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Evidence for Posttranscriptional Regulation of Transgenic Protein Kinase C–Alpha in T Cells

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Abstract Recently, we succeeded in establishing a transgenic mouse line which expressed high levels of protein kinase C (PKC)–alpha in thymocytes at the mRNA level with disproportionately small increases at the protein level. The transgenic PKC-alpha was nevertheless functionally active for inducing accelerated cell growth and IL-2 production by stimulation with anti-receptor (CD3) antibody or phorbol 12-myristate 14-acetate (PMA) in vitro. Study of the dynamics of transgenic PKC-alpha in the cells in vitro showed that the amount of PKC-alpha protein increased in the cells remarkably at ≥ 5 h after stimulation, whereas the level of PKC-alpha mRNA did not change significantly or changed slightly. This suggested that cell activation breaks the posttranscriptional regulation of the transgenic PKC-alpha in resting cells. The increase in PKC-alpha protein accompanied a prolonged membrane translocation of PKC-alpha and enhanced cell proliferation. Such a transgenic effect was inhibited completely by a PKC inhibitor, H-7, added during 0–6 h after the stimulation. These results show formally that the transgenic PKC-alpha whose production was accelerated through cell activation plays a key role in the late (for ≥ 5 h) signal delivery for disregulated cell growth.

Key words: protein kinase C, transgenic mouse, dynamics after activation, prolonged membrane translocation, disregulation of late signaling

Protein kinase C (PKC), a family of ubiquitously expressed calcium/phospholipid-dependent serine/threonine kinases consisting of at least 8 isozymes (α, βI, βII, γ, δ, ε, ξ, ζ), is believed to play a key role at several grounds in signal delivery for cell activation [Nishizuka, 1986, 1988; Osada et al., 1990; Tsutsumi et al., 1993]. Overproduction of PKC in the cell transfected with PKC genes actually causes disregulated cell growth and may lead to cell immortalization in vitro [Persons et al., 1988; Housey et al., 1988; Finn et al., 1991]. Whereas PKC is activated by agonists for translocation to the membrane, the PKC is downregulated thereafter at both protein and mRNA levels [Tsutsumi et al., 1993; Strulovici et al., 1991; Freire-Moar et al., 1991]. However, the dynamics of production and activation of PKC in vivo and in vitro, in relation to the signal transduction for normal and disregulated cell growth, still remain to be determined.

Recently, we have established a transgenic mouse line carrying a rabbit PKC alpha cDNA guided by the human CD2 promoter/enhancer [Iwamoto et al., 1992]. The overall amount of the PKC-alpha protein in the transgenic thymocytes was only slightly more than that in normal ones. This result suggested that the transgenic PKC-alpha is regulated in vivo at the posttranscriptional level. This observation corresponded with the apparently normal development of the transgene-bearing thymocytes but did not explain well the mechanism of enhanced growth and IL-2 production of the thymocytes in vitro in response to anti-receptor (CD3) antibody and phorbol 12-myristate 14-acetate (PMA). We here provide new evidence of a cell activation-linked break of the regulation of the transgenic PKC-alpha at the posttranscriptional level. Further experiments have shown that the transgenic PKC-alpha protein whose production and membrane translocation are promoted through the activation-linked break of posttranscriptional regulation plays a key role in the late signal delivery for disregulated cell proliferation.

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 14-acetate.

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MATERIALS AND METHODS

Animals and Cells

The PKC-alpha transgenic mouse line, which was previously produced by microinjecting a 14 kb ClaI-XbaI fragment from the rabbit PKC-alpha cDNA inserted into the artificial EcoRI site at 5′ untranslated region of CD2B (pCD2/PKC) into fertilized BCF_1 (C57BL/6 × BALB/c) × BALB/c mouse eggs [Iwamoto et al., 1992], was maintained by successive breeding; the transmission of the transgene into the offspring was confirmed with individual mice by examining their tail DNA. Normal BCF_1 or C57BL/6 mice were used as control.

Single cell suspensions of thymocytes in medium were prepared as described [Iwamoto et al., 1992]. For analyses by Western and Northern blots and fluorescent antibody technique, they were incubated in the presence or absence of anti-CD3 mAb (145-2C11; donated by Dr. T. Takahashi, Aichi Cancer Center, Nagoya) or PMA (Sigma, St. Louis, MO) in 24 well plates containing 2 ml of RPMI medium supplemented with 5% FCS per well for 5 min to 24 h.

Western Blot Analysis

Cells were suspended in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM [Ethylene-bis-oxyethylenenitrilo] tetraacetic acid, 1 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol containing fresh 50 uM phenylmethylsulfonyl fluoride (Sigma), 100 μg/ml leupeptin (Peptide Institute, Osaka, Japan), 500 μg/ml trypsin inhibitor (Sigma), and 500 μg/ml pepstatin A (Peptide Institute) and were solubilized by sonication (Tomy Seiko, Tokyo, Japan). For some experiments the fractions containing cytosolic and membrane-bound PKC were obtained by centrifugation at 100,000*g* followed by passing through a DEAE-cellulose column [Iwamoto et al., 1992]. Samples were electrophoresed through sodium dodecyl sulfate/polyacrylamide (8–10%) gels and electrophoretically transferred to a nitrocellulose membrane. Immunoreactive proteins were visualized by adding mAb, clone MC-3a, which specifically reacted with PKC-alpha [Hidaka et al., 1988], and horseradish peroxidase–labeled goat antimouse IgG, as described [Iwamoto et al., 1992].

Confocus Laser Microscopy and Laser Flow Cytometry

Membrane-translocated activated PKC was previously demonstrated by fluorescence antibody technique on fixed and detergent-treated cells [Diaz-Laviada et al., 1991]. We applied this technique to confocus laser microscopy and laser flow cytometry. Thymocytes (1×10^7) harvested from the culture were fixed with 4% paraformaldehyde for 30 min at room temperature and then treated with 0.2% Triton X-100 at 4°C for 3 min. They were washed three times with medium and were reacted with anti-PKC-alpha mAb (10 μg/ml) at 4°C for 40 min followed by three times washing and subsequent staining with FITC-labelled antimouse Ig goat antibody (Tago, Burlingham, CA). The stained cells were first observed under a confocus laser microscope (Bio-Lad, Richmond, CA) and were then examined on a laser flow cytometer (Coulter, Hialeah, FL), measuring the level of fluorescence (log) of 5,000 cells.

Northern Blot Analysis

Total cellular RNA was prepared by a single-step method [Iwamoto et al., 1992]. Ten micrograms of RNA was electrophoresed onto 1% (w/v) agarose gels containing 0.66 M formaldehyde and blotted to nylon membrane (Hybond N). The DNA probe used in this study was a SacI/EcoRI fragment of PKC-alpha cDNA, which was rabbit PKC-alpha transgene-specific [Iwamoto et al., 1992]. Prehybridization, hybridization, and washes were performed under the condition as described [Iwamoto et al., 1992].

Measurement by Densitometry

In order to compare individual protein/RNA bands developed by Western and Northern blots, we measured their density by use of a dual wavelength flying-spot scanner (Shimazu, Kyoto, Japan). Percent change of the value from the appropriate control was calculated for convenience, although the measured density might not lineally correlate to the protein/RNA amount.

Assessment of Cell Proliferation

Cells suspended in RPMI tissue culture medium supplemented with 10% FCS but without additional 2-mercaptoethanol (10^6 cells/200 μl) were cultured in 96-well plates added with agonist for 48 h. [^{3}H]thymidine (37 kBq/well) was added for an additional 8 h. After harvesting the cells on filter paper the radioactivity (cpm) was determined by a liquid scintillation counter. To confirm the functional role of transgenic PKC-

alpha in cell proliferation, H-7 as a PKC inhibitor [Kawamoto and Hidaka, 1984] or HA1004 as a control drug of H-7 [Asano and Hidaka, 1984] was added to the culture.

RESULTS

Cell Activation Breaks the Posttranscriptional Regulation of Transgenic PKC-Alpha

We first measured the overall protein amounts of PKC-alpha in thymocytes from PKC-alpha transgeneic mice and from normal control mice, which were stimulated in vitro with anti-CD3 mAb (Fig. 1A) or PMA (Fig. 1B) for 5 min to 24 h. Corresponding to the previous result, despite a great difference (approximately 3 times more in transgenic cells than in normal cells) in the level of mRNA [Iwamoto et al., 1992], the difference in the total amount of PKC-alpha between transgenic and normal thymocytes was minimum at 5 min after stimulation. To our surprise, however, the amount of PKC-alpha greatly increased (approximately 3–7 times) in the transgenic cells stimulated with anti-CD3 or PMA for 5–24 h. On the other hand, in normal control thymocytes the amount of PKC-alpha barely changed for 5 min to 12 h and decreased to $< 50\%$ of the initial amount at 24 h after stimulation. In addition, the amount of PKC-alpha did not change significantly after 5 h incubation in no stimulation controls (not shown). All of these results, which were confirmed by experiments repeated three times, suggest an activation-linked break of the regulation of PKC-alpha.

We next measured the amounts of PKC-alpha in subfractions of the cells stimulated with agonist. As shown in Figure 2, which representatively presents the data of thymocytes stimu-

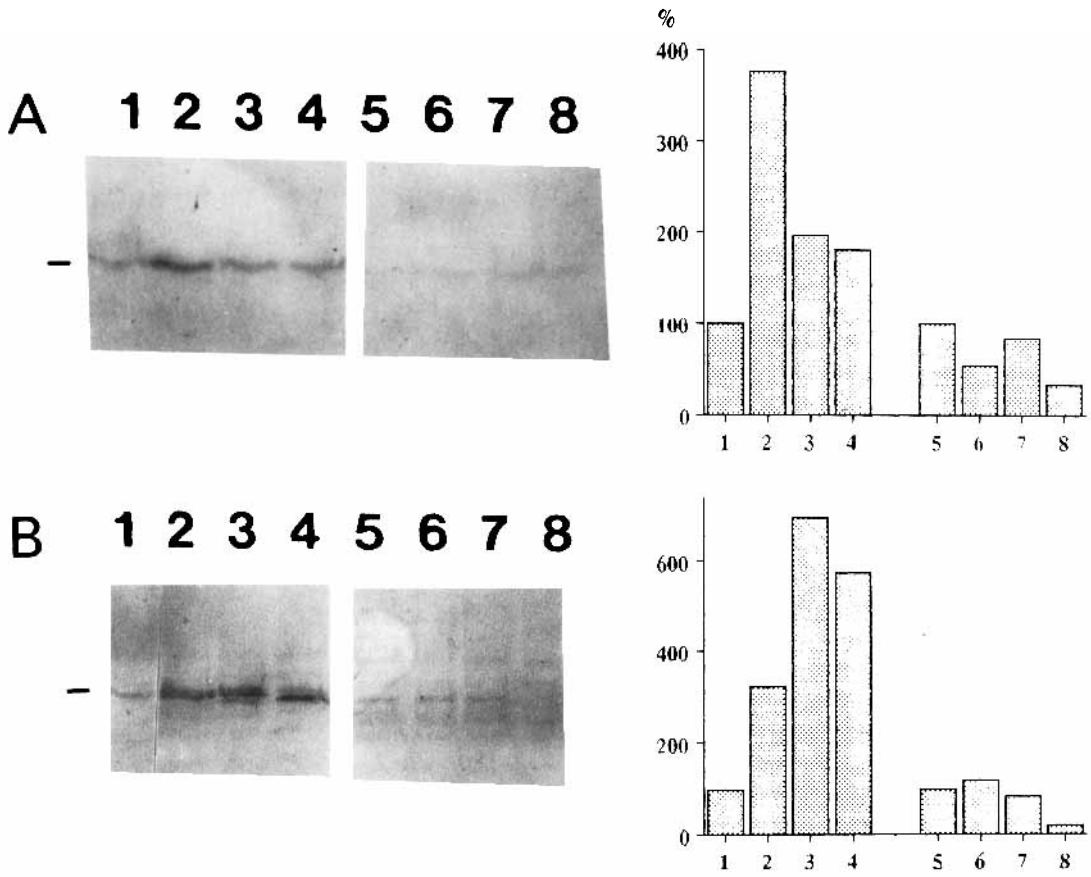

Fig. 1. Cell activation-linked break of the regulation of transgenic PKC-alpha at the protein level. Thymocytes from PKC-alpha transgenic mice (**lanes 1–4**) and normal control mice (***lanes 5–8***) were stimulated in vitro with anti-CD3 mAb (ascites 1:1,000) (**A**) or PMA (10 ng/ml) (**B**) for 5 min (lanes 1, 5), 5 h (lanes 2, 6), 12 h (lanes 3, 7), or 24 h (lanes 4, 8). The cells harvested were lysed and separated by SDS-PAGE (8%) followed by immunoblot analysis of PKC-alpha. The position of PKC (80 kDa) is indicated by the horizontal line. The density of the band for PKC-alpha was measured, and % density of each lane againt lane 1 (from lane 2 to lane 4) or lane 5 (from lane 6 to lane 8) was presented (right).

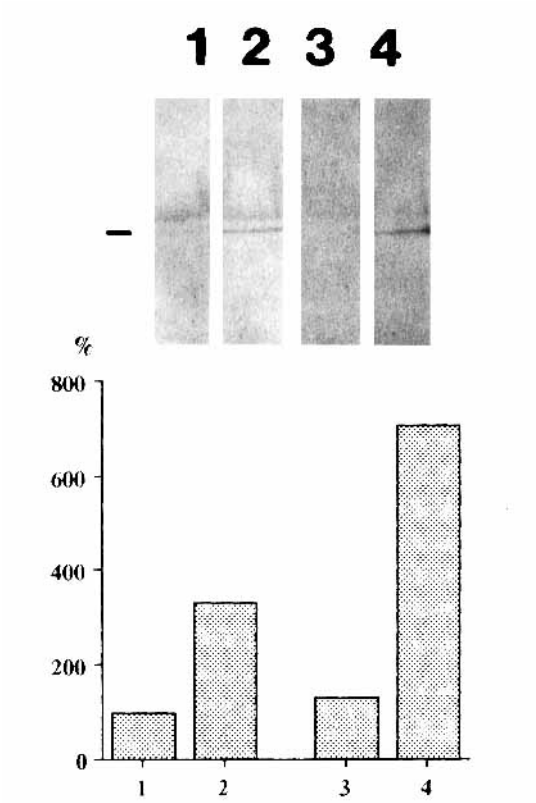

Fig. 2. PKC-alpha in both cytosol and membrane fractions from transgenic cells increased after cell activation. Thymocytes from normal control mice (**lanes 1, 3**) and PKC-alpha transgenic mice (**lanes 2, 4**) were stimulated in vitro with anti-CD3 mAb (1:1,000) for 5 h. The cells harvested were lysed, and their cytosol (lanes 1, 2) and membrane (lanes 3, 4) fractions were separated by SDS-PAGE (10% gel) followed by immunoblot analysis of the PKC-alpha. The position of PKC (80 kDa) is indicated by horizontal line. In this figure, an additional nonspecific band is seen in all lanes at a higher position than that of 80 kDa. Density of the band for PKC-alpha was measured, and % density of each lane against lane 1 was presented (bottom).

lated with anti-CD3 mAb for 5 h, the amounts of both cytosolic (lane 2) and membrane-bound (lane 4) PKC-alpha in transgenic cells were much greater than those (lanes 1 and 3) in normal cells. This suggested that the activation-linked break of the regulation of PKC-alpha implied the promotion of both production and membrane translocation.

A question arose as to whether the observed phenomenon would include acceleration of transcription of the transgene. Northern blot analysis of PKC-alpha mRNA in the transgenic cells showed that the level of the transgene transcription did not change significantly during 1–4 h stimulation with anti-CD3 antibody, although it slightly increased in the cells stimulated with PMA for 4 h (Fig. 3). This result suggests that the increase in PKC-alpha after stimulation is mainly, if not exclusively, at a posttranscriptional level.

Dynamics of Membrane Translocation of Transgenic PKC-alpha After Stimulation

Confocus laser microscopy on fluorescent antibody-labelled cells demonstrated PKC-alpha protein as diffused granules on cell membranes (representative pictures are shown in Fig. 4). This suggested that the fluorescence antibody technique reveals only membrane-associated PKC-alpha. We next quantitated the membrane translocated PKC-alpha in individual cells by laser flow cytometry. Before stimulation (peak 1 in Fig. 5) or 5 min after stimulation (not shown), all normal and PKC-alpha transgenic thymocytes expressed PKC-alpha on cell membranes at low levels with some variation among individual cells, and the overall expression level on transgenic cells was only slightly more than that on normal controls. One hour after stimulation with PMA, the level of expression of PKC-alpha increased on cell membranes of both transgenic and normal thymocytes (peak 2). However, the increase in the former was much more extensive than that in the latter. In all transgenic thymocytes, a high level of membrane expression of PKC-alpha was maintained for 5 h (peak 3 in Fig. 4), and the level at 12 h (peak 4) was still higher than that before stimulation in some though not all cells. These results corresponded partially to the data presented in Figure 2 that demonstrated increment of PKC-alpha in both membrane and cytosolic fractions of cell lysates of whole population by Western blot analysis.

Evidence of Involvement of Disregulated Transgenic PKC-Alpha in Enhanced Cell Growth

We previously showed that IL-2 production was augmented most evidently in the thymocytes stimulated with anti-CD3 mAb coupled with PMA. Under a slightly modified culture condition, we could also demonstrate a definite transgene effect to accelerate proliferation of thymocytes in response to anti-CD3 mAb plus PMA. To elucidate the role of the disregulated PKC-alpha developing as a result of a break of the negative regulation of the signal transduction, we added H-7 prior to anti-CD3 mAb plus PMA. The transgene-linked promotion of cell proliferation was partially prevented by 2–10 μM H-7 and was completely prevented by 50–200 μM H-7 (data not shown). Any concentrations of H-7 we used did not change cell viability,

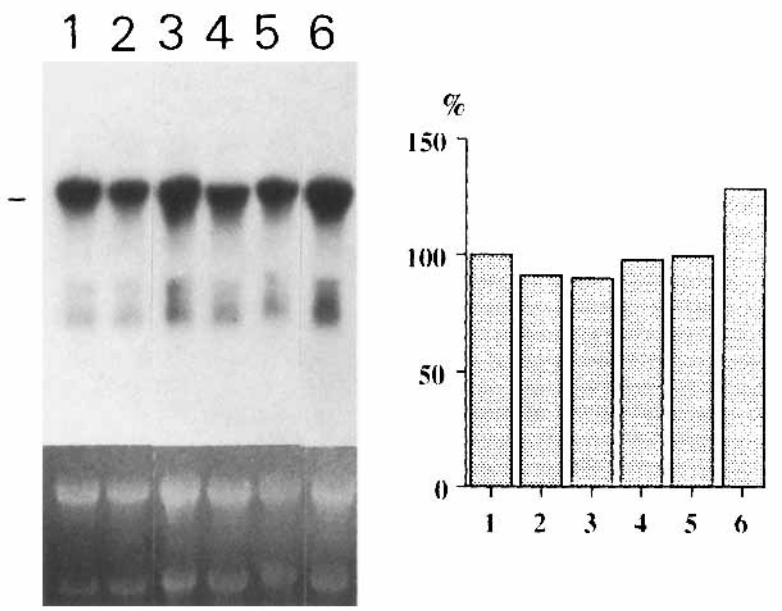

Fig. 3. Lack of remarkable up or down regulation of the transgenic PKC-alpha at the mRNA level. Thymocytes from PKC-alpha transgenic mice were stimulated with anti-CD3 mAb (1:1,000) (**lanes 2, 5**) or PMA (10 ng/ml) (**lanes 3, 6**) for 1 h (lanes 1–3) or 4 h (lanes 4–6). **Lanes 1, 4:** No stimulation controls. The cells harvested were lysed for extraction of RNA followed by Northern blot analysis of the PKC-alpha mRNA. The position of the transcript of PKC-alpha (4.0 kb) is indicated by horizontal line. The picture of ethidium bromide staining of 28 S and 18 S ribosomal RNA is shown below for comparison of the amount of RNA loaded. Density of the band for PKC-alpha mRNA was measured and corrected for the variation of the loaded RNA, and % density of each lane against lane 1 was presented (right).

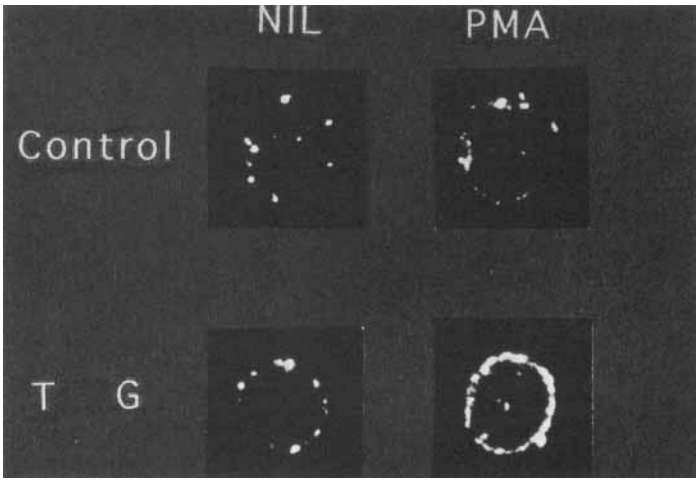

Fig. 4. Demonstration of membrane translocated PKC-alpha by confocus laser microscopy. Thymocytes from normal control mice and PKC-alpha transgenic mice (T G) were stimulated with PMA (10 ng/ml) for 1 h. These cells and unstimulated cells (NIL) were fixed and detergent-treated. They were then stained with anti-PKC-alpha mAb and FITC-labelled antimouse Ig goat antibody for analysis by confocus laser microscopy. Photographs were taken every 0.6 μm for a number of cells, but that for the center of a single cell for each group is shown as representive.

as determined by dye exclusion testing. We next tested the effect of addition of H-7 (40 μM) at different times on proliferation of PKC-alpha transgenic thymocytes in response to anti-CD3 mAb plus PMA. As shown in Figure 6, addition of H-7 during 0–6 h after the start of culture almost completely inhibited the transgene-mediated acceleration of cell growth, and addition at 12 h (not shown) or 18 h partially did so. Such inhibition was never observed when HA1004

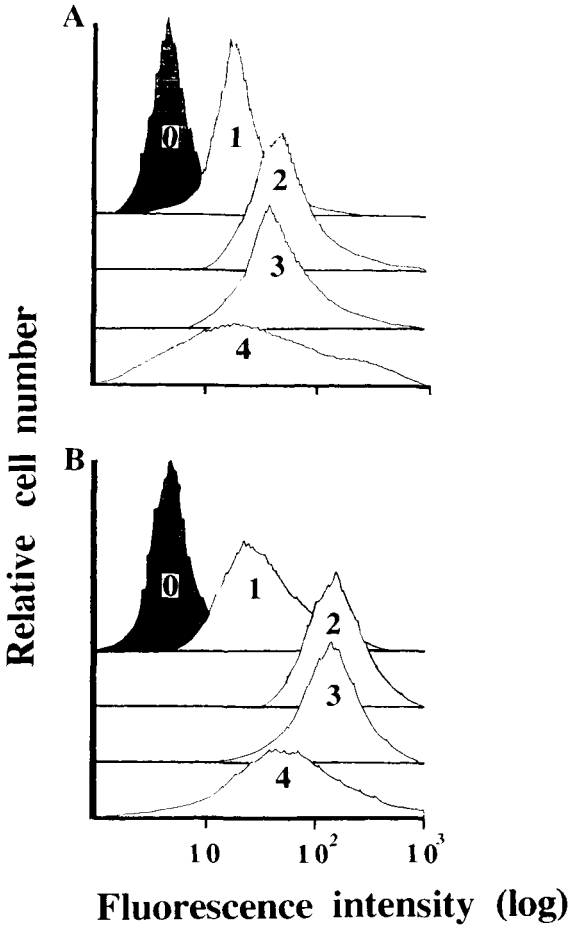

Fig. 5. Measurement of membrane-translocated activated PKC-alpha by laser flow cytometry. Thymocytes from normal control mice (**A**) and PKC-alpha transgenic mice (**B**) were stimulated with PMA (10 ng/ml) for 1 h (peak 2), 5 h (peak 3), or 12 h (peak 4). These cells and unstimulated cells (peak 1) were fixed and detergent-treated. They were then stained with anti-PKC-alpha mAb and FITC-labelled antimouse Ig goat antibody for analysis by laser flow cytometry. Shaded peaks (peak 0) show no stained (the second antibody only) controls. Shown is a representative of five experiments.

was added as a control drug for H-7, which confirmed the specificity of action of H-7. The proliferation of normal cells in response to anti-CD3 mAb plus PMA, which was low, was also inhibited by H-7 added during 0–6 h after stimulation, though much less evidently. These results demonstrate that transgene-mediated accelerated cell growth indispensably requires the action of H-7–sensitive activated PKC at a time even later than 6 h after the start of culture and correspond to the data of membrane translocation of the transgenic PKC-alpha for a longer time than 5 h after stimulation (see Fig. 5).

DISCUSSION

This study has demonstrated that stimulation of PKC-alpha transgenic thymocytes with anti-CD3 or PMA breaks the regulation of the transgene at the posttranscriptional level. The observed alteration in PKC-alpha protein expression in the transgenic cells after stimulation might be due to an alteration in the rate of translation. However, it could as well result from an altered rate of degradation of the membrane-associated PKC by calpain [Kishimoto et al., 1989]. Presently, we have no evidence for or against the alternative views. The molecular mechanism of the cell activation-linked potential alteration of the translation rate or the calpain action is not known. However, some metabolic event linked to the cell cycle transition after stimulation, potentially including the action of activated PKC itself, would be responsible. This might further suggest a mechanism of feedback positive regulation by activated PKC.

The cell activation-linked break of the posttranscriptional regulation of transgenic PKC-alpha accompanied extensive and prolonged membrane translocation/activation of PKC-alpha (Figs. 2, 4, 5). The latter accompanied accelerated cell growth, which was closely linked to the prolonged membrane translocation of PKC-alpha, whose activity was completely inhibited by H-7. This explained the reason the transgenic PKC-alpha that was only marginally expressed at the protein level in unstimulated cells was functionally active for promoting cell growth and IL-2 production in response to agonists [Iwamoto et al., 1992]. Our observation in turn elucidated a crucial function of membrane translocated PKC-alpha in the long process (for ≥ 5 h) of signal transduction for cell activation. This function was previously suggested for phorbol-activated PKC in normal cells, with a question under debate on the specificity of the phorbol action in these cells [Weiss et al., 1987; Berry et al., 1990].

Earlier studies on cell lines transfected with PKC genes showed that the transfected PKC genes induced overproduction of PKC proteins accompanied by disordered growth control [Persons et al., 1988; Housey et al., 1988] and cell immortalization [Finn et al., 1991]. These cell lines were already activated for cell growth before gene transfection and were not subjected to the regulatory mechanism that would work in

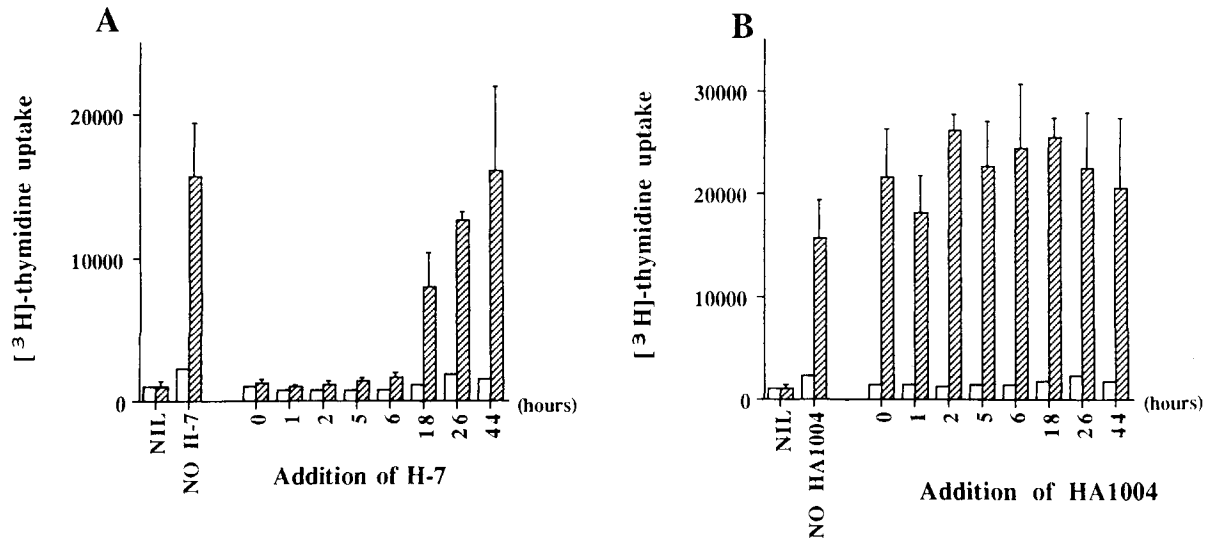

Fig. 6. Time-dependent requirements of activated transgenic PKC-alpha in the late signal delivery for accelerated thymocyte proliferation. Thymocytes from normal control mice (open columns) and PKC-alpha transgenic mice (shaded columns) were stimulated with anti-CD3 mAb (1:1,000) plus PMA (10 ng/ml) for 48 h, when [^{3}H]thymidine uptake was assayed. H-7 or HA1004 (40 μM) was added at the indicated time after the start of culture. NIL, no agonist stimulation control; no H-7/no HA1004, agonist stimulation only control. Each column shows the mean of triplicate cultures with S.D. Difference of values between no H-7 culture and culture added with H-7 at 0–18 h for transgenic cells was statistically significant. Shown is a representative of three experiments.

normal cells. Our transgenic model suggested a solid regulatory mechanism that protects the cell from overproduction of proteins at the resting stage, even when the level of mRNA is disregulated. Such a regulatory mechanism, which is lacking in already activated cell lines, might work as a physiological mechanism of protection against trivial usage of aberrant genes during ontogenic and was probably responsible for apparently normal ontogenic development of thymocytes bearing the PKC-alpha transgene [Iwamoto et al., 1992]. Our present results showed that once cells are activated by agonists, the regulatory mechanism is broken for extensive usage of the aberrant genes. This activation-linked break of normal regulation could therefore be a step towards growth disregulation by aberrant signal transmitting genes or oncogenes. Study is now in progress on what would happen in the PKC-alpha transgenic mice during aging when they might meet with activators from the environment. We are also investigating mice receiving agonists such as PMA as known tumor-promoting agents affecting PKC [Housey et al., 1988].

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REFERENCES

Asano T, Hidaka H (1984): Vasodilatory action of HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide], a novel calcium antagonist with no effect on cardiac function. J Pharmacol Exp Ther 231:141–145.

Berry N, Ase K, Kishimoto A, Nishizuka Y (1990): Activation of resting human T cells requires prolonged stimulation of protein kinase C. Proc Natl Acad Sci USA 87:2294–2298.

Diaz-Laviada I, Larrodera P, Nieto JL, Cornet ME, Diaz-Meco MT, Sanchez MJ, Guddal P, Johansen T, Haro A, Moscat J (1991): Mechanism of inhibition of adenylate cyclase by phospholipase C–catalyzed hydrolysis of phosphatidylcholine. Involvement of a pertussis toxin–sensitive G protein and protein kincase C. J Biol Chem 266: 1170–1176.

Finn OJ, Persons DA, Bendt KM, Pirami L, Ricciardi P (1991): Retroviral transduction of protein kinase C-γ into cytotoxic T lymphocyte clones lead to immortalization with retention of specific function. J Immunol 146:1099–1103.

Freire-Moar J, Cherwinski H, Hwang F, Ranson J, Webb D (1991): Expression of protein kinase C isoenzymes in thymocyte subpopulations and their differential regulation. J Immunol 147:405–409.

Hidaka H, Tanaka T, Onoda K, Hagiwara M, Watanabe M, Ohta H, Ito Y, Tsurudome M, Yoshida T (1988): Cell type–specific expression of protein kinase C isozymes in the rabbit cerebellum. J Biol Chem 263:4523–4526.

Housey GM, Johnson MD, Hsiao WLW, O'Brian CA, Murphy JP, Kirschmeier P, Weinstein JB (1988): Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. Cell 52:343–354.

Iwamoto T, Hagiwara M, Hidaka H, Isomura T, Kioussis D, Nakashima I (1992): Accelerated proliferation and interleukin-2 production of thymocytes by stimulation of soluble anti-CD3 monoclonal antibody in transgenic mice carrying a rabbit protein kinase Can J Biol Chem 267:18644–18648.

Kawamoto S, Hidaka H (1984): 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem Biophys Res Commun 125:258–264.

Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, Kuroda T, Nishizuka Y (1989): Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem 264: 4088–4092.

Nishizuka Y (1986): Studies and perspectives of protein kinase C. Science 233:305–312.

Nishizuka Y (1988): The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665.

Osada SK, Mizuno K, Saido TC, Akita Y, Suzuki K, Kuroki T, Ohno S (1990): A phorbol ester receptor/protein kinase, nPKCeta, a new member of the protein kinase–C family predominatly expressed in lung and skin. J Biol Chem 265:22434–22440.

Persons DA, Wilkison WO, Bell RM, Finn OJ (1988): Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-1 cDNA. Cell 52:447–458.

Strulovici B, Daniel-Issakani S, Baxter G, Knopf J, Sultzman L, Cherwinski H, Nestor J Jr, Webb DR, Ransom J (1991): Distinct mechanisms of regulation of protein kinase Ce by homones and phosbol diesters. J Biol Chem 266:168–173.

Tsutsumi A, Kubo M, Fujii H, Freire-Moar J, Turck CW, Ransom T (1993): Regulation of protein kinase C isoform proteins in phorbol ester–stimulated Jurkat T lymphoma cells. J Immunol 150:1746–1754.

Weiss A, Shields R, Newton M, Manger B, Imboden J (1987): Ligand-receptor interactions required for commitment to the activation of the interleukin 2 gene. J Immunol 138: 2169–2176.