Some Characteristics of an Early Protein (ICP 22) Synthesized in Cells Infected with Herpes Simplex Virus

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SUMMARY

In Vero cells incubated at 40 °C or treated with azetidine at 37 °C, synthesis of a polypeptide (‘C’) of apparent mol. wt. 66,000 was stimulated. It was not phosphorylated and was found in the cytoplasmic fraction of cell lysates. In cells infected with herpes simplex virus type 1 (HSV-1) in the presence of azetidine, synthesis of cellular proteins, including polypeptide C, was suppressed and infected cell polypeptides ICP 4, 0, 22 and 27 (apparent mol. wt. 170,000, 120,000, 75,000 and 60,000, respectively) were made. All were phosphorylated and accumulated in the nucleus. Messenger RNA for the same four polypeptides was made in cells infected in the presence of cycloheximide. Thus, ICP 22 is distinct from cellular polypeptide C and is probably a virus-specific α polypeptide, although it differs from α ICP 4, 0 and 27 in that its rate of synthesis does not decline rapidly when later polypeptides are produced. It is modified after synthesis in at least two steps, the second of which may require a later virus-specific polypeptide. In cells infected with HSV-2 the synthesis of a polypeptide analogous to ICP 22 could not be detected.

INTRODUCTION

In cells infected with herpes simplex virus (HSV) the production of virus-specific proteins (infected cell polypeptides, ICP) is regulated in at least three distinct classes (Honess & Roizman, 1974, 1975). The α polypeptides are made early and initiate the synthesis of β polypeptides, which in turn suppress the further production of α and initiate the production of γ (late) polypeptides. The synthesis of β polypeptides declines later in infection while that of γ polypeptides continues.

The α polypeptides are the most clearly defined group because their mRNAs are transcribed from the DNA of the infecting virus without prior protein synthesis. If cycloheximide or puromycin is added at the time of infection α mRNAs accumulate and are translated into protein when the inhibitor is removed (Honess & Roizman, 1974). If the synthesis of β mRNA is inhibited by adding actinomycin D at the time of reversal of the block, only α ICP are made, together with those host cell proteins whose synthesis has been incompletely suppressed by the virus.

A rather less clear identification of members of the α group can be made by adding the amino acid analogues canavanine or azetidine. Incorporation of these into α polypeptides interferes with their ability to initiate β ICP synthesis, although a few are still made (Honess & Roizman, 1975; Pereira et al. 1977).
Three polypeptides, ICP 4, 0 and 27 have been clearly identified as α polypeptides. A fourth polypeptide, ICP 22, is also made after reversal of a cycloheximide block in infected, but not in uninfected, cells (Kozak & Roizman, 1974) and in cells infected in the presence of canavanine. However, its synthesis was also stimulated in uninfected cells treated with canavanine or incubated at 38.5 °C (Honess & Roizman, 1975) and it was therefore thought to be probably a cellular protein. Stimulation of the rate of synthesis of a number of proteins by incubating cells at 45 °C or by adding amino acid analogues at 37 °C has recently been described (Kelley & Schlesinger, 1978). It was suggested that the inducing treatments inactivate a regulatory protein with the result that an excess of mRNA for the 'heat shock' proteins is synthesized. In HeLa cells the proteins had apparent mol. wt. of 120000, 95000, 93000 and 76000.

We have investigated the possibility that ICP 22 (mol. wt. approx. 75000) is one of the cellular heat shock proteins whose mRNA is synthesized in response to infection with herpesvirus in the presence of cycloheximide. Our experiments show, however, that ICP 22 is not one of these proteins but is probably an α polypeptide.

METHODS

Infection. HSV-1 (F) and HSV-2 (G), obtained from Dr B. Roizman, Chicago, were grown by low multiplicity passage in African green monkey kidney (Vero) cells and stored at −70 °C as sonicated whole cell lysates with titres of 5 × 10⁸ to 20 × 10⁸ p.f.u./ml. Infection of cells, labelling of proteins and electrophoresis were carried out as described before (Fenwick et al. 1978). Freshly confluent monolayers of Vero cells, 20 to 24 h after subculture, were infected with 10 to 20 p.f.u./cell. After adsorption at 20 °C for 20 min the virus inoculum was removed and replaced with growth medium and the cells were transferred to a 37 °C incubator at zero time.

Labelling was done with ¹⁴C-amino acids (1 µCi/ml, protein hydrolysate; The Radiochemical Centre, Amersham, Bucks.) in medium lacking amino acids or with ³²P-orthophosphate (carrier-free, 50 µCi/ml) in medium lacking phosphate. Azetidine (4 mM) was dissolved in medium containing 1/10 the normal concentration of amino acids.

Electrophoresis. Samples of whole cell lysates were subjected to electrophoresis in slab gels containing an exponential gradient of acrylamide ranging from 7 % at the top to 20 % (w/v) at the bottom. The marker proteins used for estimation of mol. wt. were trypsin inhibitor (21000), actin (45000), tubulin (55000), bovine serum albumin (68000) and RNA polymerase (39000, 155000 and 165000). Proteins were stained with Coomassie brilliant blue and the gels were dried and placed in contact with Kodak Kodirex X-ray film for 1 to 2 weeks to form autoradiograms.

Materials. Cycloheximide (actidione) and L-azetidine-2-carboxylic acid were obtained from Calbiochem, Bishops Stortford, and actinomycin D from BDH Chemicals, Ltd., Poole, Dorset.

RESULTS

Heat shock polypeptides

Uninfected Vero cells were incubated at 40 °C for periods of up to 8 h and then labelled for 2 h with ¹⁴C-amino acids in the presence of actinomycin D at 40 °C. The autoradiogram in Fig. 1 shows that the synthesis of at least four polypeptides (labelled A, B, C and D) was stimulated after 2 h at 40 °C. After longer periods at the abnormal temperature the rate of synthesis of polypeptide C declined again, although that of A, B and D did not.

A number of experiments were done to investigate the nature of the induction of specific
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Fig. 1. Polypeptides synthesized in uninfected cells at 40 °C. Cells were incubated for the times shown (h) at 40 °C before labelling with 14C-amino acids for 2 h at 40 °C in the presence of actinomycin D (2 µg/ml). An autoradiogram of electrophoretically separated polypeptides is shown in this and subsequent figures.

Fig. 2. Comparison of α and heat-shock polypeptides. Mock-infected (M) or HSV-1-infected (I) cells were incubated for 4 h with cycloheximide (50 µg/ml) and then labelled for 1 h with 14C-amino acids in the presence of actinomycin D (2 µg/ml, channels 1 and 2). Other uninfected cells were incubated at 40 °C for 2 h and then labelled for 1 h at 40 °C in the presence of actinomycin D (channel 3).

protein synthesis at high temperature and in general these confirmed the report of Kelley & Schlesinger (1978). The following observations (not illustrated) were made: (i) induction involved the synthesis of new RNA, presumably mRNA, which did not occur in the presence of actinomycin D; (ii) the mRNA for polypeptide C was unstable since C was not labelled if cells were kept at 40°C for 2 h, followed by 2 h at 35°C, before adding 14C-amino acids. The mRNAs for A, B and D, on the other hand, were relatively stable; (iii) the induced labelled polypeptides themselves were stable during a 1 h chase at 35 or 40°C in the presence of cycloheximide; (iv) they were not detectably labelled by adding either 14C-glucosamine or 32P-phosphate to the medium; (v) they remained predominantly in the supernatant (cytoplasmic) fraction after centrifuging a cell homogenate for 20 min at 20,000 rev/min.

Comparison of heat shock and α polypeptides

In order to label α polypeptides, cells were infected with HSV-1 and incubated for 4 h with cycloheximide. The inhibitor was then removed and the cells were incubated for 1 h with 14C-amino acids in the presence of actinomycin D. Mock-infected cells were similarly treated. Samples of the lysates were subjected to electrophoresis in parallel with a lysate of labelled heat-shocked uninfected cells. The resulting autoradiogram (Fig. 2) shows that ICP 4, 0, 16, 22 and 27 were made after reversal of cycloheximide (channel 2). ICP 22
Effect of azetidine on synthesis and phosphorylation of polypeptides. Cells were mock-infected (M) or infected with HSV-1 (I) and incubated in medium containing 1/10 the normal concentration of amino acids with or without 4 mM-azetidine (AZ). They were labelled from 4 to 5 h p.i. with 14C-amino acids (channels 1 to 4) in the same medium or with 32P-phosphate (channels 5 to 8) in similar medium lacking phosphate.

Fig. 3. Effect of azetidine on synthesis and phosphorylation of polypeptides. Cells were mock-infected (M) or infected with HSV-1 (I) and incubated in medium containing 1/10 the normal concentration of amino acids with or without 4 mM-azetidine (AZ). They were labelled from 4 to 5 h p.i. with 14C-amino acids (channels 1 to 4) in the same medium or with 32P-phosphate (channels 5 to 8) in similar medium lacking phosphate.

Fig. 4. Identification of ICP 22 as an α polypeptide. Cells were mock-infected (M) or infected with HSV-1 (I). Cycloheximide (CX, 50 or 100 μg/ml) was added to two cultures at zero time. All cultures were treated with actinomycin D (2 μg/ml) at 3.75 h and washed with medium containing actinomycin D at 4 h and labelled in its presence with 14C-amino acids from 4 to 5 h.

 migrated to the same region of the gel as the major heat shock polypeptide C, but is clearly distinguishable from it. We have estimated mol. wt. from electrophoretic mobilities as follows: A, 120000; B, 90000; C, 66000; D, 27000; ICP 22, 75000.

Effect of azetidine on protein synthesis in normal and infected cells

Azetidine can be incorporated into virus α polypeptides in place of proline and hydroxyproline, impairing their function in initiating β polypeptide synthesis (Honess & Roizman, 1975), and other amino acid analogues were reported to stimulate the production of heat shock proteins in uninfected cells (Kelley & Schlesinger, 1978). The autoradiogram in Fig. 3 shows the result of incorporation of azetidine in normal and infected cells. Proteins were labelled with either 14C-amino acids or 32P-phosphate from 4 to 5 h p.i. Treatment of uninfected cells with azetidine had an effect closely similar to that of exposure to high temperature. Synthesis of polypeptide C was markedly stimulated (channel 2) but it was not phosphorylated (channel 7). In infected cells, in the presence of azetidine, virus-specific polypeptide synthesis was restricted to ICP 4, 6, 0, 22 and 27 (compare channels 3 and 4) and all of these were phosphorylated (channel 5). Synthesis of cellular polypeptides, including heat shock proteins, was suppressed. In untreated infected cells (channels 3 and 6) a polypeptide migrating a little more slowly than ICP 22 was labelled suggesting that ICP 22 is normally modified after synthesis and phosphorylation and that modification is prevented by incorporation of azetidine. A similar change in mobility of ICP 22 was seen during a chase period following pulse-labelling (Fig. 4B of Pereira et al. 1977 and Fig. 6 of this paper).
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Fig. 5. Times of synthesis of virus-specific polypeptides. Cells were labelled with $^{14}$C-amino acids in the presence of actinomycin D for 1 h starting at the times shown after infection with HSV-1. The right hand channel shows polypeptides labelled from 4 to 5 h p.i. in the presence of azetidine, identifying ICP 4, 6, 0, 22 and 27.

Is ICP 22 an $\alpha$ polypeptide?

ICP 22 is made in HSV-1-infected cells after removal of cycloheximide even if actinomycin D is added immediately after washing away the cycloheximide (Fig. 2). It would seem, therefore, to be either an $\alpha$ polypeptide or a $\beta$ polypeptide which, like ICP 6, starts to be made very soon after initiation of $\alpha$ ICP synthesis. In order to reduce the risk of low levels of $\beta$ mRNA synthesis either in the presence of cycloheximide or after removing cycloheximide and before the actinomycin D had taken effect, the concentration of cycloheximide was increased and actinomycin D was added 15 min before reversing the cycloheximide block by washing with medium containing actinomycin D. The autoradiogram in Fig. 4 shows that there was no difference between the patterns of labelled polypeptides in cells treated with 50 or 100 $\mu$g/ml of cycloheximide. ICP 4, 0 and 27 were more heavily labelled than in the control cells in which infection was allowed to proceed without cycloheximide (channel 2). This is because in normal infection the synthesis of $\alpha$ ICP 4, 0 and 27 had declined as $\beta$ and $\gamma$ polypeptides were formed. ICP 22 was also conspicuously labelled after removal of cycloheximide, indicating that it is an $\alpha$ polypeptide since its mRNA is made in the presence of cycloheximide. A slightly slower migrating labelled polypeptide seen in the untreated infected cells (channel 2, Fig. 4) may be the modified form of ICP 22 (see Fig. 3). If so, it is implied that modification does not occur in the absence of $\beta$ and $\gamma$ polypeptides and also that synthesis of ICP 22 was not switched off rapidly in response to $\beta$ and $\gamma$ ICP synthesis. A trace of ICP 6 was also detected but, in contrast to ICP 4, 0, 22 and 27, much less than in normally infected cells.

The kinetics of synthesis of ICP 22 were examined by labelling polypeptides in the presence of actinomycin D added at 30 min intervals after infection with HSV-1. Fig. 5 shows that mRNAs for ICP 4, 0, 22 and 27 had already been synthesized by 1 h and were translated during the following hour. The rate of synthesis of ICP 4 and 27 declined between
Fig. 6. Modification of ICP 22. HSV-1-infected cells were incubated for 4 h with cycloheximide (50 µg/ml). After washing they were pulse-labelled (P) for 30 min with 14C-amino acids. Two cultures were incubated for a further chase period (Ch) of 2 h in non-radioactive medium. Actinomycin D (2 µg/ml) was present during pulse and chase as indicated (Act).

2 and 4 h p.i. and that of ICP 0 possibly earlier, while production of ICP 22 continued unabated during this period. Other polypeptides not discussed here are identified in Fig. 5 in order to allow comparison with earlier work using the same electrophoresis system.

Modification of ICP 22

The autoradiograms in Fig. 3 and 4 suggested that the post-translational modification of ICP 22 to a slightly slower migrating form was prevented by blocking the production of β and γ polypeptides. The result of an experiment that demonstrates this more clearly is shown in Fig. 6. Cells were infected with HSV-1 and incubated for 4 h with cycloheximide. After removing cycloheximide they were pulse-labelled with 14C-amino acids with or without actinomycin. Some cultures were further incubated for a chase period in non-radioactive medium in the continued presence or absence of actinomycin. The autoradiogram (Fig. 6) shows that there are probably three forms of ICP 22. Two (a, b) were labelled during the initial pulse in the presence or absence of actinomycin D (channels 1, 2). These disappeared during a chase and a third form appeared (c, channel 3). If actinomycin D was present, the intermediate form (b) of ICP 22 accumulated during the chase period (channel 4), suggesting that in the absence of β and γ polypeptides the transition from b to c is blocked. Under the same conditions the modification of ICP 4 (Pereira et al. 1977) appeared to occur normally (although the faster migrating forms a and b are not clearly resolved).

Infection with HSV-2

Many of the HSV-1-specific polypeptides have slightly different electrophoretic mobilities compared to similar ones made after infection with HSV-2 (Courtney & Powell, 1975; Morse et al. 1978; Preston et al. 1978). However, we have been unable to identify an ana-
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Fig. 7. Synthesis and phosphorylation of polypeptides in cells infected with (1) HSV-1 or (2) HSV-2 in the presence of azetidine. Cells were labelled during the intervals (h) shown with (a) 14C-amino acids or (b) 32P-phosphate; 4 mM-azetidine was present throughout. The arrow shows the possible position of phosphorylated ICP 22 from HSV-2-infected cells.

logue of ICP 22 in Vero cells infected with HSV-2. The autoradiogram in Fig. 7(a) compares the polypeptides made in HSV-1- and HSV-2-infected cells in the presence of azetidine. The HSV-2 polypeptides presumed to correspond to HSV-1 ICP 4, 5, 6, 0 and 27 can be seen, but ICP 22 has no obvious counterpart. Other labelling periods were also tried but ICP 22 was never detected in HSV-2-infected cells.

In a similar experiment (Fig. 7b) cells infected with HSV-1 or HSV-2 in the presence of azetidine were labelled with 32P-phosphate. The phosphorylated HSV-2-specific analogues of HSV-1 ICP 4, 6, 0 and 27 are quite distinct. In the HSV-1-infected cells ICP 22 is the most heavily 32P-labelled polypeptide. A slightly darker area in the autoradiogram (Fig. 7b, channel 3, arrow) may possibly indicate the presence of HSV-2 ICP 22 migrating just ahead of the HSV-1-specific polypeptide.

DISCUSSION

The definition and identification of *α* or 'immediate early' polypeptides in cells infected with HSV-1 has presented some problems but there seems to be no doubt that ICP 4, 0 and 27 are *α* polypeptides (Honess & Roizman, 1974). If cycloheximide is added at the time of infection these polypeptides are made, after removal of the inhibitor, at rates higher than in cells undergoing normal infection. In addition, ICP 22 was originally included in the *α* class (Kozak & Roizman, 1974) but subsequently its qualifications were questioned (Honess & Roizman, 1975).

The experiments reported here support the earlier proposition that ICP 22 is specified by the virus and is an *α* polypeptide, although one whose rate of synthesis does not decline rapidly with the appearance of *β* and *γ* polypeptides. There may have been some confusion between ICP 22 and a 'heat shock' protein whose synthesis in uninfected cells is stimulated by raising the temperature to 40°C or by adding amino acid analogues to the medium.
However, in Vero cells at least, heat shock polypeptide C differs considerably from ICP 22 in electrophoretic mobility and its synthesis, like that of other cellular proteins, is suppressed by infection with HSV-1. The heat shock proteins A, B and C may correspond to p120, p95/93 and p76 found in other mammalian cells (Kelly & Schlesinger, 1978).

Other properties of ICP 22 reported previously are that it is phosphorylated and modified to a slightly slower migrating form after synthesis (Pereira et al. 1977), and that it accumulates in the nucleus of the infected cell (Fenwick et al. 1978). In contrast, Vero polypeptide C is not phosphorylated and remains in the cytoplasm. Our experiments indicate that ICP 22, like ICP 4 (Pereira et al. 1977), is modified in at least two steps and that (unlike ICP 4) the second step may depend on a β or γ polypeptide. The nature of the function of ICP 22 and whether this β/γ-mediated modification is necessary for its performance is unknown.

A number of immediate early (IE) polypeptides of HSV-1 have been described by others (Preston et al. 1978), namely IE 175, 136, 110, 87, 68 and 63. The numbers indicate apparent mol. wt. \( \times 10^{-3} \). The probable correlation between this nomenclature and that of Honess & Roizman (1974) is shown below:

<table>
<thead>
<tr>
<th>IE</th>
<th>ICP</th>
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<tbody>
<tr>
<td>175</td>
<td>4 (170 to 174)</td>
</tr>
<tr>
<td>136</td>
<td>6 (146)</td>
</tr>
<tr>
<td>110</td>
<td>0 (125)</td>
</tr>
<tr>
<td>87</td>
<td>16 (97)</td>
</tr>
<tr>
<td>68</td>
<td>22 (75)</td>
</tr>
<tr>
<td>63</td>
<td>27 (60)</td>
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The numbers in parenthesis are our estimates of mol. wt. of the ICPs. The main discrepancy is that ICP 6 is not considered to be an α polypeptide because it is made at a much lower rate after reversing cycloheximide than in normally infected cells at the same time. IE 136 was classified as 'immediate early' since a small amount of its RNA is made in the presence of cycloheximide (Preston, 1979).

ICP 16 is invariably made in small amounts after reversing cycloheximide (Fig. 4). Like α ICP 4, 0 and 27, its synthesis starts within 1 h p.i. and declines again between 2 and 4 h (Fig. 5). On the other hand, unlike the other α polypeptides, it is not made, or only in trace amounts, in the presence of azetidine (Fig. 3, 7).

ICP 19 (mol. wt. about 87,000) should also be mentioned in a discussion of α polypeptides because of its early synthesis and sharp decline between 2 and 3 h (Fig. 5). However, it appears not to be an α polypeptide as it is not made on reversing cycloheximide, nor in the presence of azetidine.

We have not seen an obvious analogue of ICP 22 in cells infected with HSV-2, nor is one evident in the work of Powell & Courtney (1975) or Pereira et al. (1977), but Preston et al. (1978) reported a slightly faster migrating version of IE 68 in HSV-2 infection. It is possible that the relative rate of synthesis of ICP 22 varies between different strains of HSV-2.

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REFERENCES


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