Molecular Inhibition of Phospholipase Cγ Signaling Abrogates DU-145 Prostate Tumor Cell Invasion

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ABSTRACT

Up-regulated signaling from the epidermal growth factor receptor (EGFR) has been correlated with tumor invasion and metastasis in numerous human neoplasias. Recently, we have demonstrated that increased levels of EGFR promote the invasiveness of human prostate carcinoma DU-145 cells. However, the intracellular signaling pathway responsible for this enhanced tumor invasiveness has not been identified. We postulated that increased cell motility signaled via phospholipase Cγ (PLCγ) activation was critical for tumor invasiveness. Highly invasive DU-145 cells engineered to overexpress the EGFR were stably transfected with a dominant-negative fragment of PLCγ from the Z-region (PLCz) or with irrelevant peptide minigenes. PLCz was expressed only in the appropriate transfectant lines, with a concomitant decrease in inositol phosphate generation. The transfectant cell lines all formed tumors when inoculated into the peritoneal cavity of athymic mice. Tumors from the cells expressing PLCz fragment were significantly less invasive than the transfectants containing the control minigenes, as assessed by the diaphragm invasion model and invasion into abdominal soft organs. The cells expressing PLCz grew and formed colonies in soft agar at rates comparable to the cells expressing the control minigenes. These data suggest that up-regulated signaling by EGFR promotes prostate tumor invasiveness secondary to increased cell motility. Furthermore, PLCγ represents a potential therapeutic target to limit tumor progression promoted by up-regulated signaling from the EGFR and related receptors with intrinsic tyrosine kinase activity.

INTRODUCTION

Prostate cancer-related deaths are due mainly to tumor invasion and metastasis (1, 2). Curative therapies are available only if the cancer is organ confined. Once the tumor breaches the prostate capsule to invade locally or metastasize to distant sites, none of the prevailing treatments dramatically enhance patient survival (2–6). Hence, a more in-depth understanding of the basic biology of prostate cancer progression to the invasive and metastatic stages would increase the knowledge required for developing novel therapeutic approaches.

Cell properties such as proliferation, motility, and proteolysis are principal in tumor cell progression to the invasive and metastatic stage. Disruption of any one of these processes may, in turn, interfere significantly with tumor progression. Tumor cell motility is critical in this process of tumor invasiveness and metastasis. Therefore, interventions have been designed to inhibit enhanced tumor motility and prevent tumor progression (7–10). One potential target would be the motility signaling pathway induced by growth factors acting via up-regulated growth factor receptors (11). The growth factor receptor most often found up-regulated in human tumors that have progressed to the invasive and metastatic state is the EGFR (11). Up-regulated EGFR has been correlated with tumor invasion and metastasis in glioblastoma multiformes (12), gastric (13), bladder (14), non-small cell lung (15), and breast carcinomas (16). These clinical correlations are supported by experimental model systems in which metastatic potential of human colon carcinoma cells correlated with EGFR level (17, 18). Thus, EGFR up-regulation has been correlated not with simple proliferation but with tumor progression.

Cancers of the prostate present autocrine stimulatory loops in which both the EGFR and its activating ligand, transforming growth factor α, are produced by the carcinoma cells (19–23). We have demonstrated previously that increased levels of full-length EGFR promotes the invasiveness of DU-145 human prostate carcinoma cells both in vitro (20) and in vivo (24), whereas expression of an EGFR variant that is fully mitogenic but lacks the capacity to stimulate cell motility (c‘973) results in decreased invasiveness secondary to down-regulation of endogenous EGFR. The question is whether the operational signal for tumor invasiveness is that of cell motility.

PLCγ activation is required for motility signaled by the EGFR (25) as well as the related receptors for PDGF (26) and IGF-1 (27). Initial experiments demonstrated that inhibition of
PLC activity with the aminosteroid U73122 greatly diminished tumor invasiveness but not tumor growth in mice inoculated with DU-145 cells overexpressing full-length WT EGFR (24). It is possible that inhibition of tumor invasiveness by U73122 was secondary to a mechanism other than diminished growth factor-induced cell motility, either affecting a different downstream pathway or a PLC isoform other than PLCγ-1. Even if the mechanism of invasion inhibition was not via decreased motility, it would not decrease the utility of such a therapeutic intervention. However, knowledge of the precise mechanism of inhibition of invasiveness would allow for the optimal utilization of PLC inhibitors and provide a biological rationale for specifically targeting PLCγ-1.

To further define the critical role of the growth factor receptor-PLCγ signaling pathway in promoting tumor progression, targeted molecular intervention was used in the present study. We used a dominant-negative fragment of PLCγ, PLCz (25), that includes the src homology 2, src homology 3, and phospholipase-inhibitory domains and specifically inhibits activation of PLCγ and not the other isoforms (28). When expressed in fibroblasts or glioblastoma tumor cells, PLCz prevents induced cell motility and invasiveness (25). In the present investigation, a cDNA encoding this fragment was stably introduced into DU-145 prostate tumor cells being transcriptionally regulated either by a constitutively active promoter (SV40 early promoter) or a steroid hormone-responsive promoter (MMTV LTR). Expression of this dominant-negative PLC fragment resulted in greatly diminished tumor invasiveness and spread in vivo.

**MATERIALS AND METHODS**

**Animals.** Male athymic BALB/c nu/nu mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions. Mice were used at 6–8 weeks of age and weighed 20 to 27 g. Animals were maintained in accordance with established institutional guidelines and approved protocols.

**Cell Culture and Establishment of Infectant Cell Lines.** WT DU-145 prostate carcinoma cells were generated as described previously (20). These cells express levels of EGFR that do not undergo autocrine ligand-induced down-regulation because they are in excess of the degradation trafficking pathway. The cells are maintained in high-glucose (4.5 g/L) DMEM (Life Technologies, Inc., Grand Island, NY) media supplemented with FBS (7.5%), 100 units/ml penicillin, 200 μg/ml streptomycin, nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine (37°C, 90% humidity, 5% CO₂), and 1000 μg/ml G418 (Life Technologies, Inc.); all cells were cultured in the absence of G418 for at least 3 days prior to testing. Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA).

PLCz was expressed in the WT DU-145 cells by lipid-mediated transfection. Briefly, PLCz was cloned into the pXf vector for constitutive expression from the SV40 early promoter or the pDexMTX vector for steroid hormone-inducible expression from the MMTV LTR (25, 29). In addition, control pXf constructs that expressed short peptides were used to account for transfection and selection procedures (30). These were introduced into the WT DU-145 cells with the Lipofectin reagent (Life Technologies, Inc.). Stable transfectant cells were selected in the above media supplemented with methotrexate (1.2 μg/ml).

The expression of PLCz was determined by immunoblotting of whole-cell lysates using antibodies that recognize the Z-region of PLCγ-1. The endogenous PLCγ and the PLCz fragments are demarcated.

**Fig. 1** Expression of PLCz fragment in parental and transfectant WT DU-145 cells. Protein lysates were made from approximately 4 × 10⁶ cells, size-separated by 7.5% SDS-PAGE under reducing conditions, and analyzed by immunoblotting using a mixed monoclonal antibody preparation (UBI). This antibody preparation recognizes epitopes in the Z-region of PLCγ-1. The endogenous PLCγ and the PLCz fragments are demarcated.

PLC Activity Assays. PLC activity was monitored by a functional assay in which the production of IP species was measured (25, 34). DU-145 cells grown to 90% confluency were labeled in serum-free medium 199 (Life Technologies, Inc.) containing 5 mCi/ml [3H]myo-inositol, for 20 h, after which the cells were washed twice with PBS to remove unincorporated label. Due to the presence of autocrine loop, cells were maximally stimulated for 15 min with 20 nM EGF prior to the addition of LiCl (10 mM) to inhibit IP hydrolysis by inositol phosphatases. The experiment was terminated at 0 and 30 min after LiCl addition by removing the media and adding boiling distilled water to the cells. Cell lysates were collected and boiled for 5 min. The lysates were briefly centrifuged to remove particulate material, and the soluble cytosolic fraction was retained for analysis.

Inositols and IPs were separated on Dowex (AG1-X8 100–200 mesh; Bio-Rad) anion-exchange minicolumn. Inositol and glycerophosphoinositol was eluted sequentially with water and then with a 5 mM sodium borate, 60 mM sodium formate solution. IP was then eluted with 200 mM ammonium formate and 100 mM formic acid. The IP content was quantitated by scintillation counting. PLC activities were expressed as the amount of IP production over 30 min as a fraction of total inositol incorporation.

Cell Proliferation Assays. Cell proliferation was evaluated by assessing mitochondrial reduction of MTT as described (35), with the following modifications. Cells were plated at 5000 cells/well in 96-well microtiter plates in 200 μl growth medium (7.5% FBS in DMEM) and allowed to attach for 24 h. Serum-containing medium was removed, and cells were quiesced for 2 days in 0.5% dialyzed FBS in DMEM. At harvest, medium was removed from the appropriate wells, replaced with 50 μl of MTT solution (2 mg MTT/ml PBS; Sigma Chemical Co., St. Louis, MO) and incubated for 4 h at 37°C. After incubation, the MTT solution was carefully aspirated; 100 μl DMSO (Sigma) were added to each well. Data were analyzed on plate reader using the SoftMax program (Molecular Devices Corp., Menlo Park, CA).

RESULTS

Dominant-Negative PLCz Fragment, Present in Transfected Cell Lines, Diminishes PLC Activity. To determine whether inhibition of PLCγ signaling abrogates tumor progression, we required a highly aggressive model system. DU-145 cells expressing high levels of full-length Wi' EGFR were chosen as the experimental model system because they are significantly more invasive than parental DU-145 cells both in vitro and as tumors in athymic mice (20, 24). The WT DU-145 cells are syngenic with the parental DU-145 with the exception that they contain an extra copy of the human EGFR cDNA driven from a retrovirus LTR cassette (36). The dominant-negative PLCγ-1 fragment, PLCz, was introduced into these cells by lipid-mediated transfection. The PLCz peptide was detected by immunoblotting in the WT DU-145 cells transfected with the designated constructs were labeled metabolically with [3H]myo-inositol and then exposed to saturating levels of EGF (20 nM) for 15 min prior to the addition of LiCl (10 mM). PIP2 hydrolysis was terminated by removing the media and adding boiling distilled water to the cells at either 0 or 30 min after the addition of LiCl (as denoted under the columns). The amount of labeled inositol generated was calculated as the percentage of incorporated label. The results from two experiments, performed on separate days, are shown. Both basal and accumulated IPs were lower in the cells expressing PLCz; incorporation of myo-inositol was similar in all three lines.

Fig. 2 IP accumulation in the transfected WT DU-145 cells. Near-confluent WT DU-145 cells stably transfected with the designated constructs were labeled metabolically with [3H]myo-inositol and then exposed to saturating levels of EGF (20 nM) for 15 min prior to the addition of LiCl (10 mM). PIP2 hydrolysis was terminated by removing the media and adding boiling distilled water to the cells at either 0 or 30 min after the addition of LiCl (as denoted under the columns). The amount of labeled inositol generated was calculated as the percentage of incorporated label. The results from two experiments, performed on separate days, are shown. Both basal and accumulated IPs were lower in the cells expressing PLCz; incorporation of myo-inositol was similar in all three lines.
with the pXf-PLCz but not the control peptide cDNA constructs (pXf-PIP2 and pXf-gel; Fig. 1).

It is predicted that PLCz should limit EGFR-induced phospholipase activity by inhibiting PLCγ-1 hydrolysis of phosphoinositide bisphosphate (PIP2; Refs. 25, 28, and 29). Total PLC activity was assessed by accumulation over a 30-min period of IPs in the presence of LiCl (10 mM), which prevents hydrolysis by inositol phosphatases. Because these cells present an autocrine stimulatory loop that hampers measurement of EGF-induced PLC activity, we performed the assays in the presence of saturating concentrations of exogenous EGF (20 nM) to ensure maximal stimulation. PLC activity was significantly depressed in cells expressing the PLCz fragment compared to WT DU-145 cells and those transfected with a control peptide (Fig. 3).
expression on WT DU-145 prostate tumor invasiveness

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Tumor formation</th>
<th>Diaphragm tumors</th>
<th>Diaphragm invasiveness*</th>
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<tbody>
<tr>
<td>No construct</td>
<td>5/5</td>
<td>2/2*</td>
<td>3.5+ (3-4)</td>
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<tr>
<td>pXf vector controls</td>
<td>10/10</td>
<td>8/8*</td>
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<td>pDexMTX/PLCz</td>
<td>7/10</td>
<td>6/10</td>
<td>0.5+ (0-1)</td>
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* The number of mice with macroscopic tumors in the peritoneal cavity over the number of mice challenged.

Invasiveness was scored microscopically on a scale of 0 (non-invasive) to 4 (tumor obliterating the diaphragm); the number is the average invasiveness of all diaphragm tumors (not including mice which did not present tumors on the diaphragm); the range of invasiveness is shown in the parentheses.

Three mice died early with extensive tumors and were not available for histological examination to ascertain diaphragm invasiveness.

Two mice died with extensive tumors and could not be necropsied to ascertain diaphragm invasiveness.

Expression of PLCz Diminishes WT DU-145 Tumor Invasiveness and Spread. We have a series of transgenic cell lines with which to test our hypothesis that disruption of the EGFR-PLCy signaling pathway diminishes tumor progression. In addition to the pXf-PLCy transfected line, we generated a second PLCz-expressing cell line, in which the PLCz fragment is expressed from a steroid hormone-responsive MMTV LTR (29). Mice (five per group) were inoculated with WT DU-145 cells containing the various PLCz constructs. In the first experimental series, prostate tumor cells expressing the dominant-negative PLCz fragment from either transcription promoter, pXf or pDexMTX, were compared to untransfected WT DU-145 cells (Table 1). The untransfected WT DU-145 cells grew aggressively with three of the five mice dying early; the tumors in the mice sacrificed on day 45 were highly invasive and spread throughout the peritoneal cavity. The PLCz transfectant lines exhibited much less invasiveness and were spread less extensively throughout the peritoneal cavity.

In the second series, growth and invasiveness of these transfectant cells were compared to the two different control constructs. Again, the control construct transfectant cells formed aggressive and invasive tumors, killing two of the mice early. The tumor cells expressing the dominant-negative PLCz fragment were less spread and significantly less invasive as assessed by histopathology scoring of diaphragm invasion (Table 1 and Fig. 3). Tumors that expressed PLCz (pXf/PLCz and pDexMTX/PLCz) also were less invasive into abdominal organs than the control tumors (Fig. 3). As an example, significant invasion into the pancreatic parenchyma was noted in 8 of 9 animals with control tumors (Parental WT, pXf Pip2, and pXf gel) but only 2 of 12 animals with pXf/PLCz or pDexMTX/PLCz tumors found on the pancreas.

Fig. 4. Expression of PLCz fragment in parental and transfectant WT DU-145-derived tumors. Protein lysates were made from these tumors, size-separated by 7.5% SDS-PAGE under nonreducing conditions, and analyzed by immunoblotting using rabbit polyclonal antisera (Santa Cruz Biotechnology). This antibody preparation recognizes epitopes in the Z-region of PLCy-1. The endogenous PLCy and the PLCz fragments are demarcated.

PLCy Is Expressed in the Tumors from Both the SV40 Early Promoter and the MMTV LTR. The tumors from the PLCz transfectant WT DU-145 cells were significantly less invasive and spread through the peritoneal cavity than the untransfected or control transfectant lines. PLCy protein should be detectable in these tumors, if this is related to abrogation of PLCy signaling. PLCy protein was detectable in vitro only in the pXf/PLCz transfectant lines; a similarly sized Mr ~51,000 fragment was not noted in the other transfectants (Fig. 1). In vivo endogenous steroid hormones can activate transcription from the MMTV LTR in the second PLCz-transfectant line, pDexMTX/PLCz. Tumors were isolated from the diaphragm surface of the inoculated mice, and protein extracts were analyzed by SDS-PAGE followed by immunoblotting for PLCy. As noted in Fig. 4, PLCz could be detected at levels comparable to or higher than endogenous PLCy in tumors derived from both pXf/PLCz and pDexMTX/PLCz transfectant DU-145 prostate cells.

Cell Growth Rates of the Various Transfectant Lines Are Equivalent. One explanation for the differences found in tumor invasiveness would be differential growth rates of the WT DU-145 sublines. All of the sublines grew at comparable rates in vitro as assessed using the MTT dye reduction method (Fig. 5) and confirmed by a cell proliferation assay in which actual
cell numbers were enumerated. In an attempt to more closely approximate the in vivo situation, we performed soft-agar growth assays on the transfectant WT DU-145 cell lines (24). All transfectant lines presented similarly high cloning efficiencies of >85% (1035 ± 29 colonies for PLCz-containing transfectants versus 1134 ± 61 colonies for the control transfectants; n = 2 for each line). This finding confirms our previously reported separation of the PLCγ signaling pathway from mitogenic signaling by EGFR both in fibroblasts (25, 29) and in the DU-145 prostate cells (24).

**DISCUSSION**

Our previous findings implicated PLCγ signaling in prostate tumor invasiveness and subsequent metastasis (20, 24). Inhibition of PLC activity by U73122 blocked tumor invasiveness but not tumor cell growth in vitro or in vivo. However, even a rather specific pharmacological inhibitor of PLC activity, U73122, could not define the EGFR-PLCγ signaling pathway as the critical element in tumor invasiveness. A molecular approach to disruption of PLCγ activation and signaling was necessary to identify the primary factor as PLCγ and not other PLC isoforms or pathways. Herein, we present evidence that disruption of signaling by a dominant-negative PLCγ fragment, PLCz, also resulted in greatly diminished tumor invasiveness (Table 1 and Fig. 3). The concordance of the findings, that both pharmacological inhibition and molecular targeting decrease invasiveness, highlight the central role of PLCγ signaling in prostate tumor invasion.

PLCγ signaling is required for enhanced cell motility in response to EGF and other growth factors such as PDGF and IGF-1 (25–27). Fibroblast cell motility (37) and DU-145 prostate tumor cell invasiveness (20, 24) are enhanced only by EGFR constructs that activate PLCγ and not by signaling-restricted EGFR variants that fail to activate PLCγ, although they trigger the mitogen-activated protein kinase, signal transducers and activators of transcription, and other signaling pathways (25, 38). Many other cellular responses to EGFR activation are not dependent on PLCγ signaling. In fact, mitogenesis is increased in the absence of EGF-induced PLC activity in fibroblasts (29), and the growth of DU-145 cells is promoted equally by PLC-activating and -nonactivating EGFR (20). In the present study, the expression of PLCz did not limit cell growth (Fig. 5) or clonogenicity or prevent the development of i.p. tumors; these findings concur with the lack of growth inhibition by U73122 (24). That disruption of the EGFR-PLCγ signaling pathway abrogates tumor invasiveness and cell motility, without affecting other cellular responses or tumor cell growth, demonstrates that prostate tumor invasiveness is dependent on induced cell motility driven by the transforming growth factor α-EGFR autocrine loop.

We postulate that targeted interventions aimed at inhibiting PLC activity is not limited to prostate tumors but extends to other tumors in which up-regulated growth factor receptors correlate with tumor invasion and spread. Expression of dominant-negative EGFR constructs prevented metastasis but not localized tumor growth of human colorectal carcinoma cell lines

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6 L. Wong, T. B. Deb, A. Wells, S. A. Thompson, and G. R. Johnson. A differential requirement for the COOH-terminal region of the EGF receptor in amphiregulin and EGF mitogenic signaling, manuscript in preparation.
The observation that only tumor progression and not in situ growth was inhibited was as predicted by our model because these cells are not dependent on EGFR signaling for proliferation or survival. Glioblastoma multiformes present the strongest correlation between up-regulated EGFR signaling and tumor invasion of human de novo tumors (12); in these cells, disruption of growth factor receptor-PLCγ signaling pathway blocks tumor cell invasiveness. U73122 inhibited invasion of normal brain tissue by primary isolates of human glioblastomas, and PLCζ expression prevented invasion by a rat glioblastoma-derived cell line. It is interesting to note that other growth factor receptor systems, such as the PDGF system, have been implicated in glioblastoma progression. Disruption of PLCγ signaling abrogated cell motility induced by other growth factors, PDGF and IGF-1, in addition to EGF. Thus, PLCγ may serve as a point of convergence for a number of growth factor receptors to signal cell motility and tumor invasiveness.

Our studies demonstrate that PLCγ is a candidate therapeutic target for genetic as well as pharmacological interventions in limiting prostate tumor progression. By defining the critical element as PLCγ signaling in response to autocrine and externally derived growth factors, we have provided important additional information for rationale utilization of this therapeutic approach in both prostate carcinoma and other cancers. In addition to the present study, there is direct evidence that supports the validity of these concepts in prostate carcinomas (18, 24), glioblastomas, and colorectal carcinomas (17). Based upon the biological properties reported in the literature, we predict that these concepts will also apply to a wide variety of other human tumors, the spread of which may be promoted by signals from the external cell milieu such as those of the breast, lung, and thyroid. Future studies will be necessary to provide empirical evidence for the inhibition of tumor invasion and metastasis by targeted disruption of PLCγ signaling.

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REFERENCES


Molecular inhibition of phospholipase c gamma signaling abrogates DU-145 prostate tumor cell invasion.
