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Testing topological models for the membrane penetration of the fusion peptide of influenza virus hemagglutinin

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Low pH-induced binding of the bromelain-solubilized form of influenza virus hemagglutinin (BHA) to membranes occurs through the fusion peptide. From asymmetric hydrophobic photolabeling of membranes, evidence was obtained that this peptide penetrates only one leaflet of the bilayer. The asymmetrical labeling was achieved by employing a photoreactive analogue of a fatty acid whose transbilayer distribution can be manipulated by a membrane proton gradient.

Influenza virus hemagglutinin; Fusion peptide; Asymmetrical hydrophobic photolabeling; Membrane

1. INTRODUCTION

General photolabeling of the apolar phase of membranes is a valuable tool for studying the interaction of proteins with membranes [1–7]. Analyses of detailed labeling patterns have in several cases allowed one to suggest molecular models for the arrangement of membrane-associated segments [8–10]. Recently, hemagglutinin, the major spike protein of influenza virus, has been subjected to such investigation [11,12]. This protein is responsible for the acid-induced membrane fusion, and it is generally believed that this process is triggered by a mechanism enabling penetration of the hydrophobic N-terminal fusion peptide of the HA2 subunit into the target membrane. In agreement with this, we could demonstrate that the low-pH binding of bromelain-solubilized hemagglutinin (BHA) to liposomes is mediated solely by the fusion peptide which appears to adopt the structure of an amphipathic helix [12]. Two gross models have so far been considered for the membrane interaction of this segment [12]: in model I, the helix extends along the membrane-water interface whereas in model II, it assumes a transmembrane configuration and forms an oligomeric structure (see also fig.5).

In this report, a relatively simple and potentially powerful approach is described by which such topological problems can be addressed. The concept is based upon the recent finding that in large unilamellar vesicles (LUVs), transmembrane pH gradients generate an asymmetric distribution of phospholipids, fatty acids and other amphiphiles that contain ionizable functions with an appropriate pK_a value [13,14].

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2. MATERIALS AND METHODS

2.1. *Materials*

11-[4-[3-(Trifluoromethyl)diazirinyl]phenyl]-[2-^{3}H]-undecanoic acid ([^{3}H]TUA) was prepared [11] with a specific radioactivity of 22.2 Ci/mmol. Egg-phosphatidylcholine (egg-PC) (Grade I) was obtained from Lipid Products, South Nutfield, England. Disialoganglioside (G_{D1a}) was from Supelco Inc. (Bellefonte, PA). BHA was prepared from influenza virus PR8/34 as described previously [11]. All other chemicals were commercial grades and of highest purity.

2.2. *Methods*

LUVs were prepared from a mixture of lipids consisting of egg-PC (1.7 μmol), G_{D1a} (0.13 μmol) and [^{3}H]TUA (0.07 μmol) in 1 ml of 150 mM sodium borate, pH 10, by repeated extrusion through 0.2 μm polycarbonate filters [15]. The LUVs were then passed through a Sephadex G-50 column equilibrated and eluted with pH 7.2 buffer (150 mM NaCl, 15 mM sodium citrate, 10 mM Mes and 5 mM Hepes). Void volume fractions were pooled and concentrated to a volume of 1.6 ml. These vesicles were equilibrated at 37°C and then BHA (50 μl; 170 μg protein) was added to initiate the labeling experiment. At the time points shown in fig.4, 200 μl-aliquots were transferred into Eppendorf tubes and exposed to UV light for 10 sec. The light source and general equipment used was the same as described earlier [16]. After photolysis, the samples were neutralized and thereafter three volumes of chloroform-methanol (1:2, v/v) were added. The precipitated protein was collected and subjected to SDS-polyacrylamide gel electrophoresis using a 12% Tris/Tricine-buffered system as specified elsewhere [12]. The Coomassie blue-

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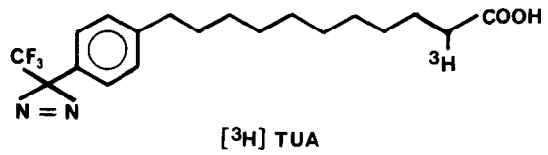

Fig.1. Chemical formula of the photoreactive fatty acid [^{3}H]TUA.

stained band representing the BHA2 subunit of BHA was excised and the protein-associated radioactivity was determined as described earlier [11].

3. RESULTS AND DISCUSSION

Hydrophobic binding of BHA to LUVs occurs spontaneously upon acidification of the medium. The respective segment (fusion peptide) interacting with the bilayer is 21 residues long as concluded from [^{125}I]TID labeling results [12]. The residues labeled with this probe are Phe-3, Ile-6, Phe-9, Trp-14, Met-17 and Trp-21 [12].

A possible approach for distinguishing between the two insertion modes (model I and II as described in section 1 and depicted in fig.5), might be based on asymmetrical labeling of the membrane that is by restricted labeling in a single (inner) leaflet of the bilayer. In fact, labeling of the fusion peptide (BHA2) could then be assumed to occur only if the segment penetrates both membrane halves (model II) but not if it interacts solely at the outer lipid-water interface (model I). In the fusion peptide of BHA, the reactive amino acid residues are distributed along the entire length of the putative helix. Thus, the interpretation of data derived from such an experiment would also seem to be particularly straightforward.

The following experiment was carried out: LUVs were prepared from egg-PC and 5% of the photoactivatable fatty acid [^{3}H]TUA (fig.1) in a sodium borate buffer (150 mM), pH 10. The external medium was then changed to a pH 7.2 buffer. As depicted in fig.2, this can be assumed to result in an asymmetric (internal leaflet) distribution of the fatty acid [13]. Following addition of BHA and a 15-min incubation at 37°C, the external pH was brought to 5.2 in order to trigger hydrophobic binding of BHA to the vesicles. At this point and during the following 5-min incubation at pH 5.2 and 37°C, aliquots were taken, photolyzed and the incorporation of label into the BHA2 subunit was determined. To assess that the fatty acid was still asymmetrically distributed, K^+ (0.1 mM), and valinomycin and nigericin (1 μg each per mg of lipid) were added to dissipate the transmembrane pH gradient and to generate a symmetrical fatty acid distribution (fig.3). Again, aliquots were subjected to photolysis and determination of BHA2 labeling.

The results are depicted in fig.4. During the 15-min incubation of BHA with LUVs at pH 7.2, virtually no labeling of BHA2 was seen, consistent with the notion that BHA does not interact hydrophobically with the bilayer under these conditions. However, following acidification of the external medium, we found labeling of BHA2 which increased with incubation time and which after 5 min corresponded to approximately 0.003% of the total label originally present. It is evident that at this point an equilibrium was not reached since subsequent dissipation of the pH gradient resulted in an immediate and approximately fourfold increase in the specific labeling. Further incubation had no effect, suggesting that the fatty acid was now equally distributed between the two leaflets.

Labeling of BHA2 was thus much heavier when labeling occurred within both monolayer halves than when it was confined to the inner leaflet. This strongly suggests that the fusion peptide of BHA mainly or solely interacts with the outer leaflet. What could be the reason for the low but increasing incorporation of label

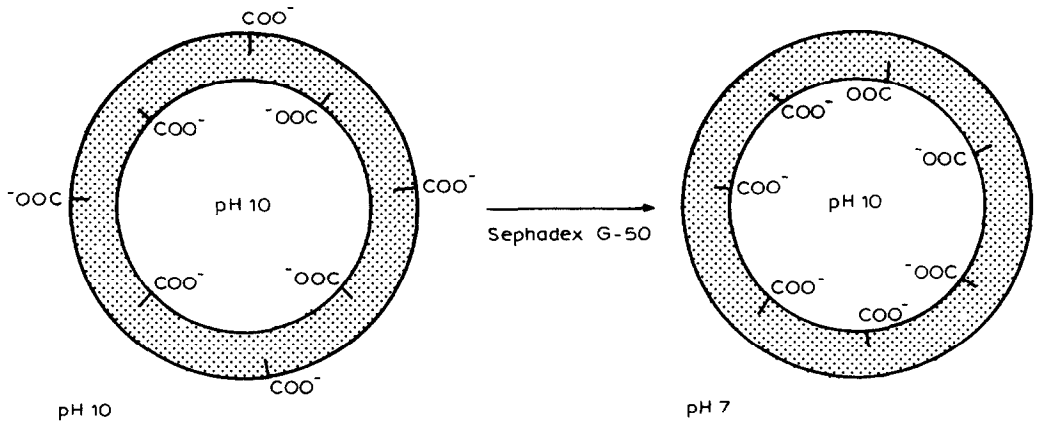

Fig.2. Schematic representation of the exchange of the external buffer leading to an asymmetric distribution of the fatty acid in the LUVs.

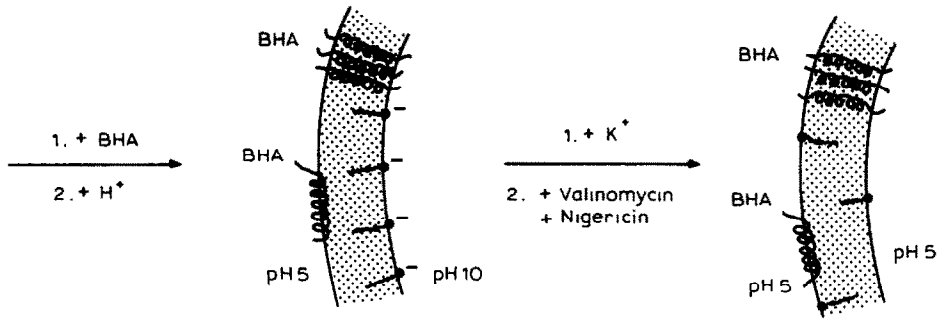

Fig.3. Scheme representing the essential features of the photolabeling experiment. LUVs containing the fatty acid within the inner leaflet (fig.2) and BHA were first incubated at pH 7.2. After 15 min, the external pH was set to 5.2 by the addition of 1 M HCl to trigger hydrophobic binding of BHA to the vesicles. The fusion peptide of BHA interacting with the membrane is depicted both according to model I (lower) and model II (upper). Addition of K^+ and of the ionophores triggered transmembrane equilibration of protons and of the fatty acid molecules.

during stage 2 of the experiment (i.e. within 15 and 20 min in fig.4)? A first possibility is that the fusion peptide reaches a central region of the bilayer and contacts the inner leaflet of the vesicles. A second explanation might be that the progressively increasing labeling of BHA reflects the time course of binding of the protein to the vesicles. However, this can be ruled out since it was established before that membrane penetration is rapid and complete within less than 2 min [17]. Finally, it is not unreasonable to assume that the interaction induces a local perturbance of the bilayer which could cause some leakage and a slow transfer of reagent molecules into the outer monolayer.

In summary, the present data strongly suggest that the interaction of BHA with the vesicles involves primarily, if not exclusively, the outer leaflet of the vesicles and, hence, makes a transmembrane configuration of the helix very unlikely. Towards further refining our ideas on the interaction of BHA with membranes, it seems now appropriate to consider the three minimal models that are depicted in fig.5.

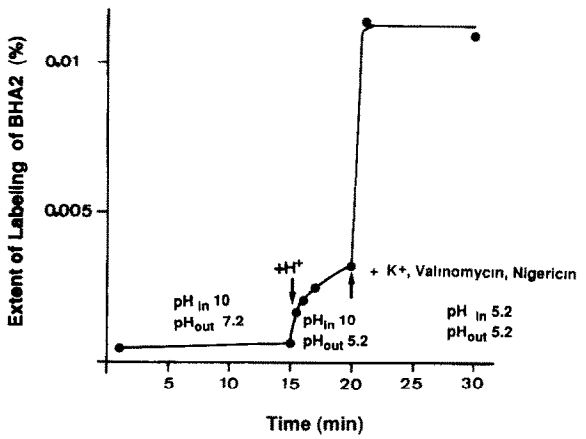

Fig.4. Extent of covalent radioactivity incorporation into the HA2 polypeptide chain of BHA at various stages of the photolabeling experiments (for details see section 2).

The pH gradient induced asymmetric distribution of acidic phospholipids, fatty acids and long chain alkylamines has interesting implications with regard to the solution of an intriguing and long-standing problem: labeling of membranes within defined depths of the bilayer [2–4]. Current attempts to solve this problem are focused on rigid molecules or on reagents which, in essence, consist of two phospholipid moieties linked together at the ω-position of either their *sn*-1 or *sn*-2 fatty acyl chain [18]. However, it has also become clear that such dimeric phospholipid molecules do not all attain the desired membrane-spanning configuration. A simpler and perhaps more straightforward solution is implied in this study, and might be based on long-chain molecules which on their ends carry ionizable groups of different pK_a values which can 'sense' a transbilayer proton gradient. For example, an ω-amino acid long enough to span the bilayer would be expected to orient itself in such a way that one end of the molecule, the carboxyl group, would be deprotonated on and confined to the alkaline side of the bilayer, whereas the (protonated) amino end would be trapped on the acidic surface. Of course, such heterobipolar molecules would have to be further equipped with a non-polar photoactivatable group (that can be attached to any desired position along the (alkylene) chain) and with a suitable radioisotope.

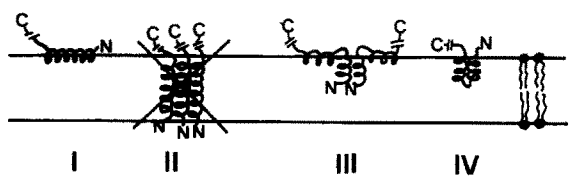

Fig.5. Scheme of 4 topological models for the interaction of the fusion peptide of BHA with membranes. On the basis of the present study, a transmembrane configuration of the helix (model II) can be excluded. All models are based on a helical secondary structure of the fusion peptide with amphipathic properties as suggested previously [12].

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