LOCALIZATION OF CELLULAR ANTIGENS
IN SODIUM DODECYL SULFATE-POLYACRYLAMIDE GELS

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ABSTRACT

A procedure is described for localizing antigen-antibody complexes in sodium dodecyl sulfate (SDS) polyacrylamide gels using 125I-labeled protein A from Staphylococcus aureus. We use the procedure to probe antigenic cross-reactivities between Strongylocentrotus and Chlamydomonas alpha- and beta-tubulins; we also demonstrate how the procedure can detect minor antibody species in an antiserum directed against a cell membrane.

KEY WORDS 125I-protein A · antigen-antibody complexes · autoradiography · tubulin · membranes

The characterization of cellular components using immunological approaches has been hampered by the fact that many polypeptides of interest (e.g., tubulin, actin) are poor antigens, eliciting weak and/or nonprecipitating antisera that are often difficult to analyze. Moreover, many structures of interest (e.g., cell membranes) carry a complex array of components, some more antigenic than others. Antisera raised against intact membranes therefore contain a complex array of antibody species at varying titers, and such antisera have heretofore been of limited experimental value.

We report here a simple, rapid, and highly sensitive method, which utilizes 125I-labeled protein A from Staphylococcus aureus for analyzing antisera against cellular components. We then show how the procedure can be applied to basic biological questions, presenting experiments which demonstrate interspecific relationships between alpha- and beta-tubulin polypeptides and experiments which reveal both the major and minor polypeptides of a cell surface membrane.

MATERIALS AND METHODS

Protein A (Pharmacia Inc., Piscataway, N. J.) was labeled with 125I (New England Nuclear, Boston, Mass.) by the chloramine-T method of Greenwood et al. (5) except that the chloramine-T was added in four aliquots at 15-s intervals. The labeled product (sp act = 1.5 x 10^6 cpm/µg protein) was separated on a G-50 Sephadex column pre-run with 20 mg of bovine serum albumin. Greater than 60% of the radioactivity was covalently bound to protein A under these conditions, and recovery from the column was essentially complete.

Sea urchin sperm tail axonemes and antitubulin antisera (raised in rabbits against vinblastine sulfate crystals of sea urchin Strongylocentrotus egg cytoplasmic tubulin [4]) were generous gifts of Dr. K. Fujiwara (Harvard Medical School, Boston, Mass.). Flagella were isolated and purified from Chlamydomonas reinhardii gametes by the pH-shock procedure of Witman et al. (12). Antiserum against C. reinhardii mt+ gametic flagella (alpha-G+) was raised in a rabbit by standard procedures; details will be described elsewhere (U. W. Goodenough and D. Jurivich, J. Cell Biol., in press).

Samples for gel electrophoresis were solubilized in lysis buffer [3% wt/vol sodium dodecyl sulfate (SDS), 5% vol/vol 2-mercaptoethanol, 10% glycerol, 0.01% wt/vol bromphenol blue, 6.25 mM Tris-HCl, pH 6.8] and boiled for 2 min before loading. Electrophoresis was carried out in slab gels (1 mm x 1 cm x 18 cm) as described in the figure legends, using the discontinuous buffer system of Laemmli (9). After electrophoresis, gels were usually treated for 5-20 min with the reversible stain xylene brilliant cyanin 6 (1) to visualize the overall quality of the gel and to facilitate slicing the gel into individual lanes with a pizza cutter. Gel lanes were then completely destained and fixed with 25% isopropanol-10% acetic acid overnight with two changes. Elimination of the cyanin staining step has no effect on the results. Before incubation with antisemur, the lanes were washed several times with distilled water and equilibrated with several changes of incubation buffer (50 mM Tris, pH
For incubation with specific antisera or pre-immune control sera, lanes were transferred to individual plastic troughs (18 x 1 x 0.5 cm) and overlayed with antisera diluted with incubation buffer (dilutions are given in the figure legends). Troughs were placed on wet paper towels in a glass baking dish which was then covered with Saran Wrap to prevent desiccation. Incubations were carried out for 12 h at room temperature with gentle agitation.

After incubation in antisera, lanes were removed from the troughs, rinsed with distilled water, and washed for at least 3 h, with several changes, in 50 ml of incubation buffer. They were then returned to their troughs, overlaid with 5 ml of buffer containing 125I-protein A (1.2 x 10^7 cpm), and incubated for an additional 12 h.

At the completion of the protein A incubations, lanes were washed several times as above and, in experiments requiring a permanent record of the stained polypeptide bands present in the sample, the gels were incubated for 3 h in 0.05% Coomassie Blue, 25% isopropanol, 10% acetic acid, destained for 30 min with 10% isopropanol, 10% acetic acid, and destained an additional 4 h in 10% acetic acid. Elimination of this staining step was also without effect on the results. The lanes were then dried down on Whatman no. 1 filter papers in a glass baking dish which was then covered with Kodak NO-Screen medical X-ray film. Radioautographs were developed for 5 min at 25°C on the antiserum) on Kodak D-19 developer and postfixed for 10 min with Kodak rapid fix.

Ouchterlony double-immunodiffusion was carried out in 5-cm disposable culture dishes (BioQuest, BBL & Falcon Products, Becton Dickinson & Co., Cockeysville, Md.) coated with 2.5 ml of a 1% agarose solution in phosphate-buffered saline. Wells were created with a micropipette tip connected to an aspirator. Isolated flagella were suspended in a small volume of 5% sucrose in 10 mM Tris, pH 7.0. To this was added ¼ vol of a 1% solution of Triton X-100 in STEEP buffer (12) to yield a final 0.25% Triton extract. Such Triton extracts served as antigen in the Ouchterlony assays.

RESULTS AND DISCUSSION

Features of the Technique

As detailed in Materials and Methods, the procedure involves three steps. (a) An SDS-polyacrylamide gel carrying the antigen(s) in question is first fixed with isopropanol-acetic acid, washed to remove SDS and adjust the pH, incubated in antiserum, and washed to remove all unreacted antibodies. (b) The gel is then incubated in 125I-labeled protein A. Protein A, a cell-wall component of S. aureus, binds specifically to the Fe regions of immunoglobulins (7, 8, 11); therefore, any antigen-antibody complexes present in the gel will form tertiary complexes with the 125I-protein A. (c) Finally, the gel is washed to remove all unreacted 125I-protein A and is subjected to autoradiography to reveal the location of radiolabeled bands.

The technique is similar to approaches using radiolabeled antiglobulin (e.g., rabbit antiserum followed by labeled goat anti-rabbit IgG [2]) but has several distinct advantages. Protein A can be labeled to high specific activity with chloramine-T without loss of its antibody-recognition properties. Moreover, because a single protein species is being labeled, no purification steps are needed and isotope use is highly efficient. Finally, a single preparation of 125I-protein A can be used to analyze and compare antisera derived from numerous animal species (8); it is not necessary to prepare different species-specific antiglobulins.

Tubulin Experiments

Fig. 1 illustrates the specificity and resolution of the procedure. The gel in Fig. 1a, stained with Coomassie Blue, displays the numerous polypeptides present in sea urchin sperm tail axonemes (10), the two prominent polypeptides being α- and β-tubulin. Fig. 1b shows an unstained companion gel which was incubated with antitubulin antisera, then 125I-protein A, and subjected to autoradiography. Antibody binding to both α- and β-tubulin subunits is observed in equivalent proportions, the β-species possibly binding slightly less than the α. Faint bands in the lower portion of the gel may represent antibodies to tubulin-associated components present both in the vinblastine crystals and in the flagellar axonemes, although breakdown of tubulin polypeptides cannot be ruled out. The specificity of the antitubulin binding is shown in Fig. 1c, where a second companion gel has been treated as above except that the antitubulin was replaced by a pre-immune serum. In this control gel, no radiolabeled bands are found.

Immunological cross-reactivity can provide valuable information about the relatedness of polypeptides. Fig. 2 illustrates that the protein-A procedure is sufficiently sensitive and selective to examine such relationships in SDS gels. The gel in Fig. 2a, stained with Coomassie Blue, displays the numerous polypeptides present in whole flagella of the unicellular alga, C. reinhardtii. Prominent are the α- and β-tubulin bands, plus a group of flagellar membrane polypeptides near the top
extant tubulins. The approach could clearly also be applied to other polypeptides. Although we have not performed such analyses in the present study, it should also be possible to quantitate the density of label in parallel samples using a gel-scanning device.

**Cell-Membrane Experiments**

Fig. 3 illustrates how the procedure can serve to characterize cell membranes, and compares its sensitivity with two other immunological techniques. Used throughout is a rabbit antiserum raised against intact flagella isolated from *C. reinhardtii* mt§ gametes. When this antiserum is presented to Triton extracts of *Chlamydomonas* flagella in Ouchterlony gels, a relatively simple pattern is encountered (Fig. 3a): two sharp and one diffuse precipitin line can be resolved. When the same antiserum is incubated with SDS-polyacrylamide gels of intact flagella and then with fluorescein-labeled goat-anti-rabbit antiserum, an apparently simple pattern is again obtained (Fig. 3b):...
FIGURE 3 Characterization of α-G⁺ antiserum (raised against isolated gametic mt⁺ flagella of C. reinhardtii) by three procedures: (a) ouchterlony double immunodiffusion analysis. The center well contains undiluted α-G⁺; outer wells carry various dilutions of gametic mt⁺ and mt⁻ Triton flagellar extract. (b) Fluorescent antiglobulin detection of immune complexes. Top tracing: Scan (550 nm) of gametic flagellar polypeptides separated on a 4-8% linear gradient gel and stained with Coomassie Blue. Middle tracing: Scan (495 nm excitation, 525 nm emission) of a companion gel equilibrated with incubation buffer, incubated with α-G⁺ for 12 h, washed, incubated for 12 h in FITC-conjugated goat-anti-rabbit IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) and washed for 2 days. α-G⁺ dilution was 1:50; anti-IgG dilution was 1:100. Bottom tracing: Fluorescent scan (see above) of a second companion gel in which pre-immune serum was substituted for α-G⁺. (c) ¹²⁵I-protein A detection of immune complexes. A gel slice containing mt⁺ gametic flagellar polypeptides (see Fig. 2b) was incubated in α-G⁺, and then in ¹²⁵I-protein A as described in Materials and Methods. The α-G⁺ dilution was 1:50 and gel exposure was 3 days.

near the top of the gel, where the prominent flagellar membrane polypeptides migrate (cf. Fig. 2a), a single major and several minor antigen peaks are resolved. The additional “blips” evident in the lower molecular weight regions of the gel scan cannot be distinguished from background noise by this procedure. In contrast, when antiserum-incubated gels are subjected to the ¹²⁵I-protein A assay and exposed to X-ray film for 72 h, a number of discrete minor bands become evident in lower portions of the gel (Fig. 3c), none of which appear in pre-immune serum controls. The
slowly migrating antigens remain most prominent and are, in fact, the only bands visible when such a gel is exposed to X-ray film for only 12 h. The technique, in other words, permits a direct identification of both strong and weak antigens present on the surface of isolated flagella.

The antigenic flagellar polypeptides visualized by the protein-A assay in Fig. 3c correspond in position to polypeptides that stain for carbohydrate by the periodic acid-Shiff procedure (3); polypeptides with the same electrophoretic mobilities are also surface-labeled by the [125I]lactoperoxidase procedure (6) (our unpublished experiments). Therefore, it appears that the full spectrum of flagellar surface glycopolypeptides in Chlamydomonas is at least weakly antigenic to rabbits, with the high molecular weight major antigens dominating most modes of antiserum characterization. Such information about the antigens present in a biological membrane permits numerous lines of experimentation. Patterns obtained with normal membranes can, for example, be compared with mutant membranes, enzyme-treated membranes, or membranes from pathogenic cells. Indeed, a "crude antiserum" can be transformed into a valuable probe of membrane structure.

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