Elongation factor-1α is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma

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SUMMARY

Overexpression of elongation factor-1α (EF1α) mRNA has been described as an actin binding protein in many divergent species, including Dictyostelium (Demma et al., 1990; Yang et al., 1990; Dharmawardhane et al., 1991; Edmonds et al., 1995), carrot (Yang et al., 1993), algae (Collings et al., 1994), Tetrahymena (Kurasawa et al., 1996), rabbit (Bektas et al., 1994), and mouse (Liu et al., 1996). The intracellular distribution of EF1α has been shown to colocalize with filamentous actin (F-actin), and is also correlated with changes in the organization of the actin cytoskeleton during chemotaxis (Dharmawardhane et al., 1991; Edmonds et al., 1995; Okazaki and Yumura, 1995). The combination of a high degree of amino acid sequence conservation across phylogeny (Riis et al., 1990) and these reports of similar actin binding activity strongly suggest that actin binding is a universal property of all EF1αs.

The control of the levels of EF1α is important for normal cell function. It has been demonstrated that increased EF1α expression is related to increased cell proliferation (Sanders et al., 1996), oncogenic transformation (Chi et al., 1992; Lew et al., 1992; Tatsuka et al., 1992) and delayed cell senescence (Shepherd et al., 1989). In addition, overexpression of EF1α mRNA was correlated with metastasis (Pencil et al., 1993; Taniguchi et al., 1992). The mechanism of the relationship between EF1α and metastasis is unclear. In one example, differential screening of cDNA libraries from metastatic and non-metastatic cell lines derived from the same parental rat mammary adenocarcinoma...
revealed a ~1.5-fold overexpression of EF1α in the metastatic compared to the non-metastatic cells (Pencil et al., 1993). In that study it was assumed that increased EF1α mRNA translated to increased EF1α protein, but this was not confirmed. If present, perhaps increased levels of EF1α protein might lead to an increased translational efficiency of metastasis-associated mRNAs in specific cytoplasmatic compartments which are associated with the conversion from a sessile to a metastatic state (for reviews, see Hesketh and Pryme, 1991; Singer, 1992). Additionally, increased levels of EF1α may help to stabilize or organize the actin cytoskeleton thereby increasing the efficiency of motility during chemotaxis (Owen et al., 1992).

The present study was undertaken to determine the actin binding properties of EF1α from two rat mammary adenocarcinoma cell lines which display different metastatic potential. The results indicate that EF1α protein from metastatic tumor cells is overexpressed, its actin binding activity is reduced compared to non-metastatic tumors, and that stimulation with EGF causes a redistribution of tumor cell EF1α into the actin cytoskeleton.

**MATERIALS AND METHODS**

All reagents were purchased from Sigma unless otherwise noted.

**Cell lines and culture conditions**

The cell lines used in this study were the MTLn3 and MTC lines (both kindly provided by Dr G. L. Nicolson, M. D. Anderson Cancer Center, Houston, TX). The MTLn3 line (high metastatic potential) was derived as a single cell clone from a lung metastasis of the 13762NF rat mammary adenocarcinoma, while the MTC line (low metastatic potential) was derived as a single cell clone from the parental tumor (Neri et al., 1982). Cell stocks were maintained according to the method of Segall et al. (1996).

**The generation of tumors**

MTLn3 and MTC cell cultures were grown to 60-80% confluence, harvested and then were resuspended at a density of 6.7×10^6 cells/ml and placed on ice. For tumor production, female Fisher 344 rats (Charles River) were injected with either MTLn3 or MTC cells. In all cases, 1×10^6 cells were injected subcutaneously into the fat pad located between the 3rd and 4th nipples. The growth of tumors was allowed to progress for 3-5 weeks.

For the purification of EF1α and histopathology analysis, tumors were harvested by excision of the primary tumor following CO2 euthanasia. To determine the metastatic activity of each cell line, the lungs and ipsilateral and contralateral axillary lymph nodes were removed and fixed in Dulbecco’s PBS (Gibco) containing 3.7% formaldehyde (Fluka). Representative sections of the fixed tissues were submitted for routine histologic examination.

**The purification of EF1α from MTLn3-generated tumors**

The purification of EF1α from vertebrate tissue was modified from the procedure of Edmonds et al. (1995). All procedures were performed at 4°C. Excised tumors were weighed, then minced and diluted 5-fold (w/v) with Buffer L (5 mM Pipes, pH 7.0, 1 mM DTT, 1 mM EGTA) containing the protease inhibitors: chymostatin, leupeptin, and pepstatin (10 µg/ml each). The tumor was homogenized by Polytron® and then further homogenized in a Teflon/glass homogenizer for 30 passes. The homogenate was spun at 100,000 g-hours in a Beckman Ti70 rotor. The supernatant was brought to 15% glycerol (v/v) and then was loaded onto a 5×20 cm DE52 (Whatman) anion exchange column equilibrated with Buffer 52 (20 mM Pipes, pH 7.0, 1 mM DTT, 0.1 mM EDTA, 15% glycerol, v/v). EF1α, as detected by SDS-PAGE and western blot analysis (see below), eluted in the flow-through fractions and was immediately loaded onto a 1.6×10 cm Poros II HS (PerSeptive Biosystems, Cambridge MA) cation exchange column equilibrated with Buffer 52. EF1α was eluted using a 200 ml 0-1 M NaCl gradient. The EF1α-containing fractions were determined by SDS-PAGE/western blot, pooled, diluted to a NaCl concentration of 100-200 mM, and then loaded onto a 1× 10 cm ceramic hydroxylapatite column (Bio-Rad) equilibrated in Buffer HTP (10 mM Pipes, pH 6.8, 10 mM KPO4, 1 mM DTT, 15% glycerol). EF1α was eluted with a 100 ml 0-1 M NaCl gradient. To rapidly concentrate the sample, the EF1α-containing fractions were again pooled, diluted to a 100-200 mM NaCl concentration, and then loaded onto a 0.46×10 cm Poros II HS column equilibrated in Buffer HTP. The EF1α sample was eluted with a 1 M NaCl pulse and then vacuum concentrated/dialyzed into Buffer S (10 mM Pipes, pH 7.0, 1 mM DTT, 0.1 mM EDTA, 20% glycerol, 0.02% NaN3) containing 0.5 M NaCl. The final EF1α sample was dialyzed against Buffer S lacking NaCl and stored under liquid nitrogen. The typical yield from an 8 g tumor was 0.5 mg EF1α with >90% purity as assessed by scanning densitometry of SDS-PAGE gels (Fig. 1B).

**Purification of EF1α from normal rat liver**

The livers from female Fisher 344 rats were excised, weighed, minced, diluted 5-fold (w/v) with Buffer L and then were homogenized in a Teflon/glass homogenizer for 30 passes. The remainder of the purification was as described above for tumor EF1α.

**Poly(U)-directed polyphenylalanine synthesis**

The procedure for the in vitro synthesis of polyphenylalanine was as described (Carvalho et al., 1984). The purified reagents for the assay: ribosomes, EF-2 and [14C]Phe-tRNA (850 cpm/pmol) from rabbit reticulocytes, and EF1α from yeast, were kindly provided by Dr William Merrick (Case Western Reserve University, Cleveland OH).

**Assessment of the specificity of an anti-EF1α antibody**

An anti-EF1α antibody was produced in chicken against a synthetic peptide, AGAGKVTSAQKAQKAK, corresponding to the carboxyl terminus of mouse EF1α which is highly conserved between all EF1αs. The peptide was coupled to poly-L-lysine (Mr 15,000-30,000) via an N-terminal cysteine residue at a molar ratio of peptide-poly-L-lysine of ~5. Total immunoglobulin Y was isolated from egg yolk by polyethylene glycol precipitation as described elsewhere (Carroll and Stollar, 1983), and was further purified by affinity chromatography against the same peptide immobilized on epoxy-activated Sepharose. Monospecific antibodies were eluted with 0.1 M glycine HCl, pH 2.2, neutralized immediately with 1 M Tris-HCl, pH 9.0, concentrated with a Centricron-30 (Amicon) and stored at −20°C in 50% glycerol.

The specificity for EF1α was assessed by western blot analysis. Samples were transferred to nitrocellulose membranes from SDS-PAGE gels using a semi-dry blotter (Bio-Rad) and the immunoreactive polypeptides were visualized with an ECL chemiluminescence kit (Amersham). As shown in Fig. 1, the antibody recognizes a single polypeptide of 50 kDa apparent Mr in homogenates from both whole tumors derived from MTLn3 cells (lanes a,b) and normal rat liver (lanes c,d). In addition, the antibody recognizes a single 50 kDa band in whole cell homogenates of MTC cells, Dictyostelium, and EF1α purified from Dictyostelium (data not shown).

To determine the specificity of the anti-EF1α peptide antibody for immunofluorescence the antibody was preabsorbed against purified rat liver EF1α transferred to nitrocellulose (Olmsted, 1981). As a parallel control, the anti-EF1α peptide antibody also was preabsorbed against nitrocellulose alone. As shown for MTLn3 cells, following preabsorption against EF1α (Fig. 3G), but not nitrocellulose (data not shown), the fluorescence intensity is greatly reduced (compare to Fig. 3B), while the rhodamine-phalloidin staining of F-actin is unaffected (Fig. 3H). Taken together the above data affirm the monospecificity of this antibody for EF1α.
The distribution of EF1α from metastatic tumors

**Immunofluorescence microscopy**

The distribution of EF1α and F-actin in resting and EGF-stimulated MTLn3 and MTC cells was assessed by confocal immunofluorescence microscopy. Cells were plated onto coverslips pre-coated with rat tail collagen I (Collaborative Biochemical) and cultured overnight. Prior to stimulation, the cells were serum-starved by incubation for 3 hours in medium supplemented with 12 mM Hepes, pH 7.0, and 0.35% BSA (MEMH). Then, the cells were stimulated for three minutes by the addition of MEMH containing 5 nM epidermal growth factor (EGF). Rapid fixation was carried out by the addition of Fix Buffer (5 mM Pipes, pH 7.2, 1.1 mM Na2HPO4, 0.4 mM KH3PO4, 137 mM NaCl, 5 mM KCl, 4.0 mM NaHCO3, 2 mM MgCl2, 2 mM EGTA, 5.5 mM glucose) containing 3.7% formaldehyde and 0.1% Triton X-100.

The cytoskeletal distribution of EF1α was imaged by reducing the amount of soluble intracellular material with mild detergent extraction prior to fixation. For these experiments cells were plated on Mattek dishes, starved and stimulated as above. After aspiration of the bathing medium, 100 μl Buffer E (20 mM Pipes, pH 6.5 with 30 mM KOH, 4 mM MgCl2, 10 mM EGTA, pH 6.5 with 20 mM KOH, 5 μM phallodynamic, 0.025% saponin) was added to the culture well. After 15 seconds of extraction the dish was flooded with Fix Buffer containing 3.7% formaldehyde. The specimens were then processed for immunofluorescence microscopy as described above.

Specimens were viewed with a Bio-Rad MRC600 scanning confocal microscope equipped with a krypton/argon laser to ensure complete channel separation of the fluorescein and rhodamine signals. Optical sections (0.3 μm thick) were imaged with a Nikon 60× flat field objective (NA=1.4) on a Nikon Diaphot microscope. Quantitative analysis and image processing were performed with NIH-Image software (version 1.55).

**The determination of the expression levels of EF1α**

Rat MTLn3 tumor, MTC tumor and normal liver tissues were excised and processed as described above. MTLn3 and MTC cultured cells were placed directly into SDS-PAGE sample buffer. Duplicate samples were separated by SDS-PAGE, and then either stained with Coomassie blue, or processed for western blotting. For quantitation of EF1α by densitometry a standard curve of purified liver EF1α concentrations was included on all western blots. Western transfer and membrane processing were performed as above except 125I-Protein A was used to detect the primary antibody. Densitometry analysis of Coomassie blue-stained SDS-PAGE gels for actin and corresponding 125I autoradiograms for EF1α was performed with a laser scanning densitometer equipped with ImageQuant software (Molecular Dynamics). To determine the total cellular protein per MTLn3 and MTC cell, a BCA protein assay (Pierce) was performed. Cultured cells were lysed as above except 0.5% Triton X-100 was used.

**The evaluation of F-actin binding by EF1α**

F-actin binding to purified MTLn3 tumor and rat liver EF1α was assessed by differential co-sedimentation analysis (Edmonds et al., 1995). EF1α (1 μM) was co-polymerized with rabbit skeletal muscle actin (3 μM; Condeelis et al., 1982) in Sedimentation Buffer (20 mM Pipes, pH 6.0-8.0 with 30-35 mM KOH, 1 mM MgOAc, 0.2 mM DTT, 1 mM ATP, 25% glycerol) overnight at 4°C. To distinguish between crosslinked versus single actin filaments, the EF1α-F-actin mixtures were centrifuged differentially in an Airfuge (Beckman). Crosslinked filaments were pelleted preferentially at 50,000 g for 4 minutes (low speed pellets). Then the resultant low speed supernatants were spun at 130,000 g for 45 minutes to pellet single actin filaments (high speed pellets). In some experiments co-sedimentation assays were performed in a buffer designed to mimic the intracellular ionic composition of mammalian cells (Alberts et al., 1994): 30 mM Pipes, pH 7.5, 130 mM K+ gluconate, 1 mM NaCl, 1 mM MgOAc, 1 mM ATP, 70 mg/ml polyethylene glycol 8000. To determine the percentage of EF1α in each sample, SDS-PAGE, western blotting and laser scanning densitometry were performed as described above.
Cell fractionation following stimulation with EGF

Cells were harvested, resuspended to a density of 5x10⁶ cells/ml in MEMH, and allowed to re-equilibrate to 37°C for 10 minutes. For cell lysis, 200 µl of the cell suspension was removed and mixed with 800 µl of ice cold Lysis Buffer (20 mM Pipes, pH 6.5, 88 mM KCl, 5 mM EGTA, 0.2 mM MgCl₂, 5 mM DTT, 1 mM ATP, 10 µg/ml each of chymostatin, leupeptin and pepstatin, 0.5% Triton X-100). Cell samples were obtained both before and after the addition of EGF (5 nM) in MEMH. As a control, instead of stimulation with EGF, an equal volume of MEMH was added and pre- and post-addition time points were collected. Triton-insoluble cytoskeletons were pelleted at 400,000 g for 20 minutes at 4°C to obtain high speed pellet (HSP) and supernatant (HSS) fractions.

SDS-PAGE gels and corresponding 125I-Protein A western blots were prepared for all time points for LSP, HSP and HSS fractions. The amount of EF1α per time point was determined by densitometry analysis of autoradiograms and the amount of actin per time point was determined by densitometry analysis of Coomassie blue-stained SDS-PAGE gels as described above. The amount of total protein in each lane was also determined by densitometry analysis of the Coomassie blue-stained SDS-PAGE gels, and the ratios for EF1α:total protein and actin:total protein were determined for each time point.

To determine if the EGF-mediated redistribution of EF1α is dependent on the integrity of the actin cytoskeleton, cells were prepared as above, then cytochalasin D (100 nM), or an equal volume of MEMH containing DMSO was added five minutes before stimulation with EGF. Stocks of cytochalasin D were prepared in DMSO and diluted into MEMH. The final concentration of DMSO was 0.1%.

RESULTS

The experiments described in the present study employed two rat mammary adenocarcinoma cell lines, MTLn3 and MTC, that previously were shown to have different metastatic potentials and differing levels of certain mRNAs (Welch et al., 1983; Taniguchi et al., 1992; Pencil et al., 1993). Prior to our detailed analysis of one of these mRNAs, it was necessary to confirm the metastatic potentials of the specific clones used in this study. As summarized in Table 1, injection of MTLn3 cells produced tumors within four weeks in all axillary lymph nodes and the lungs; however, injection of MTC cells only produced tumors in the ipsilateral lymph nodes after five weeks. Thus, in agreement with previous work, we characterize the MTLn3 cell line as highly metastatic while the MTC cell line has low metastatic potential (Neri et al., 1982). This difference in metastatic phenotype is stable with passage under our culturing conditions. It is interesting to note that the in vitro doubling time for these two cell lines is identical (14 hours); whereas, injected MTLn3 cells produce primary tumors 1-2 weeks sooner than injected MTC cells.

EF1α expression levels in tissues of differing metastatic potential

A correlation has been drawn between the metastatic potential of breast adenocarcinoma and an increased level of expression of EF1α mRNA (Taniguchi et al., 1992; Pencil et al., 1993). However, it was unclear if this increase in mRNA expression results in increased levels of EF1α protein expression. To determine the concentrations of EF1α protein within cell lines and whole tumors, homogenates were western blotted and probed with a monospecific antibody to EF1α (see Materials and Methods and Fig. 1). As summarized in Table 2 (sections I-II), in the highly metastatic MTLn3 cells, EF1α comprises ~3% of total cell protein (57 µM); whereas, in the weakly metastatic MTC cells the EF1α concentration is reduced by ~35% to 37 µM. Furthermore, the amount of EF1α protein in whole primary tumors derived from MTLn3 cells is almost twice that of normal rat liver tissue or of primary tumors derived from weakly metastatic MTC cells (Table 2, section III). These increases in the amount of EF1α protein from MTLn3 cell and MTLn3-derived tumors correlate well with the relative level of EF1α mRNA found in the MTLn3 cell line (Pencil et al., 1993). Therefore, we demonstrate that the increased amount of EF1α mRNA associated with highly metastatic tumors results in an increased level of EF1α protein and suggests a relationship between EF1α and metastasis.

Interestingly, the amounts of actin in these two cell lines also are different. As shown in Table 2, the amounts of total cellular actin (65 µM) and F-actin in the cytoskeletons (31 µM) of MTC cells are reduced by ~60% compared to MTLn3 cells (153 µM total actin, 76 µM F-actin). However, due to the decrease in EF1α and F-actin in MTC relative to MTLn3 cells, there is actually a ~30% increase in the molar ratio of EF1α:F-actin in MTC cytoskeletons compared to MTLn3 cytoskeletons (0.68 versus 0.46). This relative increase in the EF1α:F-actin ratio in the cytoskeletons of MTC cells suggests that the apparent binding affinity (Kd(app)) of MTC EF1α for the

| Table 2. Levels of expression of EF1α in cell lines and whole tumors |
|--------------------------|-----------------|-----------------|-----------------|
|                         | % Total protein | EF1α: actin* | EF1α: F-actin† | Kd(app)‡ |
| I. MTLn3                |                 |                |                |         |
| EF1α                    | 2.9             | 57             | 35             | 26 µM   |
| Actin                   | 6.5             | 153            | 0.37           | 76      | 0.46   | –     |
| II. MTC                 |                 |                |                |         |
| EF1α                    | 2               | 37             | –              | 21      | 7 µM   |
| Actin                   | 3               | 65             | 0.57           | 31      | 0.68   | –     |
| III. Rat mammary tumors and normal liver | | | | |
| MTLn3 primary tumor     | 1.86            | 1.96           | 1.06           | 1.00    |
| MTC primary tumor       | 1.00            |                |                |         |
| Normal rat liver        | 1.00            |                |                |         |

* From whole cell lysates.
† From the Triton-insoluble cytoskeletal fraction.
‡ Calculated from: apparent Kd = (EF1α)free (F-actin)free/(EF1α)bound in the Triton-insoluble cytoskeletal fraction.
§ Relative to the amount in normal rat liver.

The following table summarizes the metastatic involvement of tissues from rats inoculated with MTLn3 or MTC cell lines.

| Table 1. Metastatic involvement of tissues from rats inoculated with MTLn3 or MTC cell lines* |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Weeks† | MTLn3 | MTC |
|        | IPL   | CPL  | Lung | IPL   | CPL  | Lung |
| 3       | 2/2   | 0/2  | 0/2  | –     | –    | –    |
| 4       | 4/4   | 4/4  | 4/4  | 0/3   | 0/3  | 0/3  |
| 5       | 7/8   | 4/7  | 7/8  | 4/4   | 0/4  | 0/4  |

*Expressed as number of positive animals per total number of animals examined.
†Time following inoculation of mammary fat pads with specified cell type. IPL, ipsilateral axillary lymph node; CPL, contralateral axillary lymph node.
Fig. 2. Effect of pH on F-actin binding by EF1α purified from MTLn3-derived rat mammary tumors and normal rat liver. (A) EF1α: F-actin co-sedimentation assay at pH 6.5 where actin filaments crosslinked by EF1α (low speed pellet, LSP) were separated from EF1α bound to single actin filaments (high speed pellet, HSP) and free EF1α (high speed supernatant, HSS) by differential centrifugation. Lanes a-j, Coomassie blue-stained SDS-PAGE gel; lanes a-e, EF1α alone; lanes f-j, mixture of tumor EF1α (1 μM) and rabbit skeletal muscle F-actin (3 μM). Lanes f'-j', corresponding western blot of lanes f-j probed with an anti-EF1α antibody. Lanes a, f, f', total reaction mixture; lanes b, g, g', low speed supernatant; lanes c, h, h', LSP; lanes d, i, i', HSS; lanes e, j, j', HSP. (B) Rat tumor or (C) rat liver EF1α (1 μM) was co-polymerized with rabbit skeletal muscle actin (3 μM) at the indicated pH. (■) LSP, (●) HSS, (▲) HSP. Data are representative of 3 experiments. (D) Liver EF1α (2 μM) and muscle F-actin (12 μM) co-sedimentation assays performed under physiological conditions. The low salt buffer conditions utilized in A-C were compared to a physiological salt solution designed to mimic mammalian intracellular ionic conditions (see Materials and Methods).
containing crosslinked actin filaments (LSP). As pH is increased most of the EF1α shifts from the LSP to fractions containing single actin filaments (HSP) and unbound EF1α (HSS). In addition, the binding of F-actin by EF1α purified from rat liver displays a pattern similar to tumor EF1α (Fig. 2C): as pH increases liver EF1α redistributes from the crosslinked filament fraction to single filament or free fractions. In comparing the F-actin binding profiles of tumor and liver EF1α there is a 10-20% decrease in the amount of bound tumor EF1α at pH greater than 7.5. This difference in binding suggests that tumor EF1α has a lower affinity for F-actin compared to EF1α isolated from normal tissues. Irrespective of variable affinities, it is evident that the pH-sensitive binding of F-actin by EF1α is conserved for these two vertebrate proteins.

The ionic conditions of the co-sedimentation assays described above were chosen to allow direct comparisons with the F-actin binding activity of EF1α from Dictyostelium (Edmonds et al., 1995). These conditions are physiological for a free living amoeba, but are of low ionic strength with respect to mammalian systems. To ascertain the F-actin binding behavior of vertebrate EF1α under more appropriate mammalian physiological conditions, co-sedimentation assays were performed with liver EF1α in a buffer designed to mimic the intracellular composition of a typical mammalian cell (Alberts et al., 1994). Polyethylene glycol (M_r 8×10^3, 70 mg/ml) was included to simulate the high intracellular total protein concentration (Minton, 1983) which we measured in MTC and MTLn3 cells as 90 or 100 mg/ml, respectively. As shown in Fig. 2D, the relative binding of liver EF1α to F-actin is stimulated over 5-fold compared to the Dictyostelium-based low salt buffer. Also, the binding of liver EF1α to F-actin is sensitive to increases in pH under these generic mammalian conditions (data not shown). Thus, it appears that vertebrate EF1α has the potential to bind F-actin in vivo. In combination, the above data confirm previous results indicating similar actin binding activity by EF1α from divergent species and strongly imply that actin binding is a universal property of all EF1αs.

**Localization of EF1α in resting and stimulated tumor cells**

EF1α from Dictyostelium is associated with actin-containing cytoskeletal structures important for shepharding a chemotactic response to hormone stimulation (Dharmawardhane et al., 1991; Okazaki and Yumura 1995). MTLn3 cells, which are chemotactic to EGF, respond within minutes to exposure to EGF by extending actin-rich lamellipodial projections (Segall et al., 1996). Thus, it was of interest to determine if EF1α in MTLn3 cells displayed an association with dynamic cytoskeletal structures similar to EF1α in Dictyostelium.

The intracellular distribution of EF1α and F-actin was assessed by confocal immunofluorescence microscopy. The

![Fig. 3. Immunofluorescence images acquired by confocal microscopy of EGF-mediated changes in EF1α and F-actin distribution in MTLn3 cells.](image)
distribution of F-actin in unstimulated MTLn3 cells (Fig. 3C) is associated most strongly with prominent actin bundles resembling stress fibers and surface projections; i.e. ruffles, filopodia and lamellipodia (arrows). Within 3 minutes after stimulation with EGF, MTLn3 cells undergo a uniform flattening with the formation of broad lamellipodia that display an intense F-actin rim at the leading edge (Fig. 3F, arrowhead; see also Segall et al., 1996; Chan et al., unpublished).

The distribution of EF1α parallels that of F-actin in MTLn3 cells. In unstimulated cells (Fig. 3B), EF1α is diffuse throughout the cytoplasm with increased levels in surface projections (arrows) which contain F-actin (compare to Fig. 3C). There appears to be little nuclear staining; however, the perinuclear signal probably is associated with the endoplasmic reticulum (Sanders et al., 1996). After stimulation with EGF, a strong EF1α signal is associated with the F-actin rich zone at the leading edge of de novo lamellipods (Fig. 3E, arrowhead). Therefore, similar to results reported for *Dictyostelium* (Dharmawardhane et al., 1991; Okazaki and Yumura, 1995), the distribution of EF1α within MTLn3 cells is responsive to cytokine stimulation and co-locallizes with the actin cytoskeleton in situ.

To delineate more closely the intracellular location of EF1α with respect to F-actin it was necessary to reduce the immunofluorescence due to soluble EF1α. This reduction was accomplished by extracting cells for 15 seconds with saponin prior to fixation. After extraction, as shown in Fig. 4, EF1α in MTLn3 cells is associated with a subset of F-actin-containing fibers (arrows) in addition to the leading edge.

While the MTC cell line is not chemotactic to EGF (Segall et al., 1996) these cells do display greater overall motility than MTLn3 cells (J. Wyckoff, unpublished observations). In contrast to MTLn3 cells, the organization of the actin cytoskeleton is very different in MTC cells. The most striking difference is the absence of prominent stress fibers and an overall reduced rhodamine-phalloidin staining of F-actin in motile MTC cells (Fig. 5B,D). These observations agree well with the reduced amount of total actin measured in these cells (Table 2). Yet even though the amount of F-actin in MTC cells is reduced, there is the presence of an F-actin rich zone associated with the leading edge (Fig. 5B,D) and this region displays a strong EF1α immunofluorescence signal (Fig. 5A,C) similar to that observed in MTLn3 cells. MTC cells extracted with saponin also show a prominent staining for F-actin and EF1α in the leading edge (Fig. 5C,D).

An increase in actin polymerization is a typical response to stimulation in many motile cells (Condeelis, 1993). In *Dictyostelium* and MTLn3 cells, this increase in F-actin is associated with the formation of surface projections; i.e. filopodia and lamellipodia. In *Dictyostelium* the incorporation of EF1α into the cytoskeleton closely follows this increase in F-actin (Dharmawardhane et al., 1991; Okazaki and Yumura, 1995). Similarly, in MTLn3 cells a peak of F-actin formation at the leading edge is observed within 2 minutes of stimulation with EGF (Segall et al., 1996; Chan et al., unpublished). As shown in Fig. 6, this peak of F-actin polymerization is followed by a gradual depolymerization, or loss of F-actin, which by 5 minutes returns the total amount of cellular F-actin to pre-stimulus levels. The association of EF1α with the cytoskeletal fraction containing F-actin displays a peak at 3 minutes after EGF stimulation and then rapidly declines to prestimulus levels within 1 minute. Thus, EF1α enters the cytoskeletal fraction soon after an increase in F-actin and then leaves this fraction simultaneously with a decrease in the amount of F-actin. This result suggests a dependence on F-actin for EF1α to associate with the cytoskeletal fraction.

Further corroboration that the association of EF1α with the cytoskeleton is mediated by actin was obtained by monitoring changes in the cytoplasmic partitioning of EF1α following dis-
rupture of the actin cytoskeleton. Cytochalasin D, an agent which blocks actin polymerization and blunts the morphological and chemotactic responses of MTLn3 cells to EGF stimulation (Segall et al., 1996), prevents the increase in EF1\(\alpha\) associated with the Triton-insoluble cytoskeleton following EGF stimulation (Fig. 7B). This loss of partitioning of EF1\(\alpha\) into the cytoskeleton after stimulation with EGF appears to be specific for F-actin in that the amount of actin associated with the cytoskeleton also is reduced by cytochalasin D treatment (Fig. 7A). Exposure to buffer controls containing only DMSO did not affect the normal response to stimulation with EGF. These observations confirm that the increase in the association of EF1\(\alpha\) with the cytoskeleton following stimulation with EGF is linked to changes in F-actin.

**DISCUSSION**

**EF1\(\alpha\) is an actin binding protein in vertebrate cells**

In this study we have demonstrated that EF1\(\alpha\) from metastatic rat mammary adenocarcinoma cells is an actin binding protein. The nature of the interaction between EF1\(\alpha\) and F-actin is thought to be charge-dependent based upon the sensitivity to pH and ionic strength (Fig. 2B,C, and see Edmonds et al., 1995). The basic pI of EF1\(\alpha\) (8.5-9.5) and the acidic pI of actin (4.0-4.5) has lead to the argument that the in vitro interaction of EF1\(\alpha\) with actin is nonspecific thereby trivializing any physiological relevance. This argument is inconsistent with the following observations: (i) rat liver EF1\(\alpha\) binds tightly to F-actin under the physiological ionic conditions appropriate for mammalian cells (Fig. 2D); (ii) the intracellular concentrations of F-actin and EF1\(\alpha\) are far in excess of measured \(K_d\) suggesting the potential for a high degree of interaction. In fact, we observe ~60% of total cellular EF1\(\alpha\) in association with the cytoskeletal fraction of whole cell lysates (Table 2), and this association is reduced by cytochalasin D (Fig. 7); (iii) two specific peptide sequences from Dictyostelium EF1\(\alpha\) have been identified which bind to F-actin (Liu et al., unpublished). The pIs for these peptides are acidic while several other peptides with very basic pI do not interact with F-actin; (iv) aminoacyl-tRNA, but not deacyl-tRNA, specifically blocks actin binding (Liu et al., unpublished). Other exposed faces of the molecule with surface charge distributions similar to the tRNA binding sites do not bind to actin; and (v) EF1\(\alpha\) is a prototypical G protein where the rates of guanine nucleotide hydrolysis and exchange play crucial roles in the elongation cycle (Riis et al., 1990). GTP and GDP, but not ATP, affect the F-actin binding by Tetrahymena or Dictyostelium EF1\(\alpha\) (Kurasawa et al., 1996; Edmonds et al., in preparation). The creation of EF1\(\alpha\) mutants that fail to bind to actin, but are fully functional in protein synthesis, will help to resolve the issue of the physiological consequences of EF1\(\alpha\) binding to actin in vivo.

**The binding of EF1\(\alpha\) to actin is related to metastasis**

In a direct comparison of metastatic potential with the actin binding activity of EF1\(\alpha\), there is a 30% reduction in the ratio of EF1\(\alpha\)F-actin in the cytoskeletons of highly metastatic MTLn3 cells versus the weakly metastatic MTC cells (Table 2). At present, the explanation for this reduced affinity of MTLn3...
comparing to MTC EF1α for F-actin is unclear. Presumably, the primary sequences of MTC and MTLn3 EF1α are identical given the high degree of nucleotide sequence conservation between vertebrate EF1α genes (Riis et al., 1990), and the low EF1α gene copy number in the mammalian genome (Opde-nakker et al., 1987). Therefore, the different observed affinities for actin binding may be related to competition for actin substrate by other actin binding proteins, to different post-translational modifications, or to different intracellular pH regulation.

Indirect evidence suggests that the first seven amino acids of the amino terminus of actin may be involved partly in the interaction with Dictyostelium EF1α (Edmonds et al., 1995). This region of the actin molecule is a 'hot spot' for the binding of several other actin binding proteins, including myosin (Rayment et al., 1993). Thus, if events that induce metastasis affect the expression or activities of competing families of actin binding proteins, then EF1α may not be able to interact as efficiently with actin in metastatic cells as in nonmetastatic cells.

Alternatively, several post-translational modifications of EF1α have been reported including phosphorylation, methylation, and n-ethanolamine addition at glutamic acid residues (Venema et al., 1991; Dever et al., 1989; Sherman and Sypherd, 1989). The functional significance of these modifications is unresolved with respect to translation and their effects on actin binding have not been investigated directly. Further analysis by mass spectroscopy of intact and proteolytic fragments of each tumor EF1α will aid in determining the nature of any modifications.

Stimulation of many cell types results in cytoplasmic alkalization (Grinstein et al., 1989). In Dictyostelium, an increase in intracellular pH results in a decrease in EF1α associated with the cytoskeleton (Edmonds et al., 1995); therefore, the reduced affinity of MTLn3 EF1α for actin may reflect a higher MTLn3 cytoplasmic pH compared to MTC cells. As MTC cells lack the EGF receptor (Kaufmann et al., 1994), perhaps the amount of other protein components of the MTC cell membrane are altered compared to MTLn3 cells. Differences in the complement of membrane proteins between MTC and MTLn3 cells may include ion channels and transporters, such that proton homeostasis is different. This issue requires further study.

**Links between EF1α binding to actin and metastasis**

Given that MTLn3 EF1α binds less tightly to actin than MTC EF1α, how might this difference relate to the observed differences in metastatic potential between these two cell lines? Two possible roles for EF1α which may be related to metastatic potential are: (i) to influence the organization of the actin cytoskeleton; and/or (ii) to synthesize locally proteins important for maintaining an appropriate intracellular environment necessary for propagating the motile process.

Ultrastructural studies of Dictyostelium have shown that EF1α is associated with actin bundles in situ (Liu et al., 1996). These EF1α-F-actin bundles may exhibit an unique organization which would tend to exclude other known actin binding proteins (Owen et al., 1992). This property alone suggests that EF1α could play a unique role; however, EF1α also can affect the dynamics of actin filaments. Specifically, EF1α slows the rate of actin polymerization and depolymerization and decreases the critical concentration for actin polymerization in vitro (Murray et al., 1996). The net effect of this activity in the EF1α/actin compartment would be to produce actin filaments more slowly, and once formed those filaments bound to EF1α would remain more stable. The differences in the affinity of EF1α for actin between MTLn3 and MTC cells may alter the coordination of actin dynamics in the cytoskeleton thereby leading to a change in the efficiency of directed movement.

The compartmentation of EF1α also may have significance for EF1α function in protein synthesis. In myoblasts and fibroblasts, β-actin mRNA has been shown to localize to the leading edge, to be actively translated there, and to redistribute with actin in response to various second messenger pathways (Lawrence and Singer 1986; Klslausksis et al., 1994; Latham et al., 1994). These observations suggest a mechanism whereby cytokines could influence metastasis by compartmentalizing specific proteins required for chemotaxis through modulation of their localized synthesis. For example, if the localization of β-actin mRNA or the synthesis of actin protein at the leading edge were compromised then the efficiency of directed motility might be affected. In fact, mislocalization of β-actin mRNA has been shown to disrupt cell polarity (Klslausksis et al., 1994).

In human fibroblasts, over 70% of total poly(A)-containing mRNA is associated with the actin cytoskeleton (Tanida et al., 1992). This observation implies that actin somehow is involved in the biology of cytoplasmic mRNA. Therefore, changes in the amount of cellular actin or the organization of the actin cytoskeleton might affect the synthesis of many different proteins. Because MTC cells have less total actin (Table 2) and lack prominent stress fibers (Fig. 5) compared to MTLn3 cells, a dichotomy may exist in the complexion of proteins synthesized between these two cell types. Observations from differential hybridization screens of these two cell lines support this idea (Pencil et al., 1993). Because the leading edge is present in both cell types and stress fibers are lacking in the weakly metastatic MTC cells, perhaps the synthesis of proteins in the stress fiber compartment of the MTLn3 cells is related to their transformation to the metastatic state. In addition, as only a subpopulation of stress fibers display an association with EF1α in MTLn3 cells (Fig. 4), there is the potential for the regional synthesis of a set of actin-bound mRNAs distinct from those at the leading edge.

One current model for the involvement of the actin cytoskeleton in protein synthesis depicts the sequestration of EF1α away from other components of the translational machinery by F-actin (Liu et al., 1996). Actin and mRNA are ligands which compete for binding to EF1α, such that when EF1α is complexed with tRNA, binding to actin is excluded (Liu et al., unpublished). Under one scenario the generation of an appropriate intracellular signal (e.g. cytoplasmic alkalization) by the binding of a cytokine might weaken the interaction of EF1α with F-actin thereby favoring the binding of mRNA to EF1α and the promotion of polypeptide elongation. One prediction of this model is that the rates of translation for certain mRNAs associated with the cytoskeletal compartment may be different in MTC versus MTLn3 cells based upon the different affinities of EF1α for F-actin in these cells. However, interpretation of such experiments could be complicated by the possibility that other steps in translation may be affected by transformation to the metastatic state. It remains to be determined if protein synthetic components other than EF1α also are up-regulated in metastatic cells.

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