Interleukin (IL)1α mediates proinflammatory events through its extracellular interaction with the IL1 type I receptor. However, IL1α does not contain a conventional signal peptide sequence that provides access to the endoplasmic reticulum-Golgi apparatus for secretion. Thus, we have studied the release of the precursor (p) and mature (m) forms of IL1α from NIH 3T3 cells. We have demonstrated that mIL1α but not pIL1α was released in response to heat shock with biochemical and pharmacological properties similar to those reported for the stress-mediated release pathway utilized by fibroblast growth factor (FGF1). However, unlike the FGF1 release pathway, the IL1α release pathway appears to function independently of synaptotagmin (Syt)1 because the expression of a dominant-negative form of Syt1, which represses the release of FGF1, did not inhibit the release of mIL1α in response to temperature stress. Interestingly, whereas the expression of both mIL1α and FGF1 in NIH 3T3 cells did not impair the stress-induced release of either polypeptide, the expression of both pIL1α and FGF1 repressed the release of FGF1 in response to temperature stress. These data suggest that the release of mIL1α requires proteolytic processing of its precursor form and that mIL1α and FGF1 may utilize similar but distinct mechanisms for export.

The interleukin(IL)1-1 gene family is currently comprised of eight members, and these include the IL1 prototypes, IL1α and IL1β, as well as the IL1 receptor antagonist RA (1), IL-18, and the recently identified members, FILLδ, FILLε, FILLζ, and FILLη (2). The IL1 prototypes are translated as 31–34-kDa precursor proteins that are cleaved by two distinct specific proteases to produce mature 17-kDa forms of the IL1 prototypes from the C-terminal end of the precursor (3). Whereas precursor (p) IL1β is biologically inactive until it is processed into the mature (m) form by the IL1β-converting enzyme (ICE) (4, 5), pIL1α is biologically active (5). Precursor IL1α is recognized by a calcium-dependent protease of the calpain family, this cleavage results in the formation of the mature counterpart (6). Interestingly, the N-terminal fragment derived from pIL1α proteolytic processing contains a functional nuclear localization signal (7) and can be translocated to the nucleus (8). The ability of pIL1α to bind the IL1 type I receptors with high affinity, with subsequent activation of the signal transduction pathway, and the presence of the nuclear localization sequence anticipates the existence of a biological role for pIL1α, independent from the activity of mIL1α. Indeed, comparative studies using the pIL1α and mIL1α forms have suggested that pIL1α, but not mIL1α, is a negative regulator of cell migration (9).

Amino acid sequence (10) and x-ray crystallographic analysis (11) of the IL1 and FGF prototypes have revealed rather striking structural similarities between members of the two gene families. Like the majority of the IL1 gene family members (1, 2), the FGF gene family prototypes, FGF1 and FGF2 also lack a classical signal peptide sequence to direct secretion through the ER-Golgi apparatus (12). Whereas the release of FGF1 is regulated by a variety of stress conditions (13–18), the release of FGF2 is not regulated by stress (19), and FGF2 contains structural features that repress the stress-induced release of FGF1 (19). Because (i) the release pathways utilized by the FGF prototypes have diverged, and (ii) it is unlikely that the pathway responsible for the regulation of FGF1 would have evolved independent of the mechanisms utilized by other signal peptide-deficient gene products to gain access to the extracellular compartment, we sought to determine whether other signal peptide-deficient cytokines are also able to utilize the FGF1 release pathway for export. We focused our effort on IL1α because its precursor form is biologically functional (5, 20), and extracellular IL1α is well described as an antagonist of FGF-dependent biological activities (21–24). We report that the release pathway utilized by IL1α exhibits similar biochemical, pharmacological, and biological properties to the FGF1 release pathway. In contrast, unlike the FGF1 release pathway (16, 17), IL1α does not require the function of synaptotagmin (Syt)1 but does require the function of an intracellular protease to convert pIL1α to mIL1α, because only mIL1α is released in response to heat shock. Lastly, the stress-induced IL1α and FGF1 release pathways may be convergent, because the expression of pIL1α acts as a dominant-negative repressor of FGF1 release.
**Experimental Procedures**

**Plasmids, Transfections, and Cell Culture—Human pIL1α (1–271) and mIL1α(113–271) cDNAs (7), as well as pIL1α-β-gal and mIL1α-β-gal fusion constructs, were inserted into the expression vector, pMEXneo (25), and stable NIH 3T3 cell transfectants were obtained as previously described (7). The transfectants were grown on cell culture dishes coated with 10 μg/cm² fibronectin in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) bovine calf serum (BCS, HyClone), 1× antibiotic/antimycotic (Life Technologies, Inc.), and 400 μg/ml geneticin (G418, Life Technologies, Inc.). The Syt1 constructs, full-length rat p65 Syt1 and its deletion mutant Syt1(120–214) in the expression vector pMEXneo/hygro, were obtained as described (17). The Syt1 constructs were cotransfected into mIL1α-β-gal NIH 3T3 cell transfectants using 5 μg of DNA mixed with the multicomponent lipid-based transfection reagent FuGENE6 (Roche Molecular Biochemicals) following the manufacturer's instructions. Stable cotransfectants were grown on fibronectin-coated (10 μg/cm²) cell culture dishes in DMEM, supplemented with 10% (v/v) bovine calf serum, 1× antibiotic/antimycotic, 400 μg/ml G418, and 200 μg/ml hygromycin (Roche Molecular Biochemicals). The human FG1 construct in the expression vector, pcDNA3.1/Hygro (Invitrogen) was obtained by digesting the pXZD plasmid (32) with EcoRV and Hpal (New England Biolabs Inc.), isolating the 836-base pair fragment containing FG1β by electroelution, ligating into the pcDNA3.1/Hygro expression vector previously digested with EcoRV, and purifying by electrophoresis. The FG1β pcDNA3.1/Hygro construct was cotransfected into mIL1α-β-gal and pIL1α-β-gal NIH 3T3 cell transfectants using 5 μg of DNA mixed with the multicomponent lipid-based transfection reagent FuGENE6, following the manufacturer's instructions. Stable cotransfectants were selected and grown as described above. Whereas multiple clones were obtained in the mIL1α, FG1β, and mIL1α-FG1β backgrounds, we limited to studying a single clone in the pIL1α and pIL1α-FG1β backgrounds.

Transfectants were grown to 70–80% confluency and prior to the temperature stress, the cells were washed with DMEM. The heat shock was started by incubating the DMEM at 42 °C for 2 h (18). Prior to the temperature stress, the heat shock medium was supplemented with 4 units/ml heparin (Upjohn). When FGF1 transfectants were subjected to temperature stress, the cells were washed with DMEM. The heat shock medium was supplemented with 4 units/ml heparin (Upjohn) and was subjected to temperature stress at 42 °C for 2 h. Cell lysates were obtained in the mIL1α-β-gal and pIL1α-β-gal NIH 3T3 cell transfectants was accomplished by dividing individual cell lysates and conditioned medium in half for the detection of FG1β and IL1α immunoreactivity bands. For FG1β analysis, total cell lysates were obtained by sonication in cold lysis buffer containing 1% (v/v) Triton X-100 (Sigma) and adsorbed to a 1 ml heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech), previously equilibrated with 50 mM Tris pH 7.4 containing 10 mM EDTA (TEB). The column was washed with TEB, and the proteins were eluted with 2 ml of TEB containing 1.5 M NaCl. The volume of the eluates was reduced by using Centricon 10 concentrators (Amicon), and the eluates were analyzed by 15% (w/v) SDS-PAGE followed by immuno blot analysis using anti-human FG1β polyclonal antibodies (27) as described previously (16). IL1α was analyzed by immunoprecipitation using anti-IL1α antibody and resolved by 8% (w/v) SDS-PAGE followed by immunoblot analysis, as described above. Cell lysates from mIL1α-β-gal, mIL1α-β-gal/pIL1β-transfected NIH 3T3 cell transfectants were obtained by sonication in NP lysis buffer and processed as described previously. Conditioned medium from these cotransfectants were treated with 0.1% (w/v) DTT and divided into two equal samples. The first sample was adsorbed to heparin-Sepharose CL-6B (1 ml) and washed with TEB, and samples were eluted with 1.5 M NaCl, as reported above. Cell lysates and conditioned medium purified by heparin affinity were resolved by 10% (w/v) SDS-PAGE and subjected to immunoblot analysis using a rabbit antiserum to human IL1β and a rabbit antiserum to protein A and were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech) following the manufacturer's instructions. The activity of lactate dehydrogenase in conditioned medium was utilized as an assessment of cell lysis in all experiments and was measured by a colorimetric assay using pyruvate as a substrate (Sigma). Each experiment reported was repeated at least three times with similar results in all cases, and representative data are shown in each figure.

**Results and Discussion**

We evaluated the ability of human IL1α to enter the extracellular compartment using NIH 3T3 cells because these cells (i) are refractory to the activity of endogenous and exogenous IL1α (data not shown) and (ii) have proven to be valuable for the study of the FG1β release pathway (16–18). Thus, stable NIH 3T3 cell transfectants expressing either pIL1α or mIL1α with or without the β-gal reporter gene product were obtained and were subjected to temperature stress at 42 °C for 2 h. Cell lysates and conditioned medium treated with 0.1% (w/v) DTT were processed for IL1α immunoblot analysis. As shown in Fig. 1, A and B, mIL1α and mIL1α-β-gal were readily visible in conditioned medium in both β-gal reporter gene product, β-gal, and (iii) cell lysis does not account for the release of mIL1α because the absence of pIL1α in medium conditioned by heat shock served as a negative control.

Because FG1β (14) but not FGF2 (19) was released from NIH 3T3 cells in response to heat shock as a DTT-sensitive latent homodimer (14), and both reducing agents and (NH₄)₂SO₄ were

| protein | concentration | source |
|---------|---------------|--------|
| anti-IL1α antibody | 1:1200 | rabbit |
| anti-human IL1β | 1:200 | goat |

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able to activate the heparin affinity of latent FGF1 (16), we questioned whether these reagents would also affect the ability of IL1α to be recognized by affinity reagents. Because mIL1αβ-gal does not exhibit heparin-binding affinity (data not shown), we utilized β-gal affinity and IL1α immunoprecipitation to assess this issue. As shown in Fig. 1C, IL1α immunoblot analysis of medium conditioned by temperature stress was performed using p-aminobenzyl-1-thio-β-d-galactopyranoside (PAGT) affinity and failed to detect the presence of the IL1αβ-gal fusion protein. However, treatment of heat shocked-conditioned medium with either 0.1% (w/v) DTT or 95% (w/v) (NH₄)₂SO₄ did resolve the presence of mIL1αβ-gal in medium conditioned by heat shock from mIL1αβ-gal NIH 3T3 cell transfectants. In contrast, the use of IL1α immunoprecipitation detected the presence of IL1αβ-gal in medium conditioned by heat shock from mIL1αβ-gal NIH 3T3 cell transfectants. In contrast, the use of IL1α immunoprecipitation detected the presence of IL1αβ-gal in medium conditioned by heat shock from mIL1αβ-gal NIH 3T3 cell transfectants. Thus, it is unlikely that mIL1α utilizes Cys oxidation for export.

We also examined the kinetics and pharmacologic properties of mIL1αβ-gal release in response to temperature stress, because the FGF1 release pathway exhibits relatively slow export kinetics (14). Moreover, FGF1 is sensitive to agents that interfere with translation (12), transcription (12), ATP biosynthesis (17), and assembly of the F-actin cytoskeleton (28) but is not sensitive to Brefeldin A, an agent that interferes with the function of the ER-Golgi apparatus (14). As previously observed with FGF1 release (14), the release of mIL1αβ-gal also required at least 90 min of temperature stress before IL1α immunoblot analysis was able to resolve the presence of the fusion protein in medium conditioned by heat shock (Fig. 2A). In addition, IL1α immunoblot analysis revealed that the release of mIL1αβ-gal was not sensitive to treatment of the mIL1αβ-gal NIH 3T3 cell transfectants with Brefeldin A (Fig. 2B) but was sensitive to the presence of 2-deoxyglucose (Fig. 2C) as well as cycloheximide and actinomycin D (data not shown). Further, the release of mIL1αβ-gal was also sensitive to treatment with amlexanox (Fig. 2D), an agent which induces the Sec-dependent disassembly of F-actin stress fibers (28) and exhibits a dose-response to amlexanox similar to that reported for the inhibition of FGF1 release in vitro (18). These data suggest that the pathway utilized by mIL1αβ-gal for entering the extracellular compartment is dependent upon transcription, translation, ATP biosynthesis, and the actin cytoskeleton but is independent of the function of the ER-Golgi apparatus.

Because the kinetic and pharmacologic properties of mIL1αβ-gal release were remarkably similar to that previously reported for FGF1, we examined the potential role of p65 Syt1 in the IL1αβ-gal release pathway. Syt1 is the protein member of a gene family of vesicular transmembrane Ca²⁺- and acidic phospholipid-binding proteins (29) involved in the regulation of conventional exocytosis (30) and endocytosis (31). Because the extravesicular p40 domain of Syt1 is released as an aggregate with FGF1 in response to heat shock (16) and is associated with FGF1 in vivo (18), we utilized a p65 Syt1 mutant (p65 Syt1Δ120–214) lacking the C2A domain, which acts as a dominant negative effector of FGF1 release in re-
response to heat shock (17). To evaluate the role of p65 Syt1 in the release of mIL1αβ-gal, we obtained stable NIH 3T3 cell cotransfectants expressing either p65 Syt1/mIL1αβ-gal or p65 Syt1(120–214)/mIL1αβ-gal and examined medium conditioned by heat shock for the presence of p40 Syt1 and IL1αβ-gal using immunoblot analysis. As shown in Fig. 3A, Syt1 immunoblot analysis revealed the presence of p40 Syt1 in medium conditioned by heat shock from the p65 Syt1/mIL1αβ-gal NIH 3T3 cell cotransfectants but did not report the presence of p40 Syt1 in heat shocked-conditioned medium from either mIL1αβ-gal NIH 3T3 cell transfectants or p65 Syt1(120–214)/mIL1αβ-gal NIH 3T3 cell cotransfectants. In contrast, however, IL1α immunoblot analysis did reveal the presence of mIL1αβ-gal in medium conditioned by temperature stress independent of the expression of either p65 Syt1 or p65 Syt1(120–214) (Fig. 3B). These data suggest that unlike the stress-induced FGF1 release pathway (17), the IL1α release pathway may not utilize the function of p65 Syt1. However, it is not possible to eliminate the function of another member of the Syt gene family in the regulation of mIL1α release.

Although the data with p65 Syt1(120–214) expression suggest that neither IL1α and FGF1 release pathways may have diverged, we questioned whether the coexpression of IL1α and FGF1 in the NIH 3T3 cell could confirm this premise. Thus we obtained stable NIH 3T3 cell cotransfectants in which pIL1αβ-gal and mIL1αβ-gal NIH 3T3 transfectants were cotransfected with FGF1 and examined their ability to release mIL1αβ-gal, pIL1αβ-gal, and FGF1 in response to heat shock. As shown in Fig. 4, IL1α and FGF1 immunoblot analysis revealed the presence of both mIL1αβ-gal and FGF1 in medium conditioned by heat shock from the FGF1/mIL1αβ-gal NIH 3T3 cell transfectants. However, IL1α and FGF1 immunoblot analysis failed to detect the presence of either pIL1α or FGF1 from the FGF1/ pIL1αβ-gal NIH 3T3 cell cotransfectants in response to temperature stress (Fig. 4). Because the precursor form but not the mature form of IL1α was able to repress the release of FGF1 in response to heat shock, the precursor domain of IL1α may contain a structural feature that may function as a dominant-negative effector of FGF1 release. Although we do not know the element within the precursor domain of IL1α responsible for this event, these data do suggest that the IL1α and FGF1 release pathways may indeed be convergent.

It is well established that the precursor forms of the IL1 prototypes are cell-associated polypeptides whereas the mature forms of the IL1 prototypes are present in either biological fluids or cell culture medium (32–35); an observation which suggests that the mature forms of the IL1 prototypes may be preferred for release. Indeed, in transgenic mice manipulated to overexpress mIL1α in basal keratinocytes, high levels of mIL1α were released into the circulation (36). In contrast, however, mechanical deformation of human keratinocytes was required to induce a rapid release of pIL1α (37). Whereas both precursor and mature forms of the IL1 prototypes have also been detected in cell culture medium conditioned by mononuclear cells treated with lipopolysaccharides (34, 38), the appearance of the precursor forms correlated with the release of LDH, a cytosolic protein used to monitor the release of cytosis because of compromised plasma membrane integrity (39). It should be noted that our experimental conditions also employed LDH release, and we did not observe any significant levels of extracellular LDH in response to temperature stress. However, because the pIL1αβ-gal release studies were limited to a single clone, we cannot eliminate the possibility that a critical ratio of pIL1αβ-gal expression may enable pIL1αβ-gal to be released in response to heat shock.

**FIG. 3.** Expression of p65 Syt1(120–214) does not alter the release of mIL1αβ-gal in response to heat shock. The mIL1αβ-gal NIH 3T3 cell transfectants, mIL1αβ-gal/p65 Syt1 NIH 3T3 cell cotransfectants, and mIL1αβ-gal/p65 Syt1αβ-gal(214–214) mutant NIH 3T3 cell cotransfectants were subjected to heat shock (42 °C, 2 h), and the level of expression of the transfected proteins in CL or CM was analyzed by heparin-Sepharose affinity followed by Western blot analysis with antibodies (A) or by heparin-Sepharose affinity followed by FGF1 immunoblot analysis (B). A, Syt1 immunoblot; lanes 1–3, total CL from mIL1αβ-gal transfectants (lane 1), mIL1αβ-gal/p65 Syt1 cotransfectants (lane 2), and mIL1αβ-gal/p65 Syt1αβ-gal(214–214) cotransfectants (lane 3). Lanes 4–6, 37 °C CM from mIL1αβ-gal transfectants, mIL1αβ-gal/p65 Syt1, and mIL1αβ-gal/p65 Syt1αβ-gal(214–214) mutant cotransfectants, respectively; lanes 7–9, 42 °C CM from mIL1αβ-gal single transfectants, mIL1αβ-gal/p65 Syt1, and mIL1αβ-gal/p65 Syt1αβ-gal(214–214) cotransfectants, respectively. B, IL1α immunoblot: total CL from mIL1αβ-gal transfectants (lanes 1 and 2), mIL1αβ-gal/p65/Syt1 cotransfectants (lanes 3 and 4), and mIL1αβ-gal/p65/Syt1αβ-gal(214–214) cotransfectants (lanes 5 and 6) after IP with a goat anti-human IL-1α antibody (lanes 1, 3, and 5) or a control antibody (lanes 2, 4, and 6); lanes 7–9, 37 °C CM from mIL1αβ-gal transfectants (lane 7), mIL1αβ-gal/p65 Syt1 cotransfectants (lane 8), and mIL1αβ-gal/p65 Syt1αβ-gal(214–214) cotransfectants (lane 9). Lanes 10–12, 42 °C CM from mIL1αβ-gal transfectants (lane 10), mIL1αβ-gal/p65/Syt1 cotransfectants (lane 11), and mIL1αβ-gal/Syt1αβ-gal(214–214) cotransfectants (lane 12).

**FIG. 4.** Expression of pIL1α but not mIL1α represses the release of FGF1 in response to heat shock. The mIL1αβ-gal and pIL1αβ-gal NIH 3T3 cell transfectants, mIL1αβ-gal/FGF1, and pIL1αβ-gal/FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock (42 °C, 2 h), and the level of expression of transfected proteins in CM was analyzed by IP followed by IL1α immunoblot analysis with antibodies (A) or by heparin-Sepharose affinity followed by FGF1 immunoblot analysis (B). A, IL1α immunoblot. Lanes 1–4, total CL from mIL1αβ-gal transfectants (lane 1), mIL1αβ-gal/FGF1 cotransfectants (lane 2), pIL1αβ-gal transfectants (lane 3), and pIL1αβ-gal/FGF1 cotransfectants (lane 4). Lanes 5–6, 37 and 42 °C CM from mIL1αβ-gal/ FGF1 cotransfectants, respectively; lanes 7–8, 37 and 42 °C CM from mIL1αβ-gal transfectants, respectively; lanes 9–10, 37 and 42 °C CM from pIL1αβ-gal/FGF1 cotransfectants, respectively; lanes 11–12, 37 and 42 °C CM from pIL1αβ-gal transfectants, respectively. B, FGF1 immunoblot. Lanes 1 and 2, total cell lysates from mIL1αβ-gal/FGF1 and pIL1αβ-gal/FGF1 cotransfectants, respectively; lanes 3–4, 37 and 42 °C CM from mIL1αβ-gal/FGF1 cotransfectants, respectively; lanes 5–6, 37 and 42 °C CM from pIL1αβ-gal/FGF1 cotransfectants, respectively.
precursor IL1α blocks FGF1 release

Our data are consistent with the observation that human bladder carcinoma cells selectively release mIL1α but not pIL1α in vitro (40). Moreover, Siders, et al. (41) using a variety of different cell types transfected with either the precursor or mature forms of the IL1 prototypes observed that the mature forms of IL1α and IL1β were also preferred for release and that the release of mIL1α from pIL1α was dependent upon the presence of a calpain-like protease activity (41). Interestingly, whereas macrophages from the ICE-null mouse were able to release pIL1β but not mIL1β in response to lipopolysaccharide stimulation, these cells were also deficient in IL1α release suggesting a possible role for ICE in the release of IL1α (42). Because we were unable to observe the presence of mIL1α in cytosol derived from the pIL1α NIH 3T3 cell transfectants, even under conditions of apoptosis where ICE is functional (data not shown), it is unlikely that ICE expression is involved even under conditions of apoptosis where ICE is functional (43). Alternatively, this may also be because of the absence of calpain activation in response to heat shock. However, our data do suggest that the proteolytic conversion of pIL1α to mIL1α may be prerequisite for the appearance of IL1α in the extracellular compartment.

Although the ability of pIL1α to repress the release of FGF1 in response to heat shock suggests a connection between the IL1α and FGF1 release pathways, we do not know whether mIL1α requires the function of helper genes to gain access to the extracellular compartment. However, it is interesting to note that like FGF1 (18, 44), early studies with IL1α using gel exclusion chromatography also noted the presence of IL1α in high molecular weight complexes (34, 45). Whether these represent the IL1α equivalent of the multiprotein aggregate of FGF1(18) is not known. It is intriguing to speculate that like the FGF1 release pathway, the release of mIL1α in response to temperature stress may require the function of an unknown set of helper genes.

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