PROTEIN PHOSPHOKINASES OF CHICK MUSCLE: CHANGES DURING EMBRYONIC DEVELOPMENT

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1. Introduction

Protein phosphorylation is a widespread phenomenom [1] involving, among others, such distinct cell functions as enzyme conversions [2], changes in membrane permeability [3], and regulation of RNA transcription [4]. Protein phosphokinases with different substrate specificity have been found [5, 6] and histone phosphorylation has been particularly implicated in gene activation through increased template activity [7]. In fact, the increased rate of nucleoprotein phosphorylation is concomitant with an active cell division like that occurring e.g. in regenerating liver [8], in unilaterally nephrectomized kidney and fast growing tumoral ascites cells [9], and the developing mammary gland [10].

Protein kinase from rabbit muscle phosphorylates several proteins, including glycogen synthetase [5, 11]. Since the pattern during embryonic development of chick muscle glycogen synthetase kinase has been described [12], it was interesting to know the patterns with the other substrates. In fact, eventual differences would indicate that protein kinases with changing functions might be present during muscle differentiation.

It is the purpose of this communication to report (i) the developmental patterns of protein phosphokinase(s) from chick muscle and liver; and (ii) some differential properties of the muscle enzyme from 11-day old embryos and the adult animal. The results obtained suggest that during differentiation the embryonic enzyme(s) is replaced by new species.

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2. Materials and methods

Fertilized chick eggs (Leghorn) were incubated at 38° and, at the ages indicated, the embryos were removed to a Petri dish containing ice-cold Ringer's medium. Hind limbs were quickly dissected, freed of skin and bones, drawn over filter paper to remove excess moisture, and the muscles instantly frozen between blocks precooled with liquid air. The tissue was powdered and then homogenized in a glass-Teflon homogenizer (A. Thomas Cat. no. 4288B, size AA) with 2 vol of 40 mM Na glycerophosphate pH 6.8, 40 mM NaF, 10 mM EDTA, and 20 mM mercaptoethanol. The homogenate was centrifuged at 7000 *g* for 10 min and the clear supernatant fluid used for enzymatic determinations. Livers were removed and treated similarly. Further purification of the muscle enzyme was achieved by ammonium sulphate precipitation (0.39 g/ml). The pellet obtained by centrifugation at 16,000 *g* for 20 min was

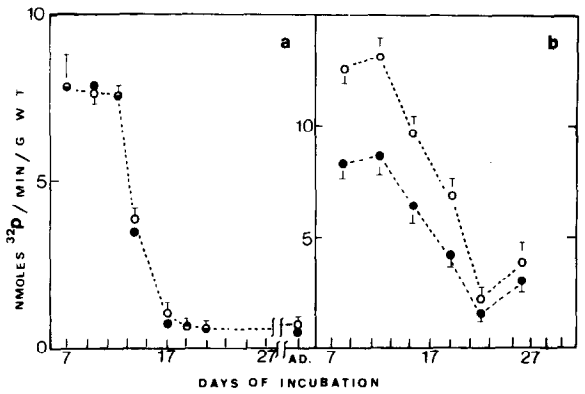

Fig. 1. The developmental pattern of protein kinase in chick muscle (a) and liver (b). Assays were carried out with homogenate supernatants and using the standard incubation mixture (see Methods) either in the presence (○- - -○- - -○) or in the absence (●- - -●- - -●) of cyclic AMP. Values shown are the mean of duplicate determinations of at least 6 and 4 independent pools of chick muscle and liver, respectively. Bars represent the S.E.M. AD = adult. Activity is expressed relative to weight of wet tissue.

resuspended in 2 mM EDTA–5 mM K phosphate pH 7, and dialyzed extensively against the same buffer.

Standard assays for determination of protein kinase activity were carried out by incubation at 30° of a mixture containing: 100 mM Na glycerophosphate pH 6.4, 20 mM KF, 1 mM EGTA, 2 mM theophylline, 6.6 mM Mg acetate, 0.8 mM [γ-^{32}P] ATP (2×10^8 cpm/μmole), 5 μM 3′,5′-cyclic AMP, 11 mg/ml casein, and enzyme in a final volume of 150 μl. Incubations were stopped by addition of 25 μl of 0.1 M EDTA–50 mg/ml albumin, followed by 2 ml of cold 5% TCA. After centrifugation for 5 min at 1200 *g* the pellet was resuspended twice in 5% TCA and then dissolved in 0.25 ml of 1 M ammonium-sodium phosphate. The solution was reprecipitated with 3 ml of 5% TCA and centrifuged. The solubilization–reprecipitation procedure was repeated once more, the final pellet dissolved in 0.1 ml of N KOH followed by 0.4 ml of water, and an aliquot counted in a liquid scintillation counter. Assays were performed under conditions of linearity against enzyme concentration and time.

[γ-^{32}P] ATP was prepared by the method of Glynn and Chappell [13] as modified by Walsh et al. [14]. Casein (Fischer Scientific Co.) was treated according to Reimann et al. [11].

3. Results

Fig. 1a shows that chick muscle protein kinase, measured with casein as substrate, decreases in activity from the high values present at the early stages of embryonic development to an activity of 0.8 nmoles/min/g of wet tissue in the adult, a value similar to that found in rabbit muscle [11]. This decrease occurs mainly from day 10–12 to the 17th, and would be even larger if expressed in terms of the protein content rather than the wet tissue weight. For comparison, the developmental pattern of liver protein kinase was also determined (fig. 1b). Although the pattern is similar to that of muscle, the actual activity is higher and the decrease is not as pronounced as in muscle during the 10 to 17 day period.

Some properties of muscle protein kinase from 11-day-old embryos and the adult animal were further explored with partially purified preparations (table 1). As can be seen, the activity of the embryonic enzyme is stimulated only slightly by cyclic AMP; sodium chloride or phosphate can further increase the activity. On the other hand, protein kinase of adult chick muscle is stimulated 2-fold by cyclic AMP* and is strongly inhibited by salts. The salt effect is the same in the presence or in the absence (not shown) of cyclic AMP. Moreover, when histone is substituted for casein, the activity of the embryonic enzyme is only 10%, while that of the adult animal is unchanged.

Further studies with the protein kinases from both developmental stages have shown that the elution profiles of DEAE-cellulose chromatography are completely different. Four activity peaks could be identified in the embryonic preparations and two in the adult, one of them (the main one) not coinciding

* The lack of activation of cyclic AMP found in crude muscle preparations might be due to various possibilities (presence of endogenous cyclic AMP, high phosphodiesterase activity, protein kinase inhibitor [15], etc.). While any of these factors might be responsible for the increased activation by cyclic AMP observed with the adult enzyme upon purification, it is worth mentioning that the embryonic protein kinases are not activated by cyclic AMP even after DEAE-cellulose purification.

Table 1
Properties of embryonic and adult protein kinases of chick muscle.

Assay medium: additions or deletion	Protein kinase activity (%)	
	11-Day embryo	Adult
None	100	100
– 3′-5′Cyclic AMP	90	50
+ Na chloride, 100 mM	130	70
+ Na chloride, 300 mM	–	40
+ Na phosphate, 100 mM	140	20
+ Na phosphate, 300 mM	–	5
+ Histone**, 4 mg/ml	10	100

* The protein kinases used were purified through the ammonium sulphate step and the assays were carried out with the standard incubation mixture, as described under Materials and methods, with the exceptions indicated in the first column. The activity determined for either the embryonic or the adult enzyme with the standard incubation mixture is taken as 100%, even though the absolute values differ (fig. 1a).

** Histone (Type II A, Sigma) was substituted for casein.

with any of the embryonic peaks. The substrate specificity, kinetic properties and activation by cyclic AMP of this peak are different from those of the others. These properties as well as the sedimentation patterns will be reported in detail elsewhere (R. Staneloni, M.M. Piras and R. Piras, in preparation).

4. Discussion

The cyclic-AMP-dependent protein kinase described by Krebs and coworkers [5, 11] in rabbit skeletal muscle acts as a kinase on phosphorylase *b* kinase and on glycogen synthetase, in addition to being able to phosphorylate casein, protamine and histone. Even after resolution of this protein kinase into distinct peaks, the broad specificity of each one of them was maintained, suggesting that specific protein kinases were not present. While more work might be necessary to elucidate this point further, it seems that at different stages of chick muscle differentiation there are multiple protein kinases with changing specificity. In fact, glycogen synthetase kinase, measured by conversion of glycogen synthetase from the I into the D form, decreases 2- to 3-fold during development of chick muscle [12], but protein kinase measured with casein as substrate decreases 7- to 9-fold (fig. 1). Moreover, the embryonic and adult enzymes differ in substrate specificity, activation by cyclic AMP, and response to salts (table 1). Therefore, the decline of protein kinase activity found during development cannot be explained only by the disappearance of a single species of the enzyme*. Rather, it would seem that while one or more of the protein kinase species decrease through the differentiation process, new ones – with different specificity and, probably, function – are formed, either by total *de novo* synthesis or by modification of the pre-existing species. Multiple molecular forms of protein kinase have been described [11, 16, 17]. In addition, changes in isozyme patterns of several enzymes have been reported in developing tissues [10, 18] and after neoplastic transformation [19, 20].

The exact role of the protein kinases found is presently unknown, but is is worth mentioning that the pattern observed with casein as substrate coincides with the decrease in mitotic index and in DNA polymerase activity observed during chick muscle differentiation [21]. A similar coincidence can be found for liver [19, 22]. Studies to identify the biological function(s) of the protein kinase isozymes are in progress.

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* The possibility that the decreased activity of the adult enzyme is due to an eventual increase of protein kinase inhibitor [15] as a function of development is unlikely since (i) activation by cyclic AMP increases with age (table 1); (ii) the protein kinase patterns depend on the substrate used, while the inhibitor is equally effective independent of the substrate used [15]; and (iii) the relative activities of the embryonic and adult enzymes are maintained even after purification through the DEAE-cellulose step (*vide Supta*).

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