BIOSYNTHESIS OF MUSCLE-SPECIFIC CREATINE KINASE DURING DIFFERENTIATION IN VITRO

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1. Introduction

Measurements of creatine phosphokinase (CPK) activity have been used for some time as an index of differentiation in muscle-cell cultures [1–4]. The developmental transition from embryonic BB isoenzyme, through the MB intermediate, to the mature muscle-specific MM form suggests that the increase in enzyme activity during myogenesis involves new gene expression [5,6]. However, we have previously emphasized the limitations on the use of total CPK activity as an index of differentiation, since quite large increases in embryonic BB-form activity also occur in cell culture [7]. Even measurements of muscle-specific CPK, by specific activity [7] or immunological methods [8], are limited as an index of new gene expression by the fact that they can only provide data on enzyme accumulation rather than rates of enzyme synthesis.

In this report, we present some experiments on the isolation of newly-synthesized CPK after incubation of cells with a radioactive amino acid as the basis for a method of determining the rate of CPK synthesis during differentiation. No measurements of rates of CPK synthesis in muscle cell cultures by amino acid incorporation studies have so far been reported, although, unlike the extensively studied myosin heavy chain [9–12], CPK is relatively easily resolved into its embryonic and mature components.

The results provide further evidence that increases in musclespecific CPK activity during differentiation involve new enzyme synthesis, but do not reveal a similar involvement of new CPK synthesis in the increases in embryonic form activity reported in early cultures [7]. The ratio of the rates of synthesis of myosin and CPK in cell cultures is comparable to the ratio of their final concentrations in mature tissue.

2. Materials and methods

2.1. *Materials*

Components of tissue culture media were obtained from Flow or Gibco-Biocult Laboratories. L-[4,5-^{3}H] leucine (22 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, England. Rabbit muscle creatine kinase was obtained from Sigma.

2.2. *Cell culture*

Cells were prepared from thigh muscle of 12 day chick embryos by mechanical dissociation [13] followed by selective plating as previously described [14]. They were plated at an initial density of 10^7/135 mm collagen-coated Petri dish in 20 ml Minimal Essential Medium (Earle's salts), containing 10% selected horse serum, 2.5% chick embryo extract, 1 mM glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml) and non-essential amino acids. Cultures were fed at 24 h and 72 h with a complete change of medium.

2.3. *Separation of isoenzymes*

Creatine kinase isoenzymes were separated by electrophoresis on 10% polyacrylamide gels with 1.6% cross-linkage, at pH 8.9, as previously described [7]. Gels were then cut into 1 mm slices and the enzyme activity in each slice was measured fluorimetrically [4]. Creatine kinase specific activity is expressed as IU/mg (1 unit converts 1 μmol creatine phosphate to creatine/min, at 37°C) of soluble protein in the original cell extract.

2.4. *Isolation and electrophoresis of radioactive creatine kinase*

After incubation with [^{3}H]leucine, cells were washed thoroughly with saline G, harvested and collected by centrifugation. The freeze-thawed cell pellets were extracted with 1.5 ml water and centrifuged at 15 000 X *g* for 15 min. Samples of the supernatant were taken for determination of protein and total acid-insoluble radioactivity and creatine kinase was extracted from the remainder by a method derived from Eppenberger et al. [15].

For SDS gels only, 100 μg of rabbit muscle creatine kinase was added as carrier at this stage. NH_4Cl was added to 0.1 M and the pH adjusted to 8.5 with NH_4OH. One and a half volumes of ethanol were added at −10°C and the mixture was stirred for 2 h at 20°C. After centrifugation, the supernatant was adjusted to 30 mM $MgSO_4$ at 0°C and stirred for 30 min. The precipitate was collected by centrifugation and resuspended in 0.2–0.3 ml of 60 mM Tris–HCl, pH 6.7 containing 10% sucrose and 1% SDS. After centrifugation, this material was subjected to electrophoresis on 10% polyacrylamide gels with 1.6% cross-linkage at pH 8.9 [16] with 0.1% SDS present in the gel and electrode buffer. After staining with Coomasie Blue, gels were cut into 1 mm slices, which were each incubated with 0.3 ml of 30% H_2O_2 in sealed tubes at 50°C for 24 h. The resulting solution was mixed with a Triton X-100 scintillation fluid for determination of radioactivity. Recovery was estimated by densitometry of stained bands of carrier CPK and comparison with standard amounts subjected to electrophoresis at the same time.

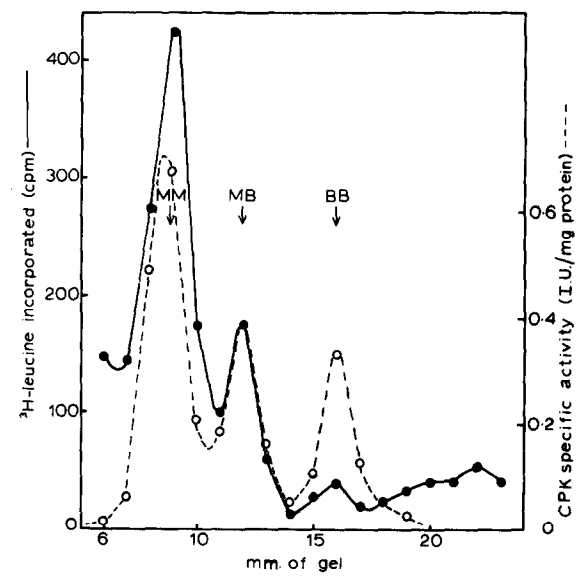

Fig.1. Separation of CPK isoenzymes by polyacrylamide gel electrophoresis. For [^{3}H]leucine incorporation (•—•), 5 cultures were each incubated with 50 μCi [^{3}H]leucine in 12 ml complete medium for 24 h, harvested at 120 h of culture and treated as described in the Materials and methods section. For CPK activity (o- - -o), 2 cultures were harvested at 114 h of culture and extracted and analysed as described previously [7].

3. Results

The M and B subunits of CPK have different isoelectric points but the same molecular weight [15,17], so the isoenzymes are separable electrophoretically at an appropriate pH but co-migrate in the presence of SDS.

Figure 1 shows both the radioactivity and enzyme specific activity gel profiles obtained, as described in the Materials and methods section, from cells labelled with [^{3}H]leucine between 96 h and 120 h and from cells at 114 h of culture respectively. Three radioactivity peaks co-migrate with the MM, MB and BB forms of CPK.

Figure 2 shows SDS–polyacrylamine gel electrophoresis of similarly-prepared samples from cultures harvested at 48 h, 72 h and 120 h, after labelling with [^{3}H]leucine for the previous 24 h. The largest peak from the 120 h culture co-migrated with rabbit muscle CPK and with a CPK preparation from 19-day embryonic chick muscle.

An average recovery of 49% was obtained for rabbit muscle CPK carrier (added in excess to each preparation at the beginning of extraction to avoid differential losses), which is similar to the value obtained by Eppenberger et al. [15] for the Mg^{2+} precipitation step. The extraction method is effective for both muscle and brain forms of the enzyme [15].

There is a significant background level of incorporation of about 70 cpm/mm throughout the gels of fig.2. At present, the only reasonable treatment is to subtract this background from the CPK peak, although

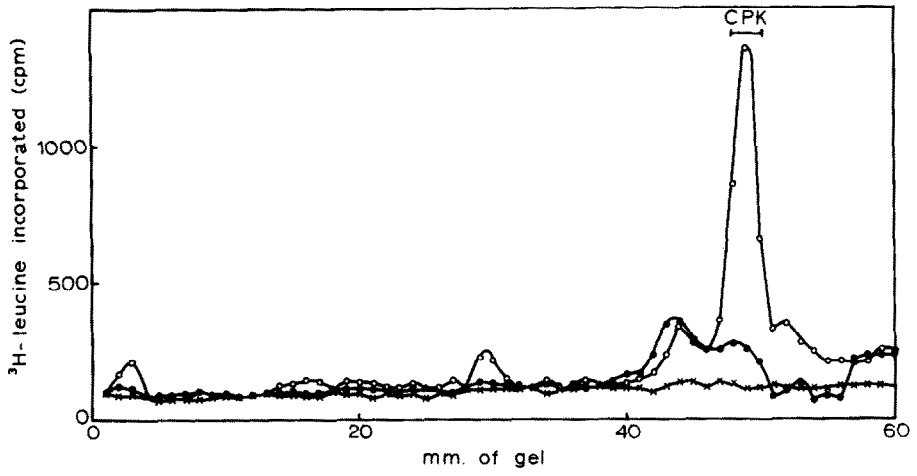

Fig.2. Appearance of newly-synthesized CPK during myogenesis in vitro. For each time-point, 2 cultures were each incubated with 50 μCi [^{3}H]leucine in 12 ml complete medium for 24 h and harvested at 48 h (X—X), 72 h (•—•) and 120 h (o—o). The position of the rabbit muscle CPK carrier on the stained gels is indicated. Polyacrylamide gels were the same as in fig.1 except for the presence of SDS.

this could result in significant underestimation at the very early culture times, which must be borne in mind when interpreting the results. In fig.3, the radioactivity in CPK, estimated from gels such as those in fig.2 after correction for both recovery and background incorporation, is expressed as a percentage of the total incorporation into protein at different periods of cell culture. At its maximum between 4 days and 5 days of culture, [^{3}H]leucine incorporation into CPK accounts for 0.5% of the total. The incorporation into CPK/mg protein is also maximal at this time. Total CPK activity/mg soluble protein in the cultures is also shown in fig.3 over the period 48–120 h when the increase in enzyme activity is due to muscle-specific CPK. Embryonic form is largely responsible for the enzyme level at 48 h [7].

Although the results are highly suggestive, aggregation problems near the top of the gels without SDS have prevented confirmation of complete identity between the main peak of fig.2 and the three peaks of fig.1., by showing that they represent the same proportion of the total radioactivity applied to each gel. We hope to avoid this by the use of guanidine hydrochloride and urea but it will first be necessary to develop an alternative method, such as specific antibody-binding [8], to detect the enzyme after such treatment.

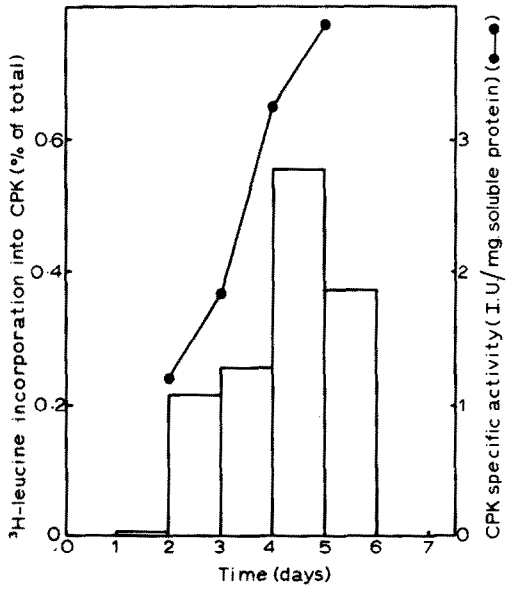

Fig.3. [^{3}H]Leucine incorporation into CPK during myogenesis as a percentage of the total incorporation (vertical columns). The data are based on experiments of the type shown in fig.2 and each point is the average of two gels (maximum range of duplicates ± 10%).

4. Discussion

Figures 1 and 2 show that, by 120 h culture, a high proportion of the radioactivity extracted by the method described has the same charge and molecular weight as CPK, although, as fig.3 shows, this represents only 0.5% of the total radioactivity incorporated.

The distribution of radioactivity between the isoenzymes in fig.1, with most of the incorporation into the M-subunit, is as one would expect at 96–120 h, when MM-form enzyme activity is increasing rapidly [7,18]. Figure 3 suggests that CPK biosynthesis reaches a significant level only after 48 h of culture and this correlates well with the observation that MM-form enzyme activity also begin to increase significantly after 48 h in our cultures [7]. This would seem to indicate that increases in embryonic BB-form activity which occur before 48 h [7] may not involve new enzyme synthesis. However, the background radioactivity on the SDS gels, referred to earlier, may obscure low levels of incorporation into CPK at these early culture times. Improvements to the purification procedure may be necessary to provide a more definite answer to this important question.

We are, as yet, unable to identify the radioactivity peak which migrates at a somewhat higher molecular weight than CPK and shows quite different kinetics of labelling (see fig.2). It is unlikely to be a precursor to CPK because of the long labelling times used in these experiments. The evidence that the M and B subunits are of almost identical molecular weight [17] would seem to rule out the possibility that we are separating the two forms on SDS gels since the minor peak has an apparent molecular weight several thousand daltons higher than M-CPK.

At about 10% [12], the proportion in myosin of the total radioactivity incorporated by late cultures is some twenty-times higher than our value for CPK. If this difference were reflected in the final levels of the two proteins in adult muscle, one would expect to find about 2% CPK since myosin constitutes nearly 40% of mature muscle protein. Our estimates for chick breast muscle based on published data vary from about 0.5% [15] to more than 2% [19], with even higher values reported for mouse muscle [20]. We have subjected aqueous extracts of 19-day embryonic thigh muscle (after centrifugation at 15 000 × *g* for 15 min) to electrophoresis in SDS–polyacrylamide gels and estimated the protein content of the stained bands by densitometry. The band co-migrating with CPK comprised 10–15% of this soluble protein (unpublished). Two-dimensional gel analysis (separating on the basis of both isoelectric point and molecular weight) has ruled out the possibilities of any major contaminant co-migrating with CPK [21]. Since 15–20% of total protein is water soluble, it would seem that even in late embryonic tissue CPK is 2–3% of the total protein.

These figures are hardly accurate enough to decide whether the final ratio of myosin to CPK is determined solely by their relative synthetic rates or whether differential rates of turnover also play a part, but they do suggest that our estimates of leucine incorporation into CPK in culture are of the expected order of magnitude.

We have expressed leucine incorporation into CPK as a percentage of the total incorporation to avoid the problem of possible variations in leucine pool size in cells at different stages. It is necessary to assume only that leucine for CPK is drawn from the same pool as that for total protein or from a pool of the same specific radioactivity. Although the use of leucine-free medium would increase incorporation and allow a more detailed analysis by reducing labelling times, we have avoided using it since there has been no detailed investigation of amino acid starvation effects on muscle-specific protein synthesis in these cultures. Even more likely, perhaps, are disturbing effects of feeding fresh serum (necessary when changing to leucine-free medium) or of changing to serum-free medium [22]. Our present studies provide the basis for investigating such effects.

Acknowledgements

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