Induction of selective phosphorylation of a fat body protein of *Sarcophaga peregrina* larvae by 20-hydroxyecdysone

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20-Hydroxyecdysone was shown to induce selective phosphorylation of a fat body protein of *Sarcophaga peregrina* larvae with a molecular mass of 30 kDa. This phosphorylation was not associated with synthesis of new protein. Fractionation of ^{32}P -labelled fat body by differential centrifugation showed that this protein was mainly present in the membrane-rich fraction, although we could not specify the membrane. Thus, 20-hydroxyecdysone may modify the function of the fat body by inducing phosphorylation of a specific membrane protein.

Previously we demonstrated that 20-hydroxyecdysone induces a cryptic storage protein receptor in the fat body of *Sarcophaga peregrina* larvae to become active in binding storage protein, and that this activation is not associated with any appreciable protein synthesis (Ueno & Natori, 1982; Ueno *et al.*, 1983; Ueno & Natori, 1984). Since the storage protein receptor is a membrane protein, it is likely that the target of 20-hydroxyecdysone in this case is the fat body membrane. On the other hand, as with the usual steroid hormones, ecdysone is known to activate specific genes. For instance, it was shown to activate the DOPA (dihydroxyphenylalanine) decarboxylase gene of *Calliphora erythrocephala* specifically, resulting in quinone tanning after puparium formation (Karlson, 1965; Sekeris *et al.*, 1971). It was also found to cause transient activation of ribosomal RNA synthesis in the fat body and salivary gland of *Sarcophaga peregrina* (Natori, 1976; Nakano & Natori, 1978).

Thus conceivably 20-hydroxyecdysone has pleiotropic actions, and its cellular targets are in both the membrane and cytoplasm. In fact, the existence of a cytoplasmic receptor for this hormone was demonstrated by use of a highly radioactive analogue of the hormone, ponasterone A (Maroy *et al.*, 1978; Yund *et al.*, 1978; Sage *et al.*, 1982).

Since we are interested in the effect of 20-hydroxyecdysone on the fat body, especially in relation to activation of the storage protein receptor, we studied biochemical events in the fat body

provoked by this hormone. We found that a protein with a molecular mass of 30 kDa was specifically phosphorylated *in vivo* when 20-hydroxyecdysone was administered to third instar larvae of *Sarcophaga*. This phosphorylation did not involve new protein synthesis. This is the first demonstration that 20-hydroxyecdysone stimulates the phosphorylation of a specific protein in target cells.

Materials and methods

Animals

The flesh-fly, *Sarcophaga peregrina*, was reared by the method of Ohtaki (1966). Third instar larvae were kept in plastic containers under wet conditions in which the secretion of ecdysone was inhibited. When transferred to dry conditions, they started to pupate after 16 h when kept at 27°C. Larvae could be used for experiments for up to 5 days after leaving their food.

Injections

Larvae were immobilized by placing them on ice. Then 5 μl of buffered saline (150 mM-NaCl in 10 mM-Tris/HCl buffer, pH 7.0) containing 1 mM-20-hydroxyecdysone (Rohto Seiyaku, Japan) or 2.2 mM-cycloheximide (Wako Junyaku, Japan) or 25 mM-emetine dihydrochloride (Sigma) was injected into the posterior part of the abdominal cavity with a microsyringe under a binocular microscope. The radioactive compound injected was 30 μCi of $^{32}\text{P}_i$ (carrier-free; New England Nuclear) dissolved in 5 μl of the buffered saline.

Abbreviation used: SDS, sodium dodecyl sulphate.

Labelling of fat body and preparation of fat body homogenate

Larvae were injected with 30 μ Ci of $^{32}\text{P}_i$ and kept at 27°C for an appropriate time. The fat bodies were dissected out under a binocular microscope, suspended in 128 μ l of buffer A [10 mM Tris/HCl buffer, pH 8.8, containing 10 mM NaH_2PO_4 , 5 mM MgCl_2 and aprotinin (0.15 trypsin inhibitor unit/ml; Boehringer Mannheim)] and homogenized in a glass homogenizer at below 4°C. Then 32 μ l of 5 mM-2-mercaptoethanol containing 2% (w/v) SDS and 1 μ l of 1 M-phenylmethanesulphonyl fluoride in acetone was added, followed by 16 μ l of nuclease solution [containing, per ml, a mixture of 6250 Kunitz units of bovine pancreatic deoxyribonuclease I (Sigma) and 7000 Kunitz units of bovine pancreatic ribonuclease A (Sigma) in 0.5 M-Tris/HCl buffer, pH 7.0, containing 50 mM- MgCl_2]. The volume was increased by adding buffer A and 8 μ l of 40 mM-EDTA solution, and the resulting homogenate was stored at -80°C.

Fractionation of fat body and enzyme assay

Fractionation was done essentially as described before (Natori & Ohtaki, 1976). Fat bodies from five larvae were homogenized in 1 ml of buffer B (10 mM-Tris/HCl buffer, pH 7.4, containing 10 mM- MgCl_2 , 4 mM-phenylmethanesulphonyl fluoride, 0.15 trypsin inhibitor units of aprotinin/ml and 0.25 M-sucrose). The homogenate was centrifuged at 1000g for 5 min at 4°C. The precipitate was washed once and suspended in 0.5 ml of buffer B (1000g precipitate fraction). The supernatant was centrifuged at 10000g for 30 min at 4°C, and the precipitate was washed once and suspended in 0.5 ml of buffer B (10000g precipitate fraction). The supernatant was further centrifuged at 100000g for 3 h at 4°C, and the pellet was washed once and suspended in 0.3 ml of buffer B (100000g precipitate fraction). The final supernatant was defined as the cytosol fraction. Each fraction was treated with 1/10 volume of nuclease solution if necessary and stored at -80°C. Marker enzymes were assayed as follows: cytochrome *c* oxidase for mitochondria was assayed as described by Wharton & Tzagoloff (1967), 5'-nucleotidase for plasma membranes was as described by Michell & Hawthorne (1965), acid phosphatase for lysosomes was as described by Michell *et al.* (1970), and glucose-6-phosphatase for microsomes was as described by Swanson (1955).

Polyacrylamide-gel electrophoresis and autoradiography

SDS/polyacrylamide-slab gel electrophoresis was performed by the method of Laemmli (1970).

The separating gel contained 12.8% acrylamide. Samples were denatured by heating at 75°C for 20 min in electrophoresis sample buffer [50 mM-Tris/HCl, pH 8.8, 10% (v/v) glycerol, 3% (w/v) SDS, 0.005% Bromophenol Blue and 7.4% (v/v) 2-mercaptoethanol]. For estimation of the molecular mass of phosphorylated proteins, the following marker proteins were used: phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (44.6 kDa), α -chymotrypsinogen (25 kDa) and cytochrome *c* (12.4 kDa). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 by the method of Fairbanks *et al.* (1971), and then dried and autoradiographed with Kodak X-Omat X-ray film.

For quantitative measurement of the phosphorylation of the 30 kDa protein, the autoradiograms were scanned at 590 nm, and from the weight of chart paper corresponding to the peak, the relative amount of phosphorylated protein was calculated on the basis of the amount of radioactivity applied to the gel. Values were determined in the range where the intensity of the band increased linearly with the amount of radioactivity applied.

Measurement of protein

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Selective phosphorylation of 30 kDa protein in fat body on pupation

Third instar larvae of *Sarcophaga peregrina* were injected with $^{32}\text{P}_i$, kept under wet conditions for 4 h, and then transferred to dry conditions. After 16 h, when the larvae were starting to pupate, their fat bodies were dissected out, homogenized and subjected to SDS/polyacrylamide-gel electrophoresis. As a control, fat bodies from larvae kept under wet conditions for the same period were examined. As is evident from Fig. 1(a), a protein with a molecular mass of 30 kDa was selectively phosphorylated in the fat body of early pupae, and no such phosphorylation was detected in the fat body of control larvae kept under wet conditions. Since there was no other significant difference between the patterns of phosphorylated protein in the two homogenates, we concluded that the phosphorylation was probably due to the effect of moulting hormone, and that the phosphorylation was an intermediate step in transmission of the hormonal stimulus. To confirm this, we injected third instar larvae, first with $^{32}\text{P}_i$ and then 4 h later with 20-hydroxyecdysone to see if the hormone induced selective phosphorylation of the 30 kDa

Fig. 1. Induction of phosphorylation of 30 kDa protein

(a) Larvae were injected with $^{32}\text{P}_i$. After 16 h, the fat body was removed, homogenized and subjected to SDS/polyacrylamide-gel electrophoresis followed by autoradiography. Lane 1, control (fat body from larvae kept under wet conditions); lane 2, fat body from larvae kept under dry conditions. (b) Larvae were injected with 20-hydroxyecdysone and $^{32}\text{P}_i$, and kept under wet conditions. After 8 h, the fat body was removed and analysed. Lane 1, control (fat body from larvae injected with saline); lane 2, fat body from hormone-treated larvae. Gels were calibrated with molecular mass markers.

protein. The larvae started to pupate 8 h after these treatments, and at this time, we examined phosphorylation of fat body protein. As shown in Fig. 1(b), phosphorylation of 30 kDa protein was detected in the fat body of hormone-treated larvae, but not of control larvae, indicating that 20-hydroxyecdysone is essential for the phosphorylation of this protein. These experiments did not show whether the 30 kDa protein was newly synthesized by the stimulus of 20-hydroxyecdysone or whether phosphorylation of pre-existing 30 kDa protein was activated by this hormone. However, the latter was probably the case since the intensity of the band corresponding to 30 kDa protein on the gel stained with Coomassie Brilliant Blue did not increase appreciably on hormone treatment (results not shown).

To determine the time course of phosphorylation, we removed the fat body from ^{32}P -labelled larvae at various times after administration of 20-hydroxyecdysone and examined the phosphorylation of 30 kDa protein by scanning the intensity of the band corresponding to the 30 kDa protein in the autoradiograms. As shown in Fig. 2, phosphorylation of the 30 kDa protein was not obvious 2 h after administration of the hormone, because slight phosphorylation of this protein was transiently detected at this stage in the fat body of control larvae injected with saline. However, the extent of phosphorylation increased thereafter, reaching a maximum level at about 6 h, and this maximal level persisted for several hours. Probably

the phosphorylation of 30 kDa protein is one of the early events induced by 20-hydroxyecdysone in the fat body.

Localization of phosphorylated 30-kDa protein in the fat body

For determination of the localization of 30 kDa protein in the fat body, larvae injected with $^{32}\text{P}_i$ were kept at 27°C under dry conditions for 16 h, and then their fat body was removed. The fat bodies from five larvae were combined, and separated into 1000g precipitate fraction, 10000g precipitate fraction, 100000g precipitate fraction and 100000g supernatant fraction as described in the Materials and methods section. Each fraction was subjected to SDS/polyacrylamide-gel electrophoresis, and the content of 30 kDa protein in each fraction was determined by measuring the radioactivity in the gel slice corresponding to 30 kDa protein. As shown in Fig. 3(a), phosphorylated 30 kDa protein was mainly recovered in the 10000g precipitate fraction. In order to characterize this fraction biochemically, we measured the distributions of four marker enzymes. As shown in Figs. 3(b)–3(e), the 10000g precipitate fraction contained a greater part of cytochrome c oxidase (a marker enzyme for mitochondria), 5'-nucleotidase (a marker enzyme for plasma membrane) and acid phosphatase (a marker enzyme for lysosomes), but no significant glucose-6-phosphatase (a marker enzyme for microsomes) was detected in this fraction. These results suggest that the 10000g pre-

Fig. 2. Time course of phosphorylation of 30kDa protein. Larvae were injected with 20-hydroxyecdysone and $^{32}\text{P}_i$. Fat bodies were removed at the indicated times and analysed by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. Autoradiograms were scanned for quantitative measurement of phosphorylation of 30kDa protein as described in the Materials and methods section. ●, Hormone-treated larvae; ○, control larvae injected with saline; Δ, ratio of phosphorylations in control and treated larvae.

citrate fraction is a membrane-rich fraction and contained a mixture of the membranes from various organelles except microsomes. Thus, it is possible that the 30kDa protein is present as a component of one of these membranes, and is selectively phosphorylated under dry conditions.

Effects of inhibitors of protein synthesis on the phosphorylation of 30kDa protein

Since phosphorylation of 30kDa protein occurs in the membrane of the fat body in the presence of 20-hydroxyecdysone, this phosphorylation was expected to take place without accompanying new protein synthesis. To confirm this, we investigated the effect of the inhibitors of eukaryotic protein synthesis, emetine and cycloheximide, on the phosphorylation of 30kDa protein.

Larvae were injected with 125 nmol/animal of emetine and $^{32}\text{P}_i$, and 4 h later with 20-hydroxyecdysone. The fat body was removed 8 h later and phosphorylation of 30kDa protein was examined by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. Under these conditions, incorporation of [^{35}S]methionine into the acid-insoluble fraction of the fat body was almost completely inhibited (results not shown). As shown in Fig. 4(a), phosphorylation of 30kDa protein was not detected when 20-hydroxyecdysone was not injected (lane 1), and was enhanced more after

injection of both 20-hydroxyecdysone and emetine (lane 2) than after injection of 20-hydroxyecdysone alone (lane 3). Therefore, it is clear that phosphorylation of the 30kDa protein occurs without accompanying new protein synthesis. It is unknown why emetine enhanced the phosphorylation of 30kDa protein in the presence of 20-hydroxyecdysone. In order to examine the effect of another inhibitor of protein synthesis, the same experiment was performed with 11.1 nmol/animal of cycloheximide. In this case, phosphorylation of the 30kDa protein was clearly detected even in the absence of 20-hydroxyecdysone (lane 1), indicating that cycloheximide itself can induce the phosphorylation of the 30kDa protein under appropriate conditions. At the moment, it is uncertain whether the protein phosphorylated in the presence of cycloheximide is the same as that phosphorylated in the presence of 20-hydroxyecdysone, but this protein showed the same mobility as the latter on polyacrylamide gels.

Discussion

In this paper we describe the selective induction by 20-hydroxyecdysone of phosphorylation of a 30kDa protein in the fat body of *Sarcophaga* larvae. This phosphorylation occurs before puparium formation, and thus is one of the early events provoked by this hormone.

There are probably two main mechanisms of action of ecdysone. Like many other steroid hormones, it probably activates specific genes and in addition it acts on the cell membrane, resulting in physiological changes in the cells. Actually, ecdysone was shown to be localized in the puffs of the salivary glands of *Drosophila melanogaster* (Gronemeyer & Pongs, 1980), and Sekeris *et al.* (1971) clearly demonstrated that it induces DOPA decarboxylase by directly activating the gene for this enzyme. Activation of the storage protein receptor by this hormone is an action on the cell membrane (Ueno & Natori, 1984). Most larval tissues are known to respond to ecdysone, and the diversity of the responses of different tissues may depend on differences in transmissions of the primary stimulus of the hormone.

Phosphorylation of 30kDa protein in the fat body of *Sarcophaga* larvae is likely to be a step in the transmission of the stimulus of 20-hydroxyecdysone. Since this reaction occurs under conditions where new protein synthesis was inhibited, it is probably independent of the activation of specific genes. Our findings are very similar to those on the effect of progesterone on *Xenopus* oocytes. Namely, during maturation of *Xenopus* oocytes, progesterone is known to induce phosphorylation of protein (Maller *et al.*, 1977; Nielsen

Fig. 3. Distributions of phosphorylated 30kDa protein and marker enzymes in the fat body

(a) Larvae were injected with $^{32}\text{P}_i$ and kept under dry conditions. After 16h, the fat body was removed, homogenized and fractionated as described in the Materials and methods section. Each fraction was subjected to SDS/polyacrylamide-gel electrophoresis and the radioactivity in the gel slice corresponding to 30kDa protein was measured. Marker enzyme activities in these fractions are shown in (b)–(e): (b) cytochrome c oxidase, (c) 5'-nucleotidase, (d) acid phosphatase and (e) glucose-6-phosphatase. Activities were normalized to those in ten larvae. The right ordinate shows the percentage of activity in the total homogenate, and the recovery (R) indicates the sum of percentage activity in each fraction. The recovery of enzyme activity was not 100% except for glucose-6-phosphatase. Enzymes may be adsorbed to fat fraction which floats up to make a thick layer on the top of the centrifugation tube during centrifugation. Column 1, 1000g precipitate fraction; column 2, 10000g precipitate fraction; column 3, 100000g precipitate fraction; column 4, cytosol fraction.

Fig. 4. Effects of emetine and cycloheximide on phosphorylation of 30kDa protein

Larvae were injected with 125 nmol/animal of emetine (a) or 11.1 nmol/animal of cycloheximide (b) and $^{32}\text{P}_i$. After 4h, 20-hydroxyecdysone was injected and the fat body was removed 8h later for analysis. Lane 1, saline was injected instead of 20-hydroxyecdysone; lane 2, 20-hydroxyecdysone was injected; lane 3, 20-hydroxyecdysone was injected but inhibitor was not injected in advance; lane 4, control (neither 20-hydroxyecdysone nor inhibitor was injected). Gels were calibrated with molecular mass markers.

et al., 1982a); the progesterone seems to act on the cell membrane and to induce phosphorylation of pre-existing proteins.

In this present paper cell fractionation experiments showed that the 30 kDa protein was concentrated in the 10000g precipitate fraction, and thus it is likely to be a membrane protein. However, it should be mentioned that ribosomal protein S6 is known to be phosphorylated in response to various growth stimuli (Gressner & Wool, 1974a; Thomas *et al.*, 1980; Lastick *et al.*, 1980). The molecular mass of S6 is 32 kDa, and it is phosphorylated when *Xenopus* oocytes are treated with progesterone (Nielsen *et al.*, 1982a). Moreover, cycloheximide was found to induce the phosphorylation of S6 (Gressner & Wool, 1974b; Nielsen *et al.*, 1982b). In these respects, 30 kDa protein is quite similar to S6. Thus, there is a possibility that the 30 kDa protein is S6, although it was concentrated in the membrane-rich fraction when the fat body was fractionated. A difference between 30 kDa protein and S6 seems to be in its isoelectric point. We found that the isoelectric point of phosphorylated 30 kDa protein is more basic than that of S6 (results not shown). However, more precise experiments are needed to clarify the exact relation of the 30 kDa protein with S6.

At the moment nothing is known about the biological significance of phosphorylation of the 30 kDa protein. The larval fat body is destined to be disintegrated during metamorphosis, and replaced by newly formed adult fat body. Therefore, phosphorylation of 30 kDa protein may be a signal to shut off fat body function.

We are very interested in the relation between phosphorylation of the 30 kDa protein and activation of the storage protein receptor on the surface of fat body cells. If the 30 kDa protein is a plasma membrane protein, its phosphorylation may be intimately related to activation of the storage protein receptor, since uptake of storage protein from the haemolymph is the last important role of the fat body before its decomposition.

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