Processing of a Pseudorabies Virus-induced Protein which is Glycosylated, Sulphated and Excreted

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(Accepted 18 August 1976)

SUMMARY

Cells infected with pseudorabies virus excrete large amounts of a glycosylated sulphated protein, mol. wt. 89,000, into the extracellular fluid. This paper reports the results of studies on the processing of this protein. Glycosylation occurs during, or very soon after, synthesis of the polypeptide chain. After a delay of several minutes the glycoprotein is sulphated; inhibition of glycosylation by high concentrations of glucosamine does not interfere with this process. The glycosylated sulphated polypeptide is then reduced in size from mol. wt. 99,000 to 89,000, possibly by proteolytic cleavage, and is excreted. Inhibition of glycosylation does not interfere with the excretion of this polypeptide, which is an energy-requiring process.

INTRODUCTION

Cells infected with pseudorabies virus, a pig herpes virus, excrete large amounts of a glycosylated sulphated protein into the extracellular fluid (Erickson & Kaplan, 1973; Erickson, 1976). Its function is unknown. This paper reports the results of investigations on the post-translational processing and excretion of this virus-induced protein.

We have studied the glycosylation and sulphation of this protein by labelling cells at late times after infection with radioactive precursors, and have investigated the effects of high concentrations of D-glucosamine (which block glycosylation), protease inhibitors and inhibitors of energy metabolism on excretion. The temporal sequence of some of the processing steps has been established. The significance of these findings is discussed.

METHODS

Cells and virus. Pseudorabies virus stocks were grown in BSC 1 cells. Monolayer cultures of these cells in 35 mm diam. Petri dishes (about 3 x 10⁶ cells/dish) were used in all experiments; cells were infected at input multiplicities of 10 to 20 p.f.u./cell.

Media. Cells were grown and maintained in Eagle’s medium (Glasgow modification) with 10% foetal bovine serum. Infected cells were maintained in the same medium with 2% foetal bovine serum.

Radiochemicals and labelling of cells. Carrier-free ³⁵S as sulphate, L-³⁵S-methionine (100,000 mCi/mmol) and D-U-¹⁴C-glucosamine hydrochloride (200 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England.
Cells were washed before labelling with Dulbecco's phosphate buffered saline (PBS). The appropriate labelled precursor was then added in PBS (0.4 ml/plate). In pulse-chase experiments the label was removed and the cells were washed and incubated further in chase medium (0.4 ml/plate). After pulse-labelling with $^{35}$S-methionine the chase medium was Eagle's medium containing 100 times the normal amount of methionine. Normal Eagle's medium was used after pulse-labelling with $^{35}$SO$_4^{2-}$ and $^{14}$C-glucosamine.

Labelling of infected cells commenced at times between 7 and 17 h post-infection; preliminary experiments had shown that under the conditions used, host polypeptide synthesis was severely inhibited and virus polypeptide synthesis was well established; excretion of the virus-induced protein under study proceeded freely at these times.

At the conclusion of experiments the medium was harvested, centrifuged at 500 g for 15 min, and analysed by polyacrylamide gel electrophoresis. Cells were scraped into 0.001 M-tris-hydrochloride, pH 9.0 (0.4 ml/plate).

Gel electrophoresis, autoradiography and fluorography. Samples were prepared for electrophoresis by boiling for 2 min in 1 % mercaptoethanol and 2 % SDS. After the addition of glycerol and tracking dye, electrophoresis was done in acrylamide gels using the discontinuous SDS buffer system described by Laemmli (1970). Tube gels containing 8 % acrylamide were run and subsequently processed for autoradiography as previously described (Pennington, 1974); gels were stained with Coomassie brilliant blue and were aligned before drying using the stained bands as markers. Unlabelled BSC 1 cells were added to samples of medium before reduction and dissociation so that these gels could be aligned in the same way. Five to fifteen % gradient slab gels were prepared and run as previously described (McCrae & Szilagyi, 1975). Autoradiography was done with Kodirex KD 54T X-ray film; fluorography was done according to the method of Bonner & Laskey (1974). Polypeptide mol. wt. were determined as previously described (Pennington, 1974).

RESULTS

Excretion of macromolecules from pseudorabies virus-infected cells and from uninfected cells

Cells were labelled with either $^{35}$S-methionine or $^{35}$SO$_4^{2-}$ for 3 h. The radioactive macromolecules excreted into the medium were analysed by polyacrylamide gel electrophoresis and a representative fluorogram is shown in Fig. 1. This shows that a number of $^{35}$S-methionine-labelled polypeptides were excreted into the extracellular fluid from uninfected cells; a small number of diffuse bands were also seen in the track containing $^{35}$SO$_4^{2-}$-labelled material, although most of the radioactivity was retained at the top of the gel. In contrast, the bulk of the radioactivity in the tracks analysing the medium from infected cells labelled with either $^{35}$S-methionine or $^{35}$SO$_4^{2-}$ was concentrated in one band which migrated with an apparent mol. wt. of 89000. This was termed polypeptide 89K. No radioactivity migrated in this region in gels analysing samples from uninfected cells.

In addition to the major band, several minor components could be seen in tracks analysing medium from infected cells labelled with either $^{35}$S-methionine or $^{35}$SO$_4^{2-}$. In $^{35}$S-methionine labelled samples these components, with one exception of high mol. wt., co-migrated with polypeptides excreted from uninfected cells; there was little correspondence, however, between the migration of $^{35}$SO$_4^{2-}$-labelled bands with $^{35}$S-methionine-labelled bands and with $^{35}$SO$_4^{2-}$-labelled bands from uninfected cells. Centrifugation at 85000 g for 2 h did not sediment polypeptide 89K (results not shown), indicating that this polypeptide was not associated to any significant extent with virus particles in the extracellular fluid.
The kinetics of appearance of polypeptide 89K in the extracellular fluid are shown in Fig. 2. Infected cells were labelled for 15 min with $^{35}$S-methionine; the label was then removed and replaced with medium containing excess cold methionine. Samples of medium were taken at various times during the chase period. Polypeptide 89K was first detected 30 min after the beginning of the chase; excretion then proceeded at a maximal rate for about another 90 min. The rate of excretion then declined.

Effect of sodium azide on the excretion of polypeptide 89K

Experiments using sodium azide, an inhibitor of energy metabolism, showed that the excretion of polypeptide 89K was an active process and could not be ascribed to leakage of
Fig. 2. Kinetics of excretion of polypeptide 89K. Cells were labelled at 17 h post-infection for 15 min with 35S-methionine. The label was removed and replaced with chase medium; samples of this were taken at the times indicated and were analysed on a slab gel. Densitometer tracings were prepared from a fluorogram using a Joyce-Loebl densitometer and the peaks were cut out and weighed.

Fig. 3. Effect of sodium azide and TPCK on the excretion of polypeptide 89K. Cells were labelled at 17 h post-infection for 30 min with 35S-methionine. The label was removed and replaced with chase medium containing various concentrations of sodium azide or TPCK. After a 3 h chase the medium was collected and analysed by tube gel electrophoresis. Densitometer tracings were prepared from autoradiograms and the peaks were cut out and weighed. ○—○, TPCK; •—•, azide.
this polypeptide from infected cells. Fig. 3 shows that addition of sodium azide to the chase medium had an inhibitory effect on the appearance of polypeptide 89K in the medium.

**Effect of cycloheximide on the excretion of polypeptide 89K**

High concentrations of cycloheximide had no effect on the excretion of polypeptide 89K when incorporated into the chase medium after a short labelling period (results not shown), indicating that protein synthesis was not required for excretion.

**Glycosylation of polypeptide 89K**

Labelling of infected cells with 14C-glucosamine and subsequent analysis of the medium showed that polypeptide 89K is glycosylated (Fig. 4). High concentrations of glucosamine are known to interfere with glycosylation of polypeptides (Klenk, Scholtissek & Rott, 1972) and the effects of this treatment on polypeptide 89K were also investigated. The results of a typical experiment are shown in Fig. 4; addition of 25 mM-glucosamine at the time of labelling with 35S-methionine led to the excretion of a major radioactive polypeptide with a...
Fig. 5. Pulse-chase experiment. Infected cells were labelled for 45 min with $^{35}$SO$_4$$^-$ starting at 16 h post-infection; chase samples were taken at the times indicated. The cell-associated polypeptides were analysed on a slab gel and densitometer tracings were prepared from the fluorogram.
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mol. wt. of 78000 and two minor radioactive components of slightly higher mol. wt. The appearance of radioactive polypeptide 89K in the medium was completely abolished. On the other hand, the addition of 25 mM-glucosamine immediately after the pulse labelling period had no effect on the appearance of radioactive polypeptide 89K in the medium. The likeliest interpretation of these results is that the decrease in mol. wt. of the major excreted polypeptide is due to inhibition of glycosylation. As this effect was only produced when glucosamine was present during the labelling period and was not seen when glucosamine was added at the beginning of the chase it was concluded that glycosylation occurred during or immediately after the synthesis of polypeptide 89K.

Identification of possible intracellular precursors of polypeptide 89K

Intracellular virus-induced 35S-methionine labelled polypeptides were examined after a short labelling period to identify possible precursors of polypeptide 89K (Fig. 4).

No major intracellular polypeptides with the same mol. wt. as the glycosylated or underglycosylated extracellular polypeptides were detected. Thus, the size of the intracellular pool of polypeptide 89K, the product of excretion under normal conditions, must be small. In addition, a second processing step leading to a change in mol. wt. must occur after glycosylation and before, or at the time of, excretion from the cell.

Sulphation of polypeptide 89K

This was investigated further by pulse-chase experiments. Fig. 5 shows densitometer tracings of fluorograms of a slab gel analysing the fate of 33SO4²⁻-labelled macromolecules in infected cells. Two major components were detected in infected cells at the end of the pulse. The predominant species had a mol. wt. of 99000 and the other species had a mol. wt. of 89000. During the chase the amount of the higher mol. wt. species in the cell declined whereas the amount of the lower mol. wt. species initially increased. Later in the chase the amount of this species also declined. These results suggest that extracellular polypeptide 89K is derived from a precursor, mol. wt. 99000, which undergoes a processing step leading to a loss in mol. wt. This step occurs after sulphation and just before excretion; no precursor was ever detected in the medium suggesting that this processing is a necessary prerequisite for excretion.

Effect of inhibition of glycosylation on the sulphation of polypeptide 89K

Infected cells were treated with 25 mM-glucosamine for various times before labelling with either 35S-methionine or 33SO4²⁻. After labelling for 3 h the medium was collected and analysed by polyacrylamide gel electrophoresis and fluorography (Fig. 6). This figure shows that 25 mM-glucosamine treatment reduced the mol. wt. of 35S-methionine-labelled polypeptide 89K very rapidly, addition of the amino sugar at the time of labelling leading to a reduction in size of the bulk of the labelled excreted molecules. Likewise, addition of glucosamine at the time of labelling with 33SO4²⁻ led to the excretion of smaller molecules. However, excretion of normal sized sulphated molecules was still apparent in samples of medium from cells pre-treated with glucosamine for as long as 30 min before labelling with 33SO4²⁻. A pre-treatment period of 1 h, however, led to the reduction in size of nearly all the excreted sulphated macromolecules. These observations contrast sharply with the effect of glucosamine on 35S-methionine-labelled excreted molecules, and are best explained by postulating that there is an interval of several minutes between the synthesis and glycosylation and between the sulphation of polypeptide 89K. Thus the residual population of normal sized 33SO4²⁻-labelled molecules excreted after short pre-treatments with glucosamine
Fig. 6. Fluorogram showing the effect of glucosamine on the sulphation of excreted polypeptides. Infected cells were treated with 25 mM-glucosamine for the times indicated (min); they were then labelled with either $^{35}$SO$_4$$^{2-}$ (S) or $^{35}$S-methionine (M) for 3 h in the presence of 25 mM-glucosamine. The medium was then analysed on a slab gel. (C), control, no glucosamine. Treatment with glucosamine started at 17 h post-infection.

are derived from a pool of molecules which were synthesized and glycosylated before the addition of glucosamine to the cells. Examination of band intensity of fluorograms of $^{35}$S-methionine- and $^{35}$SO$_4$$^{2-}$-labelled molecules showed that inhibition of glycosylation by glucosamine did not significantly alter the relative amounts of radioactivity derived from these two precursors. It is therefore reasonable to conclude that there is a strong possibility that sulphation of polypeptide 89K occurs in the polypeptide moiety, rather than the sugar chain.
**Effect of protease inhibition on the excretion of polypeptide 89K**

As no appreciable amount of an intracellular polypeptide with a mol. wt. of 89,000 was detected in short pulse labelling experiments (Fig. 4), and as sulphated polypeptide with a mol. wt. of 99,000 appeared to be a likely precursor of extracellular polypeptide 89K, we considered the possibility that polypeptide 89K could be derived from a larger precursor by one or more steps involving proteolytic cleavage. This possibility was tested in experiments in which we tested the effects of the protease inhibitors TPCK and TLCK on the excretion of polypeptide 89K. Various concentrations of the inhibitors were incorporated into the chase medium after a short pulse with 35S-methionine. Fig. 3 shows that TPCK exerted an inhibitory effect on the excretion of polypeptide 89K. TLCK (100 μM) had little effect on excretion (results not shown).

**DISCUSSION**

The results of this study have confirmed and extended the observations of Erickson & Kaplan (1973) on the fate of the major non-structural virus-induced glycoprotein found in pseudorabies virus infected cells. These workers showed that this protein is sulphated and excreted into the culture medium in large amounts.

We have shown that the post-translational processing of this protein occurs in a series of steps, and we have determined the order in which these steps take place. Firstly, during, or very soon after, polypeptide chain synthesis, the polypeptide is glycosylated. This is clearly demonstrated by the effects produced by incubating infected cells in high concentrations of glucosamine; under these conditions the mol. wt. of the excreted polypeptide (and its possible precursors) is reduced by a sizeable increment, almost certainly due to the inhibitory effects of high glucosamine concentrations on glycosylation (Klenk et al. 1972). Under normal conditions significant amounts of non-glycosylated precursors were never observed in the medium. Our conclusion that polypeptide 89K is glycosylated during, or very soon after, its synthesis contrasts with the findings of Honess & Roizman (1975). These workers studied the glycosylation of herpes simplex virus glycoproteins and concluded that the average time from the completion of the polypeptide chain to the first measurable addition of glucosamine is approx. 12 to 15 min. They restricted their observations, however, to intracellular glycoproteins, and it is possible that these proteins are processed in a different way from glycoproteins which are excreted from the cell soon after their synthesis.

Sulphation occurs after glycosylation; two major sulphated polypeptides were identified in infected cells, one with a mol. wt. identical to that of the excreted polypeptide, the other being somewhat larger. Pulse-chase experiments indicated that the larger of these was probably a precursor of the smaller, as a decrease in the amount of the larger polypeptide during a chase could be correlated with an increase in the amounts of the smaller polypeptide and the excreted polypeptide.

A lag period between glycosylation and sulphation was demonstrated in experiments which showed that when glycosylation was inhibited in infected cells, sulphation of previously glycosylated protein continued for periods of up to 30 min. A possible explanation of this lag is that protein synthesis and sulphation may occur in different places in the cell and that transport between these sites is relatively slow. The biological role that sulphation of this glycoprotein plays is not known, although it is of interest to note that the structural glycoproteins of many enveloped RNA viruses appear to be sulphated (Compans & Pinter, 1975; Pinter & Compans, 1975). The observation that the excreted polypeptide of pseudorabies virus is sulphated even when its glycosylation is inhibited...
suggests the possibility that the polypeptide rather than the carbohydrate moiety may be sulphated. This possibility is strengthened by the work of Erickson (1976) who has shown that when pronase digestion fragments of this polypeptide are analysed on Sephadex G-50 columns the bulk of the carbohydrate-containing fragments are significantly larger than the $^{35}$SO$_2^-$-labelled fragments. The $^{35}$S label in these fragments was present as sulphate ester.

Immunoprecipitation studies using antisera raised against polypeptide 89K are planned in order to identify the intracellular precursor of this polypeptide; these studies will also investigate the detailed nature of the processing steps responsible for the changes in mobility which occur during the processing of this polypeptide. The experiments with TPCK suggest that at least some of these changes are due to proteolytic cleavage; it is of interest to note that many eukaryotic secretory proteins, including polypeptide hormones, are processed in this way, and it has been suggested that this phenomenon may be in some way concerned with the secretory process (Tager & Steiner, 1974). It is likely that the precursor of polypeptide 89K is cleaved by a chymotrypsin-like enzyme, as TPCK is a substrate analogue which reacts covalently and irreversibly with proteases of this type (Shaw, Mares-Guia & Cohen, 1965).

Although the biological role of the protein excreted from pseudorabies virus infected cells is unknown, we note that virus-induced glycoproteins are also excreted from cells infected with herpes simplex virus types 1 and 2 (Kaplan, Erickson & Ben-Porat, 1975) and from cells productively infected with Friend leukaemia virus (Bolognesi, Langlois & Schafer, 1975). We have also observed the excretion of virus-induced proteins from cells infected with vaccinia virus (Pennington & McCrae, unpublished observations). We feel that in addition to the interest associated with the possible roles that these proteins may play during infection, they may also serve as a useful paradigm for the study of protein excretion from eukaryote cells.

We thank Mary Braidwood for expert technical assistance, and Professor J. H. Subak-Sharpe and Dr C. R. Pringle for their comments on the manuscript.

REFERENCES


(Received 12 July 1976)