Volume 292, number 1,2, 57–60 FEBS 10316 November 1991

ADONIS 001457939101025K

Involvement of α- and β-PKC in the differentiation of 3T3-L1 cells

Hiroo Ueda[1], Yoshihiko Ishida[2] and Hiroshi Taniguchi[1]

[1]*Second Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan* and [2]*Division of Endocrinology and Metabolism, Department of Medicine, Hyogo Medical Center for Adults, Akashi, Japan*

Received 21 August 1991

Present investigation revealed that 3T3-L1 cells contained two protein kinase C (PKC) subspecies, i.e. α- and β-PKC. These cells treated by staurosporine, a specific inhibitor of PKC, differentiated to adipocytes more remarkably than those without its treatment. Their α- and β-PKC activity decreased to 49% and 18% of the staurosporine-untreated cells respectively, and GPDH activity, a marker enzyme of adipocytes, increased to 143%. Thus, both α- and β-PKC seemed to be associated with the adipose conversion in 3T3-L1 cells.

Differentiation; 3T3-L1 cell; Protein kinase C subspecies; Staurosporine

1. INTRODUCTION

Protein kinase C (PKC) plays an important role in various cellular functions including proliferation and differentiation, and consists of several subspecies [1–6]. They have been shown to exist in many cells and tissues at various combinations and to be associated with differentiation in a certain type of cells [3,4]. 3T3-L1 cells can be induced to differentiate to adipocytes by various agents, i.e. 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin and dihydroxyvitamin D_3 [7–9]. However, in 3T3-L1 cells the subspecies of PKC had not been identified and their role in the differentiation has not been investigated.

In the present investigation we report the identification of PKC subspecies in 3T3-L1 cells, using antibodies against a subspecies-specific sequence. Furthermore, we studied the involvement of PKC subspecies to adipose conversion of 3T3-L1 cells both morphologically and biochemically through PKC inhibition by treatment with staurosporine, which is a specific inhibitor of PKC [10] and induces cellular proliferation [11] and differentiation [12].

Abbreviations: protein kinase C(PKC), Ca^{2+}/phospholipid-dependent protein kinase; IBMX, 3-isobutyl-1-methylxanthine; GPDH, glycerol-3-phosphate dehydrogenase; PMSF, phenylmethylsulfonylfluoride; PS, phosphatidylserine; DG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(aminoethylether)tetraacetic acid; MBP_{4-14}, synthetic myelin basic protein.

Correspondence address: H. Ueda, Second Department of Internal Medicine, Kobe University School of Medicine, 5-1, Kusunoki-cho 7-chome, Chuo-ku, Kobe 650, Japan. Fax: (81) (78) 382-2080.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Staurosporine was donated by Kyowa Hakko Co., Tokyo, Japan. 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, leupeptin and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylserine (PS) and diacylglycerol (DG) were from Serdary Research Laboratories, London, Ontario, Canada. Dulbecco's modified Eagle's medium (DMEM) was from Nissui Co., Tokyo, Japan. Fetal calf serum (FCS) was from Bioproducts, (Walkersville, MD). Penicillin-streptomycin and L-glutamine were from Flow Laboratories, Irvine, Scotland. [γ-^{32}P]ATP was from Du Pont/NEN Research Products, (Boston, MA).

2.2. *Cells and culture*

3T3-L1 cells were a generous gift of Japanese Cancer Research Resources Bank (JCRB)-Cell, Tokyo, Japan. They were routinely grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere.

2.3. *Assessment of differentiation*

The differentiation to mature adipocytes was induced by the addition of 0.5 mM IBMX and 0.25 μM dexamethasone to confluent 3T3-L1 preadipocytes. In 48 hours, medium was substituted with above-mentioned fresh medium without IBMX and dexamethasone. Adipose conversion was determined morphologically by the appearance of fat droplets in the cytoplasm and was quantified by the measurement of glycerol-3-phosphate dehydrogenase (GPDH), a marker enzyme of adipocytes [9,13].

2.4. *Glycerol-3-phosphate dehydrogenase assay*

GPDH assay was carried out by the method of Kozak and Jensen [14] with our modifications described elsewhere [9]. One unit of enzyme activity was defined as the oxidation of 1.0 μmol of NADH per min per mg protein. Protein concentration was determined by Lowry's method [15].

2.5. *Separation and assay of protein kinase C*

All procedures were performed at 0–4°C. 3T3-L1 cells (1.0×10^7) were suspended in 10 ml of 20 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 1.0 mM EDTA, 3.0 mM EGTA, 1.0 mM PMSF and 0.4 mM leupeptin. The cells were lysed by sonication, and centrifuged for 60 min at 100 000 × g. The supernatant was applied to a DE-52 column

(Whatman) equilibrated beforehand with buffer A consisting of 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 2-mercaptoethanol in 20 mM Tris-HCl (pH 7.5). After washing the column with 40 ml buffer A, PKC was eluted by 10 ml buffer A containing 0.5 M NaCl. The enzyme fraction was applied to a hydroxyapatite column (Koken Co., Tokyo, Japan) connected to an FPLC system and equilibrated beforehand with buffer B consisting of 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol in 20 mM potassium phosphate (pH 7.5). PKC subspecies were eluted by increasing the concentration of potassium phosphate linearly from 20 to 200 mM at a flow rate of 0.4 ml/min. Fractions of 1.0 ml were collected. PKC activity in cytosolic fractions was determined by the incorporation of ^{32}P into oligopeptide MBP_{4-14} (QKRPSQRSKYL) from [γ-^{32}P]ATP for 15 min at 30°C, and indicated as nmol phosphate incorporation per min per mg cytosolic protein. A reaction mixture of 50 μl for the assay of PKC contained tissue extracts (6 μl) along with 20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 10 μM of [γ-^{32}P]ATP (20–30 cpm/nmol), 8 μg/ml PS, 0.8 μg/ml DG, 65 μM $CaCl_2$ and 25 μM MBP_{4-14}.

2.6. *Immunoblotting*

The polyclonal antibodies were prepared against the sequence-specific oligopeptides to α-, β- and γ-PKC as well as the kinase domain specific oligopeptides [16].

3. RESULTS

3.1. *Isolation of PKC subspecies in 3T3-L1 cells*

The soluble cytoplasmic fraction of pre-differentiated 3T3-L1 cells was subjected to hydroxyapatite column chromatography using FPLC system as described above (Fig. 1). Two major peaks of the activity were detected at the fraction number 36–42 (peak 1) and 52–57 (peak 2). Furthermore, the PKC subspecies of peak 1 and 2 were determined to be β- and α-PKC, respectively, by immunoblotting (Fig. 2). γ-PKC was not found in 3T3-L1 cells and the other subspecies were not studied in the present investigation.

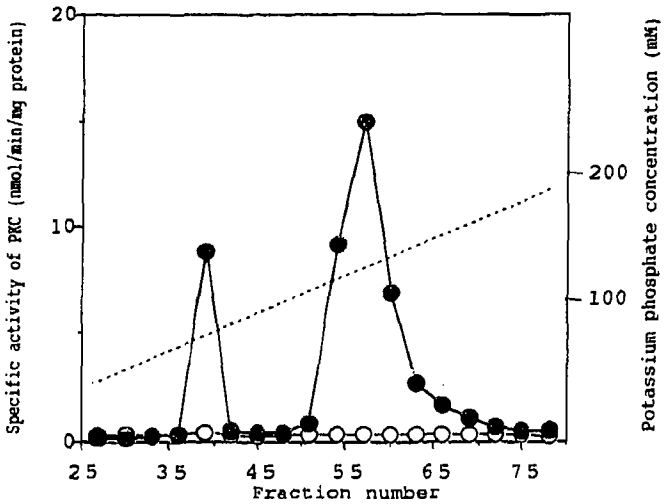

Fig. 1. Specific activity of protein kinase C of pre-differentiated 3T3-L1 cells. DE-52-purified PKC was fractionated on a hydroxyapatite column chromatography and assayed as described in section 2.5. PKC activity was assayed in the presence of 8 μg/ml PS, 0.8 μg/ml DG and 65 μM $CaCl_2$ (●), while that for the control in the presence of 5 mM EGTA (○).

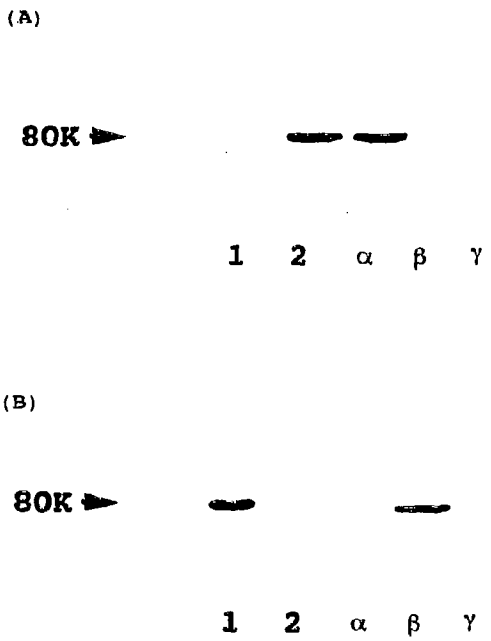

Fig. 2. PKC subspecies of 3T3-L1 cells by immunoblotting. Panel (A) and (B) are an immunoblotting by polyclonal antibody against α- and β-PKC, respectively. Fractions of peak 1 and 2 shown in Fig. 1 were dialysed and lyophilysed. 100 ng of this lyophilysate from peak 1 and 2 was applied to lane 1 and 2, respectively. Lane α, β and γ was for 100 ng of α-, β- and γ-PKC from rat brain, respectively.

3.2. *Staurosporine-untreated 3T3-L1 cells*

During differentiation of stuarosporine-untreated 3T3-L1 cells, the activity of cytosolic α-PKC decreased to 85 and 50% of pre-differentiation level of 102.3 nmol/min/mg protein on the 4th and 8th culture day after the induction of differentiation respectively (Fig. 3). On the other hand, the β-PKC activity did not change till the 8th culture day. It was 48 and 41% of pre-differentiation level of 28.3 nmol/min/mg protein on the 4th and the 8th culture day. Morphologically, however, 3T3-L1 cells on the 4th culture day after the induction of differentiation had small lipid-like droplets in the cytoplasm and became slightly rounded in shape compared with pre-differentiated cells which looked like fibroblasts (Fig. 4A). On the 8th culture day, lipid droplets in the cytoplasm became larger and the cells looked more rounded in shape, and, moreover, such cells increased in number (Fig. 4B). The activity of GPDH increased to 155 and 303% of the level of pre-differentiated cells of 41.1 U on the 4th and the 8th culture day, respectively (Fig. 3).

3.3. *Staurosporine-treated 3T3-L1 cells*

The activity of cytosolic α- and β-PKC of 3T3-L1 cells treated with staurosporine decreased to 61 and 28% of the value of pre-differentiated cells, i.e. 102.3 and 28.3 nmol/min/mg protein, on the 4th culture day after the induction of differentiation. Furthermore, they decreased to 25 and 8% on the 8th culture day (Fig. 3). Morphologically, staurosporine-treated 3T3-L1 cells on the 4th culture day after the induction of differentiation

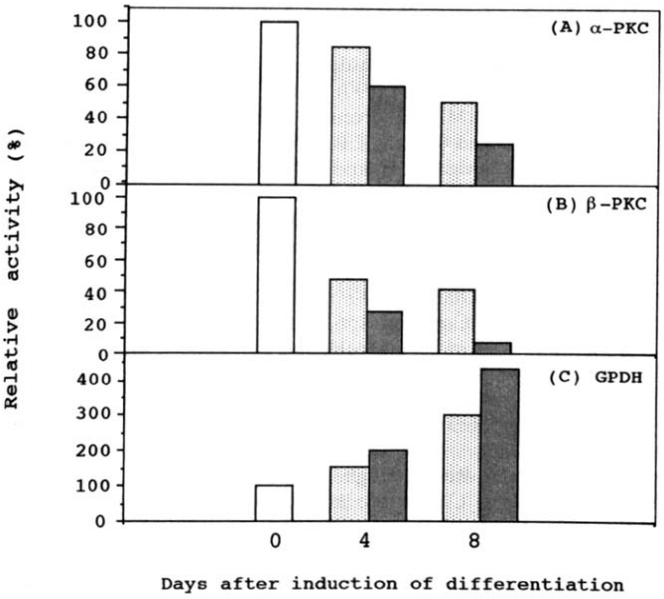

Fig. 3. Relative activity of cytosolic α- (A) and β-PKC (B) and GPDH (C) during differentiation. Activity is shown as % of that of pre-differentiated 3T3-L1 cells. Each column is indicated as follows: □, for pre-differentiated cells; ▨, for staurosporine-untreated cells; ■, for staurosporine-treated cells.

had larger lipid droplets in the cytoplasm and became more rounded in shape than staurosporine-untreated cells. On the 8th culture day, lipid droplets became much larger and the cells much more rounded, but no distinct difference was observed morphologically between staurosporine-treated and untreated cells (Fig. 4C). However, the GPDH activity increased to 204 and 433% of the pre-differentiated cells, i.e. 41.1 U, on the 4th and 8th culture day, respectively (Fig. 3).

4. DISCUSSION

This is the first report to show the presence of α- and β-PKC in 3T3-L1 cells and the decrease of these PKC subspecies might be associated with the potentiation of differentiation of the 3T3-L1 cells to adipocytes. Staurosporine, though first reported to be the most potent inhibitor of PKC [7], was also found to inhibit various protein kinases at a certain range of concentrations [17] and induce cell differentiation in some types of cells [18–20]. The concentration of staurosporine used in the present study seems to be suitable for the potentiation of PKC to differentiate the 3T3-L1 cells [7,21].

In our preliminary study 10–1000 nM of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), an activator of PKC, did not enhance adipose conversion of 3T3-L1 cells. The present study suggested that inhibition rather than activation of PKC appears to be associated with differentiation of 3T3-L1 cells. There are also some reports that other PKC inhibitors enhanced differentiation in neuroblastoma cells [18,19] and HL-60 cells [20].

Subspecies of PKC are suppressed to a different degree by different agents used [22]. In the present study, β-PKC of 3T3-L1 cells was shown to be inhibited more remarkably than α-PKC by staurosporine. Thus, this rate of suppression of PKC subspecies in the presence of β-PKC might be related to the induction of differentiation in these cells.

The inhibition of phosphorylation of a certain

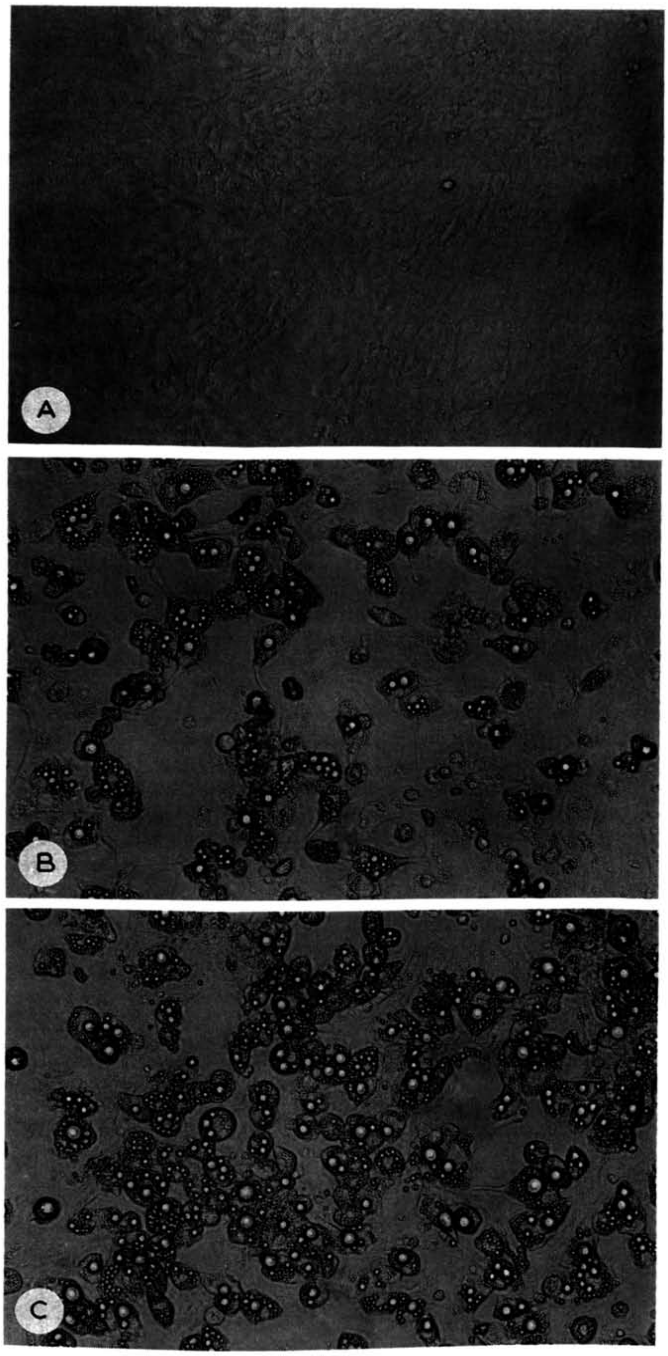

Fig. 4. Morphological change during differentiation of 3T3-L1 cells. Pre-differentiated cells, staurosporine-untreated cells and treated cells on the 8th culture day after induction of differentiation are shown in panel (A), (B) and (C) at a magnification of 312, respectively.

protein in the cell might be a trigger of differentiation through various signaling processes. However, it is not determined that the decrease of PKC activity is a cause or a result of differentiation. In order to clarify this point the investigation on PKC activity in the earlier stage of differentiation is necessary.

Meanwhile, since cyclic-AMP was reported to be involved in the cell differentiation in 3T3-L1 cells [8], it might be suggested that the factor influencing differentiation of 3T3-L1 cells is the ratio between PKC subspecies, between cyclic-AMP dependent protein kinase and PKC, or between other protein kinases and PKC.

In summary, α- and β-PKC are found in 3T3-L1 cells and the decrease of these PKC subspecies might be associated with the potentiation of differentiation of the 3T3-L1 cells to adipocytes.

Acknowledgements: We thank Prof. Yasutomi Nishizuka for his generous and valuable criticism and Dr Hiroaki Aihara, Department of Biochemistry, Kobe University School of Medicine, for his kind technical suggestion about the isolation of PKC and assay of its activity.

REFERENCES

[1] Nishizuka, Y. (1984) Nature 308, 693–698.
[2] Nishizuka, Y. (1986) Science 233, 305–311.
[3] Nishizuka, Y. (1988) Nature 334, 661–665.
[4] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31–34.
[5] Bacher, N., Zisman, Y. and Livneh, E. (1991) Mol. Cell. Biol. 11, 126–133.
[6] Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) J. Biol. Chem. 265, 22434–22440.
[7] Spiegelman, B.M. and Green, H. (1981) Cell 24, 503–510.
[8] Azarnia, R. and Russell, T.R. (1985) J. Cell. Biol. 100, 265–269.
[9] Ishida, Y., Taniguchi, H. and Baba, S. (1988) Biochem. Biophys. Res. Commun. 151, 1122–1127.
[10] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[11] Smith, C.D., Glickman, J.F. and Chang, K-J. (1988) Biochem. Biophys. Res. Commun. 156, 1250–1256.
[12] Okazaki, T., Kato, Y., Mochizuki, T., Tashima, M., Sawada, H. and Uchino, H. (1988) Exp. Hematol. 16, 42–48.
[13] Wise, L.S. and Green, H. (1979) J. Biol. Chem. 254, 273–275.
[14] Kozak, L.P. and Jensen, J.T. (1974) J. Biol. Chem. 249, 7775–7781.
[15] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Kishimoto, A., Saito, N. and Ogita, K. (1991) Methods Enzymol., in press.
[17] Herbert, J.M., Seban, E. and Maffrand, J.P. (1990) Biochem. Biophys. Res. Commun. 171, 189–195.
[18] Minana, M.D., Felipo, V. and Grisolia, S. (1990) Proc. Natl. Acad. Sci. USA 87, 4335–4339.
[19] Hensey, C.E., Boscoboinik, D. and Azzi, A. (1989) FEBS Lett. 258, 156–158.
[20] Taoka, T., Tokuda, M., Tasaka, T., Hatase, O., Irino, S. and Norman, A.W. (1990) Biochem. Biophys. Res. Commun. 170, 1151–1156.
[21] Rueggu, U.T. and Burgess, G.M. (1989) T.I.P.S. 10, 218–220.
[22] Pelosin, J.M., Keramidas, M., Souvignet, C. and Chambaz, E.M. (1990) Biochem. Biophys. Res. Commun. 169, 1040–1048.