Identification of Ribosome-binding Protein p34 as an Intracellular Protein That Binds Acidic Fibroblast Growth Factor*

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With the aim of identifying new intracellular binding partners for acidic fibroblast growth factor (aFGF), proteins from U2OS human osteosarcoma cells were adsorbed to immobilized aFGF. One of the adsorbed proteins is a member of the leucine-rich repeat protein family termed ribosome-binding protein p34 (p34). This protein has previously been localized to endoplasmic reticulum membranes and is thought to span the membrane with the N terminus on the cytosolic side. Confocal microscopy of cells transfected with Myc-p34 confirmed the endoplasmic reticulum localization, and Northern blotting determined p34 mRNA to be present in a multitude of different tissues. Cross-linking experiments indicated that the protein is present in the cell as a dimer. In vitro translated p34 was found to interact with maltose-binding protein-aFGF through its cytosolic coiled-coil domain. The interaction between aFGF and p34 was further characterized by surface plasmon resonance, giving a $K_D$ of 1.4 ± 0.3 μM. Even though p34 interacted with mitogenic aFGF, it bound poorly to the non-mitogenic aFGF(K132E) mutant, indicating a possible involvement of p34 in intracellular signaling by aFGF.

Acidic fibroblast growth factor (aFGF) belongs to the large family of FGF growth factors. It is involved in cellular processes such as stimulation of DNA synthesis and cell proliferation as well as differentiation and cell migration (1–5). In vivo, aFGF has been shown to play a role in mesoderm induction; angiogenesis; wound healing; and development of the nervous, muscular, and epithelial systems (6, 7). During the last decade, a number of proteins have been identified that interact with aFGF. Some of these proteins are known to be involved in the signal transduction pathway of aFGF, such as the transcription factor-BB (22), the nuclear protein FGF-2-interacting factor (23), and the calcium-binding protein S100A13 (18–20). aFGF has also been shown to interact with both the calcium-binding protein synaptotagmin-1 and the calcium-binding protein S100A13 (18–20). aFGF has been shown to interact with both the α- and β-subunits of protein kinase CK2. Furthermore, bFGF was reported to associate with platelet-derived growth factor-BB (22), the nuclear protein FGF-2-interacting factor (23), and the ribosomal proteins L6 and s19 (24, 25).

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1 The abbreviations used are: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; MAPK, mitogen-activated protein kinase; FIBP, aFGF intracellular binding protein; FGF-BP1, FGF-binding protein-1; FBS, phosphate-buffered saline; MBP, maltose-binding protein; DSS, disuccinimidyl suberate; GST, glutathione S-transferase; GFP, green fluorescent protein; DTT, dithiothreitol; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ER, endoplasmic reticulum.

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intracellular trafficking and functioning of aFGF remains largely unexplained. In an attempt to elucidate the intracellular role of aFGF, we precipitated proteins that bind to aFGF. We identified by precipitation and mass spectrometry two new proteins that bind to aFGF. One was protein kinase CK2, a constitutively active serine/threonine kinase (26–28) previously found to interact with bFGF (21, 29). The other one was a protein with an apparent molecular mass of 35 kDa. In this work, we report the identification of this aFGF-interacting protein as ribosome-binding protein p34.

p34 is located in the endoplasmic reticulum (30). It is a protein that contains a leucine-rich repeat domain and a coiled-coil domain (both presumably located in the cytosol) as well as a transmembrane domain close to the C-terminal tail (30). We found that although p34 binds to mitogenic aFGF, it does not bind to the non-mitogenic K132E mutant.

**EXPERIMENTAL PROCEDURES**

**Materials and Buffers**—Phosphate-buffered saline (PBS) contained 140 mM NaCl and 10 mM Na2HPO4 (pH 7.2); lysis buffer contained 100 mM NaCl, 10 mM Na2HPO4 (pH 7.2), 1% Triton X-100, and 1 mM EDTA. Protein A-Sepharose CL-4B, CNBr-activated Sepharose, glutathione-Sepharose, heparin-Sepharose, [35S]methionine, and [32P]dCTP were from Amersham Biosciences (Uppsala, Sweden). Restriction endonucleases and amyllose resin were from New England Biolabs Inc. (Beverly, MA). The Dynabeads mRNA DIRECT kit was from Dynal (Oslo, Norway). Coomasie Brilliant Blue G and reduced glutathione were from Sigma. Anti-c-Myc antibody 9E10 was from American Type Culture Collection (Manassas, VA). Anti-calpectinin antibody was from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada). Anti-MBP-FIPB antibody was obtained from Dr. Elena Kolpakova (Institute for Cancer Research, Oslo). The secondary antibodies (horseradish peroxidase-conjugated IgGs, lissamine rhodamine-labeled anti-mouse IgG, and fluorescein isothiocyanate-labeled anti-rabbit IgG) were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). SuperSignal chemiluminescent substrate and diuccimimidyl suberate (DSS) were from Pierce. PuGENE 6 reagent and Complete protease inhibitor mixture were from Roche Molecular Biochemicals. RNAsin, T7 RNA polymerase, nucleate-treated rabbit reticulocyte lysate, and canine pancreas alkaline phosphatase and amylose resin were from New England Biolabs Inc. (Beverly, MA). Anti-MBP-FIPB antibody was obtained from Dr. Elona Kolpakova (Institute for Cancer Research, Oslo). 

**Cells and Transfections**—U2OS and COS-1 cells were propagated in Dulbecco’s modified essential medium with 10% (v/v) fetal calf serum in a 5% CO2 atmosphere at 37 °C. Transient expression of the p34 protein or COS-7 cells transfected with pGEX-6P-1-p34 was determined by trypsinization and washing with PBS. The proteins were extracted with 5% trichloroacetic acid on ice for 1 h, and the pellet was extracted three times with ether. The proteins were analyzed by SDS-PAGE (12% (w/v) gel), followed by staining with Coomassie Brilliant Blue G, and the dried gel was subjected to autoradiography. The bands were analyzed with a laser densitometer and subjected to in-gel trypsin treatment, followed by either MALDI mass spectrometry alone or MALDI mass spectrometry and internal sequencing. The protein sequence data were obtained at the Rockefeller University Protein/DNA Technology Center (New York, NY) (34, 35).

**Preparation of Sepharose Beads Containing MBP Fusion Proteins**—For precipitation purposes, MBP fusion proteins were bound to either CNBr-activated Sepharose or protein A-Sepharose. Binding to protein A-Sepharose was via an antibody against MBP-FIPB, whereas the MBP fusion proteins were coupled to CNBr-activated Sepharose by incubating 1 mg (0.2–2 mg) of protein solution in PBS with 0.5 ml of prewashed CNBr-activated Sepharose. The reaction was quenched by incubation for another hour with 100 mM glycine before washing repeatedly with high salt and low pH buffers.

**Affinity Adsorption and Purification of Proteins That Bind to aFGF**—Subconfluent U2OS cells were labeled overnight with [35S]methionine/cysteine, washed with PBS, and lysed on ice for 20 min in lysis buffer (100 mM NaCl, 10 mM Na2HPO4 (pH 7.2), 1% Triton X-100, and 1 mM EDTA) with 10 mM DTT and Complete protease inhibitor mixture. The supernatants were centrifuged at 20,800 g for 3 min at 4 °C, and the supernatant was incubated for 2 h at 4 °C with CNBr-activated Sepharose without additional bound protein. The precipitation mixture was centrifuged at 3020 g for 5 min at 4 °C, and the supernatant was incubated for another 2 h at 4 °C with Sepharose-bound MBP-interferon-γ (control). After another centrifugation, the supernatant was incubated with Sepharose-bound MBP-aFGF for 2.5 h at 4 °C. The beads were then washed four times with a 1:1 mixture of lysis buffer and PBS, and the bound proteins were eluted with 2 mM NaCl in PBS on ice for 15 min. Proteins were precipitated with 5% trichloroacetic acid on ice for 1 h, and the pellet was extracted three times with ether. The proteins were analyzed by SDS-PAGE (12% (w/v) gel), followed by staining with Coomassie Brilliant Blue G, and the dried gel was subjected to autoradiography. Dried bands were excised from the gel and subjected to in-gel trypsin treatment, followed by either MALDI mass spectrometry alone or MALDI mass spectrometry and internal sequencing. The protein sequence data were obtained at the Rockefeller University Protein/DNA Technology Center (New York, NY) (34, 35).

**Coprecipitation and Western Blotting**—Transiently transfected COS-1 cells were washed with PBS and lysed on ice in lysis buffer containing 10 mM DTT and Complete protease inhibitor mixture. The lystate was centrifuged at 20,800 g for 3 min at 4 °C. The supernatant was diluted 1:1 with PBS and incubated with Sepharose-bound MBP fusion protein for 1 h at 4 °C. Precipitates were collected by centrifugation and washed three times with a 1:1 mixture of PBS and lysis buffer before SDS sample buffer was added to elute the proteins. The samples were subsequently subjected to SDS-PAGE, followed by trans-
fer to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk powder in washing buffer (PBS with 0.1% Tween 20) and incubated with mouse anti-c-Myc antibody 9E10, and proteins were visualized after incubation with a horseradish peroxidase-conjugated secondary antibody and SuperSignal chemiluminescence substrate.

In Vitro Transcription and Translation—[35S]Methionine-labeled p34 and its domains as well as CK2α and CK2β were produced in a rabbit reticulocyte lysate system as described previously (2). The template vectors were pcDNA3-Myc-p34, pRe/CMV-HA-CK2α, and pcDNA3-CK2β, respectively. In short, the plasmid was linearized downstream of the cloning sequence and transcribed for 60 min in a 20-μl reaction mixture using T7 RNA polymerase. The mRNA was precipitated with ethanol, dissolved in 10 μl of H2O containing 10 mM DTT and 0.2 units/ml RNasin, and subsequently translated for 60 min in a nuclease-treated rabbit reticulocyte lysate in the presence of [35S]methionine. The translation mixture was dialyzed against dialysis buffer (20 mM Hepes (pH 7.0), 140 mM NaCl, and 20 mM CaCl2) to remove free [35S]methionine.

In Vitro Binding Assay with Radioactively Labeled Proteins—Fifteen μg of MBP fusion protein bound to protein A-Sepharose beads was added together with 1–20 μl of in vitro translated p34, one of the p34 domain proteins, or hemagglutinin-tagged CK2α or CK2β to a 1:1 mixture of lysis buffer and PBS with 5 mM DTT. The mixture was incubated for 90 min at 4°C and washed three times with the same buffer or with the same buffer with additional NaCl. The bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE, followed by fluorography. In the competition experiments, binding was performed in the presence of the indicated amounts of unlabeled recombinant protein.

Surface Plasmon Resonance—The equilibrium dissociation constant (K_d) for the binding between aFGF and p34 was determined using a BIAcore X (BIAcore AB, Uppsala) at 25°C. GST-p34 was coupled to a CM5 sensor chip (BIAcore) using the GST kit for fusion capture (BIAcore). Anti-GST antibody was covalently linked to the carboxylated dextran matrix of the CM5 sensor chip according to the manual (GST kit for fusion capture). Thirty μl of GST-p34 (5 μg/ml) was then loaded onto the sensor chip by injection at a flow rate of 5 μl/min. The reference cell was coated similarly with recombinant GST. Injections of recombinant aFGF in containing 10 μM Hepes (pH 7.3), 0.15 M NaCl, 3 mM EDTA, and 0.25 mg/ml carboxymethyl-dextran were carried out at a flow rate of 30 μl/min, and sensorgrams were recorded. The surface was regenerated between each measurement with 10 mM glycine (pH 2.8), and the sensorgrams were analyzed using the BIAEvaluation Version 3.0 software. The means ± S.D. were calculated based on four experiments.

Northern Blot Hybridization—The human multiple-tissue Northern blot containing 1 μg of poly(A)^+ RNA/ lane was probed with a [32P]dCTP-labeled, random-primed DNA probe using the 560-bp 5'-terminal fragment of p34, the 300-bp 5'-terminal fragment of aFGF, or a probe for human β-actin. The blot was hybridized overnight in ExpressHyb hybridization solution and washed for 10 min at room temperature with 2× SSC (150 mM NaCl and 15 mM Na3 citrate (pH 7.0)) and 0.05% SDS, twice for 10 min at 50°C with 0.1× SSC and 0.1% SDS, and finally for 1 h at 50°C with 0.5× SSC and 0.2% SDS. Membranes were exposed using a phosphorimaging screen and scanned. After hybridization of the membrane with a probe, the membrane was stripped, rehybridized with an aFGF probe, and finally stripped and hybridized with a probe for human β-actin.

Immunofluorescence Microscopy—COS-1 cells were seeded on sterile coverslips and transiently transfected with pcDNA3-Myc-p34 alone or in combination with pEGFP-aFGF using FuGENE 6 transfection agent. Twenty-four h after transfection, the cells were washed three times with PBS and fixed in 3% paraformaldehyde in PBS for 15 min at room temperature. The cells were washed with PBS, and autofluorescence was quenched by incubation in 50 mM NH4Cl in PBS for 10 min at room temperature. After another wash, the cells were permeabilized with 0.5% Triton X-100 in PBS for 4 min at room temperature, washed, and incubated for 20 min at room temperature with the appropriate primary antibody (anti-calreticulin and/or anti-c-Myc) diluted in PBS, 0.1% Tween 20, and 5% nonfat dry milk powder. The cells were washed and incubated for 20 min at room temperature with the secondary antibody and then washed a final time and mounted in Mowiol. Immunofluorescence images were taken using a Leica confocal microscope and processed using Adobe Photoshop Version 5.0.

Production and Affinity Purification of Antibodies against p34—Antibodies against MBP-p34 were raised in rabbits and purified by affinity chromatography on an Affi-Gel-10 column with covalently bound MBP-p34. The antibodies were eluted with 100 mM glycine (pH 2.8), and the pH was immediately neutralized with 3 M Tris-HCl (pH 8.0).

Cross-linking—U2OS cells grown in Dulbecco's modified essential medium were either permeabilized with digitonin (40 μg/ml) or left untreated for 10 min at room temperature and then washed once with PBS. DSS was added to PBS to a final concentration of 2 mM, and the cells were kept on ice for 2 h in PBS with or without DSS. The reaction was quenched with 20 mM Tris, and the cells were kept on ice for an additional 15 min. The cells were then washed three times with PBS, scraped off, and centrifuged (20,000 × g, 3 min, 4°C), and the pellet was resuspended in SDS sample buffer. The samples were analyzed by SDS-PAGE, followed by Western blotting with an antibody against MBP-p34.

RESULTS

Identification of p34 as a Protein That Binds to aFGF—In a screen for proteins of the U2OS human osteosarcoma cell line that bind to MBP-aFGF, a promising candidate was a protein with an apparent molecular mass of 35 kDa (Fig. 1). U2OS cells were chosen because they are of human origin, which would simplify the identification process, and because they were used in our previous work (2, 8, 36). We chose to work with MBP fusion proteins because the MBP fusion (as opposed to the GST fusion) with aFGF was as potent as wild-type aFGF in binding to and activating FGF receptors (data not shown).

In addition to the 35-kDa band, specific bands with molecular masses of ~28 (later identified as the regulatory subunit of protein kinase CK2) and 17 kDa could be seen on the gels. Also some minor, but apparently specific bands with molecular masses of 29, 40, and 72 kDa could be seen in most of the experiments (data not shown). These bands could represent the catalytic subunit of protein kinase CK2 (p43), FGF-BP1 (p29), FIBP (p40), and mortaline (p72). Both FIBP and mortaline have been previously shown to bind to aFGF (15, 16), whereas FGF-BP1 has been found to bind to both aFGF and bFGF (17). The data on the interaction of aFGF with protein kinase CK2 will be published elsewhere.2

The protein was analyzed by mass spectrometry at the Rockefeller University Protein/DNA Technology Center (34, 35). No match was found in the MS data bases despite a good mass spectrum by MALDI-TOF-MS. The failure to come up
with a match most likely reflects the fact that the protein had not been previously identified. The protein was therefore purified by high performance liquid chromatography and subjected to N-terminal sequencing. The sequence NKLQQLPADFGR was identified; and by performing a FASTA search, a perfect match with the rat protein ribosome-binding protein p34 (PubMed accession number GI 480379) was obtained. Also, 11 of the detected masses found by MALDI-TOF-MS matched a hypothetical digest of this protein. The hypothetical digest was performed with the program MS-Digest. A search for the human homolog in the protein data bases yielded no results; but by performing a BLAST search with the DNA sequence of rat p34, a match with the DNA sequence of the cDNA FLJ21675, clone COL09090 (Pubmed accession number AK025328), was detected. By carrying out a conceptual translation of the DNA sequence, a high match between the human and rat proteins was observed (95% identity). By using a maximum of two missed cleavages in each fragment, the hypothetical digest came up with 70 possible masses both for the human and rat forms of the protein in the mass range given in the MALDI-TOF-MS identification. However, not all possible masses are likely to be produced by a real digest, and not all fragments are possible to detect by MS. Eleven of the detected masses found by MALDI-TOF-MS also matched a hypothetical digest of the human p34 protein. These matches confirmed that p34 was the aFGF interaction partner in the screening experiments. The sequenced fragment could also be identified in the hypothetical digest of human p34. It is unlikely that alternative RNA splicing could produce a p34 protein that has a higher similarity to the masses obtained by MS because both the protein on which the MS was performed and the one that was subjected to a hypothetical digest is the 35-kDa form. The human DNA sequence was used to design primers to obtain the human p34 cDNA from a U2OS cDNA library.

The p34 protein has previously been reported to bind to ribosomes and to be localized to the rough ER (30). To test whether aFGF binds to p34, we transfected COS-1 cells with plasmid pcDNA3-Myc-p34 and incubated the cell lysate with MBP-aFGF, with the MBP-aFGF(K132E) or MBP-aFGF(K132R) mutant, or with the MBP-interferon-γ control, all immobilized on protein A-Sepharose beads. The bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-c-Myc antibody. p34 bound to aFGF and to the aFGF(K132R) mutant, but not to the non-mitogenic aFGF(K132E) mutant or to interferon-γ (Fig. 2).

Evidence That aFGF Binds to the Coiled-coil Domain of p34—As described by Ohsumi et al. (30), the p34 protein can be divided into four different domains. We analyzed the human homolog of the p34 protein using the SMART program, which predicts known protein modules (37, 38). A schematic diagram of the protein is given in Fig. 3A. The protein contains an N-terminal leucine-rich repeat domain with four repeats of a conserved 23-amino acid stretch, a coiled-coil domain, a transmembrane domain, and a C-terminal tail. The C-terminal tail is believed to be localized inside the ER lumen.

In an attempt to identify the region of the protein involved in binding to aFGF, we made constructs containing either one or two of the domains alone and translated them in vitro in the presence of [35S]methionine. As shown in Fig. 3B (upper panel, lane 4), amino acids 141–235 were sufficient for binding to aFGF. This part of the protein contains the coiled-coil region as well as 7 amino acids on the N-terminal side and 19 additional C-terminal amino acids. By exposing the gel shown in Fig. 3B for a longer time, one can see that also the construct containing the first 235 amino acids, which include both the leucine-rich repeat domain and the coiled-coil domain, bound aFGF (data not shown). The finding that the construct containing both the

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3 Available at prospector.ucsf.edu/ucsfhtml4.0u/msdigest.htm.
leucine-rich repeat domain and the coiled-coil domain bound less strongly to aFGF than the construct containing the coiled-coil domain alone could be due to an inhibitory effect of the leucine-rich repeat domain or a failure to adopt the native conformation when this deletion construct was expressed without the last 72 amino acids. In Fig. 3B (lower panel) is shown the ability of the different domains of p34 to bind to the aFGF(K132E) mutant. Only trace amounts were bound in this case.

By conducting the opposite experiment of immobilizing MBP-p34 on protein A-Sepharose beads, we were also able to pull out in vitro translated [35S]methionine-labeled aFGF (data not shown). It may therefore be concluded that the binding of aFGF occurs with the coiled-coil domain or with the immediately adjacent amino acids.

**Ability of aFGF Mutants to Bind p34**—Klingenberg et al. (14) described a number of aFGF mutants with different mitogenic activities. We tested whether the different mutants would bind p34 with different affinity. Due to the high sequence similarity between aFGF and bFGF, we also tested for binding of bFGF to the p34 protein. MBP fusion proteins of bFGF, aFGF, and the different aFGF mutants were immobilized on protein A-Sepharose beads and incubated with [35S]methionine-labeled p34 or p34 alone. As shown in Fig. 4, p34 also bound to bFGF, but to a lesser extent than to aFGF (compare lanes 1 and 2 with lanes 3 and 4). aFGF and the aFGF(K132E) mutant bound p34 with equal affinity, whereas the S130A, S130E, and S113A mutants bound less well, and binding to the remaining mutants was almost undetectable (Fig. 4). Similar results were obtained when the coiled-coil domain was used instead of the whole p34 protein (Fig. 4).

Because aFGF bound to the highly charged coiled-coil domain of p34 and showed a requirement for a positive charge at immediately adjacent amino acids, it was possible to compete for binding to CK2α and not to aFGF(K132E) mutant, this hinted at a similarity in binding to aFGF among p34, CK2α, CK2β, and FIBP (15) and suggested that they might be able to compete for binding to aFGF. Competition experiments were conducted in which we studied binding of [35S]methionine-labeled p34, CK2α, or CK2β to aFGF in the presence of increasing concentrations of MBP-FIBP or MBP-bFGF. The binding to p34 (Fig. 6A) and CK2α (Fig. 6B) could both be competed out with FIBP, whereas the binding to CK2β could not (Fig. 6C). Instead, the amount of bound CK2β increased slightly with increasing concentrations of FIBP. The binding of aFGF to CK2α could also be competed out with excess bFGF (Fig. 6D). Because FIBP was able to compete out the binding of p34 and CK2α to aFGF and because all four proteins failed to bind to the non-mitogenic aFGF(K132E) mutant, it is likely that these four proteins bind to the same or to overlapping regions in aFGF. On the other hand, although also CK2β showed binding to wild-type aFGF, but not to aFGF(K132E), FIBP failed to compete out the binding of CK2β to aFGF. The binding was instead increased ~2-fold at the highest FIBP concentration. Because there were mainly CK2β, FIBP, and aFGF present in the precipitation mixture, this disfavors the possibility that FIBP inactivates an inhibitor of CK2β that binds to aFGF, but instead favors a possible cooperative interaction between CK2β and FIBP in binding to aFGF. The finding that bFGF could compete for binding to CK2α also indicates that the two growth factors bind to the same site in CK2α.

**Assessment of the Affinity of the Interaction between aFGF and p34**—To further describe the binding between aFGF and p34, we measured the association and dissociation kinetics using surface plasmon resonance. GST-p34 was immobilized on a sensor surface using anti-GST antibodies and sensorgrams were recorded upon injection of different concentrations of aFGF (Fig. 7). The interaction between p34 and aFGF showed characteristics of a Langmuir isotherm with a 1:1 interaction. From the association and dissociation curves, we determined the equilibrium constant (Kd) to be 1.4 ± 0.3 μM.

**Expression of p34 and aFGF in Different Tissues**—To determine the size, the possible existence of different splicing variants, and the tissue distribution of p34 mRNA, we hybridized a human multiple-tissue Northern blot containing 1 μg of poly(A)+ mRNA from different tissues with a p34 probe (Fig. 8A). At least three different splicing variants could be detected in all tissues examined. The largest splicing variant with an apparent size of 3.8 kb was the most abundant one. The smaller splicing variants had apparent sizes of 1.4 and 1.0 kb. The highest amount of p34 mRNA (both of the largest and second
largest splicing variants) was detected in placenta. Somewhat lower amounts of mRNA for p34 were found in skeletal muscle, colon, kidney, liver, and lung. p34 mRNA was clearly present also in brain, heart, thymus, spleen, small intestine, and peripheral blood leukocytes.

The membrane was then stripped and hybridized with a probe against human aFGF. The expression profile detected was in this case somewhat different (Fig. 8B). The main splicing variant of aFGF with an apparent size of 4.2 kb was detected primarily in kidney and brain and in somewhat smaller amounts in heart and skeletal muscle. A larger transcript with an apparent size of 6 kb could also be detected in kidney and skeletal muscle.

After stripping the membrane a second time, it was hybridized with a probe against human β-actin. The similar amounts of mRNA for β-actin found in the different tissues indicate that comparable amounts of total RNA were used in all cases (Fig. 8C).

Cellular Localization of p34—p34 has previously been localized to the rough ER (30), whereas aFGF is present in the cytosol and nucleus (6, 7, 15). Using confocal microscopy, we attempted to determine whether colocalization between the two proteins could be detected in vivo. In transiently transfected COS-1 cells, p34 colocalized extensively with calreticulin, which is a marker for the ER (Fig. 9, upper panels). By doubly transfecting COS-1 cells with GFP-aFGF and with a Myc-tagged version of p34, one could see that although aFGF was mostly present in the cytosol and nucleus and p34 in the ER, some colocalization around the rim of the nucleus could be detected (Fig. 9, lower panels).

Dimers of p34—Because p34 contains a coiled-coil domain, which is a domain found in many proteins forming dimers and higher multimers (39), we tested for the presence of higher complexes of p34. After performing cross-linking experiments on whole cells (Fig. 10), a band with a molecular mass corresponding to a dimer could be seen upon Western blotting. There was clearly much more monomeric than dimeric p34, which could reflect inefficient cross-linking in the whole cells. The amount of dimers observed was the same regardless of prior permeabilization of the cells with digitonin.

DISCUSSION

We have presented evidence that the ribosome-binding protein p34 binds to aFGF and to a series of different aFGF mutants with graded affinity. Although there was good binding to wild-type aFGF, there was almost no binding to the nonmitogenic aFGF(K132E) mutant. There was also a fairly good
The leucine-rich repeat domain is a module that has been found 142 human proteins containing these typical leucine-rich repeats. This domain is found in proteins with diverse cellular functions such as cell adhesion, cellular signaling, and protein translation, and it mediates reversible protein-protein interaction (40). The finding that aFGF binds to the coiled-coil domain of p34 means that the leucine-rich repeat domain is “free” to bind other proteins. One might therefore speculate whether p34 functions as an integrator of different signals, possibly signals necessary for aFGF-induced DNA synthesis.

The coiled-coil domain is highly charged and contains the majority of all arginine, lysine, and glutamic acid residues in the protein. aFGF binding to this part of the protein therefore suggests that also this interaction is electrostatic. The interaction was reduced by washing with 0.3 M NaCl and prevented by 0.5 M NaCl.

Quantification of the equilibrium constant for the binding between aFGF and p34 yielded a constant of 1.4 \( \mu M \). This represents an intermediate strength (possibly transient) binding typical between proteins involved in transitory interactions.

The high degree of similarity found in the binding of p34 and CK2 to aFGF suggested that they would bind to the same amino acids in aFGF. Many similarities were also found in the correlation between binding to p34 and mitogenic potential for the other aFGF mutants tested. Except for the S113A mutant, which has a mitogenic potential similar to that of wild-type aFGF, all the other mutants and their relative mitogenic activities have been described previously (14). The mutants that bound to p34 were the ones that retained mitogenic activity similar to that of wild-type aFGF (S130A and S130E) and the K132R mutant, which was ~3-fold less potent than wild-type aFGF, but retained the positive charge at position 132. The less mitogenic mutants did not bind p34. This correlation indicates a possible role for p34 in the mitogenic signaling of aFGF.

p34 is a protein that contains four different domains: a leucine-rich repeat domain, a coiled-coil domain, a putative transmembrane domain, and a C-terminal tail. aFGF was found to bind to the coiled-coil domain of p34. The reason why we observed less binding to the construct containing both the leucine-rich repeat domain and the coiled-coil domain could be either failure of the construct to adopt the right conformation or partial inhibition of binding by the leucine-rich repeat domain. Because the proteins are made in vitro, where exact quantification is difficult, quantification was done only by comparing the signal from the same amount of protein applied on a gel. There may therefore be some differences in the amount of proteins used in the pull-down assay, which could account for part of the observed differences in binding affinity.

The leucine-rich repeat domain is a module that has been found in a number of different proteins. The prototype member is adenylate cyclase. By searching in the SMART Database, we found 142 human proteins containing these typical leucine-rich repeats. This domain is found in proteins with diverse cellular functions such as cell adhesion, cellular signaling, and protein translation, and it mediates reversible protein-protein interaction (40). The finding that aFGF binds to the coiled-coil domain of p34 means that the leucine-rich repeat domain is “free” to bind other proteins. One might therefore speculate whether p34 functions as an integrator of different signals, possibly signals necessary for aFGF-induced DNA synthesis.

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4 C. S. Skjerpen, J. Wesche, and S. Olsnes, unpublished data.
binding of FIBP to aFGF (15). However, although the binding of p34 to aFGF was, like the binding to CK2α, competed out with increasing concentrations of FIBP, the amount of CK2β that bound to aFGF in the presence of FIBP was the same or even increased. Although these experiments point to similarities in the mode of binding to p34 and CK2α and also in binding of CK2α to both aFGF and bFGF, they suggest differences between p34 and CK2β in their mode of binding to aFGF. The finding that p34 and CK2α most likely bind to the same amino acids in aFGF might implicate p34 in the regulation of aFGF signaling through CK2. Furthermore, because FIBP does not bind to CK2β alone, the mechanism behind the FIBP-induced increase in binding of aFGF to CK2β is not known. Possibly, aFGF undergoes conformational changes after binding to FIBP, thereby exposing the binding site for CK2β. Both FIBP and CK2β are unable to bind to the K132E mutant, which points to the importance of lysine 132 in the binding. However, the competition data indicate that there are probably also amino acids involved in the binding that are not common to FIBP and CK2β.

p34 was detected in similar amounts in all tissues examined. This, together with the fact that homologs of p34 can be found in Mus musculus, Rattus norvegicus, Drosophila melanogaster, and Caenorhabditis elegans, points to a conserved function of the protein. The finding that p34 is present in similar amounts whether or not aFGF is present indicates that it probably has more functions than interaction with aFGF.

Whereas CK2 (like aFGF) is localized to the cytosol and nucleus, p34 is found almost exclusively in the ER. Some colocalization between aFGF and p34 could be detected, but the amount varied from cell to cell.

The confocal microscopy data support the data of Ohsumi et al. (30) showing that p34 is localized to the ER. However, no earlier experiments addressed the possible existence of p34 as a multimer. In the cross-linking experiment, a band with an amino acid sequence involved in the binding that are not common to CK2α and CK2β/H9252, demonstrates that there exist a number of cellular proteins that interact specifically with mitogenic aFGFs and that might be involved in the regulation of aFGF signaling and trafficking to the nucleus.

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