Specific Secretion of Polypeptides from Cells Infected with Vaccinia Virus

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The pattern of polypeptides specifically secreted by cells after infection with vaccinia virus has been analyzed. A complex pattern of apparently virus-specified polypeptides exhibiting temporal control of the type seen with intracellular polypeptides after virus infection was observed. Some of the specifically secreted polypeptides were shown to be modified by glycosylation and sulfation. The possible significance of these results is discussed.

Studies on the polypeptides synthesized after virus infection of a cell are normally confined to examining intracellular polypeptides. However, recent studies in two virus systems, pseudorabies virus (4, 5) and Rous sarcoma virus (1), have indicated that virus infection of a cell can also result in changes in the pattern of polypeptides secreted from those cells into the tissue culture fluid.

As part of a study on the sulfation of polypeptides in vaccinia virus-infected cells (M. A. McCrae and T. H. Pennington, manuscript in preparation), we have investigated, and described in detail in this report, the changes in secreted polypeptides that occur after infection with vaccinia virus.

MATERIALS AND METHODS

Virus growth and purification. The Evans vaccine strain of vaccinia was grown in, and purified from, BHK 21 clone 13 cells as previously described (7).

Virus infection and protein labeling. Virus infections and protein labeling were done as follows. Confluent 35-mm petri dishes were infected with vaccinia virus at a multiplicity of 50 PFU/cell. After adsorption for 1 h at 37°C, the inoculum was removed and replaced with Eagle medium containing 2% fetal bovine serum. Before labeling, the maintenance medium was removed, the monolayers were washed twice with phosphate-buffered saline, and then 0.2 or 0.4 ml of the appropriate label diluted in phosphate-buffered saline was added. The concentration of the isotope used were: L-[35S]methionine, 50 μCi/ml; 35SO4, 100 μCi/ml; and [14C]glucosamine, 100 μCi/ml. At the end of the labeling period either cells were harvested immediately into 0.4 ml of 10 mM Tris-hydrochloride buffer, pH 9.0, or the label was removed and replaced with 0.4 ml of Eagle medium and incubation was continued at 37°C for a further 2 h. After the chase period, the medium and cells were harvested separately. All samples were stored immediately at −80°C until required for polyacrylamide gel electrophoresis analysis. Where cytosine arabinoside (CAR) was used, it was present at a concentration of 25 μg/ml at all stages, from addition of virus to the cells until the final harvesting of the samples.

Polyacrylamide gel electrophoresis. Samples were analyzed on 5 to 15% sodium dodecyl sulfate-gradient polyacrylamide gels as previously described (7), using the Laemmli (6) discontinuous buffer system. After electrophoresis, gels were processed for fluorography, according to the method of Bonner and Laskey (2), and exposed at −80°C, using Kodak RP Royal X-Omat film.

Peptide map analysis. Peptide mapping of the 35,000 (35K)-secreted polypeptide was carried out by using a modification of the gel mapping procedure of Cleveland et al. (3), as described previously (M. A. McCrae and W. K. Jolik, Virology, in press). Digestion was with the V8 protease of Staphylococcus aureus at final concentrations of 10 and 100 μg/ml. Peptides were resolved on an 8 to 16% gradient polyacrylamide gel.

RESULTS

Temporal pattern of polypeptides secreted by virus-infected cells. The work of Kaplan (5) has shown that infection of cells with pseudorabies virus results in the secretion by the cells of large amounts of a nonstructural sulfated glycoprotein of approximately 90K molecular weight. When the pattern of proteins secreted by BSC-1 cells after infection with vaccinia virus was examined, changes were also observed (Fig. 1). The most dramatic of these at early times postinfection (2 to 2.5 h postinfection) was the secretion of a new viral-induced polypeptide with a molecular weight of approximately 35K. This protein appeared in the medium in very large amounts; if it is assumed that this protein has a normal methionine content, then the quantity observed in the medium exceeded that of any single intracellular polypeptide synthesized during the labeling period.

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FIG. 1. Polyacrylamide gel analysis showing the temporal pattern of synthesis of intracellular and secreted polypeptides in infected BSC-1 cells. Conditions of labeling with [35S]methionine and analysis of polypeptides on gradient polyacrylamide gels are given in the text. Early (E) times postinfection—labeling was for 30 min from 2 to 2.5 h postinfection, and late (L) pulses were from 5 to 5.5 h postinfection. In cases where labeling was done after inhibition of viral DNA synthesis, it occurred at 5 to 5.5 h postinfection, after the cells had been treated from the time of virus addition with CAR at a concentration of 25 μg/ml. For chase samples, the cells were harvested after a 2-h chase in the presence of 100 times the normal methionine concentration. Order of the samples: A, uninfected cell profile after a 30-min pulse; B, uninfected cell profile after a 30-min pulse in the presence of CAR following treatment for 5 h with 25 μg of CAR per ml; C, intracellular polypeptide profile after a 30-min pulse early in infection; D, intracellular polypeptide profile after a 2-h chase of C; E, intracellular polypeptide profile after a 30-min pulse late in infection; F, intracellular polypeptide profile after a 2-h chase of E; G, intracellular polypeptide profile after a 30-min pulse late in infection of cells treated with CAR; H, intracellular polypeptide profile after a 2-h chase of G; I, polypeptides secreted early in infection; J, polypeptides secreted late in infection; K, polypeptides secreted late in infection after application of a CAR block; L, polypeptides secreted by uninfected cells.
Several other changes in the secreted polypeptide profile at early times postinfection were also observed. These included the appearance of polypeptides migrating as broad diffuse bands both above and below this major polypeptide in the regions 31 to 34K and 36 to 38K, and the secretion of polypeptides with apparent molecular weights of 25K and 12K that comigrated with major polypeptides present in the infected cell at these early times (Fig. 1).

At late times in the virus growth cycle (5 to 6 h postinfection) the major secreted polypeptide of molecular weight 35K was still produced in large amounts. The broad diffuse band migrating in the 36 to 38K region, was, however, no longer produced, and the diffuse band migrating in the 31 to 35K region was replaced by a more sharply defined band with an approximate molecular weight of 32K. The levels of production of the 25K and 12K polypeptides were also reduced, and, in the case of the 25K polypeptide, this reduction could be correlated with a reduction in the synthesis of an intracellular polypeptide with the same mobility (Fig. 1). At the late times postinfection there was also a virtually complete inhibition of the normal host background of secreted proteins (Fig. 1). Other changes evident in the region below the major 35K protein in infection were (i) the sharpening into a more discernible but nevertheless diffuse band of a band in the 21 to 27K region which at early times postinfection appeared only as a smear on the gel profile and (ii) the appearance of a new band with a molecular weight of 14.5K which comigrated with a late intracellular polypeptide. In the region above the 35K protein two or three very faint new bands were seen, but these comigrated with the major virion structural polypeptides and are probably due to mature virions which would be being released at these late times postinfection.

The effect of blocking viral DNA synthesis with CAR on the secreted polypeptide profile was examined, thus allowing classification of polypeptides into the early and late classes. The addition of CAR to the medium at the time of infection was found to block the appearance of the 14.5K and 32K polypeptides, and these polypeptides can therefore be classified as classical late polypeptides in that their synthesis was prevented when viral DNA synthesis was blocked. The only other change observed when labeling at late times after CAR treatment were possible reductions in the levels of production of the 36 to 38K and 25K polypeptides.

One possible explanation of the polypeptides secreted from vaccinia virus-infected cells is that they represent leakage of intracellular polypeptides induced by virus infection. Comparison of the medium profiles in Fig. 1 with the corresponding intracellular polypeptide profiles clearly shows, however, that leakage is not the case, since at both early and late times postinfection, and at late times in the presence of CAR, there are numerous major intracellular virus-induced polypeptides that do not appear in the medium. Thus, the secretion of polypeptides into the tissue culture fluid is a highly selective process. It is also possible to exclude the possibility that the secreted polypeptide profile was merely a reflection of the production of progeny virions, since the pattern observed was completely distinct from the polypeptide profile of mature virions (7). Also, the secreted polypeptides were not sedimented under centrifugation conditions that pelleted virus particles (data not shown).

To try and identify possible intracellular precursors for the excreted polypeptides, pulse-chase experiments were carried out as detailed in Fig. 1. As has been reported previously (8), it was possible to observe numerous changes in the intracellular polypeptide profile after a chase period at both early and late times postinfection (Fig. 1). However, only in the case of the 14.5K polypeptide secreted late in infection was there a reduction during the chase period of an intracellular polypeptide of the same mobility, indicating a possible precursor product relationship. There were no intracellular polypeptides made in sufficient amounts during the pulse period and disappearing during a chase that could serve as a putative precursor of the major 35K-excreted polypeptide, even allowing for any large changes in electrophoretic mobility which could have resulted from possible modifications occurring during secretion (9). Thus it would appear that this polypeptide is synthesized and then very rapidly excreted into the medium after synthesis. This supposition was confirmed by carrying out a pulse-chase experiment in which virus-infected cells were given a 15-min pulse of [35S]methionine at 2 h postinfection. Medium samples were then collected from these cells after various chase times and analyzed on polyacrylamide gels. The results indicated (data not shown) that maximum secretion of this polypeptide was achieved even after only a 15-min chase period.

Modification of secreted polypeptides. In the RNA tumor viruses (1) and pseudorabies virus (1, 9) the major secreted polypeptides were found to be modified either by glycosylation or glycosylation and sulfation. To examine whether the secreted polypeptides associated with vaccinia virus were modified in a similar way, experiments were performed that used [35S]methionine or [14C]glucosamine as the labeled precursors.
VACCINIA-INFECTED CELL POLYPEPTIDE SECRETION

Figure 2 shows a comparison of the secreted protein profiles obtained after labeling with \[^{35}\text{S}]\text{methionine, } \[^{35}\text{SO}_4\], \[^{14}\text{C}]\text{glucosamine. It was immediately clear from this study that the major } 35\text{K-secreted polypeptide is neither glycosylated nor sulfated. In several other cases, however, the diffuse nature of the secreted polypeptide bands was at least partially explained by their post-translational modification. Thus the } 36\text{ to } 38\text{K, } 32\text{K, and } 21\text{ to } 27\text{K polypeptides appeared to be both glycosylated and sulfated, whereas the } 31\text{ to } 35\text{K polypeptide made early in infection appeared only to be glycosylated and not sulfated. It is also interesting to note from a comparison of the } \[^{35}\text{S}]\text{methionine and } \[^{14}\text{C}]\text{glucosamine tracks of uninfected cells that many of the poly-peptides normally secreted by BSC-1 cells into the medium are glycoproteins. A compilation of the data obtained on the nature of the polypeptides secreted after vaccinia virus infection, their time of synthesis postinfection, and the nature of any post-translational modification is given in Table 1.}

**Are virus-induced secreted polypeptides virus-specified polypeptides?** To investigate whether the secreted polypeptides produced after viral infection were virus specified or merely virus-induced derepressed host polypeptides, the secretion profile after infection of a second cell type was examined. Figure 3 shows a comparison of the medium polypeptides produced by uninfected and virus-infected HeLa cells.

![Figure 2: Post-translational modification of secreted polypeptides.](image)

**Fig. 2.** Post-translational modification of secreted polypeptides. Conditions of radioactive labeling and subsequent analysis of the products on gradient polyacrylamide gels are given in the text. A through D give the profiles of samples labeled with \[^{35}\text{S}]\text{methionine; } E \text{ through } H \text{ give the profiles of samples labeled with } \[^{35}\text{SO}_4\]; \text{ and } I \text{ through } L \text{ give the profiles of samples labeled with } \[^{14}\text{C}]\text{glucosamine. Order of the samples: } A, E, \text{ and } I \text{, polypeptides secreted by uninfected cells; } B, F, \text{ and } J \text{, polypeptides secreted by infected cells after labeling early in infection (2 to 2.5 h postinfection); } C, G, \text{ and } K \text{, polypeptides secreted by infected cells after labeling late in infection (5 to 5.5 h postinfection); } D, H, \text{ and } L \text{, polypeptides secreted by infected cells after labeling late in infection after treatment with CAR.}
and BSC-1 cells. It is apparent on comparing the medium profiles of the two uninfected cell types that the pattern of polypeptides secreted into the medium by a particular cell type was diagnostic of that cell type, and this has been found to be true for a number of other cell types that we have examined, including BHK cells, primary chicken embryo fibroblasts, Vero cells, and rat embryo cells (McCrae and Pennington, unpublished data). Comparison of the medium profiles of the two infected cell types showed them to be very similar, with a major secreted polypeptide of molecular weight 35K being produced in both cell types in response to virus infection. The major polypeptide of 35K was also produced by other cell types after virus infection, including BHK cells, primary chicken embryo fibroblasts, and mouse L-cells (data not shown). The identical nature of the 35K polypeptide observed in the various cell types was confirmed by V8 protease peptide map analysis. Figure 4 shows the peptide maps for the 35K polypeptide obtained from three of these cell types; the maps obtained are identical, increasing the probability that the polypeptide is virus coded.

The only clearly discernible difference between the two infected medium profiles was that the majority of the 36 to 38K polypeptide migrated more slowly in the HeLa cell sample. This polypeptide is post-translationally modified by being both glycosylated and sulfated, and it is possible that the altered mobility observed is a reflection of the fact that these post-translational modifications are performed by host-specified rather than viral-specified enzymes, which may differ in the nature of the sugar moieties that they transfer to the protein in the two cell types.

**DISCUSSION**

This report shows that, after infection of a cell with vaccinia virus, significant changes occur in the pattern of polypeptides being specifically secreted by that cell in a manner analogous to the more extensively studied changes in intracellular polypeptides. The changes exhibit the same sort of complex temporal control patterns that are observed with the intracellular polypeptides; thus, some of the polypeptides are secreted continuously throughout the virus growth cycle, whereas others can be classified as early or late polypeptides depending on their temporal pattern of production during the growth cycle.
peptides serve a useful function in normal virus growth. Two speculative possibilities for their function are: on the one hand, that they may operate to make surrounding uninfected cells more susceptible to virus infection, possibly by operating at the cell receptor level, or, alternatively, that these polypeptides, particularly the major 35K one, may be of importance in helping the virus escape the immune surveillance system of an infected animal. Thus, it could be that production of large quantities of a foreign antigen by the infected cells could partially "swamp" the host's immune system, thereby weakening the response to the unrelated structural antigens of the virus particle and so aiding the spread of the infectious virus throughout the animal.

The rather complex pattern of secreted polypeptides does of course make it quite possible that they serve both of the above functions and/or others, and, clearly, more work will be required to elucidate their function, and work toward this end is in progress. This study has, however, shown that analysis of the tissue culture fluid from virus-infected cells, which is normally simply discarded, may produce a new set of virus-specified polypeptides, the study of whose function may give new insights into the process of controlled virus infection.

ACKNOWLEDGMENTS

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LITERATURE CITED


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TABLE 1. Summary showing the classification, time of synthesis, and modifications of polypeptides secreted by vaccinia virus-infected cells

<table>
<thead>
<tr>
<th>Approx mol wt of polypeptide $\times 10^{-4}$</th>
<th>Synthesis period</th>
<th>Production at late times postinfection in the presence of CAR</th>
<th>Modification by glycosylation</th>
<th>Modification by sulfation</th>
</tr>
</thead>
<tbody>
<tr>
<td>36-38</td>
<td>Early</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>35</td>
<td>Continuous</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31-35</td>
<td>Early</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>Late</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>Early</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21-27</td>
<td>Continuous</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14.5</td>
<td>Late</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Continuous</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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The observation that a similar pattern of secreted polypeptides was produced by several cell types in response to virus infection implies that the information for these secreted polypeptides is virus coded rather than merely reflecting a derepression of cellular genes induced by viral infection. Given that the information is virus coded, it seems probable that the secreted poly-