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Calvasculin, as a factor affecting the microfilament assemblies in rat fibroblasts transfected by *src* gene

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Cell transformations accompany alterations in cell morphology and microfilament patterns. Calvasculin encodes mRNA termed pEL-98, 18A2, 42A, p9Ka, or mts1, found to be elevated in several metastatic cell lines. We report the elevation of calvasculin expression in SR-3Y1 cells, which show disappearance of ordered microfilaments, compared to that in 3Y1 cells and that the similar distribution of calvasculin to that of actin filaments. Interestingly, calvasculin co-sediments with F-actin and bundles actin filaments in a Ca^{2+}-dependent manner. This activity, along with the elevation of calvasculin following transformation, suggests that the disorganization of filaments in SR-3Y1 cell is due to the cross-linking activity of calvasculin.

Calvasculin; EF-hand; F-actin; Microfilament; Rat fibroblasts transfected by *src* gene; SR-3Y1 cell

1. INTRODUCTION

Several coordinated changes in growth control, cell shape, and cell metabolism occur upon transformation of chick fibroblasts by avian sarcoma virus [1]. In contrast to the orderly arrangements of microfilaments in untransformed cells, transformed cells indicate highly disordered patterns. There was a loss of the regular actin bundles making up stress filaments with the appearance of a more diffuse pattern of staining. Neoplastic transformation is known to alter the expression pattern for the various members of the Ca^{2+}-modulated protein family [2–6]. However, the exact physiological meanings between the members of S-100 family and the disorganization of stress fibers upon cell transformation remain unknown. In this study, we found calvasculin, with EF-hand structures and one of the members of S-100 family, is highly expressed in transformed cells. Moreover, we report an initial characterization of the interaction of calvasculin as an actin crosslinking protein.

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Abbreviations. EGTA, ethylenebis(oxyethylenenitrilo) tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PBS, phosphate-buffered saline.

2. MATERIALS AND METHODS

2.1 *Proteins purification and electrophoresis*

Actin was purified from chicken breast muscle in globulous forms described elsewhere [7]. Calvasculin was prepared from bovine aorta by the method of Watanabe et al. [8,9] One-dimensional sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Schägger and von Jagow [10]. Protein concentration was determined by the method of Bradford [11] using bovine serum albumin as a standard.

2.2. *Cells and immunocytochemical procedures*

3Y1 and SR-3Y1 cells were obtained from Dr. M. Hamaguchi (Department of Molecular Pathogenesis, Nagoya University School of Medicine). Each coverslip was fixed in 4% paraformaldehyde in PBS for 30 min and subsequently incubated in 0 1% Triton X-100 in PBS. After the incubation with 5% normal goat serum for 30 min to block the nonspecific binding sites, the coverslips were incubated with phalloidin–rhodamine (phalloidin:6.6 nM in PBS) for 30 min or anti-calvasculin serum (1:600 dilution) in PBS for 60 min followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit IgG(H +L) antibody, 1:200 dilution) for 25 min. After washing in PBS, rhodamine and fluorescein were observed.

2.3. *Electron microscopy*

The interaction of calvasculin with actin filaments was visualized by negative staining with 1% potassium phosphotungstate, pH 7.0. F-Actin (0.1 mg/ml) only and F-actin (0.1 mg/ml) plus dithiothreitol, 100 mM KCl, 1 mM $MgCl_2$, and 1 mM ATP containing 1 mM Ca^{2+}. A specimen was prepared by the negative staining technique.

2.4. *Quantitative assay for F-actin and calvasculin binding activity*

Binding activities of calvasculin were assayed by high-speed centrifugation method. Actin-filaments (3 μM) were incubated with various concentrations of calvasculin at 25°C for 30 min in 5 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 100 mM KCl, 1 mM $MgCl_2$, and 1 mM ATP containing either 1 mM Ca^{2+} or EGTA. After incubation, the mixtures (100 μl) were centrifuged at 100,000 × g (Beckman, TL-100 Ultracentrifuge) for 30 min. Supernatants and pellets were dissolved separately in equivalent volume of Sample buffer (50 mM Tris-HCl,

pH 6.8, 8 M urea, 2% SDS, 0.04% BPB) and subjected to SDS-PAGE. The Coomassie brilliant blue-stained bands were quantified by densitometry.

3. RESULTS

Staining of rat embryo fibroblast 3Y1 and SR-3Y1 (3y1 transfected by *src* gene) cells with phalloidin-rhodamine revealed striking difference in microfilaments organization as quite similar to those reported previously [12,13]. SR-3Y1 cells lacked a consistent pattern of stress fibers and instead showed a stained perinuclear aggregate with 'fluffy' protrusions and cytoplasmic granular (data not shown). Immunofluorescence staining of calvasculin in 3Y1 and SR-3Y1 cells at sub-

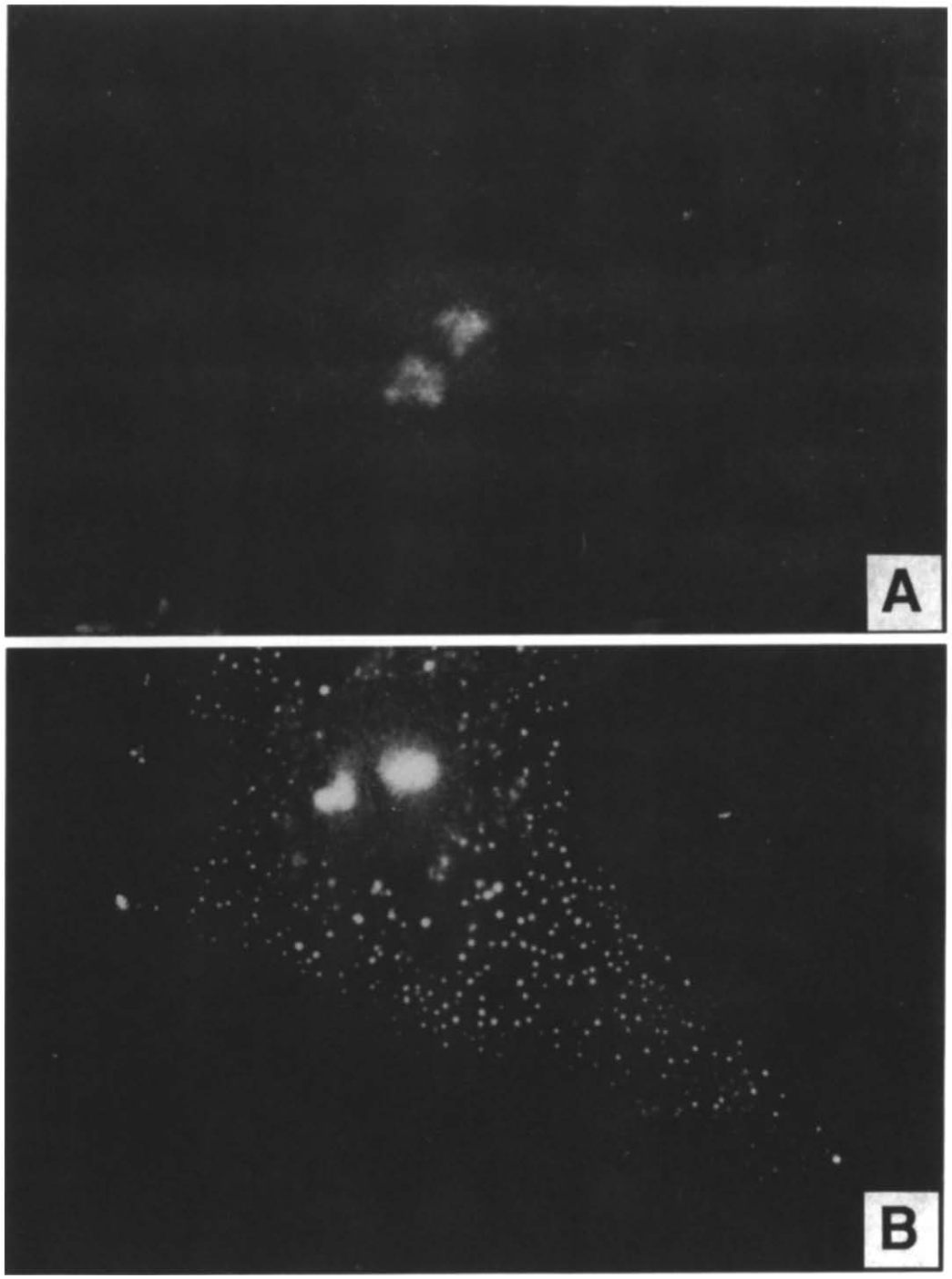

Fig. 1. Calvasculin distribution of 3Y1 and SR-3Y1 cells after staining with anti-calvasculin antibody: (A) 3Y1, anti-calvasculin: (B) SR-3Y1, anti-calvasculin. Each coverslip was fixed in 4% paraformaldehyde in PBS for 30 min and subsequently incubated in 0.1% Triton-X100 in PBS. After the incubation with 5% normal goat serum for 30 min to block the nonspecific binding sites, the coverslips were incubated with anti-calvasculin serum (1:600 dilution) in PBS for 60 min followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit IgG(H +L) antibody, 1:200 dilution) for 25 min. After washing in PBS, fluorescein was observed. The magnification was × 1,000.

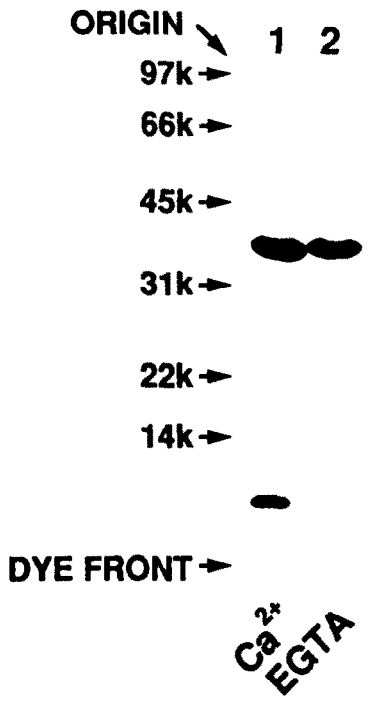

Fig. 2. Interaction of calvasculin with F-actin in the presence (lane 1) and absence of Ca^{2+} (lane 2). F-actin binding was assayed by a co-sedimentation assay. The amounts of calvasculin and F-actin were 8.4 μg and 31.5 μg, respectively, per tube. At the end of the incubation at 25°C for 30 min in 5 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 100 mM KCl, 1 mM $MgCl_2$, and 1 mM ATP containing either 1 mM Ca^{2+} or EGTA, the reaction tubes were centrifuged at 100,000 × g for 30 min at 25°C. F-Actin and F-actin-calvasculin complex were sedimented by the centrifugation. Pellets were analyzed by SDS-PAGE.

confluent densities demonstrated a cytoplasmic granular distribution of calvasculin with highly expression in SR- 3Y1 cells compared to 3Y1 cells (Fig. 1A and B). Essentially every cell was observed to stain positively with the anti-calvasculin antiserum. Distribution of calvasculin agrees well to cytoplasmic actin filaments organization in SR-3Y1 cells.

To determine whether actin filaments bind to calvasculin in vitro, we made use of high- or low-speed centrifugation method. Under the experimental conditions used, calvasculin co-precipitated with F-actin in the presence of Ca^{2+}, indicating that calvasculin is an F-actin binding protein (Fig. 2). Calvasculin alone was not sedimented by centrifugation at 100,000 × g for 30 min in the presence or absence of Ca^{2+} (data not shown). When highly purified calvasculin and F-actin in the presence of Ca^{2+} were mixed at appropriate ratios at 25°C, gel-like aggregates formed immediately, and the co-sedimentation was observed using low-speed centrifugation method (data not shown). These results indicate that the aggregation of F-actin by calvasculin was occurred in a Ca^{2+}-dependent manner. Addition of S100α or S100β, which sequence is highly homologous to calvasculin, to F-actin solution does not produce the aggregates in the presence or absence of Ca^{2+}. The interaction of calvasculin with F-actin was not inhibited by the presence of calmodulin (data not shown).

We examined the actin-binding activity of calvasculin by changing its concentrations. The amount of calvasculin bound to actin filaments was increased with increase in the amounts of calvasculin mixed with actin. The saturation was achieved at an approximate molar ratio of three molecules of calvasculin to one actin monomer (Fig. 3). The quantitative assay for calvasculin cross-linking activity with F-actin using low-speed centrifugation method revealed a similar molar ratio as well as using high-speed centrifugation method (data not shown).

The interaction of calvasculin with F-actin was further studied by electron microscopy after negative staining. Calvasculin caused F-actin to form bundles in a Ca^{2+}-dependent manner (Fig. 4).

4. DISCUSSION

In the present study, the expression of calvasculin, which encoded mRNA termed pEL-98, 18A2, 42A, p9Ka, or mts 1, is found to be elevated in SR-3Y1 cells compared to that in 3Y1 cells. The distribution of calvasculin which agrees well to cytoplasmic actin filaments organization in SR-3Y1 cells indicate the direct

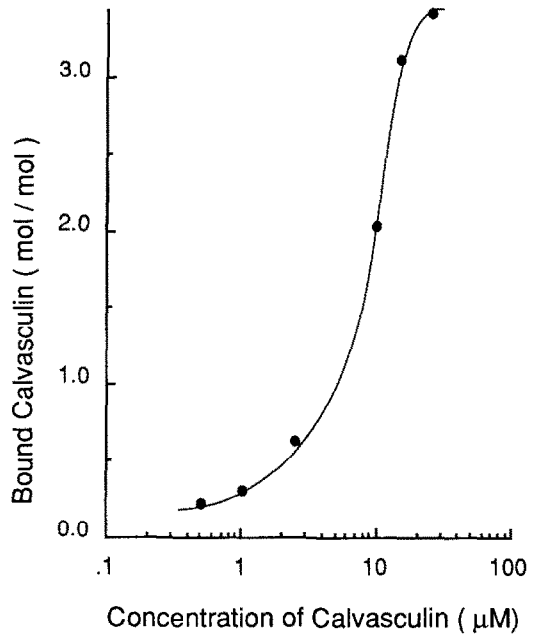

Fig. 3. Quantification of F-actin binding activities of calvasculin by high-speed centrifugation. Binding activities of calvasculin were assayed by high-speed centrifugation method. Various concentration of calvasculin were mixed with F-actin (3 μM) in 5 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 100 mM KCl, 1 mM $MgCl_2$, and 1 mM ATP containing 1 mM Ca^{2+}. After incubation, the mixtures were centrifuged at 100,000 × g for 30 min. Pellets and supernatants were separately in equivalent volumes of SDS-sample buffer and subjected to SDS-PAGE. The amount of calvasculin were determined by densitometry, as described previously.

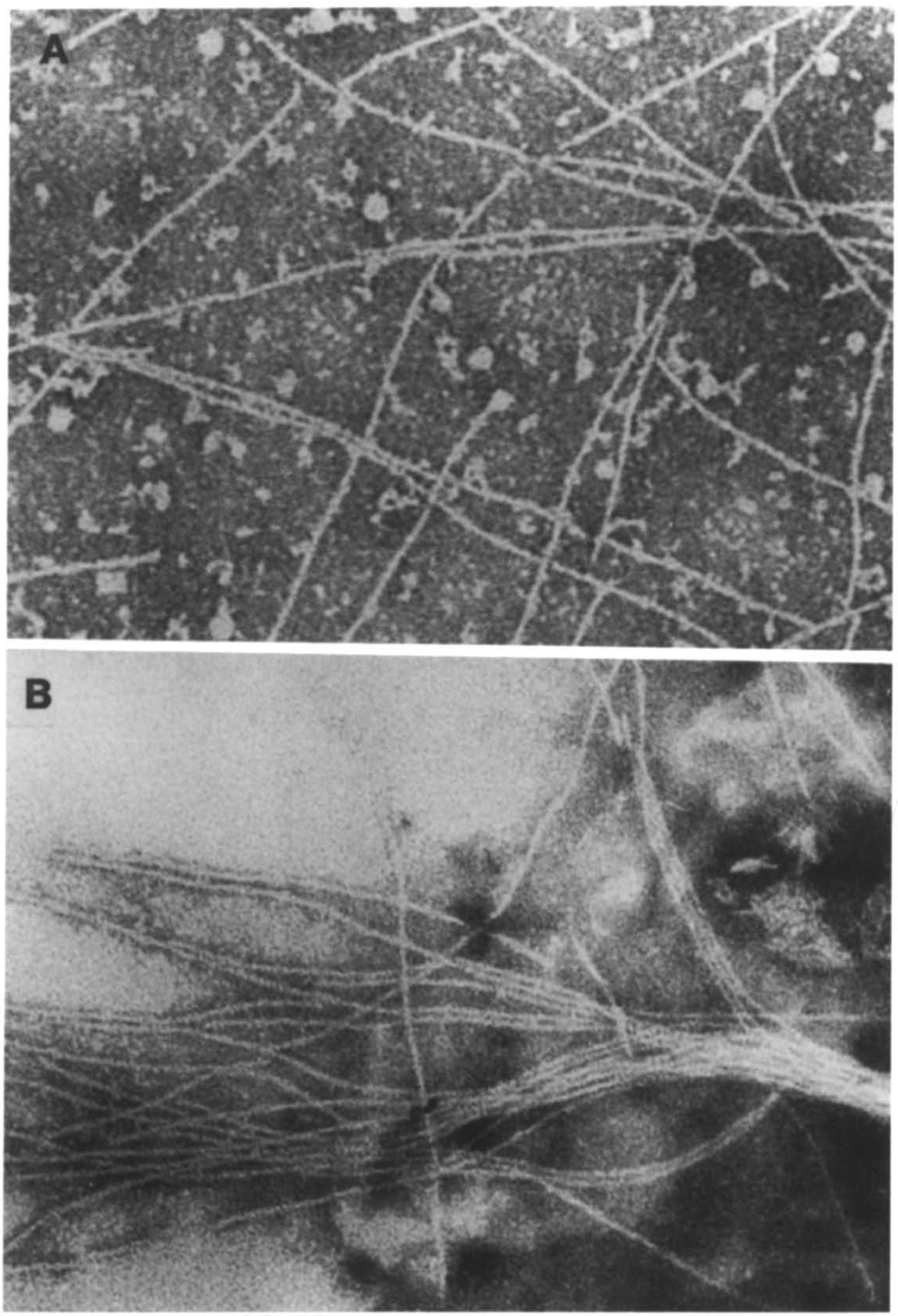

Fig. 4. Electron micrographs of actin bundles formed with calvasculin and F-actin in the presence of Ca^{2+}. The interaction of calvasculin with actin filaments was visualized by negative staining with 1% potassium phosphotungstate, pH 7.0 (A) F-Actin (0.1 mg/ml) only, and (B) F-actin (0.1 mg/ml) plus calvasculin (0.05 mg/ml) were incubated for 30 min in 5 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 100 mM KCl, 1 mM $MgCl_2$, and 1 mM ATP containing 1 mM Ca^{2+}. A specimen was prepared by the negative staining technique. Numerous actin filament bundles were observed The magnification was × 200,000.

interaction between actin filaments and calvasculin. Calvasculin also reveals a bundling activity of F-actin filaments in a Ca^{2+}-dependent manner in vitro.

mRNA corresponding to the protein pEL-98 is presented in large amounts in both chemically transformed and activated oncogene-transformed cell lines [6] and the gene mts 1 is also expressed in tumor cells higher than in normal cells [3]. The immunofluorescence staining of calvasculin in SR-3Y1 and 3Y1 cells indicates that the differences at the mRNA level are also reflected at the protein level. The expression of calcyclin, a member of the S-100 family, is also elevated in high-metastatic human melanoma cell lines in nude mice compared to low-metastatic ones [14]. Although some Ca^{2+}-binding proteins have been implicated in neoplastic progression or metastasis, little is known about the function of the members of the S-100 family in that regulation.

Cell transformation appears to accompany alteration in cell morphology and microfilament patterns. Biochemical studies have shown that many actin-binding proteins are involved in the regulation of the assembly dynamics of microfilaments through cross-linking filaments into networks or bundles [15,16]. However, the molecular mechanisms for regulating microfilament assembly are still unclear in transformed cells. The expression of 55-kDa protein, an actin-bundling protein in cultured cells, is increased in microfilaments isolated from Kirsten or Rous sarcoma virus-transformed NRK cells and 55-kDa protein/actin filament bundles appear rather stable and not to be regulated by Ca^{2+} [17]. Calvasculin/actin filament bundles was observed in the presence of Ca^{2+} in vitro. Studies related the condition of calvasculin-actin interaction in transformed cells will need to be done. As judged from the F-actin bundling activity and the intracellular localization related that of actin filaments, calvasculin is most likely involved in the dynamic formation of microfilament bundles in SR-3Y1 cells. Furthermore, our preliminary study has demonstrated that calcyclin showed evidence of interaction with F-actin. Generally, tropomyosin isoforms with high Mr are decreased or missing and tropomyosin with low M_r are increased in transformed cells. Consequently we are currently examing the modulation of actin- binding activity of calvasculin by tropomyosin isoforms.

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