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S100A13 Is Involved in the Regulation of Fibroblast Growth Factor-1 and p40 Synaptotagmin-1 Release in Vitro*

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We have previously characterized the release of the signal peptide sequence-less fibroblast growth factor (FGF) prototype, FGF-1, in vitro as a stress-induced pathway in which FGF-1 is released as a latent homodimer with the p40 extravesicular domain of p65 synaptotagmin (Syn)-1. To determine the biologic relevance of the FGF-1 release pathway in vivo, we sought to resolve and characterize from ovine brain a purified fraction that contained both FGF-1 and p40 Syn-1 and report that the brain-derived FGF-1:p40 Syn-1 aggregate is associated with the calcium-binding protein, S100A13. Since S100A13 binds the anti-inflammatory compound amlexanox and FGF-1 is involved in inflammation, we examined the effects of amlexanox on the release of FGF-1 and p40 Syn-1 in response to stress in vitro. We report that while amlexanox was able to repress the heat shock-induced release of FGF-1 and p40 Syn-1 in a concentration-dependent manner, it had no effect on the constitutive release of p40 Syn-1 from p40 Syn-1 NIH 3T3 cell extracts. These data suggest the following: (i) FGF-1 is associated with Syn-1 and S100A13 in vivo; (ii) S100A13 may be involved in the regulation of FGF-1 and p40 Syn-1 release in response to temperature stress in vitro; and (iii) the FGF-1 release pathway may be accessible to pharmacologic regulation.

The FGF* prototype, FGF-1, functions as an extracellular mitogen for a diverse population of target cells, yet it lacks a classical signal peptide sequence for secretion (1). FGF-1 is released in response to temperature stress in vitro (2)

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* The abbreviations used are: FGF, fibroblast growth factor; Gal, galactosidase; RP-HPLC, reversed phase-high pressure liquid chromatography; Syn, synaptotagmin; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; IL, interleukin.
The rabbit polyclonal antibodies against recombinant human FGF-1 and rat p40 Syn-1 were prepared as described previously (5, 13). All antibodies were used for immunoblot analysis at a concentration of 4 μg/ml in blocking buffer as described previously (2) except that an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) was used for protein detection.

**Preparation of Brain Extracts—**Ten (1.5 kg) unstripped ovine brains (Pel-Freeze® Biologicals, Rogers, AR) were homogenized in 1.3 volumes of 50 mM Tris-HCl, pH 7.4, for 2 min in a Waring blender. The homogenate was centrifuged at 10,000 × g for 1 h, and the supernatant was filtered through sterile gauze. The filtrate was subjected to stepwise salt fractionation with 50 and 95% (NH₄)₂SO₄ saturation, and the precipitates were collected by centrifugation as described (14). The 95% (NH₄)₂SO₄ precipitate was resuspended in 100 mM HEPES, pH 7.4, and dialyzed for 18 h against 50 volumes of the resuspension buffer using a Spectra/Por (M, 12–14,000) dialysis membrane (Spectrum Medical Industries Inc., Houston, TX). All purification procedures were performed at 4 °C.

**Chromatographic Analysis of Neutral Brain Extracts—**A 2.5 × 22-cm plastic column containing 25 ml of hydrated heparin-Sepharose CL-6B was equilibrated with 10 volumes of 50 mM Tris-HCl, pH 7.4, and the brain extract was adsorbed twice over the immobilized heparin. The column was washed with at least 10 bed volumes of the resuspension buffer until the absorbance of the eluate at λ = 280 nm was less than 0.01. Three batch fractions were eluted with 100 ml of 50 mM Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.7 M NaCl, and 1.5 M NaCl, and samples from each NaCl eluate (25 ml) were adsorbed to a C4 column (Vydac™, Hesperia, CA) conditioned in 0.1% trifluoroacetic acid (Pierce). RP-HPLC was performed as described (18) using a linear gradient of acetonitrile (40–100%) in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min, and the effluent was monitored at λ = 214 nm. Samples were collected as absorbance peaks independent of volume in 1.0 M Tris-HCl, pH 7.4, in an attempt to maintain aggregate integrity and analyzed by FGF-1 and Syn-1 immunoblot analysis as described (2, 5) except that the ECL system was used for protein detection. Although many peaks exhibited the presence of both FGF-1 and Syn-1 by immunoblot analysis, a 1.5 M NaCl Sepharose elution fraction contained a unique absorbance peak that contained both FGF-1 and Syn-1 by immunoblot analysis at a dilution of 1:100. This peak was re-chromatographed on a microbore 300-A C4 Aquapore RP100 column (Perkin-Elmer), and bound proteins were eluted as absorbance peaks at λ = 214 with a linear gradient (40–100%) of 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and a flow rate of 0.2 ml/min.

**Protein Sequencing—**Approximately 10 μg of protein from the indicated fractions B and C were subjected to proteolytic digestion using lysyl endopeptidase C (Boehringer Mannheim) as described (73). Peptides were isolated by RP-HPLC using an Applied Biosystems model 130 separation system. Isolated peptides were subjected to automated Edman degradation using either an Applied Biosystems model 473A or 477A protein sequenator. Proteins were identified by comparison of the amino acid sequences obtained for several of these peptides against an NCBI (National Center for Biotechnology Information) protein sequence data base using the BLAST (Basic Local Alignment Search Tool) program.

**Pharmacology of FGF-1 Release in Response to Heat Shock—**NIH 3T3 cell FGF-1 [2] transfectants and FGF-1/bgal and p65 Syn-1 cotransfectants (13) were grown to 80% confluence on fibronectin-coated dishes and subjected to temperature stress (41.5 °C, 90 min) under the presence of amlexanox for the same period were used as a control. Conditioned media were collected and filtered through 0.2-μm cellulose acetate. Aliquots (300 μl) were taken for analysis of lactate dehydrogenase activity, or cell viability. In addition, cells maintained at 37 °C in the presence or absence of amlexanox for the same period were used as a control. Conditioned media were collected and filtered through 0.2-μm cellulose acetate. Aliquots (300 μl) were taken for analysis of lactate dehydrogenase enzymatic activity in the conditioned media according to an adaptation of the original method of Bergmeyer (75) and Sigma procedure DG1340-UV. The remaining filtrate was treated with fresh 0.1% (v/v) dithiothreitol for 2 h at 37 °C and processed by heparin-Sepharose chromatography as described (2).

**RESULTS**

**Brain-derived FGF-1 Exists as a Denaturant-sensitive Aggregate with Syn-1—**Extracts of ovine brain were prepared at a neutral pH and subjected to (NH₄)₂SO₄ precipitation (50 and 95% saturation) as described under "Experimental Procedures." The 95% (NH₄)₂SO₄ saturation precipitate was resuspended as described previously (18) and separated into three batch-eluted fractions (0.4, 0.7, and 1.5 M NaCl) by heparin-Sepharose chromatography. Whereas immunoblot analysis of each eluate revealed the presence of both FGF-1 and p40 Syn-1 in all three fractions, only the 1.5 M NaCl post-heparin-Sepharose eluate was able to produce a reversed phase (RP)-HPLC peak (Fig. 1A) that contained both FGF-1 and p40 Syn-1 by immunoblot analysis (data not shown). The presence of p40 Syn-1 in the 1.5 M NaCl post-heparin-Sepharose elution fraction was unexpected since recombinant p40 Syn-1 has been observed to elute at a lower (~0.6 M NaCl) ionic strength [5]. To ensure that the post-RP-HPLC heparin-binding fraction containing both FGF-1 and p40 Syn-1 did not contain additional non-associated proteins, this fraction was again subjected to analysis by RP-HPLC. As shown in Fig. 1B, RP-HPLC analysis revealed a single symmetrical peak with a retention time identical to that previously observed (Fig. 1A). Immunoblot analysis using FGF-1 and Syn-1 antibodies confirmed the presence of both FGF-1 and p40 Syn-1 in this sample (Fig. 2, A and B).

Since this fraction (Fig. 1B) contained both FGF-1 and p40 Syn-1 and the electrophoretic mobility of the FGF-1/Syn-1 aggregate released in response to temperature stress is denaturant-sensitive (5), we anticipated that treatment of this fraction with a chaotropic agent should resolve the FGF-1 and Syn-1 components as individual peaks with RP-HPLC retention times identical to their retention times defined by both the native (Fig. 1A) and recombinant (Fig. 1D) proteins. Therefore, the brain-derived, post-RP-HPLC fraction (Fig. 1B) containing both FGF-1 and p40 Syn-1 was heated (5 min, 95 °C) in the presence of 8.0 M guanidine HCl and analyzed by RP-HPLC. As shown in Fig. 1C, the fraction containing both FGF-1 and p40 Syn-1 was present, but its absorbance was reduced significantly. In addition, numerous fractions with distinct retention times were readily visible including a major absorption peak and fractions previously defined as FGF-1 and p40 Syn-1 (Fig. 1, A and D). These data suggest that thermal and guanidine HCl denaturation of the brain-derived post-RP-HPLC fraction (Fig. 1B) containing both FGF-1 and p40 Syn-1 generates additional fractions with different retention times including peaks with retention times identical to FGF-1 and p40 Syn-1. Thus it is likely that this brain-derived, heparin-binding fraction represents an aggregate that contains FGF-1 and p40 Syn-1 as well as other temperature- and chaotrope-sensitive components.

The S100 Gene Family Member, S100A13, Is a Component of the Brain-derived FGF-1 and p40 Syn-1 Aggregate—Since the major absorption fraction containing both FGF-1 and p40 Syn-1 (Fig. 1B) demonstrated an unknown retention time, we sought to determine its structure. However, automated Edman degradation of this peak failed to yield any information. Therefore, this fraction (Fig. 1B) was subjected to LysC digestion, and the peptides were resolved by RP-HPLC. Automated Edman degradation of these peptides demonstrated that this fraction contained a member of the S100 gene family (17), the ovine homolog of human S100A13 (16). Interestingly, the structure of the S100A13 protein predicted from the bovine (GenBank™ accession number AB001567), murine, and human cDNA sequences (16) suggests that S100A13 contains 98 amino acid residues, 9 of which have cyclic side chains that absorb in the far-UV area used for detection (19, 20). Thus, the high UV
absorbance feature of the FGF-1 and p40 Syn-1 aggregate (Fig. 1B) may be due in part to the extinction coefficient of S100A13 (Fig. 1, C and D). Since it was possible to resolve a single fraction from RP-HPLC whose retention time was altered by treatment with temperature and the chaotropic agent, guanidine HCl, we suggest that S100A13 is a component of a multimeric aggregate of FGF-1, p40 Syn-1, and S100A13 containing aggregate and that S100A13 is also present in this aggregate as a protein with a blocked amino terminus.

It is important to note that the major absorption peak in Fig. 1C, resulting from the denaturation of the fraction (Fig. 1B) containing FGF-1, p40 Syn-1, and S100A13, exhibited a distinct retention time, yet immunoblot analysis of this major absorption peak demonstrated the presence of low levels of FGF-1 and p40 Syn-1 (data not shown). Since members of the S100 gene family are known to self-associate (21) and to associate with membrane phospholipids (22), it is possible that the major absorption peak in Fig. 1B may contain S100A13 aggregates as well as other peptidic and non-protein components such as acidic phospholipids. Thus, denaturation of the multimeric aggregate in Fig. 1B containing p40 Syn-1, S100A13, and FGF-1, with temperature and treatment with guanidine HCl resulted in only a partial disruption of the aggregate. In addition, we cannot eliminate the possibility that the FGF-1, p40 Syn-1, and S100A13-containing peak resolved in Fig. 1A and B, is the result of nonspecific protein aggregation under RP-HPLC conditions. However, it is noteworthy that recombinant S100A13 elutes from immobilized heparin between 0.2 and 0.4 M NaCl (data not shown), and recombinant p40 Syn-1 elutes from heparin-Sepharose at 0.7 M NaCl (5). Since the brain-derived, post-heparin-Sepharose fraction containing p40 Syn-1, FGF-1, and S100A13 was isolated as a high affinity (1.5 M NaCl elution peak) heparin-binding fraction prior to resolution by RP-HPLC, it is likely that S100A13 and p40 Syn-1 gained high heparin binding affinity through their ability to associate with FGF-1 prior to analysis by RP-HPLC.

Interestingly, automated Edman degradation of the FGF-1 fraction (Fig. 1C) derived from treatment of the FGF-1, S100A13, and p40 Syn-1 aggregate (Fig. 1B) with temperature and guanidine HCl also failed to yield information. However, automated Edman degradation of LysC fragments derived from the FGF-1 peak (Fig. 1C) demonstrated that it was present as a protein with a blocked amino terminus that has previously been characterized as FGF-1b (residues 1–154) (23). Unfortunately, however, it was not possible to collect sufficient material from the p40 Syn-1 peak described in Fig. 1C for structural analysis, but it was possible to confirm the identity of this peak by Syn-1 immunoblot analysis (data not shown). Likewise, automated Edman degradation of the remaining peaks described in Fig. 1C did not yield any information, and sufficient material was not available for analysis by LysC digestion. These data imply that the ovine brain-derived, heparin-binding and denaturant-sensitive aggregate resolved by RP-HPLC (Fig. 1B) contains at least FGF-1b, p40 Syn-1, and S100A13. Furthermore, these data also provide an in vivo correlate to the presence of FGF-1 and p40 Syn-1 as a denatured, aggregated protein.

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**Fig. 1. Purification of a heteromeric aggregate containing FGF-1, Syn-1, and S100A13 from ovine brain.** Ovine brain extract was prepared and separated into three fractions by heparin-Sepharose chromatography as described under "Experimental Procedures." Data are reported as a function of relative absorbance and retention time. The solid line on the chromatogram indicates the development of the acetonitrile gradient. A, RP-HPLC analysis of 25 ml of the post-heparin-Sepharose 1.5 M NaCl eluate. Fractions were collected as absorbance peaks, divided in two, and 0.4 M NaCl (data not shown), and recombinant p40 Syn-1 elutes from heparin-Sepharose at 0.7 M NaCl (5). Since the brain-derived, post-heparin-Sepharose fraction containing p40 Syn-1, FGF-1, and S100A13 was isolated as a high affinity (1.5 M NaCl elution peak) heparin-binding fraction prior to resolution by RP-HPLC, it is likely that S100A13 and p40 Syn-1 gained high heparin binding affinity through their ability to associate with FGF-1 prior to analysis by RP-HPLC.

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**Time and resolve it as a single peak. C, the fraction obtained after two consecutive runs containing FGF-1, p40 Syn-1, and S100A13 was treated with of 8 M guanidine HCl in 0.25 M Tris-HCl, pH 8.5, vortexed for 1 min, heated for 5 min at 95 °C, vortexed for 1 min, and allowed to cool to room temperature before separation by RP-HPLC on a microbore column. Fractions were collected as absorbance peaks, divided in two, and subjected to FGF-1 and Syn-1 immunoblot analysis and protein sequencing. D, samples of purified human recombinant FGF-1, rat recombinant Syn-1, and murine recombinant S100A13 were initially resolved by RP-HPLC to identify the retention time for each protein (data not shown) and then resolved as a mixture by RP-HPLC.**
The S100A13-binding, Anti-allergic, and Anti-inflammatory Drug, Amlexanox, Represses the Stress-induced Release of FGF-1 and p40 Syn-1 in Vitro—Because (i) S100A13 was purified as an aggregate with FGF-1 and p40 Syn-1 from ovine brain, (ii) elevated FGF-1 levels are associated with inflammatory environments in vivo (24), (iii) Syn-1 is released as a p40 fragment in response to temperature stress (5) and is required for heat shock-induced FGF-1 secretion (13), (iv) the anti-inflammatory and anti-allergic compound, amlexanox, binds S100A13 (Ref. 25; GenBank™ accession number AB001567), (v) amlexanox is able to interfere with the release of intracellular granules from basophilic and mast cells (26), and (vi) NIH 3T3 cells express the S100A13 transcript (data not shown), we sought to determine whether amlexanox would be able to modify the stress-induced release of FGF-1 and p40 Syn-1 from NIH3T3 cells in vitro. NIH 3T3 cell FGF-1:β-galactosidase (Gal) and p65 Syn-1 co-transfectants were subjected to temperature stress (90 min, 41.5 °C) as described previously (13) in the absence and presence of amlexanox. The conditioned medium was treated with dithiothreitol, adsorbed to heparin-Sepharose, and the presence of FGF-1:β-gal and Syn-1 assessed by immunoblot analysis as described previously (2). As shown in Fig. 3, A and B, amlexanox was able to repress the release of both FGF-1:β-gal and p40 Syn-1 in response to temperature stress. The inhibition of FGF-1:β-gal and p40 Syn-1 release was dependent upon the concentration of amlexanox and was within the concentration range that exhibits pharmacologic effects as an anti-allergic and anti-inflammatory agent (27–29).

Similar amlexanox concentrations were also able to inhibit the release of FGF-1 from FGF-1 NIH 3T3 cell transfectants in vitro (Fig. 5). Because of the possibility that amlexanox may also possess broad inhibitory activity upon conventional cellular secretion, we evaluated the ability of amlexanox to repress the release of a synthetic form of FGF-1 engineered to enter into the conventional ER-Golgi-mediated secretion pathway (30). Exposure of NIH 3T3 cells stably transfected with the FGF-4 signal peptide sequence: FGF-1 chimera (30) to amlexanox did not result in inhibition of the constitutive secretion of the FGF-1 chimera (data not shown). Furthermore, the forced secretion of FGF-1 is known to induce a prominent transformed NIH 3T3 cell phenotype in vitro (30), and amlexanox was unable to modify this phenotype. Additionally, the p40 extracellular fragment of Syn-1 is constitutively released in p40 Syn-1 NIH 3T3 cell transfectants, and this release is potentiated by heat shock treatment (13). Whereas this constitutive release of p40 Syn-1 occurs by an unknown pathway, the temperature-enhanced release of p40 from these cells appears to utilize the regulated FGF-1 release mechanism (13). Thus, we examined the ability of amlexanox to influence the release of p40 Syn-1 from the NIH 3T3 cell p40 Syn-1 transfectants, and we observed that amlexanox was not able to block the constitutive release of p40 from cells maintained at 37 °C (Fig. 4, lanes 1, 3 and 4). However, amlexanox was able to repress the temperature-sensitive component of p40 Syn-1 release (Fig. 4, lane 5 versus lane 2) in vitro. Finally, all experiments were monitored for cell lysis and changes in the permeability of the plasma membrane by measuring the levels of the cytosolic enzyme lactate dehydrogenase in the conditioned media of all samples, and no significant differences in extracellular lactate dehydrogenase levels were observed among the various treatments and conditions. We therefore suggest that amlexanox, the S100A13-binding compound, is able to significantly repress the temperature stress-induced release of both FGF-1 and p40 Syn-1.

Chemical Modification of Amlexanox Reduces its Ability to Repress the Release of p40 Syn-1 and FGF-1 in Response to Temperature Stress in Vitro—To provide a structural basis for the ability of amlexanox to repress the release of FGF-1 and p40 Syn-1 from its full-length (p65 Syn-1) precursor in vitro, we obtained several chemical derivatives of amlexanox (Fig. 5) with variable anti-allergic and anti-inflammatory activities. Each amlexanox derivative was evaluated for its ability to...
FGF-1: media derived from FGF-1: "Experimental Procedures." Followed by immunoblot analysis for FGF-1 and Syn-1 as described under Syn-1 by exposure to 41.5 °C for 90 min. The media were collected and ing heat shock in the presence of 0.8

Lane descriptors are identical to those described in

Media were conditioned by two 150-mm dishes of
temperature stress.

Cell FGF-1:

Gal chimera and p40 Syn-1 into media conditioned by NIH 3T3

release, derivative AA777 was not able to do so. Syn-1 immu-

derivatives AA617 and AA648 were able to repress FGF-1

amlexanox (AA673) or one of its derivatives revealed that while

in vitro
detergent- and reducing agent-sensitive, and electrophoretic
mobility shift analysis anticipated the presence of additional
components (5). In order to determine whether the information
obtained from the characterization of the FGF-1 release path-
way in vitro could be applied to an in vivo situation, we pursued
the isolation of a multiprotein aggregate from ovine brain that
contained FGF-1 and p40 Syn-1, and we report that the brain-
derived FGF-1:Syn-1 aggregate also contains the calcium-bind-

protein, S100A13 (16), a member of the S100 gene family of
EF-hand-containing Ca2+-binding modulator proteins (17).

The S100 gene family members (31) encode low molecular
weight acidic polypeptides containing two Ca2+-binding EF-
hand motifs flanked by two hydrophobic domains (17, 32).
S100A13 also exhibits these structural features, and in con-
trast with other S100 gene family members, it contains a car-
boxyl-terminal domain that is rich in basic amino acid residues
(16). Whereas all tissues express at least one member of the
S100 gene family, the cellular distribution is specific for mem-
bers of the S100 gene family (17). In this regard it is interesting
that like FGF-1 (1) and Syn-1 (33), S100 proteins are highly
enriched in neural tissue (34, 35). However, S100A13 expres-
sion is not limited to neural tissue, and with the possible
exception of leukocytes, S100A13 expression is ubiquitous in
other organs, tissues, and cell types (16) including its presence
in NIH 3T3 cells. Interestingly, some members of the S100 gene
family have been found to be associated with the nucleus (36),
although most are cytosolic and can be associated with the
endoplasmic reticulum and actin stress fibers (37). However,
like FGF-1 (2), several S100 gene family members are released
from cells in vitro (17, 38) despite the absence of a conventional
signal peptide sequence for classical ER-Golgi-mediated sec-
tion. Furthermore, even though no specific cell-surface receptor
has been identified for these extracellular S100 gene family
members (34, 39), some do possess extracellular functions in-
cluding the regulation of neuronal differentiation (40–42),
the stimulation of melanoma and glial cell proliferation and
migration (35, 43), and chemotactic activities (44, 45). Indeed,
it is interesting that FGF-1 is also able to accomplish these
functions as well (1).

Since S100A8 and S100A9 are released from monocytes
through a novel ER-Golgi-independent pathway that requires
an intact tubulin network (38), it is not perhaps surprising that
S100A13 may be a component of the FGF-1 and p40 Syn-1
ER-Golgi-independent release pathway. Indeed, the release of
S100A8 by monocytes and activated neutrophils coincides with
the release of the pro-inflammatory cytokine, interleukin
(IL)-1α by these cells (46). This is interesting since the IL-1 and
FGF gene family prototypes most likely evolved without clas-
sical signal peptide sequences from a common primordial gene
(23). The IL-1 and the FGF gene family prototypes also exhibit
sequence homologies (47, 48) including very similar crystallo-
graphic structures (49, 50). Like S100A8 and the IL-1 proto-
types, FGF-1 is also expressed during inflammatory situations
in vivo (24) and is thought to be released during these situa-
tions. Thus, it is also not surprising that the anti-allergic and
anti-inflammatory S100A13-binding compound, amlexanox
((25) GenBank™ accession number AB001567), is able to re-

FIG. 3. The effect of amlexanox on the release of the FGF-1:β-
Gal chimera and p40 Syn-1 into media conditioned by NIH 3T3
cell FGF-1:β-Gal and p65 Syn-1 co-transfectants exposed to tem-
perature stress. Media were conditioned by two 150-mm dishes of
NF 3T3 cells stably transfected with human FGF-1:β-Gal and rat p65
Syn-1 by exposure to 41.5 °C for 90 min. The media were collected and
resolved by 10% acrylamide SDS-PAGE under reducing conditions fol-
lowed by immunoblot analysis for FGF-1 and Syn-1 as described under
“Experimental Procedures.” A, FGF-1 immunoblot analysis. 1st lane,
media derived from FGF-1:β-Gal and p65 Syn-1 NIH3T3 cell co-trans-
fectants maintained at 37 °C for 90 min. 2nd lane, media derived from
FGF-1:β-Gal and p65 Syn-1 NIH3T3 cell co-transfectants following
heat shock in the absence of any drug; 3rd to 5th lanes, media derived
from FGF-1:β-Gal and p65 Syn-1 NIH3T3 cell co-transfectants follow-
ing heat shock in the presence of 0.8 × 10−6, 5 × 10−6, and 10−4 M
AA673 (amlexanox), respectively; 6th lane, 50 ng of recombinant human
FGF-1:β-Gal and 50 ng of recombinant rat p40 Syn-1. B, Syn-1 immu-

oblot analysis; the filter was stripped according to the manufacturer’s
instructions (Amersham Pharmacia Biotech) and re-probed for Syn-1.

Lane descriptors are identical to those described in A.

decrease the release of both FGF-1 and p40 Syn-1 from NIH
3T3 cell FGF-1:Syn-1 transfectants in response to temperature stress
in vitro. As shown in Fig. 5, immunoblot analysis of media
conditioned by heat shock in the presence or absence of either
amlexanox (AA673) or one of its derivatives revealed that while
derivatives AA617 and AA648 were able to repress FGF-1
release, derivative AA777 was not able to do so. Syn-1 immu-

oblot analysis of these samples also demonstrated that am-
lexanox derivatives AA617 and AA648 but not derivative AA777
were able to inhibit the release of p40 Syn-1 from endogenous
p65 Syn-1 in response to temperature stress (data not shown).
These data suggest that either substitution of the isopropyl
side chain with an acetyl group or deletion of the amino group
at the opposite end of the molecule does not reduce the ability
of amlexanox to repress FGF-1 and p40 Syn-1 release in re-
response to temperature stress in vitro. However, substitution of
the isopropyl side chain with a methyl group generates an
amlexanox derivative that is unable to repress the release of
either FGF-1 (Fig. 5) or p40 Syn-1 (data not shown) in response
to heat shock. These data suggest that the major functional
group within the structure of amlexanox that is able to modify
FGF-1 and p40 Syn-1 release is the hydrophobic side chain,
including the maintenance of at least a two-carbon unit.
press the release of FGF-1 and p40-Syn-1 in response to temperature stress in vitro.

Although we have not determined whether S100A13 is released in a brefeldin A-insensitive manner with FGF-1 and p40 Syn-1 in response to cellular stress in vitro, our data do suggest that S100A13 may be at least a component of the intracellular multiprotein aggregate involved in the regulation FGF-1 release. Interestingly, S100 proteins (22) Syn-1 (8) and FGF-1 (4) are all well characterized as acidic phospholipid-binding proteins and since the p40 Syn-1 and FGF-1 components of the aggregate released from cells in response to heat shock are sensitive to denaturant agents, it is possible that the ability of these proteins to interact with phosphatidylinerine may be a common feature of other unknown protein components of this release pathway. Indeed, gel shift analysis of the denaturant-sensitive component of the low heparin-binding affinity p40 Syn-1 and FGF-1 aggregate (5) anticipated the presence of a protein (~10 kDa) with an apparent molecular weight similar to that predicted from the S100A13 cDNA (16).

Since the FGF prototypes are well known for their angiogenic potential (47, 48) and tumor metastasis and growth is dependent upon tumor angiogenesis in vivo (51, 52), it is also not surprising that the expression of many of the S100 gene family members correlates with metastatic tumor potential (53–56), and in one instance is diagnostic for human melanoma metastatic potential (57, 58). Indeed, it is possible that S100 family members support neoplastic and pro-inflammatory situations in vivo by their ability to participate in the release pathway for extracellular signal peptide-less angiogenic and inflammatory signals such as FGF-1 and IL-1α. Interestingly, several S100 gene family members are also known to interact with members of the annexin gene family (59–61), and like Syn-1 and S100 gene family members, the annexins are Ca²⁺-dependent acidic phospholipid-binding proteins (62). Annexin II is a particularly interesting member of this family since it has been implicated in the regulation of exocytosis (63, 64), and this activity appears to be mediated by its ability to be associated with S100A10 (p11) to form an annexin II-p11 aggregate (65, 66). Whereas annexin II is known to interact with plasminogen and plasminogen activators on the cell surface (67), annexin II, like S100A13 and FGF-1, does not contain a classical signal sequence for ER-Golgi-mediated secretion (63, 68).
Although it is not known whether S100A13 is able to interact with annexin II on the inner surface of the plasma membrane, annexins I through V were identified together with S100A13 as amlexanox-binding proteins [25].

The ability of amlexanox to repress the release of FGF-1 and the extravasicular p40 domain of p65 Syn-1 from NIH 3T3 cells in response to heat shock is noteworthy since S100A13 is an amlexanox-binding protein [25]. Thus it is possible that amlexanox may interfere with the putative interaction between intracellular S100A13 and the FGF-1 and Syn-1 aggregate. Since amlexanox does not repress the appearance of the constitutively released p40 Syn-1 fragment in vitro, it is possible that amlexanox may be able to target the interaction between S100A13 and the putative FGF-1 and p65 Syn-1 aggregate. However, it is also possible that amlexanox may be interactive with other intracellular acidic phospholipid-binding proteins including members of the annexin gene family. Indeed, it is intriguing that the S100A13 [22], FGF-1 [4], and Syn-1 [8] components of this novel release pathway bind membrane phospholipids, since the ability of amlexanox to interfere with either the metabolism or the membrane release of lipid mediators of the inflammatory response, such as arachidonic acid derivatives, has served as the basis to explain the mechanism of action of amlexanox pharmacology [26-29].

Access to structural analogs of amlexanox has enabled us to define the importance of the isopropyl side chain as a functional element of its ability to repress the heat shock-induced release of FGF-1 and p40 Syn-1 in vitro. Since the modification of the amino group as well as conversion of the isopropyl side chain to an ethyl group did not influence the effectiveness of amlexanox to repress FGF-1 release in response to temperature stress, we suggest that these derivatives may serve as appropriate negative controls for these in vitro studies. Indeed, the general effect of these drugs on membrane permeability was measured by the release of lactate dehydrogenase, and amlexanox was not able to augment the presence of lactate dehydrogenase in the extracellular compartment under any condition. Interestingly, cromolyn, the parent drug used as the model for the development of amlexanox [69, 70], was also able to inhibit in a dose-dependent manner the release of FGF-1 and p40 Syn-1 from NIH 3T3 cell FGF-1 transfectants in response to temperature stress, but the data are not shown because contrary to amlexanox, cromolyn treatment resulted in significant dose-dependent increases in membrane permeability as measured by the presence of cytosolic lactate dehydrogenase in the extracellular compartment.

These data also reinforce the biological significance of the FGF-1 release pathway previously elucidated by the use of in vitro methods [2-5, 13] since insight into the role of S100A13 as a potential participant in the FGF-1 release pathway was derived from in vivo data using extracts of ovine brain. Since (i) the brain-derived aggregate appears to be partially resistant to both temperature denaturation and treatment with guanidine HCl, (ii) the known components of the brain-derived multiprotein aggregate bind acidic phospholipids, and (iii) the functional group within the structure of amlexanox responsible for the inhibition of FGF-1 and p40 Syn-1 release in vitro is the non-polar isopropyl side chain, we anticipate that phospholipid metabolism may not only play an important role in the mechanism of FGF-1 homodimer release but also in the regulation of the unknown intracellular responsibilities assumed by the FGF-1 monomer. With regard to the latter, we suggest that since 100 gene family members are well characterized as calcium-binding proteins that have calmodulin-like activities (17, 32, 35, 45), this function may involve structural aspects of the filamentous cytoskeleton and targets of the Ca²⁺/calmodulin-dependent protein kinases.
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