Differential Localization of Tropomyosin Isoforms in Cultured Nonmuscle Cells

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Abstract. We have previously shown that chicken embryo fibroblast (CEF) cells and human bladder carcinoma (EJ) cells contain multiple isoforms of tropomyosin, identified as a, b, 1, 2, and 3 in CEF cells and 1, 2, 3, 4, and 5 in human EJ cells by one-dimensional SDS-PAGE (Lin, J. J.-C., D. M. Helfman, S. H. Hughes, and C.-S. Chou. 1985. *J. Cell Biol.* 100: 692–703; and Lin, J. J.-C., S. Yamashiro-Matsumura, and F. Matsumura. 1984. *Cancer Cells* 1:57–65). Both isoform 3 (TM-3) of CEF and isoforms 4,5 (TM-4,-5) of human EJ cells are the minor isoforms found respectively in normal chicken and human cells. They have a lower apparent molecular mass and show a weaker affinity to actin filaments when compared to the higher molecular mass isoforms. Using individual tropomyosin isoforms immobilized on nitrocellulose papers and sequential absorption of polyclonal antiserum on these papers, we have prepared antibodies specific to CEF TM-3 and to CEF TM-1,-2. In addition, two of our antitropomyosin mAbs, CGβ6 and CG3, have now been demonstrated by Western blots, immunoprecipitation, and two-dimensional gel analysis to have specificities to human EJ TM-3 and TM-5, respectively. By using these isoform-specific reagents, we are able to compare the intracellular localizations of the lower and higher molecular mass isoforms in both CEF and human EJ cells. We have found that both lower and higher molecular mass isoforms of tropomyosin are localized along stress fibers of cells, as one would expect. However, the lower molecular mass isoforms are also distributed in regions near ruffling membranes. Further evidence for this different localization of different tropomyosin isoforms comes from double-label immunofluorescence microscopy on the same CEF cells with affinity-purified antibody against TM-3, and monoclonal CGβ6 antibody against TM-a, -b, -1, and -2 of CEF tropomyosin. The presence of the lower molecular mass isoform of tropomyosin in ruffling membranes may indicate a novel way for the nonmuscle cell to control the stability and organization of microfilaments, and to regulate the cell motility.

NONMUSCLE cells so far examined express multiple isoforms of tropomyosin (6, 8–11, 13–17, 23–25, 34–36, 39, 42, 48, 50, 52). For example, chicken embryo fibroblasts (CEF)[1] contain at least five isoforms of tropomyosin (called a, b, 1, 2, and 3) (34, 35), and human or rat nonmuscle cells have tropomyosin isoforms 1, 2, 3, 4, and 5 (17, 36, 39, 52, 53). CEF isoform 3 (TM-3) and human isoforms 4 and 5 (TM-4,-5) have lower apparent molecular masses and are the minor isoforms of tropomyosin in normal cells. On the contrary, CEF isoforms 1 and 2 (TM-1,-2) and human isoforms 1, 2, and 3 (TM-1,-2,-3) are the major isoforms with higher apparent molecular masses. In transformed cells, the expression of the minor isoforms increases and the amount of at least one of the major isoforms decreases or disappears (7, 19, 20, 30, 31, 34, 36, 41, 45). The differential expression of tropomyosin isoforms appears to correlate well with the morphological changes in transformed cells. Furthermore, the lower molecular mass isoforms from CEF and rat nonmuscle cells have been shown to have a weaker actin-binding ability than the higher molecular mass isoforms (34, 39). In this study, we have purified tropomyosin isoforms from human bladder carcinoma cells and characterized them in terms of actin-binding abilities. We have found that the lower molecular mass isoforms bind actin filaments more weakly than the higher molecular mass isoforms. Together, these observations suggest that multiple isoforms of tropomyosin may be necessary for nonmuscle cells to regulate the organization and function of microfilaments within the cell. However, the molecular mechanism for this regulatory function of tropomyosin isoforms remains to be determined. Knowledge concerning the intracellular localization and the biochemical properties of individual isoforms may allow us to better evaluate this regulatory function of tropomyosin.

At present, it is not known whether the different isoforms have different localizations within the cells. We have approached this question by preparing CEF isoform 3–specific and isoforms 1, 2–specific rabbit antibodies by preabsorption

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast.

and affinity-purification. These antibodies, together with CGβ6, an mAb that has been shown to recognize only the higher molecular mass isoforms of CEF tropomyosin, were used to probe the intracellular localization of tropomyosin isoforms in CEF cells. In addition, we have used two mAbs prepared previously (CG3 and CGβ6) to study the distribution of tropomyosin isoforms in human EJ cells. The CG3 antibody recognizes the tropomyosin isoform 5 (TM-5), whereas the CGβ6 reacts only to tropomyosin isoform 3 (TM-3) of human bladder carcinoma (EJ) cells. Also, results from immunofluorescence studies have suggested that only the lower molecular mass isoform (TM-5) of tropomyosin is localized in significant amounts near the peripheral ruffles of the cell.

Materials and Methods

Cell Culture

Primary cultures of CEF were prepared from the skins of 10-d-old embryos as described previously (35). A human EJ cell line was a generous gift from L. B. Chen (Danna Farber Cancer Institute, Harvard Medical School). Cells were maintained in DME containing 10% FCS and incubated at 37°C in a humidified chamber with 5% CO_2 and 95% air. Secondary and tertiary cultures of CEF cells were used for all experiments. For indirect immunofluorescence, the cells were grown on 12-mm round glass coverslips for 1 or 2 d before use.

Preparation of Tropomyosin Isoforms from CEF and Human EJ Cells

Purified tropomyosin was prepared from monolayer cultures of CEF cells as described previously (34). This CEF tropomyosin can be resolved into five bands, namely, a, b, 1, 2, and 3 isoforms on one-dimensional gels. The individual isoforms 1, 2, and 3 were further purified by electroelution of the appropriate gel bands on preparative SDS-PAGE and renatured as described previously (33). These individual isoforms were used for affinity purification of isoform-specific antibodies.

The human EJ tropomyosin isoforms were prepared by the conventional methods (19) including heat treatment, ammonium sulfate fractionation, isoelectric point precipitation, and ion-exchange column chromatography. Cells were cultured in 150-mm dishes, washed three times with PBS containing 5 mM $MgCl_2$ and 0.2 mM EGTA, and stored at −70°C. Cells in wet weight of 45 g were suspended in 5 vol of extraction buffer containing 0.3 M KCl, 50 mM imidazole buffer, pH 7.0, 1 mM EGTA, and 0.5 mM $MgCl_2$, and homogenized three times for 10 s each, using a polytron homogenizer at the middle setting. The homogenates were further stirred for 3 h at 4°C and then centrifuged at 7,000 *g* for 20 min. The supernatant was heated in a boiling water bath for 10 min and cooled on ice for 30 min. The denatured proteins were removed by centrifugation at 7,000 *g* for 15 min. The resulting supernatant was fractionated by ammonium sulfate. The tropomyosin isoforms were precipitated between 30 and 65% saturated solution. The precipitates were collected by centrifugation at 7,000 *g* for 15 min and dialyzed against 10 mM KH_2PO_4 buffer, pH 7.0, containing 1 M KCl and 0.1 mM dithiothreitol (DTT). The resulting solution was adjusted to pH 4.6 with 1 N HCl to precipitate the EJ tropomyosin, which was then collected by centrifugation at 10,000 *g* for 10 min. The pH 4.6-precipitate was dissolved in 10 mM imidazole buffer, pH 7.0, containing 30 mM NaCl, 0.1 mM DTT, and 0.1 mM EGTA, and dialyzed overnight against the same buffer. After centrifugation to remove insoluble material, the sample was applied to a DEAE-cellulose (DE-52; Whatman Inc., Clifton, NJ) column (1.2 × 10 cm) previously equilibrated with the same buffer, and eluted with a 200-ml linear gradient to 380 mM NaCl. The eluted materials containing partially purified tropomyosin were used for Western blots. Final purification was performed by chromatography on a hydroxyapatite column (Bio-Gel HTP; Bio-Rad Laboratories, Cambridge, MA). This column chromatography resolved the human EJ tropomyosin isoforms into three fractions, which were isoforms 1, 2, and 3, isoform 3, and isoforms 4 and 5. About 0.26, 0.13, and 0.54 mg of tropomyosin TM-1, -2,-3, TM-3, and TM-4,-5, respectively, were obtained from 45 g of EJ cells. Both TM-1,-2,-3 and TM-4,-5 were used for actin-binding experiments.

Preparation of Isoform-specific Antibodies

Rabbit no. 2 antiserum against chicken gizzard tropomyosin was prepared and characterized previously (35, 42). This antiserum reacted strongly with isoforms 1 and 2 but weakly with isoform 3 of CEF tropomyosin. Another rabbit was immunized with purified CEF tropomyosin by the protocol described previously (42). The antiserum obtained from this rabbit (designated as rabbit no. 6) recognized all isoforms of CEF tropomyosin with a stronger reactivity to isoform 3 than to isoforms 1 and 2. These two polyclonal antisera were used to prepare isoform-specific antibodies by the sequential absorption and affinity-purification method as described (51). Briefly, ~10 μg of the individual isoforms were spotted on 0.5-cm^2 nitrocellulose paper, air-dried, and incubated at room temperature for 1 h in TBS (Tris buffered saline: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NaN_3) containing 3% BSA. Diluted antiserum (rabbit no. 2) was preabsorbed twice with papers containing isoform 3 (1 h each) at room temperature. The remaining antiserum was further incubated with papers containing isoforms 1 and 2 for 1 h. After a wash in TBS containing 0.5% Tween 20, the paper was washed three times with elution buffer (5 mM glycine HCl, pH 2.3, 0.5 M NaCl, 0.5% Tween 20, 100 μg/ml BSA). These eluates were pooled and immediately neutralized by the addition of Na_2HPO_4 to a final concentration of 50 mM. The pooled eluates were dialyzed against one-tenth dilution of TBS overnight and lyophilized. This antibody was designated as CEF TM-1,-2-specific antibody (TM-1,-2 Ab). For the preparation of isoform 3-specific antibody, the diluted rabbit no. 6 antiserum was preabsorbed twice each with papers containing isoform 1 and isoform 2 of CEF tropomyosin to remove antibodies that were common to all isoforms as well as specific to isoforms 1 and 2. The remaining antiserum was incubated with papers containing isoform 3 and eluted as described above. This antibody was specific to isoform 3 and designated as CEF TM-3-specific antibody (TM-3 Ab).

mAbs CG3 and CGβ6 were prepared and characterized as described previously (33). The CG3 antibody reacts with all isoforms of CEF tropomyosin, whereas the CGβ6 antibody recognizes the higher molecular mass isoforms (a, b, 1, and 2) but not the lower molecular mass isoform 3 of CEF tropomyosin. However, when characterized on human EJ bladder carcinoma tropomyosin by immunoblotting, it was found that CG3 specifically reacted with the lower molecular mass isoforms 4 and 5 (EJ TM-4,-5) and CGβ6 recognized only isoform 3 (EJ TM-3) of human EJ tropomyosin. Two-dimensional gel analyses of the immunoprecipitate from labeled human nonmuscle cells by CG3 mAb further demonstrated that CG3 specifically reacted with TM-5 isoform. Thus, these antibodies could be used as isoform-specific mAbs to examine the intracellular localization of tropomyosin isoforms in human cells.

Indirect Immunofluorescence Microscopy

Cells on coverslips were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with cold acetone (−10°C) for 5 min. After being rinsed with PBS, the coverslips were incubated with various primary antibodies in a humidified chamber at 37°C for 30 min. The coverslips were washed extensively with PBS and afterwards reacted with FITC-labeled goat anti-mouse IgG or FITC-labeled goat anti-rabbit IgG antibodies. After extensive washing in PBS, the coverslips were mounted on slides with gelvatol (32). For double-label immunofluorescence, the CEF cells on coverslips were processed and reacted with a mixture of CGβ6 (the higher molecular mass isoform-specific antibody) and affinity-purified CEF isoform 3-specific antibody (TM-3 Ab) as described above. A mixture of FITC-labeled goat anti-mouse IgG (heavy and light chains) and tetramethyl rhodamine isothiocyanate-labeled goat anti-rabbit IgG antibodies was used to detect the primary antibodies. The cells were observed and photographed on a Zeiss epifluorescence photomicroscope III with a Zeiss 63× oil phase 3 lens, as described previously (32).

For the preabsorption experiments, 100 μl of each rabbit antisera were mixed with 420 (for no. 2) or 210 μg (for no. 6) of purified CEF tropomyosin isoforms. After incubation at 4°C overnight, the mixtures were centrifuged at 12,000 *g* for 5 min. The resulting supernatants were used for immunofluorescence microscopy. In the case of preabsorbing mAbs, appropriate amounts of EJ tropomyosin (2 μg for CG3 and 5 μg for CGβ6) were mixed with 0.4 μg of purified CG3 or CGβ6 antibody and incubated at 4°C for 30 min. The mixture was immediately used for immunofluorescence microscopy.

Other Procedures

Immunoprecipitation with rabbit no. 2 and no. 6 antibodies was performed

as described previously (35, 42), except using protein A-Sepharose CL-4B instead of formalin-fixed *Staphylococcus aureus*. A slight modification of this immunoprecipitation was used for IgM mAbs, such as CG3 and CGβ6. Briefly, human EJ cells were labeled in vivo with 100 μCi of [^{35}S]methionine (1052 Ci/mmol) for 16 h in 3 ml of methionine-free media containing 2.5% FCS. After washing 3 times with PBS containing 2 mM EGTA and 5 mM $MgCl_2$, the cells (100-mm dish) were lysed in 1 ml of immunoprecipitation buffer (50 mM Tris, pH 8.0, 165 mM NaCl, 0.1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and passed through a no. 27 needle at least 10 times. The lysates were clarified by centrifugation at 110,000 *g* for 30 min. To aliquot 300 μl of the lysates, 5 μl of mAb from ascites fluid was added. After incubation at 4°C for 1 h, 7 μl of goat anti-mouse IgG (heavy and light chains) antibody was added to the mixture; this mixture was then incubated for another hour at 4°C. Finally, the immune complexes were incubated with 100 μl of protein A-Sepharose CL-4B for 30 min and then pelleted by microcentrifuge. The pellet was washed 3 times with the immunoprecipitation buffer and then once with PBS. The antigen antibodies-protein A-Sepharose complexes were solubilized in 50 μl of SDS gel sample buffer and boiled at 100°C for 3 min. After centrifugation to remove protein A-Sepharose, the supernatant was mixed with purified EJ tropomyosin and analyzed by two-dimensional gel electrophoresis (34). Isolation of microfilaments from CEF cells was performed according to the method described previously with LCK16 mAb (35). Both the immunoprecipitates and the isolated microfilaments were analyzed on SDS-polyacrylamide gels. SDS-PAGE was carried out according to Laemmli (2, 27) with a low concentration of bisacrylamide (12.5% acrylamide and 0.104% bisacrylamide). Western immunoblotting was performed as described (33).

Results

The specificities of the two rabbit antisera were first examined on the total extract of [^{35}S]methionine-labeled CEF cells by immunoprecipitation. As can be seen in Fig. 1, both antibodies specifically precipitated five protein bands; i.e., tropomyosin isoforms a, b, 1, 2, and 3, which were identical to that found in the isolated microfilament fraction (lane *E* in Fig. 1). Immunoprecipitation with normal rabbit serum gave no specific bands (lane *B* in Fig. 1). The antibody no. 2 raised against chicken gizzard tropomyosin cross reacted most strongly with isoforms 1 and 2 and weakly with isoform 3 in homogenates of CEF cells (lane *D* in Fig. 1). On the other hand, the antibody no. 6 raised against CEF tropomyosin reacted strongly with isoform 3 and weakly with isoform 1 (lane *C* in Fig. 1). Therefore, these two polyclonal anti-tropomyosin antibodies (no. 2 and no. 6) were ideally suited for the preparation of higher molecular mass (TM-1,-2) and lower molecular mass (TM-3) isoform-specific antibodies by sequential absorption of common, cross-reactive antibodies on purified individual CEF tropomyosin isoforms immobilized on nitrocellulose papers as detailed under Materials and Methods. The specificities of the isoform-specific antibodies finally obtained were tested by Western immunoblots on both partially purified CEF tropomyosin and CEF total cell extracts. As can be seen in Fig. 2, the TM-3 isoform-specific antibody (TM-3 Ab) reacted only with the TM-3 isoform, whereas TM-1,-2 isoform-specific antibody (TM-1,-2 Ab) recognized TM-1,-2 isoforms but not TM-3 isoform of CEF tropomyosin.

After staining for indirect immunofluorescence microscopy, TM-1,-2 antibody revealed stress fiber staining (Fig. 3 *B*). Occasionally, periodic staining could be seen in some cells. However, the ruffling membranes seen by phase microscopy were totally negative in staining (*arrowheads* in Fig. 3 *B*), indicating that the higher molecular mass isoforms TM-1,-2 of CEF tropomyosin were not present in this area. On the contrary, TM-3 antibody stained both stress fibers and ruffling membranes (Fig. 3 *D*). However, these staining

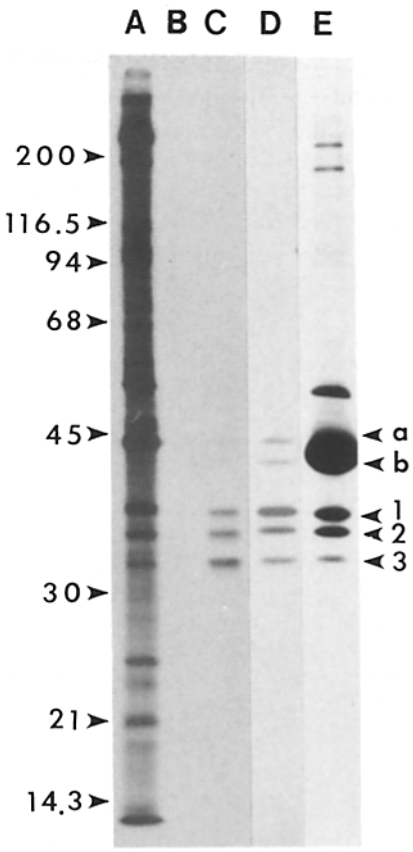

Figure 1. SDS-PAGE analysis of the immunoprecipitates of CEF total extracts with rabbit anti-tropomyosin antiserum. CEF cells were labeled with [^{35}S]methionine for 20 h in methionine-free medium, and processed for immunoprecipitation and microfilament isolation as described previously (42). The immunoprecipitates, isolated microfilaments, and total cell extracts were analyzed on 12.5% SDS-PAGE. After electrophoresis, the gels were processed for fluorography. (*A*) Total cell extracts; (*B*) immunoprecipitate with normal rabbit serum; (*C*) immunoprecipitate with rabbit no. 6 antiserum against CEF tropomyosin; (*D*) immunoprecipitate with rabbit no. 2 antiserum against chicken gizzard tropomyosin; and (*E*) the isolated microfilament fraction. CEF tropomyosin isoforms are indicated as a, b, 1, 2, and 3.

patterns were not seen in the control experiments with either preimmune rabbit serum or antibody preabsorbed with CEF tropomyosin (data not shown). Apparently, the lower molecular mass isoform of tropomyosin has a significantly different distribution within the CEF cell than the higher molecular mass isoforms. To further investigate whether different isoforms have different localizations within the same CEF cell, we have used double-label indirect immunofluorescence staining with rabbit TM-3 antibody and mouse monoclonal CGβ6 antibodies. The CGβ6 antibody has been shown to be specific to higher molecular mass isoforms (a, b, 1, and 2) of CEF tropomyosin (33). Fig. 4 shows the result from such double-label immunofluorescence microscopy. Clearly, the lower molecular mass isoform (TM-3) was distributed near the ruffling membranes (indicated by *arrowheads* in Fig. 4 *C*), in addition to stress fiber localization similar to that shown by the higher molecular mass isoforms (a, b, 1, and 2). Double-label experiments in which the primary antibod-

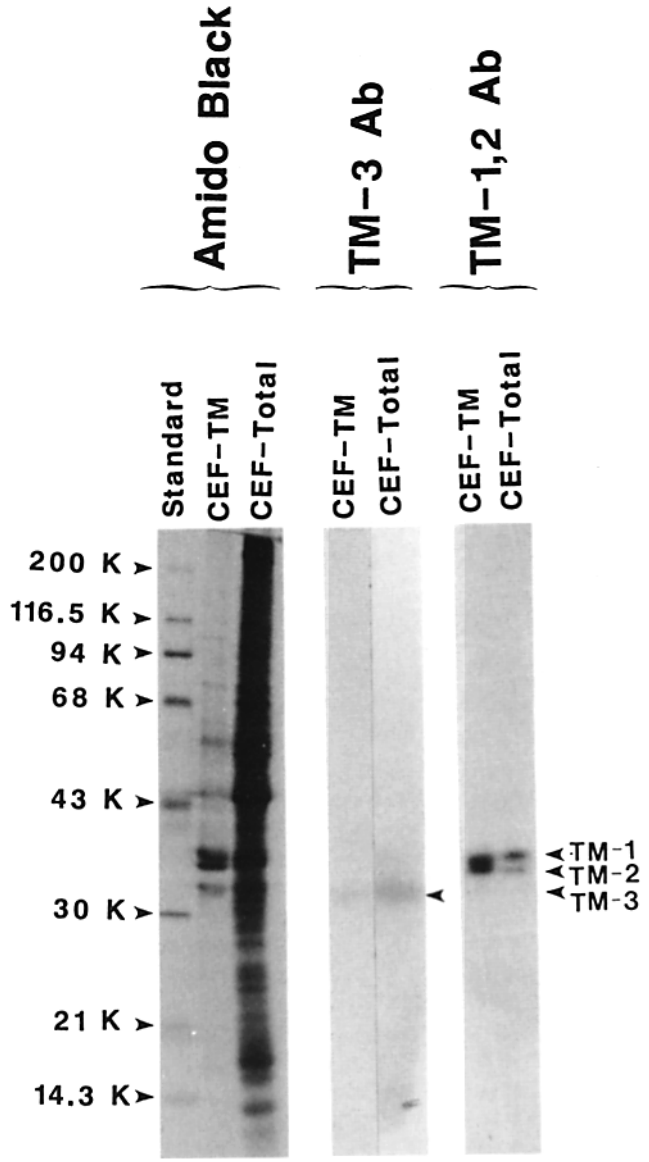

Figure 2. Western blot analysis of affinity-purified isoform-specific antibodies binding to CEF tropomyosin isoforms. Partially purified CEF tropomyosin (lane *CEF-TM*) and total extract (lane *CEF-Total*) of CEF cells were separated on 12.5% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose paper and either stained with Amido black or reacted with isoform 3-specific antibody (TM-3 Ab) or isoforms 1, 2-specific antibody (TM-1,-2 Ab), followed by ^{125}I-labeled goat anti-mouse IgG (heavy and light chains). Bound antibody was detected by autoradiography. Major CEF tropomyosin isoforms are indicated by TM-1, TM-2, and TM-3. Note that TM-3 antibody reacted only to the lower molecular mass isoform (TM-3), whereas TM-1,-2 antibody recognized the higher molecular mass isoforms (TM-1 and TM-2).

ies were applied in different orders gave essentially the same results. This suggested that the absence of fluorescence labeling of the mAb in the ruffling membrane was not due to competition or steric hindrance.

To determine whether this differential localization of tropomyosin isoforms also exists in other cell lines, we have screened all of our mAbs (33) by immunoblotting on human bladder carcinoma EJ cells to see which antibodies are specific to EJ tropomyosin isoforms. We have found that the CGβ6 antibody specifically recognized a higher molecular mass isoform (TM-3), whereas the CG3 antibody reacted to the lower molecular mass isoforms (TM-4,-5) of EJ tropomyosin (data not shown). As with the lower molecular mass tropomyosin isoforms of CEF and rat embryo cells (34, 39), human EJ TM-4,-5 also showed a weaker affinity to actin filaments when compared to the higher molecular mass isoform, including TM-3, of EJ tropomyosin (Fig. 5). Therefore, it is interesting to determine the intracellular localization of tropomyosin isoforms TM-3 and TM-4,-5 within human EJ cells by monoclonal CG3 and CGβ6 antibodies. To further identify which one of the low molecular mass isoforms of human tropomyosin was recognized by CG3 antibody, two-dimensional gel analysis of the immunoprecipitates from [^{35}S]methionine-labeled EJ cell extract (Fig. 6 *A*) was carried out. As can be seen in Fig. 6 *B*, two dimensional gel electrophoresis effectively separated the purified EJ tropomyosin into five spots. The immunoprecipitates obtained with CG3 antibody showed only TM-5 isoform (Fig. 6 *D*). Consistently, the immunoprecipitate with CGβ6 antibody contained mainly TM-3 isoform (Fig. 6 *C*), although in the case of heavily loaded gel, TM-2 isoform can also be recognized. Therefore, we concluded that CG3 recognized the TM-5 isoform, while CGβ6 reacted mainly with TM-3 isoform of human EJ tropomyosin.

Immunofluorescence microscopy was performed with these mAbs on human EJ cells. Fig. 7 *B* shows cells stained with CGβ6 antibody to reveal the localization of human tropomyosin 3 (TM-3) isoform. Few stress fibers were stained; apparently, the majority of EJ TM-3 isoform was diffusely distributed throughout the cytoplasm, except in the peripheral ruffle regions (Fig. 7 *B*). On the other hand, EJ TM-5 isoform recognized by the CG3 antibody obviously is located near the ruffling membranes, in addition to stress fibers and diffuse dots localization (Fig. 7 *D*). Both staining patterns by CG3 and CGβ6 antibodies can be abolished by preabsorption of antibodies with purified EJ tropomyosin (Fig. 7, *F* and *H*, respectively).

Discussion

In this study, we have demonstrated that within nonmuscle cells, the lower molecular mass isoform of tropomyosin distributes differently from the higher molecular mass isoforms. Although both higher and lower molecular mass isoform-specific antibodies have revealed tropomyosin staining along stress fibers (or microfilament bundles), an additional distribution near the peripheral ruffles can only be observed with antibody specific to the lower molecular mass isoform. This ruffle location of the lower molecular mass isoform of tropomyosin has been detected not only in CEF cells, but also in human bladder carcinoma cells with two different antibody probes. Therefore, it is likely that this may be a general pattern of localization for the lower molecular mass isoform of tropomyosin in normal and transformed cells.

It has been reported previously that tropomyosin could not be found in ruffling membranes of cultured cells by immunofluorescence (28, 29). This failure to detect tropomyosin in the ruffle regions might well be due to the use of an antibody that was prepared against skeletal muscle tropomyosin.

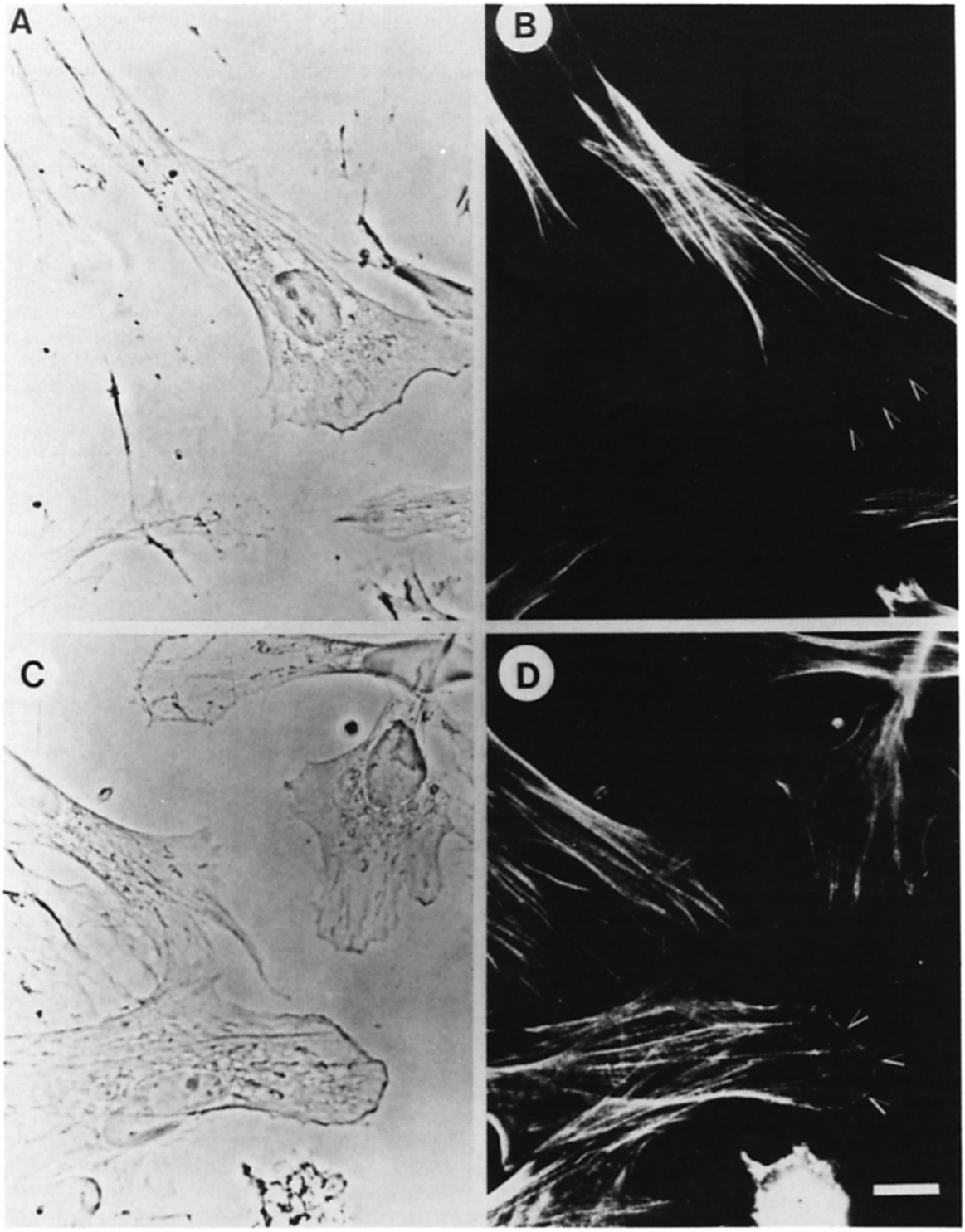

Figure 3. Indirect immunofluorescence of CEF cells with affinity-purified isoforms 1, 2-specific (A and B) and isoform 3-specific (C and D) antibodies. (*A* and *C*) Phase-contrast micrographs; (*B* and *D*) fluorescent micrographs. No peripheral ruffles (indicated by *arrowheads* in *B*) are stained with isoform 1, 2-specific antibody. On the other hand, fluorescence in *D* is found not only in stress fibers but also peripheral ruffles. Bar, 10 μm.

From our experience, an antibody prepared against muscle isoforms of tropomyosin tends to react strongly to the higher molecular mass isoforms, but only weakly to the lower molecular mass isoforms of nonmuscle tropomyosin. This may be further supported by the fact that human or rat nonmuscle tropomyosin isoform 1 (one of the highest molecular mass isoforms) and skeletal muscle tropomyosin isoform are encoded from the same gene by differential splicing mechanism (18, 37). In the present study, we have shown that antibodies specific to the higher molecular mass isoforms of nonmuscle

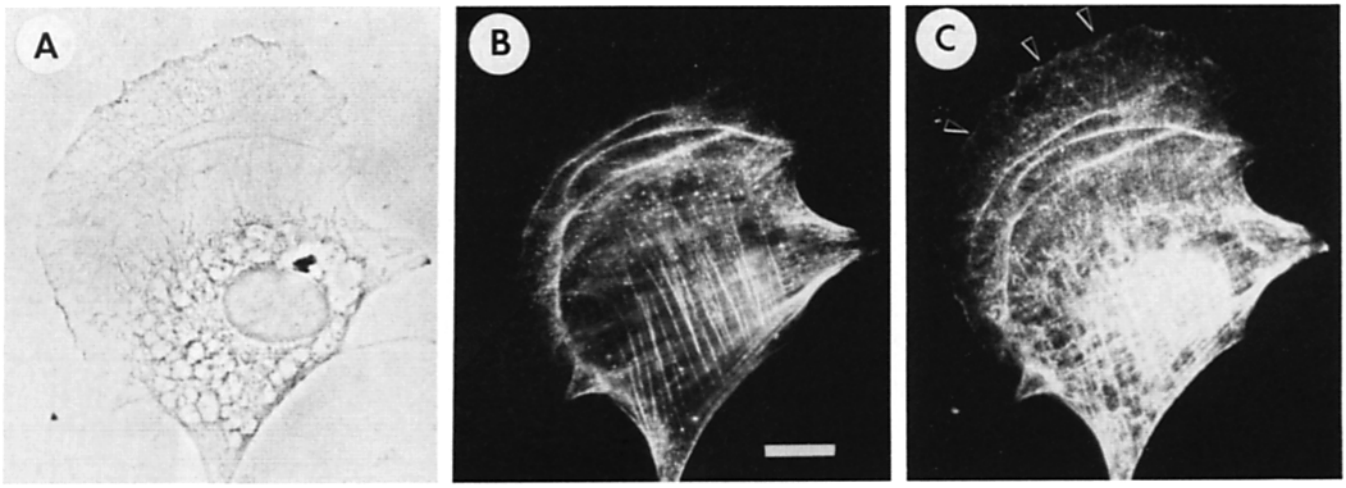

Figure 4. Indirect double-label immunofluorescence of CEF cells. Same cells were reacted with both mouse monoclonal CGβ6 antibody (*B*) against the higher molecular mass isoforms (i.e., TM-a, -b, -1, and -2) of CEF tropomyosin and affinity-purified rabbit anti-isoform 3 antibody (*C*). Fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG antibodies were used for the second antibody in the indirect immunofluorescence. (*A*) Phase-contrast micrograph; (*B* and *C*) fluorescent micrographs. Monoclonal CGβ6 antibody stained mainly stress fibers (*B*), whereas anti-isoform 3 antibody stained peripheral ruffles as well as stress fibers (*C*). In addition, more dot staining could be seen with anti-isoform 3 antibody. Bar, 10 μm.

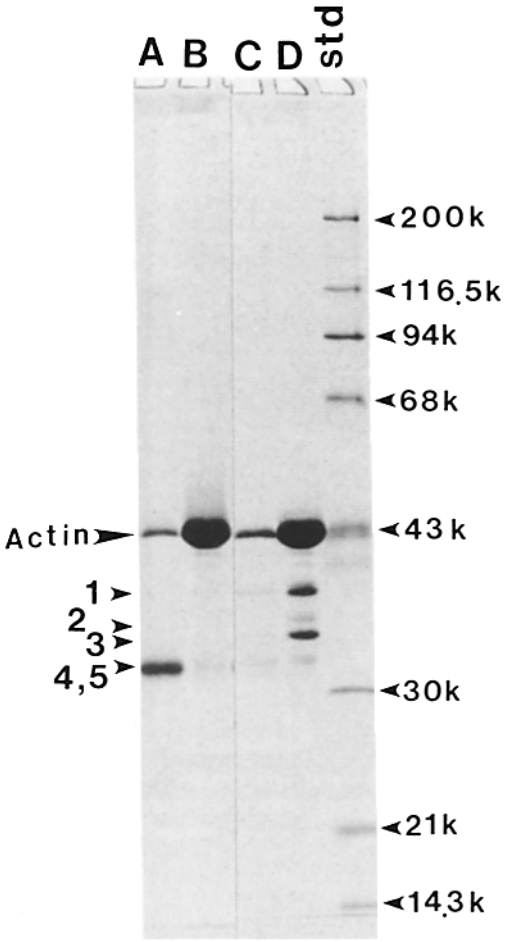

Figure 5. Tests for the actin-binding ability of human EJ tropomyosin isoforms. Actin (90 μg) and tropomyosin (15 μg) were mixed in 100 μl of a buffer solution of 10 mM Tris, pH 8.0, 30 mM KCl, and 10 mM $MgCl_2$. After incubation for 30 min at room temperature, the protein samples were centrifuged for 30 min in a Beckman airfuge at 26 psi. Aliquots of the resulting supernatants (lanes *A* and *C*) and pellets (lanes *B* and *D*) were analyzed on 12.5% SDS-PAGE. Human EJ tropomyosins are indicated by 1, 2, and 3 and 4, 5.

tropomyosin do reveal the typical tropomyosin location along stress fibers, as reported previously (28, 29). Thus, it is likely that the peripheral ruffles contain the lower molecular mass isoforms of tropomyosin but not the higher molecular mass isoforms.

Localization of nonmuscle tropomyosin in the ruffle region has also been detected by two other investigators (4, 53). Boschek et al. (4) have reported that the ruffle-like flowers, which appear transiently on the dorsal surface of chicken embryo cells undergoing transformation by Rous sarcoma virus, contain tropomyosin and other contractile proteins such as actin, myosin, and α-actinin. The other report by Warren et al. (53) has demonstrated by antitropomyosin labeling experiments and microinjection studies that tropomyosin can occur in peripheral ruffles of normal and transformed rat kidney cells. The antibody that they have used reacts with the higher molecular mass isoforms but not with the lower molecular mass isoforms of normal rat kidney cells. Using this antibody, they have observed a redistribution of the higher molecular mass tropomyosin isoforms from ruffles to stress fibers as these motile normal rat kidney cells settle down in contact with one another. However, ruffle staining with the same antibody has not been detected in the relatively nonmotile normal rat kidney 52E cells, suggesting that the higher molecular mass isoforms of tropomyosin are not present in the peripheral ruffles of these cells. It should be interesting to determine whether or not the lower molecular mass tropomyosin isoforms exist in the ruffles of these cells. From the present study, we would predict that the lower molecular mass isoforms of tropomyosin should be present

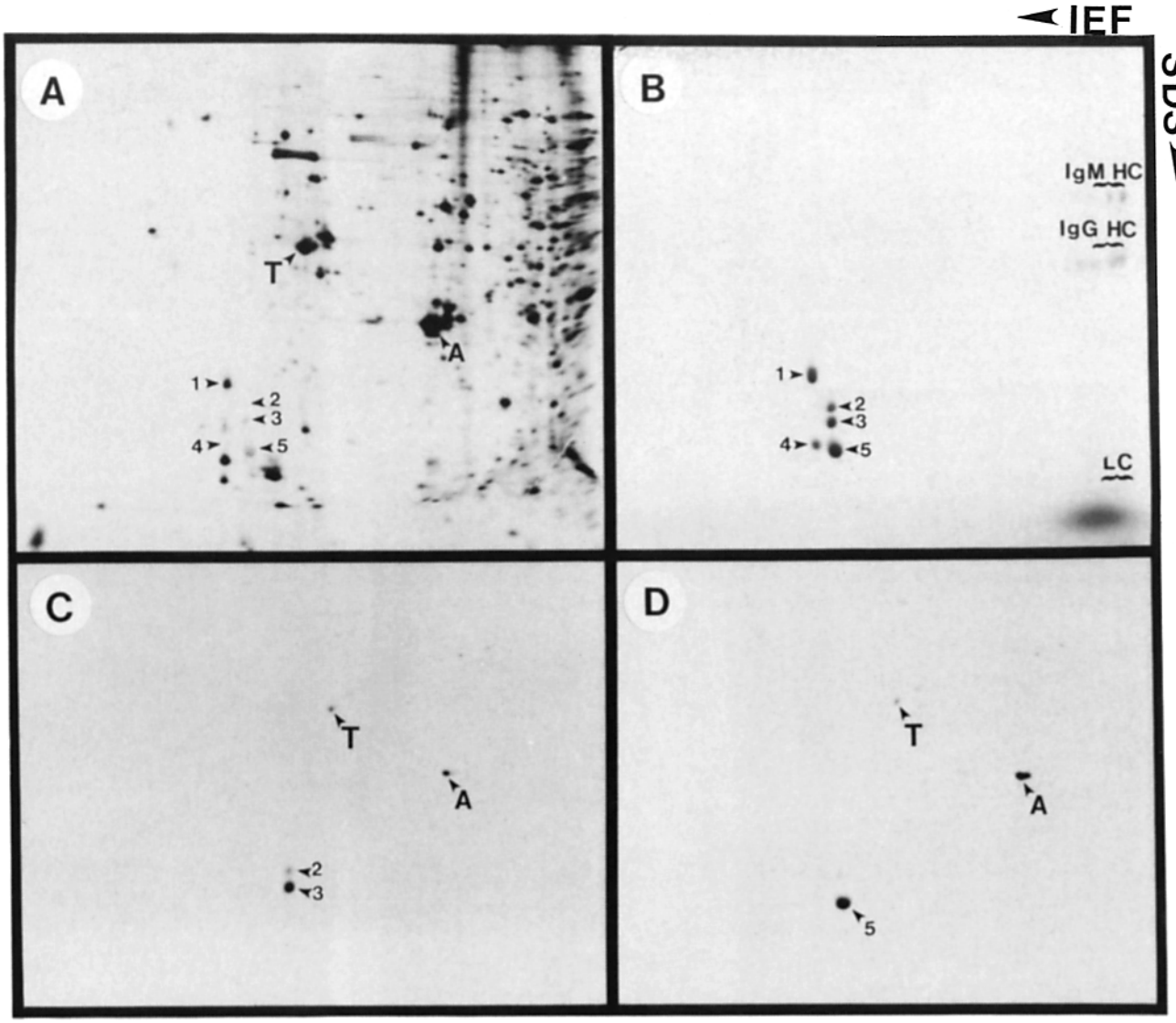

Figure 6. Immunoprecipitation analysis. Two-dimensional gel analysis of a total cell extract of human EJ cells (*A*) and immunoprecipitated proteins by monoclonal CG3 (*B* and *D*) or CGβ6 (*C*) antibodies. Cells were labeled in vivo with [^{35}S]methionine. The immunoprecipitation was performed as described under Materials and Methods. Purified human EJ tropomyosins were mixed with the labeled cell extract and the immunoprecipitates for two-dimensional gel analysis to facilitate the identification of individual tropomyosin isoforms. Two-dimensional gels were run with the first dimensional gels containing pH 4–6 ampholytes and the second dimensional slabs comtaining 12.5% polyacrylamide. Fluorographs (*A*, *C*, and *D*) and Coomassie Blue-stained pattern (*B*) of the gels are shown with the acidic ends to the left. Human nonmuscle tropomyosin isoforms TM-1–5 are indicated by numbers *1–5*, respectively. *A*, actin; *T*, tubulin; *HC* and *LC*, heavy and light chains, respectively, of mouse IgM and goat IgG (using as secondary antibody) molecules. As can be noted, CG3 preferentially precipitated TM-5, whereas CGβ6 antibody strongly reacted with TM-3 and weakly with TM-2 isoform.

in the peripheral ruffles. In this regard, Fowler and Bennett (16) have already reported the presence of tropomyosin in the membrane of the human erythrocyte, a nonmotile cell type. Based on the similarity in their apparent molecular masses, the erythrocyte membrane tropomyosin may in fact be the same as the lower molecular mass tropomyosin isoforms of nonmuscle cultured cells. Moreover, all of the erythrocyte tropomyosins are thought to associate with the short actin filaments in the membrane skeleton (16, 46). Cultured nonmuscle cells have been shown to contain cortical bundles of actin filaments (49), so it is possible that the lower molecular mass isoform of tropomyosin detected in the peripheral ruffles of these cells may be totally bound to actin filaments.

At present, the function of tropomyosin in nonmuscle cells remains unclear. The multiple isoforms of tropomyosin shown to exist in all nonmuscle cells so far examined may be necessary for these cells to carry out fine regulation of cell shape and cell motility. Although the molecular mechanism for this regulation is unknown, several lines of evidence have accumulated to support the following speculation: cells may perform this regulatory mechanism by controlling the amounts of different tropomyosin isoforms expressed. For example, differential expression of tropomyosin isoforms has been observed in transformed cells, and these changes in tropomyosin isoforms may be responsible for rearrangement of microfilament bundles and/or changes in cell shape (7, 19, 20, 30,

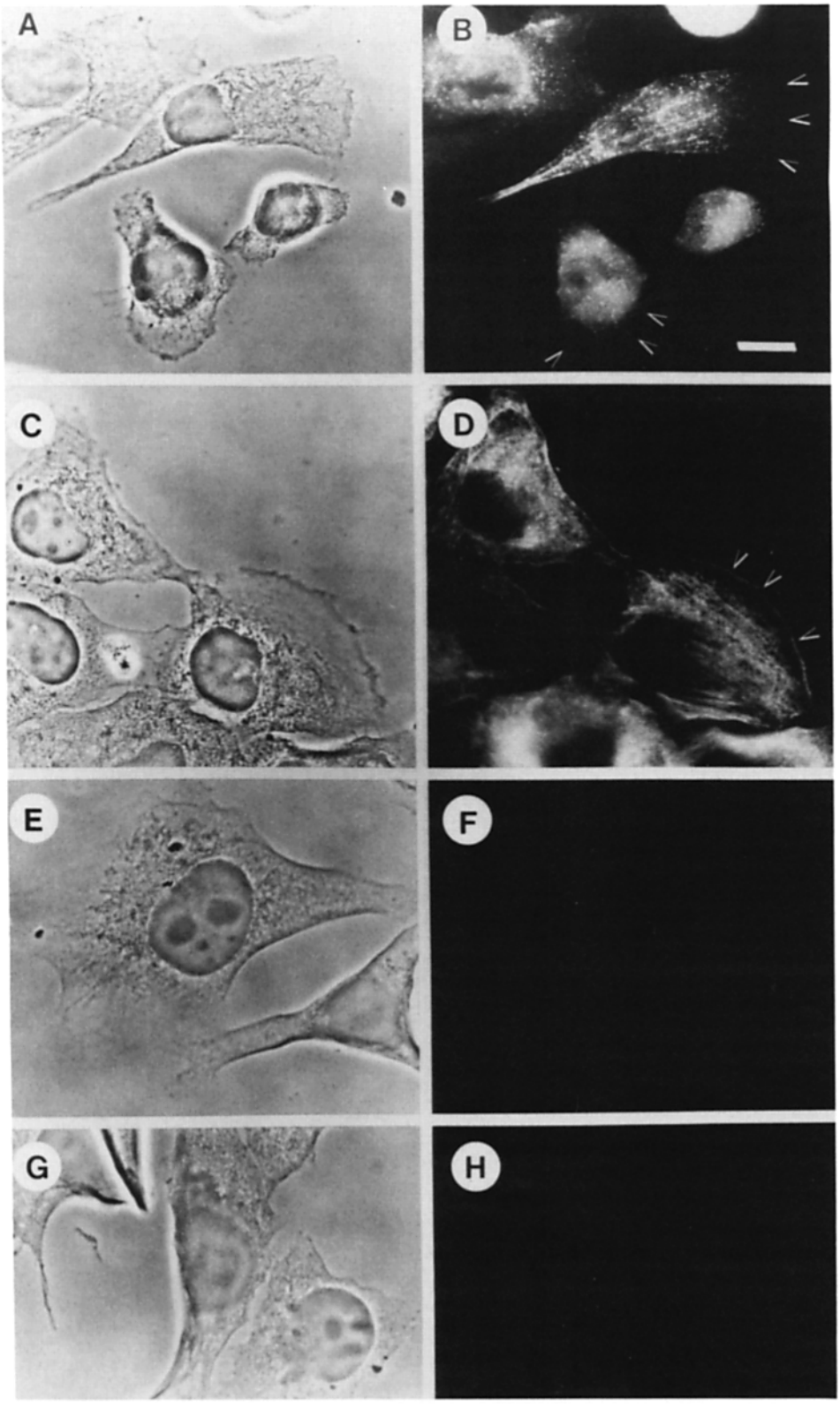

Figure 7. Indirect immunofluorescence of human EJ cells with monoclonal CGβ6 antibody against TM-3 isoform (*A* and *B*), monoclonal CG3 antibody against TM-5 isoform (*C* and *D*), and preabsorbed antibodies (*E* and *F* for CGβ6, and *G* and *H* for CG3). (*A*, *C*, *E*, and *G*) Phase-contrast micrographs; (*B*, *D*, *F*, and *H*) fluorescent micrographs. The diffuse, dot stain throughout the cytoplasm, except the ruffle regions, was the major staining pattern with CGβ6 antibody (*B*). Occasionally, few stress fiber staining was also observed. On the other hand, not only stress fiber and diffuse stains, but also ruffle stain were readily detected by CG3 antibody (*D*). Fluorescent micrographs (*F* and *H*) obtained from preabsorption experiments were taken and printed at the same exposure time as that for those shown in *B* and *D*. Bar, 10 μm.

31, 34, 36, 41, 45). Alternatively, cells may distribute specific isoforms in a localized area to affect the performance of particular microfilament functions. For example, we have now shown that the lower molecular mass tropomyosin isoforms with weaker actin-binding ability are localized more abundantly in highly motile regions (ruffles) than the higher molecular mass isoforms of tropomyosin. Another possibility is that the multiple isoforms of nonmuscle tropomyosin may play a role in modulating the organization of microfilaments in cells by regulating the interaction between actin and other

actin-binding proteins. For example, skeletal muscle and nonmuscle tropomyosins have been shown to inhibit the binding of filamin, spectrin, α-actinin, caldesmon, and 55-kD protein to actin filaments (12, 26, 38, 40, 44, 47, 54), as well as the action of fragmin, villin, gelsolin, DNase I, actin-depolymerizing protein (destrin), and actin-depolymerizing factor on actin filaments (1, 3, 5, 13, 21, 22, 43). Obviously, the differential distribution of tropomyosin isoforms within the nonmuscle cell could be very critical to this sort of a modulating function.

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