Vol. 134, No. 1, 1986
January 14, 1986

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 191-200

EXPRESSION OF A NEW ß TUBULIN SUBUNIT IS INDUCED BY 20-HYDROXYECDYSONE IN DROSOPHILA CULTURED CELLS

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Received November 12, 1985

SUMMARY : One polypeptide, P4, of molecular weight 55000 daltons and pHi, 5,1, is synthesized in Drosophila melanogaster Kc 0 % cells only when they were treated by the insect moulting hormone, 20 hydroxyecdysone (20-HE). P4 is precipitated with vinblastine sulfate and migrates as a tubulin subunit. Immunoblot experiment confirms that P4 is a ß subunit of tubulin. This ß tubulin is recovered by in vitro translation only when mRNAs are extracted from treated cells. Thus, regulation of expression of this polypeptide is at the level of transcripts and is under a steroid hormone control. The 20-HE induced ß tubulin comigrates with the ß3 subunit and like the latter, is specific to the mid period of embryogenesis.

Steroid hormones play an essential role in development and physiological regulation in eukaryotic organisms. Since they control, in part, gene expression. Experimental systems in which cytodifferentiation is under steroid hormone control are therefore important models for studying the regulation of gene expression in eukaryotic organisms.

Drosophila melanogaster cultured cell lines are used as such a model to study the action of ecdysteroid hormones. Many specific responses to 20-HE have been described in the Kc 0 % cell line. Following hormone addition, Drosophila melanogaster cultured cells undergo dramatic modification in morphology involving cell elongation, extention of thin processes and finally an arrest of cell division (1). Several enzymatic activities are greatly enhanced (2, 3, 4). Protein pattern, as analysed by two dimensional gel electrophoresis is specifically modified (5), particularly for actin. Recently, we demonstrated that three days of 20-HE treatment increase actin quantity by two fold and actin polymerization by four fold (6). Our evidence indicated that the hormone affected actin biosynthesis partly through increasing the level of actin mRNA (7).

0006-291X/86 $1.50

The contractile protein, actin, found in the cytoskeleton of all eukaryotic cells has been implicated in a variety of developmental and cellular processes (8). Another major component of cytoskeleton is tubulin ; it is a heterodimer of one α and one ß subunit. It is also, like actin, implicated in a wide variety of cellular activities including cell motility, intracellular transport, cell shape determination and modulation, exocytis, endocytosis and cell surface motility.

In Drosophila, there are several distinct genes scattered on the genome for both α and ß tubulin (four of each, (9)). In all organisms tubulin microheterogeneity is developmentally determined depending on tissue (testis for ß2, (10)) and the stage (mid embryogenesis for ß3, (11)) ; during rat brain maturation (12) and during chicken development (13), microheterogeneity of tubulin increases. Such differential expression of distinct tubulin subunits can be an interesting model to study control of protein synthesis by effectors, such as steroids as in our case.

In this paper, we describe the synthesis of a new polypeptide, previously named P4 (5), of 55000 daltons, in Drosophila Kc cells which is produced, only when the cells are treated with 20-HE. P4 is a ß tubulin subunit. It is also synthesized in vitro, (in a rabbit reticulocyte lysate), when primed mRNAs are extracted from 20-HE treated cells. This ß tubulin subunit has the same electrophoretic characteristics as tubulin isoform ß3. Like ß3 (11), P4 is specific of a stage development (8 hr-13 hr) in embryogenesis.

MATERIALS AND METHODS

Cell culture

Kc 0 % Drosophila cells were derived from the line established by Echalier and Ohanessian (14). They were cultured at 23°C at a concentration of 5 to 40 x 10^3 cells per ml in D22 medium (from Echalier and Ohanessian, (14)) without serum. When required, ecdysterone (SIMES, Milan) was added to cultures at a final concentration of 1 µM.

Labeling of cells with [^{35}S] methionine

D22 medium was removed from a bottle of exponentially growing cells and replaced with the same quantity of methionine free culture medium during twenty minutes. Then 20 µCi/ml (1220 Ci/mmole, Amersham) was added. After 30 minutes of incubation, cells were harvested and washed twice with the following buffer : (75 mM NaCl, 24 mM EDTA, 10 mM Tris HCl pH 8, 3 mM CaCl2, 2 mM MgCl2).

Cell fractionation

Cell fractionation was according to Olmsted (15), with minor modifications. All operations were performed at 4°C. Cells were homogenized in 0.24 M sucrose, 0.02 M MgCl2, 0.01 M Tris, pH 7.0 and sonicated three times during 10 seconds, centrifuged at 35 000 g for 30 minutes. The supernatant was centrifuged at 150 000 g for 15 minutes and the last supernatant used for protein fractionation.

Precipitation of microtubule protein with vinblastine

Vinblastine was added to the 150 000 g supernatant at a final concentration of 2×10^{-3} M. The mixture was incubated at 4°C for at least 30 minutes ; the precipitate was then sedimented at 150 000 g for 5 minutes. The supernatant was removed and the pellet resuspended in "lysis buffer" (9.5 M urea, 2 % w/v NP40, 2 % ampholin LKB, 5 % ß mercaptoethanol)for two dimensional gel electrophoresis as described by O'Farrel (17). The focusing time was 6 800 volt-hours. Second dimension gels were 10 % acrylamide with an acrylamide bis-acrylamide ratio of 100 : 2.7. Slab gels were fixed, stained, treated for fluorography (19), dried and exposed to Kodirex film.

Western blot and immunological detection

After a second dimension electrophoresis in 8 % acrylamide 0,08 % bis acrylamide, gels were blotted and immunologically detected as described by Towbin et al (19). After saturation in the presence of bovine serum albumine, blots were incubated with monoclonal anti ß tubulin serum (Amersham) diluted 1:3000. After washings, blots were incubated with the second antibodyindicator. As indicator anti-mouse IgG labelled with peroxidase was used. Revelation was made with DAB (3-3' diaminobenazidine tetrahydrochloride - Sigma).

RNA isolation and translation in vitro

Embryos were dechorionated by treatment with bleach, washed and homogenized in ten volumes of 0.7 % NaCl. Then they were disrupted and homogenized in 0.03 M Tris pH 8.3 ; 0.1 M NaCl ; 0.01 M CaCl2 in a Dounce homogenizer with a tight pestle. Large cellular debris and any unbroken embryos were removed by centrifugation for 10 Min. at approximately 10 000 x g at 4°C in a Sorvall centrifuge. RNAs were then prepared from the supernatant. RNAs were extracted from Kc 0 % cells and embryos by using sodium dodecyl sulphate/phenol chloroform procedure of Palmiter (20). Subsequently, poly A^+ RNAs were obtained through oligo (dT) cellulose chromatography (21). Cell free translation of mRNAs was performed using rabbit reticulocyte lysate (22) mix (Amersham) containing 10 µCi [^{35}S] methionine (25 µl). Translation products were analyzed by two dimensional gel electrophoresis. The gel was fluorographed to reveal radioactive spots.

RESULTS

1° Specific changes in polypeptide synthesis in Kc cells treated with 20-HE

Protein synthesis of Kc cells untreated or after treatment with the insect moulting hormone, 20-HE, was previously analysed (5).

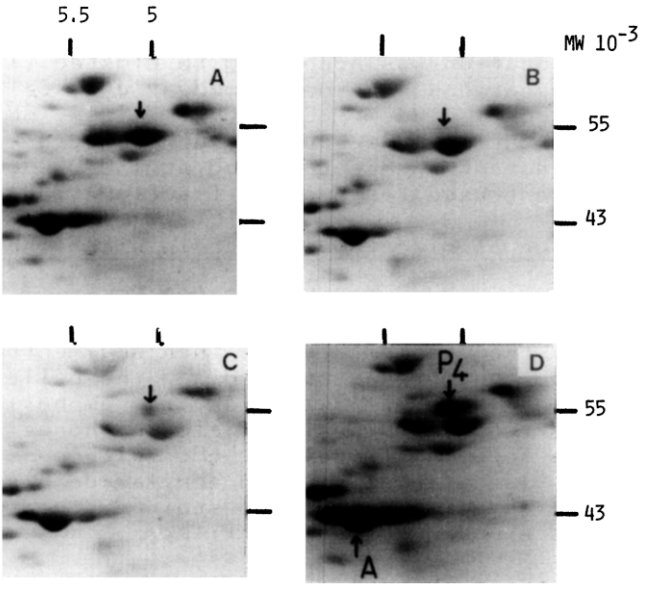

Figure 1 : Autoradiography of two dimensional gel electrophoresis of [^{35}S] methionine labelled proteins synthesized during 20-HE treatment (1 µM) of Drosophila melanogaster Kc 0 % cells. Only the region of interest of the gel is shown (between 40 000 and 60 000 daltons). "A" is actin. Untreated (A), 7 hours (B), 24 hours (C), 48 hours (D) of treatment.

Proteins were labelled with [^{35}S] methionine and separated on two-dimensional acrylamide gel electrophoresis. 20-HE induced specific changes in polypeptide synthesis : three are newly induced, two are repressed and six have increased rate of synthesis.

Now, if we focus in the range of polypeptides about 40 000 to 60 000 daltons and 4.5 to 6 of pHi, two qualitative differences are obvious (fig. 1). First, synthesis of actin (molecular weight 43 000 daltons) is increased when cells are treated with 20-HE for 7 hours (1 B), 24 hours (1 C) and 48 hours (1 D). This was previously described (5, 7). Morever one spot, P4, is present only in treated cells. Its isoelectric point is 5.1 and its molecular weight 55 000 daltons. P4 is not present in untreated cells (1 A). It is synthesized increasingly from seven hours to 48 hours of treatment (Fig 1 B, 1 C, 1 D).

2° A new ß tubulin subunit is synthesized in Kc cells treated by 20-HE

The electrophoretic properties of P4 are the same as tubulin subunits. Tubulin is known to precipitate with vinblastine sulfate

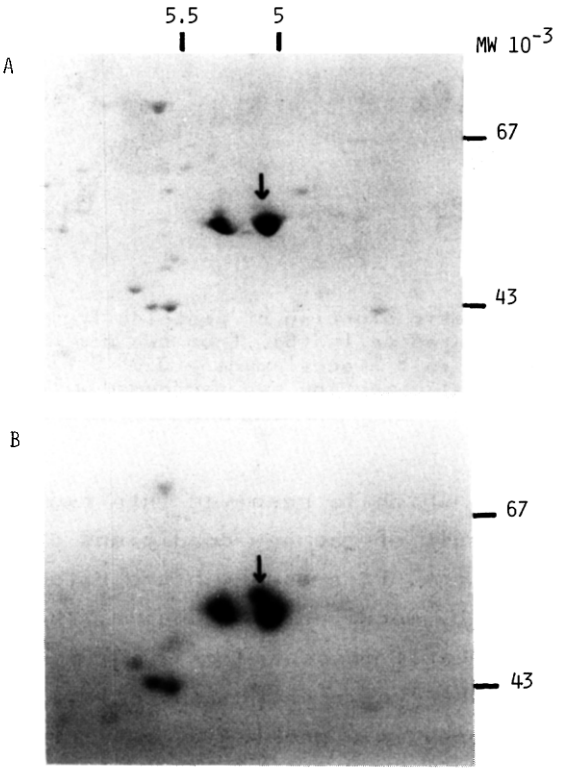

Figure 2 : Autoradiography of two dimensional gel electrophoresis of tubulin subunits after vinblastine precipitation from : untreated cells (A), 24 hour 20-HE treated cells (B). Only the area of the gel between 40 000 and 70 000 daltons is shown.

and this method, therefore, permits the rapid isolation of microtubule protein from crude supernatant of Kc cells.

Two dimensional gel electrophoresis of proteins from the pellet (Fig. 2) displays two classes of tubulin subunits : one of isoelectric point 5.3 is α and the other of isoelectric point 5.1 is ß. When Fig. 1 is compared to Fig. 2 A a purification is evident : two polypeptides are prominent on 2 A, these are tubulin subunits. On Fig. 2 B a third polypeptide is abundant, it is P4. So P4 which is newly synthesized in cells treated by 20-HE, behaves as a ß tubulin subunit.

In order to characterize P4, we have made immunoblots of gel electrophoregrams using monoclonal anti ß tubulin of mouse. The conditions of gel electrophoresis are modified. Three ß tubulin peptides (one major and two minor spots) are present in the immunoblots of proteins from untreated cells (Fig. 3A). One major spot is the ß1 constitutive tubulin subunit and the two minor spots correspond to

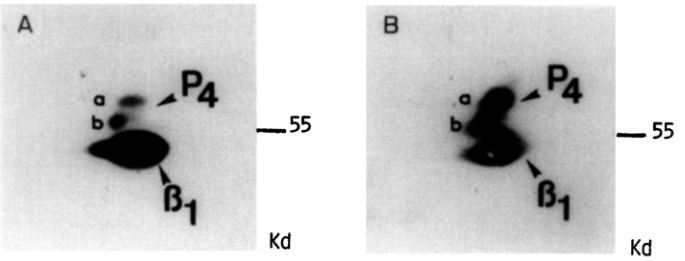

Figure 3 : Electrophoretic blotting of proteins from untreated cells (A) and treated cells (B), from two dimensional gel electrophoresis in 8 % acrylamide - 0.08 % bis-acrylamide. Immunological detection was performed with monoclonal anti ß tubulin and anti mouse IgG labelled with peroxidase.

the spot P4 of figure 1, which is resolved into two polypeptides (P4 a and P4 b) on a result of the new conditions of electrophoresis employed in this experiment. P4 a and P4 b are ß tubulin peptides since they are revealed by mouse anti ß tubulin. The concentration of these two peptides is greatly increased by 20-HE treatment (Fig 3B). The fact that we don't observe in untreated cells the presence of P4 peptides by autoradiography is a problem of sensitivity of detection in this method.

3° A mRNA is newly expressed for ß tubulin subunit synthesis

To know if regulation of expression by 20-HE of this ß tubulin subunit is at transcript level, mRNAs were prepared from untreated and treated cells and translated *in vitro* in a rabbit reticulocyte lysate system. As shown in Figure 4, translation products are analysed by two dimensional polyacrylamide gel electrophoresis, before (Fig. 4 A and 4 B) and after vinblastine precipitation (Fig. 4 C and 4 D). When mRNAs are extracted from untreated cells, this tubulin subunit is not synthesized (Fig. 4 A and 4 C). When mRNAs are derived from 20 hour treated cells, it is present (Fig. 4 B and 4 D). These results are identical to those observed in *in vivo* experiments.

These results show that a tubulin new mRNA is expressed in Kc treated cells. This expression may occur by several ways as discussed below. But *in vitro* translation results exclude the possibility of a post-translationnal modification induced by 20-HE in Kc cells.

DISCUSSION

In this paper we describe synthesis of a new ß tubulin subunit when Drosophila melanogaster Kc cells are treated with the insect

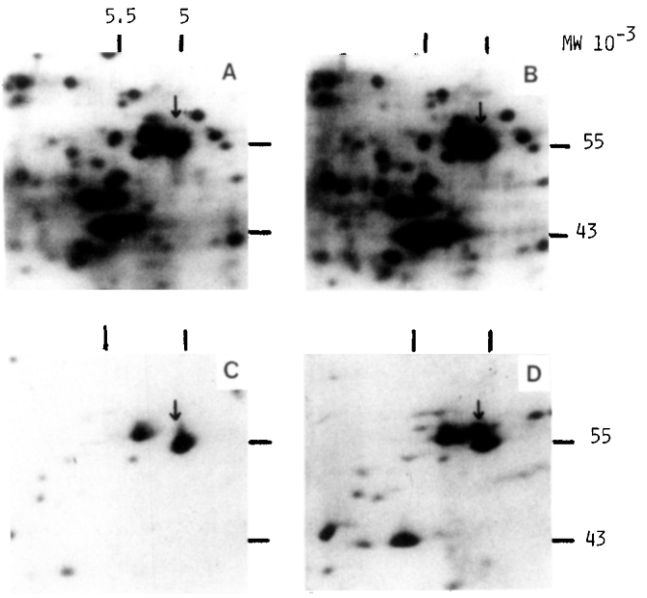

Figure 4 : Autoradiography of two dimensional gel electrophoresis of translation products obtained in a rabbit reticulocyte lysate system, in the presence of [^{35}S]methionine using mRNA extracted from untreated cells (A) and 20-HE treated cells : 20 hours (B). Translation products precipitated with vinblastine sulfate : from untreated cells (C) and 20 hour 20-HE treated cells (D).

moulting hormone, 20-HE. Present results are in agrement with Berger (23) who reported that total tubulin synthesis is not really affected by 20-HE. Based on the present results, we can conclude that there is an increase in tubulin microheterogeneity due to 20-HE treatment.

Raff et al (11) have demonstrated new synthesis of ß3 tubulin subunit at mid period of embryogenesis (stage 8 hr-13 hr). Using the same electrophoresis analysis, it seems that P4 and ß3 are the same polypeptide. In order to confirm this correlation, mRNAs are prepared from 0 hr-6 hr and 8 hr-13 hr old embryos. They are then translated *in vitro* in a rabbit reticulocyte lysate system. Translation products are analysed by two dimensional gel electrophoresis before (Fig. 5 A and 5 B) and after vinblastine precipitation (Fig. 5 C and 5 D). ß3, as Raff et al (11) have previously described is stricly synthesized in 8 hr-13 hr old embryos, it is not present in stage 0 hr-6 hr of embryogenesis. In these conditions, ß3 comigrates with P4.

Gietz and Hodgetts (24) have recently analyzed dopadecarboxylase expression as function of 20-HE titer during embryogenesis of

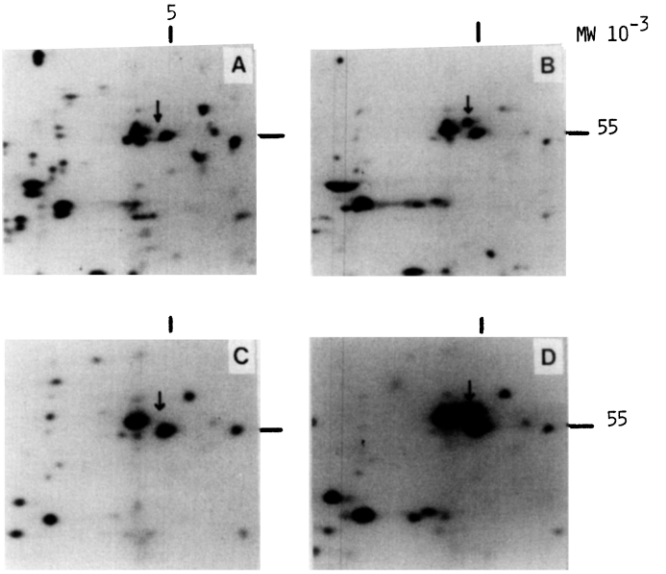

Figure 5 : Autoradiography of two dimensional gel electrophoresis of translation products obtained in a reticulocyte lysate system, in the presence of [^{35} S] methionine with mRNA extracted from 0 hr-6 hr embryos (A and C) or 8 hr-13 hr old embryos (B and D) before (A and B) and after (C and D) vinblastine precipitation.

Drosophila : ecdysteroid titers begin to increase early and reach a maximum at midembryogenesis (8 hr). This peak is followed by an increase of dopadecarboxylase transcripts followed by an increase of dopadecarboxylase activity. This activity is also stimulated by 20-HE in Kc cells (25). We observe that synthesis of ß3 occurs immediatly after the first peak of 20-HE in embryo. In our cells an ecdysone inducible tubulin comigrates with ß3. Morever Kc cells have their origi in embryos (14).

Our data clearly demonstrate that the control of this tubulin subunit expression is at the mRNA level. The response to 20-HE is very rapid, puffs appearing within minutes as described by Ashburner (26) ; so a new mRNA may be synthesized. We observe a very rapid response in embryo (ecdysone peak at 8 hours and synthesis of tubulin subunit in 8 hr-13 hr stage). In kc cells, ecdysone inducible tubulin is synthesized 7 hours after treatment and perhaps early.

We can speculate that one tubulin gene is hormonally controlled. The new mRNA may be transcribed from a gene not previously expressed or from a gene already expressed but with a different splicing in the presence of 20-HE. On the other hand, message for this

tubulin subunit may be present, but a change in its translatability or stability may occur in presence of 20-HE.

Microtubules are made up of heterodimers of one α and ß tubulin polypeptide. In Drosophila, the presence of four α and four ß tubulin gene sequences has been demonstrated, each of which is represented only one per haploid genome. Kalfayan and Wensink (27), and Raff et al (11) have shown that each of the four α genes and three of the ß genes are functionnal genes which are differentially expressed : two tubulin subunits are differentially expressed at different times in development ; the testis specific ß2 tubulin and the ß3 tubulin are expressed only during the mid period of embryogenesis. Other tubulins i.e. ß1 tubulin and α1 and α2 tubulin are expressed throughout development and in all tissues. The observation of the stage and time specific synthesis of ß3 tubulin and the effect of 20-HE on the synthesis of this particular subunit leads to the speculation that ß3 tubulin may play a functional role in differentiation and morphogenesis.

Our current work is to clarify the molecular mechanisms of differential expression of this gene. We wish to know if variation in protein synthesis is mainly due to modification in transcripts level as suggested in the present in vitro translation experiment.

Modifications of actin and tubulin synthesis are previously be correlated with morphological changes and motility observed after ecdysterone treatment of cells in culture. But cytoskeletal proteins also play an important role in intracellular organization and this role is not only structural but also functional.

ACKNOWLEDGEMENTS

This work was supported by INSERM CRE n° 83 1008 and CNRS LT/HL n°3366. The authors thank P. DENOULET and B. EDDE for their help for immunoblot and immunological detection techniques.

One of the authors (MLS) is a recipient of a fellowship from the "Fondation pour la Recherche Médicale".

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