Roles of the phosphorylation of human interleukin 1α in proteolytic processing

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Abstract To investigate the role of phosphorylation of a precursor form of interleukin-1α (pre-IL-1α), we obtained cells producing either phosphorylated or unphosphorylated pre-IL-1α. Although calcium-dependent proteolytic processing of unphosphorylated pre-IL-1α could be observed in cell lysates, proteolytic processing was not induced by treatment with calcium ionophore in intact cells producing the unphosphorylated pre-IL-1α. Further, unphosphorylated pre-IL-1α showed no calcium-dependent binding to acidic phospholipids at concentrations below 5×10^{-6} M. These results suggested that phosphorylated pre-IL-1α became susceptible to proteolytic processing by association with the cell membrane in a calcium-dependent manner.

Key words: Interleukin-1α; Phosphorylation; Proteolytic processing

1. Introduction

Interleukin-1 (IL-1) has pleiotropic biological activities implicated in a variety of physiological processes [1]. There are two distinct types of IL-1, termed IL-1α and IL-1β, both of which have been cloned [2–5]. Both types of IL-1 are primarily translated as 33-kDa molecules (pre-IL-1). Then, pre-IL-1α and pre-IL-1β are post-translationally processed into 17-kDa molecules (mature IL-1) mediated by calpain (EC 3.4.22.17) [6,7] and IL-1β converting enzyme [8,9], respectively, followed by the extracellular release of mature IL-1.

Intracellular pre-IL-1α is phosphorylated at serine residue(s) in the precursor region at levels at least 10-fold higher than intracellular pre-IL-1β [10,11]. We also described previously that processing of pre-IL-1α into mature IL-1α was induced by increases in intracellular calcium concentration [6], and that in vitro phosphorylated human pre-IL-1α bound to acidic phospholipids in a calcium-dependent manner [12]. These results suggested the possibility that the selective phosphorylation of pre-IL-1α is related to its calcium-dependent proteolytic processing. In this study, we tested this possibility by constructing a mutant pre-IL-1α lacking phosphorylation sites. Our results indicated that the phosphorylation of pre-IL-1α might facilitate calcium-dependent association with the cell membrane where pre-IL-1α becomes susceptible to proteolytic processing.

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Abbreviations: IL-1, interleukin-1; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MEM, minimum essential medium; EGTA, ethylene glycol bis-β3-aminoethyl ether *N,N,N',N'*-tetraacetic acid; BSA, bovine serum albumin; LPS, lipopolysaccharide; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

2. Materials and methods

2.1. Antibodies

Rabbit anti-IL-1α was prepared by immunizing rabbits with human rIL-1α [10], and was used for immunoprecipitation. The antigen specificity was confirmed by immunoprecipitation, Western blotting, and neutralization experiments [10], and pre-IL-1α as well as mature IL-1α was equally recognized by this antibody [13].

2.2. Plasmid construction

The IL-1α expression plasmid was constructed as follows. The *Pst*I–*Hin*cII fragment (0.9 kb) of pHL4 [10], which contains the coding region of pre-IL-1α, was inserted into pUG131 [14] treated with *Pst*I and *Eco*RV. This plasmid (pUHL(PH)) was digested with *Bgl*II and *Sma*I, following the end-filling of protruding ends, and ligated with a *Xba*I linker. The linker-ligated *Xba*I fragment (0.9 kb) was replaced with the *Xba*I stuffer fragment of pEF-BOS [15], which contains the human elongation factor 1α promoter.

For site-directed mutagenesis, the *Pst*I–*Hin*cII fragment of pHL4 was inserted into M13tg131 treated with *Pst*I and *Eco*RV. Single-stranded phage DNA was used as a template for mutagenesis by the method described by Kunkel et al. [16]. Mutated pre-IL-1α cDNA was also inserted into pEF-BOS as described above. The primers used for the mutagenesis are shown in Fig. 1.

2.3. Cell culture and DNA transfection

NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The IL-1α expression plasmid DNA (20 μg) and pSV2neo plasmid DNA (0.5 μg) carrying the neomycin resistance gene were cotransfected into NIH/3T3 cells by the calcium phosphate precipitation method [17]. Colonies resistant to the antibiotic G418 (active concentration, 250 μg/ml) were isolated and IL-1α activity in cell lysates was measured by incorporation of [^{3}H]thymidine into the IL-1- and IL-2-dependent cell line D10G4N.

2.4. Metabolic labelling of cells with [^{32}P]orthophosphate

NIH/3T3 cells producing pre-IL-1α in 35-mm dishes were rinsed with phosphate-free Hanks' balanced salt solution (HBSS), placed at 37°C for 30 min, and then labelled by incubation at 37°C for an additional 2 h with 0.1 mCi of [^{32}P]orthophosphate (2 mCi/ml; 1 Ci = 37 GBq, ICN Biomedicals Inc., Costa Mesa, USA) in 1 ml of phosphate-free HBSS. Labelled cell lysates prepared as described previously [6] were immunoprecipitated with rabbit anti-human IL-1α antibodies [10] and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18]. Gels were dried and exposed to Kodak XAR film at −80°C.

2.5. Metabolic labelling of cells with [^{35}S]methionine

NIH/3T3 cells producing pre-IL-1α in 35-mm dishes were labelled by incubation at 37°C for 2 h with 0.1 mCi of L-[^{35}S]methionine (Tran^{35}S-label, specific activity 1037 Ci/mmol; ICN Biomedicals Inc.) in 1 ml of methionine-free minimum essential medium (MEM) supplemented with 2% FCS. Labelling medium was replaced with 1 ml of DMEM supplemented with 2% FCS (chase) in the presence or absence of calcium ionophore (A23187, Sigma) and incubated for 30 min. The resultant culture supernatants and cell lysates prepared as described previously [6] were immunoprecipitated with rabbit anti-human IL-1α

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antibodies [10] and analyzed by SDS-PAGE [18]. Gels were treated with Enlightning (Dupont/NEN Research Products), dried, and exposed to Kodak XAR films at −80°C. The radioactivities of IL-1α bands in the gel were quantitated using a Bio-imaging analyzer (Fuji Photo Film Co., Ltd.).

2.6. Preparation of ^{35}S-labelled pre-IL-1α

Cells were labelled with L-[^{35}S]methionine and the resultant cell lysates were prepared as described above. Labelled pre-IL-1α was purified by immuno-affinity column chromatography [19] from the cell lysates.

2.7. Binding of pre-IL-1α to phophatidic acid

Phosphatidic acid vesicles were prepared as described previously [12] except rehydration with 10 mM Tris-buffered saline (pH 8.0) containing 2 mM ethylene glycol bis-β3-aminoethyl ether *N,N,N',N'*-tetraacetic acid (EGTA). Phosphatidic acid vesicles at a final concentration of 0.4 mg/ml were incubated with ^{35}S-labelled pre-IL-1α in the absence or presence of 1, 2, 3 or 4 mM $CaCl_2$ for 30 min at 22°C, followed by harvesting on a Millipore filter (GVWP025) presoaked in the rehydration buffer supplemented with 1 mg/ml bovine serum albumin (BSA). Each filter was washed twice with rehydration buffer for the $CaCl_2$-free samples or 10 mM Tris-buffered saline (pH 8.0) for the $CaCl_2$-containing samples, then dried, and the radioactivity was counted.

3. Results

3.1. Isolation of cells producing the unphosphorylated pre-IL-1α

We and others reported previously that most pre-IL-1α is phosphorylated in lipopolysaccharide (LPS)-activated human monocytes [10,11]. Although the precise in vivo phosphorylation sites of pre-IL-1α were unknown, our experimental data using a synthetic peptide (residues 84 to 112) showed that all or any of 4 serine residues close to dibasic/tetrabasic amino acid sequence were phosphorylated [10] as shown in Fig. 1.

To investigate the role of phosphorylation of pre-IL-1α, we constructed the S mutant in which all of 4 serine residues likely to be phosphorylated were converted into glycine residues by site-directed mutagenesis (Fig. 1). When NIH/3T3 cell clones expressing the S mutant were labelled with [^{32}P]orthophosphate, pre-IL-1α was phosphorylated in all the clones tested to the same extent as in cells expressing the wild-type IL-1α (data not shown). By limited proteolysis with trypsin, phosphorylated amino acid(s) in the S mutant were localized in the same trypsin fragment, in which phosphorylated serine residues were present [13] (data not shown). Since it was suggested that the site of phosphorylation was not present in mature IL-1α [10] and that trace amounts of phosphothreonine were detected in purified [^{32}P]orthophosphate-labelled pre-IL-1α [10], we considered that one threonine residue adjacent to these 4 serine residues might be phosphorylated. Therefore, this threonine residue was further converted into a glycine residue (ST mutant, see Fig. 1).

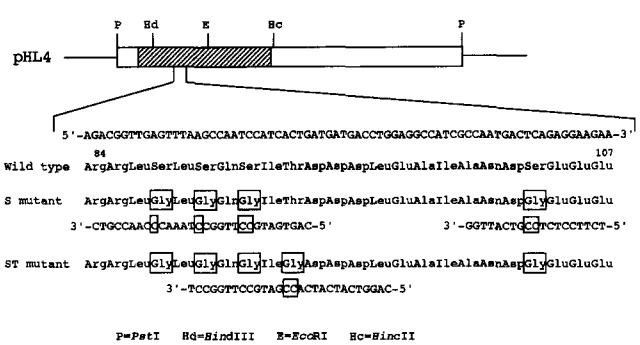

Fig. 1. Amino acid and nucleotide sequences in the phosphorylated region of pre-IL-1α. The primers used for mutagenesis are shown below the amino acid sequences of S and ST mutants. Nucleotides and amino acids replaced in the mutants are boxed. The coding region of pre-IL-1α in pHL4 is shaded.

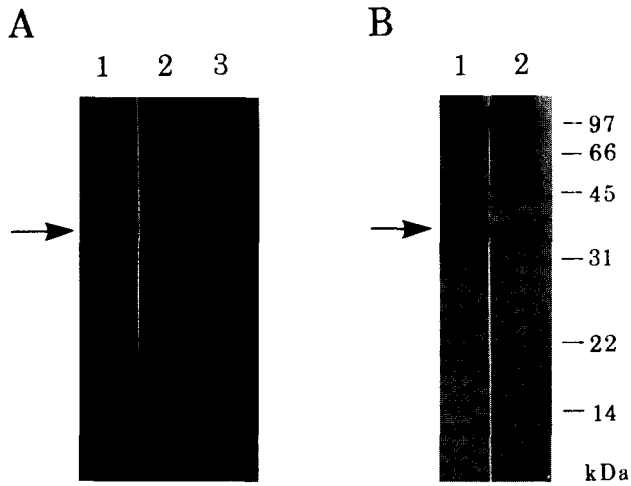

Fig. 2. Phosphorylation and synthesis of pre-IL-1α. Cells were labelled with [^{32}P]orthophosphate (A) or L-[^{35}S]methionine (B). The resultant cell lysates prepared from the NIH/3T3 cells expressing wild-type IL-1α (lane 1) and ST mutant (lane 2), or untransfected NIH/3T3 cells (lane 3) were immunoprecipitated by anti-IL-1α antibodies and analyzed by SDS-PAGE. The arrows indicate the migration posotions for pre-IL-1α.

In the NIH/3T3 cell clone expressing the wild-type IL-1α, phosphorylated forms of pre-IL-1α were detected (Fig. 2A, lane 1). In contrast, in the cell clone expressing the ST mutant, phosphorylation of pre-IL-1α was hardly detected (Fig. 2A, lane 2), although ^{35}S-labelled pre-IL-1α was present (Fig. 2B, lane 2).

3.2. Processing and release of unphosphorylated IL-1α

Although pre-IL-1α produced by LPS-activated human monocytes is inefficiently processed and released, we and others described previously that the processing of pre-IL-1α into mature IL-1α was enhanced by increases in intracellular calcium concentration [6,20]. We also described previously that processed mature IL-1α was selectively released from the cells and that the amount of pre-IL-1α released paralleled lactate dehydrogenase activity, suggesting that the release of pre-IL-1α might result from cell damage [21]. When cells producing the wild-type IL-1α were treated with A23187, proteolytic processing of pre-IL-1α was induced and accompanied by the release of mature IL-1α (Fig. 3A). In contrast, in cells producing the unphosphorylated pre-IL-1α, processing of pre-IL-1α was hardly detected by treatment with A23187, although trace amounts of processed IL-1α were observed intracellularly by treatment with 10 μM of A23187 (Fig. 3B). As a result, the release of mature IL-1α was hardly detected in cells producing the unphosphorylated IL-1α. However, when cell lysates prepared from cells producing the unphosphorylated pre-IL-1α were incubated in the presence of $CaCl_2$, processing into mature IL-1α was observed (Fig. 4, lane 4). The percentage of processing in unphosphorylated pre-IL-1α was nearly the same as that

in phosphorylated pre-IL-1α (Fig. 4). Thus, these results demonstrated that the phosphorylation of pre-IL-1α did affect its susceptibility to calcium-dependent proteolytic processing in intact cells but not in cell lysates.

3.3. Calcium-dependent binding of pre-IL-1α to phosphatidic acid

According to our preliminary results, in vitro phosphorylated pre-IL-1α was bound to membrane in the presence of $CaCl_2$ [12]. We therefore examined whether the phosphorylation of pre-IL-1α was related to the association with phospholipids. Phosphorylated and unphosphorylated pre-IL- 1α as binding substrates were affinity-purified from HTB9 5637 cells [6] and ST mutant-expressing cells. As shown in Fig. 5, phosphorylated pre-IL-1α bound to phosphatidic acid in a calcium-dependent manner. In contrast, unphosphorylated pre- IL-1α showed relatively low calcium-dependency compared to the phosphorylated form. At physiological concentrations of intracellular calcium, that is below 5×10^{-6} M, unphosphorylated pre-IL-1α showed no calcium-dependent binding, suggesting that the phosphorylation of pre-IL-1α affects the calcium-dependent association with cell membrane.

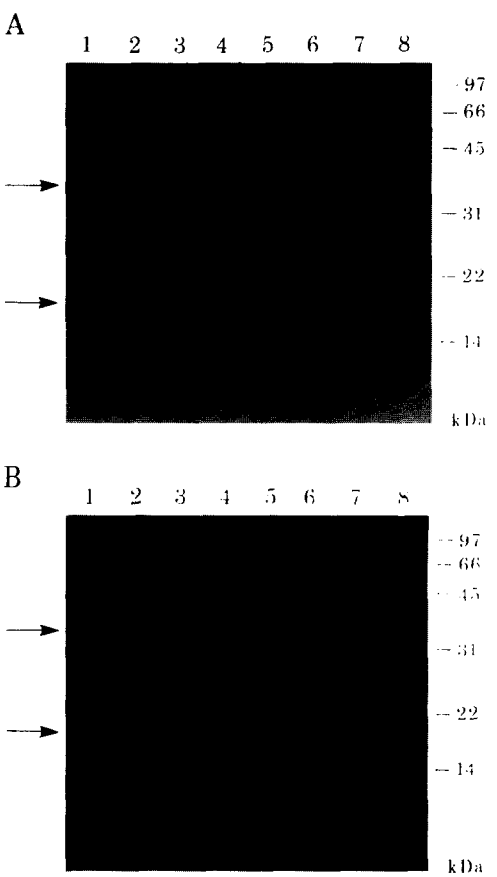

Fig. 3. Processing and release of IL-1α by treatment with A23187. Cells expressing wild-type IL-1α (A) and ST mutant IL-1α (B) were labeled with L-[^{35}S]methionine and chased in the absence (lanes 1 and 5) or presence of 2 μM (lanes 2 and 6), 5 μM (lanes 3 and 7), or 10 μM (lanes 4 and 8) A23187. The resultant cell lysates (lanes 1 to 4) and supernatants (lanes 5 to 8) were imunoprecipitated and analyzed by SDS-PAGE. The arrows indicate the migration positions for pre-IL-1α (33 kDa) or mature IL-1α (17 kDa).

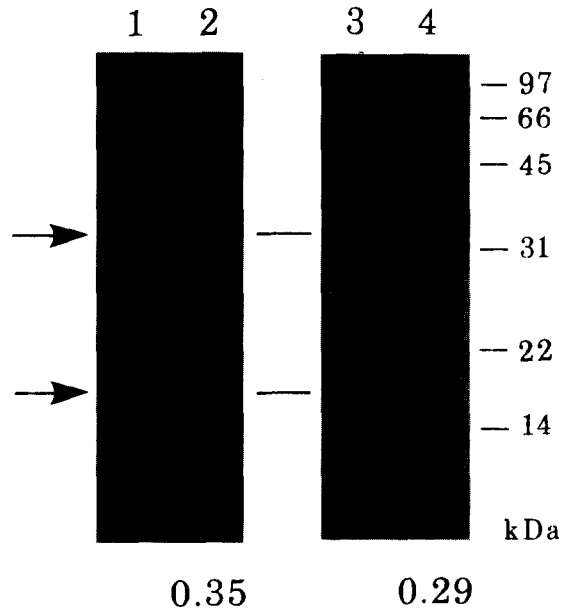

Fig. 4. Calcium-dependent processing of pre-IL-1α in the cell lysates. Cells expressing wild-type IL-1α (lanes 1 and 2) and ST mutant IL-1α (lanes 3 and 4) were labelled with L-[^{35}S]methionine as described in section 2 and lysed with 1 ml of 9 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 20 mM Tris-HCl (pH 8.0) with 5 mM EGTA (lanes 1 and 3) or 1 mM $CaCl_2$ (lanes 2 and 4). After incubation at 37°C for 30 min, each cell lysate was immunoprecipitated and analyzed by SDS-PAGE. The ratio of mature IL-1α in the presence of $CaCl_2$ was indicated below the autoradiogram. The amounts of pre- and mature IL-1α were corrected based on the differences in number of methionine residues between the two forms. The arrows indicate the migration positions for pre-IL-1α (33 kDa) or mature IL-1α (17 kDa).

4. Discussion

In this study, we explored the role of phosphorylation of pre-IL-1α by the alteration of phosphorylated residues into unphosphorylated residues. Although unphosphorylated as well as wild-type pre-IL-1α served as substrates for calcium-dependent processing protease in cell lysates, unphosphorylated pre-IL-1α was hardly processed in intact cells by increases in intracellular calcium concentration and showed no calcium-dependent binding to phosphatidic acid at physiological intracellular calcium concentrations. We and others demonstrated that calcium-dependent proteolytic processing of pre-IL-1α in cell lysates was selectively mediated by calpain [6,7]. Calpain is believed to be converted to an active form by binding to phospholipids in a calcium-dependent manner [22], although the activated form of calpain has never been isolated from tissues. Therefore our results suggested that calcium-dependent association of phosphorylated pre-IL-1α with the cell membrane might be necessary for proteolytic processing by calpain. Adachi et al. [23] also reported that phosphorylated forms of calpastatin, an endogeneous calpain inhibitor, were distributed in the membrane fraction compared to unphosphorylated forms, although they did not examine the effects of calcium on its change in distribution.

Since phosphoamino acid analysis of human pre-IL-1α revealed that phosphoserine was predominant [10], all the 4 serine residues likely to be phosphorylated were mutated (S mutant).

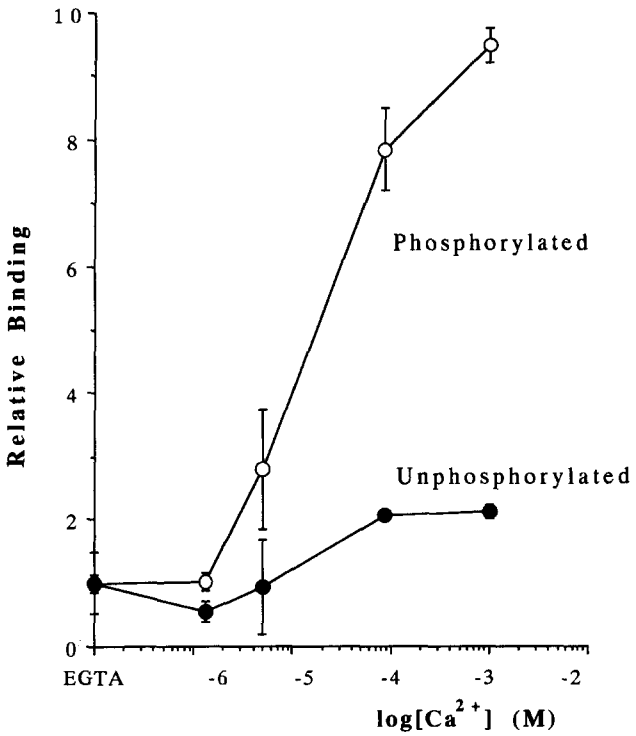

Fig. 5. Calcium-dependent binding of phosphorylated and unphosphorylated pre-IL-1α to phosphatidic acid. Affinity-purified ^{35}S-labelled phosphorylated (1216 dpm) and unphosphorylated pre-IL-1α (416 dpm) were incubated with phosphatidic acid vesicles in the absence or presence of $CaCl_2$. Relative binding activity was expressed as the ratio of binding activity to that in the presence of EGTA.

However, cells expressing the S mutant showed phosphorylation similarly to those expressing the wild-type pre-IL-1α. Although we did not determine the phosphorylated amino acid(s) in the S mutant, threonine, trace amounts of which were phosphorylated in the wild-type pre-IL-1α [10], might be phosphorylated compensating for the mutation of serine residues in the S mutant. As a result of the further alteration of the threonine residue into glycine residue (ST mutant), we obtained cells expressing the unphosphorylated pre-IL-1α.

When calpain is converted to an activated form after calcium-dependent translocation to the cell membrane, the calcium-binding domain, the E–F hand structure in calpain, is involved in the calcium-dependent translocation to the cell membrane [24]. It was also reported that cytosolic phospholipase A_2 was translocated via a calcium-dependent phospholipid-binding motif [25]. Although it was clear that calcium was required for the activation in calpain in the processing of pre-IL-1α, we did not know how pre-IL-1α translocated in a calcium-dependent manner, since pre-IL-1α has no known calcium-binding domains.

Even unphosphorylated pre-IL-1α bound to phosphatidic acid in a calcium-dependent manner, although the increase of binding induced by calcium was significantly lower than in phosphorylated pre-IL-1α. This increase of binding of unphosphorylated pre-IL-1α to phosphatidic acid might be due to a conformational change caused by the alteration of amino acids. That trace amounts of processed form of unphosphorylated pre-IL-1α were detected intracellularly in the presence of 10 μM A23187 might have been caused by this increase in binding.

In conclusion, our findings supported the model that the translocation of phosphorylated pre-IL-1α to the cell membrane was necessary for its proteolytic processing induced by calcium ionophore. Although we do not have any direct evidence supporting that the same model is applicable to LPS-activated macrophages, it is pertinent to introduce our recent observation that LPS could increase intracellular calcium concentration in murine macrophage cell line, J774.1. Although it seemed that the phosphorylation was necessary for the binding of pre-IL-1α to acidic phospholipids, we do not know whether acidic phospholipids inside the cell membrane are the main target of the binding of pre-IL-1α. We are currently investigating this point directly.

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