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Presence of a Cytoplasmic Retention Sequence within the Human Interleukin-1α Precursor

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ABSTRACT—Interleukin (IL)-1α is primarily translated as a 33 kDa molecule (IL-1$\alpha_{1\text{-}271}$), and then processed into a 17 kDa molecule (IL-1$\alpha_{119\text{-}271}$) by calpain. The precursor region of IL-1α (IL-1$\alpha_{1\text{-}118}$) contains a nuclear localization signal (KVLKKRRL, residues 79–86). We investigated the intracellular localization of IL-1α fused with green fluorescent protein or β-galactosidase. IL-1$\alpha_{1\text{-}118}$ was localized exclusively in the nucleus, but IL-1$\alpha_{1\text{-}271}$ in both the nucleus and the cytoplasm, suggesting the presence of a cytoplasmic retention signal within the mature region of IL-1α. Furthermore, the intracellular localization of IL-1α with deletions from the C terminus, internal deletions and point mutations suggested that the cytoplasmic retention signal is located within residues 168–201.

Key words: cytoplasmic retention signal, β-galactosidase, green fluorescent protein, interleukin-1α precursor, nuclear localization signal

INTRODUCTION

Interleukin (IL)-1, which is mainly produced by activated macrophages, is widely known to exhibit multiple biological activities toward various types of target cells (Oppenheim *et al.*, 1986). There are two distinct types of IL-1, termed IL-1α and IL-1β. Both types of IL-1 are primarily translated as a 33 kDa molecule (pre-IL-1). Then pre-IL-1α and pre-IL-1β are post-translationally processed into 17 kDa molecules (mature IL-1), which is mediated by calpain (EC 3.4.22.17) (Kobayashi *et al.*, 1990a; Carruth *et al.*, 1991) and IL-1β converting enzyme (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992), respectively, followed by the release of mature IL-1 extracellularly.

Intracellular pre-IL-1α is phosphorylated at serine residue(s) in the precursor region at a higher level, at least 10-fold, than intracellular pre-IL-1β (Beuscher *et al.*, 1988; Kobayashi *et al.*, 1988). We also reported previously that processing of pre-IL-1α into mature IL-1α was induced by an increase in the intracellular calcium concentration (Kobayashi *et al.*, 1990a; Watanabe and Kobayashi, 1994), and that *in vitro* phosphorylated human pre-IL-1α bound to acidic phospholipids in a calcium-dependent manner (Kobayashi *et al.*, 1990b). These results led us to propose the possibility that the selective phosphorylation of pre-IL-1α is related to its calcium-dependent proteolytic processing and release (Watanabe and Kobayashi, 1995).

The precursor region of IL-1α (IL-1$\alpha_{1\text{-}118}$) contains a sequence that resembles a nuclear localization signal (KVLKKRRL, residues 79–86) (NLS) (Wessendorf *et al.*, 1993). IL-1$\alpha_{1\text{-}118}$ and pre-IL-1α (IL-1$\alpha_{1\text{-}271}$), both of which were fused with the reporter gene β-galactosidase (β-gal), were demonstrated to be localized in the nucleus (Maier *et al.*, 1994; Wessendorf *et al.*, 1993). Upon analysis of the intracellular distribution of these green fluorescent protein (GFP) or β-gal fusions following transfection, however, we found that IL-1$\alpha_{1\text{-}271}$ is localized in both the nucleus and the cytoplasm, but IL-1$\alpha_{1\text{-}118}$ only in the nucleus. In this report, we demonstrate that IL-1$\alpha_{1\text{-}271}$ contains a cytoplasmic retention signal-like sequence and that this signal is present in the region comprising residues 168–201.

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MATERIALS AND METHODS

Plasmid construction

GFP or β-gal fusion vectors expressing different regions of IL-1α were constructed with pEGFP-N1 (Clontech, Palo Alto, CA) or pEF-BOS-β-gal, respectively. To construct plasmid pEF-BOS-β-gal, the *Eco*RI-*Sst*I fragment from pCaSpeR-βgal containing the N-terminal half of β-gal (Thummel *et al.*, 1988) and the *Sst*I-*Xba*I fragment from pCaSpeR-AUG-β-gal containing the C-terminal half of β-gal (Thummel *et al.*, 1988) were replaced with the *Eco*RI-*Xba*I stuffer fragment of pEF-BOS, which contains the human elongation factor 1α promoter (Mizushima and Nagata, 1990). DNA fragments corresponding to different regions of IL-1α cDNA were amplified by the polymerase chain reaction (PCR) method. The primer sequences and annealing temperatures used for PCR are shown in

Table 1. Primer sequences and annealing temperatures used for PCR amplification corresponding various region of IL-1α. A *Bgl*II site in sense primers and a *Bam*HI site replaced with a stop codon in antisense primers were underlined. A Kozak sequence and an ATG initiation codon were represented by a dotted line and a double line, respectively.

Region		Primer sequence	Annealing temperature (°C)
1-271	sense	CCAGATCTGCCACCATGGCCAAAGTTCCAG	55
	antisense	GAGACTCCAGGGATCCGCCTGGTTT	
1-236	sense	same as 1-271 sense	50
	antisense	AGTTTGGAGGATCCACTGATGT	
1-201	sense	same as 1-271 sense	44
	antisense	TCTCCTTCGGATCCACTGGTT	
1-167	sense	same as 1-271 sense	50
	antisense	ACTTATAAGGATCCATGTCAA	
1-118	sense	same as 1-271 sense	55
	antisense	GAGGATCCGCGAAGCTAAAAGGTGAT	
202-271	sense	AACCAGTAGATCTGAAGGAGA	57
	antisense	same as 1-271 antisense	

sense: sense primer; antisense: antisense primer

Table 1. All sense primers except the 202–271 sense one contained a Kozak sequence (GCCACC) and an ATG translation start codon. The stop codon in all antisense primers was replaced with a *Bam*HI site. PCR was carried out for 30 cycles under the following conditions: denaturation at 94°C for 0.5 min, annealing of the primer at an appropriate temperature (see Table 1) for 1 min, and extension at 72°C for 2 min. The amplified DNA fragments were digested with *Bgl*II within sense primers and *Bam*HI, and then ligated with pUG131 (Amanuma *et al.*, 1989), followed by cycle-sequencing with a DNA Sequencer LIC-4000 (LI-COR) to confirm the sequence. All the inserts except IL-1$\alpha_{202\text{-}271}$ were then inserted into pEGFP-N1 digested with *Bgl*II and *Bam*HI or pEF-BOS-β-gal digested with *Bam*HI to generate various IL-1α fusion vectors to the N terminus of GFP or β-gal, IL-1α/GFP or IL-1α/β-gal, respectively (Fig. 1A). For the construction of IL-1$\alpha_{1\text{-}167/202\text{-}271}$, the insert corresponding to IL-1$\alpha_{202\text{-}271}$ ligated with *Bgl*II and *Bam*HI was inserted into the expression plasmid for IL-1$\alpha_{1\text{-}167}$ (IL-1$\alpha_{1\text{-}167}$/GFP or IL-1$\alpha_{1\text{-}167}$/β-gal) digested with *Bam*HI. To generate an IL-1α mutant, IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$, in which all leucine, isoleucine and valine residues in the region comprising residues 180–193 were replaced with alanine residues, two complementary oligonucleotides indicated by "sense" and "antisense" in Fig. 1B corresponding to residues 168–201 were first annealed at 45°C, digested with *Bgl*II and *Bam*HI, and then inserted into IL-1$\alpha_{1\text{-}167}$/GFP or IL-1$\alpha_{1\text{-}167}$/β-gal. Finally, the resultant plasmid was ligated with the fragment corresponding to IL-1$\alpha_{202\text{-}271}$ digested with *Bgl*II and *Bam*HI.

Cell culture and DNA transfection

NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS, GIBCO BRL, Gaithersburg, MD). Cells were seeded (1.5×10^4 cells/35-mm dish) in DMEM containing 10% FCS 24 h prior to transfection. The cells were transfected with 1 μg of plasmid DNA by the calcium phosphate precipitation method (Graham and van der Eb, 1973). The cells were subjected to glycerol shock 12 h after transfection, followed by a change of the medium and culturing for a further 24 h.

Detection of GFP or β-gal fusion proteins

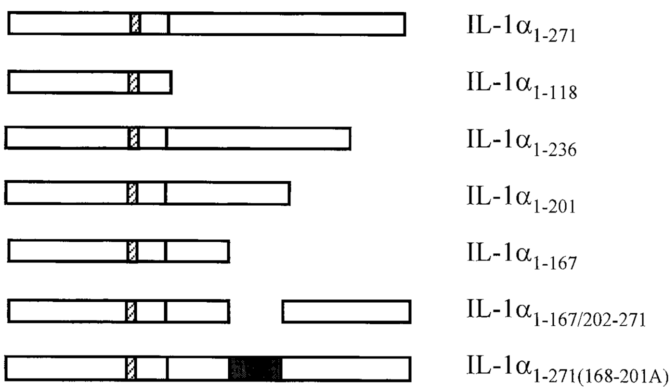
Cells in 35-mm dishes were washed three times with cold phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde and 0.2% (v/v) glutaraldehyde in PBS for 15 min at 4°C, and then washed with cold PBS twice. For the detection of GFP, the cells were examined under a confocal laser microscope (Olympus, Tokyo, Japan). For the detection of β-gal activity, the cells were incubated for 5 to 10 min at 37°C with 1 mg/ml X-gal dissolved in 2 mM $MgCl_2$, 5 mM potassium hexacyanoferrate (II) trihydrate and 5 mM potassium hexacyanoferrate (III). The blue complex formed through β-gal activity was observed under a light microscope.

RESULTS AND DISCUSSION

Intracellular localization of IL-1α

In order to determine the intracellular localization of IL-1α, we constructed expression plasmids encoding IL-1α fused to the N-terminus of GFP or β-gal (Fig. 1A). Then the localization of fusion proteins transiently expressed in NIH/3T3 cells was determined by visualizing the fluorescence of each GFP fusion protein or the enzymatic activity of each β-gal fusion protein. IL-1$\alpha_{1\text{-}118}$ (the precursor region of pre-IL-1α, pro-IL-1α) fused with GFP or β-gal, IL-1$\alpha_{1\text{-}118}$/GFP or IL-1$\alpha_{1\text{-}118}$/β-gal, was localized in the nucleus (Fig. 2, "1-118"), showing the same pattern as a transfectant harboring pENL, which expresses β-gal tagged with the NLS of SV40 large T antigen (provided by Dr. Nabeshima, Kyoto University, Japan) (Fig. 2, "pENL"). This result was compatible with that a NLS was present in the precursor region of IL-1α (residues 79–86) (Wessendorf *et al.*, 1993). On the other hand, IL-1$\alpha_{1\text{-}271}$/GFP and IL-1$\alpha_{1\text{-}271}$/β-gal were localized in both the nucleus and the cytoplasm (Fig. 2, "1-271"), raising the possibility that the intracellular localization of pre-IL-1α (IL-1$\alpha_{1\text{-}271}$) was affected by the mature region (residues 119-271). In previous papers (Maier *et al.*, 1994; Wessendorf *et al.*, 1993), it was described that pre-IL-1α as well as pro-IL-1α was localized in the nucleus. However, it seems that immunofluorescent analysis of pre-IL-1α transfected cells

A



B

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                          171                          180
                                                       Ile   ValIleLeu
                         TyrLysSerSerLysAspAspAlaLysAlaThrAlaAlaAla
sense:      5'-ATAGATCTTTATAAGTCATCAAAGGATGATGCTAAAGCTACCGCGGCTGCA
antisense:  3'-TATCTAGAAATATTCAGTAGTTTCCTACTACGATTTCGATGGCGCCGACGT

                            193                200
   Ile               Leu    Val
 ArgAlaSerLysThrGlnAlaTyrAlaThrAlaGlnAspGluAspGlnProVal
 AGAGCCTCAAAAACTCAAGCGTATGCGACTGCCCAAGATGAAGACCAACCAGTGGATCCGA-3'
 TCTCGGAGTTTTTGAGTTCGCATACGCTGACGGGTTCTACTTCTGGTTGGTCACCTAGGCT-5'
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Fig. 1. Structures of various IL-1α expression vectors analyzed. **A**, DNA fragments corresponding to different regions of IL-1α cDNA fused with GFP or β-gal are schematically shown. The shaded and grey boxes represent the nuclear localization signal (NLS) and the mutated region in IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$, respectively. **B**, The sense and antisense oligonucleotide sequences, and corresponding amino acid sequences used to generate an IL-1α mutant, IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$, are shown. *Bgl*II and *Bam*HI sites for insertion into pEGFP-N1 or pEF-BOS-β-gal are underlined. All alanine residues mutated from leucine, isoleucine or valine ones in the region comprising residues 180–193 are indicated by bold characters.

showed not only intense nuclear staining but also faint cytoplasmic staining, suggesting that a portion of IL-1$\alpha_{1\text{-}271}$ is localized in the cytoplasm.

Intracellular localization of deletion and point mutants of IL-1α

To determine whether or not the mature region affected the cytoplasmic localization of pre-IL-1α, we constructed various mutants with deletions from the C terminus (Fig. 1A). IL-1α with deletion of 35 amino acids from the C terminus fused with GFP or β-gal, IL-1$\alpha_{1\text{-}236}$/GFP or IL-1$\alpha_{1\text{-}236}$/β-gal, was localized in both the nucleus and the cytoplasm (Fig. 2, "1-236"). The localization of a mutant with deletion of 70 amino acids, IL-1$\alpha_{1\text{-}201}$/GFP or IL-1$\alpha_{1\text{-}201}$/β-gal, was also similar to that of the IL-1$\alpha_{1\text{-}271}$/GFP or IL-1$\alpha_{1\text{-}271}$/β-gal fusion (Fig. 2, "1-201"). In contrast, a mutant with deletion of 104 amino acids, IL-1$\alpha_{1\text{-}167}$/GFP or IL-1$\alpha_{1\text{-}167}$/β-gal, was associated with the nucleus (Fig. 2, "1-167"), this localization being similar to those of transfectants expressing the IL-1$\alpha_{1\text{-}118}$/GFP or IL-1$\alpha_{1\text{-}118}$/β-gal fusion protein (Fig. 2, "1-118"). These data suggested that the region comprising residues 168–201 might be involved in the cytoplasmic localization of IL-1α.

We then constructed an internal deletion mutant as to this region (Fig. 1A). The internal deletion mutant as to residues 168–201 (IL-1$\alpha_{1\text{-}167/202\text{-}271}$/GFP or IL-1$\alpha_{1\text{-}167/202\text{-}271}$/β-gal) was associated with the nucleus (Fig. 2, "id"), suggesting that the region comprising residues 168–201 could function to localize IL-1$\alpha_{1\text{-}271}$ in the cytoplasm.

Inspection of the region comprising residues 168–201 revealed that the sequence ITVILRISKTQLYV (residues 180–193, shown in Fig. 3) is rich in leucine, isoleucine and valine residues, thus being a candidate as a cytoplasmic retention signal (Hood and Silver, 2000). When all leucine, isoleucine and valine residues within residues 180–193 were replaced with alanine ones (Fig. 1B), the IL-1α mutant fused with GFP or β-gal (IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$/GFP or IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$/β-gal) was localized in the nucleus (Fig. 2, "168-201A"), further supporting that the region comprising residues 168–201 is a cytoplasmic retention signal.

We also constructed various IL-1α fusions to the C terminus of GFP (GFP/IL-1$\alpha_{1\text{-}118}$, GFP/IL-1$\alpha_{1\text{-}271}$, GFP/IL-1$\alpha_{1\text{-}}$

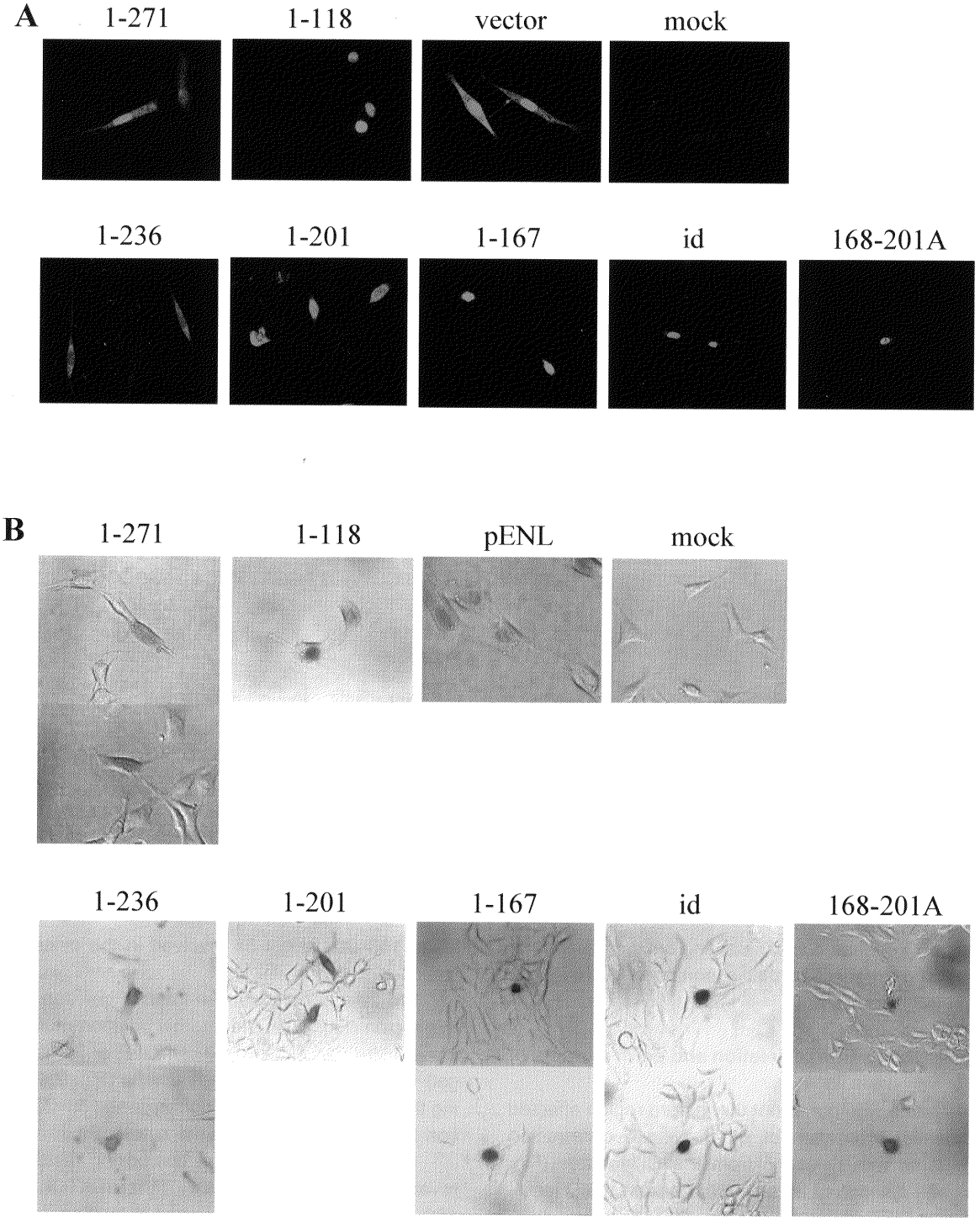

Fig. 2. Intracellular localization of different regions of IL-1α. NIH/3T3 cell transfectants were stained to visualize the fluorescence of each GFP fusion protein (A) or the enzymatic activity of each β-gal fusion protein (B) as described under Materials and Methods. 1-271, IL-1$\alpha_{1\text{-}271}$/GFP or IL-1$\alpha_{1\text{-}271}$/β-gal; 1-118, IL-1$\alpha_{1\text{-}118}$/GFP or IL-1$\alpha_{1\text{-}118}$/β-gal; 1-236, IL-1$\alpha_{1\text{-}236}$/GFP or IL-1$\alpha_{1\text{-}236}$/β-gal; 1-201, IL-1$\alpha_{1\text{-}201}$/GFP or IL-1$\alpha_{1\text{-}201}$/β-gal; 1-167, IL-1$\alpha_{1\text{-}167}$/GFP or IL-1$\alpha_{1\text{-}167}$/β-gal; id, internal deletion mutant of the region comprising residues 168–201, IL-1$\alpha_{1\text{-}167/202\text{-}271}$/GFP or IL-1$\alpha_{1\text{-}167/202\text{-}271}$/β-gal; 168–201A, IL-1α mutant in which all leucine, isoleucine and valine residues in the region comprising residues 168–201 were replaced with alanine residues, IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$/GFP or IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$/β-gal; vector, pEGFP-N1; pENL, β-gal expression vector containing the NLS of SV40 large T antigen; mock, untransfected NIH/3T3. Magnification for all photomicrographs, 200 ×.

$_{167/202\text{-}271}$ or GFP/IL-1$\alpha_{1\text{-}271\ (168\text{-}201A)}$). Localization of the IL-1α fusions exhibited the same pattern as that of fusions to the N-terminus of GFP shown in Fig. 2A (data not shown). This finding suggested that the tagged protein did not affect the intracellular localization of IL-1α.

Since the region comprising residues 119–167 is also

Human IL-1α (180-193)	**I**T**VIL**R**I**SKTQ**L**Y**V**
Rabbit IL-1α	**L**P**VT L**R**I**SQTP**L**F**V**
Horse IL-1α	**L**P**VT L**R**I**SKTR**L**F**V**
Goat IL-1α	**L**P**VT L**R**I**SKTQ**L**F**V**
Monkey IL-1α	**L**P**VT L**R**I**SKTQ**L**F**V**
Bovine IL-1α	**L**P**VT L**R**I**SKTQ**L**F**V**
Rat IL-1α	YP**VT L**K**V**SNTQ**L**F**V**
Murine IL-1α	YP**VT L**K**I**SDSQ**L**F**V**
Consensus	L-V-L-I----L-V

Fig. 3. Comparison of the cytoplasmic retention sequence with those of the IL-1αs of other species. Conserved hydrophobic amino acid residues (leucine, isoleucine and valine ones) are indicated by bold letters.

rich in leucine and isoleucine residues, the possibility remains that this region may also localize IL-$1\alpha_{1\text{-}271}$ in the cytoplasm. However, the internal deletion mutant as to residues 119–167 was not associated with the nucleus (data not shown). Furthermore, the internal deletion mutant as to residues 119–201 fused with GFP or β-gal was associated with the nucleus to the same extent as the internal deletion mutant as to residues 168–201 (data not shown), suggesting that the region comprising residues 119–167 may not function as a cytoplasmic retention sequence.

Cellular proteins containing both a NLS and a nuclear export signal (NES) have the capacity to shuttle between the nucleus and cytoplasm. The well-characterized NESs, which include the NESs in human immunodeficiency virus, type I-coded Rev protein (Fornerod *et al.*, 1997), cAMP-dependent protein kinase inhibitor (Wen *et al.*, 1995), and mitogen-activated protein kinase kinase (Fukuda *et al.*, 1996), comprise a core of closely spaced leucine or hydrophobic residues, and function by binding to an export receptor, CRM1 (Fukuda *et al.*, 1997). The sequence defined in this study was not influenced by the CRM1 specific inhibitor leptomycin B (LMB) (data not shown), suggesting that the cytoplasmic localization of pre-IL-1α is mediated by a CRM1-independent pathway as reported for several other proteins (Black *et al.*, 2001; Rashevsky-Finkel *et al.*, 2001; Vajjhala *et al.*, 2003). Consistent with this, the cytoplasmic retention sequence of IL-1α exhibits alternative spacing of leucine-rich consensus NES sequence, $LX_{2\text{-}3}LX_2LXL$ (Nakielny and Dreyfuss, 1997).

Since the sequence is highly conserved among various species of IL-1α (Gubler *et al.*, 1989) (Fig. 3), the possibility was raised that the conserved cytoplasmic retention sequence present in IL-1α species is essential for regulation of their activities under physiological conditions. Although IL-1α and fibroblast growth factor (FGF)-1 share the properties including the presence of a NLS and the absence of a signal sequence (Lin *et al.*, 1996), this study revealed that IL-1α is different from FGF-1 in terms of the presence of a cytoplasmic retention sequence.

Based on previously reported results (Carruth *et al.*, 1991; Kobayashi *et al.*, 1990a, b; Watanabe and Kobayashi, 1994, 1995), our current model is as follows. A portion of pre-IL-1α is localized in the cytoplasm, which is assisted by the cytoplasmic retention sequence described in this study. Upon cellular activation leading to calcium entry, pre-IL-1α is proteolytically processed by calpain, the mature form of IL-1α (IL-$1\alpha_{119\text{-}271}$) former being released extracellularly while IL-$1\alpha_{1\text{-}118}$ containing NLS being accumulated in the nucleus. Thus the presence of a cytoplasmic retention sequence in pre-IL-1α is suited to processing by calpain in the cytoplasm. Once released, mature IL-1α exerts its pleiotropic function via a specific receptor (Sims and Dower, 1994). On the other hand, the nuclear accumulation of IL-$1\alpha_{1\text{-}118}$ reportedly leads to transformation of perivascular mesangial cells (Stevenson *et al.*, 1997) and induces apoptosis in a range of tumor cells (Pollock *et al.*, 2003), suggesting that IL-$1\alpha_{1\text{-}118}$ plays some biological roles such as cell growth, senescence and so on. It was recently reported that pre-IL-1α also functions in the nucleus. Pre-IL-1α binds to necdin, a nuclear protein with growth suppressor activity and acts as a transcriptional activator for the synthesis of proinflammatory cytokines (Hu *et al.*, 2003; Werman *et al.*, 2004).

Although an increase in calcium concentration is certainly one of key regulators for intracellular localization of IL-1α, there may be other mechanism(s) by which intracellular localization of IL-1α would change. Much work is needed to clarify such a possibility.

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