Interaction of Frog Virus 3 with the Cytomatrix.

IV. Phosphorylation of Vimentin Precedes the Reorganization of Intermediate Filaments around the Virus Assembly Sites

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SUMMARY

Frog virus 3 (FV3) assembles in morphologically distinct assembly sites in the cytoplasm of infected cells. As the assembly sites form, the intermediate filaments (IF) aggregate, delimit the assembly sites, and remain so throughout infection. To determine the molecular basis of reorganization of IF, we analysed the vimentin of uninfected and FV3-infected cells by two-dimensional gel electrophoresis. The results showed that (i) the vimentin was more acidic in FV3-infected cells than in uninfected cells, (ii) the acidification of vimentin in FV3-infected cells was possibly due to a fourfold increase in phosphorylation, and (iii) the phosphorylation of vimentin preceded the reorganization of IF around virus assembly sites. A temperature-sensitive mutant of FV3 (ts9467), which at the non-permissive temperature neither reorganized IF nor formed assembly sites, failed to increase the phosphorylation of vimentin. Together, the above results suggest that changes in phosphorylation may modulate IF organization and that changes in IF organization are required for FV3 assembly site formation.

INTRODUCTION

Intermediate filaments (IF) measure 7 to 11 nm and contain distinct proteins, depending upon the type of cell (for review see Lazarides, 1982). One class of IF found in a wide variety of cells contains vimentin as the principal protein (Franke et al., 1978). The IF network shows changes in its organization in cells undergoing mitosis (Blose, 1979; Aubin et al., 1980), in colcemid-treated cells (Goldman & Knipe, 1973; Blose & Chacko, 1976; Starger & Goldman, 1977), during myogenesis (Granger & Lazarides, 1978, 1979) and in Sertoli cells stimulated with hormones (Spruill et al., 1983a, b). These changes in IF are coincident with the phosphorylation of vimentin (Robinson et al., 1981; Evans & Fink, 1982; Gard & Lazarides, 1982; Spruill et al., 1983a, b). Since phosphorylation controls many cellular metabolic processes (Krebs & Beavo, 1979), it was believed that changes in the organization of IF are also mediated by phosphorylation and dephosphorylation of IF proteins.

Frog virus 3 (FV3), an icosahedral DNA-containing iridovirus, induces drastic organizational changes in the cytoskeleton of a variety of tissue culture cells including those derived from mammals (Murti & Goorha, 1983; Murti et al., 1984). Soon after infecting the cell, FV3 establishes morphologically recognizable virus assembly sites (Darlington et al., 1966; Murti & Goorha, 1983). Some viral nucleic acid synthesis occurs in the host nucleus but the assembly of the virus occurs in these sites. As the assembly sites form, the IF aggregate, delimit the assembly sites and remain so throughout infection (Murti & Goorha, 1983). The significance of IF reorganization remains to be understood, but it is possible that the process may prevent the intrusion of cell components into the assembly sites and/or anchor the assembly sites close to the nucleus. Thus, FV3 induces a morphologically recognizable change in IF that may also be relevant to virus assembly.
In this study, we show that there is a fourfold increment in the phosphorylation of vimentin in infected cells compared to uninfected cells. A temperature-sensitive mutant of FV3 (ts9467) which neither reorganizes IF nor forms assembly sites at the restrictive temperature (30 °C) does not increase the phosphorylation of vimentin. Together, the above studies support the hypothesis that changes in phosphorylation modulate the IF organization and that IF reorganization is required for virus assembly site formation.

METHODS

Cells and viruses. Baby hamster kidney (BHK) cells were grown in 60 mm plastic Petri dishes in Eagle’s MEM (Flow Laboratories) supplemented with 5% foetal calf serum (KC Biologicals, Lenexa, Kan., U.S.A.). The cells were infected with a clonal isolate of FV3 as described (Murti & Goorha, 1983). The growth and assay of FV3 was done as described by Naegele & Granoff (1971). Growth and characterization of ts9467 have been described (Willis et al., 1979).

Immunofluorescence. Immunofluorescence analysis of infected cells with anti-vimentin and anti-FV3 antibodies was done as described earlier (Murti & Goorha, 1983). Isotopic labelling of proteins in infected cells. Monolayers of BHK cells were infected with FV3 at a multiplicity of 20 p.f.u./cell. After adsorbing the virus for 1 h at 23 °C, the cells were washed twice with MEM, overlaid with MEM containing 2% dialysed foetal calf serum, and kept at 30 °C. At various intervals after infection, the medium was replaced with methionine-free MEM containing 50 μCi/ml [35S]methionine (Amersham) or phosphate-free MEM containing 32P, (Amersham). At the end of the labelling period, the cells were washed three times with phosphate-buffered saline (PBS) and scraped with a rubber policeman into 1 ml PBS. The cells were pelleted in a microfuge and resuspended in 130 μl of double concentration lysis buffer [9.5 M-urea, 2% (w/v) NP40, Ampholines and 5% 2-mercaptoethanol (O’Farrell, 1975)]. The suspension was thoroughly mixed, heated at 100 °C for 5 min, and cooled to room temperature. The lysate was stored at −20 °C.

Two-dimensional gel electrophoresis. Two-dimensional (2-D) gel electrophoresis was done according to the procedure of O’Farrell (1975) with minor modifications (Mufti et al., 1985).

RESULTS

We have previously described the progressive organizational changes in intermediate filaments of FV3-infected BHK cells as observed by immunofluorescence and electron microscopy (Murti & Goorha, 1983). Immunofluorescence studies with anti-vimentin antibodies revealed that at about 6 h post-infection, the IF form bundles and outline certain spherical areas in the host cytoplasm. By 8 h, the fluorescence due to IF was concentrated around discrete spherical bodies in the cytoplasm. Electron microscopic studies confirmed the changes in IF found by immunofluorescence and in addition identified the spherical bodies as the virus assembly sites.

Qualitative change in vimentin of FV3-infected BHK cells

To determine the molecular basis for the above morphological changes in the IF network of FV3-infected cells, we analysed the vimentin of uninfected and infected cells by 2-D gel electrophoresis. The identity of vimentin in 2-D gels of lysates from FV3-infected cells was established as follows. Vimentin was purified from uninfected BHK cells following published procedures (Starger et al., 1978) for use as a marker. The unlabelled marker vimentin was mixed with [35S]methionine-labelled lysates from infected cells and the lysates were subjected to 2-D gel electrophoresis. After electrophoresis, the gels were first stained with Coomassie Brilliant Blue to locate the marker (the concentration of the cell lysate was kept low to prevent staining of other proteins), dried, and then exposed to X-ray film to locate the 35S-labelled vimentin from infected cells. In uninfected cells, the labelled vimentin had the same mobility as the marker, and its mobility relative to other major cellular proteins (e.g. actin) was similar to that previously reported (Brown et al., 1976; Ben-Zeev et al., 1979; Cabral & Gottesman, 1979; O’Connor et al., 1979). Finally, the identity of vimentin was further established by immunoprecipitation with anti-vimentin antibodies [a kind gift of Dr R. Hynes (Hynes & Destree, 1978)].

To compare the quantitative and qualitative changes in vimentin, cell extracts from [35S]methionine-labelled FV3-infected and uninfected BHK cells were analysed on 2-D gels. To compare the synthesis of vimentin in FV3-infected and uninfected cells, the vimentin spot in the gel was excised and the radioactivity was counted. No significant difference was found in the
Interaction of frog virus 3 with cytomatrix

Fig. 1. Two-dimensional (isoelectric focusing and SDS) gel analysis of cell lysate from [35S]methionine-labelled, FV3-infected BHK cells (see text for details). The cells were labelled with [35S]methionine at 6 h post-infection, for 1 h. The area outlined in black shows the position of marker vimentin. Note the shift in vimentin towards the acidic pH range.

amount of radioactivity in the two samples, which suggests that the virus did not quantitatively alter the synthesis of vimentin. However, we found a qualitative change. At 6 h post-infection, or at a time when the intermediate filaments redistribute around the forming assembly sites, most of the newly synthesized vimentin was more acidic than its counterpart in uninfected cells (Fig. 1). To determine if the shift occurred in pre-existing vimentin or in vimentin synthesized after FV3 infection, BHK cells were first labelled with [35S]methionine for 8 h, infected with FV3 for 6 h, and the cell extracts were analysed on 2-D gels (Fig. 2). The pre-labelled vimentin showed no shift towards the acidic pH range, suggesting that the shift occurs in vimentin synthesized after virus infection. Thus, FV3 infection induced a qualitative change in vimentin and the change coincided with the reorganization of intermediate filaments around the viral assembly sites.

Increased phosphorylation of vimentin in FV3-infected cells

The change in vimentin of FV3-infected cells might have been due to a post-translational modification, such as phosphorylation. To determine whether this was the case, BHK cells at 3 h post-infection were labelled for 1 h with $^{32}$P, and the cell extracts were analysed on 2-D gels. The results showed a dramatic increase in the phosphorylation (more than fourfold) of vimentin by 4 h post-infection when compared to uninfected cells (Fig. 3). Pulse-chase experiments showed no detectable differences in the turnover of $^{32}$P-labelled vimentin which suggests that the observed increase in the phosphorylation of vimentin in infected cells was not due to a decreased rate of dephosphorylation. The phosphorylation of vimentin precedes the reorganization of IF around the virus assembly site because the latter process occurs at about 6 h in BHK cells (Murti & Goorha, 1983).
Fig. 2. (a) Two-dimensional gel analysis of cell lysate from uninfected BHK cells labelled for 8 h with [35S]methionine. (b) Two-dimensional gel analysis of cell lysate from BHK cells labelled for 8 h with [35S]methionine and then infected with FV3 for 6 h. Note that the position of vimentin is the same in (a) and (b), which suggests that the vimentin synthesized before infection does not show a shift towards the acidic pH range.

Phosphorylation of vimentin is required for FV3 replication

To determine whether phosphorylation of vimentin and IF reorganization are interconnected, we studied these phenomena in BHK cells infected with a ts mutant of FV3, ts9467 (Willis et al., 1979). The mutant, at the non-permissive temperature of 30 °C, makes all early viral proteins (Willis et al., 1979), but neither reorganized intermediate filaments (Fig. 4a) nor formed assembly sites (Fig. 4b). Upon shift to the permissive temperature (25 °C), the intermediate filaments reorganized (Fig. 4c), the assembly sites formed (Fig. 4d) and the virus produces infectious progeny (Willis et al., 1979).

When 32P-labelled cell extracts from BHK cells infected with ts9467 at 30°C were analysed on 2-D gels, vimentin showed very little incorporation of the label (Fig. 5). However, upon shift to the permissive temperature, vimentin showed increased phosphorylation (data not shown) similar to cells infected with the wild-type virus (Fig. 3). The above results strongly suggest that phosphorylation of vimentin, IF reorganization and assembly site formation are all interlinked.

DISCUSSION

We have shown here that (i) the vimentin is more acidic in FV3-infected cells, (ii) the acidification of vimentin is possibly due to phosphorylation, and (iii) the phosphorylation of vimentin precedes the reorganization of IF around the virus assembly site. The above results support an earlier proposal that phosphorylation modulates the organizational changes in intermediate filaments (Lazarides, 1980). It remains to be found whether phosphorylation of vimentin is carried out by a virus-specific kinase, a cellular kinase, or a virus-modified cellular kinase. It is, however, noteworthy that in infected cells, the virus synthesizes a cAMP-independent protein kinase (Silberstein & August, 1973, 1976) and the activity of this enzyme is undetectable in ts9467-infected cells at the non-permissive temperature.
Fig. 3. Increased phosphorylation of vimentin in FV3-infected cells. The infected cells were labelled at 3 h post-infection with $^{32}$P, (1 mCi/ml) for 1 h and the lysates were analysed on 2-D gels. Note the increased phosphorylation of vimentin in FV3-infected cells (lower part) when compared to uninfected cells (upper part). Areas outlined in black denote the location of marker vimentin.
Using cells infected with a ts mutant of FV3, we have shown that phosphorylation of vimentin, reorganization of IF and formation of assembly sites are all interconnected. The results attribute an active role to IF in FV3 replication. The precise function of IF in FV3 replication remains unclear, but it is possible that their reorganization facilitates the accumulation of viral DNA and viral proteins at the assembly sites. We can also envisage two additional roles for IF in virus assembly. First, the filaments may anchor the assembly sites at a definite location within the cell such that a directed movement of viral components to the sites could occur. Second, the filaments, by surrounding the virus assembly site, may exclude cellular
components (e.g. organelles, other cytoskeletal filaments, polysomes and ribosomes) from the assembly site. Both these functions have parallels within normal cells. The IF are thought to function in the placement of cellular organelles (Lazarides, 1980). The filaments remain as a static network throughout most of the cell cycle, but during mitosis they aggregate and form a cage around the mitotic spindle (Zieve et al., 1980). The significance of this reorganization is not known but it has been suggested that the process may exclude cellular organelles from the spindle (Zieve et al., 1980) or provide structural support for it. Thus, the postulated functions for IF in normal cells are important in the formation and maintenance of FV3 assembly sites since the sites remain free from cell organelles and occur as discrete bodies near the nucleus.

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