CHARACTERIZATION OF ANTI-CLATHRIN SERUM

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1. Introduction

Coated vesicles (CVs), membrane bilayers enclosed by polygonal protein lattices, are ubiquitous cellular organelles which are involved in a variety of membrane transfer processes [1–5]. Recently, Pearse has isolated CVs from several different mammalian tissues [6,7]. They contain a major protein component, clathrin, a mol. wt 180 000 polypeptide which composes the protein coat of the CV and which makes up about 70% of the total CV protein [7,8]. Two other proteins with polypeptide mol. wt 100 000 and 55 000 are associated with the CV membrane, and each comprises ~10% total CV protein [9]. Efforts to produce an anti-clathrin serum by repeated injections of either calf brain CVs or SDS-denatured clathrin purified electrophoretically on SDS–polyacrylamide gels failed to produce any detectable antibodies. We have recently found that urea-denatured clathrin, fixed with glutaraldehyde, serves as a suitable antigen for production of an antibody which reacts with clathrin and CVs in a number of immunochemical assays.

2. Materials and methods

Calf brain CVs were purified and clathrin separated from the vesicles by incubation in 2 M urea followed by centrifugation as in [8]. To the clathrin-containing supernatant (over 90% clathrin [8]) 2.5% glutaraldehyde (final conc.) was added. After a 30 min incubation at room temperature, the solution was dialyzed for 16 h against 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.0. The solution, which contained a flocculent suspension, was combined with an equal volume of Freund's complete adjuvant, emulsified and 1.0 ml portions containing 0.5 mg protein injected subcutaneously and/or intradermally into 3 adult female New Zealand rabbits. A single booster injection containing 0.25 mg protein/rabbit was prepared and injected as described above, 21 days after the first injection. 42 days after the first injection, 50 ml weekly bleeds were obtained.

A radioimmune assay for clathrin and/or CVs was performed as follows. CVs were iodinated as in [9]. A spec. act. 5.5×10^5 cpm/μg CV was obtained. The assays were carried out in duplicate, in total vol. 0.85 ml 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.4, and 0.003% gelatin. To each tube, in the order indicated, were added 0.05 ml ^{125}I-labeled brain coated vesicles (10 000 cpm); 0.05 ml anti-clathrin γ-globulin diluted 1:240 (final dilution, 1:3840); 0.05 ml of either a serially-diluted brain coated vesicle suspension, or 0.05 ml of a serially-diluted clathrin solution. After 3 h at 37°C, 0.1 ml rabbit normal serum (Clinical Assays, Cambridge, MA) diluted 1:1 with the above buffer was added, followed by 0.1 ml of anti-rabbit IgG (Clinical Assays) dissolved in the same buffer. After an additional 14 h at 4°C, the tubes were centrifuged at $3000 \times g$ for 30 min, drained, wiped dry, and counted in a Nuclear Chicago well-type gamma counter. A pair of control assays were run in the same fashion, except that the anti-CV IgG was omitted to provide a background binding value. In another pair of tubes, both the unlabeled coated vesicles and clathrin were omitted in order to obtain a value for the maximum number of counts precipitated. The conditions were chosen so that this value was about 50% counts added.

Immune precipitates of CVs from brain and IMR

90 cells were prepared as follows. Whole calf brain or human diploid lung fibroblasts grown in monolayer culture were washed in saline, homogenized in 0.14 M NaCl, 10 mM Tris–HCl, pH 7.4. The homogenates were centrifuged for 30 min at 15 000 × *g* in a Sorvall RC2B centrifuge. The supernatants were collected and adjusted to 10 mg/ml protein. 0.15 ml of each supernatant were combined with 0.15 ml rabbit anti-clathrin serum and incubated at 4°C for 48 h. Precipitates were collected after centrifugation at 3000 × *g* for 20 min a Sorvall RC2B centrifuge, washed 3 times, dissolved in 1% SDS, 0.1% mercaptoethanol, and boiled for 1 min. Supernatants incubated with identical concentrations of preimmune serum showed no precipitation. Electrophoresis and staining of gels was performed as in [10]. Methods used for immunodiffusion were as in [8].

3. Results and discussion

Sera and γ-globulin fractions from one rabbit injected with clathrin produced a strong single precipitin band of identity when tested against calf brain CVs, purified clathrin, or a whole brain 15 000 × *g* supernatant, respectively (fig.1). This figure also indicates the purified mol. wt 100 000 and 55 000 components, the only apparent contaminants [8], show no reactivity with the antibody. The second rabbit gave a weak precipitin band, while the third was negative.

We have developed a radioimmune assay (RIA) for CVs and purified clathrin. At a titer of 1:4000 the anti-clathrin serum bound ~30% of ^{125}I-labeled CVs. We could competitively prevent binding with unlabeled CVs or urea purified clathrin. The amount of protein necessary for 50% inhibition of ^{125}I-labeled CV binding (I_{50}) is 1.8 μg using either CVs or urea purified clathrin (fig.2). The competition curves are also almost superimposable. These results were rather unexpected, since steric factors would be expected to prevent many clathrin molecules in intact CVs from binding antibody. However, we have recently found that CVs when very dilute dissociate into clathrin subunits and uncoated vesicles. Hence, we are probably measuring clathrin competition in both cases.

We have determined the cross reactivity of CVs

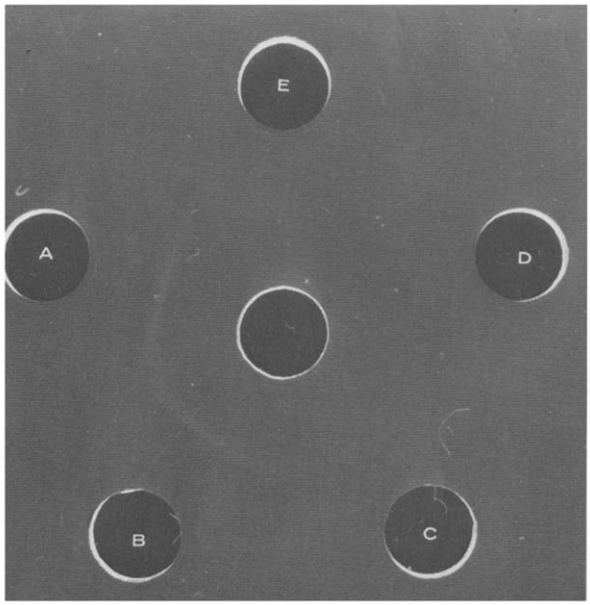

Fig.1. Double diffusion in agar, immunoprecipitate formation between anti-clathrin immunoglobulin and clathrin or CVs. The central well contained anti-clathrin immunoglobulin fraction produced by precipitation with 33% saturated $(NH_4)_2SO_4$, 5.3 mg/ml. The outer wells contained: (a) CVs in 0.1% SDS (2 μg). (b) Clathrin electrophoretically purified (2 μg). (c) A 15 000 × *g* supernatant of calf brain (100 μg). (d) 100 000 mol. wt CV protein electrophoretically purified (50 μg). (e) 55 000 mol. wt CV protein electrophoretically purified (50 μg). The outer well solutions were dissolved in 1% SDS and then dialyzed against 0.1% SDS containing 0.14 M NaCl, 10 mM Tris–HCl, pH 7.4. After 24 h at room temperature the precipitin bands which formed were photographed using indirect lighting from below.

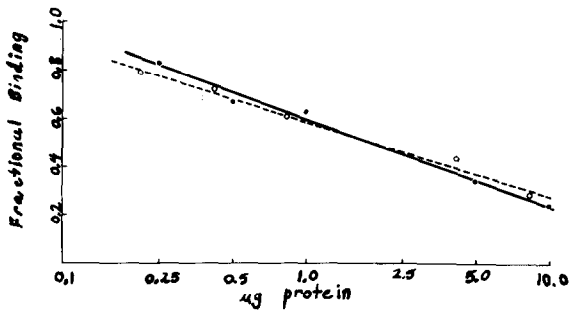

Fig.2. Competition of CVs and clathrin with ^{125}I-labeled CV using RIA.

from several sources using this assay. Purified rat brain CVs compete almost as well as do calf brain CVs (I_{50} of 2.5 μg), while purified CVs from calf adrenal medulla and parotid gland compete to a considerably smaller extent (I_{50} values of 13 μg and > 15 μg CVs, respectively). These results suggest that there are considerable immunochemical differences between clathrins from different tissues while species differences may be minimal. This result is at variance with the finding in [7] that clathrins from various tissues had very similar 1-dimensional peptide maps. It is likely, however, that the RIA is a more sensitive probe of chemical structure.

We have also used the anti-clathrin serum to precipitate CVs from crude cell extracts. We found that 0.15 ml anti-clathrin serum could quantitatively precipitate 20 μg CVs. We prepared 15 000 × *g* supernatants from calf brain and from human diploid lung fibroblasts (IMR 90). After combining the respective extracts with anti-clathrin serum, precipitates formed which were analyzed by SDS–gel electrophoresis. Figure 3 indicates that the precipitates contain major bands comigrating with clathrin, the heavy IgG chains and rabbit serum albumin (RSA), respectively. The band which comigrates with RSA probably represents nonspecific trapping of this abundant serum constituent in the immune precipitate. We also observed minor bands at mol. wt 100 000, 30 000, and 20 000 which comigrate with the CV 100 000 and 30 000 components and the IgG light chains, respectively. From comparisons of the intensities of the stained bands with those of known amounts of clathrin by densitometry, we determined that clathrin composes ~0.4% of the protein in the brain supernatant and ~0.2% in the fibroblast supernatant. Clathrin was estimated to represent ~0.1% of the protein in whole brain [6].

We feel that these results indicate that anti-clathrin serum will be a useful tool in probing the structure, distribution, quantity, and possible functions of CVs and clathrin in cells.

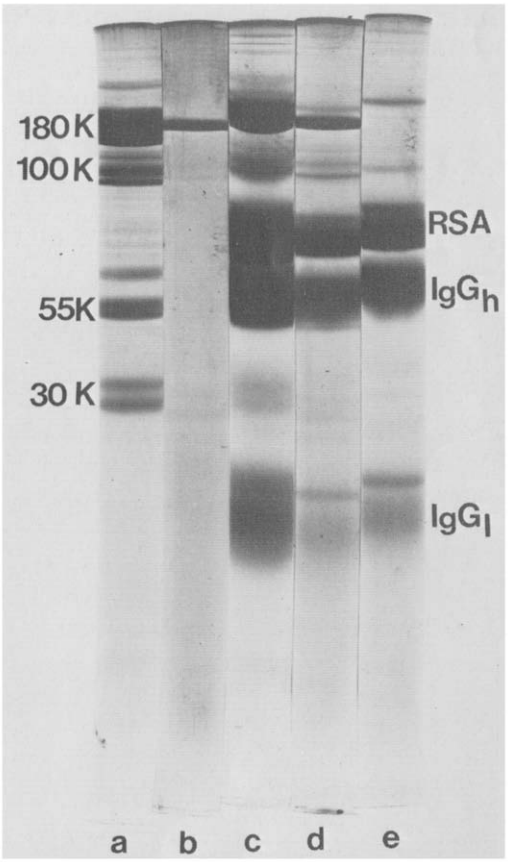

Fig.3. Polyacrylamide gels of immunoprecipitates and standard proteins. All samples were dissolved in 1% SDS, 0.1% mercaptoethanol. (a) Purified brian CVs (50 μg). (b) As in (a), (5 μg). (c) Immunoprecipitate of brain extract and anti-clathrin serum. (d) Immunoprecipitate of fibroblast extract and anti-clathrin serum. (e) Purified rabbit IgG (10 μg) + rabbit serum albumin (10 μg).

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