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Separation of Glycopeptides by High Performance Liquid Chromatography

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A method is presented for separation of tryptic glycopeptides-containing oligosaccharides of the N-asparagine-linked type. High performance liquid chromatography (HPLC) of glycopeptides on a C18 reverse-phase system eluted with a gradient of 0%-50% acetonitrile in 0.1 M $NaPO_4$ pH 2.2 resolves the two major glycosylation sites from the envelope glycoprotein (G) of vesicular stomatitis virus. Glycopeptides containing N-linked oligosaccharides of the complex type coelute with those containing N-linked oligosaccharides of the neutral, high mannose type, indicating that separation is based upon peptide rather than carbohydrate composition. The contribution of the carbohydrate component to glycopeptide elution, as determined by cleavage of the high mannose oligosaccharides with endo-β-Nacetylglucosaminidase H, is that of a significant, but minor, decrease in peptide retention time. Comparison of the tryptic glycopeptide profiles of G isolated from both wild type and mutant strains of VSV illustrates the rapid, reproducible, and quantitative nature of the technique. Through HPLC analysis of appropriately treated glycopeptides, it is possible to explore both the nature and extent of glycosylation at individual sites in glycoproteins in a single step.

Key words: glycoprotein, chromatography, high performance liquid chromatography separation, glycopeptides, N-linked oligosaccharides, vesicular stomaritis virus

In processes of cellular recognition, cell surface glycoproteins play a crucial role, whether through cell-cell binding interactions or in cell-ligand recognition. Since the oligosaccharides play an influential part in this recognition process, either directly through sugar specificity or indirectly through protein antigenic expression, a means of analyzing these species at individual glycosylation sites is highly desirable. Many techniques used previously, such as ion-exchange column separations or paper electrophoresis [1,2] lack reproducibility or resolution or produce complicated patterns due to oligosaccharide microheterogeneity. In the present work, a method based upon high performance liquid chromatography

Abbreviations used: Man, D-mannose; GlcNAc, N-acetyl-D-glucosamine; Fuc, L-fucose; gal, D-galactose; NANA, D-acetyl-D-neuraminic acid; endo H, endo-β-N-acetylglucosaminidase.

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(HPLC) is presented. The technique is rapid, reproducible, quantitative, and separates primarily by peptide rather than carbohydrate composition.

The technique was worked out using vesicular stomatitis virus (VSV) as a model system. The envelope glycoprotein (G) of VSV contains two major N-asparagine-linked oligosaccharide residues [1–4]. In fully processed virion G, both N-linked oligosaccharides are of the complex type. The basic oligosaccharide structure of G is composed of $GlcNAc_2(Fuc)Man_3$ in the peptide-linked core and GlcNAc Gal NANA in each of three outer chains [4–8]. Some variation in sugar composition, particularly with regard to sialic acid residues, has been observed for VSV grown in different host cells [2,5,7,9,10].

High performance liquid chromatography on a C18 reverse-phase column resolves both major tryptic glycopeptides of VSV G. With this technique, it is possible to compare rapidly glycopeptide profiles of G isolated from a number of VSV strains to explore variations in both peptide and carbohydrate components at individual glycosylation sites.

MATERIALS AND METHODS

Cells and Viruses

NIL-8 fibroblasts were cultured as monolayers in Eagle's Minimum Essential Medium (GIBCO) containing 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. The virus stocks used in this work were prepared in NIL-8 cells starting with a plaque-purified virus, as described by Lodish and Weiss [11].

The Indiana serotype of wild-type VSV and the ts (v) mutant derived from this parental strain, TsL513 (v), were kindly donated by H. Lodish (MIT, Cambridge, Mass).

Radioactive Labelling of Infected Cells With ^{3}H-Mannose

NIL-8 cells were infected and labelled essentially as described by Zilberstein et al [10]. Cells were plated on 60-mm culture dishes at a density of 2×10^6 cells dish in 4 ml medium and incubated for 15–20 h. The cells were then infected with ten PFU per cell of wild-type or ts mutant stocks of VSV. At 4 h postinfection, each monolayer was washed twice with 3 ml of (prewarmed) glucose-free MEM (GIBCO) containing 10% fetal calf serum and 2 mg/ml Actinomycin D. Cells were labelled with 500 μCi/ml of D-[2-^{3}H]mannose (20–30 Ci/mmole; NEN) or D-[G-^{3}H]glucosamine (20–30 Ci/mmole; NEN) in the same medium and returned to the respective temperatures. Labelling of the G protein precursor was generally for 30 min. Labelled cell monolayers were placed on ice, washed twice with 5 ml of ice-cold phosphate-buffered saline (PBS) and lysed in 0.2 ml of detergent buffer containing 2 mM Phenylmethylsulfonyl fluoride (PMSF, Sigma). The cell lysate was scraped with a rubber policeman and spun at 2,000 rpm to remove cell nuclei.

In some experiments, partially purified virions were prepared from ^{3}H-mannose-labelled cell cultures. Just before harvesting the cells, the medium was collected and filtered through a 0.22-mm diameter Millex filter unit (Millipore). The medium was layered onto a 2-ml cushion of 20% (w/v) sucrose in

PBS and centrifuged for 2 h at 76,000g, 4°C. Each pellet was resuspended in 100 μl detergent buffer prior to immunoprecipitation.

Immunoprecipitation

Labelled virus pellets and cells were lysed with detergent buffer (0.01 M sodium phosphate, pH 7.5/0.1 M NaCl/1% Triton X-100/0.5% deoxycholate/0.1% sodium dodecyl sulfate (SDS). Both viral and cellular lysates were immunoprecipitated with rabbit anti-VSV G at 20–50 μl/ml (a gift from M. Pasternack, MIT, Cambridge, Mass) and Staphylococcus aureus Cowen strain I as previously described [12], G was dissociated from the S aureus protein A-complex by boiling in 2% SDS for 2 min. Analysis by SDS polyacrylamide gel electrophoresis showed one major band upon immunoprecipitation of [^{3}H]mannose-labelled G (data not shown).

Trypsin Digestion

To reduce sulfhydryl groups, immunoprecipitated samples were adjusted to 10 mM DTT, flushed with nitrogen, and boiled for 5 min. Samples were then incubated at 37°C for 1–2 h. Similar results were obtained following reduction with iodoacetamide. Acetone was then added (acetone:sample—2:1 [v/v]) and protein precipitated at −20°C. The samples were centrifuged to pellet and the protein and the pellets dried in vacuo. Samples were dissolved in 50 mM ammonium carbonate pH 8 and incubated with 0.1 mg/ml TPCK-trypsin (Worthington) at 37°C for 2 h. Unless otherwise specified, another aliquot of 0.1 mg/ml trypsin was added and the sample incubated for an additional 2 h at 37°C. Following incubation with trypsin, samples were boiled for 5 min and lyophilized. Prior to injection onto the column for HPLC analysis, samples were dissolved in 100 μl of 0.1 M sodium phosphate, pH 2.2, buffer and centrifuged to remove any particulate material.

High Performance Liquid Chromatography

Radioactively labelled samples (100 μl) were injected onto a C18 Ultrasphere −ODS (4.6 mm × 25 cm) reverse-phase column (Beckman) at room temperature and eluted with a 0%–50% gradient of acetonitrile in 0.1 M sodium phosphate, pH 2.2 (0.1 M with respect to sodium). Samples of 0.5 ml were collected at a flow rate of 1 ml/min. A Beckman Model 334 MP liquid chromatograph equipped with two Model 110 A pumps, a Model 421 CRT microprocessor-controller, and a Model 210 sample injector was used.

Endo-β-N-acetylglucosaminidase H (endo H) Digestion

Endo H digestions were performed as previously described [12]. The samples, either before or after trypsin treatment, were incubated with 3μg of endo H per ml in 0.1 M sodium citrate (pH 5.5) at 37°C for 1 h.

Polyacrylamide Gel Electrophoresis

Proteins were analysed by polyacrylamide gel electrophoresis in SDS as described by Laemmli [13]. The stacking gel was 3.6% (w/v) acrylamide, and the

separating gel was 10% (w/v) acrylamide. Gels were fixed, dried, and subjected to autoradiography on Kodak XR-5 X-ray film. Small aliquots of ^{35}S-methionine-labelled proteins, prepared as described by Zilberstein et al [10] were added as markers for ^{3}H-labelled samples.

RESULTS

Two Tryptic Glycopeptides of VSV G Protein Are Resolved by HPLC

To determine whether the individual glycosylation sites could be resolved by high performance liquid chromatography (HPLC), the tryptic glycopeptides of the envelope glycoprotein G of vesicular stomatitis virus were analyzed. To compare tryptic glycopeptides from both newly synthesized cellular G and fully processed viral G, a temperature-sensitive mutant of VSV, ts L513(v), was used. At the permissive temperature (32–34°C), the distribution of complex type oligosaccharides in both ts L513 (v) and wild type G are qualitatively and quantitatively similar. At the nonpermissive temperature (39°C), however, the oligosaccharides of cellular G are not fully processed and remain in the newly synthesized, high-mannose form [10].

Initially, virus from ts 513(v) was isolated from NIL-8 fibroblasts that were infected and grown at the permissive temperature. To specifically label the glycopeptides of virion G, virus-infected cells were labelled with ^{3}H-mannose. The isolated virus was then immunoprecipitated with antisera to G and the precipitated protein subjected to trypsin treatment for 4 h at 37°C as described in Methods.

The resultant tryptic glycopeptides were resuspended in 0.1 M sodium phosphate, pH 2.2, buffer and injected onto a C18 Ultrasphere-ODS column for analysis by high performance liquid chromatography. Samples were eluted with a 0%–50% gradient of acetonitrile in 0.1 M sodium phosphate, pH 2.2. Free oligosaccharides elute in the void volume. The resultant profile indicates that there are two major mannose oligosaccharide-containing peaks that elute at 22% acetonitrile and 32% acetonitrile (Fig. 1A). The ratio of ^{3}H-mannose in peak I relative to that in peak II is approximately 1:1 as predicted for three mannose residues per complex-type oligosaccharide at each of the two major glycosylation sites in G protein.

Glycopeptide Separation Determined by Peptide Moiety

To determine whether the nature of the particular N-linked oligosaccharide influences the relative retention times of the glycopeptides, tryptic glycopeptides from both newly synthesized and processed forms of G were analyzed by HPLC. The N-linked oligosaccharides of newly synthesized G, like those of other viral and cellular glycoproteins, are exclusively of the neutral, high-mannose types [14]. The major high-mannose species that are present in newly synthesized G appear to be $GlcMan_9GlcNAc_2$, and $Man_9GlcNAc_2$ [10]. In contrast, the N-linked oligosaccharides of the processed G protein are of the complex type and contain 0–3 negatively-charged sialic acids. By comparing the retention times of the respective high-mannose and complex-type tryptic glycopeptides, it is possible to determine the effect of the carbohydrate residues on glycopeptide elution.

To obtain newly synthesized G, ts L513(v)-infected NIL cells were labelled with ^{3}H-mannose for 30 min at the nonpermissive temperature (34°C). Under these conditions, only the newly synthesized G-containing N-linked oligosaccha-

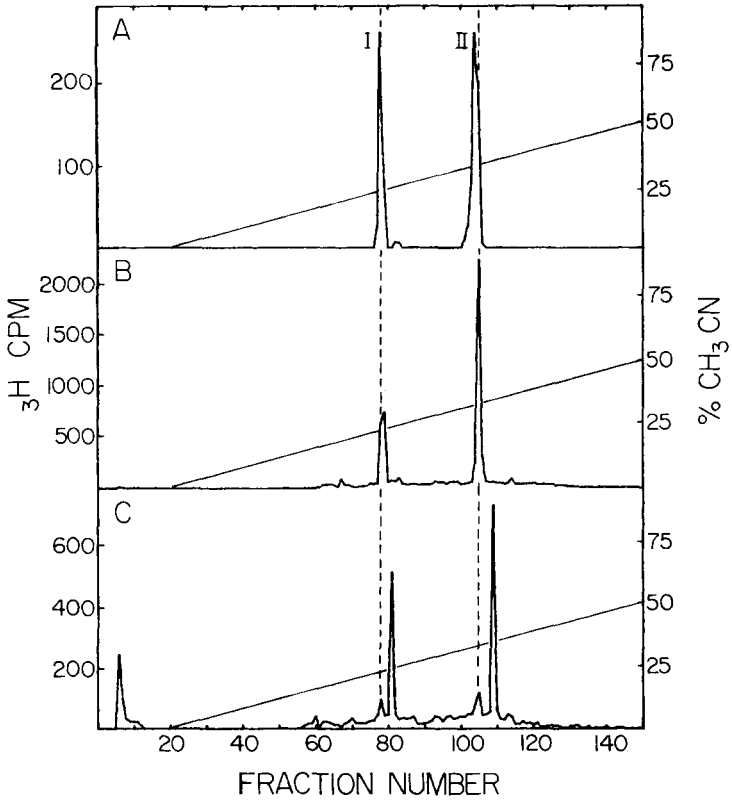

Fig. 1. HPLC profiles of tryptic glycopeptides from ^{3}H-labelled G of VSV. In these experiments, cells were infected with the temperature-sensitive mutant strain of VSV (ts L513). A. ^{3}H-Mannose-labelled G from virus. G was isolated by immunoprecipitation from ts L513-infected NIL cells that were grown and labelled at 34°C, the permissive temperature. For trypsin digestion, G (0.3 mg protein/ml) was incubated with TPCK-trypsin (0.1 mg/ml) at 37°C for 2 h. At this time, an equivalent aliquot of trypsin was added and the incubation continued at 37°C for another 2 h. B. ^{3}H-Mannose-labelled G from cells. G was isolated by immunoprecipitation from ts L513-infected NIL cells that were grown and labelled at 39°C, the nonpermissive temperature. For trypsin digestion, G (0.15 mg protein/ml) was incubated with TPCK-trypsin as indicated above. C. ^{3}H-Glucosamine-labelled G from cells after endo H treatment. G, isolated and trypsinized as above, was first digested with 3 μg/ml of endo H at 37°C prior to trypsin treatment.

rides of the high-mannnose type are labelled with ^{3}H-mannose [10]. Analysis of the tryptic glycopeptides of the G protein precursor by HPLC again showed two major peaks eluting at 22% acetonitrile and 32% acetonitrile (Fig. 1B). Comparison of the profile for newly synthesized G with that for G isolated from virus indicates that the retention times of the respective glycopeptides are identical (Fig. 1 A,B). When both samples are coinjected the same profile is obtained (data not shown). Thus, for a given size range of oligosaccharide, the retention time of the glycopeptide appears to be independent of sugar moiety or charge, and the separation is determined primarily on the basis of the amino acid content of the polypeptide.

The Contribution of the Carboyhydrate Component to Glycopeptide Retention Time Is Small

To determine the relative contribution of the carbohydrate component to the retention time of the tryptic glycopeptides, the shift in retention time of the tryptic glycopeptides following deglycosylation was determined. To achieve this goal, it was necessary to specifically label the glycopeptides and yet remove most, if not all, of the carbohydrate residues. To remove the oligosaccharides, newly synthesized G was digested with endo-N-acetylglucosaminidase H (endo H). Endo H cleaves between the proximal N-acetylglucosamine residues of high-mannose-type N-linked oligosaccharides, leaving a single N-acetylglucosamine residue linked to the polypeptide chain at each glycosylation site. Complex-type oligosaccharides are resistant to the action of end H [15–17]. Metabolic labelling of newly synthesized G with ^{3}H-glucosamine would result in specific labelling of the N-acetyl-glucosamine moiety on the deglycosylated peptides.

To label selectively the newly synthesized form of G, cells were infected with tsL513(v) and labelled with ^{3}H-glucosamine at the nonpermissive temperature. The elution positions of the respective peptides I and II corresponded to those of the ^{3}H-mannose-labelled glycopeptides (see Fig. 1B). After cleavage with endo H, the tryptic glycopeptides eluted with slightly longer retention times at 24% acetonitrile and 34% acetonitrile for peaks I and II, respectively (Fig. 1C). Thus, the contribution of the carbohydrate component to the retention times of the intact tryptic glycopeptide is relatively small.

The slight leakiness of the mutant virus at the nonpermissive temperature is illustrated by the residual peaks that remain following endo H treatment. These endo H-resistant glycopeptides represent a much smaller fraction of the oligosaccharides than is suggested by the distribution of label, since the complex-type oligosaccharides are also labelled through the external glucosamine and sialic acid residues that are added in the final steps of processing.

If wild type VSV-infected cells are labelled with ^{3}H-mannose for 30 min and the immunoprecipitated cellular G analyzed for tryptic glycopeptides by HPLC, a profile similar to that for G of ts L513(v) is observed (Fig. 2). The elution positions of the respective glycopeptides from both wild-type and mutant VSV G proteins are identical, suggesting that the temperature-sensitive defect does not lie within the amino acid region immediately surrounding the site of glycosylation. As suggested in the experiment with mutant virus, tryptic glycopeptides of newly synthesized wild-type G contain ^{3}H-glucosamine-labelled oligosaccharides of both the high-mannose and complex types (data not shown). These two types of glycopeptides can be readily separated by endo H treatment prior to HPLC, allowing quantitation of the extent of oligosaccharide processing at a single glycosylation site.

Kinetics of Trypsin Digestion

To determine the susceptibility of G protein to trypsin digestion, the kinetics of digestion with TPCK-trypsin was determined. Newly synthesized, ^{3}H-mannose-labelled G from wild-type virus was initially treated with 0.1 mg/ml trypsin at a protein:trypsin ratio of 3:1 (w/w). The sample was incubated at 37°C and at each 2-h interval another aliquot of 0.1 mg/ml trypsin was added. Initially, peak I is generated very rapidly (Fig. 3) along with two precursor peaks, III and IV (ratio

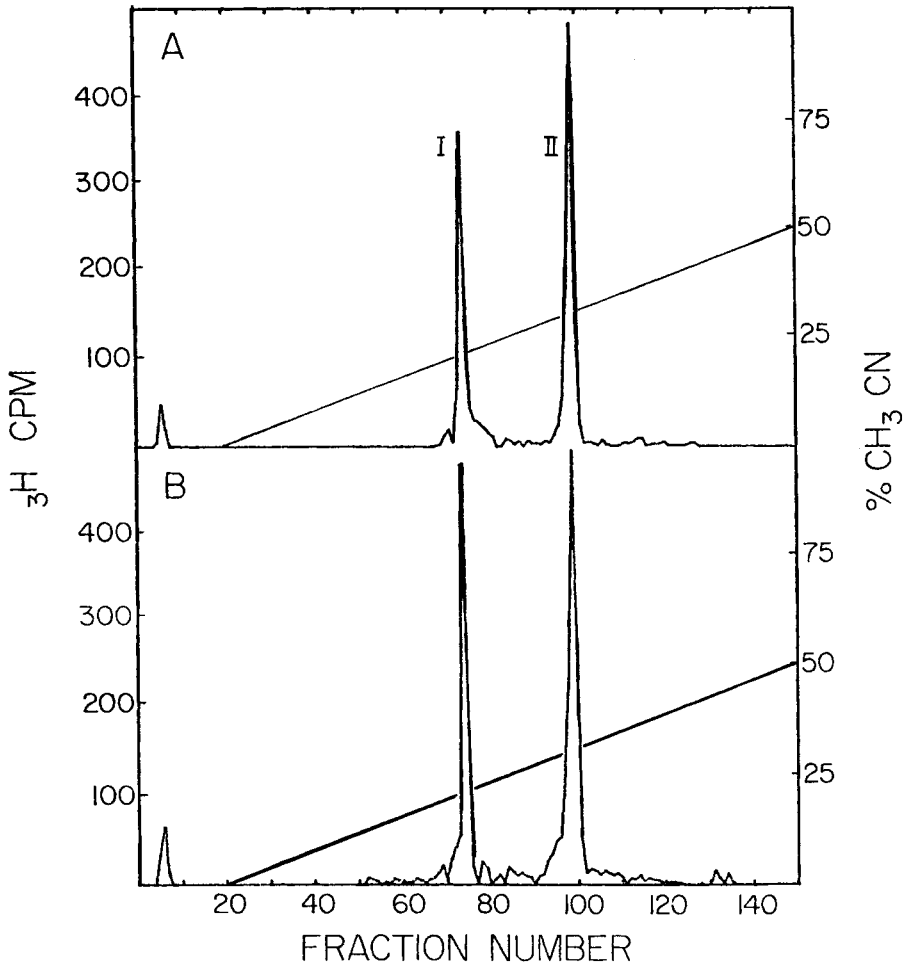

Fig. 2. HPLC profiles of tryptic glycopeptides from ^{3}H-labelled G of mutant and wild-type VSV. G was isolated from VSV-infected NIL cell lysates by immunoprecipitation. A. ^{3}H-Mannose-labelled G from the wild-type strain of VSV. Virus-infected NIL cells were grown and labelled at 37°C. Trypsin digestion was as in B. B. ^{3}H-glucosamine-labelled G from the temperature-sensitive virus [ts 513 (v)]. Virus-infected NIL cells were grown and labelled at 39°C, the nonpermissive temperature. For trypsin digestion, G (0.15 mg protein per ml) was incubated with TPCK-trypsin (0.1 mg/ml) as described in the legend to Figure 1.

of label in I:[II + III + IV] = 1.0:1.1). Following further degradation with trypsin, peaks III and IV appear to be converted to peak II since the relative proportion of label in peak I remains unchanged (Fig. 4). By 4 h of digestion, peaks I and II are the only significant peaks observed (ratio of label in I:II = 1.0:1.0). Little change in this distribution is observed after 6 h (ratio of label in I:II = 1.0:1.2). Thus, the 4-h time point under the above-specified conditions gives reproducible and quantitative results. If trypsin treatment is continued further, however, either by an increase in the trypsin:protein ratio due to sample dilution

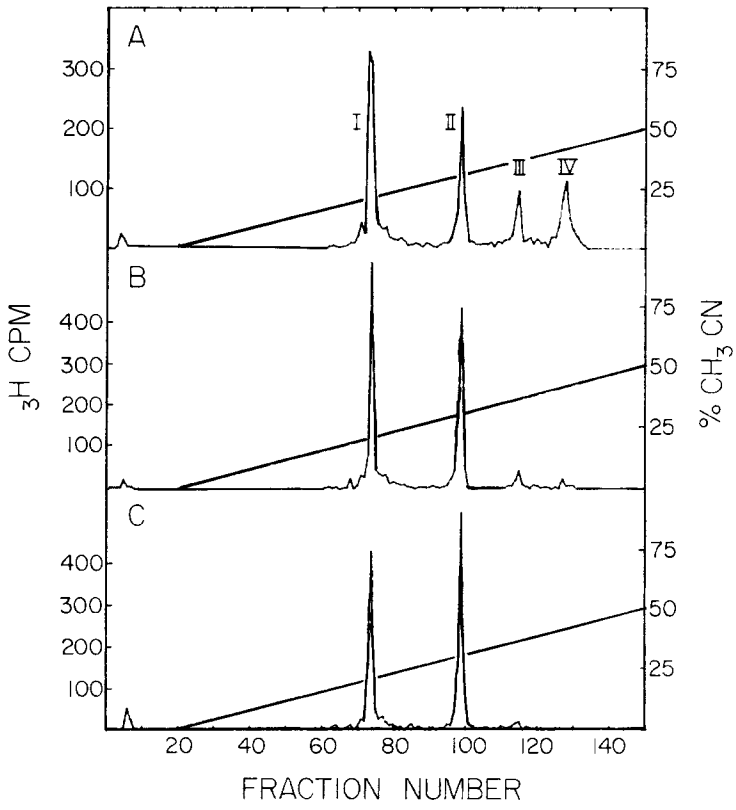

Fig. 3. HPLC glycopeptide profiles showing time course of trypsin digestion of G. ^{3}H-mannose-labelled G was isolated by immunoprecipitation from NIL cells infected with the wild-type strain of VSV and grown and labelled at 37°C. For trypsin digestion, G (0.3 mg protein/ml) was incubated with TPCK-trypsin (0.1 mg/ml) at 37°C as follows: every 2 h an additional aliquot of trypsin (0.1 mg/ml was added. Reactions were terminated by boiling. A. Incubation at 37°C for 2 h with 0.1 mg/ml of trypsin (0.1 mg/ml). B. Incubation at 37°C for 4 h with trypsin. After 2 h of incubation, trypsin (0.1 mg/ml) was added to the sample in A and incubated for an additional 2 h. C. Incubation at 37°C for 6 h with trypsin. After 4 h of incubation 0.1 mg/ml trypsin was added to the sample in B and incubated for an additional 2 h.

or further incubation with fresh enzyme, then a slow conversion of peak I to the position of peak II appears to occur (Fig. 4). Elution of peak I and separate redigestion with trypsin yields exclusively peak II (data not shown). This slow degradation of peak I is presumably due to a less accessible trypsin cleavage site, although the possibility of cleavage by contaminating chymotrypsin at these high enzyme concentrations cannot be excluded. Thus, the variation in peak I: peak II ratios (from 1:1 to 1:2) observed (see, in particular, Fig. 1) may be attributed to variation in extent of trypsin cleavage under conditions of huge trypsin excess.

To verify that the two tryptic glycopeptide peaks I and II are derived from digestion of intact VSV G rather than a contaminant or proteolytic fragment, samples purified by polyacrylamide gel electrophoresis were analyzed. ^{3}H-mannose-labelled cellular G from ts L513(v) was immunoprecipitated and then fractionated by SDS-polyacrylamide gel electrophoresis prior to digestion with trypsin and analysis by HPLC. As shown in Figure 5, peaks I and II are the only

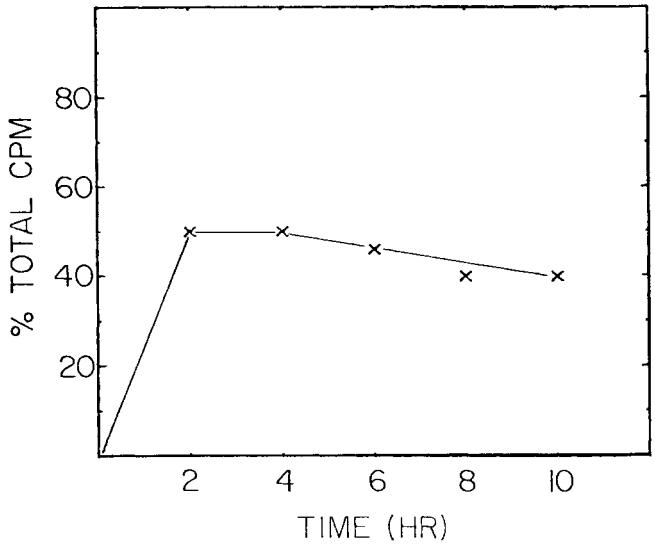

Fig. 4. Plot of kinetics of generation of tryptic glycopeptide I from G. The percentage of ^{3}H-mannose cpm in glycopeptide peak I relative to the total radioactivity in the tryptic glycopeptides of G is plotted as a function of time of trypsin digestion of G. Samples were prepared as described in the legend to Figure 3.

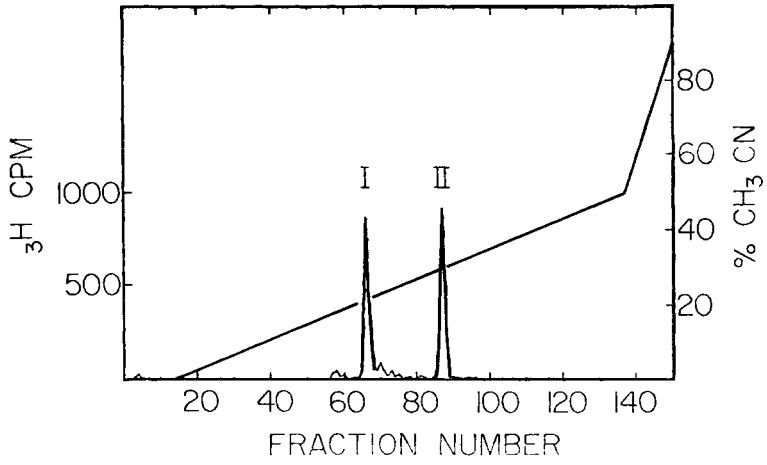

Fig. 5. HPLC profile of tryptic glycopeptides from ^{3}H-mannose-labelled G isolated by PAGE. G was initially isolated by immunoprecipitation from ts L513-infected NIL cells grown and labelled at 39°C, the nonpermissive temperature. An aliquot of the ^{3}H-mannose labelled G (30,000 cpm) and a small amount of ^{35}S-methionine-labelled G were applied to a 10% polyacrylamide gel for electrophoresis. After autoradiography, the band containing G was cut from the gel and swollen in 250 μl of TPCK-trypsin (0.1 mg/ml) in 50 mM NH_4CO_3 pH 8 at 37°C for 2 h. At this time, an equivalent aliquot of trypsin was added and the sample incubated for an additional 2 h. At this time, the sample was diluted to 50 mM trypsin, 25 μg of BSA added, and the sample left at 37°C overnight. In this experiment, the column used for HPLC was a 4.6 mm × 15 cm C18 Ultrasphere-ODS column. The flow rate was 1 ml/min, and fractions of 0.3 ml were collected.

significant peaks observed. In this case, the ratio of label in peak I:peak II is 1.0:1.1, although samples with ratios up to 1.0:1.5 under these conditions have been observed.

DISCUSSION

The goal of this work was to find a method for analyzing the nature and fidelity of glycosylation at individual sites in glycoproteins. To compare glycosylation sites among related proteins or in the same protein synthesized under varying conditions, it was necessary to find a method that is rapid, quantitative, reproducible, and insensitive to carbohydrate microheterogeneity. High performance liquid chromatography of tryptic glycopeptides in a C18 reverse-phase column fulfills these requirements.

The glycopeptides of VSV envelope G protein were analyzed by HPLC in order to calibrate the system. Two major glycosylation sites were observed after initial trypsin cleavage. When the carbohydrates are endogenously labelled with ^{3}H-mannose, the two glycopeptides are present in a ratio of approximately 1:1 as expected for two similar (if not identical) complex-type oligosaccharide chains with three mannose residues per chain. The possibility of other minor glycosylated peptides, as suggested by a number of laboratories [1,2,9,18], cannot be excluded by this data but must constitute $< 10\%$ of the total mannose label. The effect of the long-chain carbohydrate residues on the elution position of the glycopeptides is minor, since a shift of only three fractions is observed upon removal of the oligosaccharides by endo H.

At pH 2.2 glycopeptide elution is independent of the particular N-linked oligosaccharide since both the neutral high-mannose-type and the sialic acid-containing complex type co-elute. The relatively high-salt phosphate buffer (0.1 M $NaPO_4$, pH 2.2) is required for optimal resolution. When 0.16 phosphoric acid was used as the aqueous buffer system, resolution was inferior, and no improvement was obtained when triethylamine was substituted as the counter ion (data not shown). Similar conditions have been employed by Cannon and co-workers for resolution of nonglycosylated peptides from immunoglobulins [E. Cannon, private communication]. Very recently, HPLC separation of tryptic glycopeptides derived from influenza virus was reported [19]. However, no characterization either of the system or the large number of observed peaks was described.

The HPLC system described in the present work is also highly reproducible. On successive days, elution profiles varied by at most $\pm$ 1 fraction. Multiple injections of the same sample routinely produced less than 10% variability in relative peak areas. Although recoveries of injected radioactivity routinely ranged from 60%–75%, no selective loss of any particular peak was observed (data not shown). Finally, glycopeptides derived from different strains of VSV co-eluted.

The observed pattern of glycopeptide behavior is applicable to glycoproteins from other sources as well. Tryptic glycopeptides derived from murine leukemia virus envelope glycoproteins co-eluted on the basis of peptide rather than carbohydrate content (Rosner and Robbins, manuscript in preparation). Similarly, corresponding glycopeptides isolated from Sindbis grown on CHO cells (mainly complex-type oligosaccharides) and a 15b mutant (all high-mannose type oligosaccharides) also co-eluted (Hsieh and Rosner, unpublished results). Thus, the separation system described here appears to have general application.

It was observed that, at very high trypsin concentrations, a relatively slow decrease in VSV glycopeptides eluting with peak I and comparable increase in VSV glycopeptides eluting with peak II appears to occur. The kinetics of trypsin cleavage suggests that this profile results from further enzymatic cleavage of the first major glycopeptide to a smaller, more hydrophobic glycopeptide co-eluting with peak II. This interpretation is supported by the fact that sample recovery exceeded 50%, indicating that glycopeptides containing both of the major sites of glycosylation must be eluting from the column. Furthermore, previous studies have shown that the major glycosylation sites of G are on separate tryptic peptides [1,2,9]. Finally, analysis of gel-isolated G indicates that these two peaks are derived from intact G and not proteolytic fragments or contaminants. However, only direct analysis of the peptides in both peaks will resolve this point completely.

HPLC also provides a simple method for glycopeptide recovery. Glycopeptides of interest can be run on HPLC, isolated, treated with glycosidases, and then rerun on HPLC. The resultant shift in elution will eliminate any background contamination arising from unglycosylated peptides. Although resolution of peptides containing many sites may not be accomplished in a single run, analysis of peptide fragments containing a limited number of glycosylation sites should eliminate this problem.

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