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Influence of fibroblast growth factor on phosphorylation and activity of a 34 kDa lipocortin-like protein in bovine epithelial lens cells

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We examined the effect of basic fibroblast growth factor (FGF) on phosphorylation and lipocortin-like activity of the 34 kDa protein present in the basal membrane of epithelial peripheral cells which initiate growth and differentiation in the bovine lens. We found that: (i) the 34 kDa protein possesses anti-phospholipase A_2 (PLA_2) activity of lipocortin; (ii) in response to FGF, the anti-PLA_2 activity of this protein is enhanced whereas its phosphorylation is markedly decreased. It is suggested that the 34 kDa protein might represent an important biological activity in controlling FGF induction of growth and differentiation in the adult eye lens.

Lipocortin; Fibroblast growth factor; Phosphorylation; (Bovine eye lens)

1. INTRODUCTION

A recent series of investigations suggests that the mode of action of some growth factors could be intimately linked to that of lipocortins [1–5], a family of steroid-induced proteins of 34–39 kDa that inhibit phospholipase A_2 (PLA_2) and thus presumably stop inflammation [6,7]. In particular, it is proposed that the activity of lipocortin can be regulated by its dephosphorylation [8]. One of the primary signalling events leading to growth and differentiation in the eye lens was recently hypothesized to be the binding of fibroblast growth factor (FGF) to the basal surface of epithelial cells of the lens [9]. Interestingly, it was also found that a major surface protein of about 34 kDa in lens cells is structurally and immunologically related to calpactin I [10], an abundant protein which is identical with lipocortin II [3]. Here, we present evidence for a lipocortin-like anti-PLA_2 activity of the 34 kDa protein in the basal membrane of peripheral epithelial cells which initiate growth and differentiation in the bovine lens. We also provide evidence for a possible regulation of anti-PLA_2 activity due to FGF-stimulatable dephosphorylation of this protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

Eagle's minimal essential medium was obtained from Eurobio Laboratories (Paris). [γ-^{32}P]ATP (5000 Ci/mmol) and ^{14}C-labelled M_r markers for gel electrophoresis were from the Radiochemical Centre (Amersham, England) and [γ-^{35}S]thio-ATP (70 Ci/mmol) and [^{3}H]oleic acid (10 Ci/mmol) from New England), Nuclear (Dupont de Nemours, Paris). Unlabelled M_r markers for electrophoresis were from Bio-Rad (California), bicinchoninic acid (BCA) assay reagent from Pierce (Rockford, USA), Triton X-100 from BDH (Poole, England, SDS, EDTA and iodoacetic acid from Prolabo (Paris). All other products were purchased from Sigma (St. Louis, MO). Basic FGF was purified from bovine brain as in [11,12]. Lipocortin from pig lung was prepared as in [13] and kindly provided by Drs Josette Fauvel and Hugues Chap (U 101 INSERM, Toulouse).

2.2. *Isolation techniques*

Bovine lenses were dissected from eyes obtained from the slaughterhouse, each lens being incubated in a closed system for 15 h at 35°C, in 12 ml culture medium with or without basic

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FGF present (10 ng/ml). Anterior lens capsules, with adhering cells, were either sectioned along the equator of the lenses or carefully excised into two fractions: the central epithelium and circumferential (peripheral) fraction (fig.1). Each fraction was then spread over the bottom of plastic culture flasks.

2.3. *Phosphorylation assays*

In situ phosphorylation of epithelial cells attached to capsule fractions was performed in 50 μl of a reaction mixture containing 2 mM Hepes buffer, pH 7.5, 20 mM $MgCl_2$ and 10 μM [^{35}S]thioATP or 0.08 μM [^{32}P]ATP. The reaction was carried out at 0°C for the indicated times and stopped by the addition of Hepes buffer containing 800 μM unlabelled ATP. In some experiments, incubation with unlabelled ATP was continued at 25°C for increasing times of incubation.

2.4. *Preparation of basal membrane-cytoskeleton extracts*

The basal membrane-cytoskeleton complex was produced by treating epithelial cells adherent to capsule fractions, at 0°C for 30 min, with 0.5% Triton X-100 in 40 mM Tris-HCl buffer, pH 7.2, containing 100 mM NaF, 50 mM pyrophosphate and 1 mM phenylmethylsulfonyl fluoride. The detergent-resistant preparations were then washed with this medium without Triton for 30 min at room temperature. This treatment removes most of the cellular contents, whereas microfilaments and intermediate filaments remain tightly anchored to the substratum via some plasma membrane components [14,15]. Samples for gel electrophoresis were prepared as in [16]. Prior to loading, the samples were brought to an equivalent amount of protein. This was accomplished by measuring the protein content of SDS-containing samples using the assay procedure based on the Pierce BCA protein assay reagent [17].

2.5. *Preparation of 34 kDa protein*

The basal cytoskeleton material remaining adherent to capsule fractions was homogenized in a Potter homogenizer (5 strokes). The homogenization buffer consisted of Hepes (20 mM, pH 8), $CaCl_2$ (1 mM), sodium vanadate (1 mM), β-glycerol phosphate (10 mM), *p*-nitrophenyl phosphate (1 mM) and aprotinin (1 μg/ml). The resulting suspension was allowed to stand for 2 min to pellet down the debris from the capsules while leaving the fluffy supernatant intact. Following centrifugation of the supernatant at 50 000 × *g* for 30 min the 34 kDa protein was eluted from the fluffy pellet by removal of Ca^{2+} using EGTA. The elution buffer was identical to the homogenization buffer except that Hepes was adjusted to pH 7.4, NaCl (1 M), iodoacetic acid (4 mM) and EGTA (2 mM) were added and $CaCl_2$ was omitted. The suspension was allowed to remain for 4 h at 4°C, then being centrifuged at 50 000 × *g* for 30 min. The EGTA elution cycle was repeated twice, and the eluates were pooled, dialysed against water for 2 days and lyophilized. Protein concentration was determined as in [18]. Samples for electrophoresis were prepared as above.

2.6. *Phospholipase A_2 assays*

Samples were tested for PLA_2 inhibitory activity by the in vitro assay described in [8,19]. This was performed using [^{3}H]oleic acid-labelled *E. coli* membranes isolated after autoclaving.

2.7. *Gel electrophoresis*

The SDS-polyacrylamide slab gel (10% resolving gel) was as described [20]. Unstained or Coomassie blue-stained gels containing radiolabelled proteins were processed through fluorography, dried and exposed to Kodak X-ray (Omat X-AR-5) film. Autoradiograms were scanned with a Transidyne general modular densitometer.

3. RESULTS AND DISCUSSION

In the adult eye lens, two distinct cellular areas are present on the inner surface of the anterior capsule [21]: the expanding central epithelium, which does not normally undergo mitosis; and a mixed epithelial cell population at the peripheral zone, including a very small number of proliferating cells and elongating cells which differentiate into the young fiber cells of the lens bow region (fig.1). Previous studies have shown that the proliferative response of the peripheral epithelium to eye-derived growth factor, the retinal form of FGF, is very different from that of the central region [22]. In an effort to identify the early processes that control lenticular growth and differentiation, we investigated the influence of basic FGF on phosphorylation and the lipocortin-like anti-PLA_2 activity of the 34 kDa protein in basal plasma membrane of peripheral epithelial cells of bovine lens.

In initial experiments, we compared central and peripheral basal extracts prepared from control and basic FGF-treated cells with regard to their abilities to phosphorylate the 34 kDa protein from [^{35}S]thioATP. Peptide thiophosphorylation is indeed of interest, since it resists the action of protein phosphatases and can therefore prove to be a useful tool in studying the activation of protein kinases [23]. Fig.2 shows that exposure of central and peripheral cells to basic FGF leads to an increase in phosphorylation of the 34 kDa protein. This indicates that the 34 kDa protein can be phosphorylated by a protein kinase that conceivably is activated by FGF.

It could be expected that the phosphorylation state of the 34 kDa protein, and the therefore the metabolic process it controls, were also due to a protein phosphatase activity. To determine whether basic FGF could stimulate such an activity and thereby dephosphorylate the 34 kDa substrate, basic FGF-treated cells were incubated with

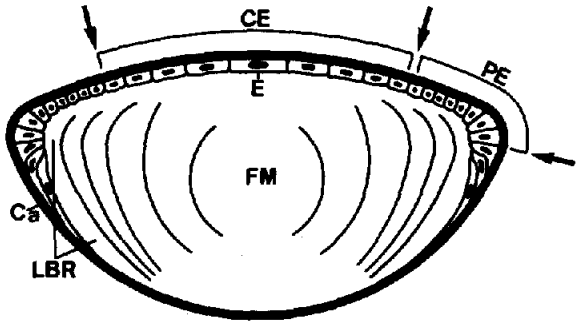

Fig.1. Diagrammatic cross-section of vertebrate lens. Ca, capsule; E, epithelium; CE, central non-proliferative epithelium; PE, peripheral epithelium; LBR, lens bow region; FM, fiber mass. Arrows show the two regions of dissection used in this study.

[^{32}P]ATP for increasing incubation periods. In some experiments unreacted [^{32}P]ATP was removed after 15 min reaction, unlabelled ATP added and protein phosphatase activity monitored. FGF pretreatment of central cells resulted only in a slight enhancement of the phosphorylation level (fig.3). In contrast, addition of FGF to peripheral cells caused a pronounced decline in ^{32}P labelling

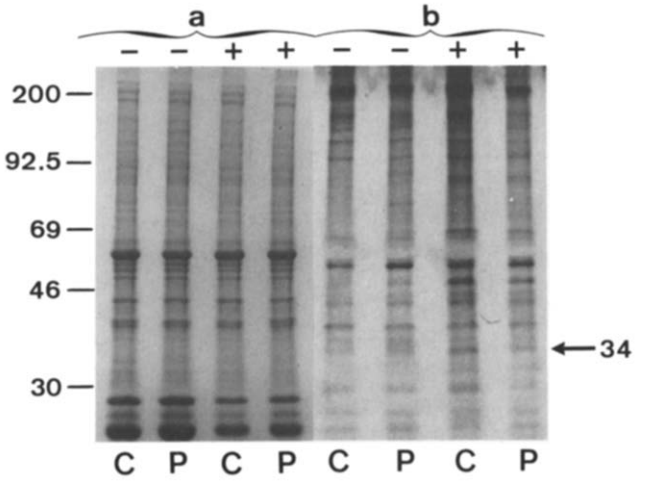

Fig.2. Basic FGF-stimulated phosphorylation from [^{35}S]thioATP of basal membrane-cytoskeleton extracts obtained from central (C) and peripheral (P) epithelial cells. Cells were preincubated for 15 h with (+) or without (–) FGF. Cells were then thiophosphorylated for 30 min and basal extracts prepared as described in section 2. Reaction mixtures were subjected to gel electrophoresis and examined with Coomassie blue stain (lanes a) and in the corresponding autoradiogram (lanes b). Molecular masses of proteins are given in kDa.

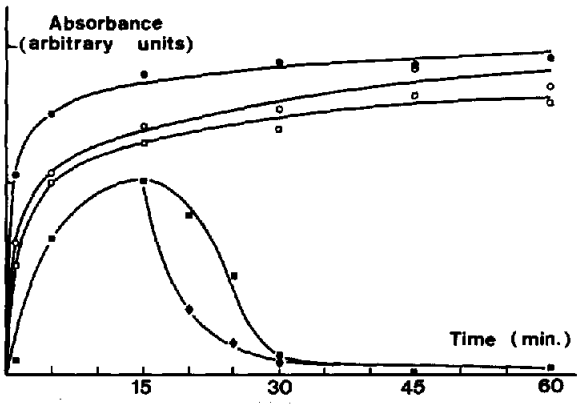

Fig.3. Time course of basic FGF-dependent phosphorylation and dephosphorylation from [^{32}P]ATP of the 34 kDa protein present in basal membrane-cytoskeleton extracts. Cells of central (○,●) and peripheral (□,■) zones were preincubated for 15 h with (●,■) or without (○,□) FGF. Phosphorylation assays were then performed for the indicated times. In a series of parallel assays, FGF-treated peripheral cells were incubated with [^{32}P]ATP for 15 min, followed by the addition of unlabelled ATP after removal of unreacted [^{32}P]ATP to follow the dephosphorylation (◆). Thereafter, basal extracts were prepared as described in section 2 and reaction mixtures were electrophoresed, autoradiographed and scanned. Peak areas corresponding to the 34 kDa protein were calculated and expressed in arbitrary units; each point is the mean of two independent experiments.

after 15 min (fig.3). Since an FGF-stimulatable kinase activity was detected when cells were labelled with [^{35}S]thioATP for 30 min (fig.2), this net dephosphorylation after 15 min can be interpreted as being the result of greater activation of phosphatase activity in the peripheral region. In addition, when unlabelled ATP was added instead of unreacted [^{32}P]ATP, a significant time-dependent decrease in bound ^{32}P was observed (fig.3). Fig.4 (lanes a) depicts the radioactive 34 kDa band obtained from control and growth factor-treated cells following 30 min incubation with [^{32}P]ATP. Taken together, therefore, the present data support the view that basic FGF strongly stimulates a protein phosphatase activity in the basal plasma membrane of peripheral cells, leading to dephosphorylation of the 34 kDa protein.

It is clear that the isolation of the 34 kDa protein could aid in understanding the function of this class of protein. Since the evidence obtained thus far indicates that this protein is bound to plasma membranes in a Ca^{2+}-dependent fashion [24], the

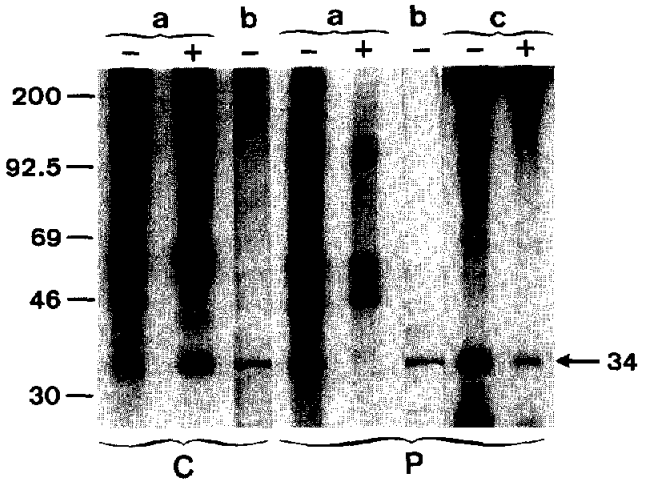

Fig.4. Purification of the 34 kDa protein and basic FGF-dependent phosphorylation and dephosphorylation from [^{32}P]-ATP of basal membrane-cytoskeleton extracts and purified 34 kDa protein. Lanes a, cells were phosphorylated for 30 min and basal extracts were electrophoresed and autoradiographed; lanes b, basal extracts prepared from non-phosphorylated cells were treated with EGTA, and the EGTA eluates were electrophoresed and examined with Coomassie blue stain; lanes c, cells were phosphorylated for 30 min, and the EGTA eluates obtained from basal extracts were electrophoresed and autoradiographed. (+) or (–) indicate whether basic FGF was present or absent during the 15 h preincubation of central (C) and peripheral (P) cells. Molecular masses of proteins are expressed in kDa.

34 kDa protein was purified from basal extracts through several elution cycles in the presence of EGTA. As expected, a prominent 34 kDa component was present in the EGTA eluates (fig.4, lanes b). To verify whether FGF-dependent dephosphorylation of this purified component agreed well with the above-reported results, the 34 kDa substrate was isolated from basic FGF-treated or untreated peripheral cells labelled with [^{32}P]ATP for 30 min. As shown in fig.4, lanes c, the ^{32}P content of the 34 kDa protein decreased dramatically compared to controls when cells were pretreated with the growth factor. Recently, it has been reported that the surface 34 kDa protein is structurally and immunologically related to calpactin [10]. On the basis of these observations, we hypothesized that the peripheral 34 kDa protein might have lipocortin-like anti-PLA_2 activity and that this activity might be regulated by the phosphorylation state of the protein, in a manner similar to that described for lipocortin in platelets [8]. To substantiate such a hypothesis, we extracted the 34 kDa protein from peripheral cells with or without basic FGF pretreatment and assayed the purified substrate for PLA_2 activity. As a positive control, lipocortin from pig lung was also assayed in a similar series of in vitro assays. Fig.5 shows that the 34 kDa protein inhibits PLA_2 in a dose-dependent manner. This activity is specific, since bovine serum albumin stimulated PLA_2 activity (fig.5, inset). Interestingly, one also observes that the anti-PLA_2 activity increases from 38 to 75% following incubation with the growth factor (fig.5). Since a large decrease in the extent of phosphorylation was detected in peripheral cells that had bound basic FGF, these results suggest that the anti-PLA_2 activity of the 34 kDa protein is closely dependent on its dephosphorylation in the peripheral epithelium.

We propose that the possible regulation of PLA_2 activity due to FGF-stimulatable dephosphorylation of the 34 kDa protein may be involved in signal transduction of FGF in the peripheral epithelium. Thus, the 34 kDa protein might represent an important biological activity in controlling growth and differentiation in the adult eye lens. Further investigations should allow us to elucidate

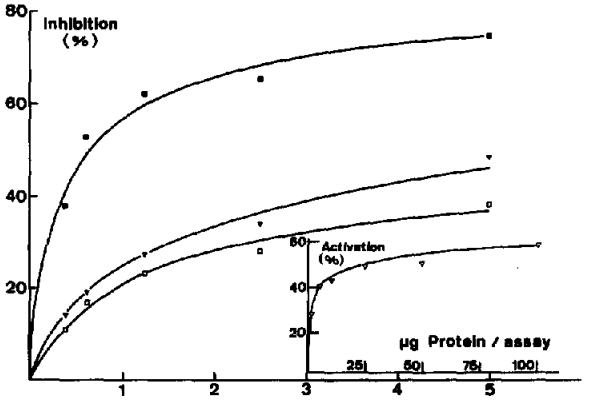

Fig.5. Effect of basic FGF on dose-dependent inhibition of PLA_2 activity by the 34 kDa protein prepared from basal extracts of peripheral epithelial cells. Cells were preincubated for 15 h with (■) or without (□) FGF. Basal extracts prepared from non-phosphorylated cells were then treated with EGTA, and the EGTA eluates were assayed for phospholipase A_2 activity as described in section 2. As a positive control, lipocortin from pig lung was also assayed in a series of parallel assays (▼). The activatory action of bovine serum albumin vs PLA_2 is shown in the inset. Results are means of two independent experiments.

the relationship between this protein and the FGF receptor and ascertain the relevance of such a molecular system to the cascade of signals that finally cause growth and differentiation in the lens.

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