USE OF THE AVIDIN-BIOTIN COMPLEX FOR THE LOCALIZATION OF ACTIN AND MYOSIN WITH FLUORESCENCE MICROSCOPY

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In the course of studies on the ultrastructural localization and functions of contractile proteins in nonmuscle cells, we have found it useful to adapt the avidin-biotin complex technique introduced by Heitzmann and Richards (7) to the specific labeling of actin and myosin components in these cells. These authors modified cell surfaces by covalent attachment of biotinyl residues, and then visualized these residues in electron microscopy by staining with a ferritin-avidin conjugate, thereby making use of the extraordinarily high binding affinity of biotin for avidin (5). We have used this general procedure to develop two specific fluorescence staining procedures, one for intracellular actin, the other for myosin. The actin procedure involves the successive reaction with biotin-labeled heavy meromyosin (HMM) followed by fluorescein-labeled avidin, which in our hands has given results superior to those obtained with the use of direct fluorescein-labeled HMM as described by Sanger (11). The myosin labeling procedure involves the successive reaction with biotin-labeled antimyosin antibody followed by fluorescein-labeled avidin, which provides an alternative and comparably effective staining procedure to the usual indirect immunofluorescence technique.

MATERIALS AND METHODS

Muscle Proteins

Actin was purified from acetone powders of rabbit back muscle by the method of Spudich and Watt (14). HMM was prepared from rabbit skeletal muscle myosin (15) by a limited tryptic digestion as described by Lowey et al. (10). These proteins were stored in 50% glycerol at −20°C. Rabbit myofibrils were prepared by the procedure of Zak et al. (18).

Biotin-N-Hydroxysuccinimide Ester (BOSu)

This reagent was synthesized by the method of Heitzmann and Richards (7), and was stored over dessicant at 4°C. It was found to be stable for at least 12 mo. Radioactive BOSu (6.4 × 10^4 cpm/μmol) was prepared by adding [14C]biotin (Amersham/Searle Corp., Arlington Heights, Ill.) to the biotin used for the synthesis.

Biotin-Labeled HMM

4 mg of fibrous actin and 4 mg of HMM were dialyzed separately overnight against 0.15 M KCl in 0.01 M potassium phosphate buffer, pH 7.5 (PBK). The two protein solutions were combined, so as to protect the actin-binding site of HMM during the chemical modification (11). The volume was adjusted to 7.5 ml with PBK, and this solution was brought to room temperature. The pH of the protein solution was then raised to 8.8 by the addition of 2.5 ml of a buffer containing 0.2 M NaHCO₃ and 0.15 M KCl, pH 8.8. The reaction was initiated by adding 5 μl (preparation A) or 8 μl (preparation B) of a freshly prepared solution of BOSu (12 mg/ml) in dimethylformamide. The solution was mixed gently and allowed to react for 5 min with periodic gentle stirring. The reaction was then quenched by the addition of 50 μl of 1 M NH₄Cl (pH 6). The modified acto-HMM was pelleted by centrifugation for 1 h at 180,000 g. This pellet was recovered, suspended in 2 ml of PBK containing sodium pyrophosphate (0.01 M) and MgCl₂ (0.002 M) (to dissociate the actin-HMM bond), and homogenized with four strokes in a glass homogenizer. This solution was then centrifuged for 1 h at 180,000 g to sediment the actin into a pellet. The supernate, containing the modified HMM (BHMM) product, was dialyzed against at least three 1-liter volumes of PBK.

Biotin-Labeled Antibody

The preparation of rabbit anti-human uterine myosin IgG has been described previously (12). A 0.4-ml solution of the IgG fraction (6.3 mg/ml) in PBK was mixed with 0.1 ml of 0.2 M NaHCO₃ in 0.15 M KCl (pH 8.8).
at room temperature. The reaction was initiated by adding 5 μl of a 12 mg/ml solution of BOSu in dimethylformamide with gentle mixing at room temperature. After 10 min the reaction was quenched by the addition of 50 μl of 1 M NH₄Cl and the product dialyzed for at least 3 days against several changes of PBK.

**Fluorescein Labeled Avidin (Fl-Avidin)**

4.5 mg of avidin (Sigma Chemical Co., Saint Louis, Mo.) was dissolved in 0.9 ml of PBK, and to this was added 0.4 ml of 0.2 M NaHCO₃ in 0.15 M NaCl (pH 8.8). Fluorescein isothiocyanate on Celite (4 mg of 8% powder, Calbiochem, San Diego, Calif.) was added in suspension, and the mixture was incubated at room temperature with constant stirring for 10 min. The reaction was then quenched by the addition of 200 μl of 1 M NH₄Cl (pH 6.0), the Celite was removed by centrifugation, and the Fl-avidin solution was dialyzed for 2 days against several changes of PBK to remove free dye. The Fl-avidin was stored with 0.02% sodium azide at 4°C and was stable for at least 3 mo.

**Cell Culture**

The cell line WI 38 was obtained from the American Type Culture Collection (Rockville, Md.). The NRK cells were provided by Dr. Peter Vogt. All cells were cultured in Dulbecco’s modified Eagle’s Medium containing 10% fetal calf serum and antibiotics under an atmosphere of 10% CO₂-90% air.

**Fluorescence Staining of Cells**

Cells were plated onto glass cover slips at low densities and cultured for at least 1 day before use. Cells were fixed with 2% formaldehyde in PBK for 20 min at room temperature. After fixation they were rinsed with three 10-min changes of PBK, the second change containing 10 mM NH₄Cl. The cells were then made permeable to proteins by freezing and thawing them once (2). For actin localization the cells were then incubated with a BHMM solution (in PBK) in the concentration range of 0.05-0.2 mg/ml for 20 min at room temperature. For myosin localization the biotinyl antimyosin IgG at 2-3 mg/ml was used for 20 min. In either case, the cells were then rinsed with four changes of PBK for a total of 20 min to wash out the unbound biotinyl protein and then incubated for 20 min in 0.06 mg/ml Fl-avidin in PBK. After a final wash with PBK for 20 min the cover slips were inverted on a drop of 90% glycerol-10% PBK. The cells were observed and photographed on a Zeiss photomicroscope, using either a dark field condenser or epi-illumination.

**Other Methods**

The actin-activated ATPase activity of the modified (prep. A) and unmodified HMM was measured by the method of Spudich (13). The EDTA-stimulated ATPase activities were measured by this assay as modified by Ash (1). Protein concentrations were determined by the method of Lowry et al. (9) as modified by Hartree (6). Polyacrylamide gel electrophoresis on 4% gels in the presence of sodium dodecyl sulfate was accomplished by the method of Fairbanks et al. (3).

**RESULTS**

**BHMM**

Figure 1 shows a sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of HMM before and after modification with biotin. The BHMM is free of actin contamination as analyzed by this technique. The degree of biotin substitution of two different preparations of BHMM and their stoichiometry of binding with Fl-avidin are summarized in Table I. As demonstrated in Table I, the modification of HMM does not impair its actin binding activity. By measuring the molar ratio of fluorescein to BHMM in an acto-BHMM-Fl-avidin complex rather than in a BHMM-Fl-avidin complex, we hoped to obtain a more accurate estimate of the binding ratio that would result when staining fibrous actin in situ.

![Polyacrylamide gel electrophoresis on 4% gels in the presence of sodium dodecyl sulfate of: (a) rabbit HMM, (b) BHMM (prep. A), and (c) actin.](image)
### TABLE I

| Stoichiometry of F1-Avidin-BHMM Complex | Prep. A | Prep. B |
|-----------------------------------------|--------|--------|
| No. of biotin molecules per HMM molecule | 8.5    | 12     |
| No. of avidin molecules bound by each HMM molecule | 4.5    | 7.2    |
| Molar ratio of fluorescein to BHMM in the acto-BHMM-F1-avidin complex | 13.2   | 20     |

* See Materials and Methods.

† Determined by using radioactive biotin.

‡ A 1-ml solution containing 0.2 mg of BHMM and 0.2 mg of actin in PBK was added to 1 ml of PBK containing 0.4 mg of F1-avidin. The acto-BHMM-F1-avidin complex was centrifuged into a pellet, and the amount of F1-avidin removed from the solution was determined from the reduction in absorbance at 495 nm (the absorption maximum of fluorescein bound to protein) of the supernate. All of the BHMM was removed with the actin as determined by three criteria: (a) the \( A_{495}/A_{280} \) ratio of the supernatant fraction was identical to that of F1-avidin alone, (b) no detectable \(^{14}C\) biotin radioactivity remained in the supernate, and (c) no BHMM was detected by gel electrophoretic analysis of the supernatant fraction. When this experiment was performed in the presence of biotin (0.02 mg/ml), less than 4% of the F1-avidin was removed by centrifugation, although all of the BHMM was pelleted with the actin.

§ The F1-avidin had an \( A_{495}/A_{280} \) ratio of 1.2. From the extinction coefficient of fluorescein (4) and the protein concentration, it was determined that an average of 2.9 fluorescein molecules were bound to each avidin molecule.

The actin-activated ATPase activities of BHMM (prep. A) and the HMM from which it was made were identical. The high-salt EDTA-stimulated ATPase activity of this BHMM was 0.42 \( \mu \text{mol} \text{ Pi/min/mg protein} \) compared to 0.52 \( \mu \text{mol} \text{ Pi/min/mg protein} \) for the unmodified HMM. These results suggest that the active site of the HMM was not seriously affected by modification with biotin under the protective conditions used.

Cells stained with BHMM and F1-avidin showed an array of clear and distinct filaments (Figs. 2 and 3). In many cases, these fluorescent filaments corresponded to the stress fibers seen with Nomarski differential contrast optics (Fig. 3). Also, as seen in Fig. 3, there was intense staining near the edges of cells in areas of ruffled membrane activity.

Cells stained in the presence of magnesium and pyrophosphate (see Fig. 2b), which prevents the binding of HMM to actin, were only dimly fluorescent. There were no filaments visible, although there was some staining of the cytoplasm and of small bodies within the nucleus. Similarly, only this dim background staining was observed if the F1-avidin was presaturated with free biotin (0.1 mg/ml) to prevent its binding to the BHMM (Fig. 2c). Cells treated with F1-avidin alone, without pretreatment with BHMM, had this same appearance, although, in cells undergoing mitosis, the F1-avidin was found to bind to the condensed chromosomes. This observation will be reported elsewhere. This low level staining with F1-avidin can be eliminated by competition with unmodified avidin.

Myofibrils stained with BHMM and F1-avidin showed intense staining of the I-band region with the Z-band region clearly unstained (Figure 2e). Myofibrils were not stained if the BHMM was added in the presence of magnesium and pyrophosphate.

Biotinyl Antibodies

Table II summarizes the degree of biotin substitution and F1-avidin binding properties of the biotinyl IgG. The molar binding ratios shown in Table II were derived from a precipitated complex of biotinyl IgG and F1-avidin. They may underestimate the ratios which occur in the fluorescent staining of cells. Cells stained with the biotinyl antimyosin antibody showed bright filamentous staining (Fig. 4). Presaturation of the F1-avidin with free biotin prevented this specific staining.

DISCUSSION

The results of experiments with the BHMM-F1-avidin reagents for actin localization are comparable to those obtained by Lazarides and Weber using a fluorescent-labeled antibody to actin (8). Our method for the localization of actin by means of HMM has two new features: (a) fixation of the cells before staining which most likely preserves structure more adequately than glycerol extraction, and (b) the amplification of the fluorescence afforded by this indirect method.

The results with the biotinyl antimyosin antibodies and F1-avidin were indistinguishable from those obtained with indirect immunofluorescence (16, 17). These results demonstrate the general usefulness of the avidin-biotin complex for localization with the fluorescence microscope, and they provide an alternative to the use of a second antibody for the amplification of the fluorescent sig-

1 Heggeness, M. H. 1977. Manuscript in preparation.
FIGURE 2 Examples of BHMM-Fl-avidin staining for actin and controls. (a) An NRK cell stained with BHMM and Fl-avidin to localize actin. In Fig. 2b–d NRK cells are stained under various control conditions, photographed and printed as in Fig. 2a. The cytoplasm adjacent to the nucleus, which contained the only detectable staining, is shown. (b) An NRK cell stained in the presence of sodium pyrophosphate (0.005 M) and magnesium chloride (0.002 M) to prevent the binding of BHMM to the cellular actin. (c) An NRK cell stained in the presence of biotin (0.1 mg/ml) to prevent the binding of Fl-avidin to the BHMM. (d) An NRK cell stained with BHMM and Fl-avidin after preincubation with unmodified HMM. (e) A rabbit skeletal muscle myofibril stained for actin. Bars, 10 μm.

FIGURE 3 An NRK cell stained for actin with BHMM and Fl-avidin (a) Image obtained with Nomarski optics. (b) Fluorescence image. Arrows indicate regions of ruffled membrane activity. Bars, 10 μm.
FIGURE 4 A W138 cell stained for myosin by using Biotinyl antimyosin and Fl-avidin. Bar, 10 μm.

| Table II | Stoichiometry of Fl-Avidin Biotinyl IgG Complex |
|----------|-----------------------------------------------|
| Exp 1    | Exp 2  |
| No. of biotin molecules per IgG molecule* | 7.8 | 7.8 |
| No. of avidin molecules bound by each IgG molecule† | 3.8 | 4.0 |
| Molar ratio of fluorescein to IgG in complex‡ | 11.1 | 11.7 |

* Determined by using radioactive biotin.
† 25 μl of biotinyl antimyosin IgG (4.5 mg/ml in PBK) was added to 1.0 ml of a 0.4 mg/ml solution of Fl-avidin in PBK. As measured by the radioactive biotin, 79% of the biotinyl IgG was precipitated by the Fl-avidin after 45 min at 0°C. The amount of Fl-avidin in this precipitate was calculated from the amount of fluorescein removed from the solution, as assayed by the change in absorbance at 495 nm. When this experiment was performed in the presence of biotin (0.02 mg/ml), no precipitation was observed.

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