Mapping the Membrane Proteins of Newcastle-Disease Virus with a Photoreactive Glycolipid Probe

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The envelope proteins of Newcastle-disease virus were preferentially labelled when a suspension of virus particles that contained the photoreactive probe 12-(4-azido-2-nitrophenoxy)stearoyl[1-¹⁴C]glucosamine was irradiated. One of the proteins labelled was not readily accessible to surface labelling with ¹²⁵I.

Fatty acid analogues containing photolabile azido groups have now been shown to satisfy the growth requirement of a fatty acid desaturase mutant of *Escherichia coli* (Greenberg *et al.*, 1976), and to effect covalent attachments to phospholipids (Greenberg *et al.*, 1976) and to the serum high-density lipoproteins (Stoffel *et al.*, 1977). In the present paper we describe a photoreactive glycolipid that is capable of labelling membrane proteins from within the membrane bilayer. The glycolipid probe is a [1-¹⁴C]glucosamine derivative of 12-(4-azido-2-nitrophenoxy)stearic acid. Spin-label studies with a nitroxide analogue suggest that it remains restricted to the outer monolayer of intact membrane systems (Wisnieski & Iwata, 1977). Other studies show that it does not react very efficiently with water-soluble proteins (Shiflett *et al.*, 1978).

The membrane system analysed was a suspension of Newcastle-disease virus, a membrane-enveloped paramyxovirus. This virus is relatively easy to isolate in pure form, reasonably well-defined with respect to component location (Miyakawa *et al.*, 1976), and is enveloped with host-derived lipids in a bilayer containing three virus-specified membrane proteins (Lenard, 1978). The viral protein designated VP1 exists primarily as a disulphide-linked dimer of a glycopeptide of about 78 000 mol.wt. Protein VP2 is also a glycoprotein (mol.wt. ~63 000), and both proteins VP1 and VP2 are accessible to surface-labelling reagents (Moore *et al.*, 1975). The predominant viral protein is VP3 (mol.wt. ~55 000), which is associated with the nucleocapsid RNA. Protein VP4 (mol.wt. ~48 000) is a minor protein component of the nucleocapsid. Protein VP5 (mol.wt. ~45 000), is an actin protein that may assume a functional role during viral assembly or may be present merely as a contaminant (Wang *et al.*, 1975). The viral protein VP6 (mol.wt. ~38 000), has been assigned to the inner side of the viral membrane envelope on the basis of surface-labelling results obtained by using ¹²⁵I-

(Miyakawa *et al.*, 1976), but it has been labelled with ³H by using pyridoxal phosphate and boro[³H]-hydride (Moore *et al.*, 1975), another surface-labelling procedure (Rifkin *et al.*, 1972). Results with various solubilizing agents are also more clear-cut with the other proteins than they are with protein VP6. For example, extraction of protein VP6 with the chaotropic agent lithium di-iodosalicylate is temperature-dependent, being relatively easier below 22°C than above, suggesting that the protein is internally disposed, but 'frozen out' of the membrane at low temperature (Miyakawa *et al.*, 1976).

The present results represent a preliminary attempt to define the location of membrane proteins, such as those discussed above, by using monolayer-restricted photoreactive probes.

Materials and Methods

Newcastle-disease virus

Newcastle-disease virus strain HP16 was propagated in eggs and isolated as previously described (Wisnieski & Iwata, 1977). A vertical superspeed rotor (Sorvall SS-90) was used to facilitate rapid isopycnic banding of the virus particles. The sucrose and renograffin gradients were spun at 20 000g for 1 h. 'Intact' virus was freshly harvested; 'stored' virus had been stored for a year at -75°C; 'freeze-thawed' virus had been freeze-thawed four times from -75°C over the course of 1 year.

Photoreactive probe

The synthesis, purification and characterization of 12-(4-azido-2-nitrophenoxy)stearoyl[1-¹⁴C]glucosamine was performed as previously described by using ether dried with sodium benzophenone and redistilled ether where appropriate (Bramhall *et al.*, 1978; Iwata *et al.*, 1978). The method involved the coupling of [1-¹⁴C]glucosamine to the *N*-hydroxy-

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succinimide ester of 12-(4-azido-2-nitrophenoxy)-stearic acid (Lapidot *et al.*, 1967). An alternate method of attaching [^{14}C]glucosamine to the aryl-azidostearic acid molecule involved the use of the coupling reagent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline ('EEDQ', Pfaltz and Bauer, Flushing, N.Y. 11368, U.S.A.). [^{14}C]glucosamine (1 μmol ; 50 mCi/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) was placed in a 0.6 ml Reactivial, and after evaporation of the solvent with a stream of N_2 , 25 μl of water was added to the vial. Red safety lights were used for the subsequent manipulations. 12-(4-Azido-2-nitrophenoxy)stearic acid (1 μmol in 50 μl of dimethoxyethane) was added to a second vial that contained 5 μmol of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. The latter compound was dissolved by rotating the vial, and the contents were then transferred to the first vial. A stir bar was added and the reaction mixture was stirred magnetically overnight at room temperature. The contents of the vial were then subjected to a modified Bligh & Dyer (1959) extraction to remove unchanged glucosamine. This involved evaporating the sample solvents under a stream of N_2 and transferring the contents to a larger tube with a total of 1.5 ml of methanol/chloroform (2:1, v/v). To this was added 0.5 ml of 0.05 M-HCl in water. The tube was vortex-mixed for 2 min and then 0.5 ml of chloroform was added, followed by vortex-mixing for 30 s; 0.5 ml of water was added, followed by vortex-mixing for another 30 s. The tube was centrifuged at 900 g for 15 min to separate the phases. The bottom organic phase was removed, and 1 ml of chloroform added to the remaining polar phase. The tube contents were vortex-mixed, centrifuged again, and the bottom organic phase was removed and pooled with the first organic fraction. After evaporation of the solvent, 400–500 μl of chloroform/methanol (2:1) were added and the sample applied to a silica-gel G chromatography plate (20 cm $\times$ 20 cm, 500 μm thick; Analtech, Newark, DE, U.S.A.). The plate was developed in benzene/ethyl acetate/light petroleum/acetic acid (100:14:10:1, by vol.). A 1 cm band around the origin was scraped from the plate and added to 5 ml of chloroform/methanol (2:1). This was filtered through sodium sulphate/glass wool followed by a wash with 5 ml of Spectro-grade acetone (Eastman, Rochester 3, NY, U.S.A.). The solvents were removed by vacuum distillation at 30–40°C. The product was stored at 5°C either dry or in 100% ethanol.

Photoactivation protocol

These experiments were carried out entirely under red safety lights until the completion of sample electrophoresis. Approx. 400 000 c.p.m. of the photo-reactive probe in ethanol was added to a tube and the solvent evaporated under N_2 . Newcastle-disease

virus (100 μg as protein) was added in about 5 ml of PBSE medium [0.9% NaCl/5 mM-sodium phosphate, (pH 7.4)/1 mM-EDTA]. Alternatively, the probe in ethanol was added directly to a tube containing Newcastle-disease virus in buffer, but the concentration of ethanol never exceeded 0.25%. The sample tube was rotated for 30 min at 37°C and the Newcastle-disease virus particles pelleted at 149 000 g for 30 min in a Beckman SW50.1 rotor. The virus pellet was suspended in 2.4 ml of PBSE medium and divided into four equal portions. Two were placed in 2 mm-path-length quartz cuvettes and irradiated in a cold-room for 6 min at 360 nm with a Mineral lamp (Ultra-Violet Products Inc., San Gabriel, CA, U.S.A.). The other two portions served as dark controls. This sequence was repeated with intact, stored and freeze-thawed virus preparations. The experiments were carried out with two preparations of probe, synthesized and purified by different methods as indicated above. All samples were pelleted in a Beckman SW50.1 rotor equipped with 0.7 ml adaptors (40 000 rev./min for 30 min). Pellets were solubilized with sodium dodecyl sulphate at 37°C for 1 h. Electrophoresis of samples was carried out in 5–20% (w/v) polyacrylamide linear gradient gels with a 2.5% acrylamide stacking gel. After drying Coomassie Blue-stained gels, each sample lane was cut into 3 mm segments, which were oxidized on a Packard Tri-Carb Oxidizer. The $^{14}\text{CO}_2$ evolved was collected and counted for ^{14}C radioactivity in a Packard liquid-scintillation counter.

Iodination procedure

The method of radiolabelling Newcastle-disease virus with ^{125}I was that of Marchalonis (1969). Approx. 20 μCi of Na^{125}I (8.5 Ci/mg; New England Nuclear) in 7–10 μl of PBSE medium was added to 100–200 μg of virus as protein in 10–20 μl of PBSE medium. The samples were incubated in an ice bath, and 1 μg of lactoperoxidase (1 mg/ml) was added, followed by 1 μl of 0.03% H_2O_2 . After 15 min, 5 μl of unlabelled NaI (50 mM) was added, then 0.6 ml of PBSE medium; the samples were pelleted as described above. This procedure was carried out with intact virus, freeze-thawed virus and virus in 0.1% sodium dodecyl sulphate. The latter sample was prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis without washing. Electrophoresis was carried out as described above. Stained protein bands were excised from the gel and assayed for radioactivity on a gamma counter.

Results and Discussion

Fig. 1 illustrates the protein profile of Newcastle-disease virus on a 5–20% sodium dodecyl sulphate/

Fig. 1. Separation of Newcastle-disease-virus proteins on a 5–20% (w/v) polyacrylamide gel. The sample was not reduced.

polyacrylamide gel under non-reducing conditions. Viral proteins designated VP1 and VP2 are glycoproteins accessible to labelling with ^{125}I and lactoperoxidase/ H_2O_2 . Proteins VP3 and VP4 are associated with the nucleocapsid RNA, and protein VP5 is similar to, or identical with, host-cell actin (Wang *et al.*, 1975). Protein VP6 is a membrane protein, purportedly localized on the inner side of the viral envelope (Miyakawa *et al.*, 1976), but accessible to radiolabelling based on pyridoxal phosphate and boro ^3H hydride (in strain Texas; Moore *et al.*, 1975). The latter method has been shown to be specific for surface-membrane components (Rifkin *et al.*, 1972); amino groups are ^3H -labelled.

Viral suspensions were incubated with the photo-reactive probe 12-(4-azido-2-nitrophenoxy)stearoyl-[1- ^{14}C]glucosamine, washed, and treated as described in the Materials and Methods section; a representative histogram showing the amount of radioactivity associated with the various gel segments of an sodium dodecyl sulphate-solubilized and electrophoresed sample is shown in Fig. 2. By comparing the histograms of irradiated and non-irradiated samples, it is obvious that there is little background radioactivity in non-irradiated virus sample protein. The peak of radioactivity at the end of the gel is due primarily to free probe migrating behind the dye front. A summary of the percentage of radioactivity associated with each virus-protein band in irradiated samples is provided in Table 1. The intact virus preparation contained a larger percentage of the radioactivity in the membrane protein VP2 than in the nucleocapsid protein VP3, which is the major viral protein. When a stored virus preparation was similarly analysed, the ratio of radioactivity in protein VP2 versus VP3 protein decreased from approx. 4:1 to 1:1. When a freeze-thawed virus preparation was analysed, the ratio of radioactivity in the glycoprotein VP2 to those in the nucleocapsid protein dropped even further, to 1:2.

In general, the ratio of radioactivity associated with membrane proteins to that associated with nucleocapsid protein VP3 correlated with the integrity of the virus as monitored by surface-labelling procedures. Interestingly the only significant decrease membrane-protein-associated radioactivity that correlated with viral disruption occurred in protein-VP2

Fig. 2. ^{14}C -labelled photoreactive probe associated with Newcastle-disease-virus proteins (a) Irradiated; (b) non-irradiated. Abbreviation used: VP, viral protein.

labelling (39–15%); and the only significant increase in protein labelling that correlated with viral disruption occurred in protein-VP3 labelling. Labelling of the other protein components did not seem to be affected to any obvious extent by freeze-thawing or by prolonged storage. Solubilized virus suspensions were not examined because our previous experience with soluble proteins indicated a 100-fold decrease in efficiency in labelling soluble (as against membrane-associated) proteins with the photoreactive probe. If this holds true for a wide variety of soluble-versus-membrane-bound systems, it would tend to suggest that the labelling of viral proteins VP3, VP4, and VP5 is not due to a constant fraction of disrupted virus in

Table 1. Radiolabelling of Newcastle-disease-virus proteins effected by irradiation of ^{14}C -labelled-probe-containing samples or by lactoperoxidase/ H_2O_2 -mediated iodination with Na^{125}I

Viral protein	Radioactivity (%)					
	^{14}C			^{125}I		Sodium dodecyl sulphate-treated
	Intact	Stored	Freeze-thawed	Intact	Freeze-thawed	
VP1*	15	20	19	64	30	19
VP2†	39	21	15	20	8	20
VP3‡	12	22	31	6	22	26
VP4	8	8	8	1	10	6
VP5	6	6	3	3	7	6
VP6§	20	23	25	6	23	23
Total c.p.m. (VP1-VP6)	301	427	322	24821	18420	36757

* Haemagglutination and neuraminidase activity.

† Fusion activity.

‡ Nucleocapsid protein.

§ Membrane-matrix protein.

all of our preparations to date, nor to penetration or diffusion of the probe to the internal nucleocapsid region of the virion; rather, the labelling of these components to the extent of roughly 25% may reflect a greater extent of membrane associations between viral nucleocapsid components and the membrane-envelope proteins and/or lipids. This type of association is suggested by the results of Miyakawa *et al.* (1976), who used reversible cross-linking reagents to analyse protein-protein associations in Newcastle-disease virus. In their studies, the protein-VP1 dimer (observed under non-reducing conditions) could be cross-linked to the nucleocapsid protein VP3 under the conditions used. Thus those authors hypothesized a steady-state interaction between these two proteins, one in the membrane, presumably a transmembrane protein, and the other associated with the nucleocapsid RNA. Such an association could be important during the viral budding process.

When we estimated the efficiency with which viral proteins were labelled as compared with envelope lipids, we found that although the photoreactive probe is more effective at labelling membrane lipids (~1% of radioactivity added), a substantial success was achieved in terms of protein labelling (~0.3%) and the assignment of these low radioactivities to specific proteins of the animal virus. This amount of radioactivity can be detected by fluorographic analysis of the gels. These studies should be pursued with other animal- and bacterial-virus systems. Probes with photoreactive groups at various positions along the fatty acid backbone could be utilized to map the position of integral membrane proteins and to monitor the movement of proteins across the membrane bilayer. Preliminary results with a cholera-toxin-membrane system and the photoreactive probe

described in the present paper are most encouraging (Shiflett *et al.*, 1978).

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