Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2927–2931, May 1982
Cell Biology

Association of a M_r 50,000 cap-binding protein with the cytoskeleton in baby hamster kidney cells

(mRNA cap/monoclonal antibody/intermediate filaments/immunofluorescence/tryptic peptide mapping)

ALBERT ZUMBÉ*, CHRISTIAN STÄHLI†, AND HANS TRACHSEL*‡

*Friedrich Miescher-Institut, P.O. Box 273, CH-4002 Basel, Switzerland; †Hoffmann-La Roche & Company AG, Grenzacherstrasse, CH-4002 Basel, Switzerland; and ‡Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Communicated by Keith R. Porter, January 20, 1982

ABSTRACT **A monoclonal antibody directed against eukaryotic mRNA 5′-cap-binding protein (anti-CBP antibody) was used to localize cap-binding protein (CBP) in BHK-21 baby hamster kidney cells by immunofluorescence microscopy. It was found that the antibody reacts with a fibrous network extending through the cytoplasm in a radial arrangement. The network behaves like intermediate filaments in colchicine-treated cells, suggesting a direct or indirect linkage of CBP with intermediate filaments. The association of CBP with a cytoskeletal element was further confirmed by isolation of proteins from Triton X-100-extracted cells and identification of CBP in the cytoskeletal fraction with anti-CBP antibody. The major polypeptide reacting with anti-CBP antibody is a M_r 50,000 component. Tryptic peptide mapping showed that this polypeptide is related to a M_r 24,000 polypeptide identified as CBP in earlier experiments [Sonenberg, N., Morgan, M. A., Testa, D., Colonna, R. J. & Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. USA* 75, 4843–4847].**

Initiation of translation of eukaryotic cellular and viral mRNAs is facilitated by the presence of the 5′-terminal cap structure $m^7G5'ppp5'N$ (1). Exceptions are the RNAs of picornaviruses (2–4) and some plant viruses (5, 6). In crude protein-synthesizing systems several polypeptides (cap-binding proteins, CBPs) were shown to crosslink specifically to the cap structure (7). In addition, a M_r 24,000 polypeptide (24-CBP) that binds to the cap structure has been identified in partially purified initiation factor fractions (8) and purified by affinity chromatography on m^7GDP-Sepharose (9). Recently, crosslinking experiments done in the presence of ATP/Mg^{2+} revealed the presence in partially purified initiation factor preparations of CBPs of M_r 28,000, 50,000, and 80,000 in addition to 24-CBP (10). These findings suggest the existence of a whole family of CBP. So far it is not known what the relative contributions to cap recognition of the different CBPs are, but it is clear that they play an important role in the initiation of translation of capped mRNAs (11, 12) and the regulation of translation in poliovirus-infected HeLa cells (13, 14).

We prepared monoclonal antibodies directed against CBP(s), using as antigen a partially purified preparation of rabbit reticulocyte eukaryotic initiation factor 3 (eIF-3) which was shown previously to contain CBP(s) (14). One of these antibodies (anti-CBP antibody) was found to react with several structurally related polypeptides, which share tryptic and chromotryptic peptides with 24-CBP (15). Here we show that one of these polypeptides, a M_r 50,000 component, is associated with the cytoskeleton in BHK-21 baby hamster kidney cells.

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MATERIALS AND METHODS

Cell Culture. Subconfluent cultures of BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO). For immunofluorescence, cells were seeded on glass coverslips in Petri dishes.

Preparation of the Monoclonal Antibody (Anti-CBP Antibody). *Immunization.* BALB/cJ female mice were injected with rabbit reticulocyte eIF-3 that had been purified through steps 1–4 as described in ref. 16. The immunization schedule of Stähli *et al.* (17) was used with 70 μg of eIF-3 per mouse for the two initial injections and 100–500 μg of eIF-3 per mouse for the booster injections just before fusion.

Cell fusion and cell culture. Spleen cells from an immunized mouse were fused with SP-2-01 myeloma cells as described (17). Culture supernatants were tested by using a solid-phase radioimmunoassay (RIA) (17) with eIF-3 at 5–10 μg/ml as antigen. Culture supernatants containing antibodies directed against eIF-3 were further tested in RIA with affinity-purified 24-CBP (9) at 1 μg/ml as antigen. Positive cultures (anti-CBP clones) were recloned by limiting dilution as described (17).

Purification of anti-CBP antibody (IgM.) Cells ($1–2 \times 10^7$) of the anti-CBP clone were injected intraperitoneally in BALB/cJ mice that had received an injection of 0.15 ml of complete Freund's adjuvant 7 days before. Two to 3 weeks later the mice were killed and the ascites fluid was collected. Antibodies were purified from the ascites fluid by $(NH_4)_2SO_4$ precipitation (1.75 M final concentration) and DEAE-cellulose chromatography (18). In the final purification step, anti-CBP antibodies were separated from IgG and lower molecular weight components by centrifugation in 12-ml 10–40% convex exponential sucrose gradients in 20 mM Hepes, pH 7.6/150 mM NaCl. Centrifugation was in the Spinco SW 40 Ti rotor at 40,000 rpm for 16 hr at 4°C.

Antisera. *Anti-tubulin antibody.* Porcine brain tubulin was isolated by using the polymerization–depolymerization method (19) and further purified by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis. The tubulin band was cut out from the gel and eluted electrophoretically. Rabbits were immunized by injecting 1 mg of tubulin in incomplete Freund's adjuvant. The injection was repeated twice with 0.5 mg of tubulin in incomplete Freund's adjuvant at weekly intervals. Rabbit anti-tubulin antibodies were isolated by affinity chromatography as described (20, 21).

Anti-decamin antibody. Rabbit antisera directed against dec-

Abbreviations: P_i/NaCl, phosphate-buffered saline; Tris/NaCl, Tris-buffered saline; CBP, cap-binding protein; eIF, eukaryotic initiation factor; RIA, radioimmunoassay; FITC, fluorescein isothiocyanate; BHK, baby hamster kidney.

amin, the intermediate filament protein of BHK cells (22), were the generous gift of R. D. Goldman (Carnegie-Mellon University, Pittsburgh).

Anti-actin antibody. Rabbit antisera directed against actin were kindly provided by G. Gabbiani (University of Geneva).

Fluorochrome-coupled antibodies. The IgG fraction from goat serum containing antibodies directed against mouse IgM (Nordic; Lausanne, Switzerland) was purified by precipitation with $(NH_4)_2SO_4$ and DEAE-cellulose chromatography (18) and then conjugated with fluorescein isothiocyanate (FITC) by using the procedure of Cebra and Goldstein (23). The reaction mixture was passed through a Sephadex G-25 column (Pharmacia) to separate the unconjugated fluorochrome from the conjugated IgG, to reestablish a pH of 7.5, and to replace the salt. Finally, the IgG was chromatographed on a DEAE-cellulose column to remove under- and over-conjugated IgG (24). Fluorescein-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-rabbit IgG were purchased from Nordic.

Immunofluorescence Microscopy. *Preparation of cells.* For fixation of microtubules, cells grown on coverslips were rinsed with phosphate-buffered saline (P_i/NaCl) and treated with freshly prepared 3.7% (wt/vol) formaldehyde in P_i/NaCl for 20 min at 4°C. Coverslips were then rinsed quickly with P_i/NaCl, treated with acetone at −20°C for 5 min, and air dried. For fixation of intermediate filaments the formaldehyde step was omitted and acetone fixation was done as described above. For fixation of microfilaments, coverslips were rinsed in P_i/NaCl, treated with ethanol at 4°C for 30 sec, and rinsed again in P_i/NaCl.

Immunofluorescent staining of fixed cells. Coverslips with fixed cells were incubated with the first antibody for 45 min at 37°C. The coverslips were rinsed with P_i/NaCl and then the appropriate fluorescein- or rhodamine-labeled second antibody was applied. After a further incubation for 45 min at 37°C and subsequent washing with P_i/NaCl the coverslips were mounted with P_i/NaCl/glycerol, 1:1 (vol/vol), on microscope slides. Antibody dilutions were made in P_i/NaCl containing 0.5% bovine serum albumin. Purified anti-CBP antibody was diluted to about 50 μg/ml. Antisera (rabbit anti-decamin antibody and rabbit anti-actin antibody) and IgG fractions (rabbit anti-tubulin antibody and commercial fluorochrome-labeled antibodies) were diluted 1:20 to 1:50. Stained cells were viewed with a Zeiss microscope equipped with epifluorescence optics. Photographs were taken with ×40 or oil immersion ×63 objectives and Ilford HP5 film. Control experiments in which the specific (first) antibody was omitted gave no staining and there was no detectable reaction of fluorochrome-coupled goat anti-rabbit IgG with mouse IgM or fluorochrome-coupled goat anti-mouse IgM with rabbit IgG (results not shown).

Preparation of Extracted Cytoskeleton. BHK-21 cells were grown in Petri dishes (four dishes, ca. 4–8 × 10^6 cells), rinsed twice in buffer A [20 mM Tris·HCl, pH 7.6/50 mM NaCl/0.3 M sucrose/2 mM $Mg(OAc)_2$/1 mM phenylmethanesulfonyl fluoride], lysed by a 30-second treatment with buffer A plus 0.5% Triton X-100, and washed in buffer A minus sucrose. At this stage the cells were detached from the dish with a rubber policeman, centrifuged (Spinco SS-34 rotor at 2,000 rpm for 10 min at 4°C) and the pellet was treated with DNase (DNase I, Worthington at 10 μg/ml) in buffer A minus sucrose for 2 hr at 4°C with occasional stirring (vol, 3 ml). After a second centrifugation as above, the sediment was taken up in 200 μl of $NaDodSO_4$ sample buffer.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis in slab gels was according to Laemmli (25). The separation gel contained 15% acrylamide (wt/wt) and 0.086% bisacrylamide.

$NaDodSO_4$/Polyacrylamide Gel–Nitrocellulose Sheet Blotting Technique (Protein Blotting). The procedure of Towbin *et al.* (26) was followed. After the electrophoretic transfer of the proteins, the nitrocellulose sheet (Millipore) was incubated for 1 hr at room temperature with 2.5% bovine serum albumin and 5% horse serum in Tris-buffered saline (Tris/NaCl; 20 mM Tris·HCl, pH 7.6/150 mM NaCl). After washing with Tris/NaCl, the sheet was incubated overnight with a solution containing purified anti-CBP antibody at 1–2 μg/ml, 0.5% bovine serum albumin, and 1% horse serum in Tris/NaCl. Bound antibody was detected by reaction with rabbit anti-mouse IgG conjugated to horseradish peroxidase, followed by incubation in *o*-dianisidine and H_2O_2, which gives a red-brown color reaction (26).

Tryptic Peptide Analysis. Polypeptide bands were cut out from fixed and stained $NaDodSO_4$/polyacrylamide gels, the protein was labeled in the slice with $Na^{125}I$ [0.5 mCi per slice (1 Ci = 3.7 × 10^{10} becquerels)] and digested with trypsin, and the peptides were analyzed (27).

RESULTS

We immunized mice with unwashed eIF-3 to obtain monoclonal antibodies directed against CBP(s) (14) and the subunits of eIF-3. Hybridoma clones were selected by screening with eIF-3 and affinity-purified 24-CBP (9) in a solid-phase RIA. Antibodies produced by one such clone were further analyzed. They belong to the IgM class, with light chains of the κ type.

We showed in a recent publication (12) that this anti-CBP antibody inhibits the translation of capped reovirus mRNA in cell-free extracts from L cells, whereas a control antibody has no effect. In contrast, anti-CBP antibody does not inhibit translation of uncapped reovirus mRNA in cell-free extracts from reovirus-infected L cells, in which initiation presumably proceeds via a cap-independent mechanism (12, 28). In addition, we showed that anti-CBP antibody inhibits the translation of capped reovirus mRNA but not of naturally uncapped encephalomyocarditis RNA in HeLa cell extracts (12). These experiments define the monoclonal antibody as anti-CBP antibody. Anti-CBP antibody was shown to react with a M_r 50,000 polypeptide in rabbit reticulocyte polysomes, and tryptic peptide analysis indicated a structural relationship between this polypeptide and 24-CBP (15). Furthermore, in postpolysomal supernatant, polypeptides with molecular weights higher than 50,000 were found to react with anti-CBP antibody (15).

Preliminary experiments indicated that this antibody also reacts with protein from HeLa cells, BALB/c 3T3 cells, and BHK cells. In order to localize CBP in cells by indirect immunofluorescence microscopy with anti-CBP antibody we chose BHK cells. Fig. 1 shows typical patterns obtained by indirect immunofluorescence microscopy with anti-actin antibody (Fig. 1*a*), anti-tubulin antibody (Fig. 1*b*), and anti-decamin antibody (Fig. 1*c*) on fixed BHK-21 cells.

Observation of BHK-21 cells by this technique, using anti-CBP antibody, reveals that CBP is distributed along a very fine network of fibers (Fig. 1*d*). These fibers extend through the cytoplasm in a radial arrangement and are more abundant around the nucleus. Identical results were obtained with antibody purified from mouse ascites fluid and hybridoma culture supernatant, and a typical staining pattern could be obtained with antibody from hybridoma culture supernatant at about 5–10 μg/ml. Furthermore, the same staining pattern was obtained by using a completely different preparation method in which cells are extracted with Triton X-100 and the resulting cytoskeleton preparation is allowed to react with anti-CBP antibody without prior fixation (results not shown). The fibrous

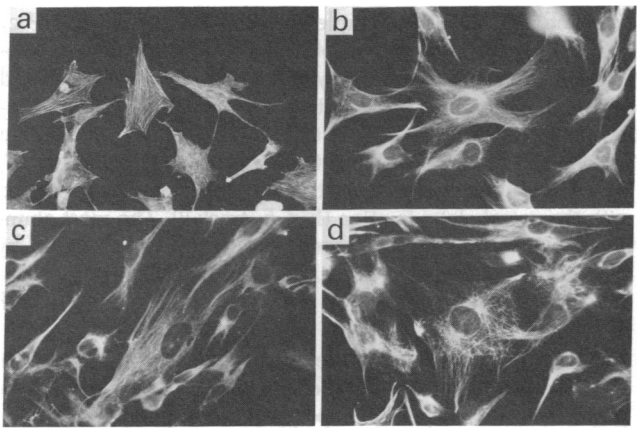

FIG. 1. Immunofluorescence microscopy (single labeling). BHK-21 cells were prepared for immunofluorescence microscopy and allowed to react with rabbit anti-actin antibody and FITC-conjugated goat anti-rabbit IgG (*a*); rabbit anti-tubulin antibody and FITC-conjugated goat anti-rabbit IgG (*b*); rabbit anti-decamin antibody and FITC-conjugated goat anti-rabbit IgG (*c*); mouse anti-CBP antibody and FITC-conjugated goat anti-mouse IgM (*d*). (×80.)

network reacting with anti-CBP antibody resembles the network stained by either anti-tubulin antibody (microtubules; Fig. 1*b*) or anti-decamin antibody (intermediate filaments; Fig. 1*c*) but has no similarities with the patterns stained by anti-actin antibody (microfilaments; Fig. 1*a*).

In order to distinguish between reaction of anti-CBP antibody with microtubules or intermediate filaments, BHK-21 cells were double stained for immunofluorescence microscopy using anti-CBP- and anti-decamin antibodies (Fig. 2) or anti-CBP- and anti-tubulin antibodies (Fig. 3). At the resolution of the light microscope, use of the double-staining technique with interphase cells shows complete superposition of the labeling patterns obtained with anti-CBP- and anti-decamin antibodies (Fig. 2) and significant but not complete superposition of the patterns obtained with anti-CBP- and anti-tubulin antibodies (Fig. 3).

After treatment of BHK-21 cells with colchicine at 20 μg/ml for 2 hr the distribution of CBP as revealed by immunofluorescence microscopy begins to change. The filaments form thicker bundles and aggregate into perinuclear bundles in the

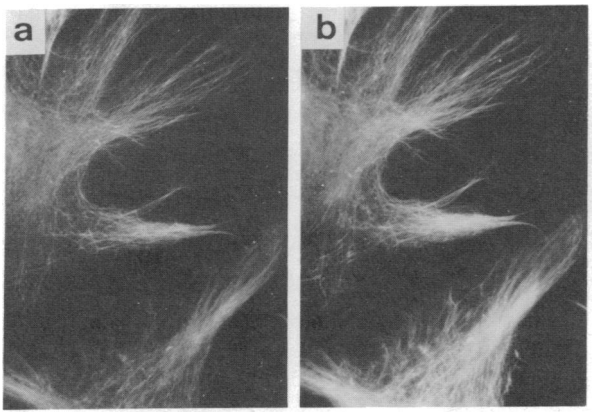

FIG. 2. Immunofluorescence microscopy (double labeling). BHK-21 cells were prepared for immunofluorescence microscopy and allowed to react with mouse anti-CBP antibody followed by rabbit anti-decamin antibody and then FITC-conjugated goat anti-mouse IgM followed by rhodamine-conjugated goat anti-rabbit IgG. (*a*) Fluorescein pattern; (*b*) rhodamine pattern of the same cell. (×400.)

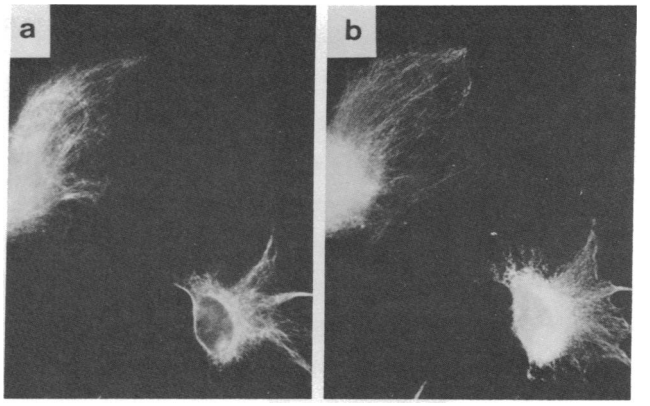

FIG. 3. Immunofluorescence microscopy (double labeling). BHK-21 cells were prepared for immunofluorescence microscopy and allowed to react with mouse anti-CBP antibody followed by rabbit anti-tubulin antibody and then FITC-conjugated goat anti-mouse IgM followed by rhodamine-conjugated goat anti-rabbit IgG. (*a*) Fluorescein pattern; (*b*) rhodamine pattern of the same cell. (×400.)

form of a ring or blob (Fig. 4 *c* and *d*). This process continues over several hours and reaches completion within 10 hr (results not shown). When cells are double-labeled for immunofluorescence microscopy using anti-CBP- and anti-decamin antibodies (Fig. 4 *c* and *d*), similar staining patterns are observed. Perinuclear filament bundles are seen, and they are similar to structures that have already been described for intermediate filaments in colchicine-treated cells (29–32). When the double-labeling technique is used with anti-CBP- and anti-tubulin an-

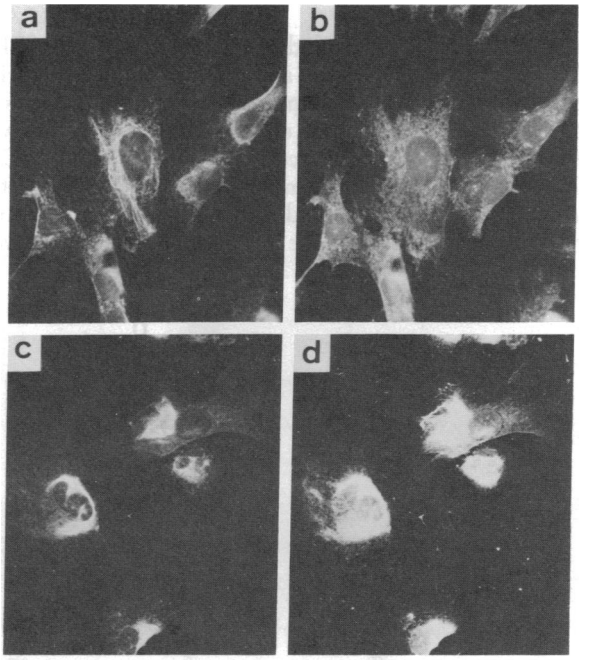

FIG. 4. Immunofluorescence microscopy (double labeling). BHK-21 cells were pretreated with colchicine at 20 μg/ml for 2 hr and prepared for immunofluorescence microscopy. They were allowed to react with mouse anti-CBP antibody followed by rabbit anti-tubulin antibody (*a* and *b*) or with mouse anti-CBP antibody followed by rabbit anti-decamin antibody (*c* and *d*). The second labeling was with FITC-conjugated goat anti-mouse IgM (*a* and *c*) or with rhodamine-conjugated goat anti-rabbit IgG (*b* and *d*). (*a* and *c*) Fluorescein patterns; (*b* and *d*) rhodamine patterns. The cells shown in *b* are the same as those in *a*; the cells in *d* are the same as those in *c*. (×200.)

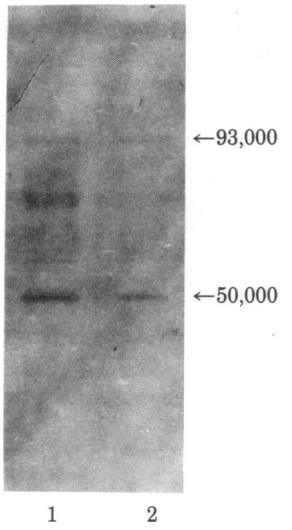

FIG. 5. $NaDodSO_4$/polyacrylamide gel–nitrocellulose sheet blot. BHK-21 cells (ca. 10^5 cells) were either dissolved directly in $NaDodSO_4$ sample buffer or first extracted with Triton X-100 and then dissolved in sample buffer. Proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis, transferred electrophoretically from the gel onto a nitrocellulose sheet, and identified by reaction with mouse anti-CBP antibody. Track 1, intact cells; track 2, Triton X-100-extracted cells.

tibodies (Fig. 4 *a* and *b*) it is evident that microtubules are depolymerized (as indicated by diffuse staining with anti-tubulin antibody; Fig. 4*b*) while CBP is present on filaments coiled around the nuclear membrane (Fig. 4*a*). These data suggest that CBP is associated directly or indirectly with intermediate filaments in BHK-21 cells.

The association of CBP with the cytoskeleton fraction of BHK cells was also demonstrated by another method (Fig. 5). BHK-21 cells grown on glass coverslips were either dissolved directly in $NaDodSO_4$ sample buffer or extracted on the coverslips with Triton X-100. Part of extracted cells were treated with anti-CBP antibody for immunofluorescence microscopy. The characteristic fibrous network described above (e.g., Fig. 1*d*) was observed (results not shown). The rest of the extracted cells were dissolved in $NaDodSO_4$ sample buffer. Total cell protein (Fig. 5, track 1) and cytoskeletal proteins (Fig. 5, track 2) were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and blotted electrophoretically onto a nitrocellulose sheet. Anti-CBP antibody was then used to identify CBP on the protein blots. The experiment shows that a major polypeptide of M_r 50,000 and a minor component of M_r 93,000 remain associated with the cytoskeletal fraction (Fig. 5, track 2). The most abundant polypeptide in this cytoskeletal fraction as revealed by staining with Coomassie blue is decamin (results not shown). This M_r 55,000 polypeptide does not react with anti-CBP antibody.

Earlier experiments (15) demonstrated that anti-CBP antibody reacts with a M_r 50,000 polypeptide in rabbit reticulocyte polysomes and a M_r 210,000 polypeptide in postpolysomal supernatant. Furthermore, polypeptides of molecular weight between 24,000 and 210,000, including components of 50,000 and 93,000, were found in partially purified eIF preparations (15). It was shown by peptide mapping that these polypeptides are structurally related and, furthermore, share tryptic and chymotryptic peptides with 24-CBP isolated by affinity chromatography on m^7GDP-Sepharose (9). Fig. 6 shows that the M_r 50,000 polypeptide in the BHK cytoskeletal fraction (Fig. 6*b*) shares tryptic peptides with 24-CBP from rabbit reticulocytes (Fig. 6*a*). The mixing experiment confirms the identity of peptides indicated by arrows (Fig. 6*c*). These data, together with the data shown in Fig. 5, demonstrate directly the presence of CBP on BHK-21 cytoskeletons as suggested above by the immunofluorescence microscopy data.

DISCUSSION

The cytoskeleton in animal cells contains three major fibrous structures, known as microtubules, microfilaments, and intermediate filaments (for a review see ref. 33). In addition, an extensive cytoplasmic meshwork was found and termed microtrabeculae (34, 35). The cytoskeleton is thought to be involved in cell movement, changes in cell shape, movement of organelles within the cell, endocytosis, exocytosis, and movement of protein in cell membranes (33).

Our immunofluorescence microscopy data (Figs. 1–4) and biochemical experiments (Figs. 5 and 6) demonstrate the as-

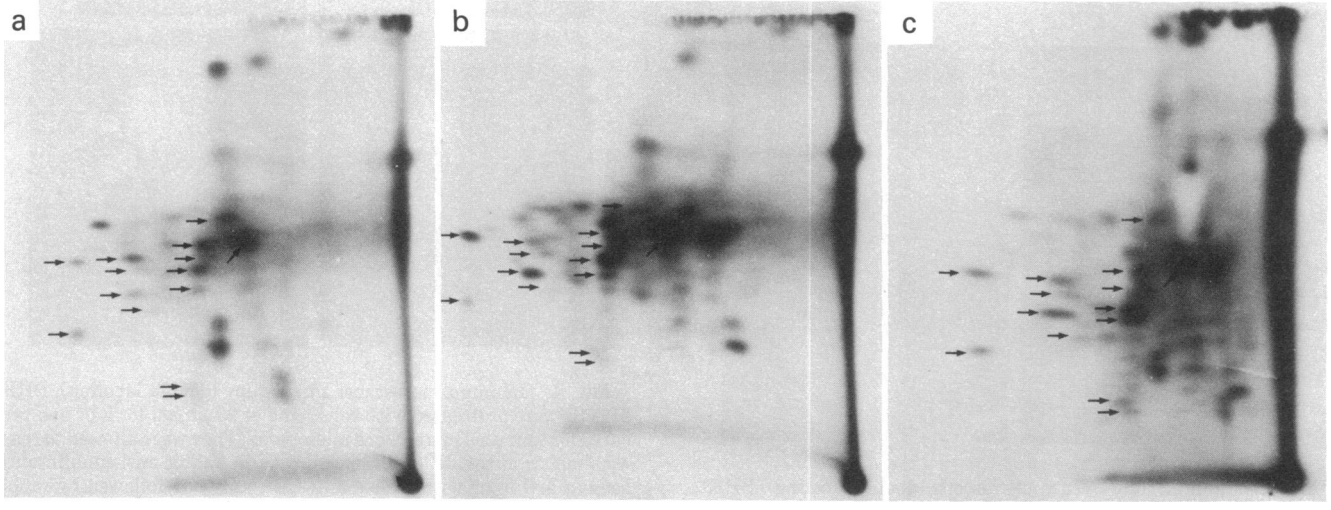

FIG. 6. Tryptic peptide patterns. The M_r 50,000 polypeptide from Triton X-100-extracted cells that was shown to react with mouse anti-CBP antibody (Fig. 5) and the M_r 24,000 CBP (24-CBP) (9) were analyzed as described in *Materials and Methods*. The autoradiograph is shown. (*a*) 24-CBP, (*b*) M_r 50,000 polypeptide, (*c*) 24-CBP plus M_r 50,000 polypeptide. Electrophoresis was from right to left (first dimension) and chromatography was from bottom to top (second dimension). Arrows indicate identical peptides.

sociation of a M_r 50,000 CBP (50-CBP) with the cytoskeleton, most likely with intermediate filaments. However, due to the low resolution of immunofluorescence microscopy we cannot distinguish whether 50-CBP is associated directly with intermediate filaments or bound to an independent structure that is linked to intermediate filaments. A likely candidate for such a structure is the microtrabecular network (34, 35). Electron microscopical evidence suggests that this network carries polyribosomes (35), and consequently we might expect to find CBP bound to it as well. The association of polyribosomes with some element of the cytoskeleton was also demonstrated by using biochemical methods (36). Lenk *et al.* (37) prepared cytoskeletons by detergent extraction of suspension-grown HeLa cells and presented evidence for the specific retention of polyribosomes with the cytoskeletal fraction. Degradation of mRNA with low levels of ribonuclease released ribosomes from the cytoskeleton, implying an attachment of polyribosomes via mRNA. More recent experiments indicate that binding of mRNA to the cytoskeleton might be a requirement for translation (38). This is further supported by the finding that shutoff of host protein synthesis in poliovirus-infected HeLa cells is accompanied by the release of host mRNAs from the cytoskeleton (39). These findings (36–39) are consistent with the observation that CBP is functionally inactivated in poliovirus-infected HeLa cells (13, 14) and indicate that CBP may be involved in linking host mRNAs to the cytoskeleton.

We thank Drs. R. D. Goldman and G. Gabbiani for providing the anti-decamin and anti-actin antibodies, Drs. R. Bürk and T. Staehelin for helpful discussions and technical advice, and R. Fessler and H. Kuster for excellent technical assistance. We also thank Dr. J. Gordon, in whose laboratory part of this work was done.

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