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Counteraction by 20-hydroxyecdysone of the effect of juvenile hormone on phosphorylation of ribosomal protein S6

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Phosphorylation of ribosomal protein S6, induced when the fat body of *Sarcophaga peregrina* (flesh-fly) larvae was incubated in vitro in the presence of ^{32}P, was found to be suppressed by juvenile hormone. This suppressive effect of juvenile hormone was counteracted by a physiological concentration of 20-hydroxyecdysone. Since 20-hydroxyecdysone is known to induce phosphorylation of S6 in vivo, this reaction was suggested to be regulated by the balance of the effects of ecdysone and juvenile hormone.

Ecdysone; Juvenile hormone; Ribosomal protein S6; Phosphorylation

1. INTRODUCTION

Steroid hormone is thought to bind to its cytoplasmic receptor, be transferred to nuclei and there activate specific genes [1–3]. The action of the insect molting hormone ecdysone may occur in the same way, because this hormone is known to cause specific activation of the DOPA-decarboxylase gene of *Calliphora erythrocephala* [4,5]. On the other hand, various larval cells are known to respond to ecdysone in different ways, suggesting that this hormone has pleiotropic functions. Thus, ecdysone may have another mode of action; namely direct interaction with cellular components, resulting in specific cellular responses without associated selective activations of genes.

Actually, we have demonstrated that 20-hydroxyecdysone activates cryptic storage protein receptors on the surface of fat body cells of *Sarcophaga peregrina* larvae in the absence of protein synthesis [6,7]. Therefore, for understanding the complex mechanisms of action of ecdysone, the direct interactions of this hormone with cellular components must be examined.

Recently, we reported that 20-hydroxyecdysone induces the phosphorylation of a 30-kDa protein in the fat body of *Sarcophaga* larvae, without associated synthesis of new protein [8]. This 30-kDa protein was identified as ribosomal protein S6 [9]. Phosphorylation of S6 has been reported to be induced in various cells in response to hormonal stimuli, but its physiological meaning is not clear. We found that the phosphorylation of S6 in vitro was suppressed by juvenile hormone and stimulated by 20-hydroxyecdysone under certain conditions. Thus, this reaction may be regulated by juvenile hormone and ecdysone in the fat body of *Sarcophaga* larvae.

2. MATERIALS AND METHODS

2.1. *Animals*

The flesh-fly, *S. peregrina*, was reared by the method of Ohtaki [10]. Third instar larvae were kept in plastic containers with a little water at room temperature. Under these conditions, the

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secretion of ecdysone was completely suppressed, and so the larvae did not pupate. Larvae could be used for experiments for up to 5 days after leaving their food.

2.2. *Phosphorylation of fat body S6 in vitro*

Each larva was treated with 20 μCi carrier-free ^{32}P dissolved in 5 μl saline. After 4 h, the fat body was excised, rinsed well in saline, and then incubated in 0.3 ml Grace's insect medium (Gibco, hemolymph-free) in the presence of 100 μCi ^{32}P for 2 h at 27°C. No significant phosphorylation of S6 was detected during the labeling period in vivo, as reported in [8]. However, this process was essential for the induction of efficient phosphorylation of S6 in the subsequent labeling experiment in vitro. An appropriate concentration of juvenile hormone II (Sigma, *cis*-10,11-epoxy-3,7,11-triethyl-*trans,trans*-2,6-tridecadienoic acid methyl ester) or 20-hydroxyecdysone (Rohto Seiyaku, Japan) was added to the culture medium. Juvenile hormone II was stored at −80°C in olive oil and diluted with ethanol just before use.

2.3. *Analysis of labeled S6 by electrophoresis*

Labeled fat bodies from 3 larvae were combined and homogenized with a Teflon homogenizer in 0.2 ml of 0.01 M Tris-HCl buffer, pH 7.5, containing 0.01 M NaH_2PO_4, 0.08 M KCl, 12.5 mM $MgCl_2$ and 0.2 M sucrose. About 10 μl of the resulting lysate was subjected to electrophoresis. Protein in an aliquot was precipitated with cold 10% trichloroacetic acid, dissolved in 20 μl of 0.19 M Tris-HCl buffer, pH 8.8, containing 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue, heated for 20 min at 75°C and then subjected to SDS-polyacrylamide slab gel electrophoresis by the method of Laemmli [11]. The separating gel contained 12.8% acrylamide. After electrophoresis, the gels were dried and autoradiographed with Kodak O-Mat X-ray film. Total radioactivity incorporated into fat body protein was determined by measuring the radioactivity of hot trichloroacetic acid-insoluble material.

3. RESULTS AND DISCUSSION

When ^{32}P was injected with 20-hydroxyecdysone into third instar larvae of *S. peregrina*, significant

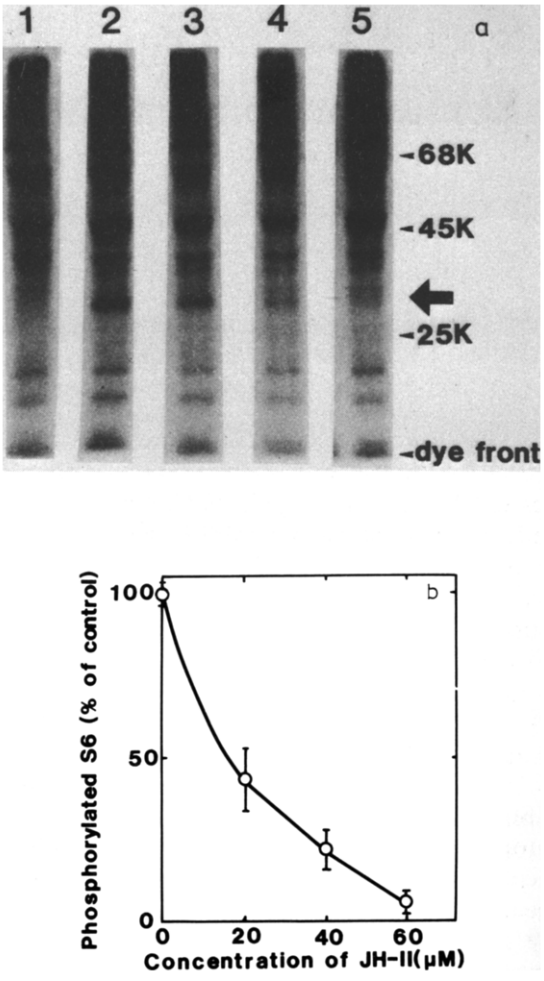

Fig.1. (a) Phosphorylation of S6 in vitro and its suppression by JH II. Fat body from third instar larvae of *Sarcophaga* prepared as described in section 2 was incubated in Grace's insect medium with increasing amounts of JH II in the presence of ^{32}P for 2 h. Then the homogenate of fat body was subjected to SDS-polyacrylamide electrophoresis and autoradiography to detect phosphorylated protein. Ribosomal protein S6 was identified as in [10]. Lanes: 1, fat body before incubation (control); 2, fat body incubated in the absence of JH II; 3–5, fat body incubated in the presence of 20, 40 and 60 μM JH II, respectively. The following molecular mass markers were used: bovine serum albumin (68 kDa), ovalbumin (45 kDa) and chymotrypsinogen (25 kDa). The arrow indicates the position of S6. (b) Densitometric scanning of the phosphorylated S6. Bands corresponding to S6 in lanes 2,3 in (a) were scanned, and relative density is plotted vs concentration of JH II.

phosphorylation of a protein of 30 kDa was detected [8]. This protein was a ribosomal protein S6, as reported before [9]. In contrast to this phosphorylation of S6 only in the presence of 20-hydroxyecdysone in vivo, phosphorylation of S6 was found to be induced even in the absence of 20-hydroxyecdysone in vitro, when the fat body from third instar larvae was incubated with ^{32}P, and the resulting labeled protein was analyzed by SDS-polyacrylamide gel electrophoresis, as shown in fig.1a (lane 2).

In these experiments, we used mature larvae, in which ecdysone should be stored in the ring gland, although it is not secreted. Thus, the fat body may have been contaminated with endogenous ecdysone while it was being excised. Another possibility was that the effect of injury of the fat body during its excision mimicked the action of ecdysone. But irrespective of the reason, it was clear that S6 was phosphorylated under these conditions.

Interestingly, this phosphorylation of S6 was suppressed by addition of juvenile hormone II (JH II) to the culture medium, as shown in fig.1a (lanes 3,4). The density of the band corresponding to S6 was plotted vs concentration of JH II. As is evident from fig.1b, phosphorylation was suppressed more than 50% by 20 μM JH II and almost completely by 60 μM JH II. Phosphorylation of other proteins was apparently not affected by JH II, indicating that JH II suppressed the phosphorylation of S6 selectively under these conditions. Conceivably ecdysone stimulated and juvenile hormone suppressed the phosphorylation of S6, and ribosomes containing phosphorylated S6 preferentially translated a certain class of mRNA, the translation products of which were required for pupation. Therefore, we tested whether 20-hydroxyecdysone counteracted the effect of JH II under these conditions. Addition of a final concentration of 10^{-7} M 20-hydroxyecdysone to the culture medium of fat body containing 100 μM JH II counteracted the inhibitory effect of JH II on phosphorylation of S6 and S6 was significantly phosphorylated, as shown in fig.2a (lane 2). However, induction of the phosphorylation of S6 under these conditions depended upon the concentration of 20-hydroxyecdysone and 10^{-6} M 20-hydroxyecdysone was much less effective, as shown in lane 3. The densitometric scanning of the band of S6, shown in fig.2b, showed that restoration of phosphorylation by 10^{-6} M 20-hydroxyecdysone was only 30% of that by 10^{-7} M 20-hydoxyecdysone, which is a physiological concentration to induce metamorphosis of this insect [12].

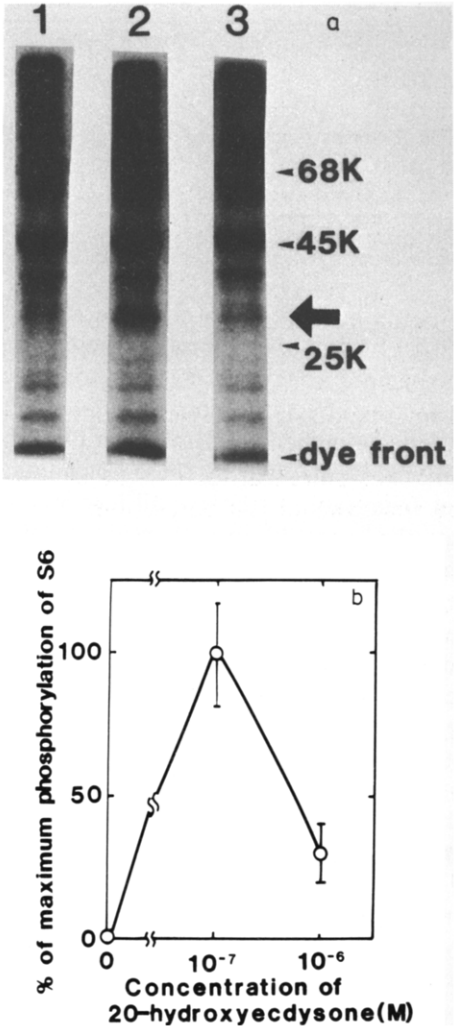

Fig.2. (a) Counteraction of the effect of JH II by 20-hydroxyecdysone. Fat body was incubated in the presence of 100 μM JH II and various concentrations of 20-hydroxyecdysone. The procedure was as described in the legend to fig.1. Lanes: 1, fat body incubated with 100 μM JH II (control); 2,3, fat body incubated with 100 μM JH II in the presence of 10^{-7} and 10^{-6} M 20-hydroxyecdysone, respectively. (b) Densitometric scanning of the phosphorylated S6. Bands corresponding to S6 were scanned and the relative density is plotted vs concentration of 20-hydroxyecdysone.

Table 1

Incorporation of ^{32}P into the hot trichloroacetic acid-insoluble fraction of fat body under various incubation conditions

Hormones added to medium	Radioactivity (cpm)
None	2048 ± 137
100 μM JH II	1916 ± 177
100 μM JH II + 10^{-7} M 20-hydroxyecdysone	2136 ± 399
100 μM JH II + 10^{-6} M 20-hydroxyecdysone	2144 ± 244

Values are averages for three fat bodies

Therefore, a higher concentration of the hormone seemed to be rather inhibitory. These results indicate that ecdysone and juvenile hormone have counteracting effects on phosphorylation of S6.

We tested whether overall protein phosphorylation in vitro is affected by these hormones. As is evident from table 1, the overall incorporation of ^{32}P into the hot trichloroacetic acid-insoluble fraction of the fat body was almost constant and not affected appreciably by the presence of these hormones in the culture medium.

From the present results, we suggest that if the concentration of 20-hydroxyecdysone in the hemolymph reaches a physiological level, it may counteract the effect of juvenile hormone and induce the phosphorylation of S6 in fat body cells, which is a prerequisite for metamorphosis of *Sarcophaga*. The phosphorylation of S6 detected in vitro does not necessarily reflect that in vivo, since it proceeded even in the absence of 20-hydroxyecdysone. However, we believe that this reaction in vitro is meaningful, because many proteins were phosphorylated, but JH II selectively inhibited the phosphorylation of only S6.

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