Phosphate incorporation into secretory protein of *Chironomus* salivary glands occurs during translation

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The Balbiani rings (BR) in *Chironomus* salivary gland cells code for giant secretory proteins, the sp-I family. During normal growth conditions the phosphorylated proteins sp-Ia and sp-Ib are formed with most phosphate present as phosphoserine. We can show that most if not all incorporation of ^{32}P into sp-I occurs in parallel with the incorporation of [^{35}S]-methionine in the giant polysomes that form sp-I and contain BR-derived mRNA. We suggest that the main function of phosphorylation of sp-Ia and sp-Ib is to provide charge neutralization of an excess of lysine and arginine residues and is therefore required during early stages of protein folding. This view is supported by the previous observation that glutamic (and aspartic) acid largely substitute for phosphoserine in a non-phosphorylated member of the sp-I family, sp-Ic, which is produced during phosphate starvation.

Key words: polysomes/giant protein/phosphoserine/Balbani rings/puromycin

Introduction

The salivary gland of *Chironomus* produces giant secretory proteins, the sp-I family, with a mol. wt. of $\sim 10^6$ (Rydlander and Edström, 1980; Hertner *et al.*, 1980), which are translated in giant polysomes with ~ 80 ribosomes (Daneholt *et al.*, 1977; Francke *et al.*, 1982). The sp-I components are coded by the large Balbiani rings (BR) (Rydlander *et al.*, 1980). Under normal growth conditions sp-Ia and sp-Ib, coded by BR1 and BR2, are produced. During phosphate starvation the production of sp-Ib ceases, that of sp-Ia decreases, and a new component, sp-Ic appears and accounts for up to 50% of the total incorporation of methionine into all cellular proteins (Edström *et al.*, 1980, 1982; Galler *et al.*, 1984). The sp-Ic is probably coded by a newly induced BR6 (Beermann, 1973).

The sp-Ia and sp-Ib proteins are phosphorylated with 8–9 phosphate atoms per 100 amino acids, mainly as phosphoserine, but there is no measurable incorporation of ^{32}P into sp-Ic, suggesting that the gene switch is an adaptation to save phosphate (Galler *et al.*, 1984). In view of the regulating effect of phosphate levels on the production of sp-I components, we wanted to elucidate the cellular localization of this phosphorylation. In cases reported so far phosphorylation is post-translational and independent of peptide bond formation, although it is not excluded that co-translational phosphorylation may also occur in cases where phosphate has a 'structural' role (see Stewart and Sharp, 1984, for discussion). For sp-I, however, most if not all phosphorylation occurs in the polysomes.

Results

Sensitivity of phosphate incorporation to protein synthesis inhibitors

In view of previous reports on insensitivity of protein phosphorylation to puromycin (Turkington and Topper, 1966; Kleinsmith *et al.*, 1966) and to cycloheximide (Schirm *et al.*, 1973) suggesting post-translational phosphorylation, we studied the sensitivity of sp-I phosphorylation to both puromycin and cycloheximide. Glands were incubated *in vitro* with [^{35}S]methionine or [$\gamma\text{-}^{32}\text{P}$]ATP, with and without inhibitor. Figure 1 shows the results of puromycin treatment. The results with cycloheximide (at a concentration of $36\text{ }\mu\text{M}$) were almost identical. Figure 1c and d show the incorporation of methionine into whole gland proteins after 15 and 60 min and Figure 1e shows the incorporation of methionine in the presence of the drug. The drug efficiently inhibits methionine incorporation into sp-I (and other proteins). Figure 1g, h and i show the ^{32}P incorporation after 15 min and 60 min without, and 60 min with the drug. The ^{32}P incorporation, which can be shown only for sp-I and sp-180, a smaller secretory protein (component II in Rydlander and Edström, 1980), is also highly sensitive to the drug. (Figure 1b and f are the separations in Figure 1d and h, respectively, after shorter exposure to display the sp-Ia and sp-Ib bands.)

These results are of interest because they differ from those of earlier inhibitor studies of protein phosphorylation. They do not, however, exclude the possibility that the phosphorylation occurs during a very short time interval after completion of translation. Also, it could be argued that kinases are inhibited by these two drugs. Further studies were therefore performed on isolated polysomes.

Labelled phosphate in isolated polysomes

To test whether phosphate incorporation occurs during translation we incubated glands with [$\gamma\text{-}^{32}\text{P}$]ATP and their sister glands with [^{35}S]methionine after which polysomes were isolated (Daneholt *et al.*, 1977). Figure 2 shows that both ^{35}S and ^{32}P label is present in polysomes from which it can be released with EDTA. Whole glands, labelled as in Figure 2, were also exposed to $200\text{ }\mu\text{M}$ puromycin for 10 min at 18°C *in vitro*, after labelling for 1 h as previously described by Hosick and Daneholt (1974). Like EDTA, the drug eliminated essentially all ^{32}P label from the polysome region. Extracts were also treated with pancreatic ribonuclease ($10\text{ }\mu\text{g/ml}$ extraction solution at 0°C for 10 min). All significant ^{32}P label was eliminated from the heavy polysome region (fractions 1–10) but some label remained in more slowly sedimenting polysome regions (puromycin and ribonuclease results not shown). Although we routinely collected radioactive protein after precipitation with trichloroacetic acid (TCA) in the cold, separate tests showed that the ^{32}P label (like the ^{35}S label) could not be released by heating precipitates in 10% TCA at 80°C for 30 min before collection on

Fig. 1. Incorporation of [^{35}S]methionine (b–e) and ^{32}P (f–i) into proteins extracted from *Chironomus pallidivittatus* salivary glands. Four glands from mid-fourth instar larvae were labelled *in vitro* in 25 μl modified Cannon solution with 10 μl [^{35}S]methionine for 15 min (c), for 60 min with the same exposure (d) and for 60 min with 200 μM puromycin (e) again with the same exposure. The sister glands were incubated in 25 μl of incubation solution to which 10 μCi [$\gamma\text{-}^{32}\text{P}$]ATP had been added for 15 min (g), 60 min (h) and 60 min with 200 μM puromycin (i). The separations in b and f are identical to those in d and h, respectively, but are shown after shorter exposure times. The extracted gland proteins were separated in 1% agarose-2.1% polyacrylamide composite, SDS gels. Autoradiograms were prepared after Coomassie blue staining, one of the stained separations is shown (a). Arrow shows origin, sp-I components have been estimated to 850 kd, sp-180 to 180 kd. The sp-180 is component II in Rydlander and Edström (1980), where the mol. wt. is given as 235 kd. This is, however, for the alkylated form. Since this protein is very cysteine-rich the true mol. wt. is closer to 180 kd (Rydlander, personal communication).

Fig. 2. Sedimentation analysis of polysomes extracted from salivary glands labelled *in vitro*. The glands were incubated for 60 min as described in the legend to Figure 1 with [^{35}S]methionine (upper) and [$\gamma\text{-}^{32}\text{P}$]ATP (lower). Polysomes were extracted and half the extracts (200 μl) were treated with EDTA (20 mM, 5 min at 0–2°C). The four aliquots, each representing four glands, were analyzed on sucrose gradients. Filled circles: without EDTA; open circles: with EDTA. ^{35}S radioactivities remaining in the glands or sedimenting to the bottom of the tubes constituted a minor part of the total radioactivity.

Fig. 3. Analysis of RNA composition in polysomes. Ten animals were labelled by bathing in medium with tritiated nucleosides. Polysomes from five glands were fractionated as described in the legend to Figure 2. Aliquots, 50 μl in size, were taken for a sedimentation profile, after which fractions 4–10, 11–16 and 17–22 were pooled, representing large polysomes (b), small polysomes (c) and post-polysomal fractions (d), respectively. RNA was extracted and separated in 1% agarose gels. Lane a represents RNA extracted directly from fixed labelled glands. The RNA extracts were heated at 65°C for 1 min before application, which converts 28S RNA to material migrating like 18S RNA. Arrow shows origin. It can be seen that the large polysomes contain undegraded 75S RNA (as well as some material in a smear preceding 75S RNA, probably degraded RNA).

filters; this excludes the possibility that the label is due to RNA.

Nature of polysomes incorporating phosphate

To determine the size of the phosphate-incorporating polysomes we used tritium-labelled monosomes which were centrifuged for different times after which the sedimentation values were determined according to Noll and Stutz (1968). The *Chironomus* monosomes have been determined to be 75S by Hosick and Daneholt (1974). We found that most of the polysomes were larger than 450S (Figure 2), in agreement with the results of Daneholt *et al.* (1977) and Wieslander and Daneholt (1977) for polysomes containing 75S RNA hybridizing to the two large BR, BR1 and BR2, in *Chironomus tentans*. Tritium-labelled RNA was also extracted from heavy polysomes, light polysomes and the post-polysomal region. We could confirm, by gel electrophoresis (Figure 3), that the large polysomes contained 75S RNA, whereas the content of such RNA was negligible in the light polysomes and the post-polysomal fractions.

Phosphate incorporation into elongating polypeptide chains

Further evidence that ^{32}P is incorporated into the sp-I proteins during translation is given in Figure 4 which shows that the bulk of incorporation in the polysomes cannot be due to phosphorylation of ribosomal protein. Heavy and light polysomes, as well as the post-polysomal region, were collected from glands labelled with either [^{35}S]methionine or [$\gamma\text{-}^{32}\text{P}$]ATP. The proteins were extracted and separated in composite SDS gels. The heavy polysomes, whether labelled with ^{35}S or ^{32}P , contain labelled protein of the typical nascent size distribution up to the position of the finished sp-I molecules (Figure 4a and b). Also, the light polysomes contain such material, which is probably due to some unavoidable shear of the giant polysomes to produce smaller pieces. However, in the case of the ^{35}S labelling there are other populations of

Fig. 4. Analysis of labelled polypeptide material from polysomes extracted from glands labelled with [^{35}S]methionine **a, c, e, g** and sister glands labelled with [γ - ^{32}P]ATP **b, d, f, h**. Glands were labelled, polysomes extracted, separated by sedimentation and fractionated as described in the legend to Figure 2. Aliquots, 50 μl in size were taken from each fraction for sedimentation profiles (not shown). The remainder of fractions 4–10 (**a, b**), 11–16 (**c, d**) and 17–22 (**e, f**) was pooled from both labelling experiments. The separations in **g** and **h** represent the post-polysomal material (fractions 17–22) from an experiment in which glands were labelled for 135 min. Carrier was added to each sample, consisting of proteins from 10 salivary glands. The samples were then diluted with distilled water so as to give a sucrose concentration <22.5% (w/v) and acetone added to 70% (v/v). After storage at 4°C overnight the extracts were sedimented at 10 000 r.p.m. in the Sorvall SS34 rotor for 20 min at 4°C, the supernatants removed, the precipitates washed with 70% acetone and dried. They were then dissolved in 100 μl of reducing, SDS containing buffer (Rydlander and Edström, 1980), heated for 4 min at 100°C after which 30 μl aliquots were separated in 1% agarose-2.1% polyacrylamide composite gels. Gels were sectioned into 1 mm thick slices which were subjected to scintillation counting. The positions of sp-I and sp-180 from the carrier gland protein are shown. HP, LP and PP designate heavy polysomes, light polysomes, and post-polysomal material, respectively.

polypeptides from the position of sp-180 and downwards in size (Figure 4c). On the other hand there is no evidence for the presence of such material among the phosphate-labelled light polysomes (Figure 4d). After 15 min of labelling the sp-I region is not yet particularly dominant in the size spectrum of the post-polysomal material (Figure 4e). This is because of the longer synthetic time for sp-I as compared with smaller proteins. Thus sp-180 is relatively pronounced at this time. After labelling with ^{35}S for 135 min the sp-I is, however, the component with most label (Figure 4g). In the phosphate-labelled post-polysomal material there is label not only in the sp-I range but also in lower mol. wt. regions, from sp-180 and downwards (Figure 4f). It is interesting to note that the low mol. wt., phosphate-labelled material in the post-polysomal region does not have a counterpart in the light polysomal separation (Figure 4d) as is the case for ^{35}S -labelled material (Figure 4c), probably because phosphorylation of such pro-

Fig. 5. Plot of label distribution due to [^{35}S]methionine or ^{32}P in nascent and finished sp-I after different times of precursor administration. Glands were labelled, polysomes extracted, separated by sedimentation and fractionated in electrophoretic gels as described in the legend to Figure 2. Gels were sectioned as described in the legend to Figure 4. To calculate sp-I label in nascent chains, all radioactivity in heavy polysomes and radioactivity in polypeptide material heavier than sp-180 in light polysomes was added together (as indicated in Figure 4c and d). For the calculation of sp-I label in finished chains, material heavier than sp-180 was used (as indicated in Figure 4e and f). Open circles, ^{35}S label; filled circles, ^{32}P label.

teins occurs post-translationally. After 135 min of ^{32}P incorporation, the low mol. wt. phosphate-labelled material is relatively inconspicuous due to accumulation of sp-I (Figure 4h). It should be mentioned, finally, that sp-I occupies a relatively broad region in the analyses of the post-polysomal, and presumably finished material, although, for example, sp-180 is present as a sharp peak. This is probably due to some shear of giant polysomes, which gives broken polysomes in more slowly sedimenting fractions, in addition to some unavoidable preparative degradation of the giant proteins. In contrast, analyses of whole glands permits sp-I to be retained in a more intact form (Figure 1).

Flow of phosphate from nascent to finished sp-I chains

The results so far do not provide information about the quantitative importance of the transitory phosphorylation of sp-I. We have, therefore, followed the distribution of protein-bound ^{32}P between nascent chains in the polysomes and finished sp-I chains in the post-polysomal region as a function of labelling time. The shift in distribution has been compared with the corresponding shift for ^{35}S given with methionine. Since there is one methionine residue per Balbiani ring repeat unit and most of the gene consists of repeat units (Wieslander and Lendahl, 1983; Galler *et al.*, 1984), methionine is likely to be uniformly distributed along the sp-I molecules. Nascent sp-I polypeptides were isolated from heavy and light polysomes and finished sp-I chains were isolated from the post-polysomal region and analyzed by electrophoresis. In the heavy polysomal separations (see Figure 4) all counts were added together. For the light polysomal and post-polysomal material only radioactivity due to chains heavier than sp-180 was used for the calculations. Figure 5 shows that after

Fig. 6. Thin layer electrophoretogram of amino acid hydrolysates of polypeptides extracted from the total polysomal region and from the post-polysomal material after labelling and sedimentation analysis of glands as described in the legend to Figure 2. Post-polysomal (F) and polysomal (N) material is shown after 20 min (F20 and N20) and 40 min (F40 and N40) of labelling. The analyses were carried out as previously described (Galler *et al.*, 1984) together with phosphoserine and phosphothreonine as carrier. Arrow shows points of application. P, PS, PT and X indicate free phosphate, phosphoserine, phosphothreonine and an unidentified component, respectively.

15 min most of the label is in polysomal nascent chains for both isotopes. After 40 min the label is about evenly distributed, whereas after 135 min, finally, the predominant amount of label is in finished material. The shift in distribution of label is similar for both isotopes. The determinations are not sufficiently accurate to exclude 10–20% lower ratios of nascent to finished chains, for ^{32}P as compared with ^{35}S . Therefore, it is quite possible that phosphorylation could occur in the polysomes somewhat later than the addition of the amino acids to the growing polypeptide chain and/or that a minor part of the phosphorylation could occur after the completion of the polypeptide chain. Nevertheless these results clearly show that most, perhaps all, of the sp-I phosphorylation occurs in the nascent sp-I chains. It could be argued that further phosphorylation could occur later than 2 h after synthesis is finished. This is, however, not likely since this is roughly the time it takes after precursor administration before the secretion reaches the lumen (Rydlander and Edström, 1980) after which further phosphorylation is improbable.

Pattern of phosphorylation in nascent and finished chains

Most phosphate in sp-I is present as phosphoserine and a minor portion in other amino acids (Case *et al.*, 1983; Galler *et al.*, 1984). If most of the phosphorylation is co-translational one would expect the pattern of phosphorylation among individual amino acids to be similar for the nascent sp-I chains and the finished chains from the post-polysomal material. This prediction was investigated by thin layer electrophoretic analyses of amino acid hydrolysates of total protein from the total polysomal material as well as from the

post-polysomal material after ^{32}P labelling for 20 and 40 min. A minor part of the post-polysomal ^{32}P label after 20 min is due to other proteins. After longer times, for example 40 min, this contribution is, however, not significant. Figure 6 shows that the distribution of ^{32}P is closely similar between three phosphorylated components, phosphoserine, phosphothreonine and an unknown component (X) for nascent and finished polypeptides at both time points. These three spots were also analyzed by scintillation counting. Of their total label, 90.2–93.5% was present in phosphoserine, 2.1–3.2% in phosphothreonine and 4.3–6.6% in X. We have also investigated the post-polysomal ^{32}P distribution in an amino acid hydrolysate after 135 min of labelling. In this case the distribution was similar with 87.8% of the label in phosphoserine, 2.3% in phosphothreonine and 9.9% in X.

Discussion

We find that nascent sp-I polypeptide chains, isolated from polysomes, are labelled after incubation of *Chironomus* salivary glands with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. These polysomes are of an unusually large size and contain Balbiani ring-derived 75S mRNA. Furthermore, the kinetic studies indicate that most if not all phosphorylation occurs during translation. The pattern of phosphorylation in the amino acid hydrolysate of nascent sp-I is more or less the same as that of the finished products.

Phosphorylation regulates the activity of many catalytic proteins in which case it occurs post-translationally and reversibly (for a review, see Ingebritsen and Cohen, 1983). Post-translational phosphorylation has also been recorded for non-catalytic proteins like ovalbumin (Sanger and Hocquard, 1962), histones (Oliver *et al.*, 1972), phosvitin (Schirm *et al.*, 1973) and casein (Turkington and Topper, 1966). In the case of casein it is not excluded that at least some phosphorylation may already occur during translation (Stewart and Sharp, 1984). On the whole there is little information available on co-translational phosphorylation. In contrast, such a process can be easily demonstrated for sp-I, mainly because of its unique size and the high degree of phosphorylation.

The present results do not permit any conclusions as to the exact site of phosphorylation in relation to the peptide bond forming site in the ribosome. Thus we cannot exclude that the co-translational phosphorylation is simply due to the large size of the sp-I components. For instance, suppose that phosphorylation can only start after residue 1000 (~15% of the size of the finished sp-I). If this was the case it would be post-translational for most proteins but not for sp-I. We think, however, that the implications of co-translational phosphorylation for sp-I structure, and perhaps also the regulation of its synthesis, are such that there are special rather than trivial reasons why phosphorylation is co-translational, as will be discussed later.

There are several reports on the presence of *O*- β -phosphoryl-tRNA in the cells of higher eukaryotes (Mäenpää and Bernfield, 1970; Sharp and Stewart, 1977; Hatfield *et al.*, 1982). So far there is, however, no evidence that such tRNA is functioning in providing phosphoserine to the growing polypeptide chain (Stewart and Sharp, 1984). For sp-I the phosphorylation during translation certainly involves more than seryl residues (Figure 6). Thus, unless more than one special tRNA is involved, a protein kinase acting on the nascent chain appears more likely.

In retrospect it is not surprising that phosphorylation occurs at an early stage for sp-Ia and sp-Ib, both of which show a high excess of the positively charged amino acids, lysine and arginine, over negatively charged amino acids (Rydlander, 1984). Serine and threonine codons are common in sub-repeats from cloned gene fragments of BR1 and BR2, often as -Arg/Lys-Pro-Ser/Thr- sequences which have -Arg-Pro-Glu- counterparts in the non-phosphorylated product of BR6 sp-Ic. The latter substitutes for sp-Ia and sp-Ib during phosphate starvation (Galler *et al.*, 1984). It can be expected that several of the seryl residues are phosphorylated. Computer 3D structure predictions by Hamodrakas and Kafatos (1984) showed that the unique part of the BR6 coded repeat unit was much more similar to that of the BR1 and BR2 products if the seryl residues were phosphorylated than if they were not. Thus, phosphoserine (and phosphothreonine) can be expected to mimic glutamic (and aspartic) acid in charge and structural properties. This would suggest that phosphate groups are needed during early stages of protein folding.

The co-translational phosphorylation of sp-Ia and sp-Ib is also of interest in view of the fact that the shift from these proteins to their non-phosphorylated counterpart, sp-Ic, occurs as a result of phosphate starvation. This is expressed as a reduction in the content of P_i in the haemolymph down to one third of the normal levels (Edström *et al.*, 1982) (haemolymph constitutes about half of the body volume). The normal phosphate export due to sp-Ia and sp-Ib is considerable and the switch is thus a phosphate-saving adaptation. Although the levels of P_i decreased only slightly or not at all in the salivary glands during BR6 induction (Edström *et al.*, 1982) it is quite conceivable that kinase activity could be controlled allosterically by means of an effector, the concentration of which is dependent on the P_i pools in the haemolymph. The coupling between the haemolymph and the glands is close in phosphate metabolism, since the glands take up ATP as such in a specific process (Egyhazi *et al.*, 1982) and the haemolymph contains ATP in considerable quantities (our unpublished data). It is, in other words, an interesting possibility that changes in the P_i pools in the animals control the gene switch primarily at the level of translation.

Materials and methods

Animals

Mid-fourth larval instar *Chironomus pallidivittatus* animals were used, raised as described by Rydlander and Edström (1980).

Chemicals, radiochemicals

Puromycin-dihydrochloride was from Sigma, actidione (cycloheximide) from Serva, ribonuclease-A (bovine pancreas, type III-A) from Sigma, agarose (ME) used for protein separations and agarose HGT(P) used for RNA analyses were both from Seakem. For labelling experiments we used adenosine [γ - 32 P]triphosphate, triethylammonium salt at 10 mCi/ml, ~3000 Ci/mmol, Amersham and L-[35 S]methionine at 10 mCi/ml, ~1300 Ci/mmol, Amersham. [3 H]cytidine and [3 H]uridine used for bathing animals in radioactive solution were 3 years old but originally at 46 Ci/mmol and 30 Ci/mmol, respectively, both from Amersham.

Gel electrophoresis

Electrophoresis of proteins in 1% agarose – 2.1% polyacrylamide composite horizontal slab gels was performed as described by Rydlander and Edström (1980). Electrophoresis of RNA in 1% agarose horizontal slab gels was done according to Daneholt *et al.* (1977).

Labelling of protein

Glands were excised and immediately placed in Cannon's modified insect medium with 17% of the normal content of methionine (Ringborg and Rydlander, 1971) (6–8 glands per 25 μ l) and incubated at 18°C. For 32 P incorporation, 10 μ Ci (1 μ l) of the precursor ([γ - 32 P]ATP) was added to 24 μ l medium; for 35 S incorporation, 10 μ Ci (1 μ l) of the precursor ([35 S]methionine) was added to 24 μ l medium.

Polysome preparations

Polysomes were prepared and analyzed essentially as described by Daneholt *et al.* (1977). For protein labelling, glands were labelled with [γ - 32 P]ATP or [35 S]methionine *in vitro* as described above and for RNA labelling by bathing whole animals (10 animals in 500 μ Ci each of tritiated uridine and cytidine in 20 ml of normal culturing medium for 4 days). Labelled glands were dissolved in 400 μ l of extraction medium (20 mM triethanolamine-HCl pH 7.6; 100 mM KCl; 1 mM MgCl₂; 0.1% β -mercaptoethanol; 0.5% Tween 80; 0.5% sodium deoxycholate) at 0–2°C for 15 min. Non-dissolved residue, mainly secretion, was removed with insect needles and the lysate layered onto a pre-cooled 15–60% sucrose gradient in 5 ml cellulose nitrate tubes. Centrifugation was at +2°C for 30 min in a Beckman SW50.1 rotor at 37 000 r.p.m. (130 000 g_{av}). The gradients were fractionated into ~22 fractions and TCA precipitation and counting was done as previously described (Daneholt *et al.*, 1977). Linearity of the gradients was checked by measuring refractive indexes. RNA was released in 0.5% SDS by heating to 56°C for 10 min. Fractions were pooled, made 0.3 M sodium acetate, 5 μ g *E. coli* tRNA/ml, given 2.5 volumes of ethanol and RNA allowed to precipitate overnight at –20°C. The RNA was recovered by centrifugation at 10 000 r.p.m. for 30 min at 4°C in a SS34 Sorvall rotor. Pellets were dried and taken up in electrophoresis buffer.

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