

## Identification of actin from hepatoma Morris 5123 cells<sup>⊙\*</sup>

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The hepatoma Morris 5123 tumor growth is accompanied by changes in actin content and polymerization (Malicka-Błaszkiwicz *et al.* (1995) *Mat. Med. Pol.*, 27, 115-118; Nowak *et al.* (1995) *J. Exp. Cancer Res.* 14, 37-40). Presently actin isoforms from cytosol and cytoskeleton fractions were separated by SDS/PAGE and identified with antibodies directed against different actin isoforms. Actin isolated from the cytosol by affinity chromatography on DNase I bound to agarose shows the presence of only one protein spot on 2D gel electrophoresis corresponding to the mobility of the rabbit  $\alpha$  skeletal muscle actin ( $M_r$  43 000) and isoelectric point equal to 5.3. It interacts only with monoclonal anti  $\beta$  actin isoform antibodies, posing the question of differential affinity of actin isoforms to DNase I.

The interest in the role of actin in cell pathology is still growing [1-3]. Actin microfilaments are components of cytoskeleton, the structure involved in many cellular functions, including cell motility, contractile ring formation in mitosis and the maintenance and changes of cell shape [4].

Data have accumulated at the alterations of cytoskeleton structure and function in malignant transformation. The observed "disintegration" of actin cytoskeleton in many transformed cell lines [5-7] can be caused by changes in actin content, and its ability to polymerize, and by a lack of physiological

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**Abbreviations:** DNase I, deoxyribonuclease I from bovine pancreas (EC 3.1.21.1); DTT, dithiothreitol; Gu-HCl, guanidine hydrochloride; IEF, isoelectric focusing; 2D PAGE, two dimensional polyacrylamide gel electrophoresis.

equilibrium between actin assembly and disassembly, so important for actin functions. Special attention has been paid to the actin microfilament architecture involved directly in adhesion and locomotion capabilities of cells in tumor growth and metastasis [1, 6, 8].

In vertebrates at least six different actin isoforms are known, differing by less than 10% of their amino-acid sequence, each encoded by a separate gene [9, 10]. The cytoplasmic  $\beta$  and  $\gamma$  actin isoforms predominate in most avian and mammalian nonmuscle cells. The two smooth muscle isoforms – actin  $\alpha$  and  $\gamma$  are found primarily in smooth muscle, whereas  $\alpha$  cardiac and  $\alpha$  skeletal actin isoforms are expressed in striated cardiac and skeletal muscle, respectively [9].

The existence of multiple actin isoforms within tissues and even a single cell suggests functional differences among them [11, 12]. It also arises the question of a correlation between particular isoform expression and some pathologies. Leavitt & Kakunaga [13] identified a variant form of actin (distinguishable from the normal actin  $\alpha$ ,  $\beta$ ,  $\gamma$  polypeptides) in malignant human fibroblasts, transformed *in vitro* by a single chemical treatment. Application of high resolution 2D gel electrophoresis and comparative examination of protein synthesis in normal and neoplastic human fibroblasts led Leavitt [14] to the discovery of a mutant  $\beta$  actin, associated with tumorigenicity of fibroblasts. Expression of a new mutant actin ( $\beta_m$ ) was also found by Shimokawa-Kuruoki *et al.* [15] in mouse melanoma B16 cell lines. It was inversely correlated with the invasiveness and metastatic potential of B16 melanoma sublines. Special attention has been drawn to the expression of the  $\alpha$  smooth muscle isoactin in non-muscle cells of several normal and neoplastic tissues [16–19]. Schmitt-Gräff *et al.* [20] reported the  $\alpha$  smooth muscle actin isoform to accompany some tumor hepatocellular damage. The same isoform, present in stromal cells of the uterine cervix, was considered by Cintorino *et al.* [21]

useful in diagnosis of cervical intra-epithelial neoplasia.

Our former studies on the experimental tumor, hepatoma Morris 5123, cells [22, 23] were directed on quantitative changes in actin content and the state of actin polymerization in these cells during tumor growth. The results indicated tumor growth process to be accompanied by changes in actin concentration and polymerization not only in the growing tumor, but also in the liver, lungs and serum of tumor bearing rats. The goal of current work was to identify actin isoforms present in the hepatoma Morris 5123 tumor cells.

## MATERIALS AND METHODS

**Materials.** The experimental tumor hepatoma Morris 5123 [24] was maintained by serial passages *in vivo*. Four-month-old inbred Buffalo rats (females) with mean body mass of 130 g, bred in the Department of Pathological Anatomy, Medical Academy of Wrocław, were used. To start the growth of the tumor, 0.3 ml of hepatoma Morris 5123 pulp was implanted intramuscularly into the left limb of the rats. Twenty-one days after tumor implantation, in the phase of extensive tumor growth, the rats were sacrificed, tumors isolated from the animals and used for further procedures.

**Sample preparation.** The cytosolic fraction was obtained after homogenization of the tumor tissue (washed with 0.9% NaCl) with 3 vol. of freshly made actin G stabilizing buffer, (10 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose, 1 mM dithiothreitol, 0.1 mM ATP and 0.1 mM CaCl<sub>2</sub>), as previously described [25]. Homogenates were centrifuged at 105 000 × *g*, and the supernatant fraction (cytosol) was used for further analysis and for actin isolation.

The cytoskeletal fraction was prepared according to Leavitt & Kakunaga [13] with 10 mM Tris/HCl buffer, pH 7.4, containing 10 mM MgCl<sub>2</sub>, 30 mM KCl, 0.01 M  $\beta$ -mercapto-

ethanol and 1% Triton X-100 used for extraction of proteins from disrupted tumor tissue. The extract was centrifuged at  $10000 \times g$  at  $4^{\circ}\text{C}$  for 15 min to separate Triton-soluble proteins. The pellet was suspended in the same buffer containing 0.1% Triton and centrifuged as above. The pellet proteins were solubilized with the sample denaturing buffer and separated in SDS/PAGE according to Laemmli [26], or saved for further analysis frozen at  $-70^{\circ}\text{C}$ .

**Affinity chromatography on immobilized DNase I.** Chromatography was performed according to the method of Lazarides & Lindberg [27]. DNase I from bovine pancreas (Sigma) was dissolved in coupling buffer (1 M phosphate buffer pH 8.6) and incubated with vinylsulfone activated agarose (Mini Leak) overnight. Free activated groups were blocked with 0.1 M ethanolamine/HCl, pH 8.5, for 3 h. Before chromatography, the cytosol fraction from hepatoma Morris was dialyzed for 18 h against 50 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM  $\text{CaCl}_2$  (buffer I), and applied to a DNase I-agarose column (0.7 cm  $\times$  2.5 cm). The column was equilibrated and elution of unbound proteins was carried out with the same buffer. Non-specifically adsorbed proteins were eluted with 0.5 M sodium acetate containing 30% glycerol and 1 mM  $\text{CaCl}_2$  (buffer II). The elution of the bound proteins was carried out with buffer II adjusted to pH 6.5, containing 0.75 M guanidine-HCl (Gu-HCl) (buffer III) and subsequently with buffer II of pH 6.5, containing 3 M GuHCl (buffer IV). The whole procedure was carried out at  $0-4^{\circ}\text{C}$ .

**Protein determination.** The standard procedures of Lowry *et al.* [28] and Bradford [29] as well as measurements of  $A_{280}$  were applied.

**SDS/PAGE.** Protein separation was performed according to Laemmli [26] on 10% gels, at 12 mA per slab, using 0.1% SDS in electrode buffer.

**2D-PAGE.** Two-dimensional electrophoresis was carried out in the Hoeffer small 2-D electrophoresis unit (SE 250), following in-

structions from the manufacturer, basically using the procedure of O'Farrell [30]. IEF was performed in the presence of 2% ampholine mixture, pH 5-8/3-10 (Pharmacia), containing 9.5 M urea, 2% Nonidet P40 and 3.3% acrylamide. The samples were prefocused for 30 min at 250 V and then were focused for 3 h at 500 V. Second dimension separation was carried out under the standard SDS/PAGE conditions [26]. After electrophoresis proteins were stained according to Wray *et al.* [31].

**Immunoblotting.** Proteins separated by SDS/PAGE were transferred to nitrocellulose sheets by the procedure of Towbin *et al.* [32] and reacted with commercially available murine monoclonal antibodies purchased from Sigma, directed against  $\alpha$  skeletal muscle actin,  $\alpha$  smooth muscle actin and  $\beta$  cytoplasmic actin, respectively. To examine the presence of the  $\gamma$  cytoplasmic actin isoform, polyclonal antibodies recognizing  $\gamma$  cytoplasmic actins, as well as  $\gamma$  and  $\alpha$  smooth muscle actins, were used (obtained as a gift from prof. Gabbiani, University of Geneva, Switzerland). Immunoreactivity was shown by the extravidin-biotin peroxidase (Sigma) technique, using 4-chloro-1-naphthol as a substrate for peroxidase.

**Sample preparation.** Rabbit skeletal muscle actin used as a control was prepared according to Spudich & Watt [33].

## RESULTS

### Identification of actin isoforms in the hepatoma Morris 5123 tumor cells

In the light of the information on distribution of actin isoforms within cells, as reviewed by Herman [12], their identification was carried out in the cytosol and cytoskeleton protein fractions, prepared from the intensively growing tumor cells and separated by SDS/PAGE.



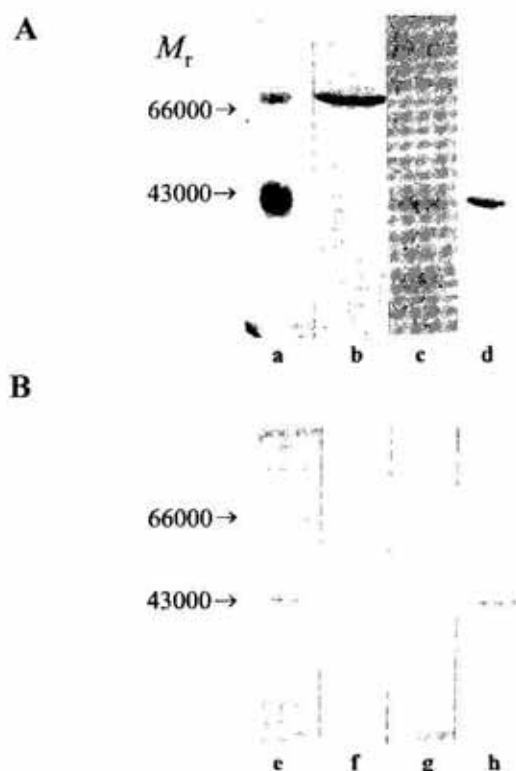
Cytosolic protein analysis evidenced the most intensive reaction in the area of  $M_r$  43 000 (i.e. the  $M_r$  of G actin), with monoclonal antibodies directed against the  $\beta$  actin isoform (Fig. 1A lane a). A distinct positive reaction in the same area was also observed when polyclonal antibodies recognizing  $\gamma$  cytoplasmic actin (besides the  $\alpha$  and  $\gamma$  smooth muscle isoforms) were applied (Fig. 1A lane d). Only a very weak, trace reaction was seen in this  $M_r$  band when antibodies to  $\alpha$  sarcomeric and  $\alpha$  smooth muscle actin were applied (Fig. 1A lane b and c).

It is interesting to note a positive reaction of different intensity also in a band of  $M_r$  a little higher than 66 000, with all applied antibodies, except for those directed against the  $\alpha$  smooth muscle actin (cf. Fig. 1A lanes a, b, c, d). This may probably result from the occurrence of proteins sharing epitopes with actin or from the presence of complexes of actin with other proteins, nondissociable under SDS/PAGE conditions.

The analysis of the cytoskeleton fraction proteins (Fig. 1B) shows a positive reaction in the area of  $M_r$  43 000 with antibodies recognizing the  $\beta$  and  $\gamma$  cytoplasmic actin isoforms (Fig. 1B lanes e and h, respectively). Very fine reaction with the use of those antibodies was observed also on the immunoblots in the area of 66 000 (almost not visible).

The  $\alpha$  smooth and  $\alpha$  skeletal isoforms could not be detected in the cytoskeletal proteins fraction (Fig. 1B lane f and g). The lack of immunoreactivity with antibodies directed against the  $\alpha$  smooth muscle isoform is consistent with a lack of  $\gamma$  smooth muscle isoactin, as the latter usually accompanies  $\alpha$  smooth muscle isoform [11].

To verify the specificity of the applied antibodies, the results were compared with those obtained with the use of the standard  $\alpha$  rabbit skeletal muscle actin. In this case the only positive reaction, and only in the  $M_r$  43 000 region, was observed when a monoclonal antibody to the  $\alpha$  rabbit skeletal muscle actin was used (not shown).

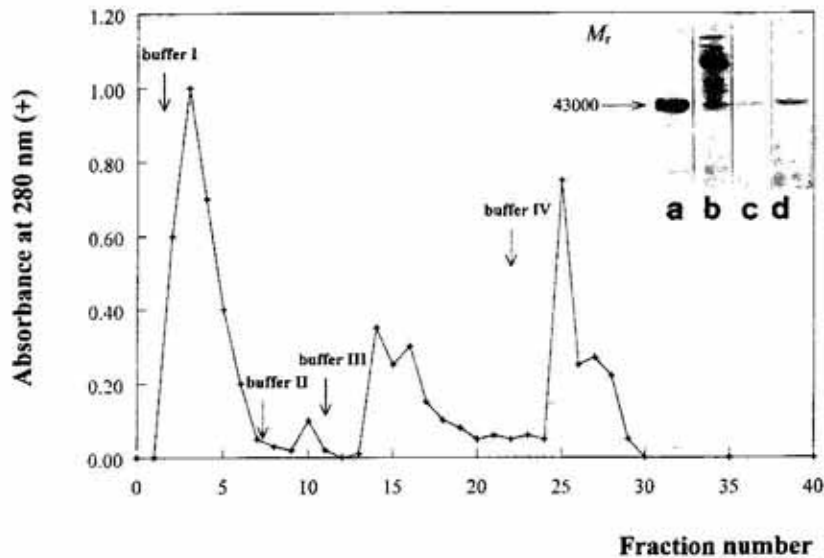


**Figure 1. Immunoblotting of hepatoma Morris 5123 proteins with antibodies directed against different actin isoforms after SDS/PAGE:**

A, cytosol; B, cytoskeleton (45  $\mu$ g protein applied in each lane of A and B); The following monoclonal antibodies were used: lanes a and e, anti  $\beta$  cytoplasmic actin; b and f, anti  $\alpha$  skeletal muscle actin; c and g, anti  $\alpha$  smooth muscle actin; d and h, polyclonal antibodies recognizing  $\gamma$  cytoplasmic actin.

#### Isolation of actin from cytosol of hepatoma Morris 5123

A further electrophoretical analysis was carried out on actin isolated from cytosol by means of affinity chromatography on DNase I [27]. Actin binds tightly to DNase I and dissociates from the affinity column only when guanidine hydrochloride (Gu-HCl) is included in the elution buffers (Fig. 2). Proteins present in the elution profile were analyzed by SDS/PAGE and the results were compared routinely with those for the standard  $\alpha$  rabbit skeletal muscle actin. The Coomassie Brilliant Blue staining patterns show (Fig. 2 insert



**Figure 2.** Affinity chromatography of hepatoma Morris 5123 cytosol proteins on DNase I-agarose column.

The column (0.7 cm × 2.5 cm) was equilibrated and unbound proteins were eluted with 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and 1 mM CaCl<sub>2</sub> (buffer I). Then elution was carried out with buffers II, III and IV (for buffers composition see Methods). Insert: protein patterns obtained after SDS/PAGE, stained with Coomassie Brilliant Blue: lane a, standard rabbit  $\alpha$  skeletal muscle actin – 15  $\mu$ g; lane b, fractions eluted with buffer I (fr. 1–7); lane c, fractions eluted with buffer III (fr. 13–17); lane d, fractions eluted with buffer IV (fr. 25–29).

lanes a, c, d) only one protein band of the standard muscle actin mobility ( $M_r$  43000) that appears always in the fractions eluted with buffer III or IV, containing 0.75 M and 3 M Gu-HCl. It has to be mentioned, however, that an additional band of  $M_r$  32000 (present in some preparations) could be observed, after silver staining according to Wray *et al.* [31], in the fractions eluted in the presence of 0.75 M Gu-HCl (not shown). It occurs that that protein might have been responsible for weaker binding of a fraction of actin to the column with DNase I and for actin release with less concentrated Gu-HCl.

The protein of  $M_r$  43000 present in fractions eluted with buffers III and IV (Fig. 2), after SDS/PAGE and immunoblotting was identified with the use of anti actin antibodies, the same as we applied for the identification of actin in the crude samples. A positive reaction was obtained always only in the presence of the monoclonal anti  $\beta$  actin antibodies (Fig. 3a, b). Actin was present also in the fraction of nonadsorbed proteins eluted with buffer I and

it interacted with antibodies directed against  $\beta$ , as well as  $\gamma$  cytoplasmic actins (not shown).

#### 2D gels electrophoretical analysis of purified actin

The purified actin preparations were further analyzed to determine the presence of actin isoforms and their isoelectric points. The results of two dimensional gel electrophoresis allowed to compare migration of actin resulting from the first (Fig. 4b, c) and after second dimension (Fig. 5A, B) with migration of the standard rabbit skeletal muscle  $\alpha$  actin (Figs. 4a and 5C). The purified actin shows on 2D gel electrophoresis a single spot with mobility similar to  $\alpha$  skeletal muscle actin. The isoelectric point of purified actin is slightly more alkaline than that of the rabbit  $\alpha$  skeletal muscle actin (cf. Fig. 4 a, b, c). It was estimated as 5.2 for the standard  $\alpha$  skeletal muscle actin and 5.3 for the hepatoma Morris 5123 actin under the experimental conditions used. There was no difference in the iso-



**Figure 3. Immunoblotting of purified actin with monoclonal antibodies directed against  $\beta$  actin isoform\*:**

(a and b) concentrated pooled fractions (separated sample protein contents were 9  $\mu$ g and 8  $\mu$ g, respectively) corresponding to the peaks (cf. Fig. 2) eluted with buffers IV and III, respectively. \*No reactivity was detected with the other applied antibodies i.e. recognizing  $\gamma$  cytoplasmic actin, anti  $\alpha$  smooth muscle actin and anti  $\alpha$  skeletal muscle actin.

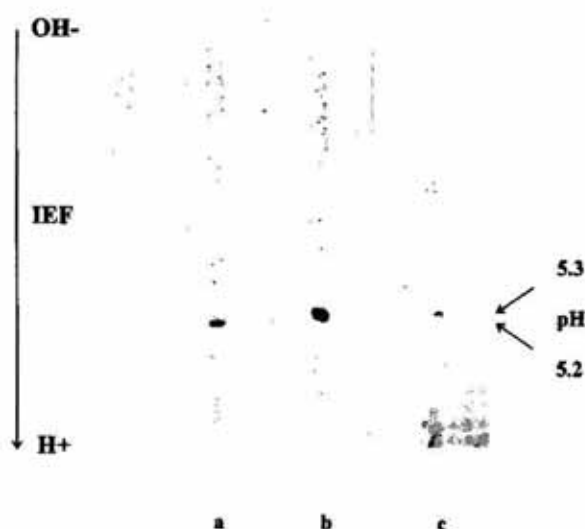
electric points determined for the actins eluted with different concentrations of Gu-HCl (0.75 M and 3 M). This proves the presence of the same actin isoform in both actin-containing peaks in the affinity chromatography elution profile. A strong reaction with anti  $\beta$  actin antibodies (Fig. 3) showed it to be the  $\beta$  actin isoform. The lack in the purified actin preparations of other actin isoforms identified in the cytosol fraction may result either from lower affinity of these isoforms than of  $\beta$  actin to DNase I immobilized on

agarose or from an excess of  $\beta$  actin over other isoforms present in the cytosol fraction causing their level in the purified preparation to be undetectable.

## DISCUSSION

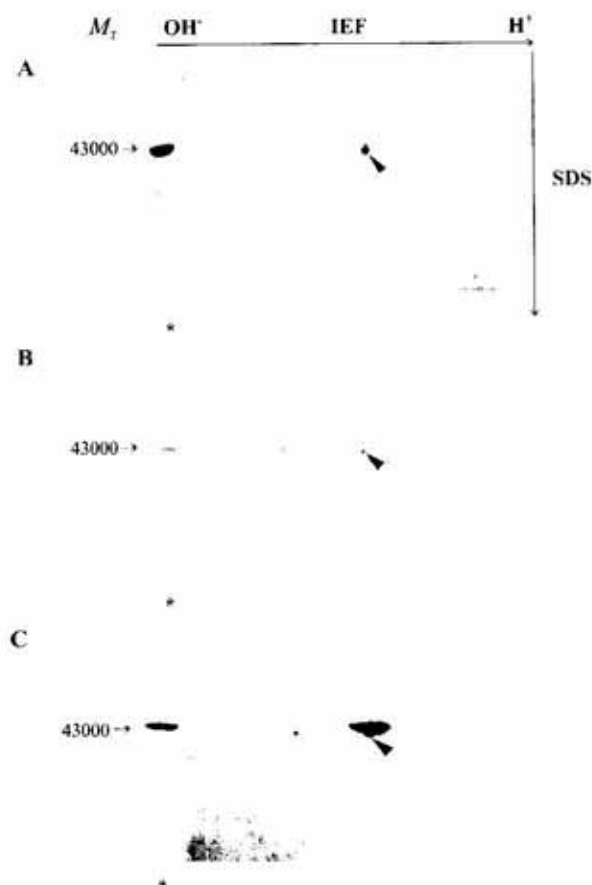
The observations of last decade, such as the existence of multiple actin isoforms within a single cell [12], subcellular sorting of actin isoforms [34], and tissue-specific and developmentally regulated expression of actin [35], suggest functional differences among actin isoforms and indicate that at the same level a cell can discriminate between them. However, the body of experimental evidence supporting the latter suggestion is still poor. Studies on changes in actin content, polymerization and isoform expression in different cellular pathologies need to be continued. Of particular interest is relation of those observations with tumor cell motility and invasiveness [6, 8, 14, 36].

We have found earlier that the hepatoma Morris 5123 growth process is accompanied by an increase in total actin content with the maximum values reached between the 2nd and 3rd week of tumor growth. It was correlated with the first metastasis of the tumor to the lungs observed in 25% of experimental animals [22, 23], suggesting a relation between



**Figure 4. Isoelectric focusing of actin isolated from hepatoma Morris 5123 tumor cells.**

a) standard rabbit  $\alpha$  skeletal muscle actin (8  $\mu$ g); b) concentrated pooled fractions 25–29 (cf. Fig. 2) (3  $\mu$ g); c) concentrated pooled fractions 13–17 (cf. Fig. 2) (1.5  $\mu$ g). Proteins were separated on the gels (75 mm  $\times$  15 mm) in ampholine gradient mixture pH 5–8/3–10. The gels were stained for protein presence according to Wray *et al.* [31].



**Figure 5. 2D PAGE of actin isolated from hepatoma Morris 5123 tumor cells:**

(A) concentrated pooled fractions of peak IV (cf. Fig. 2), 3  $\mu$ g; (B) concentrated pooled fractions of peak III (cf. Fig. 2), 2  $\mu$ g; (C) rabbit  $\alpha$  skeletal muscle actin, 10  $\mu$ g. The gels were stained according to Wray *et al.* [31]. \*standard rabbit  $\alpha$  skeletal muscle actin was used also as a protein relative molecular mass marker.

the increase of cytoplasmic actin level, tumor cell motility and the capability of metastases formation.

The present study followed the presence of actin isoforms in the hepatoma cells. As with normal liver cell [37], the main isoform showed mobility of G actin ( $M_r$  43000) and reacted with monoclonal anti  $\beta$  actin antibodies and antibodies recognizing the cytoplasmic actin isoform  $\gamma$ . However, a feeble reaction with anti  $\alpha$  smooth and anti  $\alpha$  skeletal muscle monoclonal antibodies was also observed in a band of G actin mobility on immunoblots of the cytosol proteins (Fig. 1A).

Unexpectedly a reaction with all applied antibodies (except for the anti  $\alpha$  smooth muscle antibodies), was seen in the region of  $M_r$  higher than 66 000 (Fig. 1A). We assume the reaction to be caused by strongly bound complexes of actin with cytoplasmic actin binding proteins (ABP), unable to dissociate completely under applied SDS/PAGE conditions.  $M_r$  values corresponding to the observed bands suggest the proteins to be profilin, cofilin [38], DNase [39], or actin forming dimers [40].

It should be added that the occurrence of bands of  $M_r$  higher than 66 000 on immunoblots with the different anti actin antibodies, accompanied all our experiments, performed not only with the cytosol fraction from hepatoma Morris tumor, but also with the liver cytosol and serum of the tumor bearing rats. It does not depend on the amount of protein loaded, time of separation or gel length. Further investigations have to clarify the nature of this phenomenon. The presence of polypeptides sharing epitope with actin suggested by Janssen *et al.* [41] could not be ruled out.

For further identification, actin was purified from the hepatoma Morris 5123 cytosol fraction by affinity chromatography on DNase I immobilized on agarose. This one step purification procedure has been used by others to isolate actin from different sources [27, 42]. It was interesting to note that actin was eluted from the affinity column in two peaks at two different concentrations of the dissociating agent. The presence of two peaks containing apparently identical actin isoforms can be explained by different affinity to DNase I of either pure actin and putative actin-ABP (see above) complexes or actin monomeric and filamentous forms [22, 23, 43].

Only  $\beta$  cytoplasmic actin isoform was detected in both purified actin preparations.

One possible explanation is that only the  $\beta$  isoform is able to form a complex with DNase I immobilized on the column thus raising a question of differential affinity of actin isoforms to DNase I from bovine pancreas.



Another explanation could follow from the consideration of the ratio of  $\beta$  to  $\gamma$  isoactins in the hepatoma Morris 5123 tumor cells. In nonmuscle cells this ratio was observed to vary from 6:1 to 1:8 [44, 45]. Comparison of the immunoreactivity of the cytosol fraction with different actin antibodies convinced us that in the hepatoma Morris 5123 cells the  $\beta$  actin isoform dominates over other isoforms to the extent well exceeding that reported for rat liver (the ratio of  $\beta$  to  $\gamma$  actin of 2.5:1) [9]. The latter might render the  $\gamma$  isoform undetectable in purified actin preparations under experimental condition.

The domination of  $\beta$  actin in chemically transformed cells, as presented in the case of hepatoma Morris 5123, originating from liver cells of rats bred with *N*-(2-fluorenyl)phthalamic acid [24], was reported by Leavitt *et al.* [46] and Sakijama *et al.* [47]. Also, an interaction observed with monoclonal antibodies directed against muscle isoactins confirm the presence of those isoactins, especially  $\alpha$  smooth muscle isoactin, in nonmuscle cells [16–20]. The presence of the  $\alpha$  smooth muscle isoform in the hepatoma Morris 5123 cells cultured *in vitro* has been also observed in our current experiments (not published).

The increased expression of  $\beta$  actin in transformed cells has been considered directly associated with the enhanced tumor cell motility and invasivity. Regulation of  $\beta$  actin expression and distribution might be important parameters determining pseudopodial protrusion and invasivity of tumor cells [36].

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