Xenopus lipovitellin, a new target protein for calmodulin

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1. INTRODUCTION

Vitellogenins, the yolk precursor proteins, are synthesized in oviparous vertebrate liver, released into the bloodstream upon oestrogen stimulation and sequestered by developing oocytes after receptor-mediated endocytosis [1]. They are then proteolyzed into lipovitellins and phosvitins which are deposited into crystalline yolk platelets [2], before serving as a nutrient during embryogenesis. Developmental processes are regulated by second messengers, among which Ca^{2+} have been shown to act through binding to a family of proteins, the prototype of which is calmodulin (review [3]). Calmodulin concentration of *Xenopus* oocyte cytosol was indeed shown to be high and to approximately double upon progesterone-induced meiotic maturation [4]. Therefore, we identified the Ca^{2+}–calmodulin-binding proteins of the full grown *Xenopus* oocyte and report that native lipovitellin 1 and lipovitellin 2 bind calmodulin in a Ca^{2+}-dependent manner. Moreover, the isolated subunits of both lipovitellin 1 and 2 exhibit the same property.

2. MATERIALS AND METHODS

Yolk platelet proteins were prepared from 200 full-grown *Xenopus laevis* oocytes, freed from follicular cells as in [5]. Oocytes were homogenized in 1.5 ml buffer A [0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.3 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate (Hepes) buffer, pH 7.4]. The yolk pellet was sedimented at 250 × *g* for 20 min and resuspended in 0.5 ml buffer A.

2.1. *Photoaffinity labeling technique*

A photoaffinity label for calmodulin binding proteins was synthesized, a carboxylic residue of calmodulin was substituted by nitro-azido-phenylethylene diamine following carbodiimide activation (submitted). The azido derivative was iodinated using the iodogen technique [6]. The specific activity of the ^{125}I-labeled calmodulin derivative was 10^5 cpm/pmol. The vitellus fraction (600 μg protein/sample) was incubated in 0.3 M sucrose, 10 mM Hepes buffer (pH 7.5) containing 10 mM magnesium acetate, 150 μM $CaCl_2$ and 3.5 μg [^{125}I]azido-calmodulin. Controls contained 5 mM EGTA instead of calcium or a large excess (400-fold) of unlabeled native calmodulin. After 10 min reaction at room temperature, the samples were irradiated for 20 min at 4°C by using a 350 nm UV lamp. The samples were then centrifuged for 5 min in the presence of a 5 mM EGTA, using a Beckman microfuge, in order to remove any unbound azido-calmodulin derivative.

The pellet was then washed twice with Hepes buffer (pH 7.5) supplemented with EGTA (5 mM) prior to 0.1% SDS:5–20% gradient polyacrylamide gel electrophoresis. The gel was dried

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and autoradiographed at −70°C using an intensifying screen and Kodak Min-R film.

2.2. *Calmodulin gel overlay technique*

The calmodulin gel overlay technique was performed using the procedure in [7] modified as follows: The vitellus fraction (300 μg/lane) was subjected to 0.1% SDS:5–20% polyacrylamide slab gel electrophoresis. The gel was washed in 25% (v/v) isopropanol, 10% (v/v) acetic acid to remove SDS. The solution was changed 4 times in 2 h. The gel was rinsed in distilled water and soaked for 30 min in a denaturing buffer (0.15 M NaCl, 20% (v/v) glycerol, 1 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin, 50 mM Tris (hydroxymethyl) aminomethane (Tris–HCl, pH 7.5) supplemented with 6 M guanidinium chloride, to denature all proteins prior to the renaturation step. The gel was then continuously washed for at least 24 h in the above buffer without guanidinium chloride prior to equilibration for 12 h in buffer A (0.15 M NaCl, 1 mM magnesium acetate, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 50 mM Tris–HCl, pH 7.5). The gel was then soaked in buffer A containing ^{125}I-labeled calmodulin (240 nM, 10^4–10^5 cpm/pmol, 50 ml/slab) for 24 h. Calmodulin was prepared from ram testes as in [8] and was iodinated using the iodogen technique [6]. Non-specifically bound calmodulin was removed by continuous washing for 24 h in buffer A or in buffer A containing EGTA (5 mM) instead of Ca^{2+}. The gel was then dried and submitted to autoradiography. To determine M_r-values two lanes were cut from the rest of the gel after electrophoresis and stained by the Coomassie blue technique.

3. RESULTS AND DISCUSSION

The binding of calmodulin to native lipovitellins was examined by using a photoaffinity labeling technique with radiolabeled azido-calmodulin. Yolk proteins were covalently crosslinked to the label by photolysis and separated according to size by SDS–polyacrylamide gel electrophoresis.

Yolk lipovitellin dissociates into lipovitellin 1 (LV_1) with app. M_r 115000 and lipovitellin 2 (LV_2) with app. M_r 34000 (fig.1A). Both migrate either as an unresolved broad peak (LV_1) or as several discrete species (LV_2), indicative of molecular heterogeneity [9,10]. When examined by autoradiography, the radioactivity pattern was strikingly different dependent on the presence or absence of Ca^{2+} or on the presence of an excess of unlabeled Ca^{2+}–calmodulin. In all cases, the low-M_r part of the gel (< 30000) was obscured by the excess unreacted [^{125}I]iodine-labeled azido-calmodulin. In contrast the high-M_r part of the gel was free of label when the incubation was carried out in the absence of Ca^{2+} (fig.1B) or in the presence of excess unlabeled Ca^{2+}–calmodulin (fig.1C). A major peak was observed with M_r

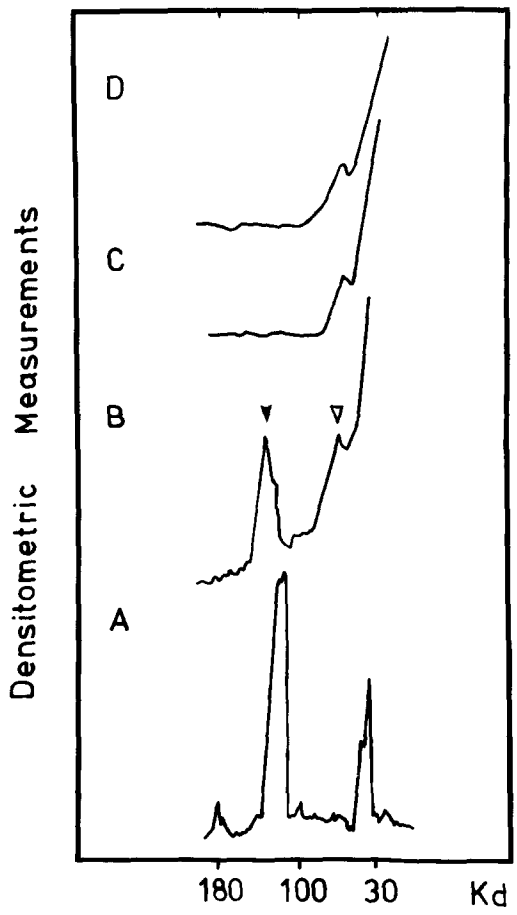

Fig.1. Covalent labeling by azido-calmodulin derivative. Densitometric measurements of Coomassie blue-stained gel (A) and autoradiograms of photoaffinity-labeled yolk platelet in the presence of Ca^{2+} (B) or controls in the presence of EGTA (C) or unmodified calmodulin (D); (▼) LV_1–calmodulin; (▽) LV_2–calmodulin.

~135000 after incubation in the presence of Ca^{2+}, indicative of the Ca^{2+}-dependent binding of 1 mol labeled calmodulin/LV_1.

A similar labeled peak was observed with migration velocity corresponding to M_r ~50000, pointing to a possible stoichiometric complex between labeled calmodulin and LV_1. However, the experiment was not clearcut:

(i) The peak was in fact a shoulder on the ascending slope of the excess unreacted labeled calmodulin;

(ii) A shoulder was also visible when the incubation was done in the absence of Ca^{2+} or in the presence of excess unlabeled calmodulin, though at a somewhat different M_r.

Such ambiguity in the calmodulin binding to LV_1 when examined by photoaffinity labeling, and the need to know whether Ca^{2+}-dependent binding of calmodulin is only a property of native lipovitellin or is also observed with separated subunits and isoforms, prompted another experimental approach which takes advantage of the gel overlay technique.

Lipovitellins were separated by SDS–polyacrylamide gel electrophoresis into LV_1 on the one hand, and the 3 polypeptides of LV_2, α, β and γ (fig.2). Denaturation was made complete by a guanidinium chloride treatment and lipovitellins were allowed to renature before incubation of the gel in the presence of [^{125}I]iodine-labeled calmodulin and in the presence or in the absence of Ca^{2+}. The radioautogram (fig.2) provides clear evidence for the Ca^{2+}-dependent binding of labeled calmodulin, not only to LV_1, but also to the 3 peptides of LV_2.

No difference was observed whether the above overlay experiment was carried out in control oocytes or after progesterone-induced meiotic maturation [4]. This report illustrates the use of two complementary techniques (gel overlay [7] and photoaffinity labeling [11]), in the identification of calmodulin target proteins. That each polypeptide chain of the lipovitellin family is capable of binding calmodulin in a Ca^{2+}-dependent manner obviously raises the question of the biological significance of the phenomenon. Taking into account the vast amount of lipovitellin known to be present in the oocyte, a significant proportion of the total calmodulin concentration in the blocked oocyte (i.e., 40 μM [4]) is likely to be bound to the yolk proteins, provided μM free Ca^{2+} is present. This would in particular explain why injection of substoichiometric amounts of anti-calmodulin antibodies into oocytes was capable of altering the biological response to the hormone [5]. Lipovitellins are thereby conferred an unexpected function, the buffering of Ca^{2+}–calmodulin upon

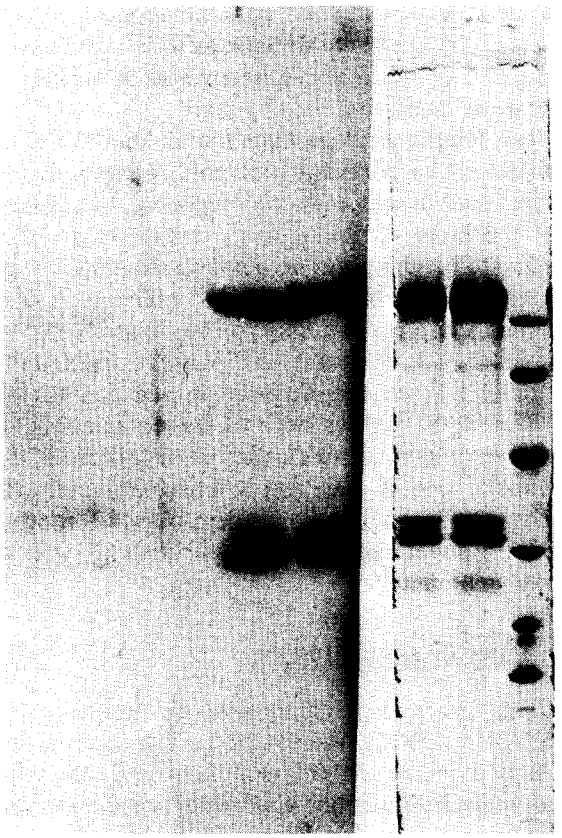

Fig.2. Labeling of calmodulin target proteins from the vitellus fraction of *Xenopus* oocytes. Lanes 1–4 represent the autoradiogram of the gel; lanes 3,4 were washed in calcium; lanes 1,2 were washed with EGTA. The corresponding Coomassie blue-stained proteins are represented in lanes 5–7; lanes 5,6 represent the vitellus fraction; lane 7 the M_r markers, phosphorylase, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin (97000, 67000, 43000, 20100, 14400, respectively).

increase in the free Ca^{2+} concentration. Such buffering is of course highly asymmetrical, being maximal in the vegetal hemisphere where yolk platelets are most dense.

It is tempting to speculate that calmodulin is involved in the breakdown of yolk platelets during embryonic development. More likely, the precursor protein, vitellogenin, may also bind calmodulin, and it has been shown that calmodulin plays a role in receptor-mediated endocytosis [12], probably through binding to the 'light chains' of clathrin [13]. Whether the putative calmodulin binding to vitellogenin helps in the endocytotic process or in further steps of the organization of yolk proteins requires further investigation. If the above hypothesis were true, calmodulin-binding to lipovitellins should be found in oocytes from all oviparous vertebrates, fish and birds as well as amphibians.

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REFERENCES

[1] Tata, J.R. (1976) Cell 9, 1–14.
[2] Opresko, L., Wiley, H.S. and Wallace, R.A. (1980) Cell 22, 47–57.
[3] Klee, C.B. and Vanaman, T.C. (1982) Adv. Protein Chem. 35, 213–321.
[4] Cartaud, A., Ozon, R., Walsh, M.P., Haiech, J. and Demaille, J.G. (1980) J. Biol. Chem. 255, 9404–9408.
[5] Cartaud, A., Huchon, D., Marot, J., Ozon, R. and Demaille, J.G. (1981) Cell Diff. 10, 357–363.
[6] Salacinski, P.R.P., McLean, C., Sykes, J.E.C., Clement-Jones, V.V. and Lowry, P.J. (1981) Anal. Biochem. 117, 139–146.
[7] Carlin, R.K., Grab, D.J. and Siekevitz, P.J. (1981) Cell Biol. 89, 449–455.
[8] Autric, F., Ferraz, C., Kilhoffer, M.C., Cavadore, J.C. and Demaille, J.G. (1980) Biochim. Biophys. Acta 631, 139–147.
[9] Ohlendorf, D.H., Barbarash, G.R., Trout, A., Kent, C. and Banaszak, L.J. (1977) J. Biol. Chem. 252, 7992–8001.
[10] Wiley, H.S. and Wallace, R.A. (1981) J. Biol. Chem. 256, 8626–8634.
[11] Andreasen, T.J., Keller, C.H., La Porte, D.C., Edelman, A.M. and Storm, D.R. (1981) Proc. Natl. Acad. Sci. USA 78, 2782–2785.
[12] Salisbury, J.L., Condeelis, J.S. and Satir, P. (1980) J. Cell. Biol. 87, 132–141.
[13] Slayter, H. (1982) Nature 298, 228.