Actinin-4, a Novel Actin-bundling Protein
Associated with Cell Motility and Cancer Invasion

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Abstract. Regulation of the actin cytoskeleton may play a crucial role in cell motility and cancer invasion. We have produced a monoclonal antibody (NCC-Lu-632, IgM, k) reactive with an antigenic protein that is upregulated upon enhanced cell movement. The cDNA for the antigen molecule was found to encode a novel isoform of nonmuscle α-actinin. This isoform (designated actinin-4) was concentrated in the cytoplasm where cells were sharply extended and in cells migrating and located at the edge of cell clusters, but was absent from focal adhesion plaques or adherens junctions, where the classic isoform (actinin-1) was concentrated. Actinin-4 shifted steadily from the cytoplasm to the nucleus upon inhibition of phosphatidylinositol 3 kinase or actin depolymerization. The cytoplasmic localization of actinin-4 was closely associated with an infiltrative histological phenotype and correlated significantly with a poorer prognosis in 61 cases of breast cancer. These findings suggest that cytoplasmic actinin-4 regulates the actin cytoskeleton and increases cellular motility and that its inactivation by transfer to the nucleus abolishes the metastatic potential of human cancers.

Materials and Methods

Cell Culture and Chemicals

The human giant cell-lung cancer cell line, Lu-65, was established previ-
ously in our laboratory (Yamada et al., 1985). Primary cultured human foreskin keratinocytes (HFK) were purchased from Seikagaku Co. (Tokyo, Japan) and were cultured as instructed by the supplier. Primary normal human uterine endometrial fibroblasts were isolated and cultured as described previously (Zhang et al., 1995). PC-10, a human squamous lung cancer cell line (Girard et al., 1973), and KU7 is a urinary bladder cancer cell line (Westley, 1979; Cavailles et al., 1988). The oral floor cancer cell line IMC2, normal embryonic lung fibroblast MRC5, and colon cancer cell line WiDr were obtained from Riken Cell Bank (Tsukuba, Japan). Colon cancer cell line SW480 was obtained from the American Type Culture Collection (Rockville, MD).

HFK were treated with 50 ng/ml wortmannin (Sigma Chemical Co., St. Louis, MO) for 15 min and then washed. After incubation for 24 h, the cells were examined by immunofluorescence microscopy as described below. For actin depolymerization, the cells were treated with 2 μg/ml cytochalasin D (Sigma Chemical Co.) for 2 h.

Production of Antibodies

BALB/c mice were immunized with a lysate of Lu-65 cells, and hybridomas were produced as described previously (Hirohashi et al., 1984). The hybridoma supernatants were then screened immunohistochemically for reactivity with acetone-fixed paraffin-embedded human tissues, as described below.

Rabbit polyclonal anti-actinin-4 antibody was raised against a KLH-conjugated synthetic peptide, MGDYMAQEDDWC (containing amino acids 1–11, Fig. 1), and affinity-purified.

cDNA Cloning and DNA Sequencing

A lambda gt11 cDNA expression library of primary cultured HFK (CLONTECH Laboratories, Inc., Palo Alto, CA) was immunoscreened with mAb NCC-Lu-632, as described previously (Young and Davis, 1983), and a positive 950-bp cDNA clone (ck-20-2) was isolated. For isolation of full-length cDNA of actinin-4 and -1, a cDNA library of mesothelioma MS-1/CDDP cells was screened with synthetic oligonucleotides specific to each actinin. A 3.5-kb actinin-4 cDNA clone, near full-length as determined by Northern blot analysis (Fig. 2B), was isolated, and both strands were sequenced using an autosequencer (model ABI 377; Perkin Elmer, Foster City, CA).

Northern Blot Analysis

Multiple tissue Northern blots I and II were purchased from CLONTECH Laboratories and hybridization was performed using a 32P-labeled oligonucleotide probe (5'-GGCTTGTAAGTTAGGGCCCACCC-3') specific to actinin-4, as described previously (Sambrook et al., 1989). The quality and quantity of electrophoresed mRNA was determined by rehybridization of the same blot with a human β-actin cDNA probe.

Extraction of Cell Lysate and Western Blot Analysis

Cells were extracted with lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% NP-40, 1 mg/ml Na2Vo4, 0.5 mM Na3Vo4, 10 mM β-glycerophosphate, 1 mM PMSF, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A) on ice for 30 min before centrifugation (12,000 g, 15 min). Cell lysates (50 μg protein) were separated by SDS-PAGE (Laemmli, 1970) and transferred to Hybond–polyvinyl difluoride membranes (Amerham International), as instructed by the suppliers. For blotting of whole cell lysates, cells were lysed directly with Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 715 mM 2-mercaptoethanol, 0.0025% bromophenol blue) (Harlow and Lane, 1988).

1. Abbreviations used in this paper: GST, glutathione S-transferase; HFK, human foreskin keratinocyte(s); PH, pleckstrin homology; P13, phosphatidylinositol 3-P; PIP2, phosphatidylinositol 4,5-bisphosphate.

Figure 1. Deduced amino acid sequence of actinin-4 (upper) in comparison with actinin-1 (lower). The nucleotide sequence of actinin-4 is available from GenBank/EMBL/DDJB under accession number D89980. Asterisks represent identical amino acids and dots represent conserved amino acids. The sequences conserved among α-actinin isoforms are underlined: actin-binding domain (amino acids 111–125, single underlining), PIP2-binding domain (150–165, double underlining), and EF-hand calcium regulation domains (742–770 and 783–811, lined boxes).

Glutathione S-Transferase Fusion Protein Production and In Vitro Translation

A cDNA fragment of actinin-4 (encoding amino acids 410–664) and the corresponding site of actinin-1 (amino acids 418–672) were amplified by PCR and subcloned into pGEX plasmids (Pharmacia Biotech, Uppsala, Sweden). Glutathione S-transferase (GST) fusion proteins expressed in Escherichia coli were affinity-purified on glutathione–Sepharose 4B (Pharmacia Biotech) as described previously (Shibata et al., 1996). 1-[35S]Methionine (Amersham International) labeled polypeptides were synthesized from cDNA clones containing the entire coding regions for actinin-1 and -4, using the TNT reticulocyte lysate system (Promega Corp., Madison, WI) as instructed by the supplier. The synthesized polypeptides were analyzed by SDS-PAGE and autoradiography as described previously (Yamada et al., 1995).
Actin-binding Assay

Purified chicken gizzard actin (Sigma Chemical Co.) was coupled to CNBr-activated Sepharose–4B beads (Pharmacia Biotech) (Prendergast and Ziff, 1991). The beads were incubated with 35S-incorporated in vitro translation products in lysis buffer overnight at 4°C. After extensive washing with lysis buffer, the beads were boiled in Laemmli buffer and analyzed by SDS-PAGE and autoradiography (Yamada et al., 1995).

A cell lysate of the lung cancer cell line PC-10 was also incubated overnight at 4°C with the actin-coupled beads. After thorough washing, the beads were boiled in Laemmli buffer and analyzed by immunoblotting as described above.

Immunoprecipitation

In vitro translation products of actinins were incubated with a monoclonal antibody against chicken α-actinin (BM-75.2, IgM, κ; Sigma Chemical Co.), anti-actinin-4 polyclonal antibody, normal mouse IgM, or normal rabbit IgG (Sigma Chemical Co.) overnight at 4°C and precipitated with anti-mouse IgM agarose (for mouse antibodies) (Sigma Chemical Co.) or protein G plus agarose (for rabbit antibodies) (Santa Cruz Biotechnology, Santa Cruz, CA). SDS-PAGE and autoradiography were carried out as described previously (Yamada et al., 1995).

Immunofluorescence Microscopy and “Wound” Assay

Cells were cultured on glass coverslips, fixed with 4% paraformaldehyde and 2% sucrose in PBS, and made permeable with 0.2% Triton X-100. After incubation with antibodies, actinins were detected with biotinylated anti-mouse IgM or biotinylated anti-rabbit IgG and Texas red–avidin D or FITC-conjugated avidin D (Vector Laboratories, Burlingame, CA). Actin filaments were visualized with FITC-phalloidin (Sigma Chemical Co.), and the cells were examined with a Zeiss LSM410 microscope (Thornwood, NY).

Cells were grown to confluency on glass coverslips, and a “wound” was then introduced in the monolayers with a plastic pipette tip (Glück et al., 1993; Glück and Ben-Ze’ev, 1994). After incubation for 24 h, the cells were fixed and examined as described above.

Immunohistochemistry

Acetone-fixed paraffin-embedded human tissues (Sato et al., 1986) were....
selected from the surgical pathology file of the National Cancer Center Hospital (Tokyo, Japan). 5-m thin sections were stained with mAb NCC-Lu-632 using an immunoperoxidase avidin-biotin complex (ABC) kit (Vector Laboratories), as described previously (Sato et al., 1986). Histological subtyping of breast cancer was done by two independent certified pathologists, according to the criteria described previously (Rosen and Oberman, 1992).

Statistical Analysis
The postsurgical survival curves for a total of 61 patients with stages I and II breast cancer (Rosen and Oberman, 1992) were expressed as described by Kaplan and Meier (1958). Statistical significance was determined by the generalized Wilcoxon test.

Results

Molecular Cloning of Actinin-4 cDNA
mAb NCC-Lu-632 (IgM, k) was selected on the basis of its intriguing immunohistochemical reactivity, as described below. A cDNA clone encoding the antigen molecule recognized by the mAb was isolated by immunoscreening of the HFK lambda gt11 cDNA library.

The 3.5-kb cDNA clone contained a 2,652-bp open reading frame encoding 884 amino acids (Fig. 1), with a predicted molecular mass of 102 kD. The mAb NCC-Lu-632 reacted consistently with the 100-kD antigen molecule in immunoblot analyses (Fig. 2A), and in vitro translation of the full-length cDNA yielded a major protein of 100 kD (Fig. 3C). This coding region and the deduced amino acid sequence showed a high degree of similarity to human α-actinin-1 (Millake et al., 1989; Youssoufian et al., 1990) (80.0% nucleotide and 86.7% amino acid similarity). It contained structures conserved among α-actinin family members, including the actin-binding domain (Kuhlman et al., 1992) (amino acids 111–125; Fig. 1), pleckstrin-homology (PH) domain containing a phosphatidylinositol 4,5-biphosphate (PIP2)-binding site (Fukami et al., 1996) (amino acids 150–165), and two EF-hand calcium regulation domains (Witke et al., 1993; Imamura et al., 1994) (amino acids 742–770 and 783–811). Following the nomenclature proposed by Beggs et al. (1992), this gene was termed actinin-4.

Expression of Actinin-4 mRNA in Normal and Neoplastic Cells
Northern hybridization was carried out with an actinin-4-specific 22-bp oligonucleotide probe to avoid cross-hybridization with actinin-1. As shown in Fig. 2B, intense expression of 3.5-kb actinin-4 mRNA was detected in the

Figure 4. Reactivity of polyclonal antibody against actinin-4 peptide. (A) Immunoblot analysis reveals that the polyclonal antibody raised against actinin-4 peptide (lane 1) reacts with a single protein of ~100 kD in the cell lysate of WiDr cells. A blot with normal rabbit IgG (lane 2) is included as a negative control. Molecular masses (in kD) are shown on the left. (B) In vitro translation products of actinin-4 (lanes 1 and 3) and actinin-1 (lanes 2 and 4) were immunoprecipitated by the anti-actinin-4 polyclonal antibody (lanes 1 and 2) or normal rabbit IgG (lanes 3 and 4). SDS-PAGE and autoradiography reveal that this polyclonal antibody is reactive with actinin-4 protein, but not with actinin-1. (C) Confocal immunofluorescence microscopy showing the subcellular localization of actinin-4 in lung fibroblasts MRC-5. Arrow indicates the nuclear staining, and arrowheads indicate linear staining of actinin-4 along actin stress fibers. Bar, 5 μm.

Figure 5. Actin-binding activity of actinin-4. (A) Direct association of actinin-4 with actin. In vitro translation product of actinin-4 was incubated with chicken gizzard actin-conjugated Sepharose 4B beads (lane 1) or Sepharose 4B beads alone (lane 2). After extensive washing, SDS-PAGE and autoradiography revealed that 35S-labeled actinin-4 protein was retained only in actin-coupled beads (lane 1). Molecular masses (in kD) are shown on the left. (B) Cell lysate of human squamous cell carcinoma cell line (PC-10) was incubated with actin-conjugated (lanes 1 and 3) or control (lanes 2 and 4) Sepharose 4B beads. Bound proteins were analyzed by immunoblotting. Approximately 100-kD proteins of actinin-1 (lane 1) and actinin-4 (lane 3) were detected with monoclonal antibodies BM-75.2 and NCC-Lu-632, respectively. Molecular masses (in kD) are shown on the left.
ovary (Fig. 2 B, lane 13) and colon (lane 15), and faint expression was detected in all tissues examined. No gross structural alterations of actinin-4 mRNA were detected in any of the human carcinoma cell lines examined (data not shown).

**Specificity of Antibodies**

To determine the specific reactivity of mAb NCC-Lu-632 with actinin-4, amino acids 410–664 of actinin-4 and a corresponding site of actinin-1 (Millake et al., 1989) (amino acids 418–672) were expressed as a GST fusion protein in E. coli. Immunoblot analysis revealed that the mAb reacted only with actinin-4 GST fusion protein, and not with actinin-1 GST fusion protein or GST alone (Fig. 3 A), confirming the specificity of mAb NCC-Lu-632 for actinin-4.

Immunoprecipitation analyses revealed that an mAb against chicken actinin, BM-75.2, was reactive with the in vitro translation product of cDNA of actinin-1, but not with that of actinin-4 (Fig. 3 D).

Immunoblot analyses revealed that the polyclonal antibody raised against the NH$_2$-terminal amino acids of actinin-4 reacted only with a single band of the same molecular weight as NCC-Lu-632 mAb (Fig. 4 A). Immunoprecipitation analyses revealed that this polyclonal antibody was reactive with the in vitro translation product of the cDNA of actinin-4, but not with that of actinin-1 (Fig. 4 B), thus confirming its specificity.

**Actin-binding Activity In Vitro**

From the deduced amino acid sequence (Fig. 1), actinin-4 was predicted to contain an actin-binding domain (Hemmings and Critchley, 1992; Kuhlman et al., 1992). To confirm this, in vitro binding experiments were performed using actin-coupled agarose beads (Prendergast and Ziff,
monoclonal antibody NCC-Lu-632 was further confirmed of actinin-4. (Fig. 7 was dispersed in the cytoplasm and nucleus (Fig. 6, cell membrane–associated localization of actinin-1, actin by phalloidin-conjugated FITC (green), and actin by phalloidin-conjugated FITC (green) is shown. Actinin-1 is concentrated specifically at focal adhesions and adherens junctions (arrowheads) (yellow), but not in the nucleus. (B) Confocal fluorescence microscopy showing nuclear localization of actinin-4 and the actin cytoskeleton in breast cancer cell line MCF7. Double fluorescence of actinin-4 detected by NCC-Lu-632 mAb (red) and actin by phalloidin-conjugated FITC (green) is shown. Actinin-4 is separated from the cytoplasmic actin cytoskeleton and localized specifically in the nucleus. (C) Immunoblot analysis of whole cell lysates from oral floor cancer cell line IMC2 (lane 1), urinary bladder cancer cell line KU7 (lane 2), and breast cancer cell lines MCF7 (lane 3) and R27 (lane 4) by mAb NCC-Lu-632 (left) and normal mouse IgM (negative control, right). IMC2, KU7, and MCF7 are cell lines showing the nuclear localization of actinin-4, and R27 is a cell line showing cytoplasmic localization. Molecular masses (in kD) are shown on the left. Bar, 5 μm.

Distinct Subcellular Localization of Two Nonmuscle Actinins

Confocal immunofluorescence microscopy revealed that actinin-1 was localized specifically at the ends of actin stress fibers (Fig. 6, A, C, and E) and adherens junctions (Fig. 7 A), as described previously (Lazarides, 1975; Wehland et al., 1979; Knudsen et al., 1995). In contrast to this cell membrane–associated localization of actinin-1, actinin-4 protein was colocalized with actin stress fibers and was dispersed in the cytoplasm and nucleus (Fig. 6, B, D, and F). The specific immunocytochemical reactivity of monoclonal antibody NCC-Lu-632 was further confirmed by the polyclonal antibody raised against the NH2-terminal amino acids of actinin-4 (Fig. 4 C). In most cancer cell lines including PC10, A431, SW480, TE4, TE6, TE7, TE10, and R27, actin stress fibers were poorly developed and actinin-4 was diffusely dispersed in the cytoplasm. Characteristically, actinin-4 was highly concentrated in the cytoplasm where the cells were sharply extended (Fig. 8, B, E, and H). Actinin-4 was stained intensely at the edges of cell clusters (Fig. 8, C, F, and I). The difference in subcellular localization between these two nonmuscle isoforms suggested that they were associated with different functions.

Upregulation of Actinin-4 upon Enhanced Cell Movement

From the above immunofluorescence observations, we speculated that the expression of actinin-4 was regulated dynamically by cell movement. An artificial linear defect was introduced in the confluent monolayers of A-431 and R27 cells, and we examined the cells forced to be motile by immunofluorescence microscopy. As shown in Fig. 9, actinin-4 was markedly induced in cells along the edges of the wound (Fig. 9 A) and in cells migrating into the wound (Fig. 9, B–D).

Nuclear Translocation of Actinin-4

In a limited number of cell lines, including the breast cancer cell line MCF7, oral floor cancer IMC2, and bladder cancer KU7, it was noticed that actinin-4 was localized exclusively in the nucleus (Fig. 7 B), in contrast to the association of actinin-1 with the cell membrane in these cells (Fig. 7 A). The nuclear staining was not due to cross-reactivity of the mAb with other nuclear proteins because NCC-Lu-632 mAb reacted only with a protein of ~100 kD in whole-cell lysates of these cell lines (Fig. 7 C).

Actinin-4 possesses a conserved PIP2-binding site. PIP2 binds α-actinin through the pleckstrin homology domain and regulates its actin-binding activity (Fukami, 1992; Fukami et al., 1996). PIP2 is one of the substrates of phosphatidylinositol 3 kinase (PI3 kinase). By treating HFK with a PI3 kinase inhibitor, wortmannin (Vemuri, 1994), actinin-4 was steadily translocated into the nucleus (Fig. 10). A bladder cancer cell line, KU7, which is deficient in...
PI3 kinase expression, also showed nuclear localization of actinin-4 without treatment (data not shown). These findings suggest that inhibition of the PI3 kinase–mediated signaling pathway is involved in the nuclear translocation of actinin-4.

Cytochalasin D inhibits actin polymerization (Casella et al., 1981). Treatment with cytochalasin D also induced nuclear translocation (data not shown), suggesting that the nuclear translocation of actinin-4 is also caused by loss of its association with the cytoplasmic actin cytoskeleton.

**Subcellular Distribution of Actinin-4 Predicts Poor Prognosis of Breast Cancer**

The distribution of actinin-4 was determined immunohistochemically in human tissue using the mAb, NCC-Lu-632. Actinin-4 was expressed in a limited population of normal cells, including erythrocytes, endothelial cells, and epithelial cells in various tissues at their border with stromal connective tissue.

In the ducts of normal mammary glands, the epithelio-stromal and epithelio-myoeipithelial borders were stained, but the nucleus of normal mammary gland cells did not stain (Fig. 11 A). Scirrhou carcinoma and invasive lobular carcinoma of the breast manifest highly infiltrative growth into the fibrous stroma, in contrast to low-infiltrative histological types including papillotubular and solid-tubular carcinomas (Rosen and Oberman, 1992). To determine the relationship between the growth pattern of breast cancer and the subcellular localization of actinin-4, 83 cases of
invasive breast cancer were examined immunohistochemically. The reactivity of mAb NCC-Lu-632 was classified into three types (Table I): nuclear (type A, Fig. 11 B), combined (type B), and cytoplasmic (type C, Fig. 10, C and D). Histologically, all type A cases were papillotubular or solid-tubular carcinomas that showed low-infiltrative extension (20/20). In type B, the majority of cases were papillotubular and solid tubular carcinomas (25/33) and fewer were scirrhous carcinomas (8/33). In contrast to these two types, most type C cases were scirrhous (15/30) (Fig. 11 C) and invasive lobular carcinomas (6/30) (Fig. 11 D), both of which extended locally in a highly infiltrative manner.

We then determined whether these staining patterns were correlated with prognosis in patients with breast cancer. The relationship between the actinin-4 staining pattern and disease-free and overall survival of 61 patients with relatively early breast cancer (clinical stages I and II [Rosen and Oberman, 1992]) was examined. A significant difference in disease-free survival was observed between types A + B and type C ($P < 0.01$) (Fig. 12 A). In addition, a significant difference in overall survival was observed between types A + B and type C ($P < 0.01$) (Fig. 12 B). These findings suggest that the subcellular distribution of actinin-4 may indicate the biological behavior of breast cancer and may be considered a new risk factor for relapse.

**Discussion**

α-Actinin is a member of the superfamily of actin-binding proteins that includes spectrin, filamin, dystrophin, and fimbrin, by virtue of the similarity of their actin-binding domains (Matsudaira, 1991). α-Actinin binds to the integrin β1 cytoplasmic domain (Otey et al., 1990, 1993) and is believed to link the actin cytoskeleton to focal adhesions in cooperation with other cytoplasmic proteins, including tensin, talin, and vinculin (Burr ridge et al., 1990; Miyamoto et al., 1995). Actinin is also localized at adherens junctions in connection with α-catenin (Knudsen et al., 1995). Assembly of these structural proteins is believed to play an important role in stabilizing cell adhesion (Burr ridge et al., 1990; Miyamoto et al., 1995) and suppressing cell motility (Glück et al., 1993; Glück and Ben-Ze’ev, 1994).

In this study, using a newly established mAb, we isolated a cDNA clone encoding a novel isoform of α-actinin, designated actinin-4. Immunofluorescence microscopy demonstrated that the classical isoform, actinin-1, was concentrated in actin stress fiber ends and adherens junctions. In contrast, this novel nonmuscle α-actinin, actinin-4, binds actin filaments at a different subcellular location, thereby exerting functions distinct from those of actinin-1. Actinin-4 is highly concentrated in sharply stretched cells. In the wound assay, the cells forced to be motile along wound edges and cells migrating into the wound overexpressed actinin-4. These findings suggest the involvement of actinin-4 in cell movement. Glück et al. demonstrated that the expression of actinin-1 was reduced in SV-40–transformed 3T3 cells (Glück et al., 1993; Glück and Ben-Ze’ev, 1994). Transfection with actinin-1 cDNA suppressed tumorigenicity. Transfection with actinin-1 cDNA in an antisense orientation increased cell motility, supporting the contradictory roles of the two nonmuscle isoforms with respect to cell movement.

Various growth factors provide stimuli for cell motility in addition to mitogenic activity (Klagsbrun, 1996). Recently, Hsu et al. (1996) identified a new murine nonmuscle α-actinin isoform, FR-17, as a fibroblast growth factor-
1–inducible gene in NIH-3T3 cells. Although it has not been fully sequenced, the deduced COOH-terminal amino acids (1–109; sequence data available from GenBank/EMBL/DDBJ under accession number U41415/6) available for FR-17 showed 97.2% identity with amino acids 776–884 of human actinin-4, suggesting that this isoform may be a murine homologue of human actinin-4. In addition to FR-17, a computer search revealed that several partial sequences identical to actinin-4 were registered in dbEST databases. These ESTs were isolated from human cDNAs of placenta (accession number AA368907), fetal brain (M85377), Jurkat T-cells (AA312012), and pancreatic islets (C06273, D83843).

The level of another actin-binding protein, thymosin β15, was reported to be elevated in human metastatic prostate cancer and correlated positively with the Gleason score (Bao et al., 1996). In contrast to actinins, thymosins inhibit actin polymerization. Cell motility is regulated by both disassembly and reformation of the actin cytoskeleton. Thymosin β15 has been considered to degrade the static anchorage of the actin cytoskeleton and liberate actin monomers. Actinin-4 may then cross-link actin filaments and reorganize the cytoskeleton, which is essential for cell movement. Further studies are necessary to elucidate how these molecules regulate the actin cytoskeleton and participate in cancer invasion and metastasis.

In this study, we found that actinin-4 existed in the nucleus of a certain population of breast cancers and in several cell lines. From the deduced amino acid sequence, it seems that actinin-4 does not possess any apparent nuclear localization signals (Boulkas, 1993). Although the precise mechanisms of the nuclear transition are still under investigation, we were able to reproduce the nuclear transition of actinin-4 in tissue culture. Actinin-4 was translocated from the cytoplasm to the nucleus by treatment with the PI3 kinase inhibitor, wortmannin (Vemuri, 1994), or by actin depolymerization using cytochalasin D (Casella et al., 1981). PI3 kinase is thought to be one of the key molecules involved in the signaling pathways of activated receptor tyrosine kinases and regulates cell growth, motility, and morphogenesis (Raffioni and Bradshaw, 1992; Royal and Park, 1995). The nuclear localization of actinin-4 was observed mainly in low-infiltrative histological subtypes of breast carcinoma. The abnormal activation of this enzyme may determine the biological behavior of cancer cells and confer metastatic potential to them.

Although histological subtyping is a common procedure in the pathological diagnosis of breast cancer, the correlation between histological subtypes and the outcome of patients in this study did not reach statistical significance (data not shown). However, we demonstrated a significantly poorer disease-free survival and overall survival in breast cancer patients with a nonnuclear localization of actinin-4 (Fig. 12). This difference was probably due to cases of papillotubular and solid-tubular carcinomas, in which the location of actinin-4 was exceptionally cytoplasmic. These cases may have had a high degree of metastatic potential that could not be detected by conventional histological examination.

| Table I. Correlation between the Subcellular Localization of Actinin-4 and Histological Subtypes of Breast Cancer |
|---|---|---|---|
| Papillotubular and solid tubular carcinoma | Scirrhous carcinoma | Invasive lobular carcinoma |
| A. Nuclear | 20 | 0 | 0 |
| B. Combined | 25 | 8 | 0 |
| C. Cytoplasmic | 9 | 15 | 6 |
| Total | 54 | 23 | 6 |
Figure 12. (A) Disease-free survival curves of 61 patients with clinical stages I and II breast cancer, according to the subcellular localization of actinin-4. Disease-free survival curves are drawn using the Kaplan-Meier method. Significant difference in disease-free survival is observed between types A + B (nuclear) and type C (nonnuclear) (P < 0.01). (B) Overall survival curves of 61 patients with clinical stages I and II breast cancer, according to the subcellular localization of actinin-4. Significant difference in overall survival is observed between types A + B (nuclear) and type C (nonnuclear) (P < 0.01).

From the above experimental and clinical observations, we conclude that a cytoplasmic localization may indicate active actin-bundling by actinin-4 and enhanced cell motility. Because no definite marker is currently available for assessment of the metastatic potential of cancer, the subcellular localization of the actinin-4 molecule may represent a potential new biological marker for cancer metastasis and may predict early relapse of breast cancer. Large-scale prospective analyses are necessary to examine this issue, not only for breast cancer but also for other cancers.

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References

Aznavoorian, S., A.N. Murphy, W.G. Stetler-Stevenson, and L.A. Liotta. 1993. Molecular aspects of tumor cell invasion and metastasis. Cancer. 71:1368–1383.

Bao, L., M. Loda, A.P. Janmey, R. Stewart, B. Anand-Apte, and B.R. Zetter. 1996. Tymosin β15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. Nat. Med. 2:1322–1328.

Beggs, A.H., T.J. Byers, J.H.M. Knoll, F.M. Boyce, G.A.P. Bruns, and L.M. Kunkel. 1992. Cloning and characterization of two human skeletal muscle α-actinin genes located on chromosomes 1 and 11. J. Biol. Chem. 267:9281–9288.

Boulakis, T. 1993. Nuclear localization signals (NLS). Crit. Rev. Eukaryotic Gene Expr. 3:193–227.

Burridge, K., G. Nukolls, C. Otey, F. Pavalko, K. Simon, and C. Turner. 1990. Actin-membrane interaction in focal adhesions. Cell Differ. Dev. 32:337–342.

Casella, J.F., M.D. Flanagan, and S. Lin. 1981. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. Nature. 293:302–305.

Cavaillé, V., P. Augereau, M. Garcia, and H. Rochefort. 1988. Estrogens and growth factors induce the mRNA of the 52Kpro-cathepsin-D secreted by breast cancer cells. Nucl. Acids Res. 16:1903–1919.

Cramer, L.P., M. Siebert, and T.J. Mitchison. 1997. Identification of novel graded polarity actin filament bundles in locomoting heart fibroblasts. J. Cell Biol. 136:1287–1305.

Felder, I.F., D.M. Gersten, and I.R. Hart. 1978. The biology of cancer invasion and metastasis. Adv. Cancer Res. 28:149–250.

Fukami, K., K. Furuhashi, M. Inagaki, T. Endo, S. Hatanan, and T. Takenawa. 1992. Requirement of phosphatidylinositol 4,5-bisphosphate for α-actinin function. Nature. 359:150–152.

Fukami, K., N. Sawada, T. Endo, and T. Takenawa. 1996. Identification of a phosphatidylinositol 4,5-bisphosphate-binding site in chicken skeletal muscle α-actinin. J. Biol. Chem. 271:2646–2650.

Giard, D.J., S.A. Aaronson, G.J. Todaro, P. Armitage, J.H. Kelsey, H. Dosik, and W.P. Parks. 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51:1417–1423.

Glück, U., and A. Ben-Ze’ev. 1994. Modulation of α-actinin levels affects cell motility and reduces tumorigenicity on 3T3 cells. J. Cell Sci. 107:1771–1782.

Glück, U., D.J. Kwidzinski, and A. Ben-Ze’ev. 1993. Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with α-actinin cDNA. Proc. Natl. Acad. Sci. USA. 90:383–387.

Hemmerly, G., and P. Strauli. 1981. In vitro motility of cells from human epithelial carcinomas. A study by phase-contrast and reflection-contrast cinemicrography. Int. J. Cancer. 27:603–610.

Harlow, E., and D. Lane. 1988. Preparing total cell lysates for immunoblotting. In Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 481 pp.

Hemmings, L.K., P.A. Kuhlman, and D.R. Critchley. 1992. Analysis of the actin-binding domain of α-actinin by mutagenesis and demonstration that dystrophin contains a functionally homologous domain. J. Cell Biol. 116:1369–1380.

Hirohashi, S., M. Watanabe, Y. Shimosato, and T. Sekine. 1984. Monoclonal antibody reactive with the stialyl-sugar residue of a high molecular weight glycoprotein in sera of cancer patients. Gann. 75:485–488.

Hosaka, S., M. Suzuki, M. Goto, and H. Sato. 1978. Motility of rat ascites hepatoma cells, with reference to malignant characteristics in cancer metastasis. Gann. 69:273–276.

Hsu, D.K.W., Y. Gou, G.F. Alberts, K.A. Peilley, and J.A. Winkle. 1996. Fibroblast growth factor-1-inducible gene FR-17 encodes a nonmuscle α-actinin isoform. J. Cell Physiol. 167:261–268.

Imamura, M., T. Sakurai, Y. Oyagawa, T. Ishikawa, K. Goto, and T. Masaki. 1994. Molecular cloning of low-Ca2+-sensitive-type nonmuscle α-actinin. Eur. J. Biochem. 223:395–401.

Kaplan, E.L., and P. Meier. 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc. 53:457–481.

Klagsbrun, M. 1996. The fibroblast growth factor family: structural and biological properties. Prog. Growth Factor Res. 16:261–268.

Knudsen, K.A., A.P. Soler, K.R. Johnson, and M.J. Wheelock. 1995. Interaction of α-actinin with the cadherin/catenin-cell–cell adhesion complex via α-catenin. J. Cell Biol. 130:877–877.

Kuhlman, P.A., L. Hemmings, and D.R. Critchley. 1992. The identification and characterisation of an actin-binding site in α-actinin by mutagenesis. FEBS Lett. 304:201–206.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680–685.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. Cell. 85:39–39.

Lazarides, E.B., and K. Burridge. 1975. α-actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. Cell. 6:289–298.

Matsudaira, P. 1991. Modular organization of actin crosslinking proteins. Trends Biochem. Sci. 16:87–92.

Millake, D.B., A.D. Blanchard, B. Patel, and D.R. Critchley. 1989. The cDNA sequence of a human placentual α-actinin. Nucl. Acids Res. 17:6725.

Miyamoto, S., H. Teramoto, O.A. Coso, J.S. Gutkind, P.D. Burbelo, S.K. Akiyama, and K.M. Yamada. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 131:791–805.

Nicolson, G.L. 1988. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. Biochim. Biophys. Acta. 948:175–224.
Nishihira, T., Y. Hashimoto, M. Katayama, S. Mori, and T. Kuroki. 1993. Molecular and cellular features of esophageal cancer cells. *J. Cancer Res. Clin. Oncol.* 119:441–449.

Otey, C.A., F.M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the β1 integrin subunit in vitro. *J. Cell Biol.* 111:721–729.

Otey, C.A., G.B. Vasquez, K. Burridge, and B.W. Erickson. 1993. Mapping of the α-actinin binding site within the β1 integrin cytoplasmic domain. *J. Biol. Chem.* 268:21193–21197.

Partin, A.W., J.S. Shoeniger, L.L. Mohler, and D.S. Coffey. 1989. Fourier analysis of cell motility: correlation of motility with metastatic potentials. *Proc. Natl. Acad. Sci. USA.* 86:1254–1258.

Prendergast, G.C., and E.B. Ziff. 1991. Mbh1: a novel gelsolin/severin-related protein which binds actin in vitro and exhibits nuclear localization in vivo. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:757–766.

Raffioni, S., and R.A. Bradshaw. 1992. Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA.* 89:9121–9125.

Rosen, P.P., and H.A. Oberman. 1992. Tumors of the mammary gland. Armed Forces Institute of Pathology, Washington, D.C. 115–175.

Royal, I., and M. Park. 1995. Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase. *J. Biol. Chem.* 270:27780–27787.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual, 2nd Ed. Cold Springs Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato, Y., K. Mukai, S. Watanabe, M. Goto, and Y. Shimosato. 1986. The AMeX method: a simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. *Am. J. Pathol.* 125:431–435.

Shibata, T., A. Ochiai, Y. Kanai, S. Akimoto, M. Gotoh, N. Yasu, R. Machinami, and S. Hirohashi. 1996. Dominant negative inhibition of the association between β-catenin and c-erbB-2 by N-terminally deleted β-catenin suppresses the invasion and metastasis of cancer cells. *Oncogene.* 15:883–889.

Stossel, T.P. 1993. On the crawling of animal cells. *Science.* 260:1086–1094.

Towbin, H., T. Stechel, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vemuri, G.S.R., S.E. 1994. Wortmannin inhibits serum-induced activation of phosphoinositide 3-kinase and proliferation of CHRF-288 cells. *Biochem. Biophys. Res. Commun.* 202:1619–1623.

Westley, B.R., H. 1979. Estradiol induced proteins in the MCF7 human breast cancer cell line. *Biochem. Biophys. Res. Commun.* 90:410–416.

Young, R.A., and R.W. Davis. 1983. Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA.* 80:1194–1198.

Zetter, B.R. 1990. The cellular basis of site-specific tumor metastasis. *N. Engl. J. Med.* 322:605–612.