Transfer of Carbohydrates on to Nascent Glycoprotein of Vesicular Stomatitis Virus

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I studied the glycosylation *in vivo* of a viral envelope protein, the glycoprotein of vesicular stomatitis virus (VSV), by pulse labelling of virus-infected HeLa cells with ^3H -labelled monosaccharides (mannose, glucosamine). Radioactivity was incorporated into the fraction of membrane-bound polyribosomes, although metabolic conversion of [^3H]-mannose into amino acids was negligible. Dissociation of bound polyribosomes revealed that the radioactively co-purified with the peptidyl-tRNA. The nascent peptides were released by alkaline hydrolysis, immunoprecipitated and analysed by polyacrylamide-gel electrophoresis. It is apparent from the size distribution of the [^3H]mannose-labelled nascent chains that attachment of carbohydrate starts when approximately half of the amino acid sequence of the viral glycoprotein has been synthesized.

The envelope of VSV (vesicular stomatitis virus) appears to be a lipid bilayer derived from the host-cell plasma membrane (Nakai & Howatson, 1968), into which a single viral protein, the glycoprotein (G-protein), has been inserted (Knipe *et al.*, 1977*b*; Atkinson *et al.*, 1976; Hunt & Summers, 1976*a*). Although the primary sequence of the G-protein is specified by the viral genome, the various glycosyl-transferases required to synthesize both identical side chains are host-cell-specific (Moyer & Summers, 1974; Robertson *et al.*, 1976). VSV-infected animal cells thus provide a useful model system in which to investigate the biosynthesis, maturation and intracellular transport of a membrane protein, since host-cell protein synthesis is suppressed subsequent to infection, and only viral proteins are synthesized and can be readily followed (Mudd & Summers, 1970).

G-protein is synthesized on ribosomes bound to the rough endoplasmic membrane (Morrison & Lodish, 1975; Grubman *et al.*, 1975) of infected cells and is inserted as a transmembrane protein into the endoplasmic reticulum (Knipe *et al.*, 1977*a*; Morrison & McQuain, 1978). The complex oligosaccharide chains (Reading *et al.*, 1978) attached at two asparagine residues of the protein (Robertson *et al.*, 1976; Moyer *et al.*, 1976) are directed into the lumen of the organelle, whereas the C-terminal segment is exposed on the cytoplasmic side of the membrane (Morrison & McQuain, 1978; Katz *et al.*, 1977). N-Acetylglucosamine and mannose are transferred

on to newly synthesized G-protein in the rough endoplasmic reticulum (Hunt & Summers, 1976*b*), most likely by transfer of an oligosaccharide *en bloc* (Hunt *et al.*, 1978; Tabas *et al.*, 1978). Kinetic studies in a translation system *in vitro* have implicated that the initial glycosylation step occurs while the polypeptide chain is being synthesized (Rothman & Lodish, 1977).

In the present paper I report the isolation of peptidyl-tRNA species from bound polyribosomes of VSV-infected HeLa cells. These isolated peptidyl-tRNA species are shown to be specifically labelled in their sugar moiety. The size distribution of the nascent polypeptide chains which served as a substrate for the cellular glycotransferases has been determined by polyacrylamide-gel electrophoresis.

Experimental

Materials

Joklik-modified Minimum Essential Medium (catalogue no. F13) was purchased from BCK Biocult-Chemie, Karlsruhe, Germany, and foetal calf serum was supplied by Flow Laboratories G.m.b.H., Bonn, Germany. Dowex 1 (X4), Dowex 50 (X4), ECTEOLA-cellulose (epichlorohydrin triethanolamine-cellulose), acrylamide, *NN'*-methylenebisacrylamide, Coomassie Brilliant Blue and SDS were obtained from Serva, Heidelberg, Germany. Puromycin, heparin, Triton X-100 and dithiothreitol were from Sigma Chemie G.m.b.H., Munich, Germany. Sucrose was from Becton, Dickinson G.m.b.H., Heidelberg, Germany. D-[1- ^3H (n)]Mannose was from NEN Chemicals, Dreieich, Germany.

Abbreviations used: VSV, vesicular stomatitis virus; SDS, sodium dodecyl sulphate.

D-[6-³H]Glucosamine hydrochloride and [³⁵S]methionine were obtained from Amersham-Buchler, Braunschweig, Germany. Instagel and Instafluor were from Packard Instrument G.m.b.H., Frankfurt, Germany. *Staphylococcus aureus* and rabbit antiserum against VSV proteins were gifts from Dr. M. Breindl, Heinrich Pette Institut, Hamburg, Germany. Paper MN 218 for high-voltage electrophoresis was purchased from Macherey-Nagel, Düren, Germany. All other chemicals were obtained from E. Merck, Darmstadt, Germany.

Cell growth, virus infection and cell labelling

HeLa S3 cells were grown at 37°C in suspension in Joklik-modified Minimum Essential Medium supplemented with 7.5% (v/v) foetal calf serum. Cultures were maintained at cell densities of 5×10^5 cells/ml. Approx. 1×10^9 HeLa cells were infected with VSV (serotype Indiana) at a multiplicity of 10 plaque-forming units/cell and labelled at 4 h after infection with ³H-labelled monosaccharides. To do this, cells were concentrated by centrifugation at 300g for 5 min and washed once with prewarmed Earle's balanced salt solution (Earle, 1943) supplemented with 5% (v/v) foetal calf serum and 100 µg of glucose/ml. The cells were resuspended in 30 ml of the same medium and labelled for 30 min with either 2 mCi of [1-³H]mannose (specific radioactivity 13.2 Ci/mmol) or 2 mCi of [6-³H]glucosamine hydrochloride (specific radioactivity 19 Ci/mmol).

Cell fractionation

Cells were washed twice with ice-cold phosphate-buffered saline (Dulbecco & Vogt, 1954), allowed to swell for 10 min in 30 ml of hypo-osmotic buffer (10 mM-KCl, 10 mM-Tris/HCl, pH 7.5, 1.5 mM-magnesium acetate), and homogenized in a Dounce homogenizer (Kontes, Vineland, NJ, U.S.A.) with eight strokes of the B pestle. The cell homogenate was centrifuged at 800g for 5 min at 4°C and the postnuclear supernatant was separated into a membrane fraction and postmicrosomal supernatant by centrifugation in a Sorvall SS34 rotor at 10000g for 10 min and 4°C. Heparin was added to the supernatant at 100 µg/ml final concentration. The membrane pellet was resuspended in hypo-osmotic buffer containing 200 µg of heparin/ml and the membranes were dissolved with 1% (w/v) Triton X-100 and 1% (w/v) deoxycholate. Particles were removed by centrifugation at 3500g for 5 min and the supernatant was layered on a cushion of 2 M-sucrose in low-salt buffer (50 mM-Tris/HCl, pH 7.5, 25 mM-KCl, 5 mM-magnesium acetate). The post-microsomal supernatant was also layered on such a cushion. The polyribosomes were recovered by centrifugation in a Ti60 MSE rotor at 170000g for 17 h at 4°C.

Sucrose-gradient analysis of polyribosomes

The isolated polyribosomal fractions were analysed on 10–50% (w/v) sucrose gradients containing high-salt buffer (500 mM-KCl, 50 mM-Tris/HCl, pH 7.5, 5 mM-magnesium acetate), 0.2% (w/v) Triton X-100 and 0.2% (w/v) deoxycholate. Centrifugation was in a Beckman SW41 rotor at 272000g for 90 min at 4°C. Fractions were collected from the top of the gradient. Fractions containing the ³H-labelled polyribosomes (Fig. 2b) were pooled and the polyribosomes were recovered by centrifugation in a Ti60 MSE rotor as mentioned above. Part of these polyribosomes were resuspended in high-salt buffer containing 0.2% Triton X-100 and 0.2% deoxycholate, and incubated with 1 mM-puromycin at 37°C for 10 min. This ribosomal suspension was loaded on a 10–40% (w/v) linear sucrose gradient in high-salt buffer containing the detergent mixture. The subunits were separated in a Beckman SW41 rotor at 272000g for 165 min at 20°C.

Isolation of peptidyl-tRNA

A column (1 cm × 5 cm) filled with ECTEOLA-cellulose was equilibrated in starting buffer (Cioli & Lennox, 1973) [100 mM-NaCl, 100 mM-sodium formate, pH 4.7, 6 M-urea, 2 mM-dithiothreitol and 0.1% (w/v) Nonidet P40]. Bound polyribosomes were dissociated in 1 ml of the same buffer containing in addition 0.5% (w/v) SDS and 500 µg of heparin/ml. After complete solubilization the sample was diluted with 9 ml of starting buffer and loaded on the column, which was subsequently washed with 30 ml of starting buffer (wash fraction). Peptidyl-tRNA was eluted by raising the salt concentration of the starting buffer to 1 M-NaCl. Fractions (2 ml) were collected. Peptidyl-tRNA was recovered by precipitation with 2½ vol. of ethanol.

Immunoprecipitation of nascent peptide chains

The peptidyl-tRNA was hydrolysed in 0.5 M-Tris/HCl, pH 9.0, for 1 h at 37°C, in order to analyse the nascent peptides by polyacrylamide-gel electrophoresis. The nascent chains were incubated with a rabbit antiserum raised against all viral proteins, and the antigen-antibody complex was adsorbed to *S. aureus* as described previously (Kessler, 1975). The adsorbed immune complexes were released from *S. aureus* by boiling in gel sample buffer.

Carbohydrate analysis of the protein fraction

Proteins of [³H]mannose-labelled HeLa cells were precipitated with 10% trichloroacetic acid and hydrolysed in 1 ml of 1 M-H₂SO₄ for 6 h at 105°C in sealed tubes (Spiro & Spiro, 1965). The hydrolysate was diluted with 6 vol. of water and passed through a column (1 cm × 5 cm) of Dowex 50 (X4; H⁺ form) connected to a column (1 cm × 5 cm) of Dowex 1

(X4; formate form). Effluent and wash fractions were freeze-dried and the sugar composition was determined by quantitative paper electrophoresis at 3500V for 30min by using 0.04M-sodium borate buffer, pH9.3, and MN 218 paper. Mannose, glucosamine and *N*-acetylglucosamine were used as standards and assayed by periodic acid/benzidine staining (Gordon *et al.*, 1959).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out in vertical slab gels consisting of 10% (w/v) polyacrylamide (acrylamide/bisacrylamide, 75:2, w/w) in a buffer containing 0.375M-Tris/glycine, pH8.7, and 0.1% (w/v) SDS (Laemmli, 1970). The samples were heated to 100°C in 0.1M-dithiothreitol/2% (w/v) SDS/80mM-Tris/HCl (pH6.8)/10% (w/v) glycerol/0.4% (w/v) Bromophenol Blue. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue in 40% (v/v) methanol/10% (v/v) acetic acid. After destaining in the same solvent the gels were either impregnated with 2,5-diphenyloxazole for fluorography (Bonner & Laskey, 1974) or cut into slices of 1 mm width. The radioautographs were scanned with an Ortec Densitometer (Munich, Germany).

Radioactivity measurements

Samples (100 μ l) of sucrose-gradient fractions and column eluates were precipitated with 5% (w/v) trichloroacetic acid (Mans & Novelli, 1961) and radioactivity was determined after addition of 5ml of Instafuor. Polyacrylamide-gel slices were dissolved by heating at 55°C in 0.3 ml of H₂O₂ in tightly capped vials for 16h. After addition of 5ml of Instagel the vials were counted for radioactivity by using the

external-standard-ratio method in a liquid-scintillation spectrometer (Packard) with an efficiency of approx. 35% for ³H.

Results and Discussion

Incorporation of [³H]mannose into G-protein and membrane-bound polyribosomes

After labelling of VSV-infected HeLa cells (4h after infection) with [³H]mannose for 30min the viral G-protein was the major protein band observed in polyacrylamide gels after fluorography (Fig. 1). A minor band, migrating slightly faster, also appeared, which is thought to be a variant of the G-protein (Hunt & Summers, 1976a), although the chemical nature of this difference has not yet been determined. It was essential to demonstrate the specificity of the radiolabelling technique and particularly to detect whether or not there was metabolic conversion of mannose into other compounds such as amino acids. To this end an [³H]-mannose-labelled protein fraction was hydrolysed and passed over a cation- and anion-exchange column (see the Experimental section). Approx. 95% of the radioactivity did not bind to the ion-exchange resins, indicating that the incorporated radioactive compound was a neutral molecule, which was later identified as mannose by high-voltage electrophoresis.

Having established that the conversion of mannose into amino acids was negligible under these labelling conditions, I investigated the distribution of radioactivity in free and bound polyribosomes in [³H]-mannose-labelled VSV-infected HeLa cells (Figs. 2a and 2b, respectively). Sucrose-gradient analysis of both free and bound polyribosome preparations was

Fig. 1. Polyacrylamide-gel electrophoresis of VSV-infected HeLa cells labelled with [³H]mannose

HeLa cells were infected with VSV and labelled 4h after infection with [³H]mannose. The proteins contained in the membrane fraction were separated on 10% SDS/polyacrylamide gels and the radioactive bands were detected by fluorography of the dried gel (cf. the Experimental section). L, N, NS and M refer to virion proteins which were used as markers. G₁ is the intracellular form of the G-protein which lacks the sialic acid. G₂ is the G-protein of the virion which contains sialic acid (Knipe *et al.*, 1977a). —, [³H]Mannose-labelled intracellular G-protein; ----, [³⁵S]-methionine-labelled VSV proteins from virus particles.

Fig. 2. Sucrose-gradient pattern of free (a) and bound (b) polyribosomes

VSV-infected HeLa cells were labelled with [^3H]-mannose and free and bound polyribosomes were isolated. The polyribosomal fractions were analysed on 10–50% (w/v) sucrose gradients containing high-salt buffer. Fractions (0.5 ml) were collected from the top of the gradient. —, A_{254} ; ●, ^3H radioactivity.

carried out in high-salt buffer and in the presence of a detergent mixture in order to avoid adsorption of the hydrophobic G-protein on to the ribosomal particles. Only the bound polyribosomal fraction showed a large peak of radioactivity at higher s values (Fig. 2b). The gradient fractions comprising the peak were recombined and the labelled material was further characterized.

When the bound polyribosomes were dissociated on incubation with puromycin, the labelled material was released from the ribosomes and appeared at the top of the sucrose gradients (Fig. 3). Since the composition of the gradient solution in this experiment was the same as was used for the polyribosome preparation, the result clearly indicated that the labelled material in Fig. 2(b) was not merely adsorbed to the ribosomes but was most probably functionally associated with the bound polyribosomes, presumably as peptidyl-tRNA.

Isolation of [^3H]monosaccharide-labelled peptidyl-tRNA

Column chromatography on ECTEOLA-cellulose (see the Experimental section) was used to confirm

Fig. 3. Sucrose-gradient pattern of bound polyribosomes after puromycin treatment

Part of the ^3H -labelled bound polyribosomes from Fig. 2(b) were resuspended in high-salt buffer containing 0.2% Triton X-100 and 0.2% deoxycholate, and incubated with 1 mM-puromycin. The ribosomal subunits were separated on a 10–40% (w/v) sucrose gradient (cf. the Experimental section). Fractions (0.5 ml) were collected from the top of the gradient. —, A_{254} ; ●, ^3H radioactivity.

Fig. 4. Isolation of peptidyl-tRNA by ECTEOLA-cellulose chromatography

[^3H]Mannose-labelled bound polyribosomes were solubilized and the peptidyl-tRNA fraction was purified by chromatography on ECTEOLA-cellulose as described in the Experimental section.

this assumption and to purify the peptidyl-tRNA fraction from bound polyribosomes (Fig. 4). In a preliminary experiment a mixture of viral M- and G-protein, prepared from [^{35}S]methionine-labelled VSV (Breindl & Holland, 1975), was chromatographed on such a column. As shown in Table 1, only small amounts of the completed viral proteins

Table 1. Isolation of peptidyl-tRNA by chromatography on ECTEOLA-cellulose

A mixture of viral G-protein and M-protein was prepared from [35 S]methionine-labelled VSV by detergent treatment (Breindl & Holland, 1975). VSV-infected HeLa cells were incubated with 3 H-labelled monosaccharides in order to label nascent G-protein chains. Peptidyl-tRNA was purified by chromatography on ECTEOLA-cellulose columns after solubilization of the bound polyribosome fraction.

	Radioactive label	Radioactivity		
		Wash fraction (c.p.m.)	Eluate (c.p.m.)	
			(c.p.m.)	(%)
G-protein + M-protein	[35 S]Methionine	4600	100	2
Polyribosomes	[3 H]Glucosamine	2350	11 500	90
Polyribosomes	[3 H]Mannose	850	27 400	97

were retarded on the ion-exchange resin. In contrast, when solubilized bound polyribosomes isolated from [3 H]glucosamine- and [3 H]mannose-labelled VSV-infected HeLa cells were fractionated on ECTEOLA-cellulose, 90–95% of the radioactivity was retarded on the column (cf. Table 1). Nascent peptides, obtained by alkaline hydrolysis or pancreatic-ribonuclease treatment of peptidyl-tRNA, did not bind to ECTEOLA-cellulose, as was demonstrated in control experiments (results not shown).

The observed binding of the labelled material to the basic ion exchanger could only be due to the tRNA molecules that were still covalently bound to the growing peptide chains. Thus this experiment demonstrated that the G-protein chains are glycosylated before the complete protein sequence is synthesized and that at least *N*-acetylglucosamine and mannose are in the carbohydrate moiety initially transferred to the nascent polypeptide chains. Labelling was also achieved by incubation of VSV-infected cells with [3 H]glucose, but a detailed analysis of the initially transferred oligosaccharide was not possible, owing to lack of material.

Size distribution of [3 H]mannose-labelled nascent G-protein

After alkaline hydrolysis of the peptidyl-tRNA, the [3 H]mannose-labelled nascent chains were recovered by immune precipitation and analysed on a 10% polyacrylamide gel (Fig. 5). A major radioactive peak was detected in the region migrating slightly ahead of the completed G-protein marker derived from virus particles. In addition, smaller polypeptides were found, the smallest having a mol.wt. of approx. 30000. In other experiments in which the immune-precipitation step was omitted or [3 H]glucosamine-labelled nascent chains were analysed, similar gel patterns were obtained. These results suggest that the polypeptide sequence of the nascent glycoprotein is approximately half finished before the addition of both monosaccharides, which are transferred most probably from a preassembled

Fig. 5. Polyacrylamide-gel electrophoresis of nascent G-protein chains labelled with [3 H]mannose. Peptidyl-tRNA was hydrolysed at alkaline pH, nascent chains were immunoprecipitated and loaded on a 10% SDS/polyacrylamide gel (cf. the Experimental section). G₂, N, NS and M refer to unlabelled virion proteins which were used as markers.

dolichyl pyrophosphoryl oligosaccharide (Hunt *et al.*, 1978; Tabas *et al.*, 1978). The gel pattern and the bound polyribosome profile indicate that the glycosylation of the second asparagine residue occurs later, most probably when the G-protein sequence is nearly completed. These observations are in agreement with the studies of translation *in vitro* by Rothman & Lodish (1977).

It is unclear, since the amino acid sequence of the viral G-protein is unknown, why the initial glycosylation step occurs so late during peptide synthesis. Late glycosylation has also been reported for nascent ovalbumin (Kiely *et al.*, 1976) and immunoglobulin heavy chains (Bergman & Kuehl, 1977). This finding can easily be understood for nascent heavy chains,

because the presumptive asparagine residue that becomes glycosylated is located in the CH2 domain. However, it is difficult to imagine why nascent ovalbumin is glycosylated so late, since there are already two asparagine residues at position 24 and 26 from the N-terminus (Palmiter *et al.*, 1978). One explanation for this surprising observation might be that these specific asparagine residues were not recognized by the cellular glycotransferase. Consistent with this interpretation are glycosylation studies *in vitro* in which lipid-linked carbohydrate chains were transferred on to exogenous oligopeptides which had the characteristic tripeptide sequences Asn-X-Ser or Asn-X-Thr (where X is one of the 20 protein amino acids) (Struck *et al.*, 1978). The previous reports on the modification of nascent secretory proteins (Kiely *et al.*, 1976; Bergman & Kuehl, 1977) suggest that the cellular glycotransferase catalysing the initial carbohydrate transfer recognizes as a signal the amino acid sequence and/or the conformation surrounding the asparagine residues of the acceptor molecule. Whether these criteria or additional topological restrictions imposed by the lipid bilayer are responsible for the late glycosylation of the nascent VSV G-protein has to be further investigated.

The experimental evidence obtained from the studies *in vivo* described in the present paper, as well as the data of the studies *in vitro* (Rothman & Lodish, 1977), demonstrate that the carbohydrate attachment to G-protein occurs co-translationally. Since the carbohydrate transfer is a rather late event in the biosynthesis of the G-protein, one would assume that glycosylation is not a prerequisite for insertion into the membrane. Studies of VSV replication in tunicamycin-treated Chinese-hamster ovary cells (Leavitt *et al.*, 1977; Gibson *et al.*, 1978) have indicated that glycosylation facilitates the subsequent steps of maturation and intracellular transport of the G-protein, possibly by stabilizing the conformation of the membrane-inserted molecule, thereby leading to a normal assembly of the viral envelope.

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