The Effect of Ethidium Bromide on the Membrane Glycopeptides in Control and Virus-transformed Cells*

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SUMMARY

Glycopeptides derived from surface and subcellular membranes of control (BHK21/C13) and virus-transformed (C13/B1) baby hamster kidney cells treated or untreated with ethidium bromide were studied. Ethidium bromide was found to mimic virus transformation with regard to the alteration of the glycopeptide patterns observed upon Sephadex G-50 chromatography. Elution profiles of glycopeptides obtained from virus-transformed cells demonstrated the existence of both a normal component (Peak B) and an enrichment of material eluting as a higher molecular weight component (Peak A). Material eluting in the Peak A region is greatly enhanced in both the control and virus-transformed cells after treatment with ethidium bromide. This alteration of the glycopeptide pattern was observed with glycopeptides derived from surface, endoplasmic reticular and mitochondrial membranes. Mitochondrial glycopeptides obtained from ethidium bromide-treated cells also seem to contain an increased amount of material eluting as a component of lower molecular weight than Peak B. This material, designated Peak C, may be located in the intramembrane space of the mitochondrion.

In previous studies in our laboratory, glycopeptides from glycoproteins of the surface membranes of control and virus-transformed cells in tissue culture have been compared. Equivalent membrane components from control cells labeled with L-[3H]fucose and from virus-transformed cells labeled with L-[3H]fucose were mixed, digested with pronase, and chromatographed together on columns of Sephadex G-50 (1-3). It has been found by this internally controlled method that there is a population of glycopeptides which is present in small amount in the control cell but is greatly increased in virus-transformed cells. The appearance of this enhanced population of glycopeptides (Peak A) is growth-dependent. It is inconspicuous in both control and virus-transformed cells in the plateau phase of growth (2). The change of the glycopeptide pattern upon viral transformation not only takes place at the surface of the cell but also takes place in the glycoproteins of the mitochondria, endoplasmic reticulum, nuclei, and lysosomes. Contamination of internal membrane fractions by surface membrane cannot account for this conclusion (4). The observed differences in the glycopeptides of control and virus-transformed cells may be explained, at least in part, by the activity of a sialyltransferase (5, 6).

Ethidium bromide, a phenanthridine dye, has been shown to bind strongly to both DNA and RNA in vivo and in vitro by intercalation between adjacent base pairs (7, 8). All studies to date implicate the mitochondrion as the primary target site of ethidium bromide at low concentrations. Ethidium bromide has been found to be an extremely effective cytoplasmic mutagen in facultatively anaerobic yeast, producing "petites" lacking cytochromes a + a3 and b (9). Treatment of cells with ethidium bromide has also been found to result in the loss or alteration of mitochondrial DNA (10-13) and RNA (14, 15), the selective inhibition of mitochondrial DNA polymerase (16), the alteration of the cytochrome system (17-19), and morphological abnormalities of the mitochondrion (17). The effects of ethidium bromide in mammalian cells (17), protozoans (20), and yeast (13) can be reversed under suitable conditions. Aside from its ability to bind to nucleic acids it has been demonstrated that ethidium bromide binds to mitochondrial membranes in a region of low polarity and is associated with energy conservation (21).

The present study deals with the effect of ethidium bromide on the carbohydrate moiety of the membrane-bound glycoproteins from normal and virus-transformed cells with special attention focused on the glycoproteins of the mitochondrion. An understanding of the glycoproteins and their origin in the mitochondrial membranes would be instrumental in our concept of the biosynthesis of the mitochondrion. We have found that membrane-bound glycoproteins from control and virus-transformed cells treated with ethidium bromide have a greatly enriched Peak A, as compared to the corresponding untreated cells. The alterations due to ethidium bromide were not confined to mitochondrial membranes but were found in the endoplasmic reticulum and surface membranes as well. The action of ethidium bromide appears to mimic the alterations observed upon viral transfor-
mation, at least, with regard to the membrane bound glycoproteins.

MATERIALS AND METHODS

Cell Culture and Cell Fractionation

Baby hamster kidney cells (BHK<sub>21</sub>/C<sub>13</sub>) and the same cells transformed with the Bryan strain of Rous sarcoma virus (C<sub>13</sub>/B<sub>4</sub>) were cultured under previously defined conditions (1-3). Control cells were grown in roller bottles for 3 days in the presence of L-[<sup>3</sup>H]fucose. The untreated transformed cells were grown for 3 days in the presence of L-[<sup>3</sup>H]fucose. Cells treated with ethidium bromide were subjected to 2 µg of ethidium bromide per ml of growth medium for 24 hours prior to the addition of L-[<sup>3</sup>H]fucose. The ethidium bromide and L-[<sup>3</sup>H]fucose treatment was continued for 3 days. Cells were harvested from the roller bottles by incubation for 5 min with trypsin (0.25%), Trypsin-Difco, 1:250 in Tris buffered saline, pH 7.5, with 0.1 µg ethidium bromide were subjected to 2 µg of ethidium bromide per ml and CsCl (refractive index 1.3880) was added to a final concentration of 1 µM E:DTA and centrifuged as previously described (1-3) to obtain a cell pellet and a supernatant. This supernatant contained surface components of the cells and was termed the cell trypsinate. The glycopeptides contained in the trypsinate are representative of those found in the plasma membrane as a whole (1, 2).

The cells were then used to obtain mitochondria and endoplasmic reticulum by a method described elsewhere (4). The inner and outer mitochondrial membranes were isolated by the digitonin method reported by Schnaitman and Greenawalt (22).

Chromatography of Glycopeptides—The cell trypsinate, mitochondria, and endoplasmic reticulum from normal cells (labeled with L-[<sup>14</sup>C]fucose) were subjected to 2 µg of ethidium bromide per ml of growth medium for 24 hours. These fractions from the transformed and ethidium bromide-treated cells (labeled with L-[<sup>3</sup>H]fucose) and digested with pronase (1). The pronase digests were then chromatographed on Sephadex G-50 (fine) columns (0.8 X 100 cm) and the fractions counted for 3H activity (model 3310). The enzyme marker for the inner mitochondrial membrane, cytochrome oxidase, was assayed by measuring the formation of benzoate from benzaldehyde (23). Absorbance readings were taken at 290 nm on a Zeiss PMQII spectrophotometer.

Enzyme Assays—The outer mitochondrial membrane marker, monamine oxidase, was assayed by measuring the formation of 2 ethidium bromide was found to alter the patterns of glycopeptides eluting from Sephadex G-50 columns of both normal, BHK<sub>21</sub>/C<sub>13</sub>, and virus-transformed C<sub>13</sub>/B<sub>4</sub>, cells. Cell growth of both cell lines was only slightly reduced during the 4-day treatment with ethidium bromide (by 20 to 40%) and no significant inhibition of labeling with L-fucose was observed. Under these conditions the mitochondrial protein content increased by 20 to 50% (12), while the covalently closed circular mitochondrial DNA was totally absent in its normal position on a cesium chloride ethidium bromide gradient. Fig. 1, a and b shows the disappearance of MI DNA upon ethidium bromide treatment, whereas nuclear DNA (N II) remains. Treatment of crude mitochondrial fractions with deoxyribonuclease to remove adherent nuclear DNA shows that the mitochondrial DNA remaining after ethidium bromide treatment was primarily shifted into the Peak II region (Fig. 1, c and d), which contains a mixture of linear DNA, circular DNA with single-strand scissions, and circular DNA with an altered superhelix density (12). This structural alteration of the mitochondrial DNA may be partially due to an ethidium bromide-induced increase in nuclease activity (12). After 4 days of exposure to ethidium bromide, the concentration of mitochondrial DNA per mg of mitochondrial protein was reduced to about one-half (Table I).

![Fig. 1. Mitochondrial DNA from BHK<sub>21</sub>/C<sub>13</sub> cells grown in the absence (a and c) and in the presence (b and d) of 2 µg of ethidium bromide per ml for 48 hours; 0.5 µCi of [PH]thymidine per ml was added to all cultures. Mitochondrial DNA was centrifuged to equilibrium in CsCl-ethidium bromide gradients. Mitochondrial fractions in c and d were treated with 50 µg of deoxyribonuclease per ml to remove adherent nuclear DNA; M I, closed circular mitochondrial DNA; M II, nicked circular and linear mitochondrial DNA; N II, nuclear DNA containing less than 5% M II.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Mitochondrial DNA per mitochondrial protein</th>
<th>Mitochondrial DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nuclear DNA&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Control (4 days)</td>
<td>2.8 ± 0.2 (2)</td>
<td>2000 ± 300 (3)</td>
<td>5740 ± 500 (3)</td>
</tr>
<tr>
<td>Ethidium bromide (2 µg/ml, 4 days)</td>
<td>1.0 ± 0.1 (2)</td>
<td>1830 ± 200 (3)</td>
<td>7190 ± 400 (3)</td>
</tr>
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<sup>a</sup> [PH]Thymidine (0.2 µCi per ml) was given to cells 24 hours prior to and during ethidium bromide treatment.
FIG. 2.
Legend on page 3017
During this period in which the normal mitochondrial genome was destroyed, the specific activity of the nuclear DNA actually increased by about 25% as determined by the incorporation of [3H]thymidine (Table I). This increase was also observed in other cell types (12). These results demonstrate that ethidium bromide, under the conditions used, affects mitochondrial and nuclear DNA differentially and that the drug penetrates the mitochondrial membrane.

The glycopeptides derived from C12/B4 cell trypsinates possess a clearly defined component that is higher in molecular weight than is found in corresponding glycopeptides from normal BHK21/C13 cell trypsinates (1-3). This early eluting material is referred to as Peak A, while the later eluting peak is designated Peak B. When the trypsinate mixture is incubated with neuraminidase, sialic acid is removed and Peaks A and B of Cl3/B4 and Peak B of BHK21/C12 are converted into glycopeptides that migrate together as lighter material termed Peak B' (5).

The two internal cellular membrane components studied in these experiments, endoplasmic reticulum and mitochondria, have patterns that are virtually identical with that of the trypsinate of the cell surface, except for the high molecular weight material that elutes in the void volume (Fig. 2, c and e). This material has not as yet been characterized.

When ethidium bromide was added to the virus-transformed cells, C12/B4, it was found that the quantity of glycopeptides eluting from a Sephadex G-50 column in the Peak A region was greatly enhanced relative to both the untreated C12/B4 and normal BHK21/C13 cells. The glycopeptides in the trypsinates obtained from ethidium bromide-treated C12/B4 cells contained, almost exclusively, material eluting in the Peak A region (Fig. 2a). This material, when treated with neuraminidase (Fig. 2b), behaved in the same fashion as the trypsinate glycopeptides obtained from untreated C12/B4 cells. The endoplasmic reticulum obtained from ethidium bromide-treated C12/B4 cells when compared to the endoplasmic reticulum from untreated C12/B4 cells also showed a greater amount of material eluting in the Peak A region (Fig. 2c). This Peak A material was also sensitive to neuraminidase treatment (Fig. 2d). Finally, the ethidium bromide-treated C12/B4 mitochondria again possess a proportionately larger amount of Peak A material (Fig. 2e) relative to the untreated C12/B4 mitochondria. There is some variability in the results especially with regard to the material in the Peak C region of the mitochondrial patterns due to the variation of growth behavior and the response to ethidium bromide. However, the qualitative results were reproducible in several experiments. The mitochondrial material eluting in the Peak C region which increased in amount after treatment with ethidium bromide was unaffected by neuraminidase treatment, while the material eluting in the Peak A and B regions was reduced to a common size (Fig. 2f).

The C12/B4 mitochondria were disrupted with digitonin and the inner and outer mitochondrial membrane isolated. The glycopeptide patterns of both the inner (Fig. 3a) and outer (Fig. 3b) mitochondrial membranes were virtually the same as that of intact mitochondria. Practically no labeled glycopeptides were released into the supernatant solution of the mitochondria after digitonin treatment and therefore, no sample could be analyzed by chromatography. Assays for cytochrome oxidase and monoamine oxidase were conducted to check the efficiency of the isolations of inner and outer mitochondrial membranes. The assay for cytochrome oxidase, an inner membrane marker, showed that the outer membrane preparation contained less than 3% of inner membrane. The assay for monoamine oxidase, a marker for the outer membrane, demonstrated that the inner membrane fraction was contaminated by less than 6.5% of the outer membrane.

Finally, when the mitochondria were subjected to 10 times more digitonin than is necessary to remove the outer membrane, the same glycopeptide pattern for the inner membrane preparation was obtained. It was found that the specific radioactivity (counts per min per mg of mitochondrial protein) of the glycopeptides of the outer membrane was three to four times higher than that of the inner membrane. This is in good agreement with the results found by deBernard and co-workers (26) which demonstrated that the outer mitochondrial membrane contained approximately four times more sialic acid per mg of protein than the inner mitochondrial membrane.

Mitochondria, obtained from C12/B4 cells treated with ethidium bromide, were separated into inner and outer mitochondrial membrane components by digitonin treatment. It was found that there were little or no glycopeptides eluting in the Peak C region from each of the membrane fractions (Fig. 3, c and d). However, the supernatant solution, after mitochondrial fractionation, contained a significant amount of solubilized, labeled glycoproteins eluting as Peak C (Fig. 3e). Virtually no Peak C material was present in the mitochondrial supernatant solution after digitonin treatment obtained from the untreated normal or virus-transformed cells, and therefore no elution patterns could be obtained. When the data from Fig. 3, c and e were combined graphically, the result was a composite pattern which was virtually identical with that for the whole mitochondria.

To investigate further the release of glycoproteins eluting in the Peak C region, a mitochondrial preparation from ethidium bromide-treated C12/B4 cells was treated with trypsin in order to disrupt partially the outer mitochondrial membrane. Mild trypsinization of isolated mitochondria was performed under conditions similar to the trypsination of the whole cell, except that the reaction was run at room temperature for 5 min. The pronase mitochondrial trypsinate consisted primarily of Peak C material and very little of Peak A (Fig. 3f), while similar trypsin treatment of whole cells release Peak A and B material. The pronase-treated mitochondrial pellet remaining after trypsination contained material eluting from a Sephadex G-50 column in the Peak A and B regions, while only a small amount of Peak C material remained from C12/B4 plus ethidium bromide cells with L-[3H]fucose. Glycopeptides of the a, trypsinates from C12/B4 and C12/B4 plus ethidium bromide cells; b, trypsinates from C12/B4 and C12/B4 plus ethidium bromide cells that had been incubated with neuraminidase prior to treatment with pronase; c, endoplasmic reticulum from C12/B4 and C12/B4 plus ethidium bromide cells; d, endoplasmic reticulum from C12/B4 and C12/B4 plus ethidium bromide cells that had been incubated with neuraminidase prior to treatment with pronase; e, mitochondria from C12/B4 and C12/B4 plus ethidium bromide cells; f, mitochondria from C12/B4 and C12/B4 plus ethidium bromide cells that had been incubated with neuraminidase prior to treatment with pronase.
FIG. 3. Sephadex G-50 elution profiles of glycopeptides released by pronase digestion of various mitochondrial fractions prepared from BHK21/C13 cells grown in the presence of L-[14C]fucose and from C13/B4 cells grown in the presence of L-[4H]fucose (±) 2 μg of ethidium bromide per ml. Conditions are the same as those described for Fig. 2. Glycopeptides of the: a, whole mitochondria from BHK21/C13 cells and inner mitochondrial membranes from C13/B4 cells; b, whole mitochondria from BHK21/C13 cells and outer mitochondrial membranes from C13/B4 cells; c, whole mitochondria from BHK21/C13 cells and inner mitochondrial membranes from C13/B4 plus ethidium bromide; d, whole mitochondria from BHK21/C13 cells and outer mitochondrial membranes from C13/B4 plus ethidium bromide; e, whole mitochondria from BHK21/C13 cells and mitochondrial supernatant (after the removal of inner and outer mitochondrial membranes) from C13/B4 plus ethidium bromide; f, whole mitochondria from BHK21/C13 cells and mitochondrial trypsinate (5 min at room temperature) from C13/B4 plus ethidium bromide; g, whole mitochondria from BHK21/C13 cells and trypsinated mitochondrial pellet from C13/B4 plus ethidium bromide; h, whole mitochondria from C13/B4 cells and mitochondrial trypsinate (30 min at 37°C) from C13/B4 plus ethidium bromide.
the material eluting in the Peak A region relative to the un-
ence of a soluble glycoprotein in the mitochondrial intramem-
tral region by gel electrophoresis.

When mitochondria were treated with trypsin under more rigorous conditions (37°C for 30 min) the trypsinate pattern is similar to that of whole mitochondria (Fig. 3a versus 2a).

Finally, the same results obtained under mild trypsination conditions were observed when mitochondria were incubated for 5 min at room temperature with Enzite-trypsin (from Miles Laboratories, trypsin bound to cellulose support, 5 mg per ml). The Enzite-trypsin presumably works solely at the outer surface of the mitochondria and does not penetrate this organelle.

Taken together, the three sets of data on Peak C material employing digitonin and trypsin just described, and the absence of reactivity with neuraminidase indicate that the mitochondrial Peak C material is derived from a soluble glycoprotein lacking any significant amount of sialic acid and is localized in the intramembrane space of the mitochondria. It may be the same material described by Sottocassa (27) who demonstrated the presence of a soluble glycoprotein in the mitochondrial intramembrane space by gel electrophoresis.

When ethidium bromide was added to normal, BHK21/C13 cells, the Sephadex G-50 elution pattern obtained with the pronase-digested trypsinate (Fig. 4a) resembled the pattern found with material from transformed cells. There was an enhancement of the material eluting in the Peak A region relative to the untreated BHK21/C13 cells. The glycopeptides eluting in the Peak A and B regions from the BHK21/C13 trypsinate could be reduced to material chromatographing in a single region, Peak B', when treated with neuraminidase, mimicking results obtained with material from the virus-transformed cells. The enhancement of the Peak A material was also found in the endoplasmic reticulum (Fig. 4c) and the mitochondria (Fig. 4d). The mitochondria from ethidium bromide-treated BHK21/C13 cells also contained, as did the C12/B4 cells treated with ethidium bromide, an enlarged component of smaller material, Peak C (Fig. 4c).

The digitonin fractionation of the mitochondria obtained from normal and ethidium bromide-treated BHK21/C13 cells yielded results similar to those observed with the corresponding virus-transformed cells previously described.

**DISCUSSION**

The addition of ethidium bromide to the growth medium of normal BHK21/C13 and virus-transformed C12/B4 cells results in a marked alteration in the elution patterns from Sephadex G-50 columns of the glycopeptides derived from the membrane glycoproteins of these cells. Glycopeptide elution patterns, obtained with pronase-digested internal cellular membranes, are not due to contamination by surface membrane fragments. This was unequivocally demonstrated by treatment of living intact cells with neuraminidase. This treatment completely removed Peak A from surface membranes and trypsinate but left Peak A intact in all internal membrane systems (4).

In the absence of ethidium bromide, glycopeptides from normal BHK21/C13 cells elute essentially as one peak (Peak B) with only slight evidence of Peak A material. Virus-transformed cells yield, upon digestion with pronase, glycopeptides that migrate as larger material in the Peak A region, in addition to the Peak B glycopeptides. Growth of cells in the presence of ethidium bromide results in a pronounced increase of the Peak A material relative to Peak B in both control BHK21/C13 and virus-transformed C12/B4 cells. Further, preliminary analysis by high voltage paper electrophoresis has shown that Peak A material migrates the same whether it is derived from C12/B4 cells either untreated or treated with ethidium bromide.1 The alteration of the glycopeptide pattern in BHK21/C13 cells caused by ethidium bromide mimics the alteration caused by virus transformation. The mechanism and significance of this are not known. However, it is known that the occurrence of Peak A glycopeptide is growth-dependent (2). Perhaps the observed perturbation of nuclear DNA synthesis by ethidium bromide (Table I) is linked in some way with increased biosynthesis of Peak A glycopeptide.

In addition to the increase of Peak A glycopeptides in the mitochondria, a third glycopeptide species, Peak C, appears to increase to varying degrees as a result of treatment with ethidium bromide. Peak C may consist of a glycopeptide(s) derived from a soluble glycoprotein(s) in the mitochondrial intramembrane space. This material is readily released into the supernatant solution under mild treatment with digitonin, trypsin or Enzite-trypsin. Our data, along with electron microscopic observations, indicate that all three methods of treatment probably rupture the outer mitochondrial membrane selectively. The glycopeptide(s) obtained from the trypsinate of the mitochondria yields Peak C material and virtually no Peak A or B glycopeptides upon digestion with pronase. The outer mitochondrial membrane has both Peaks A and B and yet, unlike the case with the plasma membrane, very little Peak A or B is released by the trypsin. This may indicate that the glycoproteins of the outer mitochondrial membrane are oriented into the intramembrane space or are

1 G. Saslau and L. Warren, unpublished data.
not accessible to the trypsin. More rigorous treatment with trypsin releases material eluting in the Peak A and B regions in ratios similar to whole mitochondria. Experiments dealing with this point are in progress.

It is improbable that the known effects of ethidium bromide on mitochondria, discussed in the introduction, could also account for the changes found in the glycoprotein patterns of the cell surface and endoplasmic reticulum. The observation that mitochondrial DNA is altered in structure and reduced in amount during the period of ethidium bromide treatment while glycoproteins persist and change suggests that mitochondrial glycoproteins are coded for by nuclear DNA.

It is evident that ethidium bromide is in some way responsible for changes in the carbohydrate component of the glycoproteins of both intracellular membranes and the plasma membrane. These changes are also observed in the inner and outer mitochondrial membranes. This would indicate a cytoplasmic origin for the mitochondrial glycoproteins, although it is possible that ethidium bromide acts in the same way both in the cytoplasm and the mitochondria. Further studies will be conducted with chloramphenicol, cycloheximide, and ethidium bromide to furnish more conclusive evidence on this point. The mode of ethidium bromide action has not been determined at the present time.

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