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Regulation of Transformed State by Calpastatin via PKCε in NIH3T3 Mouse Fibroblasts

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Ca^{2+}-activated neutral protease calpain is ubiquitously expressed and may have pleiotropic biological functions. We have previously reported that repeated treatment of NIH3T3 mouse fibroblasts with the calpain inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN) resulted in the induction of transformed foci [T. Hiwasa, T. Sawada, and S. Sakiyama (1990) *Carcinogenesis* 11, 75–80]. To elucidate further the effects of calpain in malignant transformation of NIH3T3 cells, calpastatin, an endogenous specific inhibitor of calpain, was expressed in NIH3T3 cells by transfection with cDNA. G418-selected calpastatin-expressing clones showed a significant increase in the anchorage-independent growth ability. A similar increase in cloning efficiency in soft agar medium was also observed in calpain small-subunit-transfected clones. On the other hand, reduced expression of calpastatin achieved by transfection with calpastatin antisense cDNA in Ha-*ras*-transformed NIH3T3 (ras-NIH) cells caused morphological reversion as well as a decrease in anchorage-independent growth. When NIH3T3 cells were treated with ALLN for 3 days, cell growth was stimulated by approximately 10%. This growth stimulation by ALLN was not observed in ras-NIH cells, but recovered by expression of a dominant negative form of protein kinase C (PKC)ε but not by that of PKCα. Western blotting analysis showed that an increase in PKCε was much more prominent than that of PKCα in NIH3T3 cells after treatment with ALLN. These results are concordant with the notion that calpain suppresses malignant transformation by predominant degradation of PKCε.

Key Words: **calpain; calpastatin; ras; protein kinase C; malignant transformation.**

Carcinogenesis is controlled by the expression levels and activities of many oncogene and tumor-suppressor gene products. Protein expression levels are regulated by the synthesis, processing and degradation. Therefore, intracellular proteases can differentially affect the carcinogenesis and the transformed state depending on the substrates. Three groups of intracellular proteases have been documented well, calpain, proteasome and caspase. These proteases can degrade various products from oncogenes and tumor suppressor genes (listed at home page http://www.m.chiba-u.ac.jp/class/seika1/substrate.html), and thereby promote or suppress malignant transformation.

Ca^{2+}-activated neutral cysteine proteinase calpain is one of the major cytoplasmic nonlysosomal proteases (1, 2). Many isozyme forms including μ-calpain, m-calpain, muscle-specific calpain 3 (p94), nCL-4 and calpain-10 have been reported (3, 4). The catalytic activity of calpain is specifically inhibited by an endogenous inhibitor calpastatin (5). It was reported that calpain can degrade oncogene products such as PDGF receptor, c-Mos, c-Jun, c-Fos, Src, and EGF receptor (6–10) as well as protein kinase C (PKC) which is a receptor of tumor promoter phorbol esters (11), suggesting that calpain plays a suppressive role in malignant transformation. Consistently, a recent report showed that antisense RNA-mediated deficiency of nCL-4 resulted in neoplastic transformation (12). On

Abbreviations used: ALLN/calpain inhibitor I, *N*-acetyl-Leu-Leu-norleucinal; Dex, dexamethasone; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-3,4-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKC, protein kinase C.

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the other hand, calpain can also degrade tumor suppressor gene products such as p53, NF2, and IκBα (13–15). This implies the carcinogenic role of calpain.

We have previously reported that repeated treatment of NIH3T3 mouse fibroblasts with calpain inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN) resulted in growth stimulation and induction of transformed foci (16). This raised a possibility that calpain can suppress transformation. However, ALLN also inhibits cysteine protease cathepsins and proteasome (16–18). Thus, in the present study, we examined the effects of elevated or reduced expression levels of calpastatin on the malignant transformation. The results suggest the tumor-suppressive role of calpain.

MATERIALS AND METHODS

Materials. ALLN was purchased from Nakarai Chemicals (Kyoto, Japan). 3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and G418 were purchased from Sigma. Dexamethasone (Dex) was obtained from Wako Pure Chemicals (Kyoto, Japan).

Cell culture. NIH3T3 mouse fibroblasts and their activated-Ha-*ras*-transformed cells (ras-NIH, clone F25) were provided by Takao Sekiya (National Cancer Center Research Institute, Tokyo, Japan) (19). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% calf serum.

Transfection. cDNAs for human calpastatin (20) and human calpain 30-kDa subunit (30K) (21) were inserted into the *Sma*I site of pMSG eukaryotic expression vector (Pharmacia). cDNAs for dominant negative mutants of rabbit PKCα and PKCε (K368R and K371R, respectively) were constructed in an expression vector, SRD (22, 23). These plasmids were cotransfected with *neo* gene into NIH3T3 or ras-NIH cells using LipofectAMINE reagent (Invitrogen). Transfected cells were selected in the presence of G418 (400 μg/ml) for 2 weeks.

Preparation of cell extract and Western blotting analysis. For induction of transfected genes, cells were treated with Dex at a concentration of 1 μM for 2 days. Cells were then washed with phosphate-buffered saline (PBS) three times and incubated in lysis buffer [0.5% Nonidet P-40, 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 50 μM antipain, 50 μM pepstatin A, and 50 μM ALLN] for 10 min at 4°C. The cell lysate was centrifuged at 13,000*g* for 10 min, and the supernatant was lyophilized and used as described (24). Western blotting analysis was carried out using Immunostar (Wako Pure Chemicals). The antibodies used were anti-PKCα, anti-PKCε (Transduction Laboratories), anti-calpain 30K (Chemicon International, Temecula, CA) and anti-calpastatin (Takara Biochemical Inc., Kyoto, Japan) antibodies.

Anchorage-independent growth. Cell growth in soft agar medium was examined as described previously (25). Five thousand (NIH3T3) or 1000 (ras-NIH) cells were plated in soft agar medium which contained 0.4% SeaPlaque agarose, 10% calf serum, Dulbecco's modified Eagle medium and Dex (1 μM) or the solvent, dimethyl sulfoxide (0.1%). After culture at 37°C for 2 weeks, colonies of which the diameters were larger than 0.1 mm were scored. The colony-forming efficiency was calculated by dividing the colony number by the cell number plated.

Methods for assessing cell growth. A total of 5×10^3 cells were plated in each well of 96-well plates in the absence or presence of 4 μM ALLN, and cultured for 3 days. The activity of mitochondrial succinic dehydrogenase was measured by incubation for 4 h in the presence of MTT (0.5 mg/ml) followed by measurement of absorbance at 570 nm with a reference wavelength of 655 nm according to the method of Mosmann (26) as described previously (27). Absorbance reflects the viable cell number and was expressed as a percentage of that of control cells cultured in the absence of ALLN.

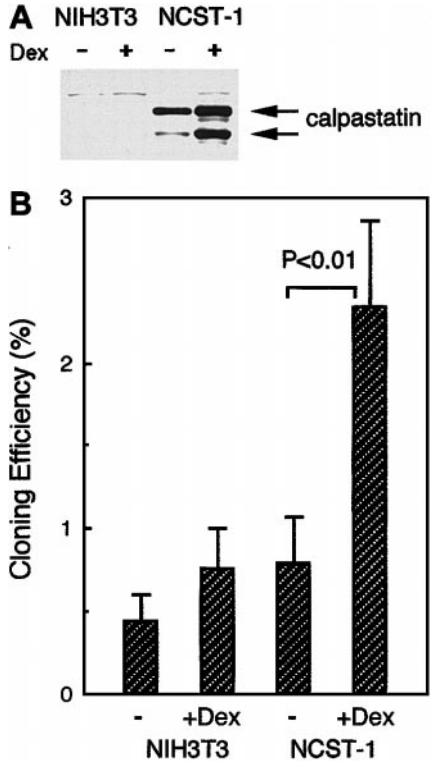

FIG. 1. Transfection of calpastatin into NIH3T3 cells. NIH3T3 cells were transfected with calpastatin, and a G418-selected clone, NCST-1, was isolated. (A) Western blotting analysis of calpastatin in NIH3T3 and calpastatin-transfected NCST-1 cells with (+) or without (−) treatment with Dex for 48 h at a concentration of 1 μM. (B) Effects of calpastatin on anchorage-independent growth. NIH3T3 and NCST-1 cells were cultured in soft agar medium in the presence (+) or absence (−) of Dex for 2 weeks, and the colonies were scored. The ordinate represents the average cloning efficiency and bars represent SD.

RESULTS

Stimulation of Anchorage-Independent Growth by Calpastatin

To investigate the effects of calpastatin, transfection with cDNA of calpastatin was carried out. cDNA for calpastatin was inserted into a downstream region of MMTV-LTR of an inducible expression vector, pMSG, and transfected into NIH3T3 cells. The transfected genes were effectively stimulated by treatment with Dex as described previously (28). Figure 1A shows the results of Western blotting analysis of the parental NIH3T3 and a calpastatin-transfected clone, NCST-1. The expression of endogenous calpastatin was undetectable in NIH3T3 cells irrespective of the treatment with Dex. On the other hand, a considerable amount of

calpastatin was expressed in NCST-1 cells and the level was further elevated by treatment with Dex. The expression level without Dex treatment varied among transfected clones, and was, therefore, due to the leakiness of the promoter of the vector as described (28).

Despite such high expression of calpastatin after treatment with Dex, phase morphology of NCST-1 cells was not appreciably changed (data not shown). The anchorage-independent growth which is one of the typical phenotypes of transformed cells was significantly higher in NCST-1 cells in the presence of Dex compared to that in the absence of Dex (Fig. 1B). Similar results were obtained in other calpastatin-expressing clones (data not shown). The cloning efficiency of the parental NIH3T3 cells in soft agar medium was kept lower than 1% even in the presence of Dex. These results imply that the high expression of calpastatin augmented the anchorage-independent growth ability.

Stimulation of Anchorage-Independent Growth by Calpain 30-kDa Small Subunit

Although regulatory roles of calpain 30-kDa small subunit (30K) were suggested, calpain 80K large subunit alone is sufficient to exhibit proteolytic activity (29). To investigate the biological roles of 30K, cDNA of 30K constructed in pMSG was transfected into NIH3T3 cells. Two of the transfected clones, N30K-8 and N30K-9, showed detectable levels of 30K protein expression without treatment and further enhanced levels after treatment with Dex (Fig. 2A). On the other hand, another clone, N30K-3, did not express a detectable level of 30K.

The colony-forming efficiencies in soft agar medium of N30K-8 and N30K-9 cells were similar to that of N30K-3 cells in the absence of Dex (Fig. 2B). However, they were much higher than that of N30K-3 cells in the presence of Dex. Thus, 30K may have a promoting role in anchorage-independent transformation.

Phenotypic Reversion of ras-NIH3T3 Cells by Calpastatin Antisense

Then, we asked whether oncogene-transformed phenotypes were reversed or not by repression of calpastatin. cDNA of calpastatin was inserted into the expression vector pMSG in antisense orientation and transfected into Ha-*ras*-transformed NIH3T3 (ras-NIH) cells. The expression of calpastatin antisense might reduce the protein expression level of calpastatin. As expected, the levels of calpastatin decreased or diminished in two transfected clones, FCST-AS-2 and FCST-AS-5, compared to that in parent ras-NIH cells (Fig. 3A). In these clones, effects of Dex treatment were only marginal (data not shown), and therefore, following experiments of these clones were performed in the absence of Dex.

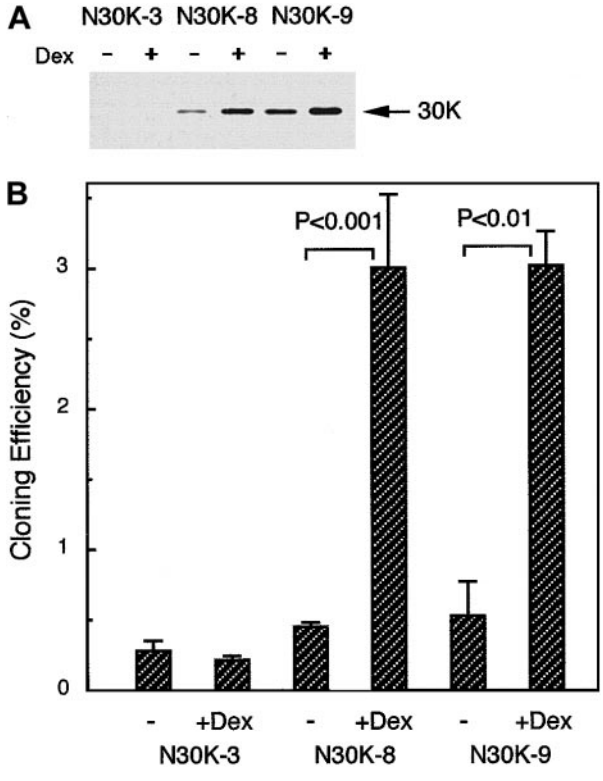

FIG. 2. Transfection of calpain small subunit 30K into NIH3T3 cells. (A) Western blotting analysis of calpain 30K in 30K-transfected clones, N30K-3, N30K-8 and N30K-9, with (+) or without (−) pretreatment with Dex. (B) Effects of 30K on anchorage-independent growth. N30K-3, N30K-8 and N30K-9 cells were cultured in soft agar medium in the presence (+) or absence (−) of Dex. The ordinate represents cloning efficiency and bars represent SD.

Intriguingly, drastic morphological changes were observed in calpastatin-AS-transfected clones (Fig. 3B). FCST-AS-2 and FCST-AS-5 cells showed flat morphology and contact inhibition which resembled to those of normal NIH3T3 cells. Cell growth ceased when the cells reached at confluency. These phenotypes were apparently contrast to those of ras-NIH cells. Colony-forming efficiency of FCST-AS-5 cells in soft agar medium was approximately 1.2%, which was much lower than that of the parental ras-NIH cells (Fig. 3C). Thus, reduced expression of calpastatin caused phenotypic reversion, likely through calpain activation.

Increase in PKCε Levels by Treatment with ALLN

Among many possible substrates of calpain, PKC has been well documented (11). When tumor-suppressor gene *DAN* (30) induced morphological reversion of ras-NIH cells, activation of calpain and concomitant down-regulation of PKCα and PKCϵ were observed (31). Thus, these PKC subtypes were suggested to be involved in the transformation of NIH3T3 cells. Western blotting analysis showed three different forms of PKCα, and the expression levels of these bands were

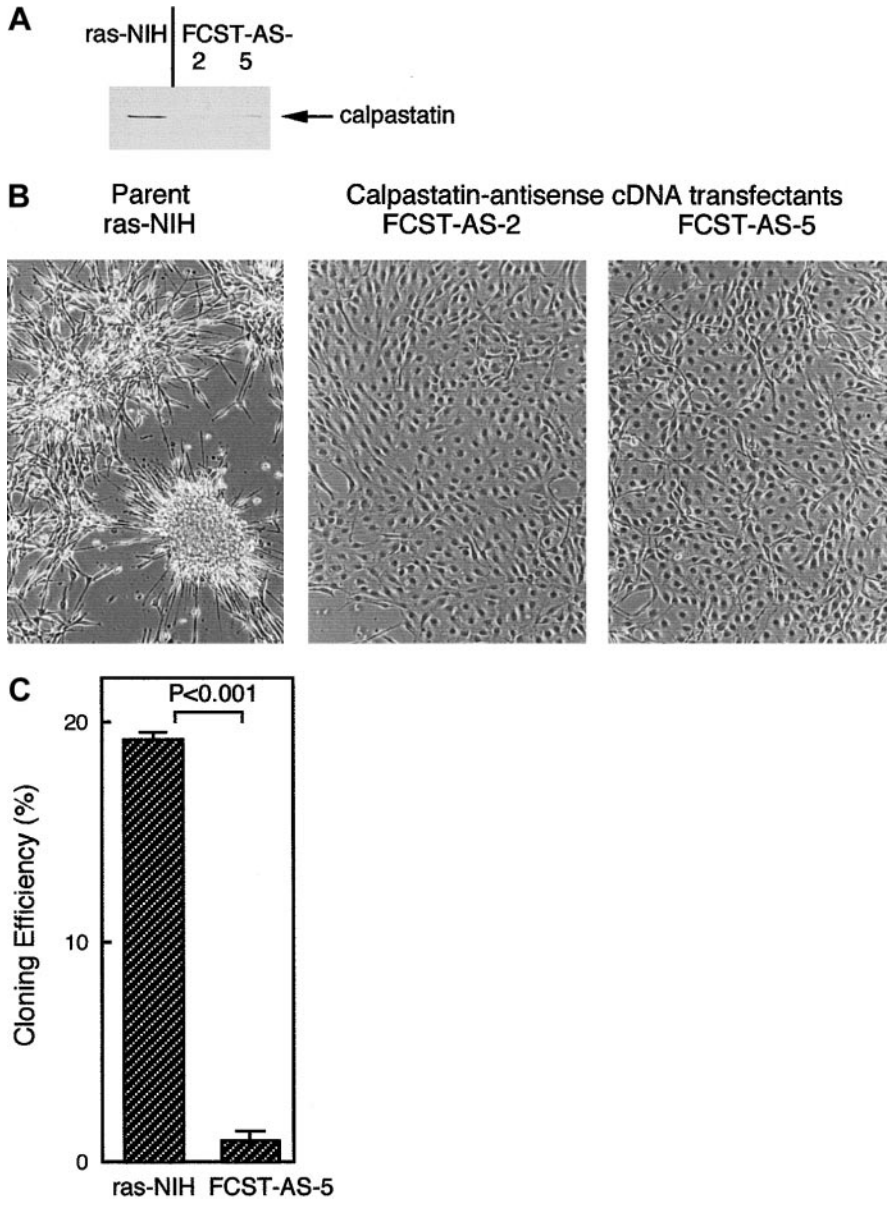

FIG. 3. Transfection of calpastatin antisense cDNA into ras-NIH cells. (A) Western blotting analysis of calpastatin in ras-NIH cells and calpastatin antisense-transfected FCST-AS-2 and FCST-AS-5 cells. (B) Phase morphology of parent ras-NIH cells and calpastatin antisense-transfected FCST-AS-2 and FCST-AS-5 cells. Original magnification, ×100. (C) Anchorage-independent growth of ras-NIH and FCST-AS-5 cells. Bars represent SD.

slightly lower in the calpastatin-transfected clone, NCST-1, than those in the vector-transfected control clone, NV-4 (Fig. 4A). After treatment with Dex to stimulate the expression level of calpastatin, the largest (uppermost) band was kept detectable while others disappeared in NCST-1 cells. The extent of this disappearance was greater than that in NV-4 cells, in which down-regulation of PKCα was regarded to be independent of transfected calpastatin. As for PKCϵ, the uppermost band of PKCϵ was rather increased by treatment with Dex in NCST-1 cells (Fig. 4B). It is conceivable that the lower two bands of PKCϵ could be partial degradation products produced by calpain and that the inhibition of calpain by calpastatin resulted in decrease in the lower bands and concomitant increase in the uppermost band.

We have previously reported that repeated treatment of NIH3T3 cells with ALLN at low concentrations (1 to 10 μM) induced transformed foci (16). It is possible that ALLN as a calpain inhibitor can suppress degradation of PKCs. Thus, the protein levels of PKCα and PKCϵ in NIH3T3 cells were examined after treatment with ALLN for 24 h at varying concentrations. The level of PKCα was increased by treatment with

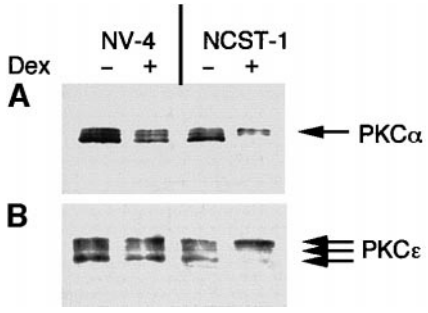

FIG. 4. Effects of calpastatin on the expression levels of PKCα and PKCϵ. NIH3T3 cells were transfected with vector alone (NV-4) or calpastatin (NCST-1), and G418-selected clones were isolated. Cells were then treated with Dex at 1 μM for 48 h, and cell extracts were analyzed by Western blotting using antibodies against PKCα (A) and PKCϵ (B).

ALLN at concentrations between 2.5 and 20 μM but decreased at 40 μM (Fig. 5A). Similar increase in the protein level was observed in PKCϵ but the extent was much higher than that in PKCα (Fig. 5B). Treatment with ALLN at 40 μM also caused marked reduction in PKCϵ expression. This was presumably due to calpain-independent toxic effects of ALLN since ALLN caused apoptosis in human prostate adenocarcinoma cells at concentrations higher than 50 μM (32).

Morphological reversion of ras-NIH cells by dominant negative PKCϵ. To examine which of PKCα and PKCϵ has a more important role in transformation, dominant negative forms of PKCα and PKCϵ were transfected into ras-NIH cells. Each one of the dominant negative PKCα- and PKCϵ-transfected clones (designated FPKCα-DN-2 and FPKCϵ-DN-12, respectively) showed much higher expression levels of the introduced genes compared to the levels of the respective endogenous wild-types in the vector-transfected clone, FV-1 (Figs. 6A and 6B).

Morphological alterations were remarkable in both transfectants. FPKCα-DN-2 cells showed notable growth retardation and shrunk morphology with long processes (Fig. 6C). On the other hand, FPKCϵ-DN-12 cells exhibited flat morphology and contact inhibition which were similar to those observed in calpastatin antisense-transfected cells and also in normal NIH3T3 cells. This suggests that maintenance of transformed morphology of ras-NIH cells required PKCϵ activity.

Recovery of Response to ALLN in PKCϵ-DN-Transfected Cells

Going back to the initial observation that ALLN stimulated proliferation of NIH3T3 cells (16), response to ALLN was compared among transfectants described above. Cells were treated with ALLN at a concentration of 4 μM for 3 days, and then the relative viable cell numbers were examined by MTT assay (Fig. 7). Proliferation of NIH3T3 cells (column 1) as well as vector-transfected NV-1 cells in the absence (column 2) or presence (column 3) of Dex was stimulated by treatment with ALLN to similar extents. ALLN failed to stimulate growth of calpastatin-transfected NIH3T3 (NCST) cells (columns 4 and 5), suggesting that calpain activity was required for ALLN-induced growth stimulation. ras-NIH cells were not significantly affected by ALLN (column 6). Low activity of calpain in ras-NIH cells might account for the unresponsiveness to ALLN. As expected, calpastatin-antisense-transfected ras-NIH cells recovered the response to ALLN (FCST-AS-5; column 7). Similar recovery from the refractoriness to ALLN was also observed in dominant negative PKCϵ-transfected ras-NIH cells (FPKCϵ-DN; column 9) but not in dominant negative PKCα-transfected cells (FPKCα-DN; column 8). These results were reproducible at ALLN concentrations between 2 and 5 μM (data not shown), and summarized in Table 1. Taken together, ALLN-induced growth stimulation of NIH3T3 cells seems to be mediated by decrease in calpain activity and the resultant increase in PKCϵ activity.

DISCUSSION

Involvement of calpain in malignant transformation was suggested by the induction of transformed foci of NIH3T3 cells after repeated treatment with calpain inhibitor ALLN (16). However, overexpression of an 80-kDa large catalytic subunit of μ- or m-calpain alone failed to achieve the increase in the calpain activity in NIH3T3 or ras-NIH cells (T. Hiwasa, unpublished observation). An alternative way to elucidate the role of calpain in malignant transformation is application of calpastatin which is an endogenous calpain-specific inhibitor (5, 33). In our present study, high expression levels of calpastatin enhanced anchorage-independent growth of NIH3T3 cells (Fig. 1B), suggesting that calpastatin promotes malignant transformation. Similar increase in anchorage-independent growth was also observed in transfected cells expressing a large amount of calpain small subunit 30K (Fig. 2B). This suggests an inhibitory role of 30K toward calpain activity, yet

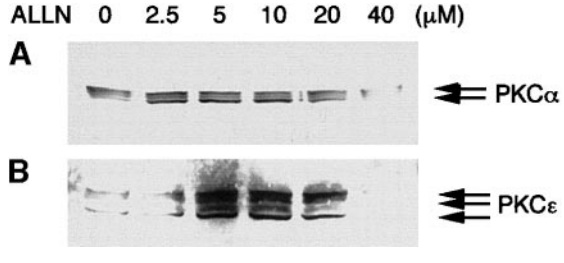

FIG. 5. Effects of ALLN treatment on the levels of PKCα and PKCϵ. NIH3T3 cells were treated with ALLN for 24 h at concentrations indicated. Cell extracts were analyzed by Western blotting using antibodies against PKCα (A) and PKCϵ (B).

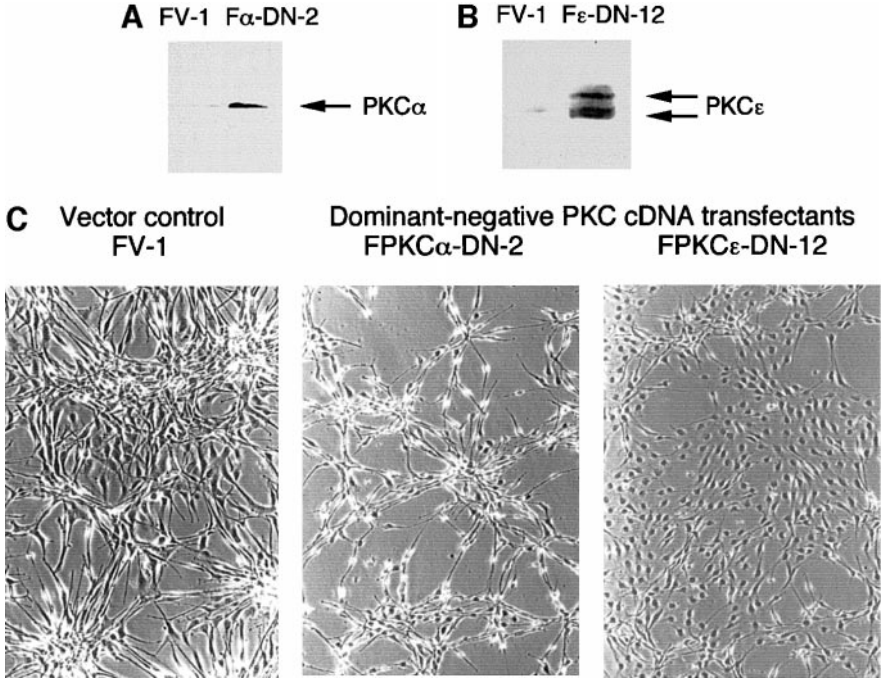

FIG. 6. Transfection of dominant negative PKCα and PKCϵ into ras-NIH cells. The expression levels of PKC in a vector-transfected clone, FV-1, a dominant negative PKCα-transfected clone, FPKCα-DN-2, and a dominant negative PKCϵ-transfected clone, FPKCϵ-DN-12, were analyzed by Western blotting using antibodies against PKCα (A) and PKCϵ (B). (C) Phase morphology of FV-1, FPKCα-DN-2 and FPKCϵ-DN-12 cells. Original magnification, ×100.

the other regulatory functions of 30K cannot be excluded.

The roles of calpastatin in an oncogene-transformed cell line, ras-NIH, were also investigated. Western blotting analysis showed that the protein expression level of calpastatin was undetectable in NIH3T3 while that in ras-NIH was clearly observed (Figs. 1A and 3A). Decrease in calpastatin expression level by transfection with calpastatin antisense resulted in phenotypic reversion (Figs. 3B and 3C). Thus, a high expression level of calpastatin leading to decreased calpain activity seems to be necessary for the maintenance of the transformed state in ras-NIH cells.

Similar phenotypic reversion of ras-NIH cells was also observed when the cells were transfected with a tumor-suppressor gene, *DAN* (30). In *DAN*-transfected cells, calpain activity was elevated and some of PKC subtypes such as PKCα and PKCϵ but not PKCλ or PKCμ were down-regulated (31). These results also imply the tumor-suppressive role of calpain and, furthermore, PKCα and PKCϵ as possible targets of calpain. Therefore, the effects of expression of dominant negative forms of PKCα and PKCϵ were examined in ras-NIH cells. Although both transfectants showed growth retardation and decrease in anchorage-independent growth ability, only PKCϵ dominant negative-transfected cells showed morphological reversion (Fig. 6C, Table 1). Thus, PKCϵ rather than PKCα plays a key role in the expression of specific transformed phenotypes in ras-NIH cells.

Then, we further investigated whether PKCϵ is involved in enhanced cell growth and transformation by treatment with ALLN. The expression level of PKCϵ increased markedly after treatment with ALLN in NIH3T3 cells (Fig. 5B). The largest form of PKCϵ increased while the smaller forms decreased after induction of calpastatin expression in the same cells (Fig.

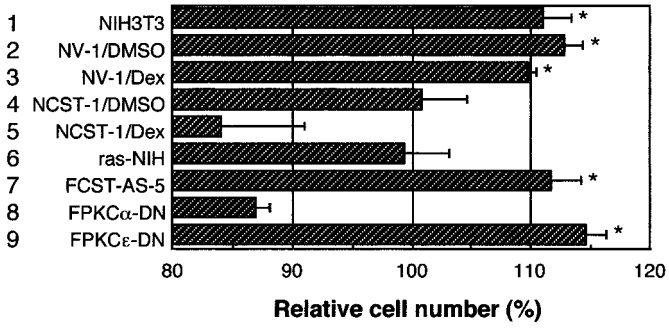

FIG. 7. Growth stimulation by ALLN. NIH3T3, ras-NIH and their transfected cells were cultured for 3 days in the absence or presence of 4 μM ALLN. Relative viable cell numbers were measured by MTT assay and expressed as percentages of that of control cells cultured in the absence of ALLN. Data are means of four experiments and error bars represent SD. Asterisks indicate statistically significant increases ($P < 0.05$) versus control cells cultured in the absence of ALLN.

TABLE 1

Summary of Characteristics of Transfected Cells

	NIH3T3	NCST	N30K	ras-NIH	FCST-AS	FPKCα-DN	FPKCε-DN
Morphology[a]	F	F	F	T	F	M	F
Anchorage-independent cell growth[b]	−	+	+	++	±	±[c]	±[c]
ALLN-stimulated cell growth[d]	+	−	±[c]	−	+	−	+

Note. Characteristics of NIH3T3, ras-NIH, and their transfected cells including NCST-1, N30K-9, FCST-AS-5, FPKCα-DN-2, and FPKCε-DN-12 cells are shown.

[a] Phase morphology was designated; F, normal flat; T, transformed; and M, intermediate, as shown in Figs. 3B and 6C.

[b] Anchorage-independent cell growth was classified; −, <1%; ±, 1–2%; +, 2–5%; and ++, >5%, as shown in Figs. 1B, 2B, and 3C.

[c] Data not shown.

[d] ALLN-stimulated cell growth was classified; +, responsive to ALLN; −, not responsive; and ±, intermediate, as shown in Fig. 7.

4B). These observations suggest that PKCϵ is predominantly degraded by calpain. Similar selective down-regulation of PKCϵ by calpain was also reported in rat pituitary cells after treatment with thyrotropin-releasing hormone (34).

Stimulation of cell growth by ALLN was not observed in ras-NIH cells (Fig. 7). Assuming that ALLN enhanced cell growth by suppression of calpain-mediated degradation of PKCϵ, why inhibition of calpain by ALLN caused no effect on ras-transformed cells? Two explanations could be proposed; first, calpain activity is almost completely suppressed in ras-NIH cells; second, PKCϵ activity is fully activated in ras-NIH cells. The latter possibility may be plausible since dominant negative PKCϵ-transfected cells recovered the response to ALLN and showed enhanced cell growth in the presence of ALLN (Fig. 7, Table 1). The recovery of response to ALLN implies that calpain activity is not completely suppressed in ras-NIH cells. Consistently, forced expression of μ- or m-calpain large subunit alone did not induce phenotypic reversion (T. Hiwasa, unpublished observation).

Then, how PKCϵ is related to Ras? Phosphatidylinositol 3-kinase (PI3K) is one of Ras effectors and is activated by binding to a GTP-bound form of Ras (35). Activation of PI3K results in enhanced production of phosphatidylinositol-3,4-bisphosphate (PIP_2) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which, in turn, work as ligands not only for PDK1 and Akt (36) but also for PKCϵ and PKCη (37). Induction of phenotypic reversion of ras-NIH cells by a dominant negative form of PKCϵ (Fig. 6C) suggests that the pathway of Ras → PI3K → PIP_2/PIP_3 → PKCϵ is necessary for maintenance of the transformed state. This is compatible with the report that transgenic mice overexpressing PKCϵ exhibited enhanced carcinoma formation (38). Thus, elevated activity of PKCϵ might induce some of tumor-specific phenotypes but not simply stimulate cell growth.

Based on the present results, it may be possible to propose the target of cancer chemotherapy. Calpain can degrade multiple substrates and promote or suppress malignant transformation depending on the substrates. PKCs are predominant targets of calpain and cause a variety of biological effects depending on the subtypes (39). The present study implies that PKCϵ plays a key role as a downstream target of calpain in promotion and maintenance of malignant transformation. Consequently, a selective inhibitor of PKCϵ, if any, may be a promising anticancer drug with little unexpected side effect.

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