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Diffusion properties of clathrin on the surface of isolated mouse liver nuclei by the fluorescence recovery after photobleaching technique

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Clathrin labeled with eosin maleimide showed physicochemical properties similar to the native clathrin. The diffusion coefficients of clathrin protomers and cages measured at 20°C by the fluorescence recovery after photobleaching technique (FRAP) were found equal to $(9 \pm 1) \cdot 10^{-8}\ cm^2 \cdot s^{-1}$ and $(3 \pm 0.4) \cdot 10^{-8}\ cm^2 \cdot s^{-1}$, respectively. After incubation with isolated mouse liver nuclei suspended in an aqueous buffer, FRAP measurements showed that 78% of clathrin was immobilized on the nuclear surface. This immobile fraction might correspond to aggregates of molecules resembling coated pits. The mobile fraction had a diffusion coefficient of $2.5 \cdot 10^{-9}\ cm^2 \cdot s^{-1}$ which was reduced seven times when the suspension medium of the nuclei contained 50% sucrose, showing that the aqueous phase exerted an important drag on the clathrin molecules motion diffusing on the nuclear surface.

Clathrin, a fibrous protein, is present on the cytoplasmic side of the plasma membrane of cells as a polymer made up of hexagonal lattices –the coated pits– through which receptor-mediated internalisation of various macromolecular ligands (viz. hormones, α_2-macroglobulin, virus, etc.) takes place [1,2]. Subsequently to the binding of the ligand to the receptor at the coated pits or to the accumulation of the ligand receptor complexes therein, the internalisation of the complex into the cell takes place by the invagination of the coated pit as coated vesicle [2]. The coated vesicle is a closed shell like structure made up of hexagonal and pentagonal lattices of the clathrin protein molecules [1,2]. The coated vesicles have also been implicated for the transfer of newly synthesized and processed proteins from Golgi to lysosome [3] and in exocytosis [4] and also for the nuclear membrane vesicularisation during cell mitosis [5].

At present no information is available on the association properties of clathrin with biological membranes in vitro. Here we report a study of fluorescently labeled clathrin attached to the membrane of isolated nuclei by the fluorescence recovery after photobleaching (FRAP) technique. The present study can be considered as a model for the behavior of clathrin molecules associated with other intracellular membrane.

Nuclei from the mouse liver were isolated following the procedure of Blobel and Potter (1966) [6] with some modifications as described by Peters (1983) [7].

Bovine brains were homogenised in Mes buffer (0.10 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$ and 3 mM NaN_3 (pH 6.5)) following the procedure of

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid; FRAP, fluorescence recovery after photobleaching; Mes, 4-morpholine-ethanesulfonic acid.

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Pearse [1]. The coated vesicle were purified in a 8% sucrose ^2H_2O-H_2O gradient following the procedure of Nandi et al. [8]. The coated vesicle pellet was resuspended in the Mes buffer and centrifuged at $20000 \times g$ for 10 min and the supernatant was used for further experiments.

Coated vesicles prepared in 0.10 M Mes (pH 6.5) was dialysed for 15 h against two changes of 0.01 M Tris (pH 8.5). The solution was centrifuged at 36000 rpm for 1 h in a type 70.1 Ti rotor in a Beckman L8-65B centrifuge. The supernatant was dialysed against 0.02 M Tris (pH 7.2) for 12 h to give a preparation of clathrin which was used for experiments with the nuclei.

Eosin maleimide was reacted with clathrin at pH 7.2 for 2 h at room temperature using 4-fold molar excess calculated for clathrin subunits ($M_r \approx 180000$) following the procedure as reported previously [9]. The labeled clathrin was purified from unreacted reagents by eluting through a small Sepharose 4B-Cl column equilibrated in a 0.02 M Tris (pH 7.2) buffer. Labeling of cages was carried at pH 6, 0.1 M Mes and purified as above. Gel electrophoresis of the labeled clathrin was carried out as described previously [9]. A value of $A^{1\%}_{280nm} = 10.9$ was used to determine the concentrations of clathrin [10].

The labeled clathrin sample, after removing the free and aggregated dyes (see above) was incubated for 10 min at 37°C with nuclei in 0.02 M Tris (pH 7.2) containing 0.15 M NaCl as a final concentration, followed by centrifugation of the complex over a sucrose gradient (discontinuous 0.25 and 2 M) for 30 min at 30000 rpm. A drop of this nuclei suspension was put in a microscopic slide for FRAP experiments.

The measurements of diffusion coefficient of labeled clathrin in solution, were performed in microslide of 0.2 mm optical path.

The FRAP curves were determined with an apparatus which has been described in details elsewhere [11,12].

For the clathrin solutions a Zeiss Neofluar 10/0.3 NA objective yielding a gaussian beam radius of 10.5 μm was used. For the clathrin-nuclei complexes the microscope was equipped with a Zeiss Neofluar 100/1.3 NA objective yielding a beam radius of 1.58 μm.

From the recovery curve, we determined as

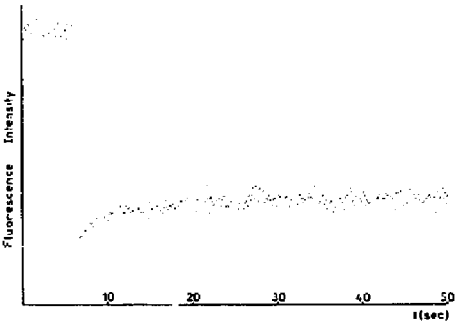

Fig. 1. FRAP curve measured on nuclei preincubated with eosin-labeled clathrin for 10 min at 37°C. Nuclei were suspended in 0.02 M Tris (pH 7.2), 0.15 M NaCl at 20°C. The laser beam radius was 1.58 μm.

usual the diffusion coefficient and the fraction of diffusing molecules L.

When the labeled clathrin was centrifuged in a 10–30% sucrose gradient at 28000 rpm for 90 min at 23°C, it showed the same band distribution as unlabeled clathrin (8 S). By electrophoresis on SDS gel, labeled and non-labeled clathrin showed the same protein bands after staining with Coomassie blue viz: 180 K of clathrin, 110–130 K of clathrin associated proteins, 50 K and 32–34 K of clathrin light chains [9]. In a separate electrophoretic experiment with labeled clathrin all these protein bands were fluorescent although the intensity was stronger in 180 K and 110–130 K bands [8].

Electron microscopic studies showed that the labeled cages had the same structure as the unlabeled cages at pH 6. The velocity sedimentation in a 10–30% sucrose gradient at pH 6 was identical for the labeled and unlabeled cages.

The diffusion coefficient of labeled clathrin obtained by the FRAP technique was equal to $(9 \pm 1) \cdot 10^{-8}$ cm$^2 \cdot$s^{-1} in dilute buffer. A value of $(12.7 \pm 0.02) \cdot 10^{-8}$ cm$^2 \cdot$s^{-1} was computed for the diffusion of human brain 8.1 S clathrin from sedimentation equilibrium measurements [10]; this value is 40% greater than the value obtained here from FRAP.

The diffusion coefficient of the clathrin cages in solution that we obtained with FRAP was $(3 \pm 0.4) \cdot 10^{-8}$ cm$^2 \cdot$s^{-1}. The value of the diffu-

sion coefficient of the isolated coated vesicles from bovine brain, which have the same diameter as the cages studied here, has been found to be $4.3 \cdot 10^{-8}$ $cm^2 \cdot s^{-1}$ (at 20 °C) by laser light scattering measurements [13], a value 60% higher than our FRAP result.

FRAP measurements were performed on the nuclei incubated with labeled protomers after removal of the free protomers as described in the method section. According to our FRAP measurements, ≃ 22% of the labeled protein was mobile on the nuclear surface with a diffusion coefficient of $(2.5 \pm 0.5) \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$. This is nearly 35-times smaller than the value of the diffusion coefficient obtained for the molecule in water. The reduced mobility of the protein in the presence of the nuclear membrane indicated that the clathrin molecule was associated with the nuclear envelope. From these results one could distinguish two protein fractions on the nuclear surface after equilibration of protomeric clathrin: (i) a diffusing fraction which might be constituted of protomers or small oligomers and (ii) a nondiffusing (nonmobile) fraction probably originating from the aggregates of the protein. The aggregates representing the immobile fraction might result from the polymerisation of the protomer clathrin induced by the nuclear membrane. It is tempting to suggest that this aggregated immobile fraction has the same structure as the clathrin coated pit found associated with the plasma membrane. However, ultrastructural studies will be necessary to prove this suggestion.

In order to learn more about the motion of the mobile clathrin fraction on the nuclear membrane, we studied the influence of the viscosity of the surrounding medium on the diffusion coefficient of clathrin moving on the nuclear surface. First we measured the diffusion coefficient of clathrin in the presence of 50% sucrose in dilute buffer and found a value of $(1.0 \pm 0.1) \cdot 10^{-8}$ $cm^2 \cdot s^{-1}$. This was 9-times lower than the value found in water. Considering that the diffusion coefficient, D, was related to the viscosity of the medium by the equation $D = \alpha(T/\eta)$ where η = viscosity of the solvent, α = a constant, and T the absolute temperature, one could see that the observed decrease in the D value in sucrose solution corresponded to a 44% sucrose solution instead of the value of 50% which was expected from the preparation.

The value of D of the cages in sucrose solution was similar (≃ 10% lower) to the value obtained for the protomer in the same sucrose solution. This might have resulted from the instability of the cage structure in concentrated sucrose solution resulting in the dissociation to protomers. Such a dissociation of the polymeric clathrin coat structure has been observed earlier [8].

Nuclei previously incubated with labeled clathrin protomer and cages as described above, were suspended in a 50% sucrose-water solution and FRAP measurements were performed. The diffusion coefficient value of clathrin, fraction mobile on the nuclear membrane surface was $(3 \pm 1) \cdot 10^{-10}$ $cm^2 \cdot s^{-1}$ in 50% sucrose solution. This was about 7-times less than the value of the diffusion coefficient found when the nuclei were suspended in water without sucrose. Furthermore the percentage of the diffusing particles on the nuclear surface was 24. This value was practically the same as was found in the absence of sucrose.

Theoretical computations of Saffman and Delbrück [14] showed that the viscosity of the surrounding liquid phase exerted a relatively small effect on the diffusion coefficient of a cylindrical macromolecule embedded in a membrane sheet. These calculations have been confirmed by FRAP measurements on bacteriorhodopsin incorporated in large vesicles of dimyristoylphosphatidylcholine [15].

According to current views, clathrin is anchored to proteins or lipid molecules embedded in biological membranes. Therefore the fluorescence recovery we measured could be attributed to the diffusion of the anchor complexed with clathrin on the nuclear membrane. A large part of the clathrin molecule in this complex is surrounded by the aqueous phase which might explain the important influence of the aqueous phase. Another plausible mechanism of clathrin movements on the nuclear surface might be the following: dissociation from a membrane site, diffusion through the aqueous phase and binding to a neighboring site. Further studies will be necessary to clarify this point.

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