Biochem. J. (1989) 264, 305–308 (Printed in Great Britain)

Regulation of cyclic AMP-dependent protein kinase levels during skeletal myogenesis

Ian A. J. LORIMER and Bishnu D. SANWAL*
Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1

We showed previously that the levels of type I regulatory subunit of cyclic AMP-dependent protein kinase increase during differentiation of L6 skeletal myoblasts as a result of a specific decrease in its rate of degradation. Studies on the rates of degradation of the catalytic subunit show that, unlike the type I regulatory subunit, catalytic subunit is degraded very slowly in myoblasts ($t_{\frac{1}{2}}$ = 29 h) and more rapidly in myotubes ($t_{\frac{1}{2}}$ = 14 h). As with the regulatory subunit, the degradation of catalytic subunit is increased by treatment of myoblasts with cyclic AMP analogues. These results suggest that the overall increase in the amount of type I cyclic AMP-dependent protein kinase holoenzyme during myogenesis is due to the increase in levels of mRNA for the catalytic subunit. This probably leads to an increase in the amount of catalytic subunit, which then stabilizes the regulatory subunit, thereby causing an increase in the levels of this protein also.

INTRODUCTION

L6 is a permanent cell line of rat skeletal myoblasts which differentiates in culture to form multinucleate myotubes, mimicking the process which occurs in the whole organism during development (Sanwal, 1979; Pearson, 1981). An essential part of this differentiation process is the co-ordinate increase in the levels of a large number of muscle-specific proteins. Most evidence shows that this increase is mainly regulated at the level of transcription. However, our previous studies on the regulation of the cyclic AMP-dependent protein kinases during myogenesis suggest that there may be exceptions to this general rule (Lorimer *et al.*, 1987).

The cyclic AMP-dependent protein kinases are multi-subunit enzymes, consisting of a regulatory subunit dimer which binds two catalytic (C) subunits (Beebe & Corbin, 1986). They are generally classified into two isoenzyme types, termed type I and type II on the basis of their order of elution from anion-exchange columns. These isoenzymes have identical C subunits, but differ in the type of regulatory subunits that they contain. We have studied the regulation of these enzymes during myogenesis, as it has been suggested that cyclic AMP may be involved in the regulation of this process (Curtis & Zalin, 1981; Wahrmann *et al.*, 1973). We have previously shown that L6 myoblasts contain both types of cyclic AMP-dependent protein kinases, and also a significant amount of type I regulatory subunit (R_I) in the free form (Rogers *et al.*, 1985). During myogenesis the total amount of cyclic AMP-dependent protein kinase activity increased about 3-fold. Analysis by anion-exchange chromatography, photoaffinity labelling (Rogers *et al.*, 1985) and immunoblotting (Lorimer & Sanwal, 1987) showed that this increase in activity was due to a selective increase in the amount of type I isoenzyme. Further studies showed that the increase in R_I was not regulated transcriptionally, but was due to a specific decrease in the rate of degradation of this subunit (Lorimer *et al.*, 1987). In the present work we have continued these studies to investigate the mechanism by which the increase in the level of C subunit takes place. We have found that this increase is probably due to an increase in the levels of its mRNA, and that changes in its rate of degradation are not involved in regulating its increase. An overall scheme for the regulation of R_I and C levels during myogenesis is presented, and the possible relevance of this scheme to the regulation of other increases in multi-subunit protein complexes during differentiation is discussed.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP (3000 Ci/mmol), L-[^{35}S]methionine (> 800 Ci/mmol) and En^{3}Hance were from DuPont–New England Nuclear. Rabbit anti-goat antibody was from Zymed Laboratories, San Francisco, CA, U.S.A. Other chemicals were from various commercial sources.

Cell culture

L6 rat skeletal myoblasts (Yaffe, 1968) were cultured in α-modified minimal essential medium containing 10% (v/v) horse serum, 16 mM-glucose and 50 μg of gentamycin/ml as described previously (Lorimer *et al.*, 1987).

Measurement of C-subunit mRNA levels

Northern blotting and hybridizations were performed as described previously (Lorimer *et al.*, 1987). C mRNA levels were measured by use of a ^{32}P-labelled 600-base-pair *Eco*R1 fragment from the plasmid pMC1 (Uhler *et al.*, 1986*a*). This sequence contains part of the *C*-terminal coding region of C and about 160 base pairs of 3′ untranslated sequence (Uhler *et al.*, 1986*b*). The mRNA levels for the R_I subunit were measured by using a 770-base-pair *Pst*I fragment from the plasmid p62C12 as described previously (Lorimer *et al.*, 1987). Blots were

Abbreviations used: R_I, regulatory subunit of type I cyclic AMP-dependent protein kinase; C, catalytic subunit of cyclic AMP-dependent protein kinase.

* To whom correspondence should be addressed.

first probed with R_1 cDNA and re-probed with the DNA complementary to C mRNA. The initial hybridization with R_1 cDNA served as a control to relate the changes in C mRNA directly with the observations which we had described previously (Lorimer, 1988) for R_1 mRNA. The autoradiograms were quantified by use of a laser densitometer. Linearity between input RNA and densitometry data was established.

Half-life measurements

Labelling of L6 cells with [^{35}S]methionine and chase conditions with unlabelled methionine were as described previously (Lorimer *et al.*, 1987), except that the cells were labelled for only 6 h and longer periods of chase were used (up to 20 h). Immunoprecipitations were also carried out as described previously, but with the following changes: instead of anti-R_1 antibody, extracts were incubated for 60 min with 1 μg of anti-C antibody which had been raised in goat (a gift from M. Murtaugh, University of Minnesota); after this incubation, a second incubation with 5 μg of rabbit anti-goat antibody was carried out for 60 min on ice. The immunoprecipitation was then completed by incubation with *Staphylococcus aureus*, pelleting and washing as described previously (Lorimer *et al.*, 1987). Samples were electrophoresed on 10% acrylamide gels (Laemmli, 1970). Gels were then treated with En^{3}Hance according to the manufacturer's instructions before being dried and autoradiographed. Films were scanned with an LKB densitometer.

RESULTS AND DISCUSSION

Fig. 1 shows a Northern blot of total L6 RNA from days 2–7 after plating which has been probed with labelled C cDNA. Throughout differentiation a single band of approx. 2.6 kilobases is labelled. A band of this size has also been reported in both mouse (Uhler *et al.*, 1986*a*) and bovine (Showers & Maurer, 1986) RNA, and has been shown to code for the C_α isoenzyme. We did not detect a band in the 4.3–4.4 kilobase range, which has been reported by others and shown to code for the C_β isoenzyme. There is a marked increase in the amount of C mRNA with differentiation, which begins at day 4 and is maximal at day 6 in parallel with fusion. No changes in the level of R_1 mRNA were discernible during this time, in conformity with our previous findings (Lorimer *et al.*, 1987). The changes seen for the C mRNA thus were not due to generalized changes in total mRNA content or other trivial causes, such as differences in extraction efficiency at different times during differentiation. By scanning densitometry the increase in C mRNA was estimated to be about 4-fold. Therefore it appears that the increase in kinase activity seen during myogenesis (Rogers *et al.*, 1985) is regulated by this change in C mRNA levels. We have attempted to perform run-off transcription experiments to determine whether this increase in C mRNA was due to an increased rate of transcription from the C gene in myotubes. However, although we were able to detect labelled transcripts for β-actin and R_1, we were unable to detect any transcripts for C, using either isolated myoblasts or myotube nuclei (results not shown). This suggests that transcription from the C gene occurs at a very low rate. We therefore were unable to prove that the increase in C mRNA is due to an increased rate of transcription, although this seems to be the most likely explanation. A second possibility,

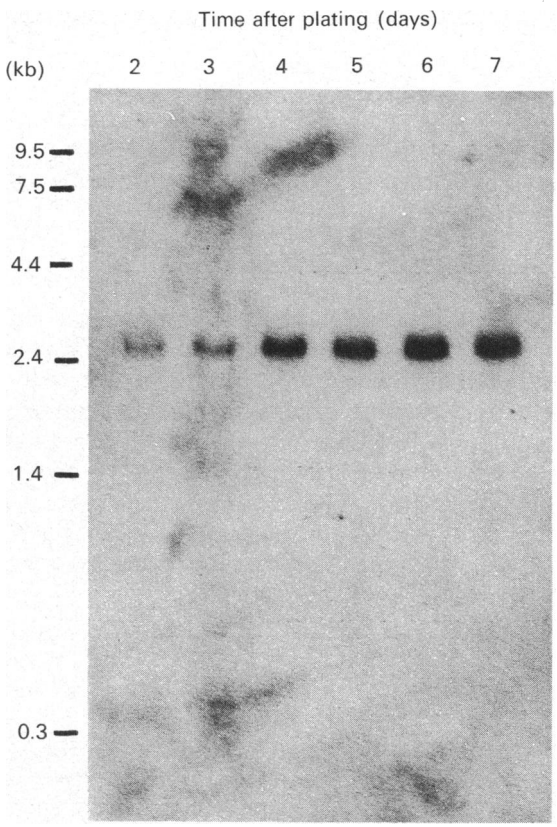

Fig. 1. C mRNA levels during L6 myogenesis

Total RNA was isolated from L6 cells on days 2–7 after plating. Fusion of the myoblasts begins on day 4 and typically proceeds such that about 95% of the nuclei are present in myotubes by day 6. Isolated RNA (20 μg/lane) was then electrophoresed on agarose gels, transferred to a nylon membrane, and probed with ^{32}P-labelled DNA complementary to C mRNA. RNA size markers (shown in kilobases, kb) were purchased from Bethesda Research Laboratories.

that C mRNA levels are increased by a specific stabilization of the mRNA, however, cannot be ruled out. Regulation of C mRNA levels during development have also been studied by Oyen *et al.* (1987), who showed that they increased in developing rat testes.

Although our results suggested that the increase in C activity during myogenesis was regulated by changes in C mRNA levels, we also chose to measure the half-life of C in myoblasts and myotubes, as we had found that control at this level regulated R_1 levels during myogenesis. L6 cells were labelled with [^{35}S]methionine and then chased with media containing unlabelled methionine. After different periods of chase, samples were isolated and C was immunoprecipitated with a specific antibody against bovine C. Immunoprecipitates were electrophoresed on SDS/polyacrylamide gels, which were then dried and radioautographed. Labelled C remaining was quantified by scanning densitometry. Fig. 2 shows representative plots of the resulting densitometry data from the autoradiograms. Calculated half-life values, together with previous data on R_1 half-lives for comparison, are summarized in Table 1. In myoblasts, C was degraded with a half-life of 29 ± 5 h. This is almost a 10-fold slower rate than that determined for R_1 in myoblasts. In

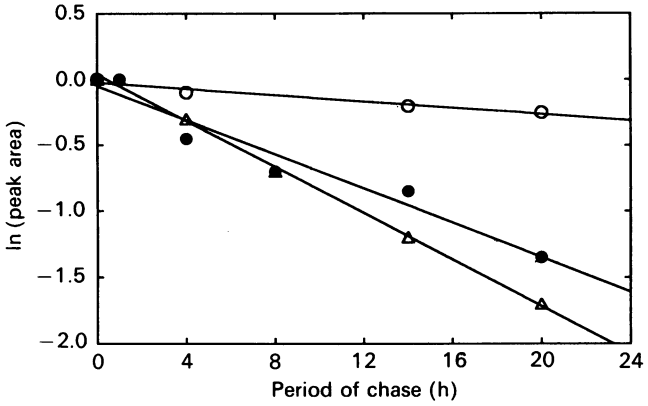

Fig. 2. Rates of degradation of C in myoblasts in the absence and presence of cyclic AMP analogues and in myotubes

Myoblasts and myotubes were labelled with [^{35}S]methionine and chased with media containing unlabelled methionine. Samples of cells were isolated at different periods of chase, and C was isolated by immunoprecipitation. The immunoprecipitates were then run on SDS/polyacrylamide gels and the amount of labelled C remaining was quantified by scanning densitometry of autoradiograms of the gels. When needed, myoblasts were treated for 6 h with 0.25 mM-dibutyryl cyclic AMP, 0.25 mM-8-bromo cyclic AMP and 0.1 mM-3-isobutyl-1-methylxanthine. Cells were then labelled and chased in media containing the same concentrations of analogues. The results shown are for one typical experiment. Each point corresponds to one actual measurement. Data were normalized to the peak-1 area determined for 1 h of chase. Slopes were determined by linear regression. Symbols: ○, myoblasts; ●, myotubes; △, myoblasts treated with cyclic AMP analogues.

Table 1. Comparison of C and R_I half-lives

Values shown are means ± S.D. of the numbers of separate determinations shown in parentheses. Each determination is the result from an entirely separate experiment and is constituted of a single set of points as exemplified by data in Fig. 2.

	Half-life (h)	
	C	R_I*
Myoblasts	29±5 (4)	3.3±0.1 (3)
Myotubes	14±4 (3)	10±2 (2)
Myoblasts+cyclic AMP	9±2 (3)	1.9±0.3 (3)

*Values for R_I half-lives have been reported previously (Lorimer *et al.*, 1987; Lorimer, 1988) and are shown again for comparative purposes.

myotubes, C was degraded with a half-life of 14±4 h, about twice as fast as in myoblasts. This is probably due to the overall increase in the rate of proteolysis with differentiation, which has been observed previously in our laboratory (Kaur, 1980). This result, however, is in contrast with the results obtained for R_I, which was found to be much more stable in myotubes. Interestingly, the rates of degradation of C and R_I are the same (within experimental uncertainty) in myotubes.

To continue our comparison of the degradation of C with that of R_I, we also studied the effects of cyclic AMP analogues on the degradation of myoblast C. The data shown in Fig. 2 and in Table 1 are for myoblasts treated with a combination of dibutyryl cyclic AMP and 8-bromo cyclic AMP, together with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. As with R_I, this treatment caused a marked increase in the degradation of C; its half-life after this treatment was 9±2 h, compared with 29 h in untreated myoblasts. However, degradation of free C is still much slower than degradation of free R_I. Several other laboratories have previously reported studies on the regulation of C levels by cyclic AMP. Alhanaty *et al.* (1981) have presented evidence for a proteolytic activity in brush-border epithelial cells, which was active against free C but not the holoenzyme. Hemmings (1986) has reported a decrease in C levels in pig epithelial cells after long-term treatment with hormones that increase intracellular cyclic AMP, and proposed that the decrease was due to an increased proteolysis of the C, although actual rates of proteolysis were not measured. Our results confirm this proposal, showing that cyclic AMP can increase the rate of proteolysis of C in intact cells.

We have shown previously (Rogers *et al.*, 1985) that, along with the increase in the concentration of R_I subunit, there is an increase in the activity of the holoenzyme during myogenesis. Since cyclic AMP shortens the half-life of R_I (Table 1), and since its concentration also decreases drastically in myotubes (from 150 pmol/mg of protein in myoblasts to less than 5 pmol/mg in myotubes; Lorimer, 1988), we suggested that the change in the stability of R_I was probably due to a decrease in cyclic AMP levels during myogenesis. In view of the findings on the regulation of C in the present paper, this possibility now seems less likely. A more plausible hypothesis would be that the increase in R_I during myogenesis is due to a decrease in its rate of degradation, caused by an increase in its association with C to form proteolysis-resistant holoenzyme, rather than by a decrease in the level of cyclic AMP *in vivo*. The increased association would be due to an increase in the amount of C in myotubes (indirectly due to an increase in the level of C mRNA) so that more is available to complex with R_I. Increased levels of C and R_I would result also in the increased formation of the holoenzyme in myotubes, a phenomenon that we have described previously (Rogers *et al.*, 1985). That R_I is probably stabilized by its association with C is also shown by the fact that conditions which facilitate dissociation of the holoenzyme into C and R_I (presence of cyclic AMP) result in their increased degradation (Table 1). A report by Uhler & McKnight (1987) has also suggested that this type of mechanism was involved in the control of type I holoenzyme levels in 3T3 cells transfected with C cDNA. In this system and in our own previous work (Rogers *et al.*, 1985), no increase in R_{II} was observed, suggesting that this role is unique to the type I regulatory subunit R_I.

In conclusion, our data suggest that the increase in type I cyclic AMP-dependent protein kinase during myogenesis is regulated such that only one subunit (C) is regulated transcriptionally, whereas the other subunit (R_I) is regulated post-transcriptionally via a decrease in its rate of degradation. This type of mechanism may have general significance for the co-ordinate regulation of multi-subunit complexes in other differentiating systems.

In the assembly of IgM during B-cell differentiation, for instance (Dulis, 1983), resting B cells synthesize only IgM heavy chains, which are rapidly degraded in the absence of light chains. Differentiation of these cells results in a transcriptionally regulated increase in the synthesis of light chains, which combine with the heavy chains, decreasing their rate of proteolysis, and thereby bringing about an overall increase in the amount of IgM complex. The assembly of the membrane cytoskeleton during avian erythropoiesis may also be regulated similarly (Blikstad *et al.*, 1983; Lazarides & Moon, 1984; Lehnert & Lodish, 1988). In this case, α- and β-spectrin are synthesized in excess of other membrane cytoskeletal components and stabilized by association with these.

This work was supported by an operational grant from the Medical Research Council of Canada to B.D.S. and a Steve Fonyo Studentship from the National Cancer Institute of Canada to I.A.J.L. We thank Dr. G. S. McKnight for providing us with the plasmid pMC1 and Dr. M. Murtaugh for providing us with antibody to the catalytic subunit of protein kinase. Also, we would like to thank Dr. E. H. Ball for his useful comments on this manuscript.

REFERENCES

Alhanaty, E., Patinkin, J., Tauber-Finkelstein, M. & Shaltiel, S. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3492–3495
Beebe, S. J. & Corbin, J. D. (1986) Enzymes 3rd Ed. **17**, 43–111
Blikstad, I., Nelson, W. J., Moon, R. T. & Lazarides, E. (1983) Cell **32**, 1081–1091
Curtis, D. H. & Zalin, R. J. (1981) Science **214**, 1355–1357
Dulis, B. J. (1983) J. Biol. Chem. **258**, 2181–2187
Hemmings, B. A. (1986) FEBS Lett. **196**, 126–130
Kaur, H. (1980) M. Sc. Thesis, University of Western Ontario
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lazarides, E. & Moon, R. T. (1984) Cell **37**, 354–356
Lehnert, M. E. & Lodish, H. F. (1988) J. Cell Biol. **107**, 413–426
Lorimer, I. A. J. (1988) Ph. D. Thesis, University of Western Ontario
Lorimer, I. A. J. & Sanwal, B. D. (1987) in Signal Transduction and Protein Phosphorylation (Heilmeyer, L. M. G., ed.), pp. 363–367, Plenum, New York
Lorimer, I. A. J., Mason, M. E. & Sanwal, B. D. (1987) J. Biol. Chem. **262**, 17200–17205
Oyen, O., Froysa, A., Sandberg, M., Eskild, W., Joseph, D., Hansson, V. & Jahnsen, T. (1987) Biol. Reprod. **37**, 947–956
Pearson, M. L. (1981) in The Molecular Genetics of Development (Leighton, T. & Loomis, W. F., eds.), pp. 361–418, Academic Press, New York
Rogers, J. E., Narindrasorasak, S., Cates, G. A. & Sanwal, B. D. (1985) J. Biol. Chem. **260**, 8002–8007
Sanwal, B. D. (1979) Trends Biochem. Sci. **4**, 155–157
Showers, M. O. & Maurer, M. A. (1986) J. Biol. Chem. **261**, 16288–16291
Uhler, M. D. & McKnight, G. S. (1987) J. Biol. Chem. **262**, 15202–15207
Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G. & McKnight, G. S. (1986*a*) Proc. Natl. Acad. Sci. U.S.A. **83**, 1300–1304
Uhler, M. D., Chrivia, J. C. & McKnight, G. S. (1986*b*) J. Biol. Chem. **261**, 15360–15363
Wahrmann, J. P., Winand, R. & Luzatti, D. (1973) Nature (London) **254**, 112–113
Yaffe, D. (1968) Proc. Natl. Acad. Sci. U.S.A. **67**, 477–483

Received 11 April 1989/14 September 1989; accepted 22 September 1989