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S100A13 mediates the copper-dependent stress-induced release of IL-1α from both human U937 and murine NIH 3T3 cells

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Summary

Copper is involved in the promotion of angiogenic and inflammatory events in vivo and, although recent clinical data has demonstrated the potential of Cu^{2+} chelators for the treatment of cancer in man, the mechanism for this activity remains unknown. We have previously demonstrated that the signal peptide-less angiogenic polypeptide, FGF1, uses intracellular Cu^{2+} to facilitate the formation of a multiprotein aggregate that enables the release of FGF1 in response to stress and that the expression of the precursor form but not the mature form of IL-1α represses the stress-induced export of FGF1 from NIH 3T3 cells. We report here that IL-1α is a Cu^{2+}-binding protein and human U937 cells, like NIH 3T3 cells, release IL-1α in response to temperature stress in a Cu^{2+}-dependent manner. We also report that the stress-induced export of IL-1α involves the intracellular association with the Cu^{2+}-binding protein, S100A13. In addition, the expression of a S100A13 mutant lacking a sequence novel to this gene product functions as a dominant-negative repressor of IL-1α release, whereas the expression of wild-type S100A13 functions to eliminate the requirement for stress-induced transcription. Lastly, we present biophysical evidence that IL-1α may be endowed with molten globule character, which may facilitate its release through the plasma membrane. Because Cu^{2+} chelation also represses the release of FGF1, the ability of Cu^{2+} chelators to potentially serve as effective clinical anti-cancer agents may be related to their ability to limit the export of these proinflammatory and angiogenic signal peptide-less polypeptides into the extracellular compartment.

Key words: Copper, Fibroblast growth factor, Interleukin 1, S100A13, Tetrathiomolybdate

Introduction

The prototype members of the IL-1 gene family are well recognized for their inflammatory and angiogenic activities in vitro and in vivo (Dinarello, 1994; Dinarello, 1998; Krakauer, 1986; Maini and Taylor, 2000). IL-1α and IL-1β are initially synthesized as precursor proteins and, whereas IL-1α is processed to its mature form by calpain, IL-1β is processed by an aspartate-specific protease from the caspase family termed the IL-1β-converting enzyme (Kobayashi et al., 1990; Kostura et al., 1989). Although these cytokines exert their biological function through high affinity receptors, these prototypes lack a signal peptide sequence to direct their export through the classical secretion pathway mediated by the endoplasmic reticulum-Golgi apparatus (Lomedico et al., 1984). Surprisingly, crystallographic studies have demonstrated that, despite their unremarkable sequence similarities (Thomas et al., 1985), the IL-1 prototypes exhibit a high level of structural homology with the pro-angiogenic signal peptide-less prototype members of the FGF gene family (Carter et al., 1988; Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991), Interestingly, whereas the FGF gene family evolved at least three genes lacking a signal peptide sequence (Burgess and Maciag, 1989; Friesel and Maciag, 1999; McKeehan et al., 1998), eight of the ten members of the IL-1 gene family lack this structural feature (Kumar et al., 2000; Smith et al., 2000). Thus, it is important to understand and define the non-classical pathways used by these signal peptide-less cytokines for export, since this information may ultimately prove to be valuable for the clinical management of inflammatory and angiogenic-dependent events.

A wide variety of cell types have been shown to synthesize both IL-1α and FGF1; however, their two main sources in mammals are the peripheral blood monocytes and tissue macrophages, which use a yet unidentified mechanism for the export of both cytokines. In NIH 3T3 cells the release of FGF1 and IL-1α is regulated by convergent yet distinct pathways that use cellular stress to mediate their release into the extracellular compartment (Tarantini et al., 1995; Tarantini et al., 2001). It is known that FGF1 is released in response to stress as a latent homodimer that requires intracellular oxidation of a conserved cysteine residue at position 30 (Tarantini et al., 1995). This event enables FGF1 to interact with the extravesicular p40

domain of synaptotagmin 1 (Syt1) and S100A13 (Carreira et al., 1998; Landriscina et al., 2001b; LaVallee et al., 1998; Tarantini et al., 1998), and these interactions facilitate the release of FGF1 as a multiprotein aggregate containing p40 Syt1 and S100A13 (Landriscina et al., 2001a). Interestingly, whereas temperature stress induces the release of the mature but not the precursor form of IL-1α from NIH 3T3 cells, the expression of precursor IL-1α represses the release of FGF1 in response to stress (Tarantini et al., 2001).

Because (1) FGF1, S100A13 and Syt1 are Cu^{2+}-binding proteins (Engleka and Maciag, 1992; Landriscina et al., 2001a; Shing, 1988), (2) Cu^{2+}-induced oxidation facilitates the cell-free self assembly of FGF1, p40 Syt1 and S100A13 as a multiprotein complex (Landriscina et al., 2001a), (3) S100A13 expression facilitates the release of FGF1 independently of transcription (Landriscina et al., 2001b), and (4) the Cu^{2+} chelator, tetrathiomolybdate (TTM) inhibits the release of FGF1 in response to stress (Landriscina et al., 2001a), it is likely that intracellular Cu^{2+} metabolism may be responsible for the stress-induced oxidative event that facilitates the assembly and release of the multiprotein FGF1 complex. Since FGF1 and IL-1α exhibit remarkably similar crystallographic structures (Graves et al., 1990; Zhu et al., 1991), and both FGF1 and IL-1α are released in response to stress from NIH 3T3 cells (Tarantini et al., 2001), we questioned whether (1) intracellular Cu^{2+} is involved in the release of IL-1α in response to stress, (2) the release of IL-1 from human blood monocytes is also induced by cellular stress, (3) Cu^{2+} affinity is able to adsorb IL-1α from media conditioned by temperature stress, (4) the release of IL-1α could be modified by the expression of S100A13 and (5) whether IL-1α, like FGF1 (Mach and Middaugh, 1995), is endowed with molten globule character.

Materials and Methods

Cell lines, recombinant proteins and Cu^{2+} affinity

U937 cells (human monocytic leukemia cell line, ATCC) were grown in suspension in PRMI 1640 medium (Sigma) containing 10% fetal bovine serum (FBS) and activated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) for 24 hours prior to each experiment. Prior to heat shock (2 hours, 42°C) cells were plated on fibronectin (10 μg/cm^3)-coated cell culture dishes. Stable NIH 3T3 cell transfectants were generated using the following cDNA constructs cloned into pMEXneo: human S100A13 with an N-terminal fusion to a 6×Myc Tag (Myc-S100A13) (Landriscina et al., 2001b); human Myc-S100A13ΔBR (Landriscina et al., 2001b), human mIL-1α-βGal (residues 113-271) with and without the C-terminal β-galactosidase tag (Tarantini et al., 2001); and precursor IL-1α (residues 1-271) with a C-terminal β-galactosidase tag (Tarantini et al., 2001). Stable U937 cell transfectants were generated using a human FGF1 cDNA construct cloned into pMEXneo. Recombinant human IL-1α (residues 113-271) was generously provided by F. Hoffmann-LaRoche (Basel, Switzerland). Recombinant human S100A13 as well as the S100A13 basic residue-rich domain (residues 88-98) deletion mutant (S100A13ΔBR) were prepared as previously described (Landriscina et al., 2001b). The chimera consisting of IL-1α (residues 113-271) and red fluorescent protein (DsRed1) was obtained by cloning human IL-1α (Tarantini et al., 2001) in-frame between the *Xho*I and *Bam*HI restriction sites in the pDsRed1-N1 vector (Invitrogen) using specific adaptor PCR primers. Transient IL-1α-DsRed1 and EGFP-S100A13 NIH 3T3 cell cotransfectants were obtained using Fugene (Roche) and the protocol of the manufacturer. EGFP-S100A13 was generous gift of C.-A. Schoenenberger (Basel, Switzerland). The Cu^{2+}-affinity of human recombinant IL-1α was performed using methods previously described for recombinant human S100A13 (Landriscina et al., 2001b).

Adenoviral transduction of NIH 3T3 cells

An adenoviral vector expressing Myc-S100A13 was prepared as described (Hardy et al., 1997) at a concentration of approximately 10^{13} viral particles per ml. NIH 3T3 cells were transduced by incubation in serum-free DMEM with approximately 10^3 viral particles and poly-D-lysine (Sigma) for two hours at 37°C. After infection, the adenovirus-containing media was removed and replaced with serum-containing media (10% FBS) for 24 hours. The transduced cells were removed by trypsin digestion and seeded for either heat shock or immunoprecipitation experiments.

Ultracentrifugation and immunofluorescence analysis

Recombinant forms of IL-1α and either S100A13 or S100A13ΔBR were incubated at molar ratios (IL-1α:S100A13) of 1:1, 1:5 and 1:10 in phosphate-buffered saline (PBS) either in the presence or absence of 1 mM $CuCl_2$ for 30 minutes at 42°C followed by centrifugation at 280,000 ***g*** for 18 hours at 4°C and resolution of the pellet fractions by S100A13 immunoblot analysis as described (Landriscina et al., 2001b). Analysis of IL-1α-DsRed1 and EGFP-S100A13 intracellular trafficking was performed using transient IL-1α-DsRed1 and EGFP-S100A13 NIH 3T3 cell cotransfectants subjected to heat shock (42°C, 2h), followed by 4% formaldehyde fixation and examination using the LTCS-SP confocal system (Leica) equipped with an inverted DMIRBE microscope using an 100× objective and the 237 μM confocal pinhole.

Analysis of protein interactions in heat-shock-conditioned media

The heat shock of human U937 and murine NIH 3T3 cells was performed as previously described (Tarantini et al., 1995) in serum-free cell medium for 2 hours at 42°C and control cultures were incubated at 37°C in serum-free cell medium for also 2 hours. Two independent clones from each transfection have been evaluated with similar results. For the analysis of the release of Myc-S100A13 and IL-1α-βGal, DTT-treated media conditioned by heat shock and cell lysates from the appropriate NIH 3T3 cell transfectants were prepared and divided into two portions, one of which was processed as described for S100A13 immunoblot analysis of the Myc reporter sequence (Landriscina et al., 2001a) and the other for IL-1α-βGal immunoblot analysis (Tarantini et al., 2001). Briefly, one portion was concentrated and immunoprecipitated with an anti-IL-1α antibody for the evaluation of mature IL-1α-βGal release, and the second portion was adsorbed to heparin-Sepharose and eluted at 1.5 M NaCl for the evaluation of Myc-S100A13 release. IL-1α, S100A13 and FGF1 from human U937 cells were resolved by Cu^{2+}-chelator affinity chromatography (Hi Trap Chelation; Amersham Pharmacia Biotech) and eluted with 60 mM imidazole. Immunoprecipitated and eluted proteins were resolved by 8% and 12% acrylamide SDS-PAGE, respectively, and evaluated by either IL-1α (Tarantini et al., 2001) or Myc (Landriscina et al., 2001b) immunoblot analysis. The activity of lactate dehydrogenase in conditioned media was used as an assessment of cell lysis in all experiments as previously reported (Tarantini et al., 2001). The effects of actinomycin D (Sigma), cycloheximide (Sigma), tetrathiomolybdate (Sigma-Aldrich) and ZLL (Biomol, USA) on IL-1α release were evaluated as previously reported (LaVallee et al., 1998).

Analysis of the molten globule character of IL-1α

Dioleylphosphoglycerol (DOPG) was purchased from Avanti Polar

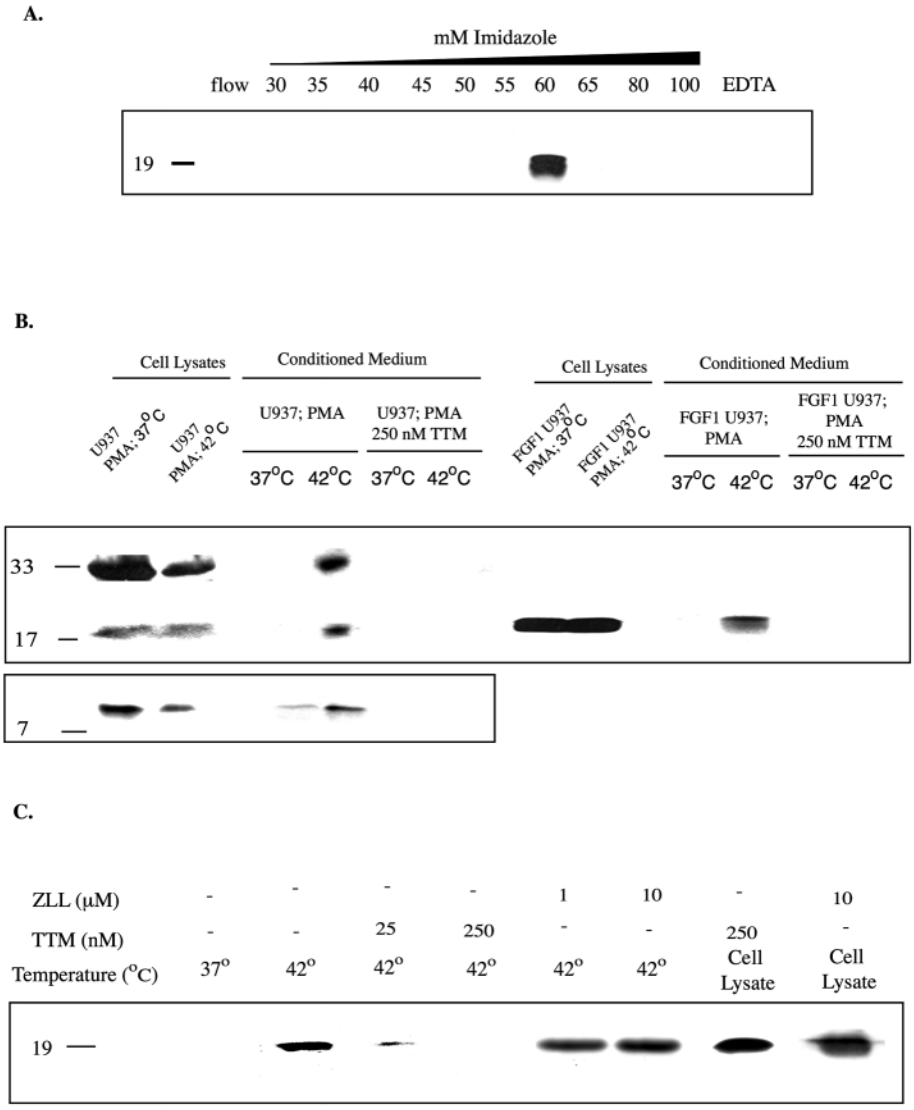

Fig. 1. The stress-induced release of IL-1α, FGF1 and S100A13 is Cu^{2+}-dependant. (A) Recombinant human IL-1α (1 μg in 100 mM Tris HCl, pH 7.2) was adsorbed to a Cu^{2+}-chelating column (Hi-Trap, Amersham), the column was washed with five column volumes of 0.2 M sodium phosphate buffer, pH 7.2 and eluted with an imidazole gradient in the wash buffer. The column was stripped with 50 mM EDTA and all fractions including the flow-through fraction were resolved by 15% acrylamide SDS-PAGE and evaluated using an IL-1α antibody. (B) Conditioned medium obtained from heat-shocked PMA-stimulated U937 and stable FGF1 U937 cell transfectants was processed by Cu^{2+} chelator affinity chromatography. Eluted proteins were resolved by 15% acrylamide SDS-PAGE, and evaluated by IL-1α (top-left panel), FGF1 (top-right panel) and S100A13 (bottom panel) immunoblot analysis**.** **(**C) NIH 3T3 cells stably transfected with IL-1α were either incubated for 18 hours at 37°C in the absence and presence of the Cu^{2+} chelator, TTM or for 2 hours in the absence or presence of the specific calpain inhibitor, ZLL, as indicated and the untreated and treated cells either maintained at 37°C or subjected to heat shock. The conditioned medium was processed and evaluated for IL-1α release as described in A. Cell lysates from TTM- and ZLL-treated cells were used to monitor the intracellular level of IL-1α expression. The TTM- and ZLL-negative control cell lysates exhibited a similar level of IL-1α expression (data not shown).

Lipids and carboxyfluorescein (CF) was purchased from Molecular Probes. For liposome preparation, DOPG was dried under a nitrogen stream and resuspended in an aquous 100 mM CF solution at pH 7.0 by vortexing and the lipid suspension sonicated for 30 minutes, extruded through an Avanti Polar Lipids Miniextruder and passed on a 10 ml dextran desalting column equilibrated in 10 mM HEPES containing 150 mM NaCl at pH 7.0 (Pierce, USA). The fluorescence of the liposomes was monitored in each experiment for 10 minutes by a Fluorolog 3 fluorescent spectroscope (Jobin Yvon, Edison, NJ) at an excitation wavelength 470 nm and emission wavelength 520 nm. The temperature was maintained at 50°C by a Peltier system. α-Chymotrypsin served as a negative control and 0.1% Triton X-100 served as a positive control for micelle lysis. Human recombinant IL-1α or α-chymotrypsin at different concentrations were added to the quartz cuvette at the 2 minute time point of each experiment.

Results

Human IL-1α is a Cu^{2+}-binding protein

Because IL-1α and FGF1 are released by a convergent stress-induced mechanism (Tarantini et al., 2001), and FGF1, as a Cu^{2+}-binding protein (Engleka and Maciag, 1992), can use intracellular Cu^{2+} to facilitate its export (Landriscina et al., 2001a), we examined the ability of human recombinant IL-1α to bind Cu^{2+}. As shown in Fig. 1A, IL-1α is able to associate with immobilized Cu^{2+} and is eluted with 60 mM imidazole. In addition, the recombinant form of pIL-1α has a Cu^{2+}-binding affinity (~60 mM imidazole) similar to the mature form. In contrast, however, the Cu^{2+}-induced mIL-1α dimer exhibits a complete lack of Cu^{2+} affinity (data not shown).

Human U937 cells release IL-1α and S100A13 in response to temperature stress in a Cu^{2+}-dependent manner

While the NIH 3T3 cell has proven to be a useful cell culture system to study the release of FGF1 and IL-1α, we sought to determine whether this pathway was also used by more physiologic cell types. Since mononuclear cells are a rich source of both FGF1 and IL-1α, we evaluated the ability of heat shock to induce the release of FGF1 and IL-1α from PMA-induced human U937 cells. As shown in Fig. 1B, IL-1α immunoblot analysis of Cu^{2+}-affinity-adsorbed cell culture media conditioned by heat shock but not at 37°C from U937 cells, exhibited the presence of both precursor (~33 kDa) and mature

(~19 kDa) forms of IL-1α as Cu^{2+}-binding proteins. In this experiment, we identified the 33 kDa protein as the precursor form of IL-1α and not as a reduction-resistant dimer of mature IL-1α since (1) the Cu^{2+}-induced dimer of the mature form of IL-1α does not exhibit any affinity for immobilized Cu^{2+} and (2) the recombinant form of pIL-1α exhibited similar Cu^{2+} affinity to the mature form of IL-1α (data not shown). Temperature stress also induced the release of FGF1 from human U937 cells stably transfected with FGF1 (Fig. 1B).

Since these data suggest that both the precursor and the mature forms of IL-1α are Cu^{2+}-binding proteins, and Cu^{2+} is able to regulate the stress-induced release of FGF1 (Tarantini et al., 1995), we assessed the ability of the Cu^{2+} chelator, tetrathiomolybdate (TTM) to repress the release of IL-1α from both U937 and NIH 3T3 cells in response to heat shock by the treatment of PMA-activated human U937 cells and murine NIH 3T3 cells for 18 hours with TTM prior to temperature stress. As shown in Fig. 1B,C, TTM was able to inhibit the release of IL-1α at 250 nM from both U937 and NIH 3T3 cells and this is consistent with the concentration of TTM used in preclinical and clinical studies for the management of solid tumor growth (Brewer et al., 2000; Cox et al., 2001; Pan et al., 2002). Interestingly, as shown in Fig. 1B, heat shock of PMA-induced U937 cells also enabled the Cu^{2+}-dependent export of S100A13, a component of the FGF1 multiprotein release complex (Landriscina et al., 2001b).

It has been extensively demonstrated that the release of the mature form of IL-1α from monocytes is highly dependent on its processing from its precursor form by calpain (Kobayashi et al., 1990). In contrast, fibroblasts including NIH 3T3 cells are not able to process the precursor forms of the IL-1 proteins (Siders et al., 1993; Tarantini et al., 2001). Because we have previously demonstrated that NIH 3T3 cells are not able to export the precursor form of IL-1α in response to temperature stress (Tarantini et al., 2001), we used the lack of precursor IL-1α processing and export in NIH 3T3 cells as a resource to further study the release of mature IL-1α-independent of precursor IL-1α processing. As shown in Fig. 1C, the stress-induced release of the mature form of IL-1α from NIH 3T3 cells does not involve intracellular calpain activity and has the same Cu^{2+}-dependent characteristics for IL-1α export as reported by human U937 cells (Fig. 1B). These observations reinforce the premise that the stress-induced pathways responsible for the regulation of IL-1α by murine NIH 3T3 and human U937 cells may indeed be similar.

IL-1α uses S100A13 for stress-induced release

Because FGF1 uses the function of the S100A13 gene product to facilitate its release in response to stress (Landriscina et al., 2001b) and S100A13 is released from activated human U937 cells in response to heat shock in a Cu^{2+}-dependent manner, we questioned whether IL-1α could also use S100A13 for its export into the extracellular compartment. To address this premise, we examined, by using a cell-free system, the ability of the recombinant forms of IL-1α and S100A13 to interact and form a Cu^{2+}- and molar ratio-dependent multiprotein aggregate that would be susceptible to ultracentrifugation. As shown in Fig. 2A (right panel), S100A13, by itself, was not susceptible to precipitation following centrifugation at 280,000 ***g*** for 18 hours either in the presence or absence of Cu^{2+}. Indeed, S100A13 was only present in the pellet fraction when incubated with the mature form of IL-1α in the presence of Cu^{2+} (Fig. 2A, left panel). In addition, the level of S100A13 present in the pellet fraction increased as a function of the IL-1α to S100A13 molar ratio with a maximum between a molar ratio of 1:5 to 1:10 (Fig. 2A), which suggests that IL-1α and S100A13 may be able to interact in a Cu^{2+}-dependent manner (Fig. 2A).

Since expression of signal peptide-less S100A13 protein in NIH 3T3 cells leads to its constitutive release at 37°C (Landriscina et al., 2001b), and IL-1α and S100A13 are able to interact in a cell-free system in a Cu^{2+}-dependent manner, we questioned whether the expression of the mature form of IL-1α in the NIH 3T3 cell could also repress the constitutive release of intracellular S100A13 at 37°C. Thus, S100A13 containing an N-terminal Myc-epitope tag was transfected into mature IL-1α-βGal NIH 3T3 transfectants (Tarantini et al., 2001) and the stable cotransfectants were either maintained at 37°C for 2 hours or subjected to heat shock. Insert-less vector and mature IL-1α-βGal cotransfectants served as controls. As shown in Fig. 2B, we observed that the expression of the mature form of IL-1α was able to repress the constitutive release of Myc-S100A13 at 37°C.

Because these results suggest that IL-1α and S100A13 may be able to associate with each other in the intracellular compartment in response to temperature stress, we performed immunoprecipitation and immunoblot analysis of cell lysates obtained from stable NIH 3T3 cell mature IL-1α transfectants, adenovirally transduced with Myc epitope-tagged S100A13. As shown in Fig. 2C, immunoprecipitation of cell lysates with an anti-Myc antibody followed by IL-1α immunoblot analysis resolves the presence of the mature form of IL-1α in the Myc immunoprecipitant fraction. However, this interaction was not observed in cell lysates obtained from the Myc-S100A13-transduced control NIH 3T3 cells (data not shown).

We also questioned whether the expression of S100A13 as a Cu^{2+}-binding protein could overcome the requirement for heat shock-induced transcription by examining the ability of actinomycin D to repress the export of an IL-1α:β-Gal chimera into the extracellular compartment when expressed in a stable S100A13 background. As shown in Fig. 2D, whereas actinomycin D was able to repress the release of IL-1α-βGal from insert-less vector and IL-1α-βGal NIH 3T3 cell cotransfectants, actinomycin D was unable to repress the export of IL-1α-βGal from Myc-S100A13 and IL-1α-βGal NIH 3T3 cell cotransfectants in response to heat shock. In addition, TTM was able to repress the release of IL-1α-βGal in response to temperature stress with data similar to those shown in Fig. 1A using human U937 cells, and similar results were obtained when cycloheximide was used to inhibit translation in the stable S100A13 background (data not shown).

Temperature stress induces the cytosolic redistribution of both IL-1α and S100A13

In order to confirm the biochemical data suggesting an interaction between IL-1α and S100A13, we obtained transient NIH 3T3 cell cotransfectants expressing IL-1α-DsRed1 and EGFP-S100A13 chimeric constructs. These cells were subjected to heat shock and examined for the localization of the reporter genes using confocal fluorescence microscopy. As shown in Fig. 3, both IL-1α and S100A13 exhibited a diffuse cytosol pattern of intracellular distribution at 37°C and this contrasted with the

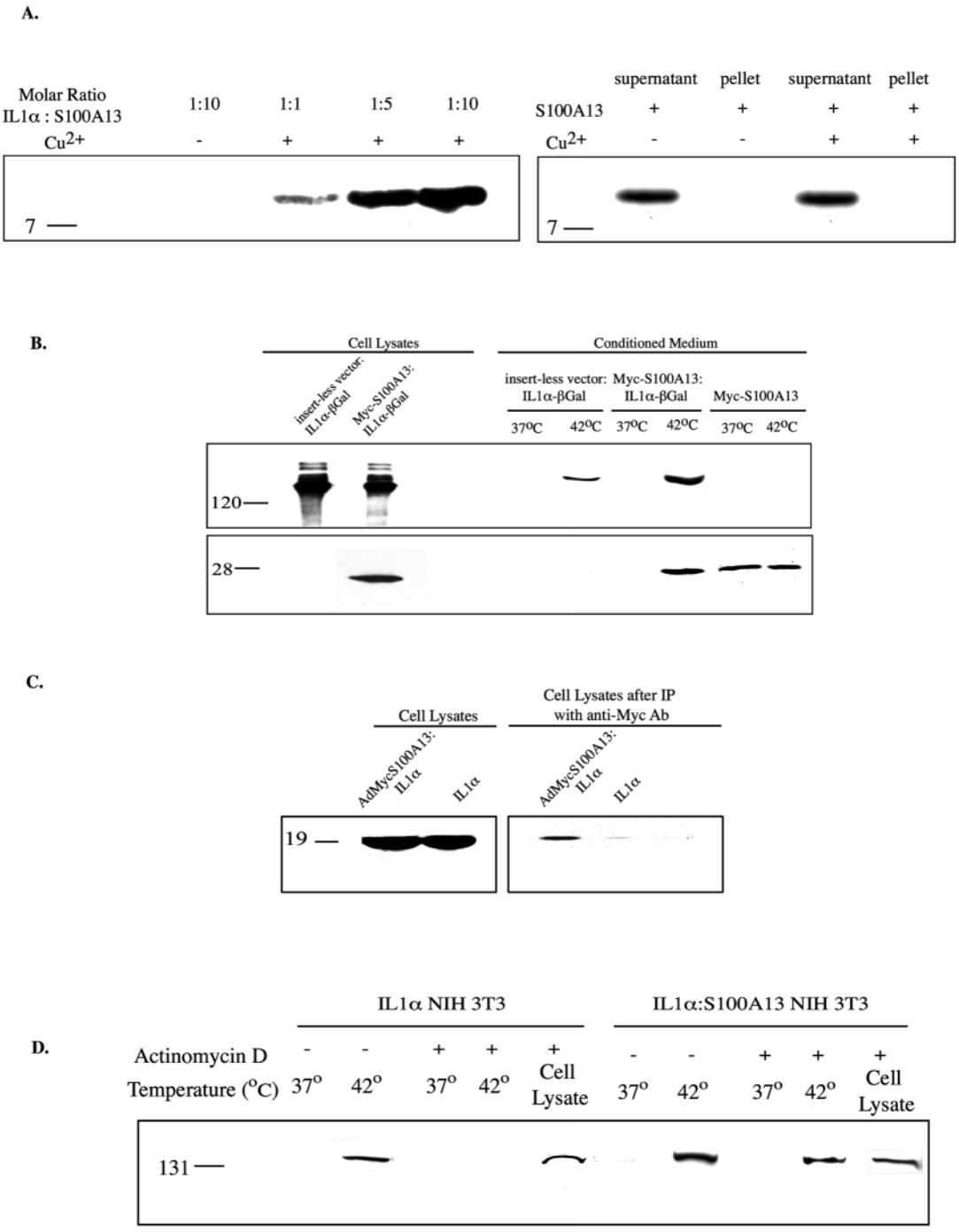

Fig. 2. S100A13 is involved in the release of IL-1α. (A) (left panel) The interaction of recombinant human IL-1α with S100A13 was assessed by the incubation of these proteins in 100 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, followed by ultracentrifugation and S100A13 immunoblot analysis of the pellet fractions; (right panel) the Cu^{2+}-dependent interaction of S100A13 with itself was assessed using the ultracentrifugation method described in the left panel. (B) Myc-S100A13 and IL-1α-βGal, insert-less vector and IL-1α-βGal NIH 3T3 cell cotransfectants were subjected to heat shock. Conditioned media were collected and processed as described (LaVallee et al., 1998). IL-1α was mmunoprecipitated with an IL-1α antibody, the immunoprecipitants were resolved by 8% and 12% acrylamide SDS-PAGE, respectively, and evaluated by IL-1α (top panel) and Myc (bottom panel) immunoblot analysis. (C) IL-1α NIH 3T3 cell transfectants were transiently transduced with a Myc-S100A13 adenoviral construct. 48 hours following transduction the cells were subjected to heat shock (42°C, 2 hours), cell lysates were obtained, immunoprecipitated with an anti-Myc antibody and resolved by IL-1α immunoblot analysis. (D) Myc-S100A13 and IL-1α-βGal NIH 3T3 cell cotransfectants and insert-less vector and IL-1α-βGal NIH 3T3 cell transfectants were evaluated for the release of IL-1α-βGal in the presence and absence of actinomycin D (10 μg/ml), as indicated, in response to heat shock. Conditioned media were processed and evaluated for IL-1α-βGal immunoblot analysis as described (Tarantini et al., 2001).

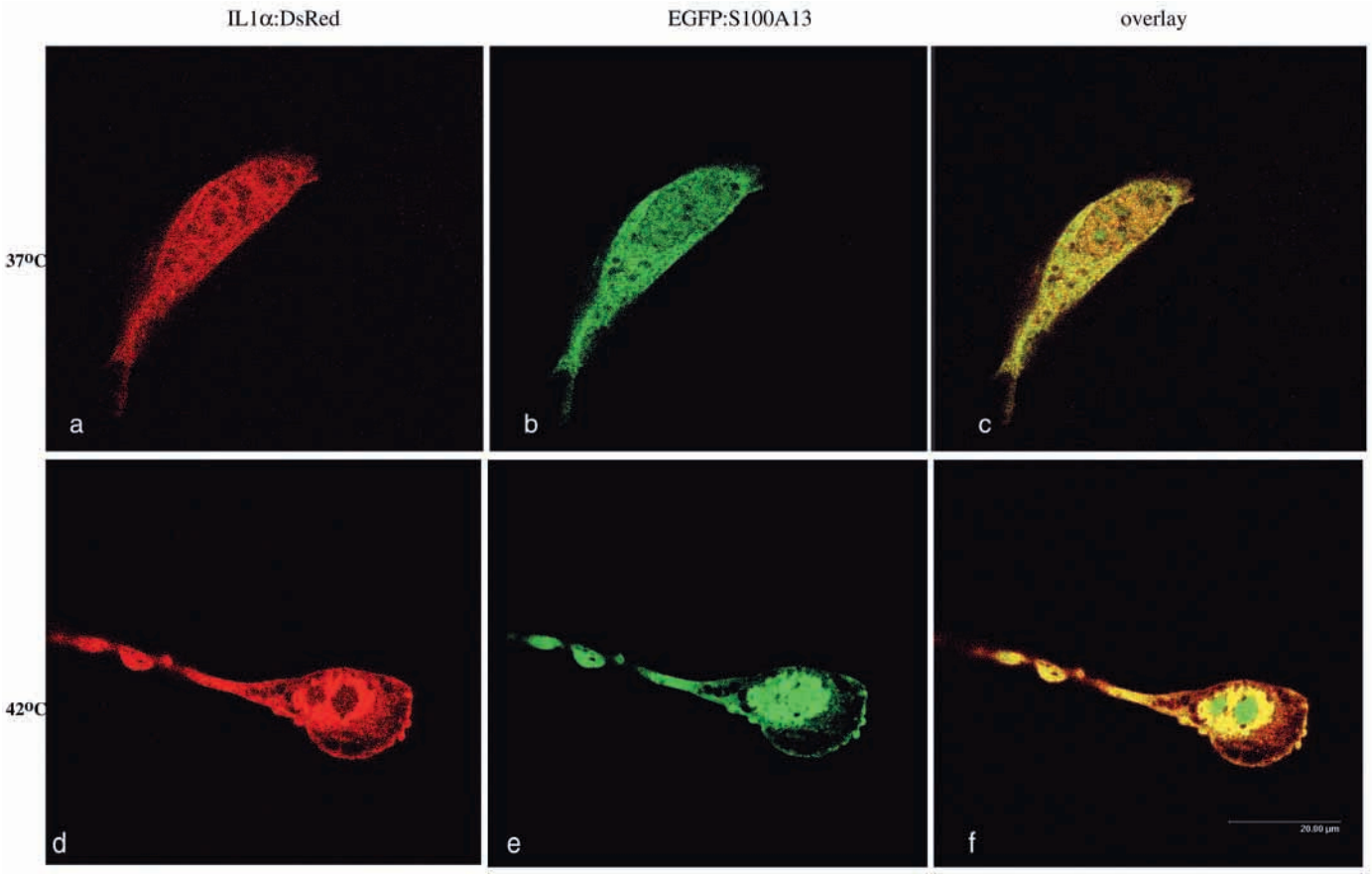

Fig. 3. Heat shock induces the cytosolic redistribution of IL-1α and S100A13. NIH 3T3 cells were transiently cotransfected with IL-1α-DsRed1 and EGFP-S100A13 constructs. 24 hours following transfection, the cells were fixed after a 2 hour incubation at either 37°C (a-c) or 42°C (d-f). Confocal images of median horizontal cell sections were obtained and the intracellular distribution of IL-1α-DsRed1 (a,d), EGFP-S100A13 (b,e) and their respective overlays (c,f) recorded.

peripheral distribution of both polypeptides in response to heat shock. This redistribution was observed in approximately 15% of the transient NIH 3T3 cell cotransfectants and the fluorescent reporter proteins, EGFP and DsRed1, did not exhibit this redistribution in response to temperature stress (data not shown). Overlay of the DsRed1 and EGFP signals demonstrated a significant level of colocalization (Fig. 3), which suggests that IL-1α and S100A13 can associate with each other near the inner surface of the cell periphery in response to heat shock.

A S100A13 mutant lacking the basic residue-rich domain is a dominant-negative regulator of stress-induced IL-1α release

Because our data argue that intracellular IL-1α and S100A13 may be able to associate in response to temperature stress, we sought to define the domain in S100A13 responsible for this association. We examined the basic residue (BR)-rich domain at the C-terminus of S100A13, since this domain is novel among the various members of the S100 gene family (Wicki et al., 1996b). Thus we deleted the last eleven residues in S100A13 and assessed the ability of the recombinant form of S100A13ΔBR to associate in a Cu^{2+}-dependent manner with IL-1α in a cell-free system. As shown in Fig. 4A, the S100A13ΔBR failed to precipitate the mature form of IL-1α in the presence of Cu^{2+}. Furthermore, like S100A13 (Landriscina et al., 2001b), the recombinant form of S100A13ΔBR eluted from immobilized Cu^{2+} at 35 mM imidazole (data not shown) suggesting that the basic residue-rich domain is not involved in Cu^{2+}-binding. In addition, we prepared a S100A13ΔBR mutant chimera containing a multiple Myc epitope tag (Myc-S100A13ΔBR), obtained stable Myc-S100A13ΔBR and IL-1α-βGal NIH 3T3 cell cotransfectants, and evaluated the ability of the IL-1α-βGal and Myc-S100A13ΔBR NIH 3T3 cell cotransfectants to release mIL-1α-βGal in response to temperature stress. As shown in Fig. 4B, IL-1α-βGal was not detected in media conditioned by heat shock from IL-1α-βGal and Myc-S100A13ΔBR NIH 3T3 cell cotransfectants. We also examined whether the expression of Myc-S100A13ΔBR could function as a repressor of S100A13 release in response to heat shock and as shown in Fig. 4C, media conditioned by temperature stress from the S100A13ΔBR and IL-1α-βGal NIH 3T3 cell cotransfectants adenovirally transduced with wild type MycS100A13, exhibit significantly reduced levels of wild type Myc-S100A13.

Recombinant mature IL-1α exhibits molten globule character

Since IL-1α and FGF1 (Prudovsky et al., 2002) translocate near

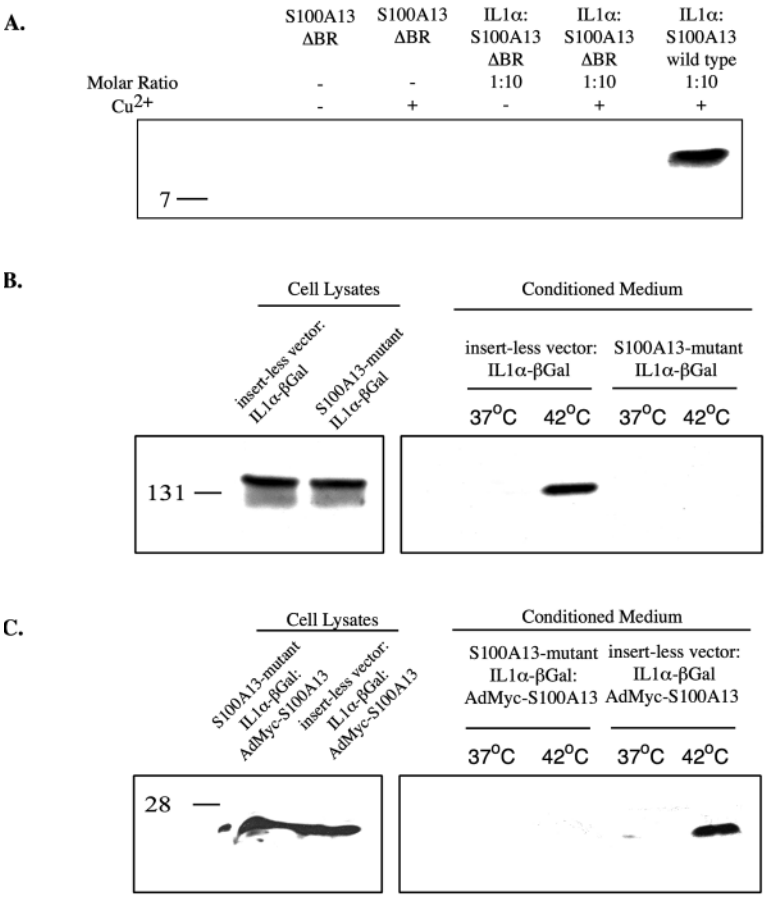

Fig. 4. Deletion of the basic residue-rich C-terminus functions as a dominant negative effector of IL-1α Release. (A) The recombinant form of S100A13 lacking the BR domain (S100A13ΔBR) was incubated with recombinant IL-1α as described in Fig. 2A at the molar ratios indicated. (B) S100A13ΔBR mutant and IL-1α-βGal NIH 3T3 cell cotransfectants were subjected to heat shock and, following DTT treatment, conditioned media were concentrated and immunoprecipitated with anti-IL-1α antibody for the evaluation of IL-1α release. Immunoprecipitated proteins were resolved by 12% (w/v) SDS-PAGE, and evaluated by IL-1α immunoblot analysis. (C) S100A13ΔBR mutant and IL-1α NIH 3T3 cell cotransfectants as well as insert-less vector and IL-1α NIH 3T3 cell cotransfectants were transiently transduced with a wild-type S100A13 adenoviral construct; 48 hours following transduction the cells were subjected to heat shock and, following DTT treatment, media conditioned at either 37°C or 42°C for 2 hours was resolved by IL-1α immunoprecipitation followed by Myc immunoblot analysis (12% acrylamide SDS-PAGE).

the inner surface of the plasma membrane prior to stress-induced release and FGF1 assumes molten globule character near 42°C (Mach and Middaugh, 1995), a novel feature that may enable it to associate with and traverse lipid bilayers (Chi et al., 2001; Samuel et al., 2000; Srimathi et al., 2002) at elevated temperatures, we questioned whether the mature form of IL-1α is also able to exibit molten globule character. We examined the ability of IL-1α to induce liposome leakage, which would reflect its solubility within the lipid bilayer. We used fluorescence spectroscopy to detect the leakage of the fluorescent probe, carboxyfluorescin (CF), from dioleyl-phosphoglycerol (DOPG) liposomes at 50°C, a temperature used to exhibit molten globule character in cell-free lipid micelles (Mach and Middaugh, 1995). In this situation, CF trapped within the vesicles is self-quenching at 100 mM but becomes highly fluorescent when released into the environment. As shown in Fig. 5, we observed a significant increase of the fluorescence signal after addition of recombinant mature IL-1α. This increase in fluorescence was dependent on the concentration of IL-1α and the addition of 0.5 μM IL-1α was as efficient as 0.1% Triton X-100 (positive control) for the release of CF from the DOPG liposomes. We used α-chymotrypsin as a negative control for these experiments and its addition (0.5 μM) to the liposomes did not exhibit increase in fluorescence (Fig. 5).

Discussion

Since S100A13 (Landriscina et al., 2001a) and FGF1 (Engleka and Maciag, 1992) have been characterized as Cu^{2+}-binding proteins, and there is a high degree of crystallographic structural conservation between the FGF and IL-1 prototypes (Graves et al., 1990; Zhang et al., 1991) including the presence of three solvent accessible histidine residues (Graves et al., 1990) that are conventionally regarded as being important for the binding of proteins to copper (Kingston et al., 1979; Kwiatkowski et al., 1977), it was perhaps not surprising that IL-1α is a Cu^{2+}-binding protein and the precursor and mature forms of IL-1α can readily be purified from media conditioned by heat shock. However, our data demonstrate for the first time that human U937 cells use intracellular Cu^{2+} for the export of the signal sequence-less polypeptides, IL-1α and FGF1, into the extracellular compartment in response to temperature stress. This finding represents a novel post-translational mechanism for the cellular response to stress. Because Cu^{2+} metabolism appears to be fundamental for the release of the signal peptide-less pro-inflammatory and angiogenic factors, IL-1α and FGF1 (Landriscina et al., 2001a), into the extracellular compartment and divalent copper is becoming increasingly recognized for its role in many physiological and pathological processes, this mechanism may offer an explanation for the ability of TTM to function as an anti-cancer agent in man (Cox et al., 2001; Brewer et al., 2000; Pan et al., 2002).

It is well established that the prototype members of the IL-1 gene family are potent inducers of the latent transcription factor, NF-κB (Baldwin, 1996; Beg et al., 1993). Indeed, human mononuclear cells are known to be: (1) a rich source of FGF1 (Wahl, 1984); (2) involved in the delivery of FGF1 to sites of inflammation (Sano et al., 1990; Remmers et al., 1991; Sano et al., 1992), and (3) are a target for extracellular IL-1α as a chemoattractant (Torisu et al., 2000). Further, IL-1α is also able to induce the expression of vascular endothelial growth factor (VEGF) and FGF2 in human endothelial cells (Ko et al., 1999; Torisu et al., 2000). Thus it is possible that the response of the tumor microvasculature to stress involves the release of IL-1α, which may be responsible for the recruitment of mononuclear cells into the tumor microenvironment in order to deliver and release FGF1. Since TTM therapy in humans significantly

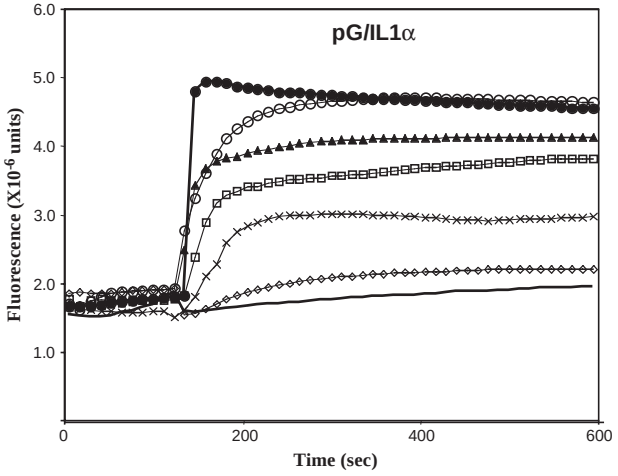

Fig. 5. IL-1α exhibits the ability to displace carboxyfluorescein from the dioleylphosphoglycerol micelles. DOPG liposomes containing CF were prepared as described in Materials and Methods; the micelle was introduced into the cuvette at a 1:60 dilution in 10 mM HEPES, pH 7.0 containing 150 mM NaCl and allowed to equilibrate. At the 120 second time point, the experiment was initiated by introducing either 0.1% Triton X-100 (positive control, ●), 0.5 μM of α-chymotrypsin (negative control, —), and different concentrations of IL-1α, 0.5 μM (○), 0.25 μM (▲), 0.125 μM (□) 0.062 μM (×) and 0.031 μM (◇).

reduces the serum levels of the FGF prototypes as well as reducing the level of the IL-1 prototypes in media conditioned by breast tumor cells (Pan et al., 2002), it is possible that TTM administration may repress the export of IL-1α within the tumor microvasculature in vivo. This may further limit the recruitment of mononuclear cells to sites within the tumor microenvironment where the release of FGF1 may foster the angiogenic potential of VEGF within the microvasculature and aid in the promotion of tumor cell proliferation and survival. This premise is consistent with the recent observation that TTM treatment is able to suppress the transcriptional activity of NF-κB in tumor cells in vivo (Pan et al., 2002), since the repression of the stress-induced release of IL-1α by TTM could significantly contribute to the downregulation of microvascular cell and tumor-derived NF-κB expression. Likewise, recent studies suggest that Cu^{2+}-chelation may represent an alternative approach to the management of β-amyloid deposition in a transgenic mouse model of Alzheimer disease (Cherny et al., 2001). Since IL-1α is significantly increased in the brains of Alzheimer disease patients (Griffin et al., 1989) and is able to induce the expression of the β-amyloid precursor gene in human endothelial cells (Goldgaber et al., 1989), it may be likely that the therapeutic effect of Cu^{2+} chelators in Alzheimer disease may be due to their ability to attenuate the release of IL-1α.

It is also well established that mononuclear cells can release both the precursor and the mature form of IL-1α as biologically active cytokines in vitro (Siders et al., 1993). However, Dinarello has suggested that, since the precursor form of IL-1α remains bound to the cell surface following its release, whereas the mature form is predominantly present in biological fluids in vivo, this may indeed represent evidence for the preferential release of the mature form of IL-1α (Dinarello, 1994). Indeed, the lack of pIL-1α release from NIH 3T3 cells in response to cellular stress (Tarantini et al., 2001) is consistent with the data of others (Siders et al., 1993) showing that a variety of different cell types including keratinocytes and fibroblasts, prefer to release the mature forms of the IL-1 prototypes. Moreover, the release of the mature forms of IL-1α as a result of pIL-1α processing may also be dependent upon the activity of intracellular calpain. Therefore the observation that pIL-1α NIH 3T3 cell transfectants do not contain the mature form of IL-1α in their cytosol (Tarantini et al., 2001) may be due to either their deficiency in the expression of the appropriate calpain gene product or the presence of high levels of the calpain inhibitor, calpastatin.

Because S100A13 and IL-1α share similar characteristics of stress-induced release from human U937 and murine NIH 3T3 cells and S100A13 is an important component of the multiprotein complex involved in the stress-induced release of FGF1, we suggest that intracellular S100A13 may be involved in the formation of a Cu^{2+}-dependant IL-1α:S100A13 heterotetramer that facilitates the export of both polypeptides. Indeed, this suggestion is reinforced by the observations that IL-1α and S100A13 are able to form a multiprotein Cu^{2+}-dependent complex in vitro as well as in a cell-free system, and IL-1α may be able to access intracellular S100A13 near the inner surface of the plasma membrane. Interestingly, experimental evidence has established a similar putative model of interaction between S100 dimers and their target proteins, the annexin gene family members (Rety et al., 1999). Thus, upon divalent ion binding, each S100A11 monomer opens up to accommodate a target annexin II monomer. In this way, an S100 dimer functionally crossbridges two homologous target molecules and forms a tight heterotetrameric complex, which is able to associate with the cytoskeleton and the lipid bilayer of the plasma membrane (Schafer and Heizmann, 1996). Interestingly, the S100:annexin 2 heterodimer complex has been reported to associate with phosphatidylserine and function as a key mediator of the extrinsic coagulation and fibrinolytic systems on the surface of endothelial cells in response to temperature stress (Kim and Hajjar, 2002). Further, phosphatidylserine is able to flip from the inner to the outer leaflet of the plasma membrane in response to cellular stress and this feature is known to be a regulator of stress-mediated vascular endothelial cell activation in vivo (Ishii et al., 2001).

Previous observations from our laboratory suggested that unlike the stress-induced FGF1 release pathway, the IL-1α export pathway does not use the function of Syt1 since a Syt1 mutant lacking the Ca^{2+}-binding C2A domain, which is able to function as a dominant-negative effector of FGF1 release (La Vallee et al., 1998), does not affect the stress-induced release of IL-1α from NIH 3T3 cells (Tarantini et al., 2001). However, the use of intracellular Cu^{2+} to facilitate the stress-induced interaction between IL-1α and S100A13 is a feature that is conserved between IL-1α and FGF1 release pathways. Although our observations further suggest that the assembly of the stress-induced IL-1α:S100A13 complex occurs prior to release, it is not yet known how this complex is able to traverse the plasma membrane. However, the ability of IL-1α to associate with and displace CF from an intra-DOPG micelle locale suggests that, like FGF1 (Mach et al., 1993), IL-1α may be endowed with molten globule character: a biophysical characteristic in which partially unfolded protein conformations formed as a result of the transition from high tertiary to low tertiary structure are able to use their secondary structure to achieve low

solubility in aqueous environments, resulting in their association with and transversion through acidic phospholipid bilayers.

Our data also suggest that the C-terminal basic-rich (BR) domain of S100A13 may be responsible for its interaction with the mature form of IL-1α but not with Cu^{2+}. Indeed, the C-terminus of other S100 gene family members have been implicated in mediating their ability to interact with proteins (Kilby et al., 1996; Pozdnyakov et al., 1998; Rety et al., 1999; Schafer and Heizmann, 1996). Interestingly, unlike other S100 gene family members, S100A13 contains a nine amino acid basic residue-rich domain, which is absent in other S100 gene family members (Schafer and Heizmann, 1996; Wicki et al., 1996a) with the exception of the recently identified S100A14, which contains a basic residue-rich domain at its C terminus (Pietas et al., 2002). Because members of the S100 gene family lacking a basic residue-rich domain at their C-terminus are also exported to the extracellular compartment (Schafer and Heizmann, 1996), we suggest that this domain may be responsible for the association of S100A13 with IL-1α. In addition, we also suggest that the remainder of the S100A13 structure may be involved in the mechanism to facilitate the traversion of the S100A13:IL-1α complex through the lipid bilayer and enable the release of the multiprotein complex. Thus we anticipate that like IL-1α and FGF1, S00A13 may also exhibit molten globule character.

We also suggest that the stress-induced interaction between the mature form of IL-1α and the BR domain of S100A13 promotes the release of both proteins as a Cu^{2+}-dependent complex in which this heterotetramer is formed by the ability of both proteins to bind Cu^{2+}. It is also noteworthy that the expression of S100A13 in an IL-1α background results in an attenuation of the sensitivity of the IL-1α release pathway to the transcription inhibitor actinomycin D. Since the transcription of the S100A13 gene is not regulated by heat shock (data not shown), it is likely that the role of cellular stress in the export of the mature form of IL-1α may not be due to the induction of a classical stress-mediated transcriptional response; rather, the stress response may involve the regulation of a post-translational activity that modifies S100A13. Although we do not know the nature of this putative post-translational activity, our data suggest that the oxidative character of intracellular Cu^{2+} is involved in the regulation of this feature and this may indeed be the responsibility of the intracellular transport of Cu^{2+} to both IL-1α and S100A13 that is susceptible to chelation by TTM.

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