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Mechanism of Protein-Induced Membrane Fusion: Fusion of Phospholipid Vesicles by Clathrin Associated with Its Membrane Binding and Conformational Change†

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ABSTRACT: The clathrin-induced fusion of liposome membranes, the membrane binding of clathrin, and the conformational states of clathrin were investigated over a wide pH range using large unilamellar and multilamellar vesicles composed of phosphatidylserine (PS), phosphatidylcholine (PC), PS/PC (2:1), PS/PC (1:1), or PS/PC (1:2). The pH profiles of clathrin-induced fusion of all types of liposomes containing PS showed biphasic patterns. Their pH thresholds were found in the pH range of 5–6 and shifted to lower pH values with decrease in the PS content. Similar shifts were observed in the pH profiles of clathrin binding to these vesicles, but the pH profiles of binding were different from the biphasic fusion patterns. With PC vesicles, only small degrees of fusion and clathrin binding were observed at pH 2–4. The pH dependences of the conformation and hydrophobicity of clathrin were determined by measuring the extent of the blue shift of the fluorescence maximum of 1-anilinonaphthalene-8-sulfonate in the presence of the protein, the fluorescence intensity of *N*-(1-anilinonaphthyl-4)maleimide bound to the clathrin molecule, the resonance energy transfer from its tryptophan to anilinonaphthyl residues, the partitioning of the protein in Triton X-114 solution, and the hydrophobicity index of clathrin using *cis*-parinaric acid. These measurements indicated that conformational change and exposure of hydrophobic regions occur below pH 6 and suggested that clathrin may adopt different conformational states in the pH region where it induced membrane fusion. In addition, the extents of inactivation of clathrin-induced fusion by preincubation of the protein below and above pH 4 were different. Clathrin formed insoluble aggregates at pH 4–6, and soluble aggregates below pH 4, suggesting that two distinct fusion-active states exist at pH 2–6, which might be related to the biphasic fusion patterns. These results suggest that clathrin-induced fusion of liposome membranes involves both protein binding to the membranes and a conformational change of clathrin accompanied by the exposure of its hydrophobic domains. Clathrin binding may induce close apposition of the membranes, while the conformational change may induce insertion of the protein molecule into the membrane to perturb the lipid bilayer. This study provides clues for elucidation of the general mechanisms of pH-dependent membrane fusion induced by proteins.

Membrane fusion is an important and controlled process in biological systems, such as endocytosis, exocytosis, fertilization, myoblast fusion, and intracellular transport. Recently, proteins have been recognized to participate in these membrane fusion processes. For instance, sperm–egg fusion may be mediated by a few kinds of proteins released from sperm (Monroy, 1985; Hong & Vacquier, 1986), and some glycoproteins are involved in myoblast fusion (Wakelam, 1985). In the exocytotic process, proteins are observed to form a pore to induce membrane fusion (Chandler, 1984), and exocytosis is regulated by dephosphorylation of phosphoproteins (Plattner, 1987). Synexin and synexin-like proteins are also involved in

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the fusion of chromaffin and specific granules in the cell (Creutz et al., 1987; Meers et al., 1987). Several attempts have been made to elucidate the role of proteins in membrane fusion (Lucy, 1984; Hong et al., 1987). However, the exact mechanism of induction or modulation of membrane fusion by proteins is still unknown. For elucidation of the mechanism, we have studied the protein-induced membrane fusion using a protein, clathrin, and liposome membrane systems.

Clathrin is the major coat protein of coated pits and coated vesicles, which are known to play a central role in receptor-mediated endocytosis and intracellular protein transport. It has a unique three-legged pinwheel structure termed triskelion, which is composed of three heavy and three light chains (Keen, 1985; Pearse & Crowther, 1987). This triskelion is an assembly unit of the basketlike coat structure. In vitro, it disassembles reversibly from coated vesicles and assembles reversibly into a polyhedral lattice structure in a pH-dependent manner (Keen, 1985; Pearse & Crowther, 1987).

Previously, we investigated the interaction between clathrin and phospholipid vesicles in the pH range of 4–7 and found that below pH 6 clathrin is capable of inducing fusion of phosphatidylserine (PS)[1]-containing vesicles and leakage of aqueous contents from the vesicles (Hong et al., 1985). We next investigated the pH dependence of the conformational states of clathrin to determine the fusogenic properties of clathrin and showed that the hydrophobic regions of the protein molecule are exposed in the pH range where it induces membrane fusion (Yoshimura et al., 1987). In the present work, the relation of clathrin-induced membrane fusion with membrane binding, conformational state, and hydrophobicity of clathrin was studied in the pH range of 2–7 using liposomes of various lipid compositions. Our results define the conditions necessary for the initiation of liposome fusion and suggest a mechanism of membrane fusion induced by clathrin.

Experimental Procedures

Materials. Bovine brain PS, egg PC, NBD-PE, and Rh-PE were purchased from Avanti Polar Lipids (Birmingham, AL). ANS and ANM were obtained from Sigma (St. Louis, MO) and Teika Seiyaku Co. (Toyama, Japan), respectively. *cis*-PnA was obtained from Molecular Probes, and Triton X-114 was from Nacalai Tesque (Kyoto, Japan). All reagents were of the highest grade of purity available.

Clathrin Preparation. Clathrin was purified from crude coated vesicles of bovine brain as described previously (Yoshimura et al., 1987). Purified clathrin in 0.5 M Tris-HCl (pH 7.5) was stored at −20 °C and dialyzed against standard buffer (10 mM TES, 100 mM NaCl, and 0.1 mM EDTA, pH 7.4) before use. The concentration of clathrin was determined spectrophotometrically using an extinction coefficient of $E^{1\%}_{1cm}$ = 10.9 at 280 nm (Nandi et al., 1980) or by the method of Bradford (1976). In this study, clathrin was used at a concentration of less than 50 μg/mL, because at higher concentrations it forms basketlike structures below pH 7 (van Jaarsveld et al., 1981).

Vesicle Preparation. LUV and LUV labeled with both NBD-PE and Rh-PE at 1 or 0.1 mol % each were prepared in standard buffer by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) with the modifications described by Wilschut et al. (1980) and then filtered through polycarbonate membranes of 0.1-μm pore size (Olson et al., 1979).

MLV were prepared by vortex mixing of the lipid film in standard buffer at 30 °C for 1 min. The vesicles were passed through polycarbonate membranes of 0.4-μm pore size and washed 4–5 times by centrifugation at 10000*g* for 5 min to remove small multilamellar and unilamellar vesicles. The vesicle concentration was determined by measuring total lipid phosphorus by the method of Bartlett (1959).

Fusion Assay. Membrane fusion was measured at 25 °C by the resonance energy transfer assay developed by Struck et al. (1981). The assay was performed in a Hitachi 650-60 fluorescence spectrophotometer, equipped with a constant-temperature cell holder and stirrer. Fusion was initiated by addition of clathrin with a Hamilton syringe to a mixture of LUV, labeled with both NBD-PE and Rh-PE at 1 mol % each, and unlabeled LUV at a molar ratio of 1:9. The rate and extent of fusion were calibrated as follows: the fluorescence of the labeled and unlabeled LUV mixture before addition of clathrin was adjusted to zero fusion level, and the 100% fusion level was set at the fluorescence intensity of LUV containing the two fluorescence probes at 0.1 mol % each, which were prepared separately.

Binding Assay. The binding of clathrin to liposome membranes was determined by the centrifugation procedure as described previously (Yoshimura & Sone, 1987). Clathrin was incubated at 25 °C for 30 min in buffer solutions of different pH values in the absence and presence of MLV. The mixtures were then centrifuged at 13000*g* for 5 min, and the amount of protein in the supernatant was determined by the method of Bradford (1976). The amount of membrane-bound clathrin at a given pH was expressed relative to the amount of protein in the supernatant at the corresponding pH in the absence of MLV.

The binding of clathrin was also estimated by measuring resonance energy transfer from tryptophan in the clathrin molecule to NBD groups in LUV. Clathrin was incubated at 25 °C for 5 min in buffer solutions of different pH values with and without LUV labeled with 2 mol % NBD-PE, and the fluorescence intensity at 340 nm was measured, using an excitation wavelength of 275 nm. Percent quenching was estimated from the fluorescence intensity of the protein with or without NBD-labeled LUV.

Other Procedures. The experimental procedures for labeling clathrin with ANM and measuring the maximum emission wavelength of ANS in the presence of clathrin, resonance energy transfer from tryptophan to ANM groups in the protein molecule, clathrin hydrophobicity using *cis*-PnA, clathrin partitioning in Triton X-114, and clathrin self-aggegation were as described previously (Yoshimura et al., 1987). The buffer solutions used were 10 mM TES (pH 7–7.5), MES (pH 6–7), acetic acid (pH 3.6–5.8), or glycine (pH 1.8–3.3), each containing 100 mM NaCl and 0.1 mM EDTA.

Results

Clathrin-Induced Membrane Fusion. Membrane fusion was measured by monitoring the fluorescence increase produced by reduction in the efficiency of resonance energy transfer between NBD-PE and Rh-PE incorporated into liposome membranes. The fusion reaction reached almost a plateau within 10 min after additon of clathrin to liposomes. The extent of fusion (percent fusion) and the rate of fusion (hereafter denoted as fusion parameters) were proportional to the

[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; ANM, *N*-(1-anilinonaphthyl-4)maleimide; LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; *cis*-PnA, *cis*-parinaric acid; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)-ethanesulfonic acid; TES, *N*-[tris(hydroxylmethyl)methyl]-2-amino-ethanesulfonic acid.

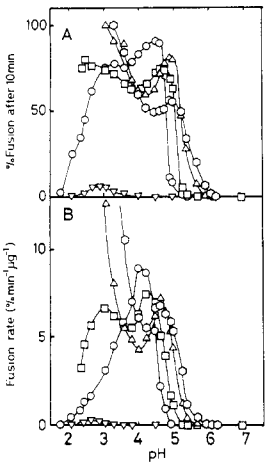

FIGURE 1: pH dependence of clathrin-induced fusion of liposomes composed of PS (○), PS/PC (2:1) (△), PS/PC (1:1) (□), PS/PC (1:2) (○), and PC (▽). Clathrin (9.5 μg/mL) was added to a mixture (50 μM phospholipid) of LUV labeled with both NBD-PE and Rh-PE at 1 mol % each and unlabeled LUV in a molar ratio of 1:9, and the fluorescence increase was monitored at 25 °C. The extent of fusion (A) was expressed as percent fusion 10 min after initiation of the fusion reaction and the rate of fusion (B) as the initial velocity of fusion. The 100% fusion level was set at the fluorescence intensity of LUV labeled with the two fluorescence probes at 0.1 mol % each. The excitation and emission wavelengths were 475 and 530 nm, respectively.

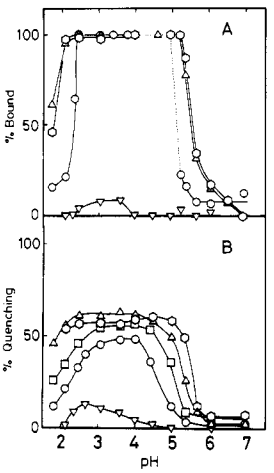

FIGURE 2: pH dependence of clathrin binding to MLV (A) and the quenching of tryptophan fluorescence of clathrin in the presence of NBD-labeled LUV (B) composed of PS (○), PS/PC (2:1) (△), PS/PC (1:1) (□), PS/PC (1:2) (○), and PC (▽). For (A), a mixture of clathrin (30 μg/mL) and MLV (180 μM) was incubated at 25 °C for 30 min, and the amount of bound clathrin was analyzed as described under Experimental Procedures. Since the values at pH 4–5 were inaccurate (see text), dotted lines are used in this pH range. For (B), a mixture of clathrin (17 μg/mL) and LUV (40 μM) labeled with 2 mol % of NBD-PE was incubated at 25 °C for 5 min, and fluorescence spectra were measured as described under Experimental Procedures.

concentration of clathrin added.

The pH dependences of the fusion parameters for liposomes of various lipid compositions are shown in Figure 1. The results may be summarized as follows. (1) Clathrin induced the fusion of liposomes containing PS below pH 6. The pH threshold of the fusion reaction was shifted to lower pH values with decrease in the PS content of the liposome membranes. (2) The pH dependences of the fusion parameters of PS, PS/PC (2:1), and PS/PC (1:1) vesicles were biphasic. The fusion parameters of these three types of vesicles increased and then decreased with a maximum in the pH range of 6–4. Below pH 4, the fusion parameters of PS and PS/PC (2:1) vesicles increased steeply, whereas that of PS/PC (1:1) vesicles displayed another maximum at about pH 3. Although the pH dependence of the fusion rate of PS/PC (1:2) vesicles was monophasic with a maximum at pH 4, the pH profile of the percent fusion showed a biphasic pattern with maxima at pH 4.5 and 3.3. (3) The pH profile of fusion of PC vesicles was different from those of the other vesicles and showed only one small peak between pH 2 and 4.

Membrane Binding of Clathrin. The initiation of fusion by clathrin is likely to be related to its binding to liposome membranes. We, therefore, examined the pH dependence of the binding of clathrin to liposomes. Figure 2A shows the results of centrifugation analysis on the pH dependence of clathrin binding to MLV with various lipid compositions. The amounts of clathrin bound to vesicles containing PS increased below about pH 6, and again decreased below about pH 3. In the pH range of 3–5, protein binding was complete.

Since clathrin formed self-aggregates at pH 4–5, as described later, estimation of its binding at pH 4–5 by this centrifugation procedure seemed to be inaccurate. Therefore, we next estimated the membrane binding of clathrin by measuring the extent of resonance energy transfer from tryptophan residues in the protein molecule to the NBD groups in LUV. Figure 2B shows the pH dependence of the percent quenching of tryptophan fluorescence caused by addition of clathrin to liposomes. The binding of clathrin to PS and PS/PC vesicles occurred below about pH 6 and decreased below about pH 3. The pH threshold of quenching was observed at pH 5–6 and shifted to lower pH values with decrease in the PS content. This pH threshold was consistent with the pH thresholds of the fusion parameters (Figure 1). Clathrin bound to PC vesicles at pH 2–5, but to a much lesser extent, also indicating that the pH profile of binding was quite similar to that of fusion.

Conformational Parameters of Clathrin. We previously investigated the conformational states of clathrin and found that the hydrophobic domains of clathrin are exposed through conformational changes below pH 6, where clathrin induces fusion of liposomes (Yoshimura et al., 1987). Here we studied the conformational states of clathrin in the pH range of 2–7.

First, we examined the pH dependence of the fluorescence maximum of ANS in the presence of clathrin. Since ANS fluorescence is sensitive to microenvironmental changes (Stryer, 1968), it has been widely used to follow the conformational changes of proteins. The fluorescence maximum of ANS in 35% ethanol solution was not affected by pH. As shown in Figure 3A, the fluorescence maximum in the presence of clathrin was shifted to lower wavelengths with decrease in pH. The extent of this shift increased gradually in the pH range of 7–6 and steeply in the pH range of 6–4, and decreased below pH 4.

Next, we examined the pH dependence of the fluorescence intensity of ANM covalently bound to clathrin (Figure 3B). Clathrin labeled with ANM had similar fusion activity to the unlabeled protein. The fluorescence intensity of ANM increased steeply in the pH range of 6–4 and decreased below pH 4. This profile was similar to that of the blue shift of the ANS fluorescence maximum. The pH dependence of resonance energy transfer from tryptophan residues in the clathrin molecule to covalently bound ANM groups was also investigated (Figure 4A). The fluorescence intensity of tryptophan residues decreased steeply in the pH range of 6–4 and was constant below pH 4, while the fluorescence intensity of sensitized ANM increased steeply in the pH range of 6–4 and decreased below pH 4. The fluorescence intensity of tryptophan residues in unlabeled clathrin was fairly constant above pH 5 but decreased slightly below pH 5 (Figure 4B). This

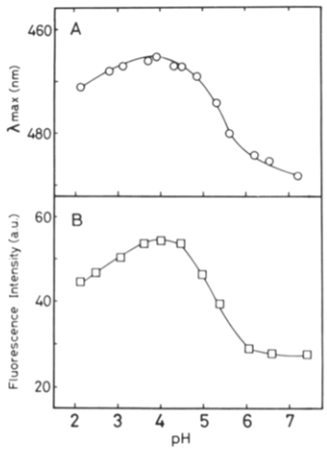

FIGURE 3: pH dependence of the ANS fluorescence maximum in the presence of clathrin (A) and the fluorescence intensity of ANM covalently bound to clathrin (B). A mixture of ANS (2.3 μM) and clathrin (35 μg/mL) for (A) or ANM-labeled clathrin (4.7 labels per clathrin heavy chain) at a concentration of 20 μg/mL for (B) was incubated at 25 °C for 30 min in buffer solutions of various pH values, and fluorescence spectra were measured at an excitation wavelength of 350 nm. Slit widths were set at 10 nm (excitation) and 5 nm (emission).

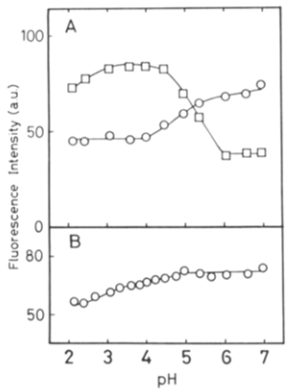

FIGURE 4: pH dependence of the fluorescence intensity of tryptophan (○) and sensitized ANM (□) in the clathrin molecule (A) and the fluorescence intensity of tryptophan of unlabeled clathrin (B). ANM-labeled clathrin (5.8 labels per clathrin heavy chain) or unlabeled clathrin at a concentration of 15 μg/mL was incubated at 25 °C for 30 min, and fluorescence spectra were measured at an excitation wavelength of 280 nm. Slit widths were set at 10 nm (excitation) and 3 nm (emission).

observation suggests that if the fluorescence intensity of tryptophan residues in *unlabeled* clathrin had not decreased below pH 5, the fluorescence intensity of tryptophan residues in *labeled* clathrin would have increased below pH 4, instead of apparently remaining constant.

The results of these three fluorescence experiments suggest that clathrin undergoes conformational changes in the pH range of 2–6 and that the protein may adopt different conformational states in this pH region.

Hydrophobic Parameters of Clathrin. Proteins that are soluble and insoluble in water are known to be generally dissolved in Triton X-114 solution but partitioned into the aqueous and detergent phases, respectively, when the temperature of the medium is raised above its cloud point (Bordier, 1981). This partitioning is due to the apparent hydrophobicity of proteins, and when hydrophobic regions are exposed, the protein should be partitioned into the detergent phase. This technique has, therefore, been used to detect the exposure of hydrophobic regions of various proteins (Kielian & Helenius, 1985; Escuyer et al., 1986; Yoshimura et al., 1987). Figure 5 shows the pattern of SDS–polyacrylamide gel electrophoresis

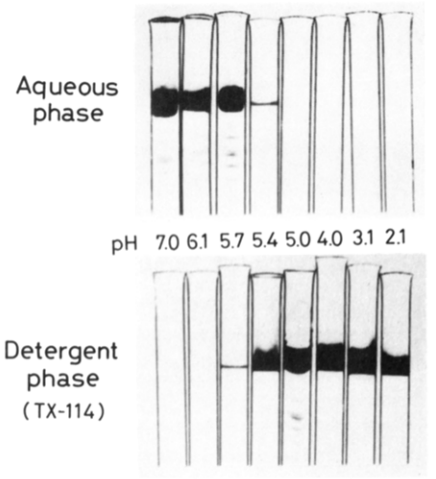

FIGURE 5: Partitioning of clathrin in Triton X-114 solution. Clathrin (100 μg) was incubated at 0 °C for 30 min in buffer solutions of various pH values, containing 1% (w/v) Triton X-114, and then at 30 °C for 10 min. The mixtures were centrifuged at low speed, and the resulting aqueous and detergent phases were analyzed by SDS–polyacrylamide gel electrophoresis.

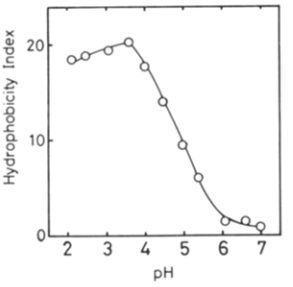

FIGURE 6: pH dependence of the effective hydrophobicity of clathrin. An ethanolic solution of *cis*-PnA (1.8 μM) was added to clathrin solution of different pH values, containing 5×10^{-4}% SDS. After incubation at 25 °C for 1–2 min, the fluorescence intensity at 420 nm was measured at an excitation wavelength of 320 nm. The hydrophobicity index was estimated from the slope of the plot of the clathrin concentration vs fluorescence intensity (Kato & Nakai, 1980).

of clathrin after its partitioning in Triton X-114 at various pH values. Clathrin was found in the aqueous phase at neutral pH, in both the aqueous and detergent phases at about pH 5–6, and entirely in the detergent phase below pH 5.

Kato and Nakai (1980) have shown that the hydrophobicity index of proteins can be estimated from the slope of the plot of fluorescence intensity of *cis*-PnA vs protein concentration. The change in hydrophobicity of clathrin was also examined by this procedure. Figure 6 shows the pH dependence of the hydrophobicity index of clathrin. This parameter increased steeply in the pH range of 6–4 and decreased slightly below pH 4. This pH dependence was very similar to those of the ANS fluorescence maximum and ANM fluorescence intensity (Figure 3). These results indicate that the hydrophobic regions of clathrin were exposed below pH 6.

Fusion Capacity of Preincubated Clathrin. The above results suggested that the conformational state of clathrin below pH 4 may be different from that at higher pH values. We have reported that preincubation of clathrin at a pH at which it could induce fusion inactivated its fusogenic capacity (Hong et al., 1985). To obtain an insight into the fusogenic activities of clathrin in different states, we measured the extent of inactivation of fusion caused by preincubation of the protein at various pH values. Figure 7 shows the ratios of the fusion rates of clathrin preincubated for 10 min to that without preincubation at various pH values. Irrespective of the lipid composition, clathrin-induced fusion was inactivated less by

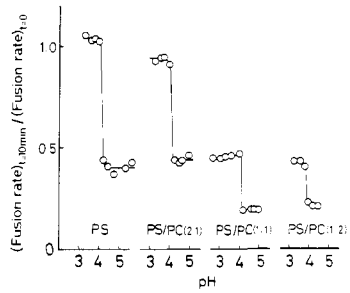

FIGURE 7: pH dependence of the ratios of the fusion rate of preincubated clathrin to that of clathrin without preincubation. Clathrin (9.5 μg/mL) was incubated at the indicated pH values at 25 °C for 10 min in a cuvette, then a mixture (50 μM phospholipid) of LUV labeled with both NBD-PE and Rh-PE and unlabeled LUV, composed of PS, PS/PC (2:1), PS/PC (1:1), or PS/PC (1:2), was added, and fluorescence increase and the rate of fusion were measured at the same pH values as the preincubation as described for Figure 1. The fusion rate of clathrin without preincubation was measured by addition of clathrin to the mixture of labeled and unlabeled LUV at the indicated pH values.

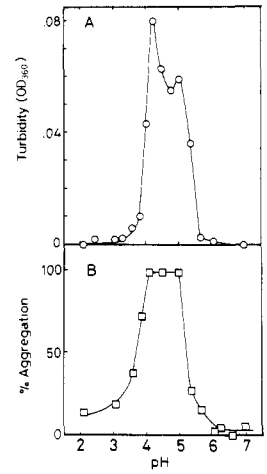

FIGURE 8: Effect of pH on the turbidity of a suspension of clathrin (A) and clathrin self-aggregation (B). For (A), clathrin turbidity at 360 nm was measured after incubation of clathrin (26 μg/mL) at the indicated pH values and 25 °C for 30 min. For (B), clathrin (20 μg/mL) was incubated at the indicated pH values and 25 °C for 30 min. The solution was then centrifuged at 10000*g* for 5 min, and the amount of protein in the supernatant was determined by the method of Bradford (1976). Percent aggregation at a given pH was expressed as the amount of clathrin relative to that at pH 7.4.

preincubation below pH 4 than at pH 4–6: in other words, the ratio of the fusion rate below pH 4 was consistently higher than that above pH 4, and values were constant both below and above pH 4, suggesting the existence of two distinct fusion-active states.

Self-Aggregation of Clathrin. Clathrin self-aggregates on decrease in pH (Yoshimura et al., 1987). Its self-aggregation at pH 2–7 was examined by measuring the change in turbidity. As shown in Figure 8A, the turbidity of a suspension of clathrin increased steeply in the pH range of 6–4 with a shoulder at about pH 5. Interestingly, the turbidity decreased steeply below pH 4, and the solution was clear below pH 3. The extent of clathrin aggregation was also measured directly by centrifugation (Figure 8B). The percent aggregation increased steeply in the pH range of 6–5, and almost all the clathrin molecules aggregated at pH 4–5. Below pH 4, the extent of aggregation again decreased, like the turbidity. However, analytical ultracentrifugation experiments showed that soluble aggregates formed at pH 2–4, whereas no aggregates were found at pH 7 (data not shown), indicating that clathrin exists in different states in these two pH ranges. Thus, we can conclude that clathrin adopts two distinct fusion-active states at pH 2–4 and 4–6, and a fusion-inactive state above pH 6.

Discussion

In the present study, we investigated the pH dependence of clathrin-induced fusion of phospholipid vesicles over a wide pH range and compared the results with the pH profiles of binding of clathrin to the membranes and its conformational states. Below pH 6, clathrin induced fusion of liposomes composed of PS, PS/PC (2:1), PS/PC (1:1), PS/PC (1:2), or PC. The pH threshold of vesicles containing PS depended on their PS content, and their pH profiles showed biphasic patterns.

The pH dependence of binding of clathrin to liposome membranes also depended on the PS content of the liposomes. The pH threshold of clathrin binding was observed at pH 5–6 and shifted to lower pH values with decrease in the PS content of the liposomes. These thresholds were similar to those for fusion, suggesting that the induction of membrane fusion at pH 5–6 is regulated by clathrin binding to the membranes. The extents of fusion and clathrin binding of PC liposomes were much lower than those of PS-containing liposomes. Nevertheless, the pH dependence of fusion of PC vesicles was parallel with the pH-dependent binding of clathrin to these vesicles, again suggesting that membrane fusion occurs when clathrin binds to the vesicle membranes.

The fusion of liposomes may be regulated by clathrin binding to liposomes membranes, but binding alone cannot explain the pH profiles of fusion at acidic pH values, that is, the biphasic pattern of fusion shown in Figure 1. It seemed possible that there was some relation between these biphasic patterns and the conformational states of clathrin. We, therefore, measured the conformational and hydrophobic parameters of clathrin using fluorescent probes and detergent at pH 2–7 and obtained several lines of evidence that the conformational change of clathrin occurs below pH 6 and that different conformational states of clathrin may exist in this pH region. The conformational states at pH 2–4 and 4–6 have fusion activity, and those at pH 6–7 do not. The existence of the two distinct fusion-active states at pH 2–6 was confirmed by the following observations: (1) the relative fusion rates of preincubated clathrin were markedly different above and below pH 4, irrespective of the lipid composition of the liposomes (Figure 7); (2) clathrin aggregates were insoluble in water at pH 4–5 but soluble at pH 2–4 (Figure 8). In the fusion-active states, clathrin may have different protein–lipid interactions with vesicles of different PS contents. This might explain the various pH profiles of the fusion parameters depending on the lipid composition of the vesicles (Figure 1). Thus, the fusion of PC vesicles could be induced by clathrin in only one of these fusion-active states at pH 2–4. In addition, lipid packing in the liposome membranes may be altered by change in pH and/or lipid composition. Changes in the surface charge of the head groups of phospholipid have been reported to affect the lipid bilayer packing density (Israelachvili et al., 1980).

The increases in the extent of the blue shift of the ANS fluorescence maximum and the fluorescence intensity of ANM at pH 2–6 suggest an increase in the area of exposed hydrophobic regions of clathrin in this pH range (Figure 3). Partitioning of the clathrin molecule into the detergent phase (Figure 5) and increase in the hydrophobicity index of clathrin (Figure 6) at pH 2–6 clearly confirmed the increase in hydrophobicity. This hydrophobic property of clathrin in the two fusion-active states must be related to its ability to induce membrane fusion.

There is a possibility that a low pH induces denaturation of clathrin which causes membrane fusion. However, this possibility seems unlikely, since its completely denatured forms at high temperature or in 8 M urea showed no fusion inducibility, and since the terminal domain of clathrin was not fusogenic but the residual central part of the protein retained "intact" fusogenicity (S. Maezawa et al., unpublished results). Thus, exposure of certain hydrophobic regions through local unfolding of clathrin is likely to occur at low pH, causing membrane fusion.

As a whole, the present results suggest that both binding of the protein to the membranes and exposure of hydrophobic regions through conformational changes of clathrin are necessary for the induction of fusion. Since the isoelectric point of clathrin is reported to be about 5.8 (Nandi & Edelhoch, 1984), it could bind to membranes electrostatically and hydrophobically. The electrostatic interactions are thought to occur between positively charged groups on the clathrin molecule and negatively charged head groups of PS molecules, while the hydrophobic interactions probably occur between the exposed hydrophobic regions of clathrin and hydrocarbon parts of the lipid bilayer. We envisage the fusion process as follows: Upon lowering the pH, protonation occurs in the clathrin molecule. This protonation and loss of negative charge lead to a conformational change and exposure of hydrophobic regions of clathrin. As a result, clathrin binds to the vesicle membranes electrostatically, while the exposed hydrophobic regions become inserted into the membrane and interact with the hydrophobic regions of the membrane. Binding of clathrin to membranes and penetration of the hydrophobic segments into bilayers should eventually produce close apposition of membranes, possibly due to neutralization of the membrane surface charge and formation of a bridge between two adjacent membranes. These events cause destabilization and perturbation of the vesicle membranes and facilitate mixing of lipids at points of close contact, thus inducing membrane fusion.

Virus membrane fusion has been studied extensively. Virus envelope proteins, such as the F protein of Sendai virus, the HA protein of influenza virus, the G protein of vesicular stomatitis virus, and the E protein of Semliki Forst virus, have been shown to mediate fusion between the virus membranes and liposomes (White et al., 1983). Most of these envelope proteins have short hydrophobic stretches of amino acids, which are assumed to be exposed by the action of proteases and pH-dependent conformational changes of the proteins, and then to interact directly with the lipid bilayer to induce membrane fusion. Recently, the participation of proteins in membrane fusion in various cellular systems has been reported (Lucy, 1984; Hong et al., 1987). Various proteins and peptides, such as synexin (Hong et al., 1982), bovine serum albumin (Schenkmann et al., 1981), and its fragments (Garcia et al., 1984), clathrin (Blumenthal et al., 1983; Hong et al., 1985), cytochrome *c* (Gad et al., 1982), melittin (Eytan & Almary, 1983; Morgan et al., 1983), diphtheria toxin (Cabiaux et al., 1984), tetanus toxin (Cabiaux et al., 1985), insulin (Farías et al., 1985), α-lactalbumin (Kim & Kim, 1986), and lysin (Hong & Vacquier, 1986), have been found to induce fusion of liposomes, and their fusion mechanisms have been studied. Results have shown that protonation, change in conformation, exposure of hydrophobic moieties of the proteins, insertion of hydrophobic segments into the membrane, and close apposition of membranes definitely contribute to the fusion of liposomes. However, it is unclear how these factors are involved in the process of membrane fusion. Some attempts to solve this problem have recently been reported (Parente et al., 1988; Meers et al., 1988).

In receptor-mediated endocytosis, clathrin is released from coated vesicles before formation of endosomes (Pearse & Crowther, 1987). Thus, the clathrin coat may inhibit fusion of the vesicles, and its removal may allow fusion to occur (Altstiel & Branton, 1983). Since the pH for initiation of membrane fusion by clathrin is lower than the cytoplasmic pH, and since negatively charged liposomes are able to fuse with uncoated vesicles at neutral pH in the absence of clathrin (Lawaczeck et al., 1987), the biological significance of the fusogenic property of the protein is obscure. However, our data showed that clathrin molecules adopt different conformational states under different conditions, implying that some effectors, such as pH, ion flux, ATP energy, and membrane potential, might activate clathrin to function in the fusion process of uncoated vesicles. Further studies on this point seem to be necessary to establish the role of clathrin in membrane fusion at the cellular level.

Previously, we found that clathrin can induce fusion of vesicles containing PS at below pH 6 (Hong et al., 1985) and that its hydrophobic regions are exposed in the pH range where it induces membrane fusion (Yoshimura et al., 1987). From these and the present studies, we conclude that membrane fusion induced by clathrin occurs in association with its membrane binding and conformational change associated with exposure of its hydrophobic domains. This finding is helpful in understanding the general mechanism of protein-induced membrane fusion.

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Patterns of Alternative Splicing of Fibronectin Pre-mRNA in Human Adult and Fetal Tissues†

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ABSTRACT: Alternative splicing of fibronectin pre-mRNA at two distinct regions, termed ED-A and IIICS, was investigated with human adult and fetal tissues by the nuclease S1 protection assay. A clear tissue specificity was observed in the splicing pattern at the ED-A region. More ED-A$^+$ than ED-A$^-$ mRNAs were identified in lung, whereas ED-A$^-$ mRNAs were predominantly expressed in liver. Endometrium contained nearly equal amounts of ED-A$^+$ and ED-A$^-$ mRNAs. The splicing pattern at the ED-A region was also different between adult and fetal liver but not between adult and fetal lung. Tissue type specific splicing was also observed at the IIICS region. Although the mRNA species containing the complete IIICS sequence comprised 40-65% of the total fibronectin mRNAs irrespective of tissue types, expression of the mRNA species lacking a part or all of the IIICS sequence was more pronounced in adult liver than in other tissues including fetal liver. These results strongly suggest that the alternative splicing of fibronectin pre-mRNA in vivo is regulated in a tissue type specific manner at both the ED-A and IIICS regions and that it is developmentally regulated in liver but not in lung. On the basis of these and other observations reported previously, a possibility that a part of the fibronectins synthesized and secreted by hepatocytes is deposited in the tissue matrix is discussed.

Fibronectins (FNs)[1] are multifunctional adhesive glycoproteins present in the extracellular matrix and plasma. FNs bind to fibroblasts and many other cell types, thereby mediating attachment and spreading of cells, and they also bind to collagens, heparin, fibrin, DNA, and certain types of bacteria [for recent reviews, see Yamada (1983), Mosher (1984), Hakomori et al. (1984), Hynes (1985), and Ruoslahti and Pierschbacher (1986)]. FNs consist of two subunits with molecular weights of about 250 000 which are connected by

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[1] Abbreviations: FN, fibronectin; ED, extra domain; IIICS, type III connecting segment; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); ED-A$^+$ mRNA, FN mRNA containing the ED-A sequence; ED-A$^-$ mRNA, FN mRNA lacking the ED-A sequence.

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