Fibroblast growth factor-1 interacts with the glucose-regulated protein GRP75/mortalin

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Fibroblast growth factor-1 (FGF-1), which lacks a signal peptide and is intracellularly localized as a result of endogenous expression or endocytosis, is thought to be involved in regulating cell growth and differentiation. In the study reported here, we purified proteins that bind intracellular FGF-1. Affinity adsorption was used to purify FGF-1-binding proteins from rat L6 cells expressing FGF-1. One of the isolated proteins was identified as the glucose-regulated protein GRP75/mortalin/PBP-74/mthsp70, a member of the hsp70 family of heat-shock proteins known to be involved in regulating glucose responses, antigen processing and cell mortality. The interaction of FGF-1 and GRP75/mortalin *in vivo* was confirmed by co-immunoprecipitation, immunohistochemical co-localization in Rat-1 fibroblasts and by using the yeast two-hybrid system. Moreover, a binding assay *in vitro* with the use of recombinant FGF-1 and mortalin demonstrated a direct physical interaction between the two proteins. These results reveal that GRP75/mortalin is an intracellular FGF-1-binding protein in cells and suggest that GRP75/mortalin is involved in the trafficking of and/or signalling by FGF-1.

Key words: co-localization, heat-shock protein, intracellular binding protein.

INTRODUCTION

The fibroblast growth factor (FGF) family of proteins consists of more than fifteen members, including FGF homologous factors, and its members have important functions in numerous physiological and pathological events such as embryogenesis, angiogenesis and wound repair [1–4]. Extracellular FGFs induce growth and differentiation in a broad range of cell types by interacting with both high-affinity tyrosine kinase transmembrane receptors and cell-surface heparan sulphate [5–7], whereas intracellular FGF-1 introduced into receptor-negative cells by means of conjugation with the A chain of diphtheria toxin induces DNA synthesis [8].

The nuclear localization sequence (NLS) contained in the primary structure of FGF-1 (NYKKPKL) is important for its mitogenic activity [9–13]. When extracellular FGF-1 binds to cell-surface receptors or to heparan sulphate, it is internalized intact into cytosol via an uncharacterized pathway, and then translocated from the cytosol to the nucleus in a cell-cycle-dependent manner [14,15]. Both the activation of FGF receptors and the internalization of FGF-1 are necessary for extracellular FGF-1 to induce proliferation [16]. This suggests that the internalization and subsequent nuclear localization of FGF-1 constitute a component of the signal transduction pathway leading to proliferation, and that intracellular trafficking of FGF-1 is a regulated phenomenon [10]. In addition to internalized FGF-1, the endogenously expressed FGF-1 is also found predominantly in the cytosol, which is consistent with the absence of a signal sequence in its primary structure [17]. Export of the intracellular FGF-1 from the cells is induced when the cells receive a heat shock [18–20]. It is also suggested that the intracellular FGF-1 modulates differentiation of myoblasts.

Taken together, these findings suggest the existence of intracellular FGF-1-binding proteins involved in the trafficking of and/or signalling by intracellular FGF-1. In the present study we sought to gain further insight into this issue by purifying FGF-1-binding proteins from cells expressing FGF-1. One of the isolated proteins was identified as the glucose-regulated protein GRP75/mortalin, a heat-shock protein (hsp) known to be involved in regulating glucose responses, antigen processing and cell mortality [18–21].

EXPERIMENTAL

Reagents

Anti-hsp/hsc70 monoclonal antibody was purchased from Stress Gen Biotechnology (Victoria, BC, Canada). Anti-mitochondrial hsp70 antibody was purchased from Affinity Bioreagents (Golden, CA, U.S.A.). FITC-conjugated mouse IgG antibody and Texas-Red-conjugated rabbit IgG antibody were purchased from Amersham Pharmacia Biotechnology (Uppsala, Sweden).

Cell culture

Rat L6 cells obtained from the American Type Culture Collection as well as Rat-1 fibroblasts were cultured with Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 10 % (v/v) fetal bovine serum (Filton, Brooklin, Australia).

FGF-1 affinity beads

Recombinant FGF-1 was prepared as described previously [9] and then coupled to activated CH-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) by following the manufacturer's instructions. In brief, 1 mg of recombinant FGF-1 was

Abbreviations used: FGF, fibroblast growth factor; hsp, heat-shock protein; NLS, nuclear localization sequence.

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mixed with 1 ml of activated CH-Sepharose in a solution containing 0.5 M NaCl and 0.1 M $NaHCO_3$, pH 8.3, and incubated overnight at 4 °C. The resultant FGF-1–Sepharose beads were incubated with 0.2 M glycine, pH 8.3, overnight at 4 °C to block remaining activated residues, then washed alternatively with 0.5 M NaCl/0.1 M formic acid (pH 4.0) and 0.5 M NaCl/0.1 M Tris/HCl (pH 8.0) at least three times.

Affinity adsorption and gel purification of FGF-1-binding proteins

Subconfluent L6 cells (10^{10}) were washed three times with PBS, lysed for 20 min on ice in 300 ml of lysis buffer consisting of 0.5 % (v/v) Triton X-100, 0.1 % sodium deoxycholate, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin, then harvested with a cell scraper. The lysate was centrifuged at 10000 ***g*** for 60 min at 4 °C. The supernatant was first incubated for 3 h at 4 °C with 50 ml of Sepharose beads, after which the beads were removed by centrifugation. The resultant supernatant was incubated for an additional 3 h at 4 °C with 20 ml of FGF-1–Sepharose, after which the beads were extensively washed with lysis buffer. The beads were then treated for 10 min at 95 °C in SDS/PAGE sample buffer, and the extracts were subjected to SDS/PAGE [7.5 % (w/v) gel]. When necessary, the samples were stored at −70 °C before electrophoresis. The gels were stained and destained with a Coomassie Brilliant Blue staining kit (Wako, Tokyo, Japan). Visible bands were excised from the gels and subjected to in-gel peptide digestion. For analytical purposes, separate gels were stained with silver after SDS/PAGE.

Determination of the peptide sequences

In-gel peptide digestion was performed essentially as described previously [21]. In brief, gel pieces containing the protein band of interest were soaked three times for 15 min each in 25 % (v/v) propan-2-ol and then three times for 15 min in 10 % (v/v) methanol. The gel pieces were then dried with an infrared lamp and rehydrated with 20 ml of solution containing 25 mM Tris/HCl, pH 8.5, 1 mM EDTA and 5 mg of Lys-C endoproteinase (Boehringer Mannheim, Indianapolis, IN, U.S.A.). After 15 min, 80 ml of Tris/HCl (pH 8.5)/1 mM EDTA/0.5 % (v/v) CHAPS was added to the reaction mixture, which was then incubated overnight at 37 °C. Finally, the reaction mixture was centrifuged and the supernatant was collected. The digested peptides in the reaction buffer were fractionated by reverse-phase HPLC on a Wakosil-II 5C18 HG C_{18} cartridge (2.0 mm × 50 mm; Wako). Chromatography was performed at 0.3 ml/min for 60 min with a linear gradient (0–60 %) of solvent B [acetonitrile containing 0.09 % (v/v) trifluoroacetic acid] in solvent A [water containing 0.1 % (v/v) trifluoroacetic acid]. The fractionation was monitored as a function of the A_{215}. Individual peaks were collected and subjected to N-terminus microsequencing with an ABI 477A protein sequencer.

Immunoblotting

FGF-1-binding proteins resolved by SDS/PAGE were electrotransferred to nitrocellulose membranes. The membranes were blocked overnight with 5 % (v/v) skimmed milk in 150 mM NaCl/10 mM Tris/HCl (pH 7.4) (TBS) at 4 °C, incubated with the respective monoclonal antibody at room temperature for 90 min, washed with 5 % (v/v) skimmed milk in TBS/0.05 % (v/v) Tween 20 (TBS-T) for 20 min, then incubated with peroxidase-labelled goat anti-mouse IgG (Chemicon, Temecula, CA, U.S.A.) diluted in TBS-T buffer. The membranes were washed with TBS-T buffer four times and the signals were detected by enhanced chemiluminescence (ECL®; Amersham Life Science, Little Chalfont, Bucks., U.K.) and Kodak X-Omat AR film (Kodak, Rochester, NY, U.S.A.).

Immunoprecipitation

Rat-1 cells were rinsed with ice-cold PBS, then lysed for 20 min on ice before being scraped; cell debris was removed by centrifugation. Either anti-(FGF-1) polyclonal antibody or control antibody was added to aliquots of supernatant, incubated at 4 °C for 1 h and subsequently incubated with Protein A + G–Sepharose (Oncogene Science, Cambridge, MA, U.S.A.) for 1 h with gentle mixing. The reaction mixtures were then centrifuged; the precipitates were washed three times in lysis buffer and the bound proteins were eluted by treating in SDS sample buffer. The samples were then resolved by SDS/PAGE, transferred to nitrocellulose membranes and blotted with anti-mthsp70 antibody. The signals were detected by peroxidase-labelled rabbit anti-mouse IgG and enhanced chemiluminescence (Amersham Life Science).

Immunofluorescence and fluorescence digital imaging microscopy

Cells were double-labelled with polyclonal anti-mortalin [22] and monoclonal anti-(FGF-1) antibodies, then detected by secondary labelling with Texas-Red-conjugated donkey anti-rabbit IgG and FITC-conjugated sheep anti-mouse IgG (Amersham) respectively. The distributions of the two fluorescent antibodies were imaged with a 40× Plan-Neofluor objective on a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) set up for confocal laser scanning microscopy [23]. The extent to which the distributions of FGF-1 and mortalin overlapped was assessed by combining corresponding images of the distributions of the individual proteins by using image-analysis software.

Detection of mortalin–FGF-1 binding with the use of a yeast two-hybrid system

The Matchmaker yeast two-hybrid system (Clonetech, Palo Alto, CA, U.S.A.) was used to determine the interaction *in vivo* between FGF-1 and mortalin. All the procedures were performed in accordance with the manufacturer's protocol. In brief, full-length FGF-1 cDNA was subcloned into the pAS2-1 yeast expression vector, thereby encoding a fusion protein with the GAL4 DNA-binding domain (FGF-1/pAS2-1). Mortalin cDNA [24] was similarly subcloned into the pACT2 vector to encode a fusion protein with the GAL4 activation domain (mortalin/pACT2). The pAS2-1 vector also encodes a gene that compensates for a deficiency in tryptophan biosynthesis in the yeast host cells, whereas the pACT2 vector encodes a gene that compensates for a deficiency in leucine biosynthesis. Y190 strain reporter yeast, which are unable to synthesize leucine, tryptophan or histidine, were co-transfected with FGF-1/pAS2-1 and mortalin/pACT2, or with other combinations of the vectors, and selected on tryptophan(−) and leucine(−) SD agar plates.

The interaction of FGF-1 and mortalin was assayed in transformed cells as a function of either histidine compensation or β-galactosidase activity. For histidine compensation assays, selected cells were streaked on agar plates containing yeast medium deficient in leucine, tryptophan and histidine, and colony formation was monitored. β-Galactosidase activity was assayed in cells streaked on agar plates containing yeast medium deficient in leucine and tryptophan but containing histidine so that all transfectants grew. After a period of cell growth, replicas of the cell colonies were produced on paper filters, then assayed for β-galactosidase activity with 5-bromo-4-chloroindol-3-yl β-D-

galactopyranoside ('X-Gal') as a substrate. Enzyme activity was indicated by the development of a blue colour.

Binding of recombinant mortalin to FGF-1–Sepharose

Recombinant His-mortalin-1 (50 ng) was incubated with FGF-1–Sepharose beads (5 ml) in 50 μl of lysis buffer containing 1 % (w/v) BSA. After 60 min at 4 °C, the FGF-1–Sepharose beads were collected by centrifugation, washed three times with lysis buffer and treated for 5 min at 95 °C in SDS/PAGE sample buffer. The extracted protein was resolved by SDS/PAGE, transferred to membranes and blotted with anti-mthsp70 antibody, which reacted with mortalin.

RESULTS

Identification of GRP75/mortalin as an FGF-1-binding protein

FGF-1 immobilized on Sepharose beads was used to isolate FGF-1-binding proteins in Rat L6 cell lysates by affinity adsorption. Three proteins with molecular masses of 75, 56 and 50 kDa were bound by FGF-1–Sepharose beads but not by control beads (Figure 1A, lanes 4 and 2 respectively). When excess recombinant FGF-1 (10 mg/ml) was included as a competitor during the adsorption procedure, the binding of the three proteins to the beads was strongly inhibited (Figure 1A, lane 3). The 75 kDa protein (p75), which was detected as a minor band in cell extracts (Figure 1A, lane 1), was particularly enriched in the FGF-1-binding fraction (lane 4). We therefore selected this protein for characterization.

After the FGF-1-binding proteins had been prepared on a large scale and resolved by SDS/PAGE, the gel portion corresponding to the p75 band was cut out and the protein contained in the gel piece was treated with Lys-C endopeptidase. The resultant peptide fragments were extracted from the gel and purified on a reverse-phase column (Figure 1B). Microsequencing of the N-terminal amino acids of peaks 13 and 18 yielded the sequences KLYSPSQI and KVQQTVQDLFG respectively. Figure 1(C) shows that the same sequences have also been seen in the primary structures of rat GRP75 (a member of the hsp70 family of proteins), mouse mortalins (GRP homologues) and human mthsp70, which all share very high overall sequence similarity [25–28]. Immunoblot analysis with antibodies against mthsp70

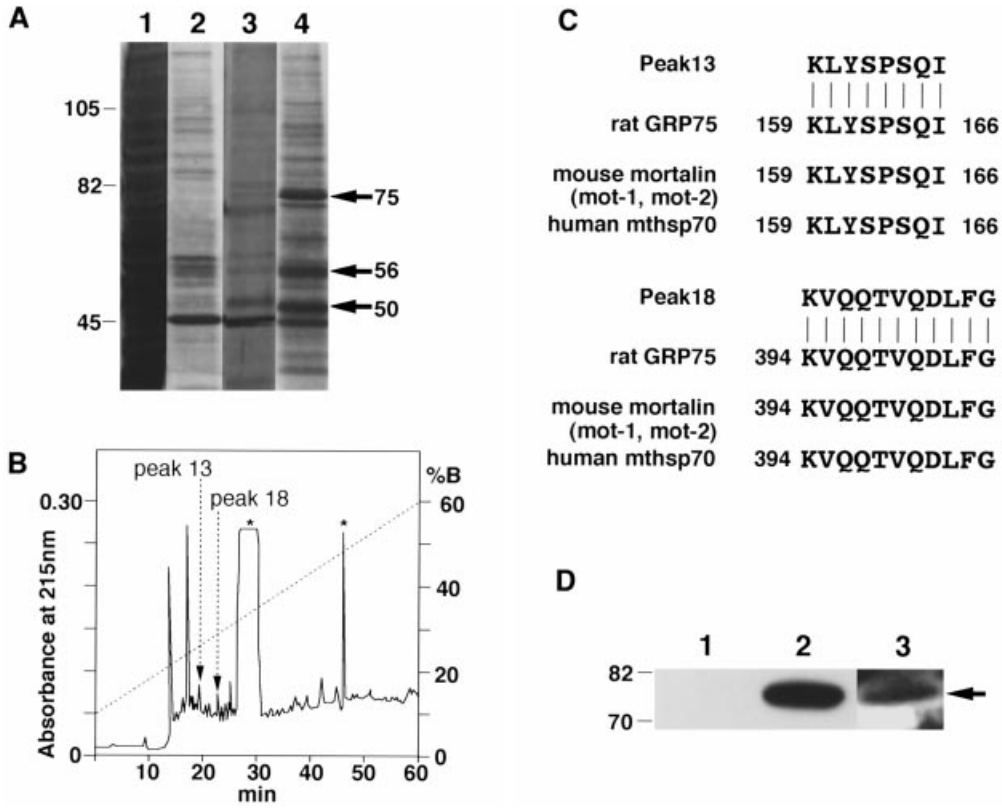

Figure 1 Identification of GRP75/mortalin as an FGF-1-binding protein

(**A**) Affinity adsorption of FGF-1-binding proteins. Proteins extracted from L6 cells (lane 1) were incubated with immobilized FGF-1–Sepharose beads in the absence (lane 4) or presence (lane 3) of excess free FGF-1. As a control, unmodified Sepharose beads were treated similarly (lane 2). The bound proteins were eluted with sample buffer; equivalent aliquots were resolved by SDS/PAGE [10% (w/v) gel] and the proteins were stained with silver. The positions of molecular-mass standards are indicated (in kDa) at the left. Proteins specifically bound to FGF-1–Sepharose, as indicated by the bands in lane 4, are marked by arrows at the right, together with the estimated molecular masses. (**B**) Reverse-phase chromatogram of the p75-derived peptide fragments. The p75 protein band shown in lane 4 of (**A**) was excised from the gel and subjected to in-gel digestion with lys-C endopeptidase. The resultant peptide fragments were extracted and fractionated on a C_{18} reverse-phase HPLC column. A_{215} was monitored. Peaks marked with asterisks were derived from the peptide extraction. (**C**) Sequences of the purified peptides aligned with those of GRP75, mortalin and mthsp70. Two peptides were successfully sequenced: KLYSPSQI and KVQQTVQDLFG [(**B**), peaks 13 and 18 respectively]. A homology search of the protein database yielded complete matches with GRP75. The sequences determined are aligned with the corresponding regions of GRP75, mortalin and mthsp70. (**D**) Anti-mthsp70 and anti-mortalin antibodies recognize p75. FGF-1-bound proteins, prepared as in (**A**), lane 4, were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and separately blotted with anti-mthsp70 (lane 2) and anti-mortalin (lane 3) antibodies; the bands were then detected by enhanced chemiluminescence. As a control, Sepharose-bound proteins prepared as in (**A**), lane 2, were also resolved and blotted with anti-mthsp70 (lanes 1). The positions of molecular-mass standards are indicated (in kDa) at the left; the position of GRP75/mortalin is indicated by the arrow at the right.

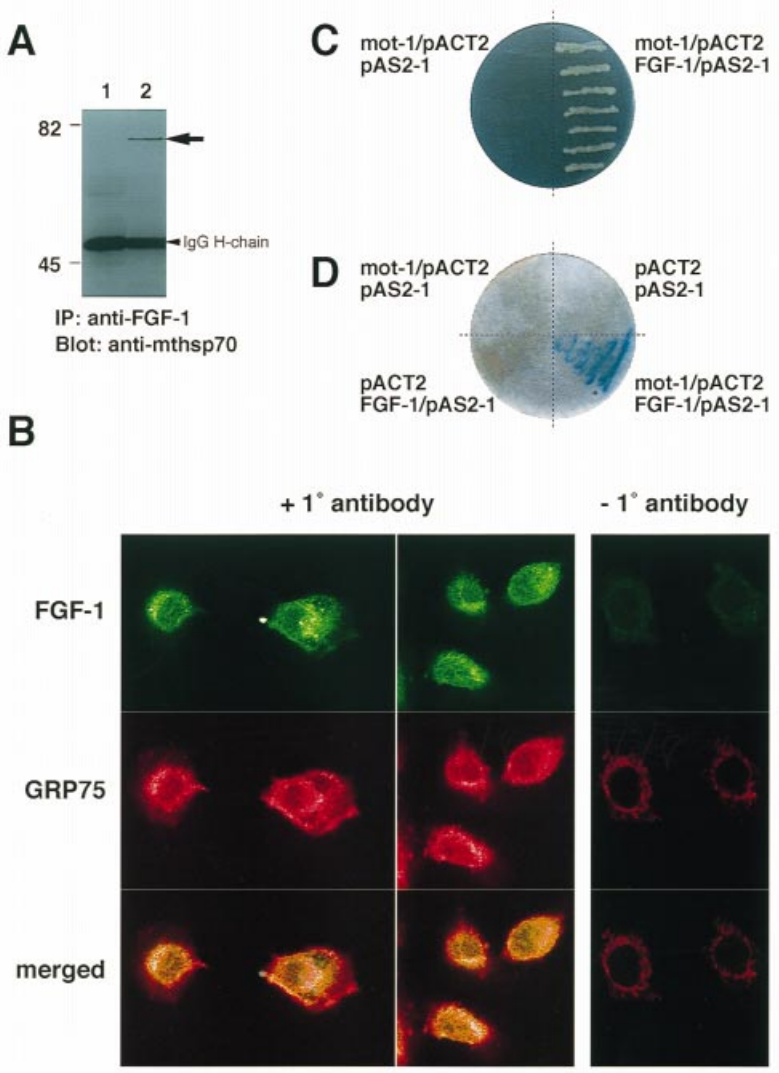

Figure 2 FGF-1 binds to GRP75/mortalin *in vivo*

(**A**) FGF-1 co-precipitates with GRP75/mortalin. Rat-1 fibroblasts endogenously expressing both FGF-1 and mortalin were lysed; the lysates were mixed with polyclonal anti-(FGF-1) antibody and Protein A–Sepharose beads. The beads were then washed; the bound proteins were resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with anti-mthsp70 antibody and the bands were detected with a peroxidase-labelled secondary antibody and enhanced chemiluminescence. The positions of molecular mass standards are indicated (in kDa) at the left. GRP75 is marked by the large arrow at the right; the small arrowhead indicates the heavy chain of rabbit IgG. (**B**) Distribution of FGF-1 and GRP75/mortalin in the Rat-1 cells. Rat-1 fibroblasts cultured on a glass coverslip were fixed and double-stained with monoclonal anti-(FGF-1) antibody and polyclonal anti-mortalin antibody and the corresponding secondary antibodies labelled with FITC and Texas Red respectively. The specimens were observed by fluorescence digital imaging microscopy. Images for each wavelength were recorded individually and are shown as a merged image. FGF-1 and GRP75/mortalin signals are shown by green (top panels) and red (middle panels) respectively; co-localized regions appeared yellow with the program used (bottom panels). For control samples, separate coverslips were similarly stained and observed except that the primary (1°) antibodies were not included (right panels). (**C**) Yeast two-hybrid system: FGF-1 binding to mortalin as indicated by histidine compensation. Yeast cells were co-transfected with an expression vector encoding the GAL4 activation domain fused with mortalin and with an expression vector either encoding the GAL4 DNA-binding domain alone (left side) or its fusion protein with FGF-1 (right side). The transfectants were streaked on a plate lacking histidine, which could be compensated for by the interaction between the products of the two vectors. The colonies were photographed after a period of cell growth. (**D**) Yeast two-hybrid system: FGF-1 binding to mortalin as indicated by β-galactosidase activity. Yeast cells were co-transfected in various combinations with expression vectors encoding either the GAL4 DNA-binding domain alone or its fusion protein with FGF-1, as well as with expression vectors encoding either the GAL4 activation domain alone or its fusion protein with mortalin. The transfectants were cloned and streaked on an agar plate containing histidine, which permitted the growth of all transfectants. A replica was made on a filter, which was then incubated with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal'), a substrate of β-galactosidase that develops a blue colour when acted on by the enzyme.

(Figure 1D, lane 2) and mortalin (lane 3) confirmed that these antibodies also recognized the p75 bound to FGF-1–Sepharose beads; in contrast, the antibody did not recognize control beads (Figure 1D, lane 1).

FGF-1 binds to GRP75/mortalin *in vivo*

Three experimental approaches were used to examine the interactions *in vivo* between FGF-1 and GRP75/mortalin: co-immunoprecipitation, double labelling with specific antibodies and a functional assay with a yeast two-hybrid system. Spontaneously immortalized Rat-1 cells express high levels of both FGF-1 and mortalin. When Rat-1 cell lysates were immunoprecipitated with anti-(FGF-1) antibody, FGF-1 immunocomplexes were found to contain GRP75/mortalin (Figure 2A, lane 2); this was not seen when the same procedure was performed with preimmune IgG (Figure 2A, lane 1). In addition, FGF-1 and mortalin were immunohistochemically localized within Rat-

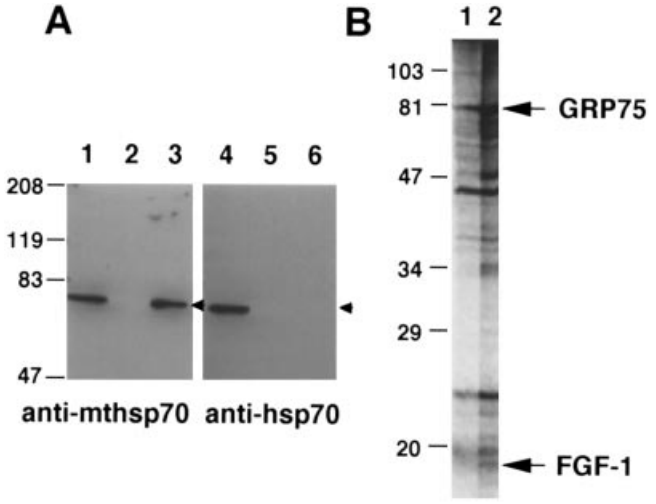

Figure 3 Binding of FGF-1 to GRP75/mortalin is selective

(**A**) FGF-1 binds to GRP75/mortalin, but not to hsp70. Total proteins of Rat-1 cells (lanes 1 and 3) or proteins that bound to Sepharose (lanes 2 and 4) or FGF-1-immobilized Sepharose beads (lanes 3 and 6) were resolved by SDS/PAGE, transferred to a membrane and blotted with anti-mthsp70/GRP75/mortalin antibody (lanes 1–3) or with anti-hsp70/hsc70 antibody (lanes 4–6); the signals were detected by chemiluminescence. The positions of molecular mass standards are indicated (in kDa) at the left; the positions of GRP75/mortalin and hsp70 are indicated by arrowheads at the right. (**B**) GRP75/mortalin co-precipitates with several proteins, including FGF-1. Cells were labelled metabolically with [^{35}S](Cys + Met) for 4 h and washed; proteins were then extracted. The extracted proteins were immunoprecipitated with mouse IgG (lane 1) or with anti-mthsp70/GRP75/mortalin antibody (lane 2), resolved by SDS/PAGE and transferred to a membrane. The signals were detected with the use of a radioimage analyser. The positions of molecular mass standards are indicated (in kDa) at the left; the positions of GRP75/mortalin and FGF-1 are indicated by arrows at the right.

1 cells by analysing digital images depicting the respective distributions of fluorescently labelled anti-(FGF-1) and anti-mortalin antibodies (Figure 2B, top and middle panels respectively). By computationally merging these images, we were able to observe co-localization of FGF-1 with GRP75/mortalin (Figure 2B, bottom panels).

We also investigated the interaction between FGF-1 and GRP75/mortalin with a yeast two-hybrid system (Figures 2C and 2D). Y190 strain reporter yeast was co-transfected with the plasmid FGF-1/pAS2-1, which encodes a fusion protein composed of FGF-1 and the GAL4 DNA-binding domain, and mot-1/pACT2, which encodes a fusion protein composed of mortalin and the GAL4 activation domain. Yeast cells transformed in this way grew on histidine(−) plates (Figure 2C, right side) and exhibited β-galactosidase activity (Figure 2D, lower right quadrant). In contrast, yeast transfected with mot-1/pACT2 and pAS2-1 alone did not grow on histidine(−) plates (Figure 2C, left side) or express β-galactosidase (Figure 2D, upper left quadrant). Similarly, yeast co-transfected with pACT2 and pAS2-1 or with pACT2 and FGF-1/pAS2-1 did not express β-galactosidase (Figure 2D, upper right and lower left quadrants).

Binding of FGF-1 to GRP75/mortalin is selective

To examine whether FGF-1 binds to other members of the hsp70 family of proteins, total proteins of Rat-1 cells were extracted; proteins that bound to FGF-1-immobilized Sepharose beads were analysed for the presence of hsp70. As shown in Figure 3(A), hsp70 was not detected in the FGF-1-bound protein fraction (lane 6), whereas GRP75/mortalin was indeed detected

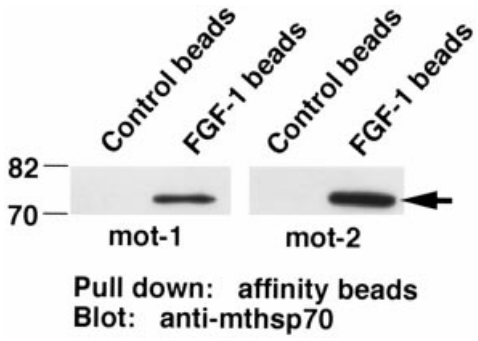

Figure 4 FGF-1 binds mortalin *in vitro*

FGF-1–Sepharose beads were incubated with recombinant mortalin proteins (mot-1 and mot-2) fused with histidine tags, then washed extensively. As a control, unmodified Sepharose beads were treated similarly. The proteins bound to the beads were extracted, resolved by SDS/PAGE [7.5% (w/v) gel], transferred to a membrane and blotted with anti-mthsp70 antibody. Co-precipitation with control Sepharose beads was analysed similarly. The positions of molecular-mass standards are indicated (in kDa) at the left; the position of GRP75/mortalin is indicated by the arrow at the right.

(lane 3). In the unfractionated cell extract, both GRP75 and hsp70 were detected at similar levels (Figure 3A, lanes 1 and 4 respectively). Thus FGF-1 binding to GRP75/mortalin is selective.

To examine further the specificity of FGF-1 binding to GRP75/mortalin, the Rat-1 cell proteins that co-precipitated with GRP75/mortalin were analysed. The cell proteins were metabolically labelled with [^{35}S](Cys + Met) and extracted; those that co-precipitated with GRP75/mortalin were analysed (Figure 3B). It was shown that FGF-1 was one of several proteins that co-precipitated with GRP75/mortalin (Figure 3B, lane 2). This observation agrees with the earlier finding of mortalin-binding proteins [24]. Therefore FGF-1 is one of the several GRP75/mortalin-binding proteins in Rat-1 cells.

FGF-1 can bind directly to mortalin

Our findings confirmed that FGF-1 and GRP75/mortalin interact *in vivo*. To determine whether FGF-1 binds directly to mortalin, His-tagged recombinant mouse mortalins (mot-1 and mot-2) were prepared and mixed with FGF-1–Sepharose or control beads. After the beads had been extensively washed, the remaining bound proteins were analysed for mortalin. We observed that mortalin proteins were bound to the FGF-1 fractions but not to the control fractions (Figure 4), confirming a direct physical interaction between FGF-1 and mortalin *in vitro*.

DISCUSSION

The results presented show that a cell-associated 75 kDa protein binds in a specific manner to FGF-1 immobilized on Sepharose beads. The protein was identified as GRP75/mortalin/PBP74/mthsp70 (hereafter termed GRP75/mortalin). We found that FGF-1 bound directly to GRP75/mortalin *in vitro*; the two proteins formed a complex *in vivo*, and they partly co-localized within expressing cells.

The various names applied to this single protein (GRP75 in rats [25], mortalin and PBP74 in mice [22,29] and mthsp70 in humans [26]) describe the identified functions of the protein in

different experimental systems. It is a member of the hsp70 family (mitochondrial Hsp75) and is known to be involved in pathways regulating glucose responses (GRP75), cell mortality (mortalin) and antigen processing (PBP74). Many hsp70 family members are involved in the folding and subcellular trafficking of proteins [27]. The mitochondrial localization of GRP75 and mthsp70 [25,26], the cytoplasmic vesicle localization of PBP 74 and the mortality-stage-dependent differential localization of mortalin strongly suggest that the specific functions of the protein are closely related to its subcellular localization [24].

The primary structure of FGF-1 contains no known signalling motif, but it does contain a cytoplasmic retention domain [17], which is consistent with the notion that FGF-1 functions as a cytoplasmic regulatory protein. The present results suggest that intracellular FGF-1 interacts with other cellular proteins that, in turn, control the subcellular trafficking of FGF-1 and/or transduce FGF-1 signal(s). It is therefore interesting that FGF-1 interacts with GRP75/mortalin, because hsps are thought to be involved in the regulation of signal transduction; for instance, they affect signalling indirectly by targeting pp60 Src and Ras to the plasma membrane [13,28,30]. In addition, the distribution of mortalin is highly dependent on cellular senescence [24], which affects the cells' reactivity to proliferative stimuli. Therefore the interaction between GRP75/mortalin and FGF-1 might have a role in regulating the reactivity of cells to proliferative signals; however, this possibility remains to be examined further.

The intracellular localization of FGF-1 and its association with other intracellular proteins is not exceptional among FGF family members. The primary structures of both FGF-2 (or basic FGF) and FGF-3 (or Int-2) contain the NLS; consequently they are both localized in the nucleus [31–33]. Moreover, the nuclear localization of FGF-3 is ensured by its binding to karyopherin α, which associates with the NLS and facilitates translocation through the nuclear pore complex [33,34]. In addition, FGF-2 binds to casein kinase II *in vitro* [35], although the biological significance of this binding is not yet clear. In this context, the interaction between FGF-1 and GRP75/mortalin might be involved in the cell-cycle-associated subcellular trafficking of FGF-1 that has been observed previously [14]. GRP75/mortalin might also have a role in FGF-1 export from the cells, as has been reported to occur with heat-shock stress [18,19].

We showed that FGF-1 and GRP75/mortalin co-localize in growing Rat-1 cells (Figure 2B). Although the direct interaction between FGF-1 and GRP75/mortalin *in vitro* suggests that post-translational modification is not necessary for their binding, further investigation will be required to assess this issue further because the results of preliminary experiments suggest that distribution of these proteins is regulated in some way (E. Mizukoshi, unpublished work). While this manuscript was in preparation, another intracellular protein, FIBP, which bound FGF-1, was identified [36]. The other proteins, ie. Synaptotagmin-1 and S100A13, were also shown to aggregate with FGF-1 intracellularly [19,37]. Characterization of all of the intracellular FGF-1-binding proteins should enable us to define the functional roles of intracellular FGF-1 as well as to understand the underlying mechanisms involved.

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REFERENCES

1 Baird, A. and Klagsbrun, M. (1991) Cancer Cells **3**, 239–243
2 Emoto, H., Tagashira, S., Mattei, M. G., Yamasaki, M., Hashimoto, G., Katsumata, T., Negoro, T., Nakatsuka, M., Birnbaum, D., Coulier, F. and Itoh, N. (1997) J. Biol. Chem. **272**, 23191–23194
3 Smallwood, P. M., Munoz-Sanjuan, I., Tong, P., Macke, J. P., Hendry, S. H., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 9850–9857
4 Mason, I. J. (1994) Cell **78**, 547–552
5 Jaye, M., Schlessinger, J. and Dionne, C. A. (1992) Biochim. Biophys. Acta **1135**, 185–199
6 Imamura, T., Tokita, Y. and Mitsui, Y. (1988) Biochem. Biophys. Res. Commun. **155**, 583–590
7 Klagsbrun, M. and Baird, A. (1991) Cell **67**, 229–231
8 Wiedlocha, A., Falnes, P. O., Madshus, I. H., Sandvig, K. and Olsnes, S. (1994) Cell **76**, 1039–1051
9 Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J. A., Hla, T. and Maciag, T. (1990) Science **249**, 1567–1570
10 Imamura, T., Tokita, Y. and Mitsui, Y. (1992) J. Biol. Chem. **267**, 5676–5679
11 Komi, A., Suzuki, M. and Imamura, T. (1998) Exp. Cell Res. **243**, 408–414
12 Lin, Y. Z., Yao, S. Y. and Hawiger, J. (1996) J. Biol. Chem. **271**, 5305–5308
13 Wartmann, M. and Davis, R. J. (1994) J. Biol. Chem. **269**, 6695–6701
14 Imamura, T., Oka, S., Tanahashi, T. and Okita, Y. (1994) Exp. Cell Res. **215**, 363–732
15 Zhan, X., Hu, X., Friedman, S. and Maciag, T. (1992) Biochem. Biophys. Res. Commun. **188**, 982–991
16 Wiedlocha, A., Falnes, P. O., Rapak, A., Munoz, R., Klingenberg, O. and Olsnes, S. (1996) Mol. Cell. Biol. **16**, 270–280
17 Shi, J., Friedman, S. and Maciag, T. (1997) J. Biol. Chem. **272**, 1142–1147
18 Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R. and Maciag, T. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10691–10695
19 Tarantini, F., LaVallee, T., Jackson, A., Gamble, S., Carreira, C. M., Garfinkel, S., Burgess, W. H. and Maciag, T. (1998) J. Biol. Chem. **273**, 22209–22216
20 Shin, J. T., Opalenik, S. R., Wehby, J. N., Mahesh, V. K., Jackson, A., Tarantini, F., Maciag, T. and Thompson, J. A. (1996) Biochim. Biophys. Acta **1312**, 27–38
21 Jeno, P., Mini, T., Moes, S., Hintermann, E. and Horst, M. (1995) Anal. Biochem. **224**, 75–82
22 Wadhwa, R., Kaul, S. C., Ikawa, Y. and Sugimoto, Y. (1993) J. Biol. Chem. **268**, 6615–6621
23 Fay, F. S., Carrington, W. and Fogarty, K. E. (1989) J. Microsc. **153**, 133–149
24 Wadhwa, R., Kaul, S. C., Sugimoto, Y. and Mitsui, Y. (1993) J. Biol. Chem. **268**, 22239–22242
25 Massa, S. M., Longo, F. M., Zuo, J., Wang, S., Chen, J. and Sharp, F. R. (1995) J. Neurosci. Res. **40**, 807–819
26 Bhattacharyya, T., Karnezis, A. N., Murphy, S. P., Hoang, T., Freeman, B. C., Phillips, B. and Morimoto, R. I. (1995) J. Biol. Chem. **270**, 1705–1710
27 Martin, J. and Hartl, F. U. (1997) Curr. Opin. Struct. Biol. **7**, 41–52
28 Rutherford, S. L. and Zuker, C. S. (1994) Cell **79**, 1129–1132
29 Domanico, S. Z., DeNagel, D. C., Dahlseid, J. N., Green, J. M. and Pierce, S. K. (1993) Mol. Cell. Biol. **13**, 3598–3610
30 Kimura, Y., Yahara, I. and Lindquist, S. (1995) Science **268**, 1362–1365
31 Quarto, N., Finger, F. P. and Rifkin, D. B. (1991) J. Cell. Physiol. **147**, 311–318
32 Kiefer, P., Acland, P., Pappin, D., Peters, G. and Dickson, C. (1994) EMBO J. **13**, 4126–4136
33 Antoine, M., Reimers, K., Dickson, C. and Kiefer, P. (1997) J. Biol. Chem. **272**, 29475–29481
34 Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E. and Laskey, R. A. (1995) Nature (London) **377**, 246–248
35 Bonnet, H., Filhol, O., Truchet, I., Brethenou, P., Cochet, C., Amalric, F. and Bouche, G. (1996) J. Biol. Chem. **271**, 24781–24787
36 Kolpakova, E., Wiedlocha, A., Stenmark, H., Klingenberg, O., Falnes, P. O. and Olsnes, S. (1998) Biochem. J. **336**, 213–222
37 Carreira, M. C., LaValee, T. M., Tarantini, F., Jackson, A., Lathrop, J. T., Hampton, B., Burgess, W. and Maciag, T. (1998) J. Biol. Chem. **273**, 22224–22231

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