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VIROSOMES RECONSTITUTED FROM HUMAN IMMUNODEFICIENCY VIRUS PROTEINS AND LIPIDS

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Purified Human Immunodeficiency Virus (HIV) was solubilized in octylglucopyranoside. After centrifugation, the supernatant was added to lipid-detergent mixed micelles. Formation of virosomes occurred during overnight dialysis. Centrifugation on a continuous glycerol gradient showed that envelope glycoproteins (gp120 and gp41) and matrix protein p17 but not core protein p25 were associated to virosomes. Proteolytic treatment of virosomes indicates that gp120 is oriented toward the outside as in the virus particles, whereas p17 protein is anchored on both sides of the liposomal membrane. Virosomes are spherical vesicles with approximately the size of the virus as shown by electron microscopy. © 1990 Academic Press, Inc.

The human immunodeficiency virus (HIV) has been proved to be the etiological agent of the acquired immunodeficiency syndrome (AIDS) (1). The genome of HIV, an enveloped retrovirus, codes for four major structural proteins. The external glycoprotein gp120 which interacts non covalently with transmembrane glycoprotein gp41 (2, 3). p17 protein is a matrix protein interacting with the lipid bilayer by its N-terminal myristoyl chain while the p25 protein participates to the nucleocapsid building (4).

Although a great deal of effort has been made to understand the genetic (5) and immunological (6) aspects of infection by HIV, little is known about its molecular mechanism of entry into the host cell.

Reconstitution of viral membrane into virosomes could shed light on the role of viral proteins in binding and fusion

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with the host cell. This reconstitution methodology was successfully applied to several enveloped viruses such as influenza (7), sendai (8) and Friend murine leukemia virus (9).

In this paper, we describe a procedure of reconstitution of the HIV viral membrane from HIV viral lysate using a detergent dialysis technique.

METHODS

Purification and solubilization of virus. The HTLV-III_b strain of HIV (10) was produced in Molt 3 cells. After removing the cells, the supernatant containing the viral particles has been concentrated in a hollow fiber concentrator (Amicon, H1 X 50 cartridge) and centrifuged at 45000g for 90 min. The pelleted virus was resuspended in 2 ml of TNE (Tris-HCl 10 mM pH8, NaCl 100 mM, EDTA 1 mM) buffer. Finally the resuspended virus was layered on a 4% to 36% (w/v) linear metrizamide gradient in TNE and centrifuged to equilibrium at 21000g for 16 h. The virus band, as established by the position of the reverse transcriptase activity in the gradient, was found at a density of 1.12 g/ml. The purified virus, solubilized in 25 or 68 mM β-D-octylglucopyranoside (OGP), was centrifuged at 160000g for 1 h at 4°C and the supernatant was analysed by Western-Blot.

Origin of lipids. The lipid mixture (Sigma) used had the composition described for the HIV membrane by Aloia and co-workers (11) (17.1% phosphatidylcholine, 19.3% phosphatidyl-ethanolamine, 20% sphingomyeline, 11.4% phosphatidylserine, 1.6% phosphatidylinositol, 0.4% phosphatidic acid and 30% cholesterol, expressed as weight percent). For virosomal preparations, a small amount of tritiated cholesterol (0.4 μCi, 4.8 ng/ml; Amersham) was added to lipids and the mixture was dried under nitrogen and placed under vacuum overnight (1 mm Hg). Lipids were resuspended in phosphate-buffered saline (PBS) containing 4% OGP.

Formation of virosomes. The purified virus was lysed in PBS containing 2% OGP (68 mM) (incubation during 20 min at 4°C) and centrifuged at 160000g for 50 min at 4°C. Solubilized proteins were then added to lipids in PBS - 4% OGP and the solution was mixed during 30 min at 4°C (protein/lipid = 1.3 in weight). Virosome formation occured during overnight dialysis against PBS. Liposomes (control experiment) were formed by the same procedure but without addition of viral proteins.

Isolation of virosomes. After dialysis, virosomes were loaded on a continuous (10% → 6% glycerol (Merck)) or discontinuous (2 ml of 36% metrizamide (Nyegaard) + 0.6 ml of 10% glycerol) density gradient and then centrifuged at 120000g for 16 h at 4°C. Lipids were detected by radioactivity counting and protein by ELISA in presence of 1% Triton X100. A small metrizamide cushion, under the continuous glycerol gradient, prevented the pelleting of virosomes.

Enzyme-linked immunosorbent assay (Sandwich ELISA). The first antibody* was adsorbed overnight at 4°C in 96 wells immunoplate (Nunc). After saturation of the plastic with PBS containing 4% newborn calf serum and 1% bovine serum albumin

(saturation buffer), the sample was incubated in the coated wells for 2 h at 20°C. The second antibody** was then added for 90 min at 37°C. After several washes with 0.1% Tween 20 in PBS, 50 µl of biotinylated goat anti-mouse antibodies (Amersham, RPN 1021) 500-fold diluted in saturation buffer was added (if the second antibody is not biotinylated**) for 90 min at 37°C. Detection was performed with 50 µl of a streptavidin-peroxydase complex (Amersham, RPN 1051) diluted 1:1000 in saturation buffer (incubation for 30 min at 37°C, in the dark) and the chromogenic substrate (ortho-phenylenediamine dihydrochloride, Sigma) at 0.4 mg/ml in 0.1 M citrate buffer pH 4.5 in presence of 0.03% H_2O_2. The optical density was measured within 1 h at 492 nm (Titertek Multiscan).

* The first antibody was a polyclonal sheep anti-gp120 (Biochrome) for the detection of gp120, a polyclonal sheep anti-gp41 (Biochrome) for the detection of the gp41 and a mouse monoclonal anti-p25 (1H12-A1) or anti-p17 (8A6-A7) purified from ascitic fluid for the detection of the p25 and the p17 (12).

** The second antibodies for the detection of respectively the gp120, gp41, p25 and p17 (12) were: a mouse monoclonal anti-gp120 (Dupont), a mouse monoclonal anti-gp41 (Epitope), a pool of biotinylated purified human immunoglobulins from seropositive patients and a biotinylated mouse monoclonal anti-p17 (6D6-F6).

Electron microscopy. Isolated virosomes or HIV particles were adsorbed to carbon-coated formvar grids hydrophilized with pepton broth. Specimens were negatively stained with 2% uranyl acetate.

Proteolysis treatment. Virosomes isolated on a discontinuous gradient were digested at 37°C with 0.25 mg/ml trypsin (Sigma) in PBS. The reaction was stopped (at different times) by adding 5 mg/ml of soybean trypsin inhibitor (SBTI, Sigma) in PBS and the resistant proteins were detected by ELISA after lysis of the virosomes in 1% Triton X100. The possible resistance of protein to trypsin treatment was analysed by the same procedure except that 1% of Triton X100 was present during proteolysis in order to allow access to protein domains which are normally protected from proteolysis by the lipid membrane barrier.

RESULTS

Solubilization of the viral proteins. A purified virus preparation has been lysed in 25 mM OGP, in the presence or absence of 0.5 M NaCl, and centrifuged for 1 h at 160000g in order to eliminate any particulate material. The supernatant has been analysed by gel electrophoresis and Western-Blot. Although the lowest concentration in OGP, required for the complete disruption of a lipid membrane (13) has been used here, the internal viral proteins p25 and p17 as well as the envelope glycoproteins have been solubilized by this treatment (Fig.1). As a selective solubilization of gp120 and gp41 could not be obtained with 25 mM OGP, further

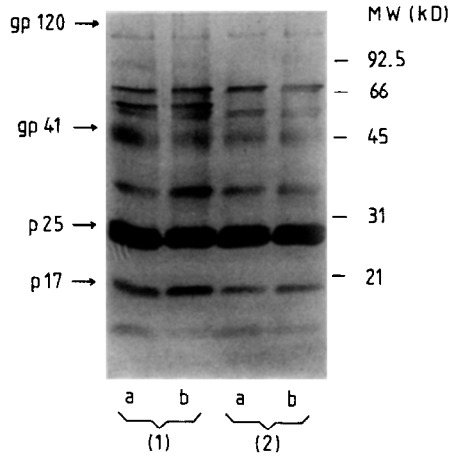

Fig.1. Western-blot analysis (detected by a pool of human antibodies from seropositive patients) of **(a)** viral lysate (25 mM OGP) and **(b)** the supernatant of centrifuged viral lysate (160000g, 1h, 4°C). **(1)** = PBS medium; **(2)** = PBS/0.5M NaCl medium.

experiments were performed in 2% (68 mM) OGP to achieve maximum solubilization.

Virosomes. The detergent-soluble virus proteins were added to lipid-detergent mixed micelles containing tritiated cholesterol as lipid tracer. The virosomes formed during overnight dialysis were examinated by centrifugation on a continuous glycerol gradient. The fractionation of this gradient has shown that the liposomes, containing lipids only, were mainly concentrated at the top of the gradient (Fig.2b). The association of viral proteins to these structures gave rise to virosomes of various densities, most of them accumulating on the top of the 36% metrizamide layer, as shown by the distribution of the tritiated cholesterol (dotted curve, Fig.2a). The analysis of the viral protein content in the gradient fractions by specific ELISA's has indicated that the viral envelope proteins gp120 and gp41, and the p17 matrix protein were associated with the lipids. On the other hand, the nucleocapsid p25 protein was found in the upper part of the gradient, being apparently not associated with lipids in virosomes. Part of the p17 protein has been found in the first fraction of the gradient which might be due to a partial aggregation. Centrifugation of virosomes on a discontinuous density gradient (36% metrizamide and 10% glycerol) has clearly

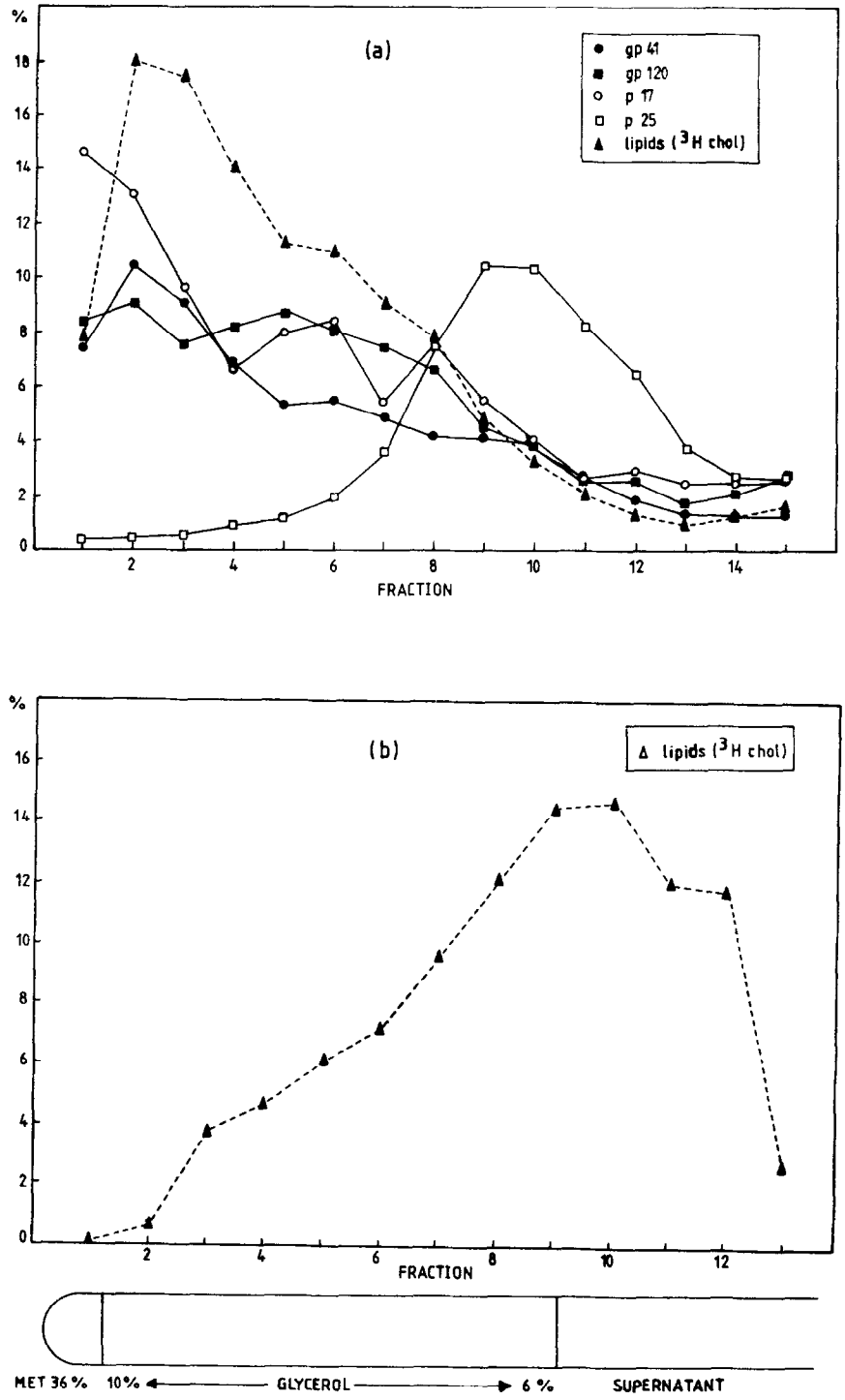

Fig.2. Centrifugation (120000g for 16 h at 4°C) of **(a)** virosomes formed from HIV lysate or **(b)** liposomes (control experiment) on a linear glycerol gradient (10% → 6% in PBS). Viral proteins were detected by ELISA and lipids by radioactivity counting (^{3}H cholesterol). Units on the ordinate refer to the percentage of recovered material as compared to the sample input.

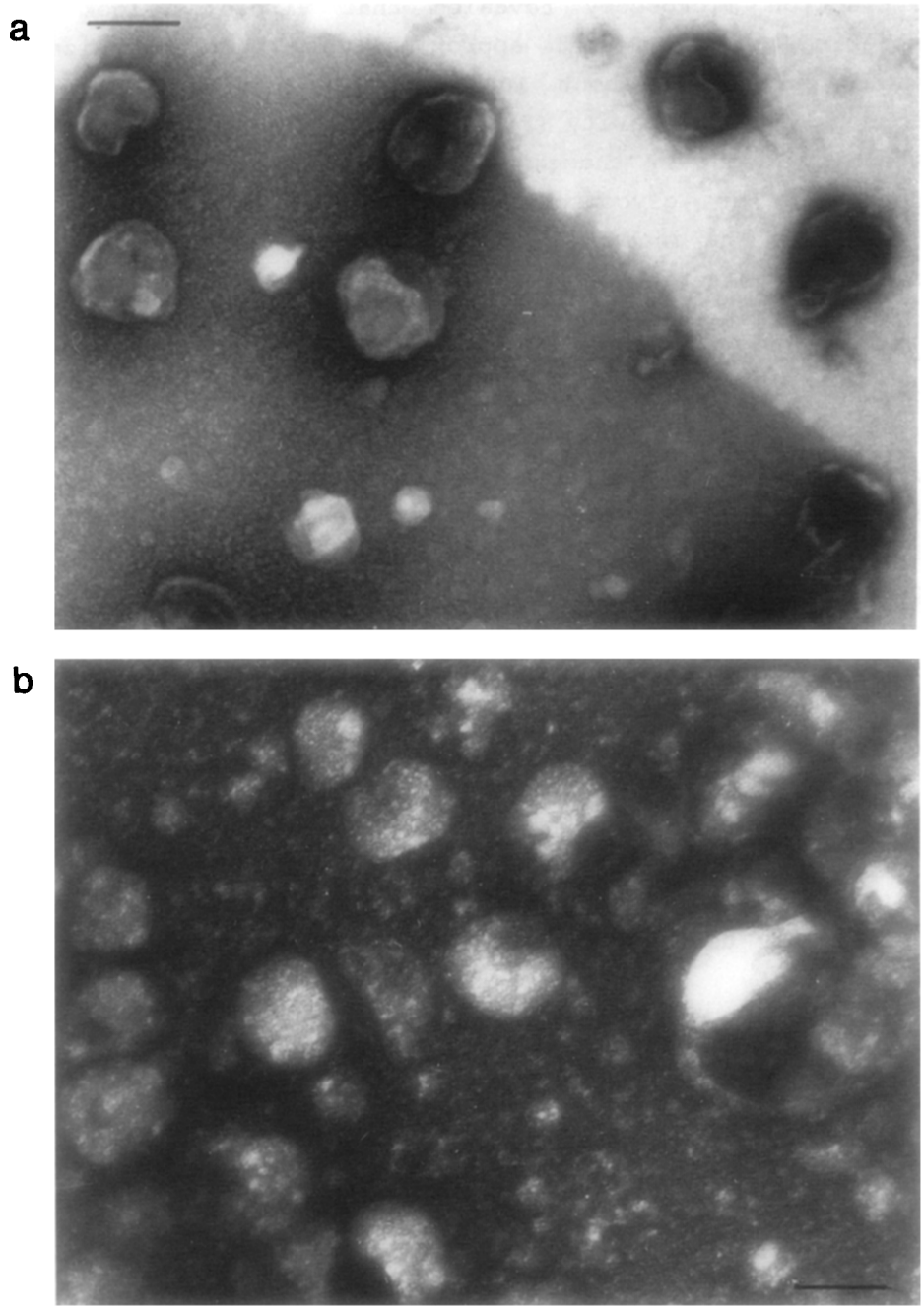

Fig.3. Electron microscopy analysis (negative staining) of **(a)** HIV and **(b)** virosomes constructed from HIV proteins and exogenous lipids. Bar represents 100 nm.

indicated that p17 was preferentially associated with lipids and does not migrate to the bottom of the gradient as it was observed for pure protein aggregates (data not shown).

Electron microscopy revealed that virosomes look like spherical vesicles with approximatively the same size as viral particles (about 100 nm, (2)) but with an empty aqueous internal cavity (Fig.3). The uranyl acetate negative staining used in these experiments did not permit easy detection of glycoprotein spikes at the surface of virosomes. The internal cavity is empty due to the absence of genetic material and nucleocapsid.

To determine the topology of viral proteins in the lipid vesicles, virosomes were submitted to an enzymatic proteolysis. Trypsin, which does not cross the lipid bilayer (14), was added to virosomes to cleave external domains of viral proteins. The reaction was stopped at different times and the resistant proteins were detected by ELISA (Fig.4). ELISA signal of gp120 disappears completely during proteolysis. On the other hand, only 50% of ELISA signal of p17 disappear even after prolonged exposure to trypsin. Control experiments performed in the same conditions show that, when vesicles are disrupted with a detergent (Triton X100), p17 is fully accessible to proteolysis (data not shown).

DISCUSSION

Non ionic detergents such as octylglucoside have been extensively used for membrane protein solubilization (15).

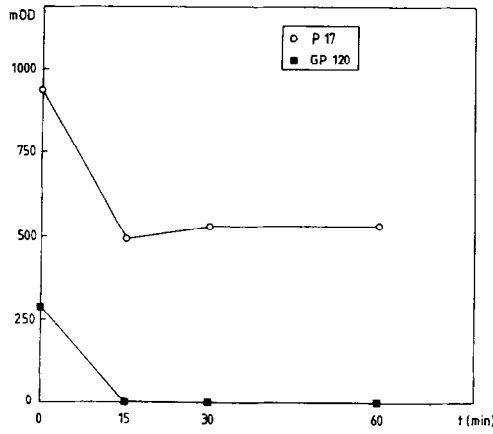

Fig.4. Trypsinolysis of viral proteins gp120 and p17 associated with virosomes. Virosomes were centrifuged on a discontinuous density gradient (2 ml of 36% metrizamide overlayered with 1 ml of 10% glycerol, in PBS) at 120000g for 16 h at 4°C. Proteins were detected by ELISA as described in Methods. Optical density was measured at 492nm.

Most enveloped viruses, especially paramyxoviruses, retained their nucleocapsid intact while loosing their envelope glycoproteins after the detergent treatment (16). Here, octylglucopyranoside solubilizes not only the envelope glycoproteins of HIV but also the p24 and p17 core proteins. Even near the critical micellar concentration (21 - 25 mM) which is the minimum concentration for complete disruption of lipid membranes (13), octylglucopyranoside is able to disintegrate the nucleocapsid, suggesting that the interactions between these capsid proteins, responsible for the core organization, are weak. In fact, the viral protein p17, which is probably anchored at the inner face of the viral lipid envelope through its N-terminal myristoyl chain (17), should play a major role in the interaction between the viral membrane and the nucleocapsid (p25). Once the viral membrane is lysed, the interactions between core proteins are not strong enough to keep the nucleocapsid structure intact.

The viral envelope was reconstituted by a detergent dialysis technique (15) and the formation of virosomes was analyzed by centrifugation on a continuous density gradient. We observe that three different viral proteins are able to bind to lipids and form virosomes: gp41 because of its transmembrane character (18); gp120 probably because its association to gp41 by non-covalent interactions (which explains the partial loss of gp120 during centrifugation of virosomes in density gradient) and p17 which could anchor in the virosomal membrane with its N-terminal myristoyl chain (17). On the other hand, the nucleocapsid protein p25 shows no tendency to associate to virosomes confirming its hydrophilic character (19).

Morphology of isolated virosomes was characterized by electron microscopy using uranyl acetate negative staining. We observed that virosomes appears as spherical vesicles with approximatively the same size as viral particles but with an empty internal cavity which could be due to absence of genetic material and nucleocapsid.

The topology of the viral proteins in the virosomal membranes was studied by proteolytic treatment. Complete disappearance of ELISA signal of gp120 after trypsinolysis indicates that this protein is fully accessible to the

enzyme and is thus mainly present at the outer face of the virosome membrane since trypsin is unable to cross the membrane (14). Asymmetric reconstitution is frequently observed with viral envelope glycoprotein, possibly because of their hydrophilic domains, their size being incompatible with the steric constraints imposed by the strong curvature of the vesicles (100 nm diameter). Another possible explanation would be that the glycoproteins would remain soluble for a long time before complete removal of detergent and then anchor on already formed lipid vesicles (20, 21). Unlike large glycoproteins, p17 protein can be inserted in lipid membrane of virosomes on the internal as well as on the external face. Indeed, only 50% of ELISA signal of p17 disappear during proteolysis suggesting that a part of p17 protein is not cleaved by trypsin being oriented to the internal face of vesicles. Orientation of the transmembrane glycoprotein gp41 was not investigated in this study; we might predict that it is mainly oriented with its NH_2-end facing the exterior since the N-terminal moiety is required for gp120 binding (3, 22).

Virosomes could be used to present viral proteins to the immune system in their natural environment (especially for the transmembrane protein gp41). This would be especially useful when using genetically engineered envelope glycoprotein for immunization. The enhancement of immunogenicity by use of liposomes has been already demonstrated (23, 24, 25).

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