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Herpes simplex virus-1-specific proteins are involved in alteration of polyphosphoinositide metabolism in baby-hamster kidney cells

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Herpes simplex virus type 1 (HSV-1) induces altered phosphoinositide metabolism in baby hamster kidney (BHK) cells, measured as incorporation of [^{3}H]inositol or [^{32}P]P_i [Langeland, Haarr & Holmsen (1986) Biochem. J. **237**, 707–712]. We now report that this response in the inositol phospholipids is dependent on virus-specific proteins synthesized in the β (early) stage of virus protein synthesis. This was demonstrated both by resistance to the inhibitory effect of cycloheximide after this stage of infection, and by the use of temperature-sensitive (ts) mutants of HSV-1; ts mutants in which protein synthesis was blocked so that only the α proteins were expressed showed a PIP_2/PIP (phosphatidylinositol 4,5-bisphosphate/phosphatidylinositol 4-monophosphate) ratio similar to uninfected cells, while ts mutants which were defective in protein synthesis at a late β stage or later showed increased PIP_2/PIP ratios similar to cells infected by wild type HSV-1.

INTRODUCTION

We have previously shown that herpes simplex virus type 1 (HSV-1) infection of baby-hamster kidney (BHK) cells leads to altered polyphosphoinositide (PPI) metabolism (Langeland *et al.*, 1986). An increase in [^{32}P]- and [^{3}H]inositol labelling of phosphatidylinositol 4,5-bisphosphate (PIP_2) and a corresponding decrease in phosphatidylinositol 4-monophosphate (PIP) labelling was observed, starting 4–5 h post-infection. The significance of this altered PPI labelling is unknown. Phosphoinositides are well known as substrates for phospholipase C, and thus as precursors of the second messengers inositol trisphosphate and diacylglycerol (reviewed by Berridge, 1985). It was recently reported that the herpes virus Epstein–Barr virus induces protein kinase C activation, probably through phospholipase C activation (Dugas *et al.*, 1988). The human immunodeficiency virus (HIV)-1 glycoprotein gp120 can induce increased levels of inositol trisphosphate in CD4-positive lymphocytes (Kornfeld *et al.*, 1988). Elevated PPI turnover has also been observed in other virus infections (Macara *et al.*, 1984; Sugimoto *et al.*, 1984). In these transforming retrovirus infections, the observed increase in PPI labelling has been related to the viral oncogenes and their ability to transform cells. HSV-1 and HSV-2 also have the capacity to transform cells *in vitro*, but the mechanism is poorly understood (reviewed by McNab, 1987). It has been suggested that instead of expressing oncogenes in the traditional sense, HSV may turn on specific cellular functions (MacNab, 1987).

In this context, the altered PPI turnover which we observed after HSV-1 infection (Langeland *et al.*, 1986) may be of significance. The present work was initiated in an attempt to classify the virus functions involved. HSV proteins are synthesized in a sequential manner, where the latest proteins synthesized, the γ-proteins, depend on the previous synthesis of the β-proteins, which in turn are dependent on the synthesized α-proteins. We now report that the alterations in PPIs can be abolished by inhibition of virus-induced proteins at the β-protein stage of protein synthesis.

MATERIALS AND METHODS

Materials

Eagle's minimum essential medium (EMEM) and newborn calf serum were purchased from Flow Laboratories (Irvine, Ayrshire, U.K.). The cells used were BHK 21 clone 13 (Macpherson & Stoker, 1962). [^{32}P]P_i (10 mCi/ml, carrier-free) and [^{3}H]inositol (1 mCi/ml) were purchased from Amersham International (Amersham, Bucks., U.K.). Cell culture dishes were from Nunc (Roskilde, Denmark). T.l.c. plates and chemicals (chromatography grade) were obtained from E. Merck (Darmstadt, Germany). Autoradiography films were Fuji RX medical X-ray films (Fuji Photo Film Co., Tokyo, Japan).

Virus strains and mutants

The HSV-1 wild type used was strain 17 *syn*$^+$ (Brown *et al.*, 1973). The temperature-sensitive (ts) HSV-1 mutants tsK and tsH have been isolated and described earlier (Marsden *et al.*, 1976; Preston, 1979). The HSV-1 ts mutant ts1201 was isolated and described by Preston *et al.* (1983).

Radiolabelling and infection of cells

In experiments where the ts mutants were used, nearly

Abbreviations used: PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PPI, (poly)phosphoinositide; HSV, herpes simplex virus, BHK, baby-hamster kidney; ts, temperature sensitive, EMEM, Eagle's minimum essential medium; V_M 175, virus-induced polypeptide of apparent M_r 175000.

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confluent BHK cells grown in 32 mm cell culture dishes were prelabelled for 16 h with [^{32}P]P_i (3 μCi/ml) or [^{3}H]inositol (3 μCi/ml). Immediately before infection, the medium was removed, and replaced by fresh EMEM without isotope. The temperature of cells and virus was then adjusted to 38.5 °C (non-permissive temperature) or 31 °C (permissive temperature) before virus was added. In the cycloheximide experiments, the isotope (5 μCi/ml) was added immediately before infection, and the temperature was kept at 37 °C throughout the experiment. The cycloheximide concentration was 180 μM. All infections were performed at a multiplicity of infection of 20 plaque-forming units per cell. It should be noted that zero time is the time when the virus is added, not at the end of the adsorption period.

Extraction of lipids

The extraction procedures have been described previously (Langeland *et al.*, 1986). Briefly, medium was removed, and methanol/$CHCl_3$/HCl (49:20:1, by vol.) was added directly to the cell culture dishes. Samples were transferred to test tubes, further chloroform and water were added before the organic phase was collected and dried.

Thin layer chromatography

Lipid residues were dissolved in 30 μl of chloroform and applied on the t.l.c. plates. The inositol lipids were separated in the solvent system $CHCl_3$/methanol/40% methylamine/water (60:36:5:5, by vol.) as described by de Chaffoy de Courcelles *et al.* (1984). After visualization by autoradiography, the labelled lipids were scraped off the t.l.c. plates and radioactivity measured by scintillation counting.

RESULTS

Infection in the presence of cycloheximide

We wanted to investigate whether the HSV-1-specific changes in polyphosphoinositides were induced by the virus-specific proteins. In the first set of experiments (Figs. 1*a* and 1*b*) cycloheximide was present from 30 min before infection and until the cells were harvested at the times indicated. The data show that upon cycloheximide treatment the labelling of both PIP (*a*) and PIP_2 (*b*) are generally reduced both in infected and mock-infected cells. However, the HSV-1-specific alterations in the PPIs are abolished in the cycloheximide-treated cells, suggesting that the inhibition of the PPI response may be related to the inhibition of protein synthesis. To further examine whether the effect of cycloheximide was limited to a certain period of infection, the inhibitor was added at various times after infection, and was present until harvesting at 10 h post-infection (Fig. 1*d*). This end point was chosen because previous experiments had consistently shown a definite and maximal PIP_2 response at this time. In parallel, cells which had not been exposed to cycloheximide were harvested at the times indicated (Fig. 1*c*). The end points of Fig. 1(*c*) are thus untreated cells corresponding to the cycloheximide-treated cells of

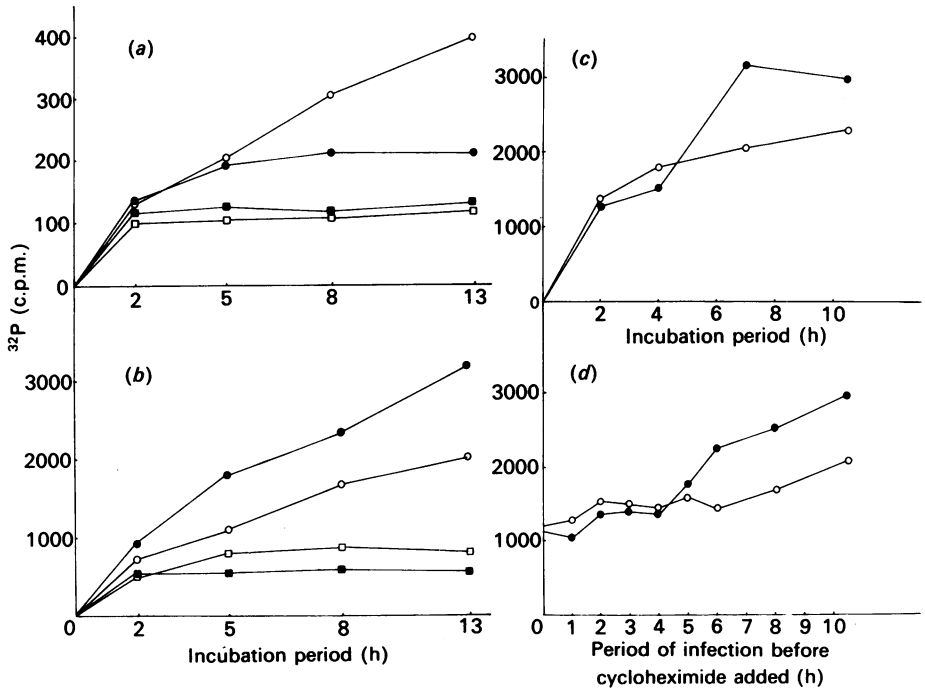

Fig. 1. Effect of cycloheximide on [^{32}P]P_i incorporation into PIP and PIP_2 in HSV-1 or mock-infected cells

Confluent BHK cells were incubated in EMEM containing 5 μCi of [^{32}P]P_i/ml from the time of infection. Cycloheximide (180 μM) was present as indicated. (*a*), (*b*) Cycloheximide (□, ■) or vehicle (○, ●) were added 30 min before HSV-infection (■, ●) or mock infection (□, ○). Cells were harvested at the times indicated after infection and labelling of PIP (*a*) and PIP_2 (*b*) was measured. (*c*) [^{32}P]PIP_2 in infected (●) or mock-infected (○) cells which had not been exposed to cycloheximide. Cells were harvested at the times indicated. Simultaneously, (*d*), HSV-1 infected (●) or mock infected (○) cells were exposed to cycloheximide from the time indicated and until all samples were harvested 10 h postinfection, and the contents of [^{32}P]PIP_2 measured, corresponding to the end points of (*c*). Data are given as means of triplicate incubations. Three separate experiments were performed, and all gave similar results.

Fig. 1(*d*). The data show that when cycloheximide was added later than 5 h after infection, it had little effect on the PIP_2 response, whereas addition at any time up to 4 h post-infection abolished the HSV-1-induced PIP_2 response. When the curves for the cycloheximide-treated cells (Fig. 1*d*) are compared with the untreated cells (Fig. 1*c*), it appears that the time after which cycloheximide no longer has an inhibitory effect (*d*) and the time when the PIP_2 response starts (*c*) are very similar. The data represent means of three parallel incubations; within-experiment variability was calculated to be less than 15%. The effect of cycloheximide on the HSV-1-induced PIP response was also tested, but data were not sufficiently consistent to be significant (results not shown).

Infection with temperature-sensitive HSV-1 mutants

To study further whether specific virus-induced proteins are responsible for the altered turnover of PIP and PIP_2, temperature-sensitive HSV-1 mutants were used. At the permissive temperature the synthesis of virus-encoded proteins is normal, but at non-permissive temperature it is blocked at a specific stage depending upon the site of mutation. Thus, the tsK mutation in the α-gene for the virus-induced polypeptide of apparent M_r 175000 (V_M 175) results in synthesis of immediate early proteins at non-permissive temperature, but β- and γ-proteins are absent (Preston, 1979, 1981). Correspondingly, the tsH is deficient in the early (β) gene for the DNA polymerase (Dargan & Subak Sharpe, 1984), and the ts1201 affects a late (γ) protein involved in getting DNA into pre-made, empty nucleocapsids (assembly protein) (Preston *et al.*, 1983). Thus, at non-permissive temperature, the tsK mutant allows the synthesis of the α-proteins, while β- and γ-proteins are strongly inhibited or absent; in the tsH mutant, both α- and β-proteins are expressed, but few or no γ; and finally in the ts1201 mutant, α-, β- and γ-proteins up to the synthesis of the assembly protein are expressed. In order to lessen the effect of different temperatures on the uptake of isotopes, these experiments were performed with prelabelled cells. Incubations were continued for 10 h, and data presented as PIP_2-to-PIP ratios (Fig. 2). Separate experiments were performed with [^{3}H]inositol and [^{32}P]P_i labelling (Figs. 2*a* and 2*b* respectively). The data show that all mutant infections gave increased PIP_2/PIP ratios similar to, or even greater than, the wild type virus when incubations were performed at permissive temperature (31 °C). At non-permissive temperature (38.5 °C), however, the tsK gave no increase in PIP_2/PIP ratio as compared with mock-infected cells, whereas tsH and ts1201 both showed increased ratios similar to wild type virus (Figs. 2*a* and 2*b*). Labelling with [^{3}H]inositol or [^{32}P]P_i gave similar results.

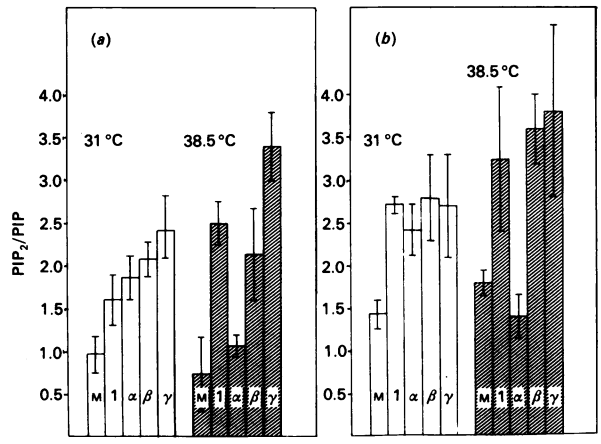

Fig. 2. PIP_2/PIP ratio, measured as [^{3}H]inositol or [^{32}P]P_i incorporation, in cells infected with HSV-1 wild type (1), tsK (α), tsH (β), ts1201 (γ) or mock infected (M)

Prelabelled cells were infected, and incorporation of radiolabel into the phosphoinositides was measured 10 h post-infection. The ratio of [^{3}H]inositol-labelled PIP_2/PIP (*a*) or [^{32}P]PIP_2/[^{32}P]PIP (*b*) were measured after infection and incubation at 31 °C (open bars) or 38.5 °C (hatched bars). Data are means ± S.E.M. of four to six separate incubations.

DISCUSSION

The aim of the present work was to reveal whether the changes in cellular PPI metabolism after HSV-1 invection previously observed (Langeland *et al.*, 1986) were mediated by virus-specific proteins. It has been observed that HSV infection leads to a shut-off of host protein synthesis (Sydiskis & Roizman, 1967; Fenwick, 1984). A small number of cellular proteins, however, accumulate during lytic infection (La Thangue *et al.*, 1984). To reduce the possible influence from uninfected cells with normal protein synthesis, we chose a high multiplicity of infection; all experiments were performed with 20 plaque-forming units per cell. Cycloheximide has been widely used to inhibit virus-specific protein synthesis in HSV infection, but it also affects cellular protein synthesis (Read & Frenkel, 1983). Bearing in mind possible unspecific effects, or effects directly on the phospholipids, we chose cycloheximide as a general inhibitor of protein synthesis. The results illustrated in Figs. 1(*c*) and (*d*) demonstrate that addition of the drug after a certain stage of infection had no effect on the virus-induced increase in [^{32}P]PIP_2. This time, approximately 5 h post-infection, coincides with the time when the PIP_2 increase is first observed in the absence of cycloheximide (Fig. 1*c*). Furthermore, the time-course of synthesis of α-, β- and γ-proteins during normal lytic infection suggests that one or more β-proteins affect the metabolism of the phospholipids. The peak of α-proteins is significantly before 5 h, and γ-proteins continue to increase after this time (Honess & Roizman, 1974).

To avoid a misinterpretation of these data due to unknown side effects of cycloheximide, we also approached the problem by using temperature-sensitive mutants of HSV-1. They synthesize viral proteins normally at permissive temperature, but at non-permissive temperature the sequence of synthesized proteins is stopped at specific points. The results in Fig. 2 show that the synthesis of the α-proteins is not sufficient to induce the wild-type-specific increase in PIP_2/PIP ratio. However, when the synthesis of β-proteins was allowed, but no active DNA polymerase made, the ratio was the same as with a normal wild-type infection. The same was observed when infection was stopped at the synthesis of the assembly protein (Fig. 2). Taken together, the data indicate that the observed response in the PPIs was dependent upon one or more virus-specific proteins most likely synthesized after the V_M 175 but before the DNA polymerase. It must be noted that the synthesis of some of the earliest γ-proteins starts before the synthesis of the DNA polymerase. However, since these are increasingly

synthesized after the β phase of protein synthesis, the data concerning cycloheximide treatment argue against a role for γ-proteins; there is no difference in the cycloheximide effect on the [^{32}P]PIP_2 level at 6 h (early γ phase) and at 10 h (late γ phase) (Fig. 1*d*).

The possibility also remains that the changes in the PPIs are caused by a cellular protein which is turned on or amplified at early stages of virus infection. We have not been able to show any phospholipase C activation during infection, as measured by formation of [^{3}H]diacylglycerol or [^{32}P]phosphatidic acid (results not shown). Whether the involved protein(s) acts as a PIP kinase, or has more indirect influence on the PIPs, remains to be shown.

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