Isolation of a high molecular weight actin-binding protein from baby hamster kidney (BHK-21) cells

(Actin crosslinker/cultured cells/microfilament organization)

JEFFERY A. SCHLOSS† AND ROBERT D. GOLDMAN‡

Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213

Communicated by David Marshall Prescott, June 7, 1979

ABSTRACT A high molecular weight protein (HMWP) with properties similar to those of both actin-binding protein (ABP) and filamin has been isolated from cultured baby hamster kidney (BHK-21) cells. The protein was present in an actomyosin-depleted sucrose extract of the cells and was eluted, upon gel chromatography on Sepharose 4B, near the void volume. The subunit migration on sodium dodecyl sulfate/polyacrylamide gels and the amino acid composition of HMWP were similar to those of ABP and filamin. HMWP bound to and crosslinked F-actin from rabbit muscle, as shown by its formation of a high molecular weight gel. HMWP has been isolated from cultured baby hamster kidney (BHK-21) cells. HBMWP has chemical properties similar to those of ABP and filamin, and induces the gelation and crosslinking of muscle F-actin.

MATERIALS AND METHODS

Cell Culture. Baby hamster kidney (BHK-21/C13) cells were grown in roller bottles and harvested as described (23).

Isolation of BHK HMWP. Actomyosin was prepared as described by Yerna et al. (23). The supernatant remaining after actomyosin was pelleted was centrifuged for 3 hr at 100,000 × g. Subsequent purification steps were based on procedures described for isolation of gizzard filament (7, 8). To the clarified supernatant, ammonium sulfate was added to 55% saturation, at pH 7.5. The precipitate was collected and dialyzed against 0.5 mM EDTA/0.2 mM dithiothreitol/50 mM potassium phosphate, pH 7.5 (4B buffer). The sample was applied to a 2.6 × 95 cm column of Sepharose 4B (Pharmacia) equilibrated in 4B buffer. If cells from more than 30 roller bottles were used, the sample was divided so as not to exceed a 30-ml equivalency of protein per column run. Fractions were collected at a flow rate of 20 ml/hr. Fractions from the descending portion of the large first peak (which included the void volume) were applied to a NaDodSO4/polyacrylamide slab gel to determine which fractions contained the desired protein. Appropriate fractions were pooled, dialyzed against 0.5 mM EDTA/0.2 mM dithiothreitol/20 mM potassium phosphate, pH 7.5 (DEAE buffer), and applied to a 2.6 × 10 cm column of DEAE-cellulose (for 60 roller bottles) (DE-52, Whatman) that had been equilibrated with DEAE buffer. After washing with 100 ml of DEAE buffer, protein was eluted with 500 ml of a linear gradient consisting of 0-0.4 M NaCl in DEAE buffer, at a flow rate of 40 ml/hr. The first major peak (75-110 mM NaCl) was monitored by NaDodSO4/polyacrylamide slab gel electrophoresis to determine which fractions were enriched for HMWP. These fractions were dialyzed against DEAE buffer and concentrated by application to a 1.5 × 3 cm DE-52 column.

Abbreviations: ABP, actin-binding protein; NaDodSO4, sodium dodecyl sulfate; HMWP, high molecular weight protein; BHK-21, baby hamster kidney cell line 21/c13; S-1, heavy meromyosin subfragment 1.

† Present address: Department of Biology, Kline Biology Tower, Yale University, New Haven, CT 06520.

‡ To whom reprint requests should be addressed.
followed by elution with 0.3 M NaCl in DEAE buffer. The 10 ml containing the most concentrated HMWP were applied to a 2.6 X 95 cm Sepharose 4B column and eluted with 4B buffer. Fractions across the single symmetrical peak obtained were once again monitored on a slab gel, and the most highly enriched ones were pooled.

**Isolation of Rabbit Skeletal Muscle Proteins.** Actin and myosin subfragment 1 (S-1) were prepared as described (24). The actin was free of regulatory proteins and the S-1 contained neither actin nor undigested myosin.

**Assay of HMWP Binding to Actin.** The proteins were dialyzed against 30 mM KCl/0.5 mM MgCl2/0.2 mM ATP/10 mM potassium phosphate, pH 7.0, for several hours. At the concentrations given in the text, aliquots of the proteins were mixed at 4°C and then incubated for 30 min in a 24°C water bath (or other temperature sequence as described). The tubes were then centrifuged for 10 min at 17,400 X g. Supernatants were removed. Pellets were rinsed in 0.5 ml of dialysis buffer at room temperature and centrifuged again. The pellets were then resuspended in sample buffer for NaDodSO4/polyacrylamide gel analysis.

**NaDodSO4/Polyacrylamide Gel Electrophoresis.** Gels were run in 5-mm diameter glass tubes (25, 26) or 1-mm thick slabs (26) and stained for protein or carbohydrate (25). Densitometer traces were obtained at 550 nm with a Zeiss PM46 spectrophotometer.

**Protein Determination.** Protein concentrations were estimated by the biuret procedure (27) as described (24).

**Amino Acid Analysis.** Analysis was performed by using a Durrum D-500 analyzer after 24-hr hydrolysis in 6 M HCl at 110°C under reduced pressure. Values obtained for threonine and serine residues were increased by 5 and 10%, respectively, to correct for hydrolysis.

**Microscopy.** A small piece of an actin-HMWP gel was flattened between a glass slide and coverslip and was observed under a Zeiss Photomicroscope III equipped with polarized light optics and a 1/20 compensator.

**RESULTS**

Actomyosin-depleted sucrose extracts of BHK-21 cells contained a protein whose molecular weight was appropriate for a high molecular weight actin-binding protein (Fig. 1). This fraction was therefore used as a starting material. The ammonium sulfate fractionation and chromatographic procedures described resulted in a fraction, 80-90% of which migrated on NaDodSO4/polyacrylamide gels with an approximate molecular weight of 250,000 (Fig. 1). A band with the same relative mobility was present on a gel containing whole BHK-21 cell homogenate, in an amount similar to that of myosin heavy chain (not shown). The recovery from 60 roller bottles (30 ml of packed cells) was 2 to 4 mg of HMWP.

BHK HMWP was soluble at high (0.6 M KCl) and low (30 mM KCl/10 mM phosphate) ionic strength at neutral pH. It contained no detectable carbohydrate, as determined by the absence of periodic acid/Schiff-staining material on NaDodSO4/polyacrylamide gels loaded with 60 µg of HMWP. Under these conditions, 50 µg of BHK-21 cell homogenate contained several periodic acid/Schiff-positive bands (results not shown). The amino acid composition of BHK HMWP was similar to that of ABP and filamin (Table 1).

When isolated BHK HMWP was mixed with purified rabbit skeletal muscle F-actin, a gel formed within 60 sec. The gel did not occupy the entire solution volume, but appeared as a light-scattering bubble-trapping mass suspended in the clear solution. This mass decreased in size during a 1-hr incubation in a fashion similar to the reported coagulation of filamin-actin complexes (11).

The interaction of BHK HMWP with actin was studied by mixing the proteins under various conditions, collecting the resulting gel, and analyzing the pellet by electrophoresis on NaDodSO4/polyacrylamide. An example of such an experiment is shown in Fig. 2. Actin and HMWP were combined at 4°C in the ratios shown, incubated at 24°C for 30 min, and centrifuged at 24°C. The gelled pellets contained both proteins (Fig. 2, lanes G-K). Very little actin (Fig. 2, lane F) and no HMWP (Fig. 2, lane E) was pelleted in control tubes. Incubation and centrifugation at 4°C (Fig. 2, lane D) or incubation at

### Table 1. Amino acid composition of BHK HMWP: Comparison with filamin and ABP

<table>
<thead>
<tr>
<th>Residue</th>
<th>BHK HMWP</th>
<th>Mammalian filamin*</th>
<th>Avian filamin†</th>
<th>Macrophage ABP†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>8.9</td>
<td>9.2</td>
<td>7.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Thr</td>
<td>6.6</td>
<td>6.4</td>
<td>5.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Ser</td>
<td>7.5</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Glu</td>
<td>11.0</td>
<td>10.8</td>
<td>9.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Pro</td>
<td>8.0</td>
<td>8.7</td>
<td>8.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Gly</td>
<td>10.7</td>
<td>12.0</td>
<td>12.5</td>
<td>11.8</td>
</tr>
<tr>
<td>Ala</td>
<td>6.9</td>
<td>7.7</td>
<td>8.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Val</td>
<td>9.6</td>
<td>9.8</td>
<td>10.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Met</td>
<td>1.3</td>
<td>1.1</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Ile</td>
<td>4.4</td>
<td>4.1</td>
<td>3.3</td>
<td>4.4</td>
</tr>
<tr>
<td>Leu</td>
<td>5.8</td>
<td>6.0</td>
<td>6.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.8</td>
<td>2.7</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Phe</td>
<td>2.8</td>
<td>2.2</td>
<td>2.9</td>
<td>3.2</td>
</tr>
<tr>
<td>His</td>
<td>2.1</td>
<td>2.1</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Lys</td>
<td>6.0</td>
<td>6.4</td>
<td>4.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Arg</td>
<td>3.3</td>
<td>3.5</td>
<td>5.5</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* Ref. 9. † Ref. 7. † Ref. 10.
regulated in this range of free calcium concentration, ref. 23).

When observed by polarized light microscopy, the actin-HMWP gel was anisotropic (Fig. 3 a and b). This demonstration of molecular order prompted us to examine the ultrastructure of the gel. Thin sections of the gel revealed crosslinked arrays of actin (Fig. 3 d, e, and f). However, only a small proportion of the actin in the gel (10%) was a very rough estimate from observations of thin sections) was involved in these arrays. Within the arrays, laterally aggregated actin filaments were joined by electron-dense material. The axial spacing of the crosslinks was measured as 33.9 ± 0.9 nm (SD, n = 147). Careful examination of thin sections of a pellet of the actin used for these experiments revealed uniform actin structure without any evidence for crosslinked arrays (Fig. 3 c). Thus the crosslinking structures probably contained HMWP.

**DISCUSSION**

HMWPs that interact with actin have been implicated in cytoplasmic consistency changes (3, 10, 11, 13–15, 19). Macrophage ABP and smooth muscle filamin have been isolated and appear to be similar (7–10). The present investigation has made possible the comparison of HMWP isolated from cultured fibroblasts with ABP and filamin. We have adopted the general term “HMWP” (19) to avoid additional terminology: precise comparison of the three proteins awaits amino acid sequence determination to unequivocally demonstrate their proposed identity (9, 31).

BHK HMWP was chemically similar to ABP and filamin on the basis of (i) its subunit and native molecular weight as determined by NaDodSO₄ electrophoresis and gel permeation chromatography, (ii) its solubility, (iii) the absence of carbohydrate, and (iv) its amino acid composition. It bound to actin, resulting in gel formation. Additional similarity to purified filamin was shown by the insensitivity of gelation to temperature and calcium concentration (cf. ref. 11). On the other hand, gelation of purified ABP and actin, or of extracts from several cell types, is temperature and calcium sensitive (10, 13–15). The different observations may be due to changes in the HMWP during isolation, such as partial denaturation or proteolysis, or to loss of required regulatory factors. Attempts to obtain initial enrichment of BHK HMWP by precipitation in a complex with actin (as in ref. 5) were unsuccessful. Thus we used the somewhat laborious procedures described for filamin isolation (7, 31).

The role of actin-gelling proteins in cell movement is not yet completely understood. It would be useful to know the ultrastructural arrangement of actin filaments that are in the crosslinked state. Our micrographs have demonstrated that HMWP can form regular arrays along laterally aggregated F-actin. The axial period of the crosslinks is close to the half pitch of the actin helix, suggesting that HMWP recognizes a specific aspect of the F-actin structure. The ability of HMWP to occupy closely spaced sites on F-actin is consistent with a possible competitive interaction between HMWP and myosin, with actin. This competition has been discussed by others (12, 14, 31–34), appeared in our preliminary results with S-1, and was inherent in the preparative scheme. Perhaps actin–HMWP complexes represent the cytoskeletal form of actin, in contrast to the contractile form that complexes with myosin.

On the structural level, these differences may be reflected in the two interconvertible states of microfilament organization that have been described, namely bundles and meshworks (see ref. 35). It is of interest to determine the possible role of the HMWP in microfilament bundle and meshwork formation. Experiments that combine examination of the ultrastructure of
actin-HMWP gels formed in vitro with elucidation of mechanisms for regulating crosslinking should contribute to our understanding of in vivo changes in microfilament organization. These studies can be aided by the ability to work with purified proteins. This report has shown that a gel-inducing protein from cultured fibroblasts is similar to such proteins isolated from other cells. It remains to be determined which factors control the cytoskeletal organizational changes in which this protein may function.

This work was done in partial fulfillment of the Ph.D. requirements of J.A.S. and was presented at the 1978 American Society for Cell Biology Meeting in San Antonio. The authors thank Ms. B. Chojnacki and Ms. W.-D. Liao for technical assistance. The work was supported by Grants AG-00890-02 and CA17210-04 from the National Institutes of Health.