Membrane Glycoproteins and Antigens Induced by Human Cytomegalovirus

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SUMMARY

Early after infection of cells with cytomegalovirus, the membranes were modified with respect to both glycoprotein composition and immunological specificity. Virus-specified antigens were inserted into the plasma membrane at 24 h after infection, as much as 2 days before virion and dense body maturation. Although at least four virus-induced glycoproteins were synthesized and bound to plasma and microsomal membranes between 20 and 24 h after infection, virus-specified antigen accumulated primarily on the plasma membrane. In contrast, at late times (72 h) after infection when virus nucleocapsids can be detected in the nucleus, virus-specified antigen was prominent on the plasma, endoplasmic reticulum and nuclear membranes. It is proposed that the virus-specified glycoproteins and antigens of this herpesvirus accumulate first on the plasma membrane and then on internal membranes. The appearance of virus-specified antigen on internal membranes coincides with the commencement of virion and dense body envelopment.

INTRODUCTION

Cytomegalovirus (CMV), like other members of the herpes virus group, causes persistent and latent infections (Rapp et al. 1975). There is generally little or no overt disease in the natural adult host but infection of neonates and immunologically compromised individuals frequently occurs (Plummer, 1973). Reactivation of the virus in pregnant or immunologically compromised adults may be associated with an impairment of cell-mediated or humoral immunity. Immunity to virus antigens on the cell surface is probably the primary immunological mechanism of host resistance. Cytomegalovirions and dense bodies are bound by a membrane which contains several species of glycosylated proteins (Stinski, 1976). Since the virus membrane-associated antigens induce neutralizing antibodies (Stinski, 1976), virus-specified glycoproteins may play a major role in eliciting a host immune response.

Recently, we have reported the synthesis of a novel set of glycoproteins in CMV-infected cells (Stinski, 1977). These glycoproteins were regarded as virus-specific because they were immunoprecipitated from infected cell extracts by antibody prepared against the membrane antigens of virions and dense bodies (Stinski, 1977). They are presumably associated with the various cellular membranes and acquired by virions and dense bodies during the envelopment phase of morphogenesis. Envelopment of virions occurs at either nuclear or internal cytoplasmic membranes, while envelopment of dense bodies occurs exclusively at internal cytoplasmic membranes (Smith & DeHarven, 1973). Although envelopment is...
first observed at approximately 72 h post infection (p.i.) [Smith & DeHarven, 1973], virus-induced glycoprotein synthesis is detectable as early as 15 h p.i. (Stinski, 1977).

The purpose of this investigation was to characterize virus-induced membrane modifications in CMV-infected cells. Virus-induced antigens were preferentially associated with the plasma membrane of infected cells early after infection and did not accumulate on internal membranes until late after infection. A direct link was established between the specificity of membrane antigens of CMV-infected cells and the membrane antigens of virions and dense bodies.

METHODS

**Virus and tissue culture.** The Towne strain of human CMV was propagated in human fibroblast cells as previously described (Stinski, 1976). Plaque purified, low multiplicity-passage virus was prepared as previously described (Stinski, 1978). The amount of infectious virus was determined by assays for plaques (Wentworth & French, 1970) or tissue culture infective doses (Furukawa et al., 1973). Virus and cells were routinely tested for mycoplasma contamination by methods previously described (Stinski, 1976).

**Virus infection and radioisotope-labelling.** Confluent cultures of human fibroblast cells were infected at input multiplicities of 10 to 20 plaque forming units (p.f.u.)/cell. Cells were labelled with 3H-glucosamine (20 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.), 20 μCi/ml, from 20 to 24 h after infection.

For analysis of virus antigens associated with cells using the immunoperoxidase technique, subconfluent cultures of human fibroblast cells were infected at an input multiplicity of 1 p.f.u./cell.

**Plasma and microsomal membranes.** The membranes of uninfected and infected human fibroblast cells were fractionated into plasma and microsomal membranes by the method of Tweto & Doyle (1976) with the following modifications. Lactoperoxidase catalysed incorporation of 125I-iodine by the method of Phillips & Morrison (1970) was selected as a valid marker for plasma membrane according to the criteria of Tweto et al. (1976) and Hubbard & Cohn (1975). The enzyme nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome c reductase was selected as a marker for microsomal membranes (Knipe et al., 1977) and assayed as described by Sottocasa et al. (1967). There was approximately a 3% contamination of microsomal membranes by plasma membranes and no detectable contamination of plasma membranes. Cells were suspended in 0.01 M-tris-hydrochloride, pH 8.5, containing 0.005 M-KCl and 0.001 M-MgCl₂ at a concentration of approx. 3 x 10⁶ cells/ml for 3H-glucosamine-labelled cells. Total protein was determined by the method of Lowry et al. (1951).

**PAGE.** Slab polyacrylamide gel electrophoresis (PAGE) was done in 9% gels as described previously (Stinski, 1977) according to a modification of the method of Laemmli (1970). The gels were stained by the method of Fairbanks et al. (1971). For detection of 3H-glucosamine-labelled glycoproteins by fluorography, gels were impregnated with 2,5-diphenyloxazole before drying, as described by Bonner & Lasky (1974) and exposed to Kodak XR-5 X-Omat R film at −70 °C. It should be noted that the image obtained by fluorography generally represents the true distribution of radioactivity in the gel (Lasky & Mills, 1975), although the relative amounts of a particular glycoprotein can only be considered an approximation.

**Antisera and F(ab')₂ fragments.** Antigens associated with the membranes of purified virions and dense bodies of CMV were prepared by extraction with Triton X-100 as previously described (Stinski, 1976). To avoid interaction of antibody with the CMV-induced IgG Fc
CMV membrane glycoprotein antigen

receptor in infected cells (Keller et al. 1976; Rahman et al. 1976), F(ab')\textsubscript{2} fragments were prepared from both the specific anti-CMV membrane antigen serum and a non-specific rabbit serum by methods previously described (Stinski, 1977). One ml samples of antibody fragments were adsorbed once with approx. 10\textsuperscript{7} lysed human fibroblast cells and once with approx. 10\textsuperscript{7} whole human fibroblast cells. For the appropriate experiments, F(ab')\textsubscript{2} fragments were also absorbed with CMV-infected fibroblast cells as described above.

Antibody conjugation with peroxidase. Horse radish peroxidase (type VI; Sigma Chemical Co., St Louis, Mo.) was conjugated to F(ab')\textsubscript{2} fragments by the method of Nakane & Kawaoi (1974).

Light and electron microscopy of cells treated with peroxidase-conjugated antibody. For staining plasma membrane antigens on unfixed cells, the cells were kept in PBS and not allowed to dry during antibody exposure. Peroxidase-conjugated anti-CMV membrane antigen F(ab')\textsubscript{2} fragments or peroxidase-conjugated non-specific F(ab')\textsubscript{2} fragments at equal protein concentrations were placed on the cells for 30 min at room temperature and subsequently the cells were washed continuously for 10 min with ice-cold phosphate buffered saline (PBS), pH 7.2. The presence of peroxidase-conjugated antibody was detected by adding 0.2 mg/ml diaminobenzidine-hydrochloride (Sigma), 0.005 % H\textsubscript{2}O\textsubscript{2} in 0.05 M-tris-hydrochloride, pH 7.6 and incubating for 15 min at room temperature. The cells were washed with PBS for 10 min and mounted in PBS-glycerol (1:9) for light microscopy.

For electron microscopy, cells on glass coverslips were either left unfixed and viable for detection of membrane surface antigen(s) or fixed with periodate-lysine-paraformaldehyde solution for 3 h by the method of McLean & Nakane (1973). The problem of antibody penetration was overcome by use of F(ab')\textsubscript{2} fragments and freezing and thawing the cells quickly in the presence of cryoprotective agents as follows: the cells were washed for 15 min with 0.5 M-sodium phosphate buffer, pH 7.2, containing 7% sucrose, for 15 min with 15% sucrose in the above buffer and for 15 min with 25% sucrose, 10% glycerol in the above buffer. While in the 25% sucrose, 10% glycerol-buffer solution, the cells were frozen to −80 °C and quickly thawed to 37 °C. Both unfixed and fixed cells were treated with pre-immune rabbit serum for 15 min at room temperature before adding either peroxidase-conjugated anti-CMV membrane antigen F(ab')\textsubscript{2} fragments or peroxidase-conjugated non-specific F(ab')\textsubscript{2} fragments for 2 h at room temperature. Infected cells were also treated with peroxidase-conjugated anti-CMV membrane antigen F(ab')\textsubscript{2} fragments that were adsorbed with CMV-infected cells to test for the removal of specific antibody. The cells were washed three times with PBS and then continuously for 2 h with PBS. Detection of peroxidase-conjugated antibody was with a diaminobenzidine-H\textsubscript{2}O\textsubscript{2} solution as described above for light microscopy. The cells were then fixed with 1% osmium tetroxide. The reaction products of peroxidase were sufficiently osmiophilic to provide the electron density needed to visualize cell detail without further staining. Cells were embedded in a mixture of Epon and Araldite after dehydration in graded ethanol. Thin sections were cut parallel to the plane of the growth surface, mounted on 200-mesh grids, and examined at 60 kV in a JEOL 100B electron microscope.

RESULTS

Virus-induced glycoproteins associated with plasma and microsomal membranes early after infection

A direct comparison of uninfected and infected cell glycoproteins (gp) indicated that the infected cell glycoproteins differ in number and electrophoretic mobilities from those of
uninfected cells at early and late times after infection (Stinski, 1977). To determine whether the membranes of infected cells were modified by the insertion of virus-induced glycoproteins at early times after infection, membranes of both infected and uninfected cells were fractionated and analysed by SDS-PAGE as described in the Methods. Equal amounts of radioactivity from both the plasma and microsomal membranes of infected cells were analysed but approximately half as much radioactivity was analysed for uninfected cell membranes due to the lower level of $^{3}H$-glucosamine incorporation in these cells. In infected cells, $^{3}H$-glucosamine was selectively incorporated into distinct species of glycoproteins with electrophoretic mobilities and relative rates of synthesis that were different from the host cell glycoproteins (Fig. 1). The plasma and microsomal membranes contained at least two major virus-induced glycoproteins, gp145 and gp100, and two minor virus-induced glycoproteins, gp71 and gp12, in addition to some host cell glycoproteins (Fig. 1 b and c). These data indicate that there are virus-induced glycoproteins synthesized early after infection and these glycoproteins are inserted into the plasma and microsomal membranes of the infected cell.

Detection of CMV-specific antigen(s) on the surface of infected cells

The synthesis of virus-induced gp145 and gp100 was first detectable at approx. 15 h p.i. by SDS-PAGE of cell lysates, and fluorography (Stinski, 1977). These glycoproteins were detected at the onset of CMV DNA replication which is first detectable at approx. 15 h p.i. (Stinski, 1978). To detect surface antigen(s), viable, unfixed cells at 24 and 48 h p.i. were treated with peroxidase-conjugated anti-CMV membrane antigen F(ab')$_2$ fragments or peroxidase-conjugated non-specific F(ab')$_2$ fragments. Even though non-specific F(ab')$_2$ fragments were at the same protein concentration as the specific F(ab')$_2$ fragments, no
Fig. 2. Photomicrograph of virus-specified antigen(s) on the surface of infected cells. Viable, unfixed cells were treated with peroxidase-conjugated F(ab')2 fragments as described in the text: (a) 48 h p.i., non-specific F(ab')2 fragments; (b) 24 h p.i., anti-CMV membrane antigen F(ab')2 fragments; (c) 48 h p.i., anti-CMV membrane antigen F(ab')2 fragments. The arrows indicate infected cells. Magnification × 250.
peroxidase reaction product was detected at 24 (data not shown) or 48 h p.i. with non-
specific F(ab')2 fragments (Fig. 2a). Peroxidase reaction product on the surface membranes
of infected cells treated with peroxidase-conjugated anti-CMV membrane antigen F(ab')2
fragments was detected at 24 h p.i. (Fig. 2b) and the intensity of the peroxidase staining
increased at 48 h p.i. (Fig. 2c). These results indicated that the specific antibody reacts with
a virus antigen(s) inserted in the plasma membrane early in the virus replication cycle.
Previous experiments have demonstrated that infectious virus is not produced until approx.
72 h p.i. (Furukawa et al. 1973; Stinski, 1977).

Ultrastructural analysis of CMV-specific antigen(s) on the plasma and
microsomal membranes

The insertion of CMV-specified membrane antigens into plasma and microsomal mem-
branes was studied at the ultrastructural level using peroxidase-conjugated F(ab')2 fragments
and electron microscopy as described in the Methods. For plasma membrane studies, viable,
infected cells were treated with peroxidase-conjugated F(ab')2 fragments at 14, 48, and 72 h
p.i. Fig. 3 illustrates the detection of CMV antigens on the outer surface of the plasma
membrane when antibodies against the membrane antigens of virions and dense bodies were
employed. Peroxidase reaction product was not detected on uninfected cells (data not
shown) or on the plasma membrane at 0 h p.i., but a virion absorbed to the plasma mem-
brane was highly stained by the peroxidase-conjugated specific antibody (Fig. 3a). Peroxi-
dase reaction product was detected on the outer surface of the plasma membrane at 24 h p.i.
(Fig. 3b) and the intensity of the reaction product increased at 48 h p.i. (Fig. 3c). Virions
and dense bodies were not detected at 24 and 48 h p.i. By 72 h p.i., virions and dense bodies
could be seen in extracellular spaces and peroxidase reaction product was detected on their
membranes as well as the plasma membrane (Fig. 3d).
Viable cells treated with specific antibody shed their plasma membranes and these
membranes had CMV antigen(s) detected by the peroxidase-conjugated specific antibody
(Fig. 3e). The phenomenon of cellular membrane shedding was observed when viable,
unfixed cells were treated with specific antibody but not when periodate–lysine–para-
formaldehyde fixation preceded antibody treatment. Non-specific F(ab')2 fragments con-
jugated with peroxidase did not react with plasma membranes or with the virions and dense
bodies and no membrane shedding was observed from viable cells (Fig. 3f). These data
illustrate at the ultrastructural level that CMV-specific antigen(s) appears at the outer surface
of the plasma membrane 1 to 2 days before the release of virions and dense bodies. In
addition, the specificity of the anti-CMV membrane antigen F(ab')2 fragments is dramatically
demonstrated by its specific reaction with the membranes of virions and dense bodies.

To study the intracellular distribution of CMV membrane-associated antigen(s) at an
ultrastructural level, infected cells were fixed at 24, and 72 h p.i. and treated as described in
Methods. Peroxidase reaction product was detected on the plasma membrane at 24 h p.i.,
but there was less antigen, if any, on the endoplasmic reticulum and none on the nuclear
membranes (Fig. 4a). In contrast, at 72 h p.i. nuclei were observed to have nucleocapsids but
not in all cells. The cells that lacked nucleocapsids had peroxidase reaction product on the
plasma membrane and appeared similar to the cells at 24 h p.i. in that there was little or no
staining of the nuclear membrane (Fig. 4b). In contrast, cells with nucleocapsids always
had staining on nuclear membranes (Fig. 4b). Cells productively infected and treated at
72 h p.i. with peroxidase-conjugated anti-CMV membrane antigen F(ab')2 fragments had
staining that was detectable on the plasma membrane, endoplasmic reticulum, and nuclear
membranes but not on mitochondrial membranes (Fig. 4c). Virions and dense bodies in the
cytoplasm of infected cells appeared to be contained within intracytoplasmic membrane
Fig. 3. Electron micrographs of virus-specified antigen(s) on the outer surface of the plasma membrane of infected cells at various times after infection and on the membranes of virions and dense bodies. Viable, unfixed cells were treated with either peroxidase-conjugated anti-CMV membrane antigen F(\(ab\)')\(_2\) fragments (a to e) or peroxidase-conjugated non-specific F(\(ab\)')\(_2\) fragments (f) and prepared for electron microscopy as described in the text. (a) At 0 h p.i., the outer surface of the plasma membrane does not have a peroxidase reaction product but a virion (V) adsorbed to the surface does. At (b) 24, (c) 48, and (d) 72 h p.i., the outer surface of the plasma membrane (PM) has a reaction product. Virions and dense bodies (DB) in extracellular spaces at 72 h p.i. (d and e) have reaction product on their membranes. (e) Membranes (M) shed from viable cells after treatment with anti-CMV membrane antigen F(\(ab\)')\(_2\) fragments at 72 h p.i. have reaction product. (f) There was no staining of the plasma membrane of infected cells at 72 h p.i. or of the extracellular virions and dense bodies treated with peroxidase-conjugated non-specific F(\(ab\)')\(_2\) fragments.
Fig. 4. Electron micrograph of virus-specified antigens in cellular membranes of CMV-infected cells. Cells were fixed with periodate-lysine-paraformaldehyde solution, treated with cryoprotective agents, frozen and thawed and treated with either peroxidase-conjugated anti-CMV membrane antigen F(ab')2 fragments (a, b, and c) or peroxidase-conjugated non-specific F(ab')2 fragments (d) as described in the text. (a) Reaction product appears clearly in the plasma membrane (PM) and less antigen, if any, was detected in the endoplasmic reticulum (ER) and nuclear (NU) membranes at 24 h p.i. (b) At 72 h p.i., cells with nucleocapsid (NC) have reaction product on the nuclear membrane (NU) and cytoplasmic membranes. (c) At 72 h p.i., endoplasmic reticulum and nuclear membrane have reaction product but not the mitochondrial (MI) membrane. Virions (V) and dense bodies (DB) in the extracellular spaces have reaction product on their membranes but virions (not shown) and dense bodies in the cytoplasm appear to be protected by a membrane vesicle. (d) At 72 h p.i., there was no staining of cellular membranes or of virions and dense bodies when infected cells were treated with peroxidase-conjugated non-specific F(ab')2 fragments (d).
vesicles and, consequently, they were protected from interaction with antibody. In contrast, extracellular virions and dense bodies were highly stained (Fig. 4c). Non-specific peroxidase-conjugated F(ab')2 fragments caused no detectable staining of extracellular virions and dense bodies or of cellular membranes at 72 h p.i. (Fig. 4d). Specific peroxidase-conjugated antibody adsorbed with CMV-infected cells also caused no detectable staining (data not shown).

**DISCUSSION**

Early during the replicative cycle of CMV, the membranes of infected cells become modified with respect to morphology (Stinski, 1977), glycoprotein composition and immunological specificity. This alteration of host cell membranes occurs one to two days before the release of progeny virus and is associated with the insertion of CMV-induced glycoproteins. The anti-CMV membrane antigen F(ab')2 fragments detected antigen on the plasma, endoplasmic reticulum and nuclear membranes of infected cells, but not on uninfected cells. Since it was previously established that virus-induced glycoproteins are immunoprecipitated from infected cell extracts with the same antibody (Stinski, 1977), it is proposed that the infected cell membranes contain virus-specified glycoprotein antigens similar to those present in virions and dense bodies of CMV. Studies with herpes simplex virus have also demonstrated the presence of virus envelope glycoproteins (Heine et al. 1972) and antigens (Nii et al. 1968) on the plasma membrane of infected cells.

Cytomegalovirus-specified antigen was detected first on the plasma membrane and the relative amount of antigen increased from 24 to 48 h p.i. This may reflect an increase in the relative amount of gp145 and gp100 or the presence of additional virus-induced glycoproteins that are synthesized after the initiation of virus DNA replication (Stinski, 1977). Late CMV genome expression requires virus DNA replication (Stinski, 1977) which is expected to amplify the relative amount of both early and late virus-specific antigens associated with the cellular membranes because of increased available template for transcription of virus messenger RNA.

The reason for clear detection of antigen at 24 h p.i. on the plasma membrane presumably resides in its selective accumulation of antigen during the early phase of infection rather than its accessibility to antibody. There is no reason to believe that antibody penetrated cells more easily at late times, when internal membrane staining was clear. Therefore, the pattern of staining observed early is taken to indicate qualitatively little or no antigen accumulation on internal membranes during the early phase of infection.

The ultrastructural analysis demonstrated the presence of antigen on endoplasmic reticulum and nuclear membranes when virus nucleocapsids were detected in the nuclei. Virions and dense bodies were also detected at 72 h p.i. and were frequently found within cytoplasmic vesicles. These virions and dense bodies lacked peroxidase reaction product, apparently because the integrity of the membrane-bound vesicles was not disrupted and, therefore, the antibody could not penetrate. In contrast, extracellular virions and dense bodies had peroxidase reaction product on their membranes. The virions and dense bodies of CMV probably acquired both early and late virus-induced glycoproteins during the envelopment stage of morphogenesis. Virus glycoproteins and host cell glycoproteins may be in juxtaposition on the membrane and, consequently, the virus glycoproteins may occupy a topologically unique space from which all host cell proteins are excluded.

Antibody against membrane antigens of CMV-infected cells can be found in the sera of patients with CMV infection (Thé & Largenhuysen, 1972). In addition, lymphocytes from CMV-seropositive individuals are stimulated by purified virus or infected cells, whereas lymphocytes from seronegative individuals do not respond (Moller-Larsen et al. 1976). These reports point to the importance of CMV-specified antigens present on the surface of infected
cells. The appearance of virus antigens on the plasma membrane during the early phase of virus replication may be recognized by host immunological defence mechanisms and lead to the elimination of the virus-infected cell.

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REFERENCES


CMV membrane glycoprotein antigen


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