Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 95-101

APOLIPOPROTEIN A-1 INTERACTS WITH THE N-TERMINAL FUSOGENIC DOMAINS OF SIV (SIMIAN IMMUNODEFICIENCY VIRUS) GP32 AND HIV (HUMAN IMMUNODEFICIENCY VIRUS) GP41: IMPLICATIONS IN VIRAL ENTRY

I. MARTIN, M.-C. DUBOIS, T. SAERMARK, * AND J.-M. RUYSSCHAERT

Laboratoire de Chimie-Physique des Macromolécules aux Interfaces CP206/2, Université Libre de Bruxelles, 1050 Brussels, Belgium

*EEC concerted action/Medical Physiology B, Panum Institute, Blegdamsvej 3C, DK-2200 Copenhagen, Denmark

Received May 14, 1992

Previous studies showed that apoA1, the major protein component of HDL (High Density Lipoprotein), inhibited HIV infectivity and virus-induced syncytia formation. The mechanism of inhibition is unknown. We bring here evidence that the amphipathic helices of apoA1 interact with the N-terminal peptides of SIV gp32 and HIV gp41. These peptides have been shown to be associated with the initial steps of the fusion between the host cell and the virus. Binding of apoA1 to these peptides prevents the insertion of the fusogenic domains into the cell membrane and inhibits the fusion and the entry of the virus into the host cell.

Owens et al. (1) indicated that apoA1, the major protein component of high density lipoproteins (HDL) inhibits cell fusion in HIV-1 infected T cells and in recombinant vaccinia virus-infected $CD4^+$ HeLa cells expressing HIV envelope protein on their surface. This inhibition was also observed with the herpes simplex virus (2) and seems to be specific of enveloped virus since no inhibition of poliovirus infection was observed (1).

The exact mechanism of this inhibitory effect is not known. Several possibilities could be envisaged. The amphipathic helices associated to apoA1 may disorganize the lipid bilayer constituting the viral envelope or the cell membrane and modify its fusogenic properties. ApoA1 may interact with putative amphipathic helices involved in the gp120-gp41 interaction and disrupt the gp120-gp41 association leading to the loss of gp120 in the extracellular medium. Another possibility is an association between the amphipathic helices of apoA1 and the fusogenic domain of gp41, preventing its interaction with the target membrane (1).

We bring here evidence that apoA1 does not bind to lipid membrane but interacts specifically with the N-terminal domains of SIV gp 32 and HIV gp 41.

0006-291X/92 $4.00

MATERIAL AND METHODS

Materials

Egg phosphatidylethanolamine (PE), egg phosphatidylcholine (PC), cholesterol (Chol), sphingomyelin (SM) were purchased from SIGMA Chemical Company (Saint Louis, USA).
N-(Nitrobenzo-2-oxa 1,3-diazol) phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids Inc. (Birmingham, Ala, USA).
HIV16aa (ALA-VAL-GLY-ILE-GLY-ALA-LEU-PHE-LEU-GLY-PHE-LEU-GLY-ALA-ALA-GLY) is a gift from Dr P. Horal (University of Göteborg, Sweden) and SIV12aa (GLY-VAL-PHE-VAL-LEU-GLY-PHE-LEU-GLY-PHE-LEU-ALA) is a gift from Dr T. Saermark (University of Copenhaguen, Danemark).
Apolipoprotein A-1 is an U.C.B. (Bruxelles, Belgium) product.

Methods

HIV16aa and SIV12aa were dissolved in DMSO at a final concentration of 1 mg/ml. ApoA1 was dissolved in a Tris/HCl buffer pH 7.4 at a final concentration of 2mg/ml.

Vesicles preparation: Large unilamellar vesicles (LUV) were prepared according to the extrusion procedure (3) using an Extruder (Lipex Biomembranes Inc., Vancouver, Canada). The lipid composition used corresponds to the major neutral lipids of a typical plasma cell membrane. Briefly, freezed and thawed MLV (Multilamellar Vesicles) obtained by vortexing a lipid film in a buffer (10 mM TRIS, 150 mM NaCl, 0.1mM EDTA, 0.02% NaN_3, pH 7.4) were extruded 10 times through two stacked polycarbonate membranes with a pore size of 0.1μm (Nuclepore Corp., Pleasanton, CA, USA.).

Lipid mixing assay: Lipid mixing was determined by measuring the fluorescence energy transfer between the probes NBD-PE and Rh-PE, as described (4). Fluorescence was monitored using a SLM 8000 spectrofluorimeter with excitation and emission slits of 4nm. Both probes were added to the lipid film and LUV were prepared as described above.
Liposomes containing both probes at 0.6% (molar ratio) each, were mixed in a 1/9 mole ratio with probe free liposomes at a final lipid concentration of $3\ 10^{-4}$M. The initial fluorescence of the 1/9 (labeled/unlabeled) suspension was taken as 0% fluorescence and the 100% fluorescence was determined using an equivalent concentration of vesicles prepared with 0.06% of each fluorescent phospholipid. The suspensions were excited at 470nm and the NBD fluorescence was recorded at 530nm.

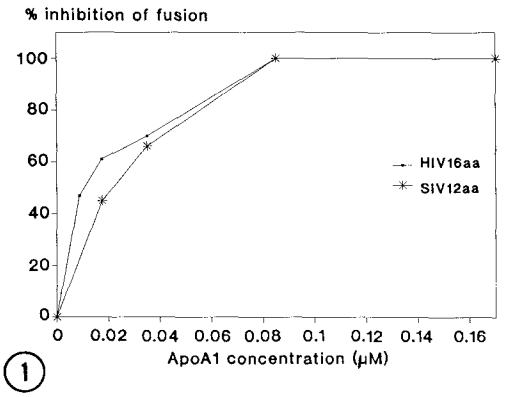
ApoA1-LUV interaction: ApoA1 and LUV were incubated at molar lipid/peptide ratio of 200 ($1.5\ 10^{-6}$ M lipid and $7\ 10^{-9}$ M protein in a total volume of 200 μl). After 3 hours incubation at 37°C, the lipid-protein complex was separated from the free protein and the free liposome on a (30%-2%) sucrose gradient (centrifugation at 120000g, 4°C for 16 hours in a SW60 Beckman rotor). The gradient was fractionated and the fractions were tested for the presence of PC (using the enzymatic colorimetric test of Boehringer Mannheim) and for the presence of apoA1 detected by Trp fluorescence.

SDS page electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% gel in a Biorad mini-protean II apparatus by the method of Laemmli (5). Proteins were visualized by staining with silver using the periodic acid procedure.

RESULTS

Inhibition of peptide-induced liposomes fusion by apoA1

A possible way to elucidate the molecular mechanism of membrane fusion is to synthesize peptides corresponding to the amino terminal domain of viral protein and to



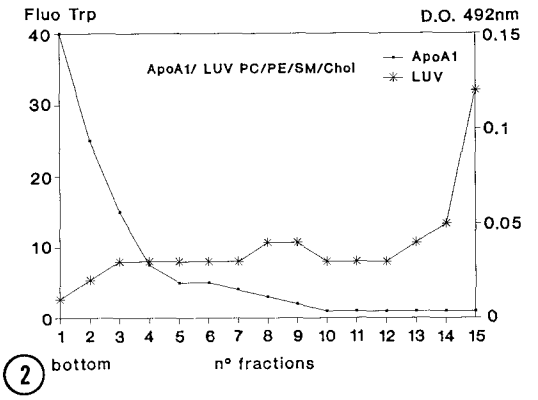


Figure 1: ApoA1 inhibition of the fusion of LUV PC/PE/SM/Chol (1,1,1,1.5 moles) induced by SIV12aa and HIV16aa fusion peptide. The percentage of inhibition is calculated 10 minutes after addition of apoA1 and fusion peptide. The total lipid concentration is 3×10^{-4}M, and the fusion peptide concentration is 1.3×10^{-5}M. Fusion is measured as described (4) at 37°C and pH 7.4.

Figure 2: Dosage of lipids (by an enzymatic colorimetric test specific to choline) and of apoA1 (by Trp fluorescence) in ApoA1/LUV PC/PE/SM/Chol (1,1,1,1.5 moles) samples after centrifugation on a continuous sucrose (2%-30%) density gradient (centrifugation at 120,000g for 16h at 4°C).

study their capacity to induce the fusion of large unilamellar vesicles (LUV). The curvature and stability of these vesicles better mimic the cell membrane as compared to small unilamellar vesicles (SUV) whose small radius of curvature perturbs the lipid organization and facilitates the peptide penetration. Although such fusion events observed with SUV do reveal peptide induced bilayer destabilization, they do not necessarily reflect the intrinsic fusogenicity of the peptide (6).

In the absence of apoA1, liposome fusion (LUV of PC/PE/SM/Chol (1,1,1,1.5 moles)) was observed after addition of a 12 residues synthetic peptide (SIV12aa) corresponding to the fusogenic sequence of SIV gp32 (7), (8) and of a 16 residues synthetic peptide (HIV16aa) corresponding to the fusogenic sequence of HIV gp41 (9). On the contrary, peptide-induced fusion of the lipid vesicles was completely inhibited in the presence of 0.1 μM apoA1 (fig.1). The inhibition percentage was identical whether liposomes were treated with apoA1 before addition of the peptide or whether the fusion peptide and apoA1 were pre-incubated before addition to LUV. The inhibition was dose dependent (fig.1) and no fusion was observed when apoA1 was added to LUV.

In order to identify the apoA1 target, we have examined the interaction of apoA1 with LUV and with the synthetic fusion peptides.

Interaction of ApoA1 with LUV

ApoA1 was incubated with LUV of PC/PE/SM/Chol (1,1,1,1.5 moles) during 3 hours at 37°C. Separation of the proteoliposomes on a sucrose gradient (30%-2%) demonstrated that no apoA1 was associated to the liposomes. LUV are concentrated at the top of the tube, whereas the free protein is concentrated at the bottom of the tube (fig.2).

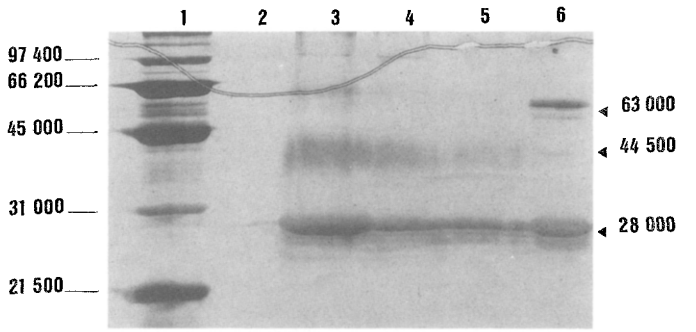


Figure 3: SDS-PAGE analysis of ApoA1 associated with SIV12aa fusion peptide. Proteins were separated by electrophoresis through 12% gel prepared by the method of Laemmli (5). The position of standard molecular weight marker proteins are indicated on the left (lane 1). Lane 6 corresponds to 2.5 μg of apo A-1. Lane 2 corresponds to 15μg of SIV12aa. Lanes 3, 4 and 5 correspond to apo A-1 (10, 5, 2.5 μg) incubated with SIV12aa (15μg).

Interaction of apoA1 with SIV or HIV fusion peptide

ApoA1 was incubated with HIV or SIV fusion peptide for 1 hour at the concentrations used for the fusion experiments. SDS-PAGE analysis shows, for apoA1 (lane 6), 2 majors bands corresponding to 63kDa (dimer of apoA1), and 28kDa (monomer of apoA1). No band was observed for the fusion peptide (HIV and SIV) alone (lane 2). When apoA1 was preincubated with SIV12aa fusion peptide at different peptide/protein molar ratios (140, 70, 35), a new band appears at 44.5 kDa (lanes 3, 4 and 5) corresponding to an association of one apoA1 with 13 SIV12aa (fig.3). A new band was also observed when apoA1 was preincubated with HIV16aa at several peptide/protein molar ratios (110, 60 and 20 (data not shown)), corresponding to one apoA1 associated with 9 HIV16aa. The disappearance of the 63kDa band indicates the elimination of the apoA1 dimer.

DISCUSSION

Owens et al. (1) have shown that apoA1 at physiological concentration (1μM) and amphipathic peptides analogues of apoA1 inhibit HIV-induced syncytium formation and HIV infectivity. The level of envelope glycoprotein (gp160) synthesis, processing and expression was identical in apoA1 and amphipathic peptides-treated cells and untreated cells suggesting that the inhibitory effect is exerted directly upon the fusion process (1). The incubation of $CD4^+$ cells with amphipathic peptides did not inhibit the HIV infection suggesting that apoA1 or amphipathic peptides may interact directly with some component of the virus (1).

It has previously been shown that short peptides homologue to the hydrophobic amino terminal fusogenic sequences of myxo-and paramyxoviruses glycoproteins could inhibit virus infection and virus-induced cell fusion (10), (11). These peptides raise the bilayer to hexagonal phase transition temperature in model membrane systems by almost 10°C,

and stabilize the vesicle bilayer. The ability of these peptides to inhibit hexagonal phase formation has been proposed as a factor contributing to their potency as inhibitors of viral replication (12), (13).

Our results suggest that apoA1 does not interact with the lipid bilayer of the liposomes. On the contrary, the SDS-PAGE electrophoresis reveals that several HIV and SIV fusogenic peptides bind to apoA1. ApoA1 contains several amphipathic helical segments which enable hydrophobic interaction of one side of the helix with phospholipid acyl chains and polar interaction of the other face, containing the charged residues with the phospholipid headgroup or water (14).

The distribution of the amino acids on the hydrophilic and hydrophobic faces of the putative fusogenic domain of retrovirus (HIV, SIV) has been shown to be quite specific and has been observed for several viral proteins (15). Since both apoA1 and HIV, SIV fusogenic sequences contain amphipathic helices, one can expect an interaction between the non polar faces of apoA1 helices and the hydrophobic face of the putative fusogenic peptides.

The binding of several fusogenic peptides to apoA1, is an agreement with the structure of apolipoproteins which contain several internal sequence repeats predicted to form amphipathic helices (14), (21). Since the amino acid sequences of the SIV and HIV fusogenic domains are quite different, the recognition of the apoA1 helices should proceed through an interaction between apolar faces and therefore the inhibition here observed should be observed for a large number of viruses which fuse with the host cell membrane. Consistent with this observation, apoA1 was found to inhibit herpes simplex virus-induced cell fusion at physiological concentration (1μM) (2).

Studies by Kowalski et al (16), and Freed et al. (17) have shown that the fusogenic capacity of the viral glycoprotein is not limited to the presence of an hydrophobic stretch at the N-terminus of its transmembrane subunit (gp41 of HIV and gp32 of SIV). Several antibodies directed against the V3 loop, which is the major immunogenic epitope of HIV-1, inhibit viral penetration without affecting the attachment of the virus to its receptor. This post binding neutralization is thought to act at the level of the fusion step (19), (20). These observations could be explained if one assume that the V3 domain or other domains are complementary to the fusion domain and protect it from the aqueous phase. Conformational change resulting from interaction with the CD4 receptor would lead to a dissociation of the V3 (or other region) and the fusion domain, resulting in an exposure of the fusion domain in its active form. ApoA1 or amphipathic helices will have an opportunity to interact with the fusogenic domain and to disrupt its normal function.

To the best of our knowledge, it is the first time that an inhibitory agent of HIV and SIV induced syncytia formation is shown to interact specifically with the fusogenic N-terminal peptide of a retrovirus. From a molecular knowledge of the structure of the peptides domains involved in this interaction, antiviral agents capable of inhibiting the fusion step could be synthesized on a rational basis. If it is quite clear that although amphipathicity is required for the inhibitory activity (1), a knowledge of the role of the

hydrophobic/ hydrophilic balance and of the distribution of the amino acids on the hydrophobic and hydrophilic domains of the amphipathic helix will be crucial in designing antiviral agents that inhibit the retrovirus penetration. This work is currently in progress in our group. Finally, recent clinical investigations have shown that a low plasma level of HDL was found in AIDS patient (18). However such a study should be extended to individuals at high risk for the development of infection before considering HDL as a risk factor for HIV infection.

ACKNOWLEDGMENTS

This work was performed with the financial support of BNB (Banque Nationale de Belgique), CGER (Caisse Générale d'Epargne et Retraite), FNRS (Fonds National de la Recherche Scientifique), and Smith Kline Beecham Biologicals. We gratefully thank NIH (NIAID grant A1-27136-01A1) and the Commission of the European Communities (SC 1000195) for continued financial support.

REFERENCES

1. Owens R.J., Anantharamaiah G.M., Kahlon J.B., Srinivas R.V., Compans R.W., Segrest J.P. (1990) J. Clin. Invest. 86: 1142-1150.

2. Srinivas R.V., Birkedal B., Owens R.J., Anantharamaiah G.M., Segrest J.P., Compans R.W. (1990) Virology 176: 48 -57.

3. Hope M.J., Bally M.B., Webb G., Cullis P.R. (1985) Biochim. Biophys. Acta. 812: 55-65.

4. Struck D.K., Hoekstra D., Pagano R. (1981) Biochemistry 20: 4093-4099.

5. Laemmli U. K. (1970) Nature 227: 680-686.

6. Rafalski M., Ortiz A., Rockwell A., van Ginkel L., Lear J., DeGrado W., Wilschut J. (1991) Biochemistry 30: 10211-10220.

7. Martin I., Defrise-Quertain F., Mandieau V., Nielsen N. -M., Saermark T., Burny A., Brasseur R., Ruysschaert J. -M., Vandenbranden M. (1991) Biochem. Biophys. Res. Comm. 175: 872-879.

8. Martin I., Defrise-Quertain F., Nielsen N., Saermark T., Burny A., Brasseur R., Vandenbranden M., Ruysschaert J.-M. (1992) Advances in Membrane fluidity vol. 6, pp 365 -376, ed. R. Aloia, Curtain C., Gordon L. Wiley-Liss, Inc.

9. Martin I., Defrise-Quertain F., Decroly E., Saermark T., Brasseur R., Vandenbranden M., Ruysschaert J.-M. (1992) Eur. J. Biochem., submitted.

10. Richardson C.D., Choppin P.W. (1983) Virology 131: 518 -532.

11. Richardson C.D., Scheid A., Choppin P.W. (1980) Virology 105: 204-221.

12. Epand R.M. (1986) Biosc. Rep. 6: 647-653.

13. Kelsey D., Flanagan T., Young J., Yeagle P. J. (1990) J. Biol. Chem. 265: 12178-12183.

14. Brasseur R., De Meutter J., Vanloo B., Goormaghtigh E., Ruysschaert J.-M., Rosseneu M. (1990) Biochim. Biophys. Acta 1043: 245-252.

15. Brasseur R., Vandenbranden M., Cornet B., Burny A., Ruysschaert J.-M. (1990) Biochim. Biophys. Acta 1029: 267-273.

16. Kowalski M, Potz J., Basiripour L., Dorfman T., Goh W., Terwilliger E., Dayton A., Rosen C., Haseltine W., Sodroski (1987) J. Science 237: 1351-1355.

17. Freed E.O., Risser R. (1990) Bull. Inst. Pasteur 88: 73 -110.

18. Falkenbach A., Klauke S., Althoff P.-H. (1990) Medical hypothesis 33: 57-61.

19. Looney D., Hayashi S., Nicklas M., Redfild R., Broder S.,Wong-Staal F., Mitsuya H. (1990) J. of AIDS 3, 649-657.

20. Skinner M., Langlois A., McDanal C., McDougal S., Bolognesi D., Matthews T. (1988) J. Virology 62 n°1, 4195-4200.

21. Brasseur R., Lins L., Vanloo B., Ruysschaert J.-M., Rosseneu M. (1992) Proteins, in press.