JOURNAL OF VIROLOGY, July 1979, p. 253-256
0022-538X/79/07-0253/04$02.00/0

Vol. 31, No. 1

NOTES

Carbohydrates of Influenza Virus
III. Nature of Oligosaccharide-Protein Linkage in Viral Glycoproteins

WALTER KEIL, HANS-DIETER KLENK,* AND RALPH T. SCHWARZ

Institut für Virologie, Justus-Liebig-Universität, 63 Giessen, West Germany

Received for publication 11 January 1979

Four different glycopeptides can be distinguished after pronase digestion of influenza A virus glycoproteins: Ia and Ib, containing *N*-acetylglucosamine, mannose, galactose, and fucose, and IIa and IIb, containing mannose and *N*-acetylglucosamine. All glycopeptides yielded *N*-acetylglucosaminyl-asparagine after mild acid hydrolysis. There was no evidence for *O*-glycosidic bonds. Thus, the carbohydrate complement is linked to the polypeptide exclusively by *N*-glycosidic linkages between *N*-acetylglucosamine and asparagine.

In glycoproteins of animal origin, mainly two linkage types between carbohydrate and polypeptide have been described: *O*-glycosidic bonds to serine and threonine and *N*-glycosidic linkages to asparagine (7, 12). In the present study an analysis was carried out to answer the question of whether both or only one linkage type exists in the glycoproteins of influenza virus.

Two different influenza A viruses have been analyzed, the Dutch strain of fowl plague virus (FPV) (Hav1Neq1), grown in chicken embryo fibroblasts, and the vaccine strain MRC12 (H3N2), derived from eggs. Virus growth, labeling with radioactive isotopes, virus purification, isolation of viral glycoproteins, and preparation of glycopeptides by pronase digestion followed by chromatography on Bio-Gel P6 columns were performed essentially as described previously (13, 14).

In previous studies two major types of glycopeptides could be distinguished in influenza virus glycoproteins: type I, containing *N*-acetylglucosamine, mannose, galactose, and fucose, and type II, containing only mannose and *N*-acetylglucosamine (5, 11, 14). Presumably due to a new batch of Bio-Gel P6, better resolution of the glycopeptide peaks was achieved in the present study, and types I and II could be further separated into subtypes Ia, Ib and IIa, IIb, respectively. Figure 1 shows the glycopeptide profiles obtained from FPV. Molecular weights were determined as described previously (14) and found to be 3,000, 2,400, 1,800, and 1,100 for Ia, Ib, IIa, and IIb, respectively. The profiles demonstrate that glucosamine and mannose are present in all glycopeptides even though in different proportions. Galactose and fucose (data not shown) are incorporated only in types Ia and Ib. The glycopeptides can also be labeled with amino acids, and Fig. 1C shows the incorporation of radioactive asparagine. Similar results were obtained with [^{14}C]threonine and [^{14}C]serine (data not shown). Acid hydrolysis and thin-layer chromatography revealed that the label was present in its original form in the glycopeptides. Analysis of isolated glycoproteins demonstrated that hemagglutinin fragment HA_1 contains only types Ia and Ib, whereas all four types are present on HA_2 and on the neuraminidase.

Four glycopeptides were also obtained when unlabeled MRC12 virus was analyzed. Their molecular weights (3,200, 2,400, 1,700, and 900, respectively) were similar but not identical to those of FPV. Again, type II glycopeptides showed a particularly high mannose content (Fournet and Schwarz, unpublished data). Amino acid analysis by the Dansyl-Edman procedure described by Chen (3) revealed that Ia contained valine, proline, asparagine, and threonine, Ib contained asparagine, threonine, proline, and isoleucine, and IIa contained asparagine and threonine. Thus, even though only four amino acids are found, asparagine is present in each glycopeptide. Analysis of the *N*-terminal and subterminal amino acids revealed heterogeneity in the peptide structure of both types Ia and Ib. This heterogeneity reflects variability either in the site of pronase cleavage (4) or in the amino acid sequence at the site of carbohydrate attachment.

Due to their different chemical properties, *N*- and *O*-glycosidic linkages can be characterized

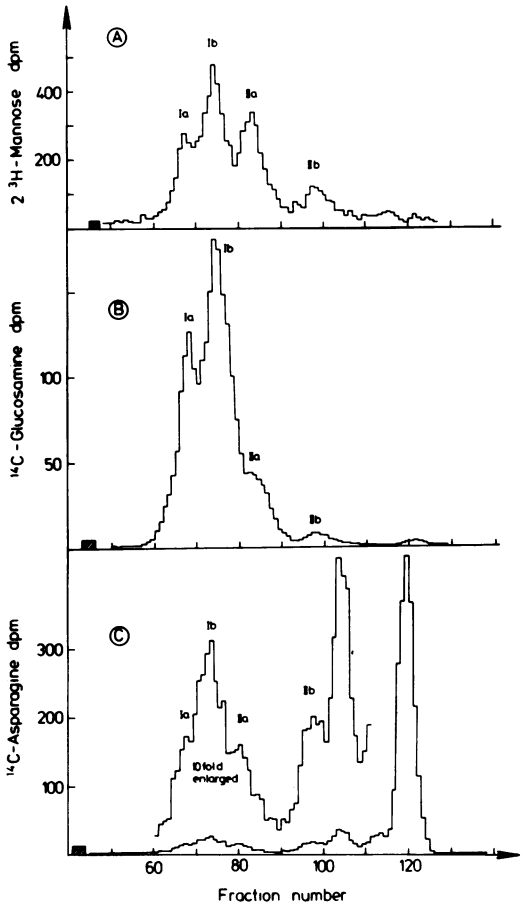

FIG. 1. *Chromatography of FPV glycopeptides on Bio-Gel P6. Purified virus particles labeled with either* D-*[2-³H]mannose,* D-*[1-¹⁴C]glucosamine, or* L-*[U-¹⁴C]asparagine were purified and treated with pronase. Fractions of 0.5 ml were collected.*

by specific hydrolysis and derivation of the resulting products. The *N*-glycosidic bond is relatively stable to acid treatment when compared to *O*-glycosidic bonds occurring in oligosaccharides (12). It is therefore possible to obtain *N*-acetylglucosaminyl-asparagine by partial acid hydrolysis (8, 12). This compound can be identified by chromatographic procedures either directly or after chemical derivation. Such an analysis was performed on nonlabeled glycopeptides Ia, Ib, and IIa of MRC12 virus. Figure 2 shows that under appropriate conditions dansylated *N*-acetylglucosaminyl-asparagine can be obtained from type IIa glycopeptide. Similar results were obtained with types Ia and Ib.

N-Acetylglucosaminyl-asparagine can be identified without dansylation if one of its constituents is radioactively labeled. Such an analysis has been performed on glycopeptides derived from purified glycoproteins labeled with [¹⁴C]glucosamine. Figure 3 shows the results obtained with glycopeptide IIa of glycoprotein HA_2. The formation of glucosaminyl-asparagine and *N*-acetylglucosaminyl-asparagine demonstrates the existence of an *N*-glycosidic linkage. Similar results were obtained for all glycopeptides except IIb (which was present in too small amounts) isolated from each individual glycoprotein. Quantification of the data by scanning of the thin-layer plates gave information on the ratio of total glucosamine to asparagine-bound

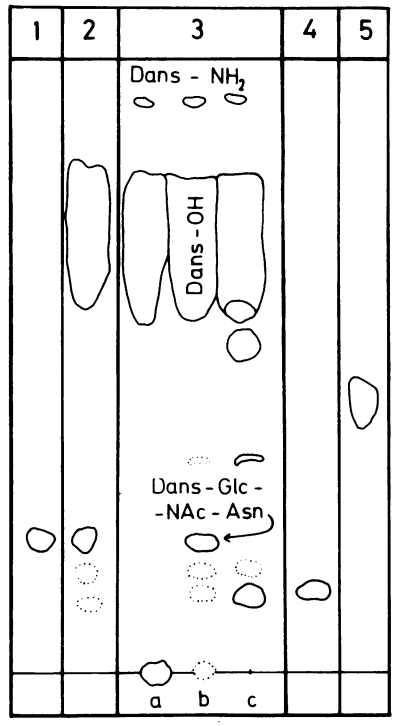

FIG. 2. *Detection of N-acetylglucosaminyl-asparagine in glycopeptide IIa from MRC12. Dansylation was carried out by the method of Chen (3). Thin-layer chromatography was performed on silica gel plates (Merck, Germany) using methyl acetate–isopropanol–25% aqueous ammonia (45:30:20) as a solvent (15). N-Acetylglucosaminyl-asparagine was obtained from Vega-Fox Biochemicals, Tucson, Ariz. (1) N-Acetylglucosaminyl-asparagine reference, dansylated. (2) N-Acetylglucosaminyl-asparagine reference treated with 2 N HCl for 20 min at 100°C prior to dansylation. (3) (a) Glycopeptide heated with water for 20 min at 100°C prior to dansylation; (b) glycopeptide hydrolyzed with 2 N HCl for 20 min at 100°C prior to dansylation; (c) glycopeptide hydrolyzed with 6 N HCl for 2 h at 100°C prior to dansylation. (4) Aspartic acid reference after dansylation. (5) Asparagine, dansylated.*

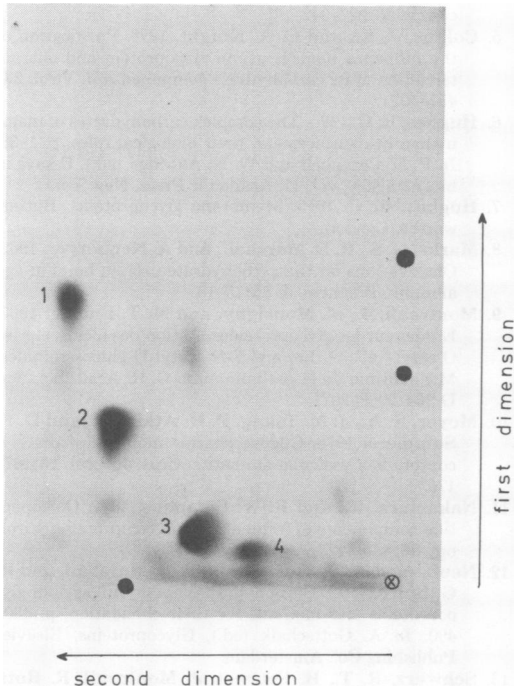

FIG. 3. *Detection of N-acetylglucosaminyl-asparagine in glycopeptide IIa from HA_2 of FPV. Glycopeptide IIa labeled with [^{14}C]glucosamine was subjected to mild hydrolysis and subjected to two-dimensional chromatography on cellulose thin-layer plates. First dimension: isopropanol–formic acid–water (60:4:20). Second dimension: 75% phenol, gas phase equilibrated with vapors of a 3% solution of aqueous ammonia (2). Plate was exposed for 5 weeks on Kodak X-Omat R film XR5. ⊗, Origin; 1, glucosamine; 2, N-acetylglucosamine; 3, N-acetylglucosaminyl-asparagine; 4, glucosaminyl-asparagine.*

glucosamine. For types Ia, Ib, and IIa the ratios were 7:1, 5.7:1, and 2.7:1, respectively. Thus, there appear to be about seven glucosamine residues in type Ia, five to six in Ib, and two to three in IIa.

Carbohydrate side chains linked to serine and threonine by *O*-glycosidic bonds can be released from the polypeptide by treatment with alkali and reducing agents in a β-elimination reaction (Fig. 4) (12). Serine is converted under these conditions into alanine, and threonine is converted into α-aminobutyric acid. Serine and threonine not involved in *O*-glycosidic bonds are not converted. When FPV glycopeptides labeled with either [^{14}C]threonine or [^{14}C]serine were subjected to such treatment, rechromatography on Bio-Gel P6 revealed that about 15% of the radioactivity was converted into short peptides, as they might be expected to occur after β-elimination. However, analysis of the hydrolysates of these peptides revealed that the label was still present as serine and threonine, respectively. *O*-Glycosidic bonds to serine and threonine show an increased stability against alkali treatment when these amino acids are terminal in a peptide (9, 12). Therefore, we have also analyzed tryptic peptides in which these amino acids are less likely to be terminal. However, the results were similar to those obtained with the glycopeptides resulting from pronase digestion.

Glycopeptides are fragments of glycoproteins which contain only a small proportion of the polypeptide moiety, namely, the attachment sites of the carbohydrate side chains. Glycopeptides are thus a suitable substrate for studies on the linkage between protein and carbohydrate. Our data show that acid hydrolysis yields *N*-acetylglucosaminyl-asparagine that can be detected either in the form of its dansyl derivative or by radioactive labeling. Prolonged treatment with mild alkali also leads to some degradation of the glycopeptides. However, this degradation was not due to β-elimination involving serine or threonine. We have therefore not obtained evi-

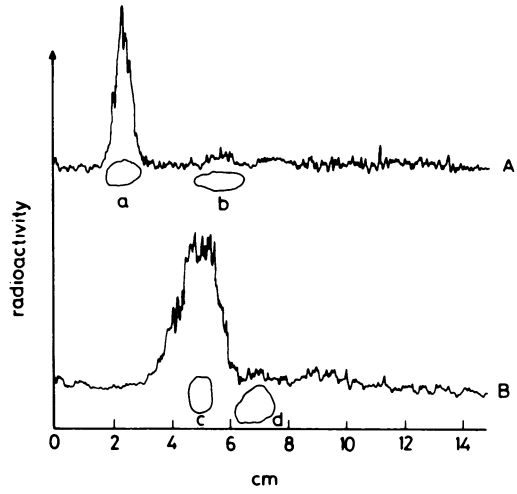

FIG. 4. *Amino acid analysis of degradation products obtained after alkali treatment of FPV glycopeptides. Mixtures of FPV glycopeptides labeled with either [^{14}C]serine or [^{14}C]threonine were treated for 4 days with 0.1 N NaOH–0.3 M $NaBH_4$ at 4°C. In the case of threonine-labeled material, $PdCl_2$ was present. The reaction mixture was rechromatographed on Bio-Gel P6, and newly formed low-molecular-weight material was hydrolyzed with 6 N HCl for 15 h at 105°C. Chromatography was done in isopropanol–formic acid–water (80:4:20) on cellulose thin-layer plates (Merck, Germany) (2). (A) Material obtained from glycopeptides labeled with [^{14}C]serine. (B) Material obtained from glycopeptides labeled with [^{14}C]threonine. Reference substances: a, serine; b, alanine; c, threonine, d, α-aminobutyric acid.*

dence for the existence of *O*-glycosidic linkages. Thus, we have demonstrated here that in influenza virus glycoproteins the carbohydrate side chains are attached to the polypeptide exclusively by *N*-glycosidic linkages between asparagine and *N*-acetylglucosamine. The absence of *O*-glycosidic linkages in the influenza virus neuraminidase has been shown before (1), but direct analysis of the *N*-glycosidic linkage was not done in that study. The influenza virus glycoproteins resemble, therefore, egg albumin, orosomucoid, ovomucoid, thyroglobulin (12), and the G protein of vesicular stomatitis virus (10) in containing only *N*-glycosidic linkages, and they differ from some types of immunoglobulins and glycophorin (6), which contain both *N*-glycosidic and *O*-glycosidic linkages.

We thank R. Rott for his support and interest in this work. We also thank T. Islei and E. Otto for excellent technical assistance. MRC12 virus was a gift of the Behringwerke AG (Germany).

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 47).

LITERATURE CITED

1. **Allen, A. K., J. J. Skehel, and V. Yuferov.** 1977. The amino acid and carbohydrate composition of the neuraminidase of B/Lee/40 influenza virus. J. Gen. Virol. **37**:625–628.
2. **Brenner, M., A. Niederwieser, and G. Pataki.** 1967. Aminosäuren und Derivate, p. 696–749. *In* E. Stahl (ed.), Dünnschichtchromatographie. Springer-Verlag, Berlin.
3. **Chen, R.** 1976. The sequence determination of a protein in micro scale: the sequence analysis of ribosomal protein L 34 of Escherichia coli. Hoppe-Seyler's Z. Physiol. Chem. **357**:873–886.
4. **Cheron, A., B. Fournet, G. Spik, and J. Montreuil.** 1976. Structure complète de glycopeptides isolés des immunoglobulines IgI du colostrum de vache. Biochimie **58**:927–942.
5. **Collins, J. K., and C. A. Knight.** 1978. Purification of the influenza hemagglutinin glycoprotein and characterization of its carbohydrate components. J. Virol. **26**: 457–467.
6. **Hughes, R. C.** 1975. The complex carbohydrates of mammalian cell surfaces and their biological roles, p. 1–36. *In* P. N. Campbell and W. N. Aldridge (ed.), Essays in biochemistry, vol. 11. Academic Press, New York.
7. **Hughes, R. C.** 1975. Membrane glycoproteins. Butterworths, London.
8. **Marks, G. S., R. D. Marshall, and A. Neuberger.** 1962. Observations on the carbohydrate-protein bond in egg albumin. Biochem. J. **85**:15–16.
9. **Montreuil, J., M. Monsigny, and M. T. Buchet.** 1967. Etudes sur les glycoproteides. Action des alcalis sur les O-seryl et O-thréonyl-β-N-acetyl-D-glucosaminides. Mechanisme de la β-elimination. C. R. Acad. Sci. Ser. D **264**:2068–2071.
10. **Moyer, S. A., J. M. Tsang, P. H. Atkinson, and D. F. Summers.** 1976. Oligosaccharide moieties of the glycoprotein of vesicular stomatitis virus. J. Virol. **18**:167–175.
11. **Nakamura, K., and R. W. Compans.** 1978. Glycopeptide components of influenza viral glycoproteins. Virology **86**:432–442.
12. **Neuberger, A., A. Gottschalk, R. D. Marshall, and R. G. Spiro.** 1972. Carbohydrate-peptide linkages in glycoproteins and methods for their elucidation, p. 450–490. *In* A. Gottschalk (ed.), Glycoproteins. Elsevier Publishing Co., Amsterdam.
13. **Schwarz, R. T., B. Fournet, J. Montreuil, R. Rott, and H.-D. Klenk.** 1978. The carbohydrates of influenza virus. II. Gas chromatographic analysis of glycopeptides derived from viral glycoproteins and mucopolysaccharides. Arch. Virol. **56**:251–255.
14. **Schwarz, R. T., M. F. G. Schmidt, U. Anwer, and H.-D. Klenk.** 1977. Carbohydrates of influenza virus. I. Glycopeptides derived from viral glycoproteins after labeling with radioactive sugars. J. Virol. **23**:217–226.
15. **Seiler, N., and J. Weichmann.** 1964. Zum Nachweis von Aminosäuren im 10^{-10} Mol-Maβstab. Trennung von 1-Dimethylamino-naphthalin-5-sulfonyl-aminosäuren auf Dünnschichtchromatogrammen. Experientia **20**: 559–560.