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ASSEMBLY OF CLATHRIN MOLECULES ON LIPOSOME MEMBRANES: A POSSIBLE EVENT NECESSARY FOR INDUCTION OF MEMBRANE FUSION

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Below pH 6, clathrin induces fusion of liposomes containing phosphatidylserine (PS) [Maezawa et al. (1989) Biochemistry 28, 1422-1428]. Under similar conditions clathrin forms self-aggregates, suggesting that the associated form of clathrin may be involved in the fusion process. For examination of this possibility, the extent of fluorescence energy transfer from *N*-(*p*-(2-benzimidazolyl)phenyl)maleimide (BIPM)-labeled clathrin to *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM)-labeled clathrin in the presence of liposomes and the number of binding sites for clathrin in one liposome were examined in the pH region inducing membrane fusion. A high degree of transfer was observed, and the area on the membrane surface occupied by a clathrin molecule was estimated to be much less than that expected from its size, indicating that clathrin binds to the liposome membrane as an associated form, which may be essential for induction of membrane fusion.

Membrane fusion is an important process in many biological systems, such as endocytosis, fertilization, intracellular transport and virus infection. Recent studies have shown that proteins participate in these membrane fusion processes (1-3). However, despite many studies on the mechanism of membrane fusion induced by proteins, and especially viral spike proteins (4), the mechanism is still unknown. Membrane fusion induced by viral spike glycoproteins has been suggested to involve clusters or oligomers of fusion-active proteins (5,6), but direct evidence for this idea has not yet been obtained.

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Abbreviations: BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide; HA, hemagglutinin; LUV, large unilamellar vesicle(s); NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SUV, small unilamellar vesicle; TES, *N*-[(tris(hydroxymethyl)methyl)amino]-ethanesulfonic acid.

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Clathrin is a major coat protein of coated pits and vesicles formed in receptor-mediated endocytosis and has a unique skeletal structure named a triskelion (7). We have found that clathrin induced fusion of liposomes containing PS below pH 6 (8) and have studied the molecular mechanism of membrane fusion induced by this protein (9-11). In the present study, we examined whether clathrin in the fusion reaction is in a monomeric or associated form by measuring fluorescence energy transfer in a mixture of BIPM-labeled clathrin and DACM-labeled clathrin in the presence of PS-containing liposomes and comparing the area on liposome membranes occupied by one clathrin molecule with the size of the clathrin molecule in the pH region inducing membrane fusion. The results showed that in the fusion-inducible pH region clathrin molecules assemble in the presence of liposomes, suggesting that the assembly is a possible prerequisite for induction of membrane fusion.

Materials and Methods

Materials: Phosphatidylserine (PS) from bovine brain, egg phosphatidylcholine (PC) and NBD-phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Birmingham, AL). BIPM and DACM were from Teika Seiyaku Co. (Toyama, Japan). The compositions of buffers were as described in our previous paper (10). All other reagents were standard commercial products.

Preparation of clathrin: Clathrin was purified from coated vesicles of brain as described previously (10). The concentration of clathrin was determined using an extinction coefficient of $E^{1\%}_{1cm} = 10.9$ at 280 nm (12).

Preparation of liposomes: Large unilamellar vesicles (LUV) containing PS and PC and those labeled with 2 mol% NBD-PE were prepared by reverse-phase evaporation (13) and then extruded by a polycarbonate membrane of 0.1 μm pore size (14). The vesicle concentration was determined by measuring total lipid phosphorus by the method of Bartlett (15).

Labeling of clathrin by fluorescent probes: Clathrin solution (8.9 μM with respect to the heavy chain) was incubated with 38 μM BIPM or 30 μM DACM dissolved in acetone in 10 mM TES, 100 mM NaCl, 0.1 mM EDTA (pH 7.4) at 0°C for 20 and 60 min, respectively. The reaction was stopped by addition of 5-fold excess of 2-mercaptoethanol, and the solution was dialyzed against the same buffer at 4°C overnight. The amounts of BIPM and DACM incorporated into the protein were determined spectrophotometrically to be 2.37 or 2.58 mol per mol of clathrin heavy chain (Mr~180K), respectively, using a molar extinction coefficient of 2.80×10^4 at 314 nm for BIPM (16) and of 1.98×10^4 at 380 nm for DACM (17).

Fluorescence measurements: Resonance energy transfers in a mixture of BIPM-clathrin and DACM-clathrin and in a mixture of clathrin and NBD-labeled liposomes were measured at 25 °C in a Hitachi 650-50 fluorescence spectrophotometer, equipped with a temperature-controlled cell holder and stirrer.

Clathrin binding to membranes: The amount of clathrin bound to liposome membranes was determined by measuring the extent of resonance energy

transfer from tryptophan residues in the clathrin molecule to NBD groups in the liposomes. The fluorescence intensity of the mixture of clathrin (3-80 μg/ml) and NBD-liposomes (50 μM) was measured at 25°C before and after adjusting the pH of the medium to 4.47 by addition of a trace amount of 5M acetic acid. The extinction and emission wavelengths were set at 275 and 339 nm, respectively. A correction was made for the contribution of membrane aggregation and fusion to fluorescence quenching by subtracting the value for change in intensity in a mixture of clathrin and unlabeled liposomes. The net extent of quenching of fluorescence was analyzed by the Klotz equation (18):

$$1 / (1 - R) = (C_0 / R - nV_0) / K_d$$

where R is the fractional saturation, C_0 and V_0 are the initial concentrations of clathrin and liposomes, respectively, n is the number of binding sites for clathrin in a liposome, and K_d is the dissociation constant. R was estimated from the ratio of the extent of fluorescence quenching of tryptophan at a given concentration of clathrin to that at infinite concentration of the protein. The latter was evaluated from a double-reciprocal plot of the extent of fluorescence quenching *vs.* the concentration of clathrin.

Results and Discussion

Fig. 1 shows the excitation and emission spectra of BIPM-labeled and DACM-labeled clathrin at pH 7.4. The emission spectrum of BIPM-clathrin overlapped the excitation spectrum of DACM-clathrin over the range of 350-400 nm, suggesting that fluorescence energy transfer from BIPM to DACM should occur when the two probes were in close contact. We have reported that clathrin molecules form self-aggregates below pH 6 as a result of close contact (10). Thus, we first measured the extent of resonance energy transfer in a mixture of BIPM-clathrin and DACM-clathrin below pH 6. As shown in Fig. 2A, fluorescence increase, that is, a greater extent of resonance energy transfer from the BIPM to the DACM probe, was clearly observed

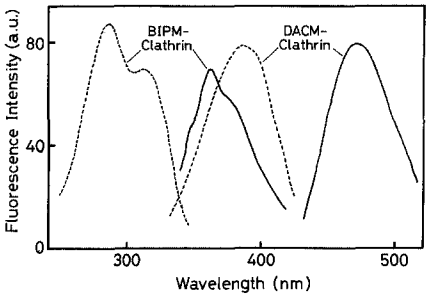


Figure 1. Excitation (dashed line) and emission (solid line) spectra of BIPM- and DACM-clathrin. The concentrations of BIPM-clathrin and DACM-clathrin were both 45 μg/ml. The excitation spectra for BIPM- and DACM-clathrin were measured at emission wavelengths of 365 and 470 nm, and their emission spectra at excitation wavelengths of 315 and 390 nm, respectively, in 10 mM TES buffer containing 100 mM NaCl, 0.1 mM EDTA and 0.01 % NaN_3 (pH 7.4).

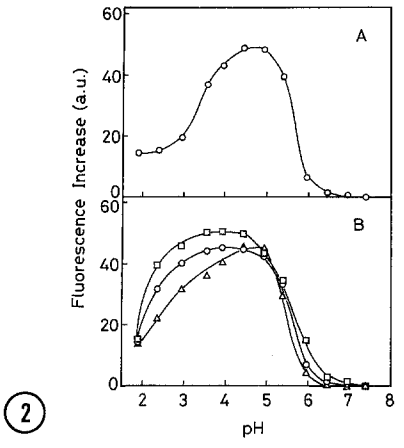
below pH 6. The transfer was pH-dependent, and the pH-profile was similar to that of clathrin self-aggregation reported previously (10). This result demonstrates that resonance energy transfer from BIPM to DACM occurs when BIPM-clathrin and DACM-clathrin come in close contact and that the transfer can be used as an indicator of assembly of clathrin molecules.

We therefore examined the energy transfer in a mixture of BIPM-clathrin and DACM-clathrin in the presence of PS-containing liposomes in the fusion-inducible pH region. As shown in Fig. 2B, a high degree of resonance energy transfer occurred below pH 6 in the presence of liposomes composed of PS, PS/PC(2:1) or PS/PC(1:2). This result shows that clathrin molecules assemble in the presence of liposomes in the fusion-inducible pH region.

The pH-dependent changes in the fluorescence increase in the presence and absence of liposomes were apparently similar; the increase in the presence of liposomes might be due to aggregation of clathrin not bound to liposome membranes. However, the pH-profiles below pH 5 in the presence of liposomes differed somewhat from that in the absence of liposomes and were markedly dependent on the lipid composition of the liposomes. Moreover, we found previously that most of the clathrin molecules bind to these types of liposomes at pH 3-5 (10); the pH profiles of the binding of clathrin to different types of liposomes were similar to those of the fluorescence increase in their presence. We also found that when the pH of the medium was changed from 7 to 4-5 in the presence of liposomes, the initial rates of fluorescence increase were quite different from those in the absence of liposomes (unpublished data). Thus, these results indicate that the increase in energy transfer is caused by the assemblies of clathrin bound to liposome membranes.

Next we measured the number of binding sites for clathrin in one LUV composed of PS/PC(2:1) in the pH region inducing membrane fusion, and compared the area of one binding site calculated from the number of sites, that is, the area occupied by one clathrin molecule, with the size of the protein molecule. In this experiment, we determined the concentration of liposomes from the amount of lipid phosphorus, assuming that, [1] LUV are 1,000 Å in diameter and homogeneous, [2] the thickness of the bilayer is 37 Å, as determined by Huang and Mason using SUV (19), and [3] the volume occupied by a lipid molecule in a liposome is 1,253 $Å^3$, as also determined using SUV (19). On the basis of these assumptions, the number of lipid molecules in one PS/PC(2:1) LUV was calculated to be 85,900. From this value, 50 µM phospholipid was estimated to be equivalent to 5.82×10^{-10} M liposomes, assuming that one LUV is one molecule.

Fig. 3 shows a typical Klotz plot for the binding of clathrin to liposome membranes at pH 4.45, the pH at which membrane fusion is most extensive (10). The number of binding sites on the membrane surface (n) and the



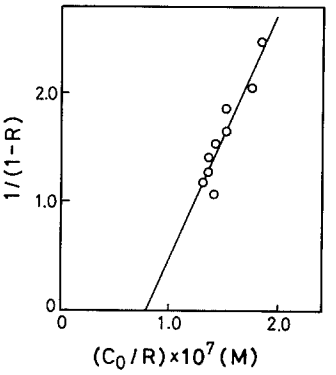


Figure 2. Fluorescence energy transfer from BIPM-clathrin to DACM-clathrin in the absence (A) and presence (B) of liposomes. BIPM-clathrin and DACM-clathrin were added in equimolar concentrations of 15 µg/ml. The concentration of PS (□), PS/PC(2:1) (○) or PS/PC(1:2) (△) liposomes was 50 µM. The differences of fluorescence intensities at the indicated pH and pH 7.4 are plotted against pH. The excitation and emission wavelengths were 320 and 470 nm, respectively.

Figure 3. Klotz plot for the binding of clathrin to PS/PC(2:1) liposomes at pH 4.47. The concentration of PS/PC(2:1) LUV was 50 µM of lipid phosphorus, which corresponds to 5.82 x 10^{-10} M liposomes (see text).

dissociation constant (K_d) were estimated to be 170 and 1 x 10^{-8} M, respectively. Thus, the area of one binding site, that is, the area of the liposome surface occupied by one clathrin molecule was calculated to be 18,500 $Å^2$, which corresponds to the area of a circle with a radius of 76.7 Å.

The clathrin triskelion is known to have a unique three-legged pinwheel-like structure, which is composed of three heavy and three light chains (7). The heavy chain is constructed from the terminal domain and the distal and proximal segments, and the light chain binds to the proximal segment. The total length of the leg is about 500 Å, and the distal and proximal segments are 255 Å and 170 Å, respectively (20). The Stokes radius of the clathrin molecule is 175 Å (21). Judging from the size and shape of clathrin and the area of the liposome surface occupied by a clathrin molecule, clathrin molecules are in close contact with each other on the liposome membrane, suggesting that clathrin binds to the liposome membranes in an associated form(s) in the fusion-inducible pH region.

Recently, the assembly or clustering of fusion proteins has been suggested to be involved in viral spike protein-induced membrane fusion. Blumenthal (5) proposed a model for membrane fusion induced by G protein of vesicular stomatitis virus, in which the proteins are assumed to be arranged as oligomers in viral membranes. Morris *et al.* (22) explained the

mechanism of fusion between hemagglutinin(HA)-expressing cells and red blood cells, assuming a fusion complex formed by some HA molecules. Hoekstra *et al.* (6) suggested that fusion between Sendai virus and erythrocyte ghosts might be regulated by clustering of the viral proteins. Moreover, Ellens *et al.* (23) suggested that fusion between HA-expressing cells and liposomes might be caused by several assembled HA molecules rather than only one. Diphteria toxin is also thought to induce fusion of SUV by interacting with the membranes as aggregates of more than one molecule (24). Our direct evidence together with these speculations indicate that the general fusogenic state of fusion-active proteins is the assembled, clustered or associated form rather than the monomeric form, and that this associated form may be essential for induction of membrane fusion.

We showed previously that membrane fusion induced by clathrin includes at least the following steps: conformational change of clathrin involving exposure of hydrophobic regions, binding of clathrin to the membranes, and close apposition of liposome membranes (9,10). The present study demonstrated that a step of association of clathrin molecules on the liposome membranes is also involved in the fusion process. For elucidation of the molecular mechanism of membrane fusion, it will be necessary to examine the sequence and roles of these steps in the fusion process. Studies on these problems are now under way.

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