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Published July 22, 2002

JCB Report

The intracellular translocation of the components of the fibroblast growth factor 1 release complex precedes their assembly prior to export

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The release of signal peptideless proteins occurs through nonclassical export pathways and the release of fibroblast growth factor (FGF)1 in response to cellular stress is well documented. Although biochemical evidence suggests that the formation of a multiprotein complex containing S100A13 and Synaptotagmin (Syt)1 is important for the release of FGF1, it is unclear where this intracellular complex is assembled. As a result, we employed real-time analysis using confocal fluorescence microscopy to study the spatio-temporal aspects of this nonclassical export pathway and demonstrate that heat shock stimulates the redistribution of FGF1 from a diffuse cytosolic pattern to a locale near the inner surface of the plasma membrane where it colocalized with S100A13 and Syt1. In addition, coexpression of dominant-negative mutant forms of S100A13 and Syt1, which both repress the release of FGF1, failed to inhibit the stress-induced peripheral redistribution of intracellular FGF1. However, amlexanox, a compound that is known to attenuate actin stress fiber formation and FGF1 release, was able to repress this process. These data suggest that the assembly of the intracellular complex involved in the release of FGF1 occurs near the inner surface of the plasma membrane and is dependent on the F-actin cytoskeleton.

Introduction

The majority of secreted proteins contain a cleavable NH_2-terminal signal peptide sequence which allows their release through the secretory pathway mediated by the ER and Golgi apparatus (Blobel, 1995). However, a group of proteins, including transglutaminase 2 (Steinhoff et al., 1994), HIV-TAT (Chang et al., 1997), interleukin (IL)* 1α and 1β (Siders and Mizel, 1995; Tarantini et al., 2001), fibroblast growth factors (FGFs) 1 and 2 (Jackson et al., 1992; Mignatti et al., 1992; Florkiewicz et al., 1995), Sphingosine-1-Kinase (Ancellin et al., 2002), the extravesicular fragment of Synaptotagmin (Syt)1 (LaVallee et al., 1998), Annexin2 (Kim and Hajjar, 2002), and S100 proteins (Donato, 2001) are devoid of a signal peptide sequence but are released into the extracellular compartment. The mechanisms underlying the release of these signal peptideless polypeptides has recently become the subject of investigation as a result of their physiological significance as regulators of angiogenesis, tumor growth, inflammation, cell proliferation, and differentiation (Stylianou and Saklatvala, 1998; Friesel and Maciag, 1999; Donato, 2001). Although the precise mechanism responsible for the release of these signal peptideless polypeptides is not known, recent evidence with FGF1 suggests that it requires the assembly of a multiprotein complex that may function to facilitate transport to the inner leaflet of the plasma membrane (Landriscina et al., 2001a).

The release of FGF1 in response to heat shock and hypoxia (Friesel and Maciag, 1999) requires the formation of a Cys 30-mediated FGF1 homodimer (Jackson et al., 1995; Tarantini et al., 1995) as well as the association of FGF1 with the extravesicular p40 fragment of p65 Syt1, an integral transmembrane protein participating in secretory vesicle docking (LaVallee et al., 1998; Tarantini et al., 1998), and S100A13, a member of the family of intracellular calcium-binding S100 proteins (Mouta-Carreira et al.,

The online version of this article contains supplemental material.

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*Abbreviations used in this paper: FGF, fibroblast growth factor; IL, interleukin; SDS, sodium dodecylsulfate; Syt, Synaptotagmin; TTM, tetrathiomolybdate.

Key words: fibroblast growth factor; heat shock; S100A13; Synaptotagmin1; confocal microscopy

Supplemental Material can be found at:
http://jcb.rupress.org/content/suppl/2002/07/19/jcb.200203084.DC1.html

Published July 22, 2002

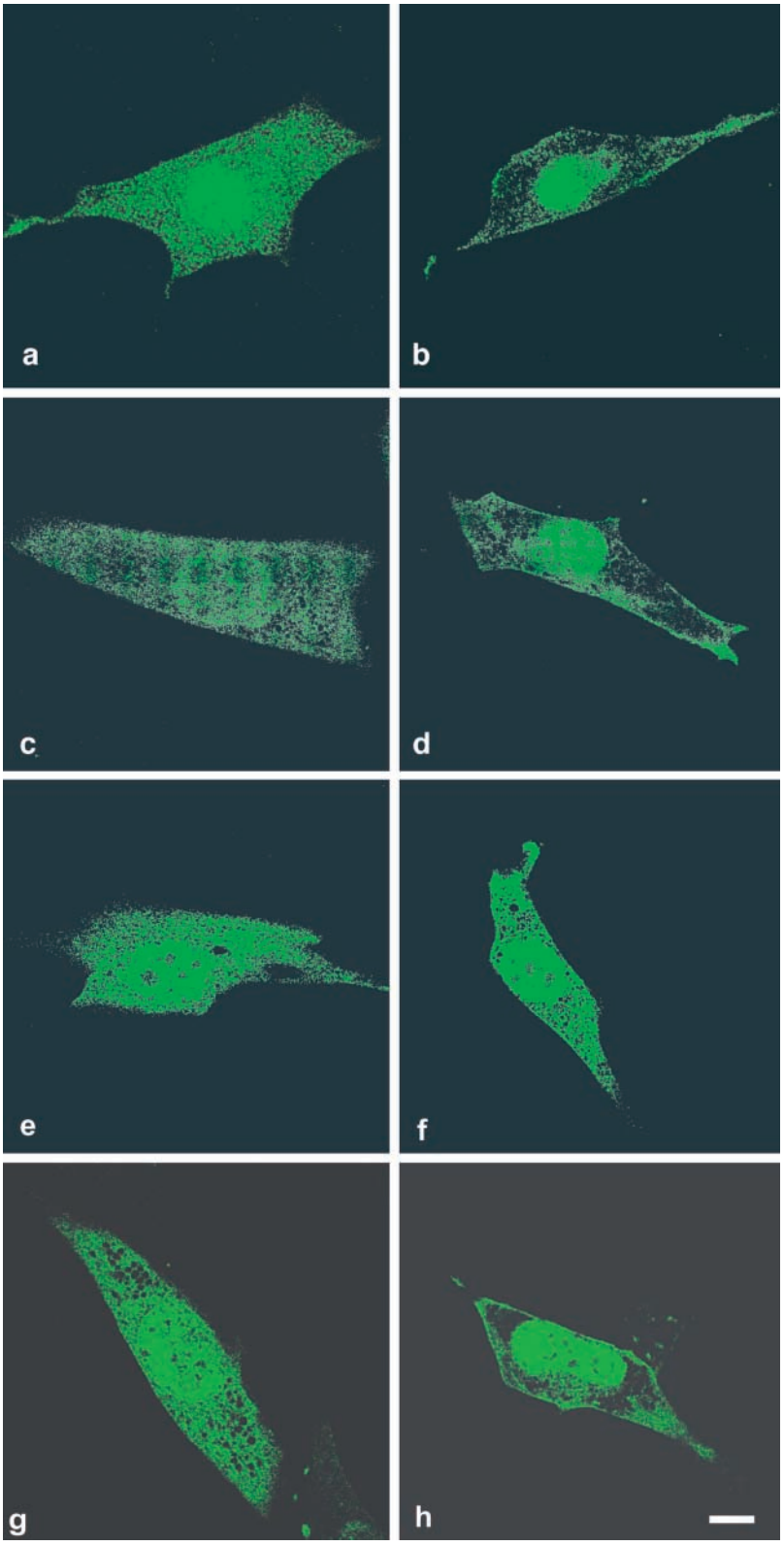

Figure 1. **The heat shock–induced redistribution of cytosolic FGF1.** FGF1:HA NIH 3T3 cell transfectants, (a and b), FGF1:GFP NIH 3T3 cell transfectants (c, d, g, and h), and GFP NIH 3T3 cell transfectants (e and f) were fixed after 2 h incubation at 37°C (a, c, and e) or at 42°C (b, d, f, g, h) in the absence (a–f) or presence of either amlexanox (g) or TTM (h) and processed for fluorescence microscopy as described in Materials and methods. Confocal images of median horizontal cell sections were taken using the 100× objective. Bar, 10 μM.

1998; Landriscina et al., 2001b). Recently, it has been demonstrated that the formation of a multiprotein release complex composed of FGF1, S100A13, and p40 extravesicular domain of Syt1 requires the oxidative function of copper (Landriscina et al., 2001a). Interestingly, the export of IL1α, another signal peptideless protein with striking crystallographic and structural similarities to the FGFs, is also induced by temperature stress (Tarantini et al., 2001) and involves the Cu^{2+}-mediated formation of a protein complex containing S100A13 (unpublished data). The stress-induced export of both FGF1 and IL1α is energy dependent (Jackson et al., 1995; Tarantini et al., 2001), requires de novo transcription and translation (Jackson et al., 1992; Tarantini et al., 2001), and is inhibited by amlex-

Published July 22, 2002

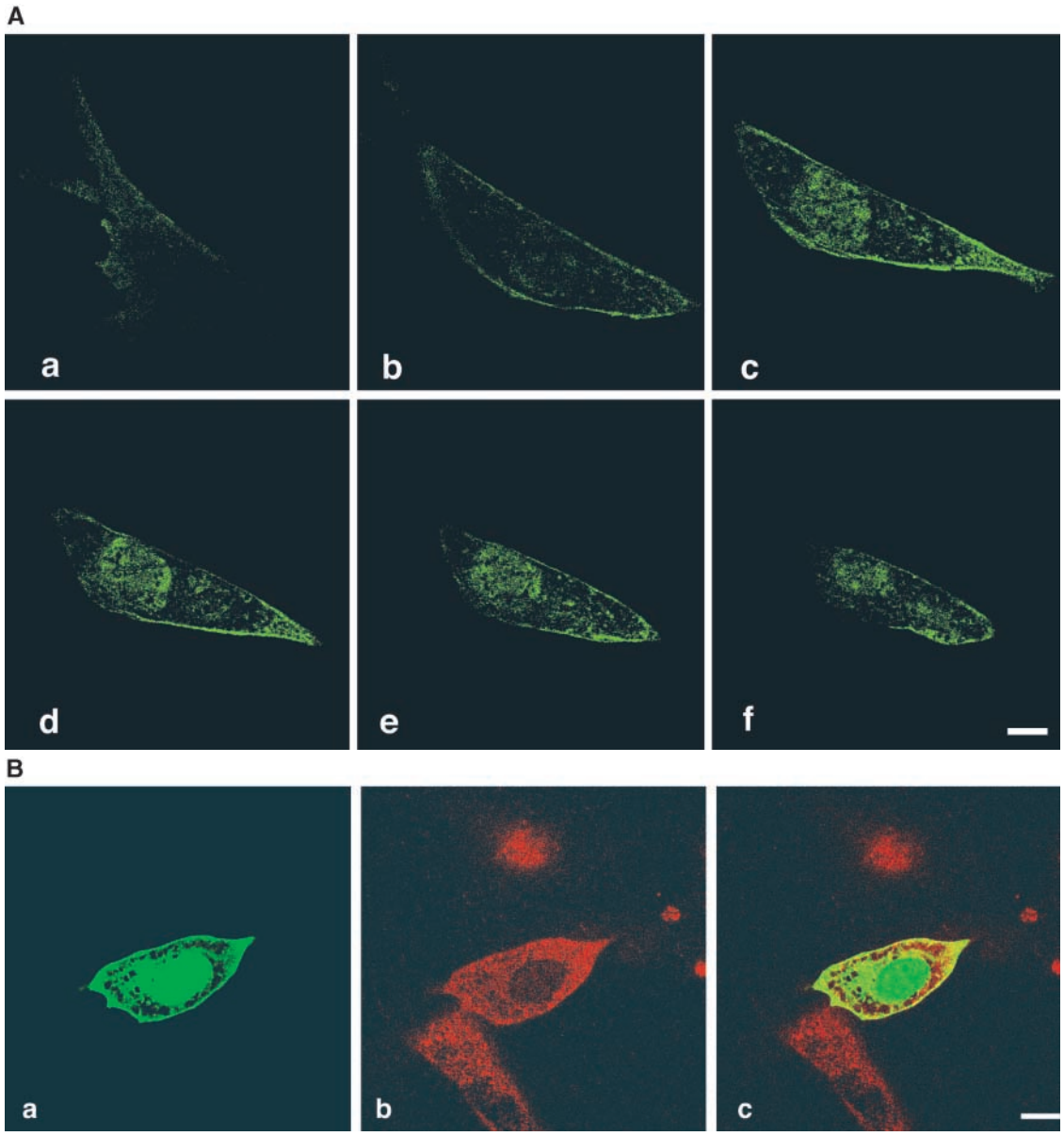

Figure 2. **The three-dimensional redistribution of cytosolic FGF1:GFP.** (A) FGF1:GFP NIH 3T3 cell transfectants were fixed after 2 h of heat shock and a series of six horizontal confocal sections were taken starting from the bottom to the top (a–f) of a cell using a 100× objective. Bar, 10 μM. (B) Heat shock does not induce the peripheral redistribution of Erk1 and Erk2 in FGF1 NIH 3T3 cell transfectants. NIH 3T3 cells were transiently transfected with FGRF1:GFP, fixed after 2 h of heat shock, and stained with antibodies against Erk1 and Erk2 and by a TRITC-conjugated secondary antibody. Confocal images of median horizontal cell sections were taken using the 100× objective and the intracellular distribution of FGF1: GFP (a), Erk1 and Erk2 (b), and their overlay (c) are shown. Bar, 10 μM.

anox (Mouta-Carreira et al., 1998; Tarantini et al., 2001), a reagent known to bind S100A13 (Shishibori et al., 1999) and attenuate the assembly of the actin cytoskeleton (Landriscina et al., 2000). In addition, the stress-induced release of both proteins is resistant to brefeldin A, an inhibitor of ER-Golgi–mediated secretion (Jackson et al., 1992; Tarantini et al., 2001).

Although insight into the mechanism responsible for the release of FGF1 has been achieved, the spatio-temporal properties of this process have remained obscure. Here we describe the release of FGF1 using a real time imaging approach and demonstrate that heat shock induces a significant alteration in the cytosolic distribution of FGF1 that is manifested by the accumulation of this protein at the cell periphery, near the inner surface of the plasma membrane where it colocalizes with S100A13 and Syt1.

Results and discussion

Before performing the confocal fluorescence microscopy studies of the stress-mediated redistribution of intracellular FGF1, we examined the heat shock-induced release of the various FGF1 chimeric polypeptides from NIH 3T3 cell transfectants using immunoblot analysis in order to determine whether the GFP or HA tag was able to impair stress-induced release. We observed that after 2 h at 42°C, both the FGF:GFP and FGF1:HA chimeric proteins were present in media conditioned by temperature stress (unpublished

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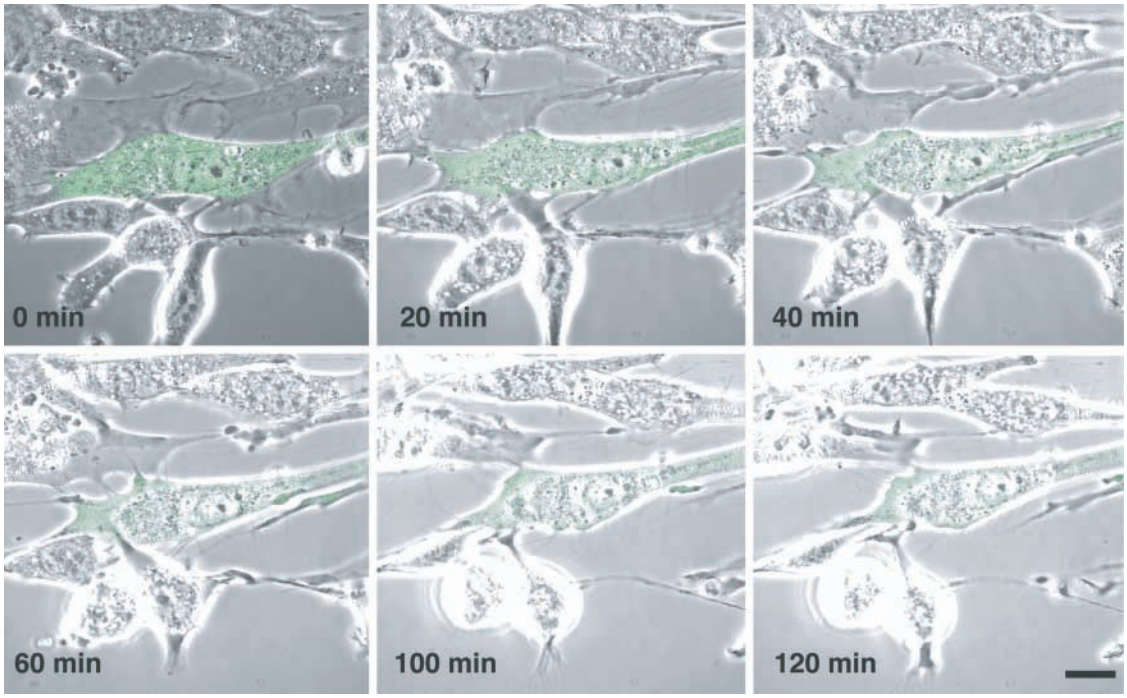

Figure 3. **The real-time visualization of the redistribution of cytosolic FGF1:GFP.** FGF1:GFP NIH 3T3 cell transfectants were incubated for 2 h in an environmentally controlled microscope chamber under heat shock conditions and the real-time imaging of an individual cell was performed combining phase contrast and confocal fluorescence microscopy using the 100× objective. Images were taken at 2-min intervals during the heat shock period. Selected images over this time period are presented. Bar, 10 μM. Video 1 is available at http://www.jcb.org/cgi/content/full/jcb.200203084/DC1.

data), and these results were similar to those observed with either the FGF1 or FGF1:β-galactosidase NIH 3T3 cells transfectants (Jackson et al., 1992; Shi et al., 1997).

In the first series of experiments, the influence of heat shock on the intracellular distribution of FGF1 was studied using cells stably transfected with either FGF1:HA (Fig. 1, a and b) or FGF1:GFP (Fig. 1, c and d). Under normal cell culture conditions, both FGF1:HA and FGF1:GFP were diffusely distributed throughout the cytosol of the NIH 3T3 cell transfectants (Fig. 1, a and c). Interestingly, after 2 h of heat shock, we observed a significant redistribution of both FGF1:HA (Fig. 1 b) and FGF1:GFP (Fig. 1 d) to the cell periphery in ~15 to 25% of the cells as suggested by an increase in staining intensity at this site. Cells were considered to exhibit the peripheral redistribution of FGF1 if the majority (>66%) of the perimeter demonstrated more intense fluorescence than the adjacent internal area. In contrast, <1% of the NIH 3T3 cells transfected with GFP demonstrated peripheral localization of the GFP reporter after heat shock (Fig. 1, e and f). Kinetic analysis of this redistribution process revealed that ~5% of FGF1:GFP NIH 3T3 cell transfectants displayed FGF:GFP at the cell periphery following 60 min of heat shock and this ratio increased to ~10–15% and 15–25% by 90 and 120 min, respectively. However, we should note that even though we utilized clonal populations of stable FGF1 NIH 3T3 cell transfectants for these studies, we observed heterogeneity of FGF1 expression within this population. Therefore, the peripheral redistribution of FGF1 was recorded from those cells which exhibited high levels of FGF1 protein expression, although similar changes were also observed with cells exhibiting lower levels of FGF1 protein expression.

In order to study the three-dimensional distribution of FGF1:GFP under heat-shock conditions, we obtained serial stacks of confocal sections of individual cells encompassing the entire cell volume, from bottom to top. The serial sectioning of the FGF1:GFP NIH 3T3 cell transfectants after 2 h of heat shock revealed a stress-induced peripheral redistribution of FGF1 throughout the entire volume of the cytosol (Fig. 2, A and B).

The FGF1:GFP NIH 3T3 cell transfectants were also used to assess the effect of heat shock on intracellular distribution of FGF1 in living cells using real time confocal microscopy in vivo. Individual FGF1:GFP NIH 3T3 cell transfectants were selected under the inverted fluorescence microscope and phase contrast microscopy was used to focus approximately on a median horizontal section. At this point, heat-shock conditions were established in the environmental chamber and a real-time record that combined phase contrast and confocal fluorescence was obtained. In agreement with the previous results acquired using fixed cells, ~20% of the FGF:GFP NIH 3T3 cell transfectants exhibited stress-induced redistribution of FGF1:GFP to a peripheral locale near the plasma membrane. Within 1 h after the onset of heat shock, FGF1:GFP redistributed itself from its diffuse cytosolic locale to the cell periphery (Fig. 3), and after 2 h, the majority of intracellular FGF1:GFP was concentrated near the inner surface of the plasma membrane and this staining was especially exaggerated in cytoplasmic extensions (Fig. 3). In contrast, we did not observe any redistribution of FGF:GFP to the cell periphery when the FGF1:GFP NIH 3T3 cell transfectants were incubated under control conditions (37°C, 2 h). In addition, NIH 3T3 cells transfected with GFP also demon-

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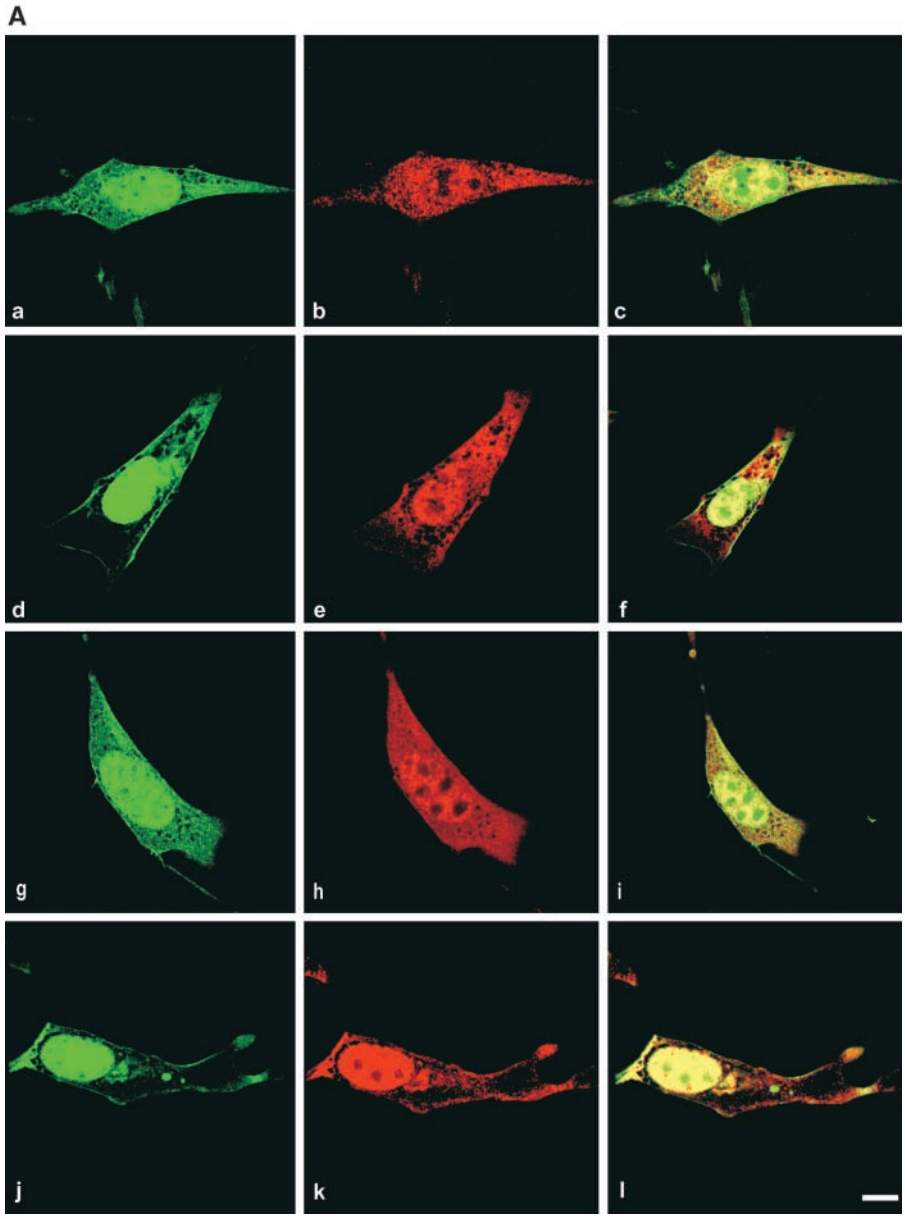

Figure 4. **Dominant-negative mutants of S100A13 and Syt1 do not affect the heat shock–induced redistribution of cytosolic FGF1:GFP.** (A) NIH 3T3 cells were transiently cotransfected with FGF1:GFP and either S100A13:Myc (a–f) or S100A13Δ88–98:Myc (g–l), and 48 h later the cells were fixed after a 2-h incubation at either 37 (a–c and g–i) or 42°C (d–f and j–l). The cells were stained with an anti-Myc antibody followed by an Alexa-647–conjugated secondary antibody. Confocal images of median horizontal cell sections were taken using the 100X objective and the intracellular distribution of FGF1:GFP (a, d, g, and j), S100A13:Myc (b and e), S100A13Δ88–98Myc (h and k), and their respective overlays (c, f, i, and l) are shown. Bar, 10 μM. (B) NIH 3T3 cells were transiently cotransfected with FGF1:GFP and either Syt1:Myc (a–f) or Syt1Δ120–214:Myc (g–l) and 48 h later the cells were fixed after 2 h of incubation at either 37 (a–c and g–i) or 42°C (d–f and j–l). The cells were stained with either an anti-Syt1 (b and e) or an anti-Myc (h and k) antibody followed by an Alexa 647–conjugated secondary antibody. Confocal images of median horizontal cell sections were taken using the 100× objective and the intracellular distribution of FGF1:GFP (a, d, g, and j), Syt1:Myc (b and e), Syt1Δ120–214:Myc (h and k), and their respective overlays (c, f, i, and l) are shown. Bar, 10 μM.

strated a homogenous cytosolic distribution of GFP at all the stages after heat shock (unpublished data).

The similarity between the kinetics of FGF1 release into media conditioned by heat shock and the peripheral redistribution of intracellular FGF1 observed in vivo prompted us to examine the pharmacology of FGF1 redistribution using reagents known to inhibit FGF1 export including amlexanox (Mouta-Carreira et al., 1998) a known attenuator of actin stress fiber formation (Landriscina et al., 2000) which is able to specifically bind S100A13 (Shishibori et al., 1999), an important component of the FGF1 release complex. We observed that amlexanox (0.3 mM) was able to completely repress the temperature-sensitive redistribution of the cytosolic FGF1:GFP (Fig. 1 g). In addition, brefeldin A (1 μg/ml), an inhibitor of classical ER-Golgi–mediated protein secretion which does not interfere with FGF1 release in vitro (Jackson et al., 1992), failed to prevent the heat shock–mediated redistribution of cytosolic FGF1:GFP to the inner surface of the plasma membrane (unpublished data). These data suggest that traditional ER-Golgi organelle traffic is not involved in the release of FGF1 in response to temperature stress, whereas the actin cytoskeleton may be a critical component for intracellular FGF1 redistribution. We have also recently demonstrated that the stress-induced release of FGF1 is dependent on the oxidative function of intracellular copper ions (Landriscina et al., 2001a) and tetrathiomolybdate (TTM), a specific copper chelator represses the heat shock–dependent export of FGF1 from NIH 3T3 cell transfectants (Landriscina et al., 2001a). Additional data obtained using a cell-free system suggest that the oxidative character of copper ions may facilitate the formation of a multiprotein FGF1 release complex which contains the FGF1 homodimer (Engleka and Maciag, 1992), p40 Syt1, and S100A13 (Landriscina et al., 2001a). Based on these observations, we decided to investigate whether intracellular copper oxidation was involved in the stress-induced peripheral translocation of FGF1. Interestingly, the incubation of FGF1:GFP NIH 3T3 cell transfectants with 500 nM TTM

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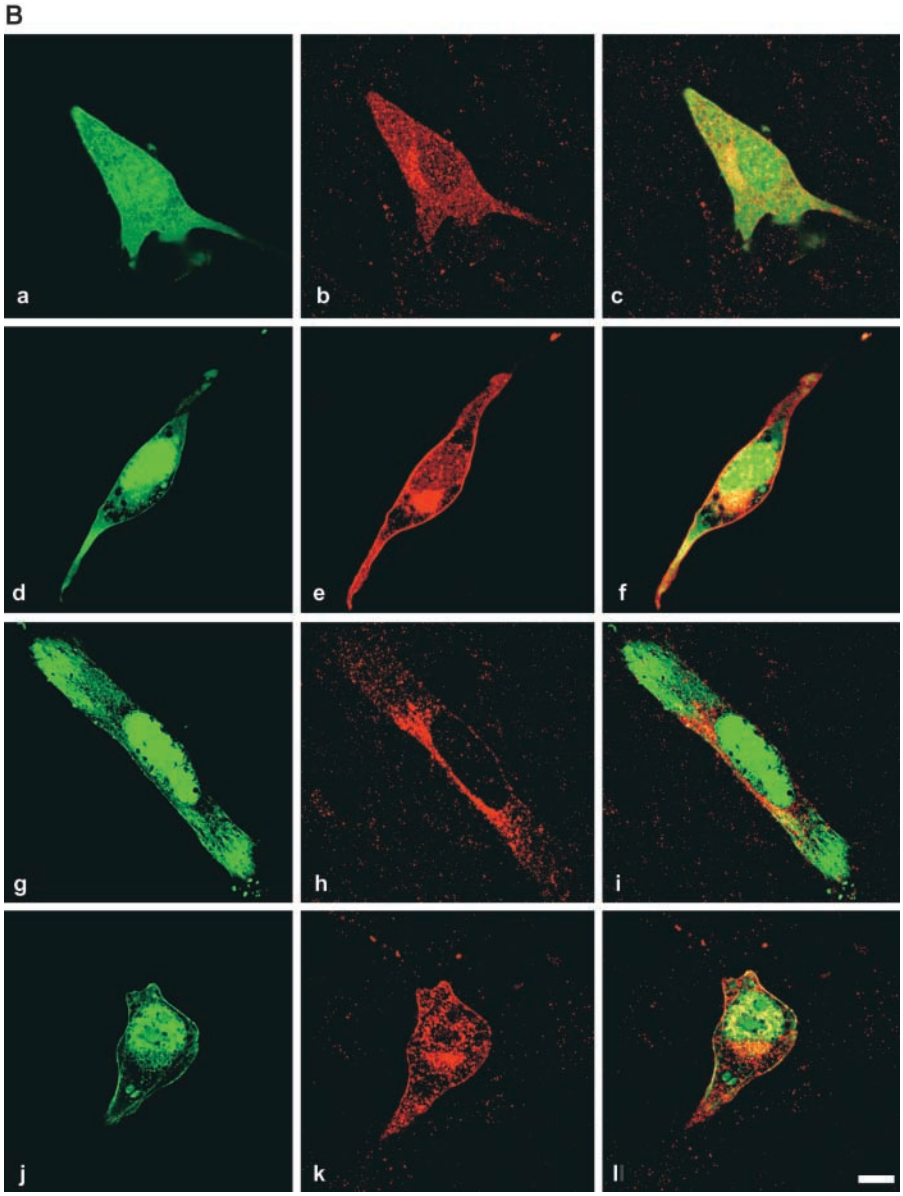
Figure 4 (continued from previous page)



24 h before and during cellular stress did not attenuate the peripheral redistribution of cytosolic FGF1:GFP (Fig. 1 h).

The observation that intracellular copper is apparently dispensable for FGF1 transport to the cell periphery prompted us to investigate whether Syt1 and S100A13 proteins, which are indispensable for the assembly and export of the FGF1 release complex, also exhibit similar intracellular trafficking properties. Transient cotransfection experiments using equimolar amounts of FGF1:GFP and S100A13:Myc cDNA constructs followed by immunofluorescence confocal microscopy demonstrated a diffuse cytosolic distribution of both proteins in the majority of all cells at 37°C (Fig. 4 A, a–c). After heat shock, ~20% of the transient cotransfectants demonstrated a peripheral accumulation of FGF1 which was accompanied by a detectable, but less pronounced redistribution of S100A13 near the inner surface of the plasma membrane (Fig. 4 A, d–f). In addition, the peripheral locale of FGF1:GFP and S100A13:Myc exhibited significant colocalization (yellow) as suggested by the GFP (green) and Myc (red) epitopes for FGF1 and S100A13, respectively (Fig. 4 A, f). We have previously observed that the deletion of the COOH-terminal basic residue-rich domain from S100A13 (amino acid residues 88–98) represses the stress-induced release of FGF1 (Landriscina et al., 2001b). Interestingly, NIH 3T3 cells cotransfected with FGF1:GFP and the S100A13Δ88-98:Myc dominant-negative mutant did not exhibit an attenuation of the redistribution of either FGF1:GFP or S100A13Δ88-98:Myc to the cell periphery (Fig. 4 A, j–l). Similar results were also obtained using FGF1:GFP and Syt1 or the FGF1:GFP and dnSyt1 mutant, Syt1Δ120-214:Myc NIH 3T3 cell cotransfectants, which is known to repress the heat shock–induced release of FGF1 (LaVallee et al., 1998). As shown in Fig. 4 B, FGF1:GFP colocalized with Syt1 near the plasma membrane and the expression of the dnSyt1 mutant did not prevent the stress-induced peripheral redistribution of FGF1:GFP (Fig. 4 B). However, we did not observe any change in the intracellular distribution of either β-gal or GFP (Fig. 1, e and f; unpublished data) as well as

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Erk1 and Erk2 (Fig. 2 B) in response to heat shock, suggesting that the stress-induced intracellular redistribution of FGF1, S100A13, and Syt1 are novel properties of these proteins and are not artifacts of the stress-induced response.

These data suggest that the accumulation of FGF1 near the inner surface of the plasma membrane precedes its assembly and release as a multiprotein complex. The translocation of FGF1 to the cell periphery may be associated with one of the systems required for intracellular vesicle transport such as components of the cytoskeleton including microtubules and actin filaments (Rogers and Gelfand, 2000). The inhibition of the redistribution of intracellular FGF1 with amlexanox suggests that actin stress fibers may be used to direct the temperature-sensitive transport of FGF1 to the inner surface of plasma membrane. It is also interesting to note that amlexanox is able to specifically bind S100A13 (Shishibori et al., 1999) and some members of the S100 gene family have been demonstrated to be associated with actin stress fibers (Mandinova et al., 1998). Thus, it is possible that the multiprotein FGF1 release complex containing S100A13 and Syt1 (LaVallee et al., 1998; Mouta-Carreira et al., 1998; Landriscina et al., 2001a) uses S100A13 for the interaction with the cytoskeletal elements. However, the failure of the dnS100A13 mutant to inhibit the redistribution of FGF1 to the cell periphery suggests that either S100A13 may not participate in the stress-induced redistribution of FGF1 or at least, that the COOH-terminal basic-rich domain of S100A13, although crucial for FGF1 export, is dispensable for the transport of FGF1 to plasma membrane. Interestingly, similar observations were also made using the dnSyt1 mutant in which expression of the Syt1 mutant did not attenuate the redistribution of FGF1 to the inner surface of the plasma membrane yet did prevent the export of FGF1 in response to stress (LaVallee et al., 1998). While these data suggest that neither S100A13 nor Syt1 play a role in the intracellular translocation of FGF1 to the plasma membrane, it is interesting that both S100A13 and Syt1 are indeed redistributed and colocalized with FGF1 near the inner surface of the plasma membrane Thus, it is possible that the individual components of the FGF1 release complex may rely on independent pathways for their redistribution in response to stress which precedes the assembly of the multiprotein complex on the inner leaflet of the plasma membrane. Indeed, this is not only consistent with the ability of FGF1, Syt1, and S100A13 to associate with acidic phospholipids (Mach and Middaugh, 1995; Fernandez et al., 2001) which may be involved in the copper-sensitive assembly of this complex (Landriscina et al., 2001a), but also with the stress-induced flipping of acidic phospholipids especially phosphatidylserine which may be involved in facilitating transmembrane translocation of the complex (Arduini et al., 1989). In addition, it is also possible that the molten globule character of FGF1 (Mach and Middaugh, 1995) may enable Syt1 and S100A13 to traverse the plasma membrane. In any event, this may be analogous to the basic principle of military science: The units use separate roads to get together in the decisive point at the decisive moment (Clausewitz, 1989).

Materials and methods

DNA constructs

Human FGF1 constructs with COOH-terminal fusions of either HA or GFP were both provided by Andrew Baird (Kings College, London, UK). The FGF1:HA and FGF1:GFP chimeras were cloned into the pCR3.1 vector. As a control, we utilized GFP cloned also into pCR3.1. Wild-type rat Syt1 and the dominant-negative deletion mutant Syt1Δ120-214 were both fused at their COOH termini with a single Myc tag and cloned into the pMEXhygra vector as described (LaVallee et al., 1998). Wild-type murine S100A13 and the dominant negative deletion mutant S100A13Δ88-98 were both fused at their COOH termini with a tag containing six Myc epitopes and cloned into the pcDNA3.1 (Hygro) vector as described (Landriscina et al., 2001b).

Cell culture and transfections

The genes of interest were expressed in NIH 3T3 cells (American Type Culture Collection). Cells were grown in DME (Cellgro) supplemented with 10% bovine calf serum (Hyclone) and an antibiotic-antimycotic mixture as described (Jackson et al., 1992). The Fugene transfection kit (Roche) was used according to the manufacturer's protocol to generate stable transfectants of NIH 3T3 cells expressing FGF1:HA, GFP, and FGF1:GFP and transient cotransfectants of NIH 3T3 cells with FGF1:GFP and Syt1:Myc, S100A13:Myc, and their mutants. Stable NIH 3T3 cell transfectants were selected in medium containing 800 ug/ml G418. Clonal populations of FGF1:GFP and GFP NIH 3T3 cell transfectants were chosen on the basis of green fluorescence detected using an inverted fluorescence microscope (Olympus). The expression of FGF1:GFP and FGF1:HA in transfectant clones was verified by FGF1 immunoblot analysis and the heat shock–induced release of FGF1:GFP and FGF1:HA was studied as previously described (Jackson et al., 1992).

Immunofluorescence and confocal microscopy

Stable NIH 3T3 cell transfectants were plated on fibronectin-coated (10 $\mu g/cm^2$) glass coverslips in 6-well TC plates at 10^5 cells/well. 24 h later, the culture medium was removed, serum-free DME supplemented with 10 U/ml heparin (Sigma-Aldrich) was added, and cells were further incubated for various time periods at either 42 or 37°C as described previously (Jackson et al., 1992). Cells were subsequently fixed for 10 min with 4% formaldehyde in PBS. Transient transfections were performed 12–24 h after plating the NIH 3T3 cells on coverslips at a density of 2×10^4 cells/well, and the heat shock experiments were conducted 72 h later which was followed by 4% formaldelyde fixation and immunofluorescence staining.

After fixation for 10 min, the cells were washed twice with PBS, blocked for 1 h in blocking buffer (PBS containing 10% bovine serum albumin, 0.1% Tween 20, 0.1% Triton X-100, and 0.1% NaN_3), incubated for 1 h with a monoclonal anti-HA antibody (Covance) diluted at 1 μg/ml in blocking buffer, washed three times with PBS, incubated for 1 h with a fluorescine-conjugated anti–mouse IgG antibody (Sigma-Aldrich) diluted ×100 in blocking buffer, washed three times with PBS, and embedded in PBS containing 10% glycerol and DABCO (1 mg/ml). The detection of Myc-tag epitope in wild-type and mutant S100A13 as well as in the Syt1 mutant in NIH 3T3 cell cotransfectants was performed using a similar method but employing a monoclonal anti-Myc tag antibody (Oncogene) and an Alexa 647–conjugated anti–mouse IgG antibody (Molecular Probes). A monoclonal antibody to the extravesicular fragment of Syt1 (Synaptic Systems) followed with an Alexa 647–conjugated anti–mouse IgG antibody was used to detect wild-type Syt1 and rabbit antibodies against Erk1 and Erk2 were obtained from Santa Cruz Biotechnology, Inc. The LTCS-SP confocal system (Leica) equipped with an inverted DMIRBE microscope was used for these studies. Cells were examined using a 100× objective and the 237 μM confocal pinhole of the Leica 2000 confocal software program.

The real-time in vivo observations were performed using FGF1:GFP NIH 3T3 cell transfectants plated on fibronectin-coated (10 $\mu g/cm^2$) 30-mm Petri dishes with glass coverslip bottoms (MatTec) seeded at 10^5 cells/dish. After 24 h, the medium was removed and serum-free DME supplemented with 10 U/ml heparin was added. The cell culture dishes were placed in a microscopy chamber (Leica) equipped with CO_2 and temperature control and mounted on the stage of the inverted microscope DMIRBE (Leica), a component of the confocal system LTCS-SP system (Leica). For the real-time in vivo observations, confocal pictures of either heat-shocked (42°C) or control (37°C) FGF1:GFP NIH 3T3 cell transfectants were taken every 2 min over the course of 2 h.

Online supplemental material

The real time visualization of the redistribution of cystolic FGF1:GFP. FGF1:GFP NIH 3T3 cell transfectants were incubated for 2 h in an environmentally controlled microscope chamber under heat shock conditions

and the real time imaging of an individual cell was performed combining phase contrast and confocal fluorescence microscopy using the 100× objective. Images were taken at 2-min intervals during the heat shock period. Selected images over this time period are presented as Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200203084/DC1.

The authors thank Andrew Baird for providing the expression constructs for FGF1:HA, FGF1:GFP, and GFP, and Norma Albrecht and Gloria Ledoux for expert administrative assistance.

This work was supported by National Institutes of Health grants RR15555, HL35627, and AG07450 to Thomas Maciag.

Submitted: 19 March 2002
Revised: 28 May 2002
Accepted: 30 May 2002

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