoprotein present on carcinoma cell plasma membranes, has been shown to enhance the fibroblastic synthesis of some MMPs, including MMP-1, -2, -3, and -9 (47, 48). Integrins and N-cadherin have been also proposed to play important roles in tumor-stromal cell interactions and invasion processes, notably via MMPs production (49–53). However, preliminary experiments indicated that co-culture of fibroblasts with EMMPRIN

transfected Chinese hamster ovary cells or addition of neutralizing Abs against N-cadherin or against a large panel of integrin subunits does not modulate ST3 expression (data not shown). Additional studies are therefore required to identify the specific factors involved in this process.

We have next confirmed the functional implication of these PKC isoforms in the regulation of ST3 expression in two different models. Using first an RNAi approach in the co-culture model, we provide evidence that the molecular inhibition of PKC α or PKC ϵ in normal fibroblasts significantly alters tumor cell-dependent ST3 induction. Second, a tetracyclin-inducible system demonstrated that the expression of CA PKC α and CA-PKC ϵ strongly increased ST3 protein expression and its promoter activity, whereas the expression of the DN form of PKC α , and to a lesser extent that of PKC ϵ , abolished the PMA-mediated induction of the proteinase. Taken together, these data clearly demonstrate the involvement of these two PKC isoforms in the regulation of ST3 expression. In addition, when the possible implication of PKC δ was investigated, we have found that the expression of CA PKC δ also increased ST3 expression, but this induction was less effective compared with that of PKC α and PKC ϵ . On the other hand, siRNA down-

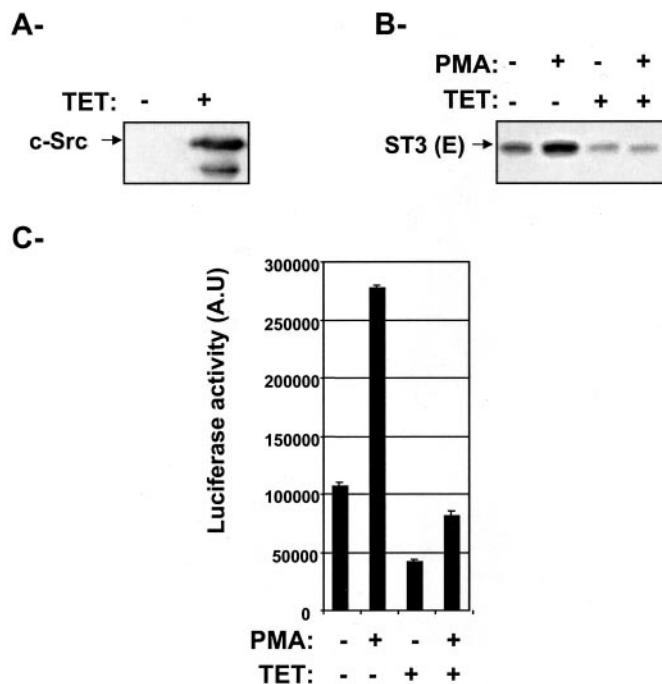


FIG. 11. Expression of CA c-Src inhibits PMA-mediated ST3 expression. *A*, selective induction of CA c-Src after a 24-h Tet treatment. *B*, RD-TR cells, stably transfected with a CA c-Src construct were treated with 20 ng/ml PMA, 4 μ M Tet, or a combination of the 2 drugs for 48 h. Analysis of ST3 protein in CM was performed by Western blot. *C*, RD-TR cells were transiently transfected with 2.5-*ST3*-LUC luciferase reporter plasmid and then treated with PMA, Tet, or a combination of the 2 drugs for 32 h. Cells were then harvested and assayed for luciferase activity. The values are representative of two independent experiments performed in triplicate on two clones.

regulation of this isoform in the co-culture model had no effect on ST3 induction and transfection of DN PKC δ in RD cells did not significantly modulate PMA-mediated ST3 induction, suggesting that this isoform does not play a crucial role in this process.

Our results seem to be consistent with a role of PKCs in the transcriptional regulation of other *MMP* genes (54, 55). Indeed, PKC-mediated pathways converge at the AP-1 binding site also called TRE (TPA responsive element) that is present in the proximal promoter region of most inducible *MMP* genes. However, although an AP-1 binding site is present in a distal part of the *ST3* promoter, this site was shown to only control the baseline *ST3* promoter activity. The *ST3* promoter is also activated by PMA, but its activation is mediated by a C/EBP binding site and by a TPA-inducible complex including the C/EBP β transcription factor (23). This factor has been shown to be phosphorylated at Ser-105 by ribosomal S6 kinase (56) that belongs to downstream targets of PKCs (57). It is therefore tempting to speculate that C/EBP β could mediate the stromal induction of ST3 by cancer epithelial cells through a PKC-mediated mechanism. Our models including co-cultures, as well as the Tet-inducible PKC isoforms should provide useful tools to identify downstream targets of specific PKC isoforms such as transcription factors recruited for the stromal induction of ST3 by cancer cells.

Concerning other signaling effectors, our study strongly supports a role for Src kinases in the control of ST3 expression. First, Src kinase inhibitors significantly potentiated both co-culture and PMA-mediated ST3 induction. Second, the expression of CA c-Src totally abolished PMA-mediated ST3 expression in RD cells. Interestingly, numerous studies have demonstrated that several PKC isoforms including PKC α and PKC ϵ can form functional signaling modules with c-Src and

v-Src (58–60). Considering the negative control of c-Src on ST3 expression, these observations suggest that c-Src may participate in a negative feedback loop in which PKC α and/or PKC ϵ are phosphorylated and down-regulated. Pharmacological studies also suggested that stress kinases could represent an important mediator of the signaling pathways leading to ST3 expression. Activation of MAPK pathways by PKCs has been described in various models, including normal fibroblasts and RD cells (43, 61). Experiments performed in the co-culture model did not allow us to detect a significant modulation of MAPK pathways in fibroblasts (data not shown). However, Western blot analysis of these proteins requires selective sorting of purified fibroblasts (33) that could affect these phosphorylation processes. In RD cells, we found significant differences between the PKC isoforms in their ability to activate the different MAPK pathways while they were all able to induce ST3 expression. Further work will be necessary to precisely determine the nature and the hierarchy of the specific pathways activated downstream of PKCs. Nevertheless, our data represent a starting point for a better understanding of the molecular pathways leading to ST3 expression in the tumor stroma.

ST3 represents a marker of the tumor stroma in virtually all invasive human carcinomas. Many human carcinomas are associated with a stromal response termed desmoplasia characterized by pronounced modifications in the phenotype of proliferating fibroblasts. In this respect, it is usually accepted that the tumor stroma supplies a structural support for cancer cells adhesion and migration, as well as the angiogenic network required for cancer cell survival (62–64). In agreement with this role, our recent studies have shown that the gene expression profile of human pulmonary fibroblasts after their interaction with non-small cell lung cancer cells is strongly modified and has revealed changes in the expression of genes involved in matrix degradation, angiogenesis, cell growth, and survival (33). Manipulating host-tumor interactions thus provides an opportunity to control tumor growth but the factors controlling tumor-induced changes in the microenvironment and the reciprocal modifications of the tumor by the microenvironment, as well as the intracellular pathways resulting from these interactions, are largely unknown (65). The heterotypic cell contact-dependent activation of selective PKC isoforms described in the present study appears to be a central signaling pathway. PKCs have been already proposed as therapeutic targets because of their role in tumor angiogenesis and tumor cell survival (66–69) and our data provide another rationale for the potential involvement of PKCs in the establishment of a permissive microenvironment.

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