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Identification of four lipocortin proteins and phosphorylation of lipocortin I by protein kinase C in cytosols of porcine thyroid cell cultures

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Four proteins of the lipocortin family, lipocortin I (35 kDa), lipocortin II (36 kDa), lipocortin V (32 kDa) and lipocortin VI (67–70 kDa), were identified in the cytosols of 2-day-old cultures of thyroid cells Only lipocortin I was phosphorylated in vitro in fully differentiated, thyroid stimulating hormone-treated cells (0 1 mU/ml) Protein kinase C was the only kinase activity which phosphorylated lipocortin I Phosphorylation shifted its p*I* from 6 9 to 6 6 The in vitro phosphorylation of lipocortin I was impaired in cultures exposed for 2 days to phorbol ester (10^{-7} M), although it was present in both the cytosol and the particulate fraction of these cells

Lipocortin, Protein kinase C, Protein phosphorylation, Thyroid, Cell culture

1. INTRODUCTION

A recent series of investigations suggests that lipocortins I and II are the best substrates for receptor (Epidermal growth factor and insulin) and oncogene-encoded tyrosine kinases [1–6] and for protein kinase C (PKC) [7–9].

These proteins are Ca^{2+}-precipitable and bind to phospholipids [2]. They inhibit phospholipase A_2 activity in vitro and in vivo [10–13] and this property decreases after their phosphorylation [14]. The exact mechanism of this inhibition remains unknown [15].

We recently described the purification and characterization of endonexin (32 kDa) and the presence of lipocortin I (35 kDa) in the pig thyroid gland [16]. We also demonstrated that the major endogenous substrates for thyroid PKC were soluble cytosolic proteins with molecular masses between 35 and 38 kDa [17].

The present study provides evidence that, among these proteins, lipocortin I (35 kDa) is the best endogenous substrate for the PKC in thyroid stimulating hormone (TSH)-treated 2-day-old cultures of thyroid cells, and that the in vitro phosphorylation of lipocortin I is impaired in cultures exposed to phorbol esters for 2 days

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2. MATERIALS AND METHODS

2 1 *Cell culture*

Thyroid cells were isolated from pig glands by discontinuous trypsinization and cultured as previously described [18] TSH (0 1 mU/ml) or phorbol ester (0 1 μM) were added from the start of the culture period

At the end of the 2-day culture period, the cells were washed twice by centrifuging at $200 \times g$ for 7 min, and the pellet was resuspended in 25 mM Earle-Hepes buffer, pH 7 2 Cells were kept frozen overnight in dry ice before the protein kinase activity was measured

Thyroid cell cytosols and particulate extracts were prepared as described in [17]

2 2 *Endogenous protein phosphorylation in vitro*

Cytosolic and particulate proteins were phosphorylated in vitro with protein kinases A and C as previously described [17]

2 2 1 *One-dimensional gel electrophoresis*

Aliquots of incubation mixtures were subjected to one-dimensional SDS/polyacrylamide gel electrophoresis (5–15% gel gradients) as described by Laemmli [19] Lipocortins were identified by Western Blot analysis as described by Towbin et al [20] and modified by Rothhut et al [21]

The radioactive phosphorylated proteins were visualized by autoradiography

2 2 2 *Two-dimensional gel electrophoresis*

Samples for two-dimensional gel electrophoresis were prepared as were those for one-dimensional electrophoresis, except that the concentrations of proteins were doubled The phosphorylation reaction was stopped according to O'Farrell [22] in the presence of 9 5 M final concentration of urea The first dimension separation was by isoelectric focusing on an ampholine pH gradient from 4 to 8 8 The second dimension was electrophoresis in 12 5% acrylamide gel The

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proteins were then transferred to nitrocellulose sheets and identified with specific antibodies and diaminobenzidine staining. Phosphorylated proteins were visualized (after exposure to X-ray films) by autoradiography.

3. RESULTS

3.1. *One-dimensional gel electrophoresis (SDS-PAGE)*

3.1.1. Western blot analysis

Lipocortin I (35 kDa) was present in the soluble and particulate fractions of TSH- and phorbol ester-treated cells (fig.1a). The 33 kDa core protein was also observed. This core protein was more abundant in the particles from phorbol ester-treated cells than in those from TSH-treated cells. It was also present in PKC-activated cytosolic fractions from both phorbol ester and TSH-treated cells.

Lipocortin II (36 kDa) and its core protein were also present in soluble and particulate fractions, but their distribution was slightly different from that of lipocortin I (fig.2a). The core protein was more abundant in particles, while the lipocortin II was predominant in cytosolic fractions from both phorbol ester and TSH-treated cells. The core protein of lipocortin II was not enhanced in cytosolic fractions in the PKC-activated assay, as was the core protein of lipocortin I.

Both lipocortins were present in higher concentrations in the cytosolic than in the particulate fractions, when evaluated by diaminobenzidine staining under the same experimental conditions.

A 56 kDa protein was detected by the lipocortin I antibody and a 110 kDa protein band was detected with the lipocortin II antibody in the cytosolic fractions in some experiments.

3.1.2. *Autoradiography*

The above two Western blot nitrocellulose sheets were autoradiographed (figs1b and 2b). A radioactive band with an apparent molecular mass of 35–36 kDa was present in the PKC-activated assay of the cytosolic fraction of TSH-treated cells. This radioactive band was not present in the other fractions of assays of TSH-treated cells or in any of the fractions and assays of phorbol ester-treated cells.

However, the electrophoretic resolution on the one-dimensioonal gels was too low to discriminate between

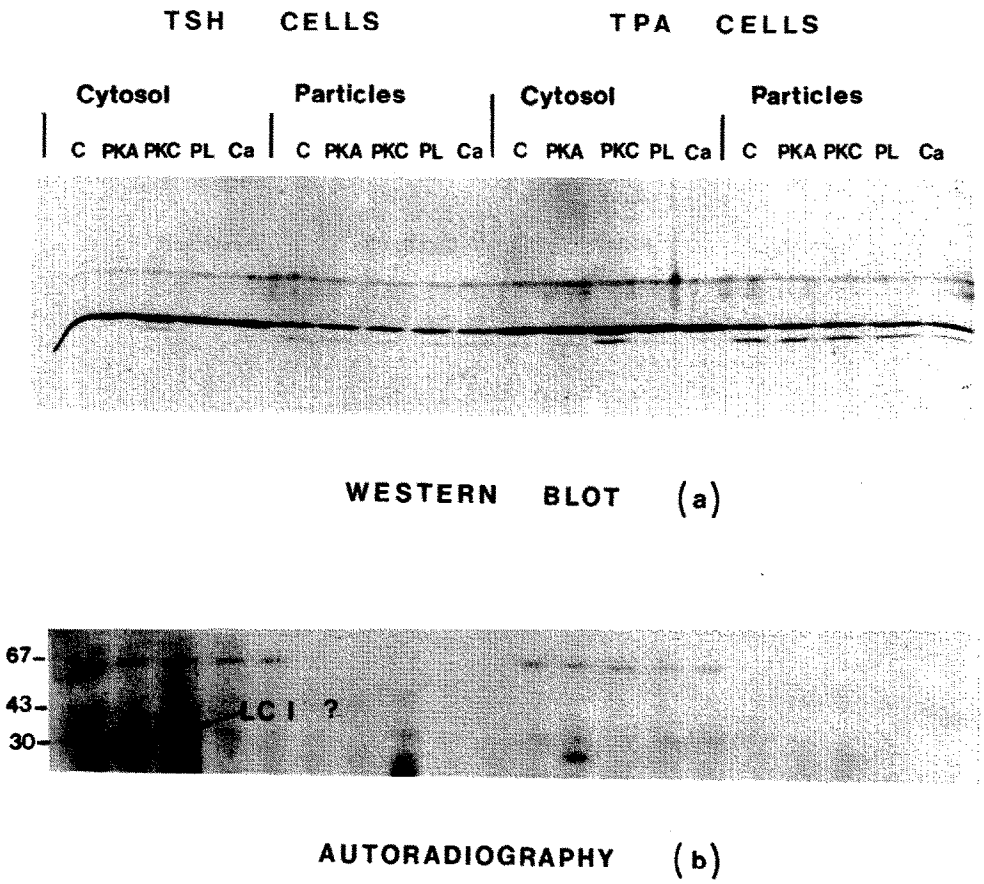


Fig.1. One-dimensional gel electrophoresis. (a) Western blot analysis with lipocortin I (35 kDa) antibody of the cytosolic and particulate fractions from TSH-treated cells (left) and tetradecanoyl phorbol acetate (TPA)-treated cells (right). (b) Autoradiography of nitrocellulose sheets. Cytosolic and particulate fractions from TSH- and TPA-treated cells were phosphorylated under basal conditions (C), or in the presence of cAMP (PKA), calcium + phosphatidyl serine + dioleine (PKC), phosphatidyl serine + diolein (PL) or calcium alone (Ca) as described in section 2.

the two lipocortins (35 kDa and 36 kDa). We therefore performed a two-dimensional gel electrophoresis of cytosol extracts from TSH-treated cells.

3.2. *Two-dimensional gel electrophoresis*

Four antibodies were used, recognizing lipocortin I (35 kDa), lipocortin II (36 kDa), lipocortin V (32 kDa) and lipocortin VI (67–70 kDa). The in vitro phosphorylations of endogenous proteins were carried out under basal (fig.3) or PKC-activated (fig.4) conditions. Analysis of Western blots and autoradiographs showed that lipocortins II, V, and VI were not phosphorylated either under basal or PKC-activated conditions. Lipocortin I was strongly phosphorylated only in PKC-activated conditions (fig.4b). It appeared in two forms, phosphorylated and unphosphorylated (fig.4a,b). The phosphorylation apparently shifted its isoelectric point (p*I*) from 6.9 to 6.6. The pI of lipocortin V (32 kDa) is the same in cultured cells and intact glands (pI 5.2) [16]. Thyroid lipocortin VI (67–70 kDa) has a pI of 5.9 and the p*I* of lipocortin II (36 kDa) is 5.0, which is lower than in other tissues.

Other unidentified endogenous PKC substrates with a relative mass of 35 kDa were present in the cytosolic extracts of TSH-treated cells (fig.4b). Thyroid lipocortins were not substrates for the cAMP-dependent protein kinase in any of the conditions tested.

4. DISCUSSION

We recently demonstrated the presence of lipocortin V in the cytosols of porcine thyroid cells. This protein had an amino acid composition similar to that of monocyte lipocortin (32 kDa) and was recognized by antibodies raised against the monocyte protein. Lipocortin I (35 kDa) and its core protein (33 kDa) were also identified in thyroid cytosols using a recombinant lipocortin I antibody [16]. Here we report that cultured thyroid cells also contain these proteins plus two other molecules of this family, which were identified as lipocortin II and lipocortin VI by specific antibodies.

Antibodies against lipocortin I and lipocortin II were used to show that these proteins were present in both the cytosol and the particulate fraction, but that their distribution between the two compartments was different, as it is in some other cell systems [23]. Lipocortin I was found in both compartments, while lipocortin II was mainly present in the cytosol.

Phosphorylation experiments showed that only the cytosolic lipocortins are phosphorylated, and that PKC is the only kinase activity which phosphorylated them in our experimental conditions. It is interesting to note that particulate lipocortins were not phosphorylated, despite the presence of considerable PKC activity in this compartment [17]. This raises the question of

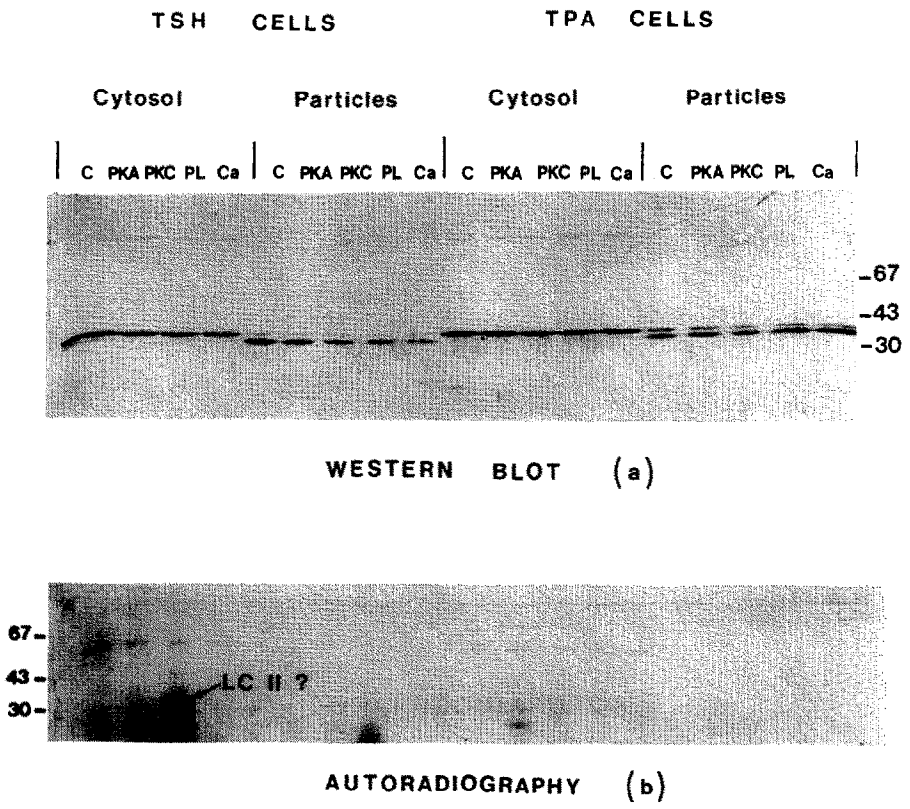
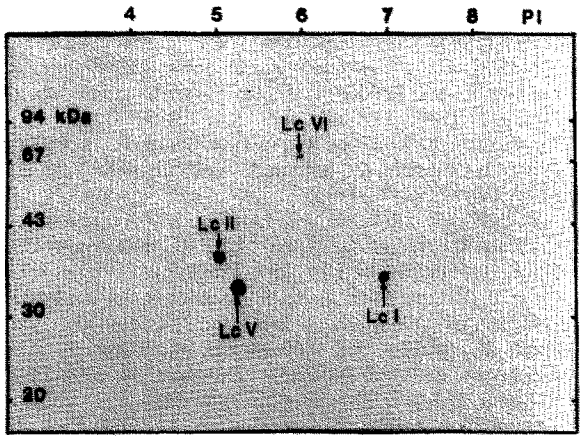
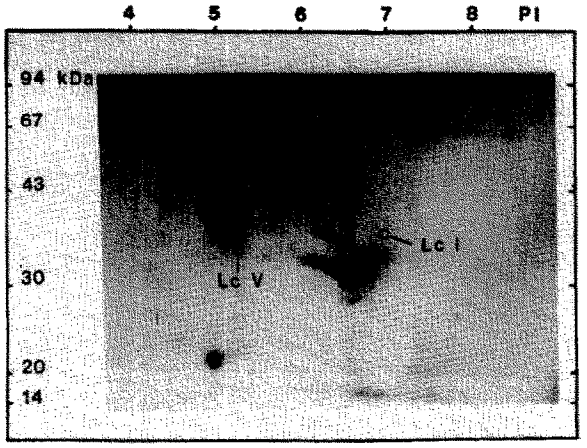


Fig.2. One-dimensional gel electrophoresis. Same conditions as in fig.1 except that lipocortin II (36 kDa) antibody was used.

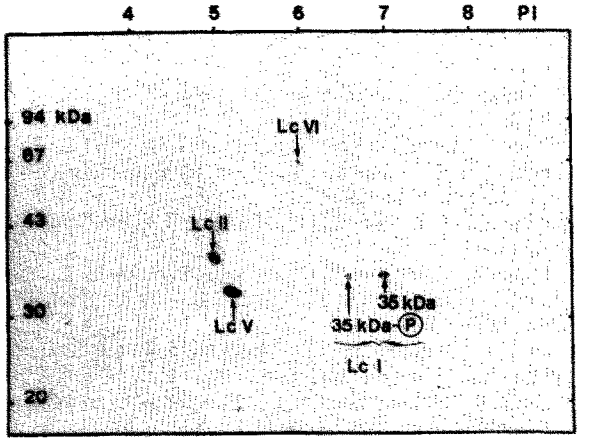


WESTERN BLOT (a)

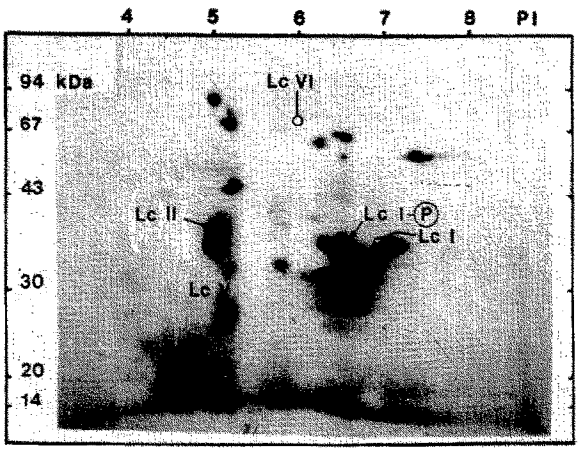


AUTORADIOGRAPHY (b)

Fig.3. Two-dimensional gel electrophoresis of cytosol from TSH-treated cells phosphorylated under basal conditions. (a) Western blot analysis with antibodies against four lipocortins. The same blot was exposed successively to the four antibodies. (b) Autoradiography of nitrocellulose sheets.



WESTERN BLOT (a)



AUTORADIOGRAPHY (b)

Fig.4. Two-dimensional gel electrophoresis of cytosol from TSH-treated cells phosphorylated under PKC-activated conditions. (a) and (b) as in legend of fig.3.

whether the particulate and cytosolic lipocortins are identical entities or distinct isoforms, with different characteristics. Three isoforms of cytosolic PKC (α, β, γ) are all able to phosphorylate lipocortin I [24]. It is, however, not known whether particulate PKC is one of these isoforms.

Of the four lipocortins present in thyroid glands, only lipocortin I was phosphorylated in the cytosols of TSH-treated cells which are well-differentiated cells with a high PKC activity [25]. The p*I* of the phosphorylated form of lipocortin I was different (6.6) from that of the non-phosphorylated molecules (6.9). This may indicate either that all molecules were not phosphorylated under our experimental conditions (3 min) or that the lipocortin I molecule population is heterogeneous. The latter could explain the lack of phosphorylation of particulate lipocortins.

Thyroid cells are dedifferentiated after treatment with phorbol ester and their PKC activity in the two compartments (cytosol and particulate) is completely desensitized in 2-day-old cultures [17]. Lipocortin I is present in these cells, as revealed by Western blot analysis, but it was not phosphorylated, probably because of the lack of enzyme. It is, however, possible that lipocortin molecules are modified in the presence of phorbol esters, preventing their phosphorylation, as has recently been suggested [24].

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