FEBS Letters 450 (1999) 210–216 FEBS 21922

Suppression of the neoplastic phenotype by transfection of phospholipase C β 3 to neuroendocrine tumor cells

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Received 12 February 1999

Abstract The expression of phospholipase C β 3 (PLCB3) is low or absent in several neuroendocrine neoplasias. To investigate the role of PLCB3 in the neuroendocrine tumorigenesis, we transfected a *PLCB3* construct to three neuroendocrine tumor cell lines with a low PLCB3 expression. The growth rate and tumorigenicity were assessed in vitro by [^{3}H]thymidine incorporation and cell counting, in vivo, by xenografting to nude mice. In vitro, PLCB3 expressing clones showed a significant growth inhibition. The tumor weight was reduced for one of the two xenografted *PLCB3*-transfected cell lines and in both, a reduced number of proliferating (Ki-67 positive) cells was observed. This study implies an essential role for PLCB3 in the neuroendocrine tumorigenesis.

Key words: Phospholipase C β 3; Neuroendocrine tumor; Transfection; Xenograft; Growth inhibition

1. Introduction

The PLCB3 transcript is widely expressed in human tissue but absent in some multiple endocrine neoplasia type 1 (MEN 1)-associated as well as sporadic neuroendocrine tumors [1,2]. The *PLCB3* gene is located on chromosome 11q13, which also harbors the *MEN 1* gene [3,4]. Loss of heterozygosity (LOH) at 11q13 has been found in a low frequency in sporadic pituitary tumors [5] and in about 30–70% of sporadic tumors of the parathyroids and the endocrine pancreas. A subset of the tumors with LOH at 11q13 exhibits mutations in the *MEN 1* gene ranging from 30–58% in different studies [6–8]. LOH at 11q13 not encompassing the *MEN 1* gene has been reported, indicating the existence of another suppressor gene in the region [9].

The specific functions of the isoenzyme PLCB3 are unclear but different members of the phospholipase family are involved in signal transduction of the seven transmembrane receptors and are widely expressed in normal tissue. Upon binding activated subunits of heterotrimeric G proteins, PLCB generates the second messenger molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate by hydrolysis. IP_3 is involved in the intracellular Ca^{2+} homeostasis whereas DAG activates protein kinases C (PKCs) [10]. PKCs have been shown to be involved in cell growth activation and differentiation [11]. In order to investigate to what extent PLCB3 is involved in the neuroendocrine tumorigenesis, we identified three endocrine tumor cell lines (RIN m5F [12], BON-1 [13] and Lcc18 [14]) with a low protein expression of PLCB3 and stably transfected them with a *PLCB3* construct [1]. The growth rate in vitro was assessed by [^{3}H]thymidine incorporation [15] and cell counting, tumorigenicity in vivo, by xenografting to nude mice. PLCB3 expressing clones were compared to, respective, wild-type (wt), an antisense control as well as cells transfected with the vector without insert. Restoration of PLCB3 expression led to a significantly reduced growth rate in vitro for all three cell lines when compared to, respective, wt and controls. The tumor weight in nude mice was reduced for tumors formed by xenografted *PLCB3*-transfected Lcc18 cells (RIN m5F could not be used due to its insulin production). Further, the xenografted tumors formed by both *PLCB3*-transfected BON-1 and Lcc18 cells show a significantly reduced staining for the proliferation marker Ki-67 [16] and lack signs of invasive growth behavior as compared to controls.

2. Materials and methods

2.1. Cell lines and culture conditions

Three endocrine tumor cell lines were chosen for the experiments because of their low but variable PLCB3 protein expression (Western blots) compared to other cell lines and normal endocrine pancreatic tissue (data not shown). RIN-m5F (RIN) (rat insulinoma) had no expression, BON-1 (BON) (human endocrine pancreatic tumor) showed a low expression whereas Lcc18 (Lcc) (human hindgut carcinoid) had a higher expression, although lower than controls. A culture for *Mycoplasma* was negative for both RIN and Lcc18 whereas BON-1 was positive and treated for 7 days with *Mycoplasma* Removal Agent (MRA) (ICN Biomedicals). After treatment, the BON-1 cells were checked *Mycoplasma*-free on culture, DNA staining and DNA probing. RIN cells were cultured in F12-modified (Nordcell) medium supplemented with 1% non-essential amino acids (Biochrom KG) and 10% fetal bovine serum (FBS) (HyClone). To culture BON cells, a 1:1 mixture of F12K (Nordcell) and DMEM (Biochrom KG) medium was used and supplemented with 5% FBS. Lcc cells were cultured in RPMI 1640 (Biochrom KG) supplemented with transferrin 0.5%, insulin 133 I.E./l (Monotard, Novo Nordisk), selenium 0.01%, hydrocortisol 0.01%, β-estradiol 0.01% and FBS 5%. All cells received 1% penicillin-streptomycin and 1% L-glutamine (Biochrom KG). The cells were housed in an incubator maintaining an atmosphere of 90% humidity and 5.0% CO_2 at 37°C.

2.2. mRNA analysis

Total RNA was isolated from neuroendocrine tumor cell lines [17]. 400 μg was used to isolate mRNA using PolyAtract (Promega) and examined for PLCB3 expression by a Northern blot analysis. Briefly, mRNA was size-fractionated by agarose/formaldehyde gel electropho-

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Abbreviations: PLCB3 (protein) and *PLCB3* (gene), phosphatidylinositol-specific phospholipase C β 3 (human); IP_3, inositol 1,4,5-trisphosphate; wt, wild-type; vector, plasmid vector without insert

PII: S0014-5793(99)00457-3

resis and then transferred to a nylon membrane (Hybond N, Amersham) for analysis. For the detection of PLCB3 mRNA, cDNA excised from a pBluescript II SK+ plasmid (Stratagene) containing human *PLCB3* cDNA [1] was used as a probe. The probe was labelled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) using the Quick prime kit (Pharmacia) according to the manufacturer's protocol. Membranes were hybridized overnight in CHURCH buffer (7% SDS, 0.5 M Na_2HPO_4, 1 mM EDTA and 50% formamide) at 42°C, then subjected to a high stringency wash. Storage phosphor screens (Molecular Dynamics) were used for exposure. Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics). To control for variations in loading and transfer among samples, the PLCB3 signal was normalized to β-actin signals obtained on the same blot. Results showed an overexpression of PLCB3 in the clones transfected with the *PLCB3* construct ranging from 5 to 15 times higher than that of wt and vector only (data not shown).

2.3. Protein analysis

Total cell lysates from the different clones were prepared and 20 μg of protein sample from each clone was analyzed for PLCB3 expression by Western blotting using an anti-PLCB3 antibody (diluted 1:250) (Santa Cruz Biotechnology). Western blotting and filter development were performed as earlier described [18]. Poinceau-red staining was used as a control for variations in the loading and transfer of protein. The expression of PLCB3 was estimated to be 15–20 times higher in the PLCB3-transfected clones compared to the basal wt level of the respective cell line, except for RIN/PLC: cl. 3 which showed an approximately five times higher expression.

2.4. Transfections

For transfections, the encoding region of *PLCB3* cDNA [1] was inserted into the pCEP-4 (vector) plasmid (Invitrogene) using standard cloning techniques. A vector antisense *PLCB3* control (antisense) was constructed by inserting the encoding region in the antisense orientation. The antisense reading frame revealed several early stop codons, thus, would not be translated to any known functional protein when it was matched against BLAST (NCBI). The different cell lines were transfected with vector, antisense and vector-*PLCB3* (PLC) according to the Lipofectin reagent protocol (Life Technologies) and stable transfectants were selected in hygromycin B (Boehringer Mannheim) at a concentration of 400 μg/ml, 125 μg/ml and 1.2 mg/ml for RIN, BON and Lcc, respectively. After clonal expansion, transfectants were maintained at the same selective concentrations and used at passages 3, 4 and 5 for RIN and BON and at passage 3 for Lcc clones. The PLCB3 expressing clones (cl.) were named RIN/PLC/cl. 1, 2 and 3, BON/PLC and Lcc/PLC/cl. 1, 2 and 3.

2.5. [^{3}H]thymidine incorporation assay

The RIN cells were used to assess the stability of the test system and all three expressing clones were used at passages 3, 4 and 5. For each experiment, 5×10^4 cells of the wt, controls and PLCB3 expressing clone of RIN were seeded in 2 ml of the appropriate medium into 35 mm petri dishes (18 dishes of each). The cell viability was assessed by trypan blue dye exclusion. After 48, 72 and 96 h, the medium was changed in six dishes of each cell type and supplemented with 0.2 μCi/ml [^{3}H]thymidine (Amersham). The RIN cells were allowed to incorporate [^{3}H]thymidine for 6 h before they were put on ice for 5 min, precipitated with 10% ice cold TCA (10 min), washed three times with ice cold PBS before and finally lysated in 0.3 M NaOH, 1% SDS at room temperature (20 min). The lysate was transferred to scintillation tubes containing Optiphase Highsafe II scintillation fluid (Wallac) for the assessment of β-radiation. The radiation was expressed as disintegrations per minute (dpm) (WinSpectral 1414, Wallac). Essentially the same procedure was repeated for BON and Lcc but due to a longer doubling time of the two cell lines, the [^{3}H]thymidine incorporation took place after 48, 96 and 144 h and in the case of BON, the [^{3}H]thymidine was allowed to incorporate for 12 h.

2.6. Cell counting

Cells were counted at the same respective time points as used for the [^{3}H]thymidine incorporation assay. 15×10^3 cells of RIN/PLC: cl 1, 2 and 3, wt and vector were seeded in triplicates onto 24 well plates (Nunc) at $t=0$. When counting, cells were washed once with PBS and then trypsinized in 0.5 ml of trypsin/EDTA and counted in triplicates, using a Coulter cell counter. The same procedure was used to count the BON/PLC clone (20×10^3 cells/well) and the Lcc/PLC: cl 1 (60×10^3 cells/well), comparing the growth to respective wt and vector controls.

2.7. IP_3 assay

The PLC activity was investigated by comparing basal levels of inositol phosphates between RIN wt and RIN/PLC: cl 1 and between BON wt and BON/PLC (Lcc18 was not investigated as the assay only could be used for adherent cell lines). 1×10^6 cells of the different clones, in triplicates, were incubated overnight (20 h) with 2 μCi D-myo[^{3}H]inositol (Amersham) in 2 ml of the appropriate growth medium (cells could not be depleted of FCS without extensive cell death). The following day, the cells were incubated with medium containing 20 mM LiAc and incubation continued for 15 min at 37°C. After washing once with ice cold PBS, 2 ml of acidified ice cold methanol was added to each well (methanol/HCl 100:1). The quenched samples were collected and the dishes were rinsed with an addition of 1 ml of acidified methanol. To each sample, 1.5 ml water and 3 ml chloroform were added. The tubes were mixed vigorously and left on ice for 30 min. The water soluble phase was collected and diluted with two volumes of water, centrifuged for 5 min and finally, the upper phase was applied to 0.5 ml AGI-X8 columns (formate form, 100–200 mesh). The samples were washed with 10 ml of 5 mM sodiumtetraborate, 60 mM sodiumformate. The total inositol phosphates were eluted with 5 ml of 0.1 M formic acid and 1.0 M ammonium formate and subjected to scintillation counting. The chloroform phase samples containing the phospholipids were dried, redissolved in methanol and subjected to scintillation counting. The IP_3 release was calculated as the ratio between total inositol phosphates and the total incorporated D-myo[^{3}H]inositol [19].

2.8. Animals and xenografting

Female balb/c nude mice weighing 20–30 g (Bomholtsgaard) were housed in cages, fed standard laboratory food and water ad libitum. They were injected subcutaneous (s.c.) on the hindleg with 2×10^6 cells in 0.5 ml of the appropriate growth medium. BON/PLC, BON/wt and BON/vector were injected s.c. in 14 animals in each group. Lcc/PLC/cl. 1 together with Lcc/wt and Lcc/vector were injected in 18 animals in each group (RIN was not suitable because of its insulin production). Tumors were allowed to grow for 7 weeks before the mice were killed. The tumors were thoroughly dissected, weighed, snap frozen in liquid nitrogen and finally cryosectioned for further analysis. Approval of this study was obtained from the Uppsala Ethics Committee for Animal Care and Use (Uppsala, Sweden).

2.9. Immunohistochemistry, antibodies and histology

6 μm thick cryosections were fixed in ice cold acetone for 10 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 30 min and endogenous avidin binding activity was blocked by sequential incubation with avidin and biotin for 15 min, each using a Blocking kit (Vector Laboratories). Unspecific binding of secondary antibody was blocked by incubation with normal goat serum, diluted 1:5 in PBS. Incubation with the primary antibody was performed overnight at 4°C in a humidified chamber and the sections were then incubated with biotinylated goat anti-rabbit immunoglobulin (Vector laboratories). The immunoreaction was visualized using a Vectastain Elite ABC kit (Vector Laboratories) with 3-amino-9-ethylcarbazole in dimethylsulfoxide as chromogen and 0.02% hydrogen peroxide as substrate. Finally, the sections were counterstained with Mayer's hematoxylin.

We used a polyclonal chromogranin A rabbit antibody (diluted 1/2000) to confirm that the tumors actually were composed of neuroendocrine cells [20]. As a marker for proliferation and malignancy, a polyclonal Ki-67 antibody (diluted 1/200) (Dakopatts) which reacts with a nuclear antigen expressed in all phases of the cell cycle except G0 [16] was used. Sections were analyzed blinded at a 1000× magnification and positive cells were counted at three different 'hot spots' (defined as areas of maximum number of positive cells compared to negative cells) using a 1 cm^2 grid. The mean percentage of positive cells of the total cell number was calculated. A pathologist analyzed the tumors histologically, scoring the abundance of necrosis and the incidence of capsular invasion (van Gieson as well as hematoxolyn/eosin staining). Necrosis less than 10% of the tumor area was scored (+), 10–50% (++) and over 50% (+++).

2.10. Statistics

All values of mean, range and standard error of the mean (S.E.M.) were calculated using StatView 4.01 (Abacus Concepts). *P* values were calculated using Student's *t*-test and considered significant when <0.05.

3. Results

Effects on the growth rate in vitro. Three transfected clones of RIN m5F (RIN/PLC: cl. 1, 2, 3) and Lcc18 (Lcc/PLC: cl. 1, 2, 3) cells, as well as one clone of BON-1 cells (BON/PLC), were identified as expressing PLCB3 mRNA. Protein expression was confirmed by Western blots (Fig. 1). As expected, no protein was detectable in the antisense controls. The PLC activity measured as IP_3 release showed increased levels by mean 11 and 23% in RIN/PLC: cl. 1 and BON/PLC, respectively, as compared to wt controls. The growth rate, by means of DNA synthesis, of the PLCB3 expressing clones was compared to that of the three controls (untransfected wt, wt transfected with the vector without an insert (vector) and antisense) by using the [^{3}H]thymidine incorporation assay. PLCB3 expressing clones showed growth inhibition at all three time points chosen for [^{3}H]thymidine incorporation assessment (Fig. 2). The mean inhibition of RIN/PLC (three clones, seven passages) at the third time point of measurements compared to wt, vector and antisense controls were 52% (range 39–86), 38% (21–67) and 33% (3–70), respectively. The BON/PLC clone (three different passages) showed a mean growth inhibition of 44% (36–56), 43% (31–56) and 48% (37–64) compared to wt, vector and antisense controls, respectively. Lcc/PLC: cl 1, 2, 3 (three different passages) were inhibited by an average of 77% (71–82) compared to wt and by 58% (57–58) compared to vector (comparison to antisense was not performed in the Lcc18 experiments). The growth inhibition was significant and reproducible in all experimental series (*P* value ranging from 0.0001 to 0.05), with the exception of RIN/PLC: cl. 2: passage 4 compared to the antisense control ($P=0.656$), with a very low variation between samples within the individual experiments (S.E.M., mean 4.8%, range 0.5–21.7%) (Table 1). Cell counting was performed comparing the PLCB3 expressing clones of RIN, BON and Lcc/PLC: cl. 1 to respective wt and the vector control. Growth inhibition was recorded in the PLCB3 expressing clones with a mean final reduction in the cell number compared to wt of 50% (range 43–60%) for RIN/PLC (three clones), 38% (range 33–42%) for BON/PLC and 48% (range 44–51%) for Lcc/PLC: cl. 1. No growth inhibition by means of a reduced cell number was seen for the vector controls (Fig. 2). The *PLCB3*-transfected clones of the adherent cell lines RIN m5F and BON-1 seemed to exhibit some morphological changes when cultured in vitro. wt and vector controls showed dendritic expansions and formed a homogenous cell layer, whereas the PLCB3 expressing clones tended to grow in colonies and were more rounded up.

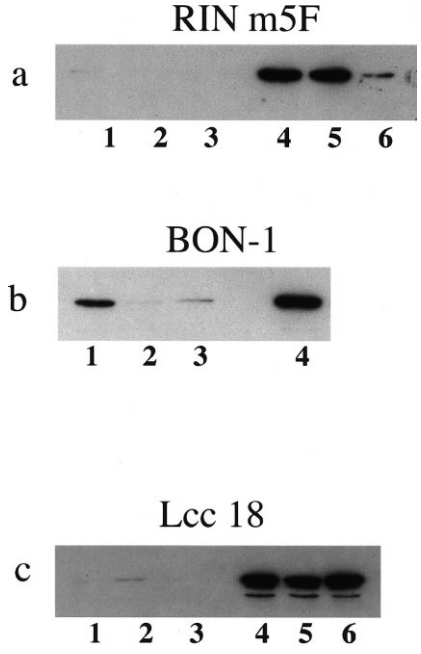

Fig. 1. Western blot for PLCB3. Lane 1: wt, lane 2: vector and lane 3: antisense. a: RIN m5F, lanes 4–6: PLCB3 clones 1–3. b: BON-1, lane 4: PLCB3. c, Lcc18, lanes 4–6: PLCB3 clones 1–3.

Tumor formation in nude mice and phenotype changes. BON/PLC and Lcc/PLC: cl. 1 cell clones as well as respective wt and vector controls were xenografted to altogether 96 nude mice. The transplants were left to grow for 7 weeks. A tumor take was obtained in 12/14 mice xenografted with BON/PLC and BON/wt, 11/14 with BON/vector, 14/18 animals harboring the Lcc/PLC: cl. 1 and 17/18 in the mice transplanted with Lcc/: wt and Lcc/vector. During dissection of the tumors, a substantial difference of invasiveness was macroscopically observed. None of the tumors formed by PLCB3 expressing clones showed macroscopic signs of invasion and all seemed to be surrounded by intact capsules whereas 33% of BON/wt and 83% of Lcc/wt mice tumors as well as 67% of the Lcc/vector and 76% of the BON/vector tumors grew invasively into the surrounding soft tissue. The tumor weight was significantly lower ($P<0.01$) in Lcc/PLC: cl. 1 compared to wt and vector. BON/PLC tumors, however, showed no weight difference compared to controls (Fig. 3). Immunohistochemical staining for chromogranin A (CgA) confirmed the neuroendocrine status of the formed tumors. Staining for proliferation marker Ki-67 indicated a significant ($P<0.001$) decrease in the number of proliferating cells in the PLCB3 expressing tumors of both BON-1 and Lcc18 compared to wt and vector. BON/PLC tumors displayed 21 ± 2 (S.D.)% Ki67 positive cells, BON/wt showed a positive cell count of 73 ± 1 (S.D.)% and BON/vector of 51 ± 2 (S.D.)%. Ki-67 staining was positive in 23 ± 5 (S.D.)% of the cells in the tumors formed by Lcc/PLC: cl. 1 and in 51 ± 5 (S.D.)% and 50 ± 2 (S.D.)% of cells from Lcc/wt and Lcc/vector xenografts, respectively (Fig. 4). Microscopic examination showed invasive growth in 4/12 BON/wt tumors whereas none of the vector or BON/PLC tumors revealed such features. However, only seven BON/wt and two BON/PLC specimens could actually be accurately scored, due to incomplete capsular material included on the slides provided for the pathologist. Necrosis was abundant ($>50\%$) in 7/12 BON/PLC, 3/12 BON/wt and 6/12 BON/vector tumors. Intermediate necrosis (10–50%) was found in 2/12 of the lesions formed by BON/PLC, 4/12 of BON/wt and 5/12 BON/vector tumors. Less than 10% necrosis was observed in 3/12, 5/12 and 1/12 in the tumors consisting of BON/PLC, BON/wt and BON/vector cell clones, respectively. The material was too limited for accurate scoring of capsular microinvasion in the Lcc18 xenografts, but thorough assessments of necrosis revealed no significant differences between Lcc/PLC and controls.

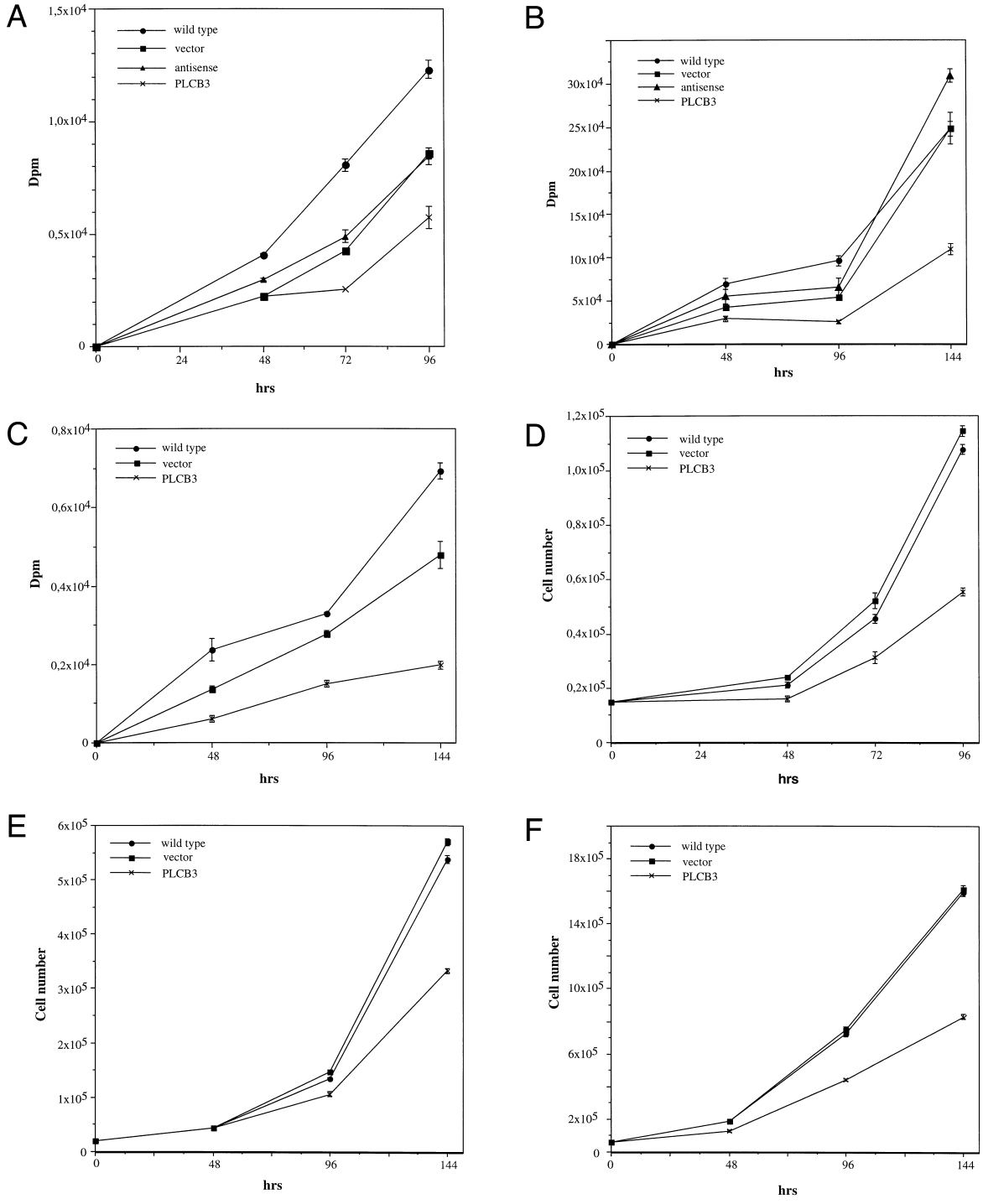

Fig. 2. Graphs visualizing the mean [^{3}H]thymidine uptake (A–C), measured as dpm and cell number (D–F). PLCB3 expressing clones show a significantly reduced [^{3}H]thymidine uptake compared to controls (wt, vector and antisense) as well as a significantly reduced cell number. Graphs showing individual experiments. Bars: S.E.M. A: RIN m5F with RIN/PLC: cl 3 passage 5. B: BON-1 with BON/PLC passage 5. C: Lcc18 with Lcc/PLC: cl 3 passage 3. D: RIN m5F with RIN/PLC: cl 1. E: BON-1 with BON/PLC. F: Lcc18 with Lcc/PLC: cl 1.

4. Discussion

We have found that the restoration of PLCB3 expression in neuroendocrine tumor cell lines substantially inhibits the growth rate and suppresses the neoplastic phenotype both in vitro and in vivo, suggesting that PLCB3 may be important in the neuroendocrine tumorigenesis. The *PLCB3*-transfected clones revealed increased levels of IP_3 compared to wt controls when grown with serum. Measurement of IP_3 is an established method to verify PLC-mediated intracellular signaling [19]. Since a complete depletion of serum is fatal to the neuroendocrine cell lines, we performed the IP_3 assay with serum present in the medium. Growth factors and G-protein activators in the serum lead to continuous intracellular signaling in the cell lines. This might explain why the difference in IP_3 levels between the PLCB3 expressing clones and their wt

Table 1
DNA synthesis rate of PLCB3 expressing clones compared to wt, vector and antisense

	PLCB3	wt	Vector	Antisense	Passage number	Statistical significance in [^{3}H]thymidin uptake for PLCB3 versus:		
						wt	Vector	Antisense
RIN	5 642 ± 61 cl. 1	1 0075 ± 1048	7 591 ± 225	8 715 ± 343	4	$P < 0.01$	$P < 0.001$	$P < 0.001$
	667 ± 87 cl. 2	4 765 ± 270	2 013 ± 437	2 219 ± 319	3	$P = 0.0001$	$P < 0.05$	$P < 0.01$
	3 700 ± 177 cl. 2	6 058 ± 175	4 685 ± 162	3 830 ± 184	4	$P < 0.001$	$P < 0.01$	$P = 0.656$
	4 100 ± 66 cl. 2	6 888 ± 209	8 354 ± 621	5 732 ± 284	5	$P = 0.0001$	$P < 0.001$	$P < 0.01$
	3 504 ± 164 cl. 3	6 292 ± 259	4 547 ± 151	5 699 ± 341	3	$P < 0.001$	$P < 0.01$	$P < 0.01$
	4 306 ± 152 cl. 3	9 496 ± 58	7 488 ± 364	5 752 ± 293	4	$P = 0.0001$	$P < 0.001$	$P < 0.01$
	5 714 ± 494 cl. 3	12 299 ± 408	8 562 ± 122	8 474 ± 380	5	$P < 0.01$	$P < 0.01$	$P < 0.01$
BON	279 040 ± 4 945 cl. 1	436 381 ± 5 280	401 742 ± 4 365	383 300 ± 5 162	3	$P = 0.0001$	$P < 0.001$	$P < 0.01$
	146 916 ± 1 713 cl. 1	248 040 ± 8 472	248 792 ± 17 846	309 244 ± 7 533	4	$P < 0.07$	$P < 0.01$	$P = 0.0001$
	109 192 ± 6 567 cl. 1	248 040 ± 7 888	249 214 ± 17 109	304 838 ± 9 798	5	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
Lcc	1 437 ± 57 cl. 1	6 843 ± 161	3 421 ± 275		3	$P < 0.001$	$P < 0.05$	
	1 235 ± 11 cl. 2	6 779 ± 265	2 969 ± 258		3	$P < 0.01$	$P < 0.05$	
	1 979 ± 90 cl. 3	6 937 ± 206	4 793 ± 355		3	$P < 0.01$	$P < 0.05$	

Mean of dpm ± S.E.M. at the third timepoint for [^{3}H]thymidine incorporation. Significant differences were between PLCB3 expressing clones and wt, vector and antisense, respectively. Passages are numbered. Clone 1: cl. 1, clone 2: cl. 2 and clone 3: cl. 3

controls is relatively moderate, however obvious. The increase of IP_3 in the transfected clones possibly reflects an increase of the PLC activity due to the transfected PLCB3 construct. [^{3}H]thymidine incorporation is a reliable and reproducible method for the assessment of cell growth and reflects both the cell number and DNA synthesis rate [15]. Together with the reduced growth rate by the cell number and the reduced tumor weight in vivo for Lcc/PLC: cl. 1, our experiments show that induction of PLCB3 expression by transfection significantly reduces the growth rate in three different neuroendocrine tumor cell lines which lack endogenous PLCB3 expression. The highly significant reduction of the [^{3}H]thymidine uptake compared to wt, vector and antisense controls was reproducible in 12/13 experimental series and showed a very low internal variation. Interestingly, the vector, to some extent, seems to impair the DNA synthesis recorded as reduced thymidine incorporation but does not, according to the results from cell counting, inhibit the cell growth. We have no indication that the construct per se produces toxic effects leading to cell death, as the clones could be kept alive for over 15 months and were able to grow in nude mice. Instead, PLCB3 expressing xenografts reveal morphological features suggesting that PLCB3 is capable of altering the neoplastic phenotype.

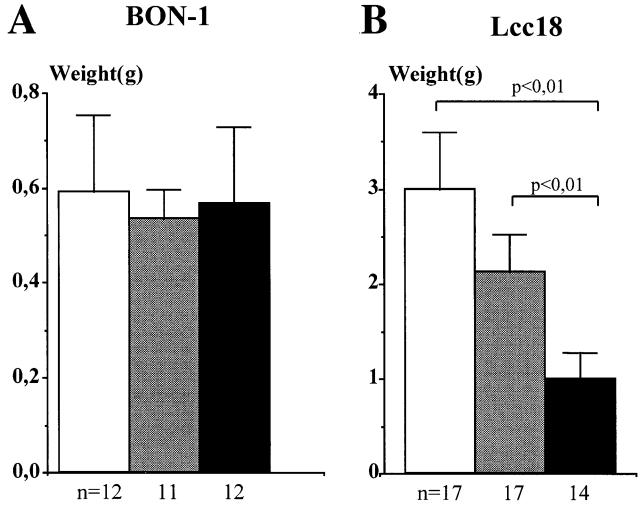

Fig. 3. Weight (g) of xenografted tumors. A: BON-1. B: Lcc18. Black stacks: tumors formed by *PLCB3*-transfected clones. White stacks: wt cells. Gray stacks: tumors consisting of cell clones transfected with the vector control. Bars: S.E.M.

Suppression of the growth in vivo by means of a significantly lowered tumor weight was observed for Lcc/PLC: cl 1 compared to controls but not for BON/PLC, which could be explained by a lower proliferation rate of the BON-1 cell line making differences more difficult to observe. However, a significantly smaller proportion of proliferating Ki-67 positive tumor cells was observed in both BON/PLC and Lcc/PLC: cl 1 compared to controls, which indicate that the proliferation rate and the malignant phenotype were suppressed [16]. Although the mechanism remains to be explained, other observations, such as a lack of macroscopic signs of invasive behavior in vivo, support the interpretation that PLCB3 may induce phenotypic changes. Unfortunately, the incomplete microscopic examination of the xenografted BON-1 tumors failed to support the macroscopic findings of a more non-invasive behavior of the PLCB3 expressing tumors. The amount of necrosis was higher in both the vector control and the PLCB3 expressing tumors of BON-1. This might be an effect of the plasmid vector itself. The proliferation rate by means of Ki-67 positive cells was, however, equally abundant in the wt tumors as in the vector controls. In consistency with interpretation principles applied in other suppressor gene studies, our findings support that PLCB3 might harbor suppressor characteristics [21].

It has been shown that PLCB1 activates PKC which in turn activates raf via a p21ras-independent mechanism [11]. The dependency on the different pathways for mitogenic signaling seems to differ between cell systems and it might be that the loss of PLCB3 function only affects the differentiation and growth regulation in certain cell types. It has been reported that PLCB4 becomes upregulated upon introduction of wt *p53* into *p53* deficient mouse M1 myeloid leukemia cells. This finding may suggest that PLCB4 comprises a downstream target in mediating the apoptotic effect of p53 [22]. Sequencing of *PLCB3* in neuroendocrine tumors has not revealed mutations [23] but silencing by hypermethylation or imprinting has not yet been excluded [24,25]. Homozygous inactivation of the *Plcb3* gene in mice resulted in early embryonic lethality. No increased incidence of tumors was observed in mice heterozygous for *Plcb3*, however, challenging of the

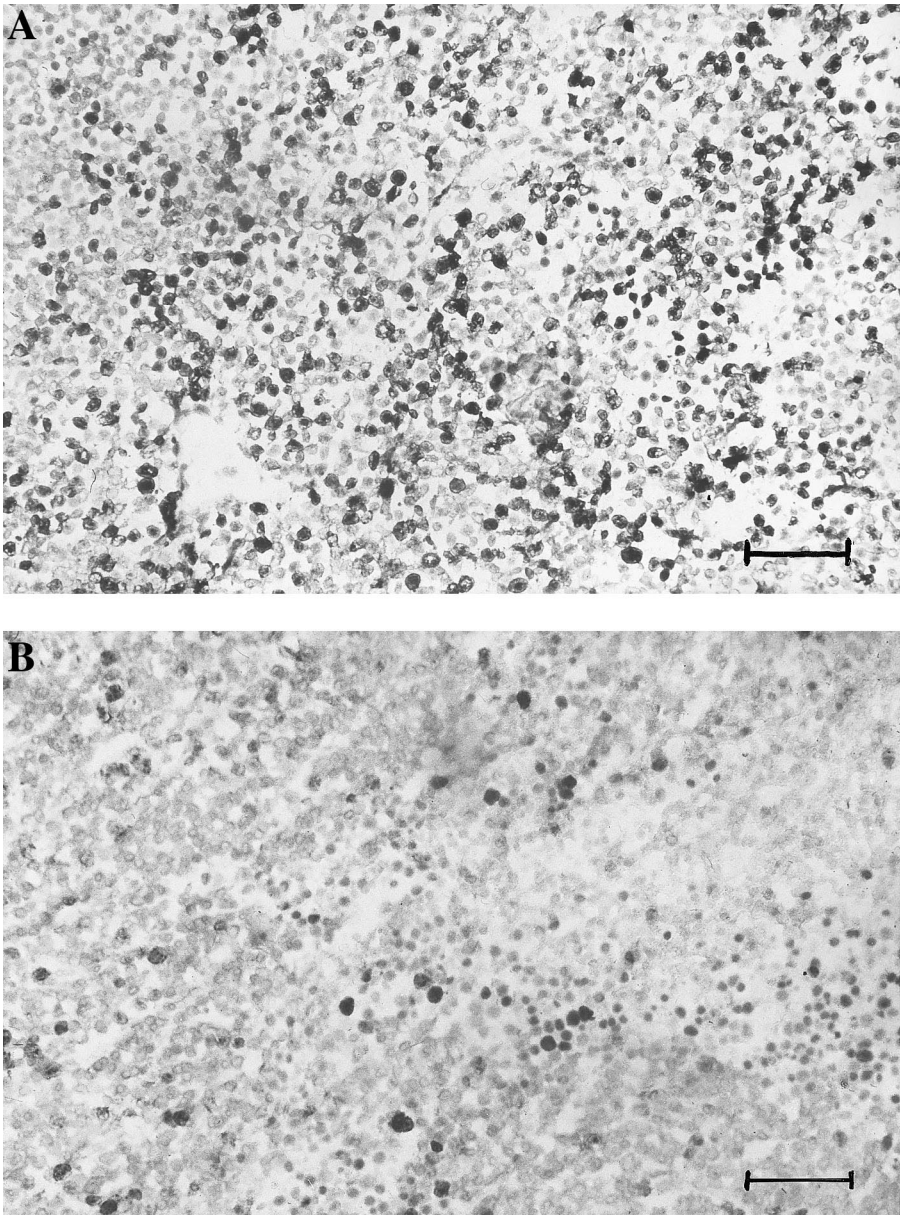

Fig. 4. Differences in immunohistochemical staining with the Ki-67 antibody in tumors formed by Lcc18 cell clones transplanted to nude mice. Photographs at 100× magnification, indication on a photograph = 200 μm. A: wt. B: *PLCB3*-transfected clone.

heterozygous mice with chemical carcinogens might reveal a haploid insufficiency as they express lower levels of *Plcb3* than their wt littermates [26,27]. Further, one might speculate whether a homozygous inactivation of the *MEN 1* gene [4] in neuroendocrine tumors could result in the downregulation of PLCB3 expression and affect a previously unknown global tumor suppressor pathway. In conclusion, the data presented here do imply that *PLCB3* may be involved in the neuroendocrine tumorigenesis and have properties of a tumor suppressor gene.

Acknowledgements: Lotti Rorsman and Lars Rönnstrand at the Ludwig Institute for Cancer Research, Uppsala, for teaching and performing the IP_3 assay. Prof. Lars Grimelius for histologic examination of the tumors and Rajni Dyal for excellent work in the lab. This work was supported by grants from the Swedish Cancer Society, Lions Foundation for Cancer Research, Swedish Medical Council, Swedish Medical Society and Torsten and Ragnar Söderbergs Foundation.

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