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(Article begins on next page)

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The Extravesicular Domain of Synaptotagmin-1 Is Released with the Latent Fibroblast Growth Factor-1 Homodimer in Response to Heat Shock*

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The heparin-binding fibroblast growth factor (FGF) prototypes lack a classical signal sequence, yet their presence is required in the extracellular compartment for the activation of cell-surface receptor-dependent signaling. Early studies with FGF-1 demonstrated its presence in bovine brain as a novel high molecular weight complex, and subsequent studies identified a second heparin-binding protein that co-purified with FGF-1. Polypeptide sequence analysis revealed that this heparin-binding protein corresponded to the extravesicular domain of bovine synaptotagmin (Syn)-1, a transmembrane component of synaptic vesicles involved in the regulation of organelle traffic. Since FGF-1 is released in response to heat shock as a mitogenically inactive Cys-30 homodimer, we sought to determine whether this heparin-binding protein was involved in the release of FGF-1. We report that a proteolytic fragment of the extravesicular domain of Syn-1 is associated with FGF-1 in the extracellular compartment of FGF-1-transfected NIH 3T3 cells following temperature stress. By using heparin-Sepharose affinity to discriminate between the monomer and homodimer forms of FGF-1 and resolution by conventional and limited denaturant gel shift immunoblot analysis, it was possible to identify FGF-1 and Syn-1 as potential components of a denaturant- and reducing agent-sensitive extracellular complex. It was also possible to demonstrate that the expression of an antisense-Syn-1 gene represses the release of FGF-1 in response to heat shock. These data indicate that FGF-1 may be able to utilize the cytosolic face of conventional exocytotic vesicles to traffic to the inner surface of the plasma membrane where it may gain access to the extracellular compartment as a complex with Syn-1.

Fibroblast growth factor (FGF)[1]-1 (acidic) and FGF-2 (basic) are the prototype members of the FGF gene family, and these proteins have been extensively characterized as potent angiogenic and neurotrophic factors (1). The FGF prototypes lack a conventional signal peptide sequence for secretion through the endoplasmic reticulum-Golgi-mediated pathway, yet their interaction with high affinity tyrosine kinase receptors on the cell surface implies the function of an alternative secretion pathway to mediate their release.

We have previously reported that FGF-1 is actively released from FGF-1-transfected NIH 3T3 cells by a transcription- and translation-dependent mechanism in response to temperature stress (2). Analysis of the effects of brefeldin A on the release of FGF-1 in this system demonstrated the unconventional nature of the FGF-1 release pathway since disruption of endoplasmic reticulum-Golgi communication (3) did not inhibit the appearance of extracellular FGF-1 in response to heat shock (4). In addition, we have also reported the following: (i) FGF-1 is released as a latent homodimer with reduced affinity for immobilized heparin (5); (ii) the FGF-1 homodimer can be activated by treatment with either $(NH_4)_2SO_4$ (2) or reducing agents (4); (iii) FGF-1 homodimer formation utilizes residue Cys-30, and dimer formation is important for FGF-1 release (5); (iv) FGF-1 contains a phosphatidylserine-binding domain between amino acid residues 114 and 137 (5); (v) the release of FGF-1 involves the function of a carboxyl-terminal domain which is not present in FGF-2 (6); and (vi) the FGF-1 secretion pathway does not restrict the release of high molecular weight forms of FGF-1 since an FGF-1:β-galactosidase chimera is released as a structurally intact protein (6). The ability of FGF-1 to interact with phosphatidylserine (5), an important component of the inner leaflet of the plasma membrane (7), implies that this interaction may play a role in its release. Finally, analysis of FGF-1 release using FGF-1:FGF-2 chimeric constructs demonstrated that FGF-1, but not FGF-2, is released in response to temperature stress (6).

Early studies on the purification of FGF-1 from bovine brain tissue demonstrated the presence of high and low molecular weight forms of the protein; the high molecular weight form of FGF-1 represents an acid-sensitive complex (8). During the characterization of brain-derived FGF-1 as a heparin-binding protein, a second polypeptide was identified that co-eluted with FGF-1 from immobilized heparin (8). This protein was isolated, sequenced, and identified as the extravesicular portion of syn-

aptotagmin (Syn)-1, an integral component of synaptic vesicles (9). Because Syn-1 has been implicated in the regulation of exocytotic (9) and endocytotic organelle trafficking (10), we questioned whether Syn-1 may also be involved in the temperature-dependent release of FGF-1. We now report the presence of denaturant- and reducing agent-sensitive forms of FGF-1 and Syn-1 in media conditioned by heat shock from FGF-1-transfected NIH 3T3 cells and have identified a p40 fragment of Syn-1 as a potential component of the extracellular FGF-1 complex.

EXPERIMENTAL PROCEDURES

Cell Culture—Stable NIH 3T3 FGF-1 cell transfectants were obtained as described previously (2) and maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) on human fibronectin (10 μg/cm^2)-coated dishes containing 10% (v/v) bovine calf serum (HyClone), 1× antibiotic/antimycotic (Life Technologies, Inc.), and 400 μg/ml G418 (Life Technologies, Inc.). A confluent monolayer of transfected cells was subjected to heat shock (42 °C for 2 h) in serum-free DMEM, containing 4 units/ml heparin (The Upjohn Co.), as described previously (2). Human umbilical vein endothelial cells were grown as described previously (11).

Processing of Heat Shock Conditioned Media and Conventional and Limited Denaturant Immunoblot Analysis—Conditioned medium was collected after temperature stress (42 °C for 2 h), processed by filtration, and activated with either $(NH_4)_2SO_4$ (2) or 0.1% (w/v) dithiothreitol (DTT, Sigma) (4). The DTT-treated conditioned medium was adsorbed directly to a 1-ml heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech), previously equilibrated with 50 mM Tris, pH 7.4, containing 10 mM EDTA (TEB), and the column was washed with TEB and eluted with TEB containing increasing concentrations of NaCl. The elution fractions were concentrated using a Centricon 10 concentrator (Amicon, Inc.) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as described previously (4). The $(NH_4)_2SO_4$-treated conditioned medium (90% saturation) was recovered by centrifugation (9,000 × g, 40 min), resuspended in TEB, and processed over a 4-ml heparin-Sepharose column previously equilibrated with TEB. The column was washed with TEB and batch-eluted as 10-ml fractions with TEB containing increasing concentrations of NaCl. Each post-heparin elution fraction was divided into two samples to be analyzed by immunoblotting with either the anti-FGF-1 or the anti-Syn-1 antibodies. Each sample was divided again into two fractions of equal volume, concentrated using a Centricon 10 concentrator (Amicon, Inc.) to a final volume of 200 μl. Of the first 200 μl, half was resuspended in an equal volume of SDS-PAGE sample buffer (125 mM Tris, 2% SDS, 10% glycerol, and 0.1% bromphenol blue) containing 2-mercaptoethanol (2-ME) and resolved by 15% (w/v) conventional SDS-PAGE, and half was resuspended in SDS-PAGE sample buffer without 2-ME and resolved by 15% conventional SDS-PAGE. The second 200 μl were also divided in half, resuspended in an equal volume of SDS-PAGE sample buffer with and without 2-ME, and resolved using a limited denaturant electrophoretic gel shift system (15% (w/v) acrylamide), where SDS was present in the sample and running buffers but eliminated from the resolving gel (limited SDS-PAGE).

Immunoblot analysis of the samples resolved by conventional and limited SDS-PAGE was performed as described previously (2). Briefly, after transfer to nitrocellulose filters, the filters were incubated in 24 mM Tris, pH 7.4, containing 136 mM NaCl, 2 mM KCl, and 0.1% (v/v) Tween 20 (TCB) which also contained 5% (w/v) milk for 2 h at 42 °C and were then washed 3 times with TCB. The filters were probed with either a rabbit anti-human FGF-1 antibody (12) or with a rabbit anti-rat Syn-1 antibody, at a concentration of 1 μg/ml, for 1 h at room temperature and then washed 3 times with TCB. The proteins were detected using the ECL detection system (Amersham Pharmacia Biotech) following the manufacturer's instructions.

Purification of Recombinant Syn-1, Preparation of Syn-1 Antibody, and Analysis of the Recombinant Syn-1 Heparin Binding Activity—A rat Syn-1 construct (p65IIs) encoding amino acid residues 96–421 in the expression vector pGEX-KG and two additional constructs, p65–123 (encoding amino acid residues 96–265) and p65–345 (encoding amino acid residues 248–421), as glutathione *S*-transferase fusion proteins were kindly provided by Dr. Richard Scheller (13) and used for the preparation of antisera. The fusion proteins were generated and purified as described (13) except that a 50 mM Tris buffer, pH 8.8, containing 10 mM EDTA, 10 mM glucose, and 10 μg/ml lysozyme was used to lyse

BL21 *Escherichia coli*. The thrombin-cleaved proteins were further purified by adsorption to heparin-Sepharose previously equilibrated in 50 mM phosphate buffer, pH 7.5, and eluted with a 0–1.5 M NaCl gradient in 50 mM phosphate buffer, pH 7.5. Female, 6-month-old, New Zealand White rabbits (Hazelton Research Animals) were injected intradermally with 1 mg of protein suspended in Freund's complete adjuvant (Calbiochem), boosted with 200 μg of protein suspended in incomplete Freud's adjuvant (Calbiochem), and the antibodies purified by incubating the serum with immobilized p65IIs on a Problot polyvinylidene difluoride membrane (Applied Biosystems, Inc.) previously blocked at 42 °C with TCB containing 5% (w/v) bovine serum albumin. The membrane was washed 3 times with 0.05% (v/v) Triton X-100 in 50 mM Tris, pH 7.4, containing 150 mM NaCl, and the antibody was eluted from the membrane with 0.2 M glycine, pH 2.8, and neutralized with 1 M K_2HPO_4, pH 7.4.

For the analytical study of the recombinant Syn-1 heparin-binding affinity, 1 μg of thrombin-cleaved, p65IIs recombinant protein was adsorbed to a 1-ml heparin-Sepharose column, previously equilibrated with TEB. The column was washed with TEB and batch-eluted with 2.5 ml of TEB containing increasing concentrations of NaCl. The post-heparin elution fractions were concentrated by Centricon to a final volume of 50 μl and analyzed by conventional SDS-PAGE, under reducing conditions. The protein was detected by immunoblot analysis with a rabbit anti-rat Syn-1 antibody using the ECL detection system.

RT-PCR Analysis of the Syn-1 Transcript and Preparation of the NIH 3T3 Cell FGF-1 and Antisense-Syn-1 Co-transfectants—Total RNA from FGF-1-transfected NIH 3T3 cells and human umbilical vein endothelial cells (HUVEC) was isolated using the guanidinium isothiocyanate method (14). As described previously (15), cDNA was obtained from 1 μg of total RNA and diluted to a final volume of 500 μl with H_2O. PCR was performed on 5 μl of cDNA in a 10 mM Tris buffer, pH 8.3, containing 1 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 2 units of *Taq* polymerase, and 0.5 μg/each of sense (5′-CCATTGCCACCGTGGGC-CTT-3′) and antisense (5′-TCCAAAACAGTTACCACCAC-3′) oligonucleotide primers designed to recognize the human and the mouse Syn-1 sequences. PCR was performed for 35 cycles as follows: 1 min at 94 °C, 2 min at 54 °C, and 3 min at 72 °C. The PCR products were separated from the oligonucleotide primers, unincorporated nucleotides, and *Taq* polymerase using a Qiagen tip-5 column according to the manufacturer's instructions (Qiagen PCR purification kit, Qiagen Inc.) and cloned into a pCR 2.1 vector (TA cloning kit, Invitrogen) following the manufacturer's instructions. The identity of the PCR fragments was then confirmed by sequencing using the dideoxy sequencing method (Sequenase, U. S. Biochemical Corp.).

For the Syn-1 antisense gene construct, a rat Syn-1 gene into the PCB1 vector (a gift from T. C. Sudhof (16)) was used to obtain the Syn-1 open reading frame that was cloned into a pCR™ II cloning vector (Invitrogen) by PCR method, using the following primers: 5′-GGGAGA-CACGGGATCCTTACTTCTTGACAGCCAG-3′ (sense oligonucleotide, containing a *Bam*HI restriction site) and 5′-GCGACAGGAGCATATG-GTGAGTGCCAGTCATCC-3′ (antisense oligonucleotide, containing a *Nde*I restriction site). A fragment encompassing the AUG initiator codon and the first 640 nucleotides of the rat Syn-1 sequence was obtained from the Syn-1/pCR™ II construct by digestion with the restriction enzyme *Eco*RI, filling with DNA polymerase I large (Klenow) fragment (Boehringer Mannheim) to obtain blunt ends, and a second digestion with *Kpn*I restriction enzyme. The product was purified by agarose gel electrophoresis and ligated (T4 ligase, Life Technologies, Inc.) in an antisense direction into *Hpa*I/*Kpn*I compatible ends of the eukaryotic expression vector p3′SS (LAC switch inducible mammalian expression system, Stratagene). The anti-Syn-1/p3′SS vector was transfected into NIH 3T3 FGF-1 transfectants using the calcium phosphate precipitation method. DMEM, containing 200 μg/ml hygromycin (Boehringer Mannheim) and 20% bovine calf serum, was used to select hygromycin-resistant colonies. Colonies were screened for the expression of the anti-Syn-1 gene by Northern blot analysis using the anti-Syn-1 fragment as probe.

Processing and Analysis of Heat Shock Conditioned Medium from FGF-1 and Anti-Syn-1 Co-transfectants and Growth Assay—The FGF-1 transfectants and the FGF-1 and anti-Syn-1 co-transfectants were subjected to temperature stress as described previously (2). Conditioned media were treated with 0.1% DTT and adsorbed over a 1-ml heparin-Sepharose column previously equilibrated with TEB. The column was washed with TEB and the protein eluted with TEB containing 1.5 M NaCl. The elution fractions were concentrated by Centricon, analyzed by conventional SDS-PAGE under reducing conditions, and immunoblotted with anti-FGF-1 antibody as described previously. The proteins recognized by the antibody were detected with an ^{125}I-protein A probe.
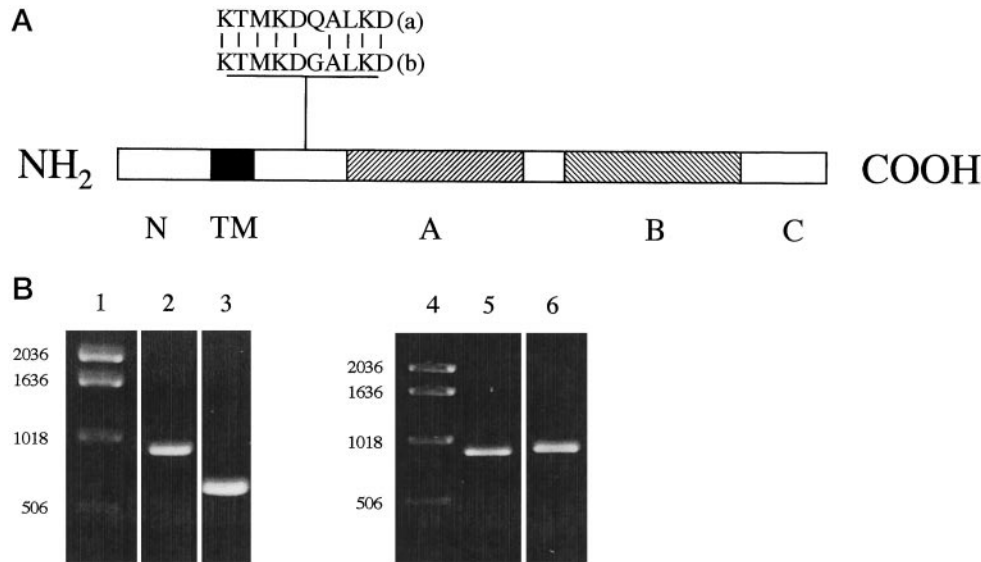
FIG. 1. **The Syn-1 domain structure and the expression of the Syn-1 transcript in non-neuronal cells.** *A,* the domain structure of Syn-1 is represented. *N* represents the Syn-1 NH_2-terminal region (intravesicular oriented); *TM,* the transmembrane region; *A,* the first C2 domain, homologous to the regulatory region of protein kinase C; *B,* the second C2 domain, and *C,* the COOH-terminal region (extravesicularly oriented). The amino-terminal sequence of the p40 fragment that co-purified with FGF-1 from bovine brain preparations (*b*) is aligned with the published sequence of bovine synaptotagmin-1 (*a*). As demonstrated by the alignment, the amino-terminal sequence of the p40 fragment corresponds to the extravesicular portion of synaptotagmin-1, cleaved near its transmembrane region in the extravesicular domain. *B,* total RNA was purified from a confluent monolayer of HUVEC or FGF-1-transfected NIH 3T3 cells. RNA (1 μg) was used for the reverse transcriptase reaction. Sense and antisense primers for the human and the mouse Syn-1 sequences, as described under "Experimental Procedures," amplified a 928-base pair PCR product. Human glyceraldehyde-3-phosphate dehydrogenase or human β-actin-specific primers were also used to amplify the same cDNA as control. The products of amplification were resolved on a 1% agarose gel and stained with ethidium bromide. *Lanes 1* and *4,* molecular weight markers; *lane 2,* Syn-1 PCR product from HUVEC cDNA; *lane 3,* glyceraldehyde-3-phosphate dehydrogenase control; *lane 5,* Syn-1 PCR product from FGF-1 transfectants; *lane 6,* β-actin control.

For the growth assay experiments, FGF-1 transfectants and FGF-1 and anti-Syn-1 co-transfectants were plated on 6-well cell culture dishes at a concentration of 2×10^3 cells/well in DMEM, containing increasing concentrations of bovine calf serum (0–20%). When the effect of FGF-1 on the growth curve was analyzed, 5 ng/ml recombinant FGF-1 and 4 units/ml heparin were added to the culture medium. At day 7 the cells were trypsinized and counted with a Coulter counter.

RESULTS

Characterization of Bovine Brain-derived Synaptotagmin (Syn)-1 as a Heparin-binding Protein Present in FGF-1 Preparations—FGF-1 was purified by heparin-Sepharose affinity from bovine brain as described previously (8). The presence of a second heparin-binding protein within the FGF-1 fraction, which eluted with FGF-1 at 1.0 M NaCl, was detected by reversed phase high pressure liquid chromatography. The co-eluted protein was characterized as a 40-kDa polypeptide by SDS-polyacrylamide gel electrophoresis and subjected to automated Edman degradation. Interestingly, the amino-terminal sequence of the p40 fragment corresponded to the extravesicular domain of synaptotagmin-1 (Syn-1) (9), starting in the α-helical stretch proximal to its transmembrane domain (Fig. 1A). Syn-1 has been described as a neuronal-specific, integral component of synaptic vesicles (9). It is considered to be the calcium sensor of the regulated neurotransmitter secretory pathway, and it displays potential docking properties through its syntaxin-binding domain (17, 18). Interestingly, Syn-1 is also able to interact with the clathrin AP-2 adaptor, participating in the clathrin-coated pit endocytotic pathway (10).

Since Syn-1 expression is considered to be neuron-specific, we examined two non-neuronal cell culture systems for the presence of the Syn-1 transcript. Analysis of human umbilical vein endothelial cells and FGF-1 NIH 3T3 cell transfectants by reverse transcription-polymerase chain reaction (RT-PCR) using human and murine Syn-1 primers demonstrated the presence of the Syn-1 transcript in both cell types (Fig. 1B). Sequence analysis of the human and murine RT-PCR products

confirmed the identity of these fragments as human and murine Syn-1 (data not shown).

A p40 Fragment of Syn-1 and the FGF-1 Monomer Are Present in Heat Shock Conditioned Medium as Heparin-binding Proteins—In order to define further the mechanism utilized by FGF-1 for release, we employed stable FGF-1-transfected NIH 3T3 cells to determine whether the p40 proteolytic fragment of Syn-1 was present in medium conditioned by temperature stress. FGF-1 and Syn-1 immunoblot analysis was performed on heparin-Sepharose fractions of medium conditioned by FGF-1 transfectants during heat shock (42 °C, 2 h). Since FGF-1 is released as a FGF-1 Cys-30 homodimer (4), the conditioned medium was initially treated with 0.1% w/v dithiothreitol (DTT) and resolved by heparin affinity-based chromatography prior to conventional immunoblot analysis under reducing conditions. As shown in Fig. 2A, the FGF-1 monomer was detected in the high NaCl elution fractions (0.6 to 1.5 M NaCl). Because the FGF-1 homodimer has been shown to elute from immobilized heparin near 0.4 M NaCl (19) and FGF-1 was undetectable in the low NaCl elution fractions (Fig. 2A), we suggest that DTT treatment of media conditioned by heat shock was effective in reducing the FGF-1 homodimer to monomer. In addition, Syn-1 immunoblot analysis of the same heparin-Sepharose elution fractions by conventional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions also resolved the presence of a p40 fragment of Syn-1 in the high NaCl elution fractions (0.8 to 1.0 M NaCl) (Fig. 2B). These data suggest that p40 Syn-1 and FGF-1 were both present in DTT-treated heat shock conditioned medium from FGF-1-transfected NIH 3T3 cells and define their respective heparin-Sepharose elution profiles.

Recombinant Rat p40 Syn-1 Exhibits Similar Heparin Binding Affinity to the Protein Derived from NIH 3T3 Cell Medium Conditioned by Heat Shock—Since Syn-1 has been previously described as a heparin-binding protein with very high heparin
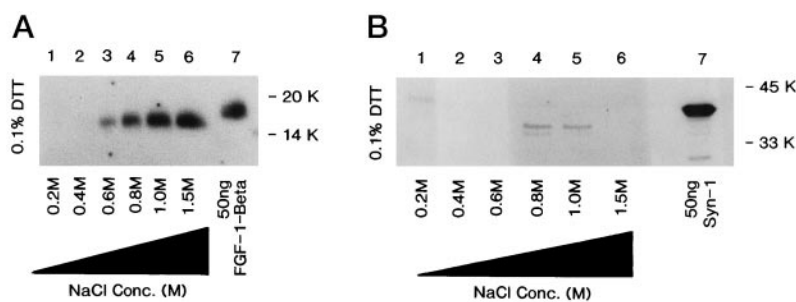
FIG. 2. **Immunoblot analysis of FGF-1 and Syn-1 in medium conditioned by heat shock after DTT treatment and heparin-Sepharose affinity chromatography.** Heat shock conditioned medium from NIH 3T3 FGF-1 transfectants was collected, treated with 0.1% DTT, and processed over a heparin-Sepharose column as described under "Experimental Procedures." Fractions were eluted with increasing concentrations of NaCl and concentrated by Centricon to a final volume of 100 μl. Each fraction was resuspended with SDS-PAGE loading buffer containing 2-ME and resolved by conventional 15% acrylamide SDS-PAGE. Immunoblot analysis was performed using an ECL detection system as described under "Experimental Procedures." *Lane 1,* 0.2 M NaCl elution fraction; *lane 2,* 0.4 M NaCl elution fraction; *lane 3,* 0.6 M NaCl elution fraction; *lane 4,* 0.8 M NaCl elution fraction; *lane 5,* 1.0 M NaCl elution fraction; *lane 6,* 1.5 M NaCl elution fraction; *lane 7,* 50 ng of FGF-1β recombinant protein (A), 50 ng of Syn-1 recombinant protein (B). *A,* FGF-1 immunoblot analysis; *B,* Syn-1 immunoblot analysis.

affinity, eluting from immobilized heparin near 1.5 M NaCl (20), we sought to confirm its heparin-binding characteristics. A rat p40 Syn-1 fragment encoding amino acid residues 96–421, which is very similar to the extravesicular domain of the p40 Syn-1 fragment isolated from bovine brain (Fig. 1), was expressed as a prokaryotic recombinant protein, and its heparin affinity was examined. As shown in Fig. 3, the recombinant form of rat Syn-1 eluted from heparin-Sepharose between 0.6 and 0.8 M NaCl (Fig. 3). These data demonstrate that treatment of heat shock conditioned medium with DTT generates a p40 fragment of Syn-1 with heparin binding affinity similar to the prokaryotic recombinant p40 fragment.

Treatment of Heat Shock Conditioned Medium with (NH₄)₂SO₄ Reveals the Presence of Reducing Agent-sensitive Forms of p40 Syn-1 and FGF-1 with Low Heparin Binding Affinity—It has been demonstrated previously that the latent, dimeric form of FGF-1 released into conditioned medium under temperature stress is not biologically active in mitogenic assays and requires activation with either DTT or (NH₄)₂SO₄ to restore its mitogenic potential (4). Since DTT treatment of medium conditioned by heat shock is able to generate a p40 Syn-1 fragment with similar heparin affinity as that exhibited by the recombinant p40, we sought to determine the heparin elution profile of extracellular FGF-1 and p40 Syn-1 when medium conditioned by temperature stress was treated with (NH₄)₂SO₄. The samples obtained from heparin-Sepharose elution after treatment of conditioned medium with (NH₄)₂SO₄ were independently examined for the presence of FGF-1 (Fig. 4) and Syn-1 (Fig. 5) by immunoblot analysis. Whereas immunoblot analysis performed after conventional SDS-PAGE under reducing conditions revealed the presence of monomeric FGF-1 in the low and high NaCl elution fractions (Fig. 4A), the appearance of p40 Syn-1 was limited to the low NaCl elution fractions (Fig. 5A). In contrast, FGF-1 (Fig. 4B) immunoblot analysis performed after conventional SDS-PAGE under nonreducing conditions demonstrated that the presence of the FGF-1 monomer was restricted to the high NaCl elution fractions and revealed the presence of high molecular weight FGF-1 immunoreactive bands in the low NaCl elution fractions (Fig. 4B). Similarly, Syn-1 immunoblot analysis of the heat shock-conditioned, post-heparin-Sepharose samples resolved by conventional SDS-PAGE under non-reducing conditions (Fig. 5B) also detected the presence of high molecular weight bands in the low NaCl elution fractions. These data indicate the following: (i) a p40 fragment of Syn-1, as well as FGF-1, is detected in the low NaCl elution samples of conditioned media treated with (NH₄)₂SO₄, but not DTT, and (ii) the electrophoretic mobility of both FGF-1 and p40 Syn-1 present in the
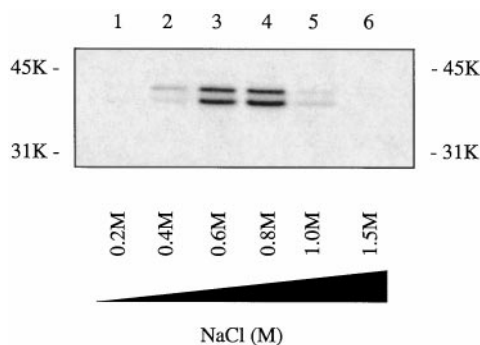

FIG. 3. **Heparin binding characteristics of recombinant p40 Syn-1.** A recombinant p40 Syn-1 fragment encoding amino acid residues 96–421 expressed as a glutathione *S*-transferase (*GST*) fusion protein was obtained as described under "Experimental Procedures." The thrombin-cleaved material (2 μg) was adsorbed to the heparin-Sepharose resin and eluted with increasing concentrations of NaCl. The elution fractions were concentrated by Centricon, resuspended in SDS-PAGE sample buffer containing 2-ME and resolved by 15% SDS-PAGE. Syn-1 immunoblot analysis was performed using an ECL detection system as described under "Experimental Procedures." *Lane 1,* 0.2 M NaCl elution fraction; *lane 2,* 0.4 M NaCl elution fraction; *lane 3,* 0.6 M NaCl elution fraction; *lane 4,* 0.8 M NaCl elution fraction; *lane 5,* 1.0 M NaCl elution fraction; *lane 6,* 1.5 M NaCl elution fraction. The doublet character for the recombinant rat Syn-1 is similar to that previously described (27).

low salt elution fractions is sensitive to the presence of reducing agent. Thus, it is likely that the monomeric forms of FGF-1 and Syn-1 detected as high affinity heparin-binding proteins in the high salt elution fractions of conditioned medium following DTT treatment may represent the reduced products of the low affinity heparin-binding forms of FGF-1 and Syn-1 present in the low NaCl elution fractions from medium treated with (NH₄)₂SO₄.

Treatment of Heat Shock Conditioned Medium with (NH₄)₂SO₄ Reveals the Presence of Denaturant-sensitive Forms of p40 Syn-1 and FGF-1—Because both Syn-1 (16) and FGF-1 (5) are able to associate with the acidic phospholipid, phosphatidylserine, we examined the possibility that the electrophoretic mobility of p40 Syn-1 and FGF-1 detected in the low NaCl elution fractions was also sensitive to the presence of the detergent, SDS. Medium conditioned by heat shock was treated with (NH₄)₂SO₄, and the post-heparin-Sepharose elution samples were resolved under limited denaturant electrophoretic conditions (limited SDS-PAGE) in which SDS was removed from the running gel but was present in the sample and running buffers. As shown in Fig. 4C, immunoblot analysis of the post-heparin-Sepharose elution fractions resolved by limited SDS-PAGE detected the presence of the FGF-1 monomer in

FIG. 4. **FGF-1 immunoblot analysis of reducing agent- and denaturant-sensitive forms of FGF-1 in medium conditioned by heat shock after $(NH_4)_2SO_4$ treatment and heparin-Sepharose affinity chromatography.** Heat shock CM from NIH 3T3 FGF-1 transfectants was collected, treated with $(NH_4)_2SO_4$, and processed over a heparin-Sepharose column as described under "Experimental Procedures." Fractions were collected, divided into two samples, and concentrated by Centricon to a final volume of 200 μl as described under "Experimental Procedures." The first sample was further divided in half, resuspended in an equal volume of SDS-PAGE loading buffer with 2-ME (*A*) or without 2-ME (*B*), and resolved by traditional 15% acrylamide SDS-PAGE. The second sample was further divided in half, resuspended in an equal volume of SDS-PAGE loading buffer with 2-ME (*C*) or without 2-ME (*D*), and resolved by limited SDS-PAGE. FGF-1 immunoblot analysis was performed using an ECL detection system as described under "Experimental Procedures." *Lane 1,* 0.2 M NaCl elution fraction; *lane 2,* 0.4 M NaCl elution fraction; *lane 3,* 0.6 M NaCl elution fraction; *lane 4,* 0.8 M NaCl elution fraction; *lane 5,* 1.0 M NaCl elution fraction; *lane 6,* 1.5 M NaCl elution fraction; *lane 7,* 50 ng FGF-1 recombinant protein.
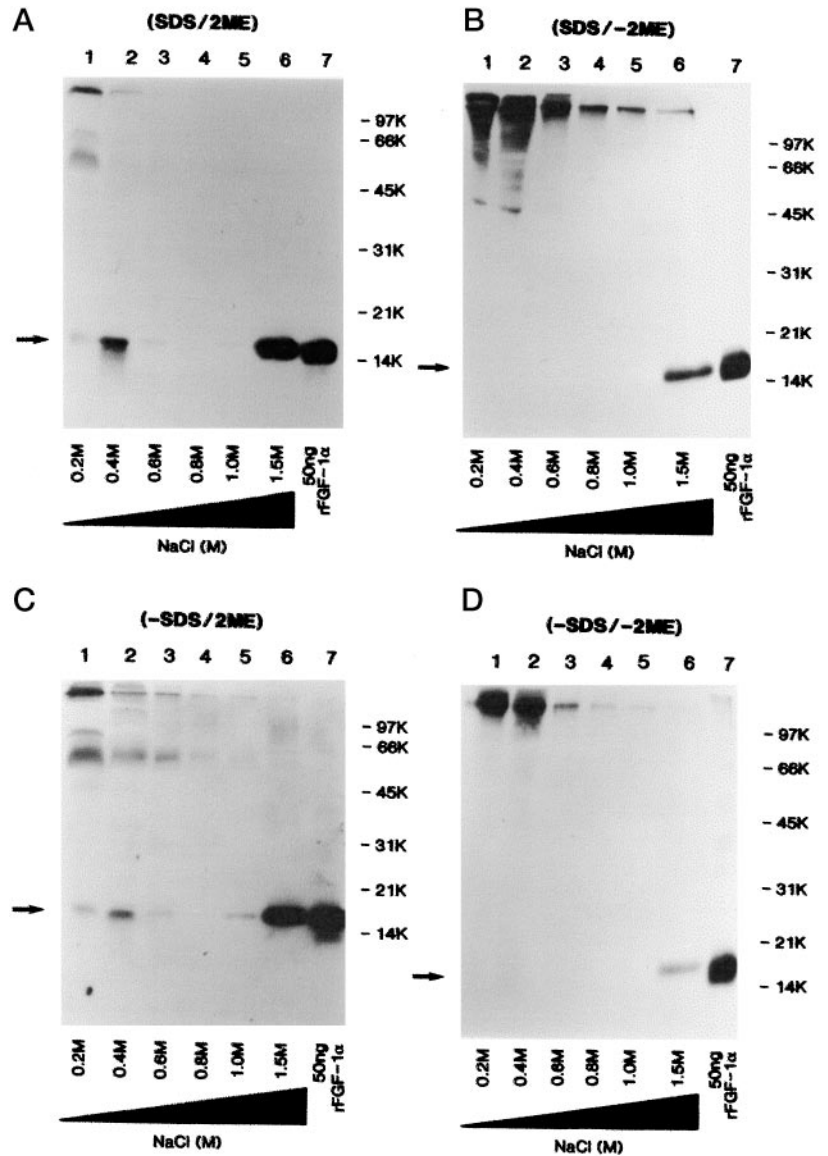
both the low and high NaCl elution fractions. Similarly, Syn-1 immunoblot analysis under the same conditions also detected the presence of the p40 Syn-1 fragment in the low NaCl elution fractions (Fig. 5*C*). Thus, both conventional and limited denaturant SDS-PAGE analysis performed under reducing conditions demonstrated the presence of the FGF-1 monomer and the p40 Syn-1 fragment in the low NaCl elution fractions. However, when FGF-1 and Syn-1 immunoblot analysis was performed under limited denaturant, non-reducing conditions, the electrophoretic mobility of both FGF-1 (Fig. 4*D*) and Syn-1 (Fig. 5*D*) present in the low NaCl elution fractions was significantly impaired. In contrast, the electrophoretic mobility of human FGF-1 (Fig. 4*D*) and rat p40 Syn-1 (Fig. 5*D*) recombinant proteins as well as the molecular weight standards were not altered by these electrophoretic conditions. These data suggest that the electrophoretic mobility of FGF-1 and p40 Syn-1 present in the $(NH_4)_2SO_4$-treated, low-heparin affinity fractions was sensitive to the presence of both denaturant and reducing agents.

The FGF-1 (Fig. 4, *A–C*) and Syn-1 (Fig. 5, *A–C*) immunoblots also revealed the presence of additional bands running between the 43- and the 67-kDa molecular mass standards. These bands were not detected in the FGF-1 (Fig. 4*D*) and Syn-1 (Fig. 5*D*) elution samples resolved under limited denaturant, non-reduc-

ing conditions in which all the bands run above the 97-kDa molecular mass marker. Since the mobility of either recombinant FGF-1 or recombinant Syn-1 was not altered by these electrophoretic conditions, we suggest that the additional bands detected in Figs. 4 and 5 (*A–C*) may represent either reducing agent- and/or denaturant-sensitive complexes between monomeric FGF-1 and the p40 fragment of Syn-1 or may reflect the presence of an additional moiety complexed with FGF-1 and/or Syn-1. Alternatively, both Syn-1 and FGF-1 are able to associate with phosphatidylserine (5, 16), and as a result, it is possible that the presence of phosphatidylserine in these samples may be responsible for the electrophoretic behavior of some of these bands. Since only monomeric FGF-1 and p40 Syn-1 were detected by immunoblot analysis in the post-heparin elution fractions of media treated with DTT, the presence of any additional high molecular weight band in Figs. 4 and 5 (*A–D*) confirmed the decreased efficiency of $(NH_4)_2SO_4$ to release FGF-1 and Syn-1 as monomeric high affinity heparin-binding proteins.

The Co-expression of an Antisense Syn-1 Construct in NIH 3T3 Cell FGF-1 Transfectants Represses the Release of FGF-1 in Response to Heat Shock—Since the extravesicular p40 fragment of Syn-1 and FGF-1 appears to be released into the extracellular compartment in response to heat shock as a de-

FIG. 5. **Syn-1 immunoblot analysis of reducing agent- and denaturant-sensitive forms of Syn-1 in medium conditioned by heat shock after $(NH_4)_2SO_4$ treatment and heparin-Sepharose affinity chromatography.** Heat shock CM from NIH 3T3 FGF-1 transfectants was collected, treated with $(NH_4)_2SO_4$, and processed over a heparin-Sepharose column as described under "Experimental Procedures." Fractions were collected, divided into two samples, and concentrated by Centricon to a final volume of 200 μl as described under "Experimental Procedures." The first sample was further divided in half, resuspended in an equal volume of SDS-PAGE loading buffer with 2-ME (*A*) or without 2-ME (*B*), and resolved by traditional 15% acrylamide SDS-PAGE. The second sample was further divided in half, resuspended in an equal volume of SDS-PAGE loading buffer with 2-ME (*C*) or without 2-ME (*D*), and resolved by limited SDS-PAGE. Syn-1 immunoblot analysis was performed using ECL detection system as described under "Experimental Procedures." *Lane 1,* 0.2 M NaCl elution fraction; *lane 2,* 0.4 M NaCl elution fraction; *lane 3,* 0.6 M NaCl elution fraction; *lane 4,* 0.8 M NaCl elution fraction; *lane 5,* 1.0 M NaCl elution fraction; *lane 6,* 1.5 M NaCl elution fraction; *lane 7,* 50 ng of Syn-1 recombinant protein.
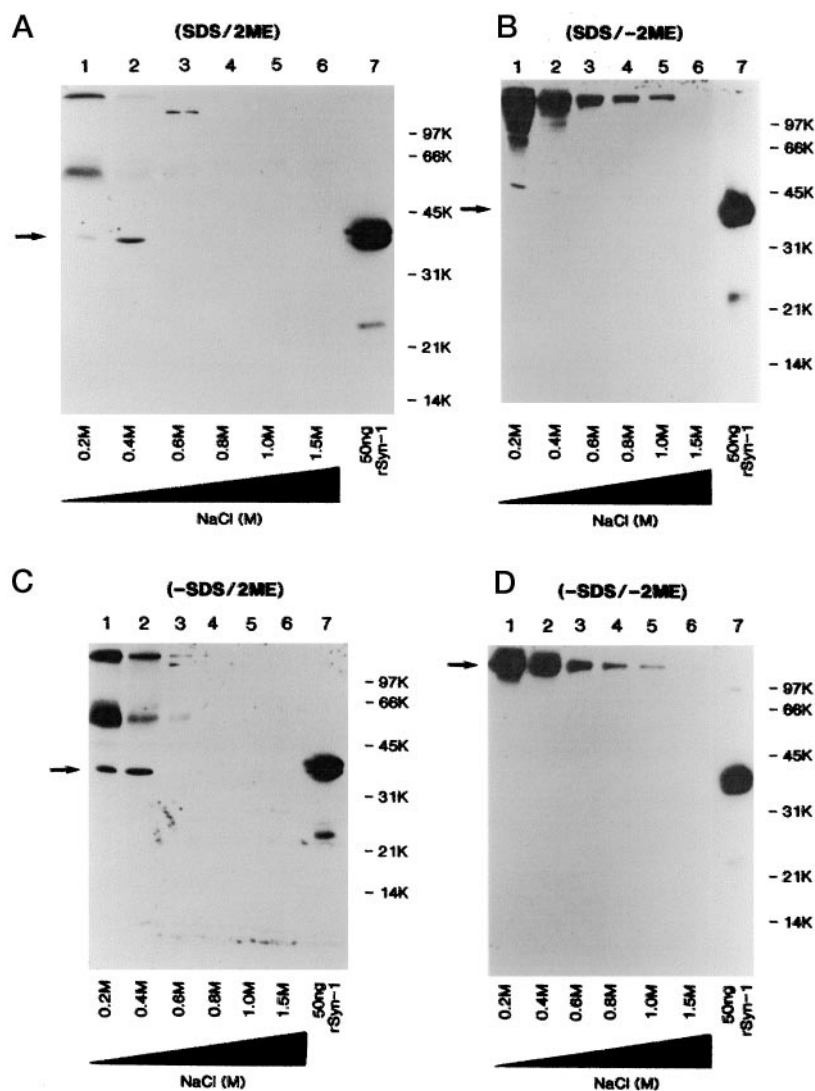

naturant- and reducing agent-sensitive complex, we sought to determine whether the release of FGF-1 required the expression of Syn-1. We employed an antisense Syn-1 strategy to repress Syn-1 protein expression in FGF-1-transfected NIH 3T3 cells and utilized an antisense rat Syn-1 transcript encompassing the AUG initiator codon and 640 nucleotides of the Syn-1 open reading frame. Stable co-transfectants were expanded and screened by Northern blot analysis using a rat Syn-1 sense probe (data not shown). While under selection, it became evident that the NIH 3T3 cell co-transfectants were growth-impaired, and as a result, the FGF-1 and antisense Syn-1 co-transfectants were analyzed for their proliferative potential (Fig. 6*A*). Although NIH 3T3 cell FGF-1 transfectants responded to the addition of serum, the NIH 3T3 cell FGF-1 and antisense Syn-1 co-transfectants exhibited a significant attenuation of growth in response to serum. Interestingly, the addition of exogenous FGF-1 to the NIH 3T3 cell FGF-1 and Syn-1 co-transfectants resulted in a 30% increase in their ability to proliferate in response to serum (Fig. 6*B*). To our knowledge, this is the first report of an FGF-1-independent cell line exhibiting sensitivity to this growth factor in the presence of high concentrations of serum. Moreover, the FGF-1 and antisense Syn-1 co-transfected NIH 3T3 cells were not able to release FGF-1 in response to heat shock (Fig. 6*C*). These data indicate that Syn-1 expression is not only important for the serum-dependent proliferation of NIH 3T3 cells but also for the release of FGF-1 in response to temperature stress.

DISCUSSION

Eleven members of the Syn gene family have been identified in both neuronal as well as non-neuronal tissues (21). As shown in Fig. 1, Syn-1 contains a short intravesicular domain, a transmembrane region, and a larger extravesicular, intracytosolic domain. The extravesicular domain contains two repeats that are homologous to the C2 regulatory domain of protein kinase C (9). The first C2 region (C2A) includes the Ca^{2+}-dependent phospholipid-binding domain of Syn-1 (16), and the second C2 domain (C2B) is responsible for the interaction with the clathrin AP adaptor that enables Syn-1 to participate in the clathrin-coated pit endocytotic pathway of cell-surface receptor recycling (10). In addition, Syn-1 has the potential to function as a docking protein through a carboxyl-terminal syntaxin-binding domain (18). A role for Syn-1 in the regulation of conventional endoplasmic reticulum-Golgi apparatus-dependent secretion has already been suggested in pancreatic endocrine cells (22), and most of the proteins that comprise the neurotransmitter secretory machinery have homologs in yeast (23). These observations suggest that Syn-1 may be fundamentally involved in the regulation of more general endocytotic and exocytotic processes other than the highly specialized neurotransmitter release pathway. Indeed, structural confirmation of the Syn-1 transcript in NIH 3T3 cell FGF-1 transfectants and in human umbilical vein endothelial cells supports this premise.
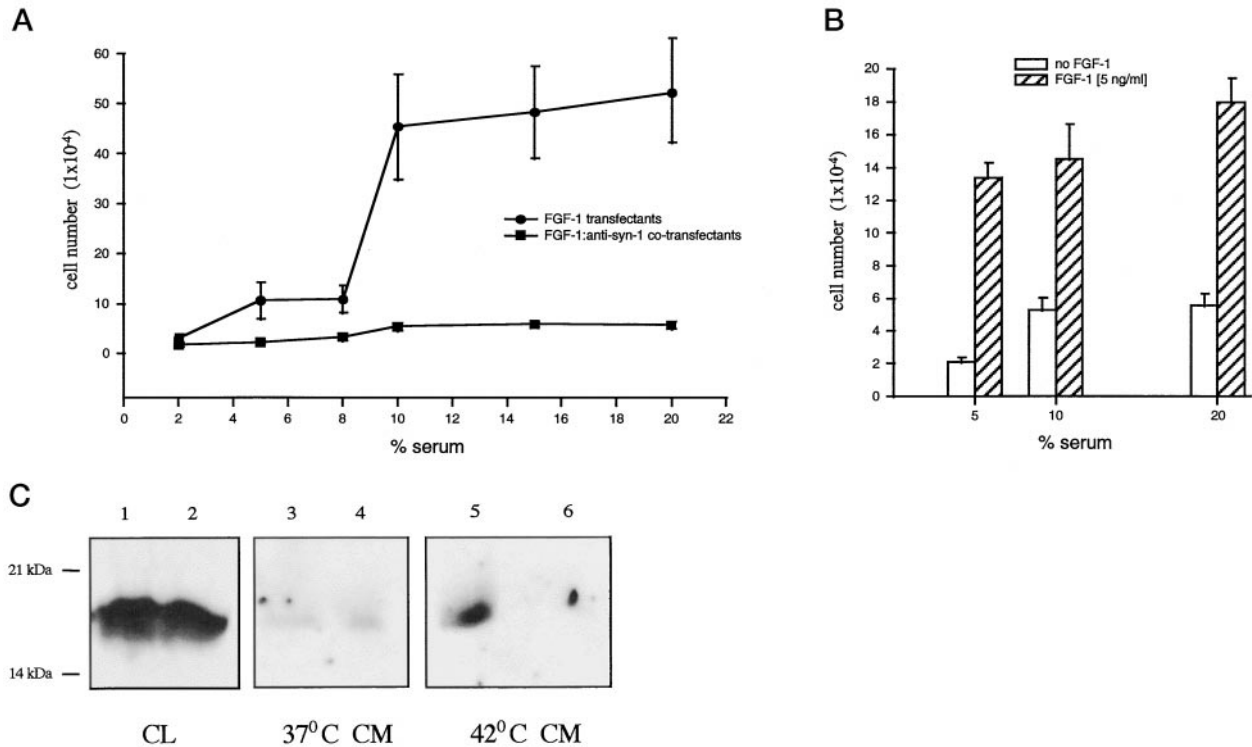
FIG. 6. **Growth response and FGF-1 immunoblot analysis of medium conditioned by heat shock from NIH 3T3 cell FGF-1 and FGF-1 and anti-Syn-1 co-transfectants, following DTT treatment and heparin-Sepharose affinity chromatography.** *A,* FGF-1 and FGF-1 and anti-Syn-1 NIH 3T3 cell co-transfectants were plated into 6-well tissue culture dishes and grown in DMEM containing increasing concentrations of BCS as described under "Experimental Procedures." After 7 days *in vitro,* the cells were harvested and quantitated as described under "Experimental Procedures". *B,* recombinant FGF-1 (5 ng/ml) heparin (4 units/ml) were added to the medium to evaluate the effect of FGF-1 on the growth curve. After 7 days the cells were harvested by trypsin treatment and quantitated as described under "Experimental Procedures." *Open bars* represent the FGF-less control, and *closed bars* represent the FGF-1 response. *C,* heat shock CM from NIH 3T3 FGF-1 and FGF-1 and anti-Syn-1 co-transfectants were collected, treated with 0.1% DTT, and processed over a heparin-Sepharose column as described under "Experimental Procedures." Elution fractions containing increasing concentration of NaCl were collected, concentrated by Centricon to a final volume of 100 μl, resuspended with SDS-PAGE loading buffer containing 2-ME, and resolved by conventional 15% acrylamide SDS-PAGE system. The FGF-1 released in CM was detected using a ^{125}I-protein A detection system as described under "Experimental Procedures." Medium from cells maintained at 37 °C served as a negative control. FGF-1 immunoblot analysis of FGF-1 transfectants lysate (*lane 1*); FGF-1 and anti-Syn-1 co-transfectants lysate (*lane 2*); FGF-1 transfectants 37 °C CM (*lane 3*); FGF-1 and anti-syn-1 co-transfectants 37 °C CM (*lane 4*); FGF-1 transfectants 42 °C CM (*lane 5*); and FGF-1 and anti-syn-1 co-transfectants 42 °C CM (*lane 6*).

The presence of a p40 fragment of Syn-1 in medium conditioned by heat shock is interesting since Syn-1 exists as a p65 transmembrane protein (Fig. 1*A*). Since the Syn-1 antibody used in this study was raised against the recombinant extravesicular domain of Syn-1 containing residues 96–421, it is likely that the p40 Syn-1 fragment observed in the medium conditioned by heat shock represents the extravesicular domain of the p65 Syn-1 translation product. This is especially noteworthy because it implies the function of a cytosolic protease that may be responsible for the generation of extravesicular p40 Syn-1 fragment under temperature stress. The protease activity would result in the cleavage of the p65 Syn-1 translation product in a region adjacent to its transmembrane domain with subsequent release of the p40 fragment from its transmembrane constraint. This event would also serve to dissociate p65 Syn-1 from its normal role in intracellular vesicular trafficking. In addition, the sequence of the NH$_2$-terminal region of the p40 fragment that was originally isolated from bovine brain in association with FGF-1 indicates that this proteolytic cleavage site does correspond to the known trypsin cleavage site already described for p65 Syn-1 in bovine chromaffin granules (24). This observation further supports the hypothesis that a novel intracellular protease may be involved in the regulation of the FGF-1 release pathway.

The data obtained from the NIH 3T3 cell FGF-1 and antisense-Syn-1 co-transfectants are also significant since they

suggest that not only is Syn-1 expression important for FGF-1 release in response to heat shock, but Syn-1 expression is important for the serum-induced proliferation of the NIH 3T3 cell. Since Syn-1 is involved in the regulation of cell-surface receptor recycling through the conventional clathrin-coated pit endocytotic pathway (10), it is possible that the repression of Syn-1 expression may interrupt the recycling of receptors involved in the maintenance of cellular nutrition. Thus, the attenuation of Syn-1 expression may obstruct transferrin receptor and/or the low density lipoprotein receptor systems and possibly others, which may result in a non-lethal nutritional defect with a proliferation-impaired phenotype. Alternatively, the repression of Syn-1 expression may also prevent the endogenous release of FGF-1 from the NIH 3T3 cell which may be important for the maintenance of cell survival. Indeed, the FGF prototypes are well recognized as cell survival factors for a variety of cell types *in vitro* (1), and since the NIH 3T3 cell expresses levels of FGF-1 below the sensitivity of our analytical methods (25), it is experimentally difficult to examine this premise. However, because the addition of exogenous FGF-1 to the NIH 3T3 cell FGF-1 and antisense-Syn-1 co-transfectants only rescued approximately 30% of their serum-induced proliferative potential, it is likely that a combination of these events is responsible for the impaired proliferative potential of these co-transfectants.

While the mechanism utilized by the FGF-1 homodimer and

the p40 Syn-1 fragment to pass through the lipid bilayer remains unknown, our data do indicate that in response to temperature stress, FGF-1 may associate with the cytosolic face of Syn-1-containing exocytotic vesicles and may utilize this extravesicular orientation for transport to the plasma membrane. Since both FGF-1 and Syn-1 are able to associate with phosphatidylserine, an acidic phospholipid present on the inner surface of plasma membranes (5, 16), it is possible that this orientation may facilitate their release. Indeed, it will be interesting to determine whether the p40 Syn-1 fragment is able to attain molten globule character as described previously for FGF-1 (26) which may enable it to associate with, and possibly transverse, acidic phospholipid-rich membranes.

Our data demonstrate that the extracellular FGF-1 complex released from NIH 3T3 cell FGF-1 transfectants in response to temperature stress is likely composed of at least the p40 fragment of Syn-1 and the FGF-1 homodimer. Whereas treatment of this complex with DTT efficiently generates the p40 Syn-1 fragment and the FGF-1 monomer as high affinity heparin-binding proteins, treatment with $(NH_4)_2SO_4$ does not. Interestingly, $(NH)_2SO_4$ treatment yields both the FGF-1 and the p40 Syn-1 fragment as low affinity heparin-binding proteins. However, FGF-1, but not the p40 fragment of Syn-1, is detected as monomeric high affinity heparin-binding protein after the $(NH_4)_2SO_4$ treatment of media. Indeed, the high affinity heparin-binding form of p40 Syn-1 is not present following treatment with $(NH_4)_2SO_4$. Although it is possible that the effect of $(NH_4)_2SO_4$ treatment may be derived from the ability of $(NH_4)_2SO_4$ to alter an unknown redox system in the extracellular compartment, it is unlikely. Although we do not know the mechanism utilized by $(NH_4)_2SO_4$ to release the FGF-1 monomer from the extracellular complex, we suggest that perhaps both the FGF-1 monomer and homodimer may be associated with the p40 Syn-1-containing complex. However, it is important to emphasize that if the FGF-1 monomer is also present in the extracellular complex, it is maintained in a form that does not enable it to associate with cell-surface FGF receptors and/or the heparin sulfate proteoglycan, since the FGF-1 present in medium conditioned by temperature stress is not able to associate with immobilized heparin and is not mitogenic (2).

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