LOCALIZATION OF CELLULAR ANTIGENS IN SODIUM DODECYL SULFATE-POLYACRYLAMIDE GELS

W. STEVEN ADAIR, DONALD JURIVICH, and URSULA W. GOODENOUGH. From The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT

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

KEY WORDS ^{125}I-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 ^{125}I-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 α- and β-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 ^{125}I (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 = 1.5×10^5 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 reinhardi* gametes by the pH-shock procedure of Witman et al. (12). Antiserum against *C. reinhardi mt*$^+$ gametic flagella (α-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 × 1 cm × 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 antiserum, the lanes were washed several times with distilled water and equilibrated with several changes of incubation buffer (50 mM Tris, pH

7.4, 0.15 M NaCl, 0.1% sodium azide) (2) for at least 3 h.

For incubation with specific antisera or pre-immune control sera, lanes were transfered to individual plastic troughs (18 × 1 × 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 ^{125}I-protein A (1.2×10^7 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 stainable 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 paper and autoradiographed for 2 h–5 days (depending on the antiserum) on Kodak NO-Screen medical X-ray film. Radioautographs were developed for 5 min at 25°C with 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 1/4 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 ^{125}I-labeled protein A. Protein A, a cell-wall component of *S. aureus*, binds specifically to the Fc regions of immunoglobulins (7, 8, 11); therefore, any antigen-antibody complexes present in the gel will form tertiary complexes with the ^{125}I-protein A. (*c*) Finally, the gel is washed to remove all unreacted ^{125}I-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 ^{125}I-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. 1*a*, 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. 1*b* shows an unstained companion gel which was incubated with antitubulin antisera, then ^{125}I-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. 1*c*, 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. 2*a*, stained with Coomassie Blue, displays the numerous polypeptides present in whole flagella of the unicellular alga, *C. reinhardi*. Prominent are the α- and β-tubulin bands, plus a group of flagellar membrane polypeptides near the top

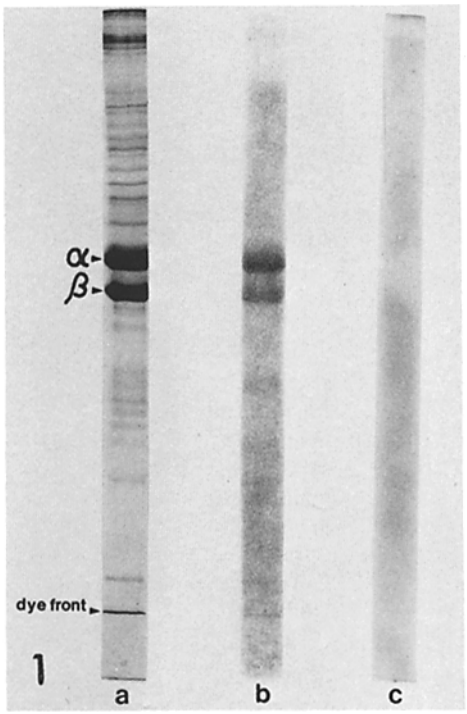

FIGURE 1 Detection of sea urchin tubulin-antitubulin complexes in an SDS-polyacrylamide gel. Sea urchin axonemal polypeptides were separated on a 7.5% polyacrylamide gel. (*a*) Coomasie Blue; (*b*) antitubulin/^{125}I-protein A; (*c*) pre-immune serum/^{125}I-protein A. Lanes *b* and *c* were exposed for 4 days. Antibody dilutions were 1:10 in incubation buffer.

of the gel (12). Fig. 2*b* shows an identical unstained gel, incubated in the same sea urchin antitubulin antiserum as was used in Fig. 1*b* and then subjected to ^{125}I-protein A and autoradiography; Fig. 2*c* shows a pre-immune serum control. It is evident that the *Chlamydomonas* α-tubulin associates far more avidly with the sea urchin antiserum than does the *Chlamydomonas* β-tubulin. It can also be shown, by exposing parallel gels to X-ray film for comparable periods of time, that a given amount of α-tubulin from sea urchin flagella binds an equivalent quantity of sea urchin antibody (and hence ^{125}I-protein A) as does the α-tubulin of *Chlamydomonas* flagella. It is likely, therefore, that the α-tubulins have not diverged significantly in the alga-to-sea urchin interval but that the β-tubulins have, a conclusion we plan to test by peptide mapping. Presumably, repetition of this kind of experiment with numerous tubulin and antitubulin combinations could be used to probe evolutionary relationships between 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. reinhardi* mt^+ gametes. When this antiserum is presented to Triton extracts of *Chlamydomonas* flagella in Ouchterlony gels, a relatively simple pattern is encountered (Fig. 3*a*): 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. 3*b*):

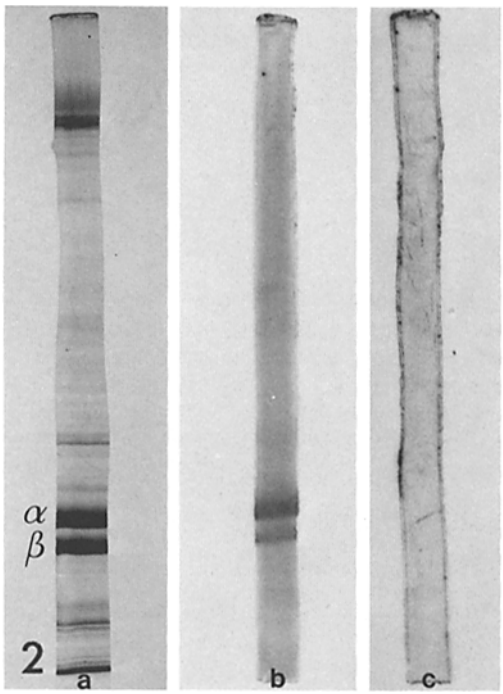

FIGURE 2 Association of sea urchin antitubulin with *C. reinhardi* α- and β-tubulin subunits. Polypeptides from gametic mt^+ flagella were separated on a 4–8% linear gradient gel. (*a*) Coomassie Blue; (*b*) anti-tubulin/^{125}I-protein A; (*c*) preimmune serum/^{125}I-protein A. Exposure and antiserum dilutions as in Fig. 1.

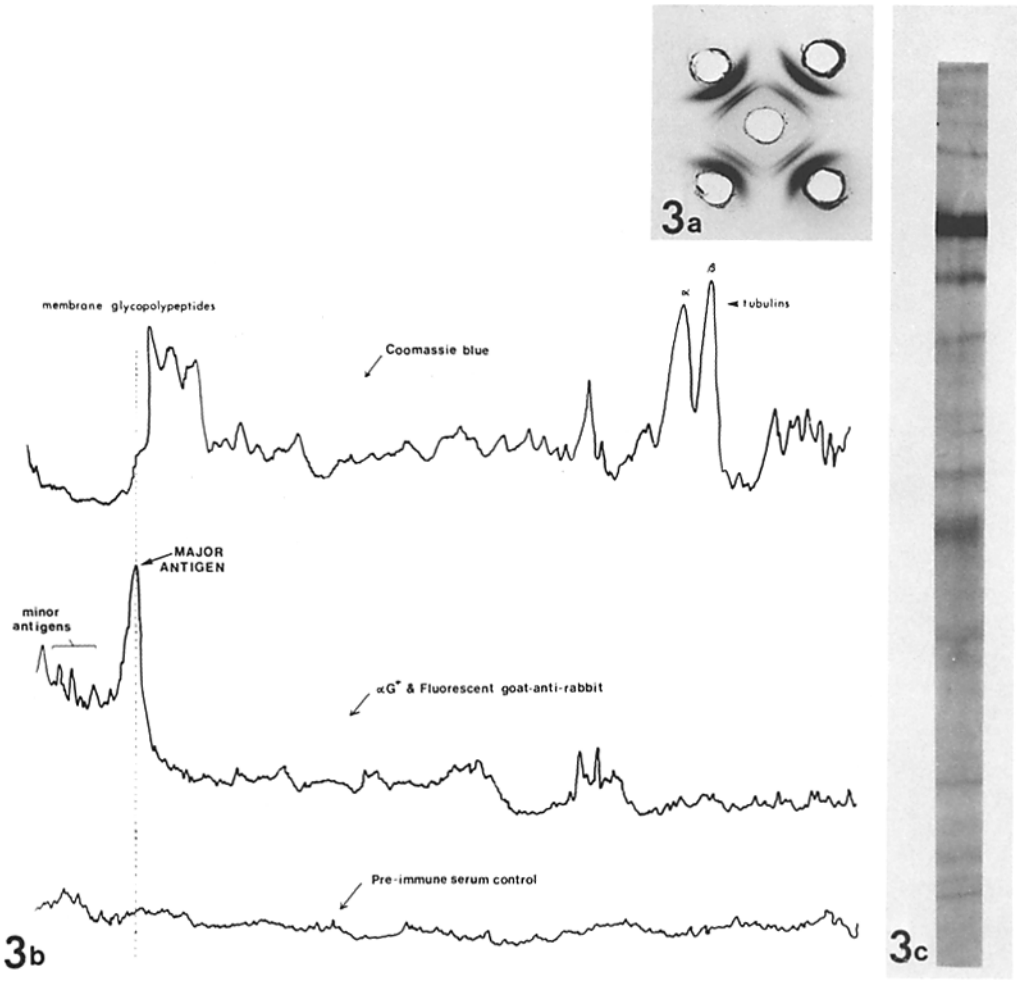

FIGURE 3 Characterization of α-G$^+$ antiserum (raised against isolated gametic *mt*$^+$ flagella of *C. reinhardi*) by three procedures: (*a*) ouchterlony double immunodiffusion analysis. The center well contains undiluted α-G$^+$: outer wells carry various dilutions of gametic *mt*$^+$ and *mt*$^-$ Triton flagellar extract. (*b*) Fluorescent antiglobulin detection of immune complexes. Top tracing: Scan (550 nm) of *mt*$^+$ 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: Fluorescnet scan (see above) of a second companion gel in which pre-immune serum was substituted for α-G$^+$. (*c*) ^{125}I-protein A detection of immune complexes. A gel slice containing *mt*$^+$ gametic flagellar polypeptides (see Fig. 2*b*) was incubated in α-G$^+$, and then in ^{125}I-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. 2*a*), 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 ^{125}I-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. 3*c*), 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. 3*c* 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 [^{125}I]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.

This work was supported by grant GM 23058 from the National Institutes of Health.

Received for publication 19 June 1978, and in revised form 27 July 1978.

REFERENCES

1. Blakesley, R. W., and J. A. Boezi. 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue 6250. *Anal. Biochem.* **82**:580–582.
2. Burridge, K. 1976. Changes in cellular glycoproteins after transformation: Identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels. *Proc. Natl. Acad. Sci. U. S. A.* **73**:4457–4461.
3. Fairbanks, G., T. L. Steck, and D. F. W. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* **10**:2606–2617.
4. Fujiwara, K., and T. D. Pollard. 1976. Simultaneous intracellular localization of myosin and tubulin during cell cycle by direct double immunofluorescence. *J. Cell Biol.* **70** (Abstr.):181*a*.
5. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114–123.
6. Hogg, N. M. 1974. A comparison of membrane proteins of normal and transformed cells by lactoperoxidase labeling. *Proc. Natl. Acad. Sci. U. S. A.* **71**:489–492.
7. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a *Staphylococcal* protein A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617–1624.
8. Kronvall, G., U. S. Seal, J. Finstad, and R. C. Williams, Jr. 1970. Phylogenetic insight into evolution of mammalian Fc fragment of G globulin using staphylococcal protein A. *J. Immunol.* **104**:140–147.
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophaghe T4. *Nature (Lond.).* **227**:680–685.
10. Linck, R. W. 1976. Flagellar doublet microtubules: Fractionation of minor component proteins and α-tubulin from specific regions of the A-tubule. *J. Cell Sci.* **20**:405–440.
11. Peterson, P. A., L. Rask, K. Sege, L. Klareskog, H. Anundi, and L. Ostberg. 1975. Evolutionary relationship between immunoglobulins and transplantation antigens. *Proc. Natl. Acad.Sci. U. S. A.* **72**:1612–1616.
12. Witman, G., B. K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, mastigonemes, and membranes. *J. Cell Biol.* **54**:507–539.