Nuclear Translocation of the N-terminal Prodomain of Interleukin-16*

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Interleukin-16 (IL-16) is a pleiotropic cytokine that functions as a chemoattractant factor, a modulator of T cell activation, and an inhibitor of human immunodeficiency virus (HIV) replication. These diverse functions are exclusively attributed to the secreted C-terminal peptide of 121 amino acids (mature IL-16), which is cleaved from the precursor protein (pro-IL-16) by caspase-3. Human pro-IL-16 is comprised of 631 amino acids with three PDZ domains, one of which is present in secreted mature IL-16. No cellular localization or biologic functions have been ascribed to the unusually large and highly conserved N-terminal prodomain formed as a result of proteolytic release of the third PDZ domain of pro-IL-16. Here we show that the N-terminal prodomain of pro-IL-16 translocates into the nucleus following cleavage of the C-terminal segment. The nuclear localization signal of pro-IL-16 consists of a classical bipartite nuclear targeting motif. We also show that the nuclear targeting of the IL-16 prodomain induces a G1 arrest in the cell cycle. Taken together, the high degree of conservation of the prodomain among species, the presence of two PDZ motifs, and the nuclear localization and subsequent inhibitory effect on cell cycle progression suggest that pro-IL-16 is cleaved into two functional proteins, a C-terminal-secreted cytokine and an N-terminal product, which affects the cell cycle.

Experimental Procedures

cDNA Constructs—Pro-IL-16 cDNA was subcloned into vector pXM (Genetics Institute). An N-terminal GFP pro-IL-16 fusion construct was produced by inserting the GFP coding sequence from pEGFP-1 (CLONTECH) into pXM-pro-IL-16 using the 5′-KpnI site in the vector and a 3′-BglII site introduced after the first methionine of pro-IL-16 by polymerase chain reaction. For the FLAG-tagged constructs, we used polymerase chain reaction to add the FLAG sequence to either the N- or C-terminal of pro-IL-16 either after the first methionine or right before the stop codon, respectively. Truncated pro-IL-16 constructs were created using recombination polymerase chain reaction to delete the selected portion of pro-IL-16 as listed in Fig. 3A and cloned back into the pXM vector. All constructs were confirmed by sequence analysis.

Transfections—For the immunoblotting experiments, transfections were performed, and cells were collected as described previously (8). For the immunocytochemistry experiments, cells were plated on chamber slides 24 h before transfection. The transfections were performed on the slides with SuperFect reagent (Qiagen) according to the manufacturer's protocol.

Immunoblotting and Immunocytochemistry—Anti-IL-16 (monoclonal antibody: clone 14.1) (10) and anti-FLAG antibodies (Sigma) were used for immunoblotting. Immunoblots were performed as described previously (8). For immunohistochemistry, only the anti-FLAG antibody was used. Briefly, 48 h after transfection, cells growing on the chamber slides were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min. After washing twice with PBS, fixed cells were permeabilized with 0.5% Triton X-100 for 5 min, washed twice with PBS, and then incubated with 5% milk and 50 μg/ml normal goat serum for 1 h at room temperature. After removing the blocking milk, the cells were incubated with anti-FLAG M2 antibody (Sigma) for 1 h at room temperature.
temperature. The slides were washed three times (5 min each) with PBS/Tween (0.05%) before incubation with rhodamine-conjugated goat-anti-mouse IgG (1:500) in 1% milk for 1 h. The slides were then washed two times with PBS/Tween, the chamber and gasket were disassembled, the slides were washed once more with PBS/Tween, and were finally rinsed with distilled water. After removal of excess water the slides were mounted with Pro Long antifade solution (Molecular Probes) covered with coverslips and sealed with nail polish. Slides were examined either with confocal microscopy or an Axioplan microscope (Zeiss) with a x 40 objective. Images of cells were captured using 400 ASA slide film (Eastman Kodak Co.).

Cell Fractionation—Forty-eight hours after transfection, COS cells grown in P100 plates were harvested by trypsin treatment and resuspended in ice-cold PBS. Cells were rinsed two times with cold PBS prior to incubation with Buffer I (20 mM Tris-HCl, 0.5 mM dithiothreitol, 10 mM β-glycerol phosphate, 300 mM sucrose, 0.2 mM EGTA, 5 mM MgCl₂, and 10 mM KCl and the protease inhibitors aprotinin, chymostatin, antipain, and pepstatin at 10 μg/ml) on ice for 15 min. 20 μl of 10% Nonidet P-40 were added into the 400-μl cell suspension and hand-mixed for 10 s. The sample was then centrifuged at 2000 rpm for 5 min at 4 °C using a desk-top microcentrifuge to pellet the nuclei. The supernatant was further fractionated by centrifugation at 14,000 rpm for 20 min at 4 °C. The supernatant fraction of this centrifugation was collected and classified as the cytoplasmic fraction. The nuclear pellet was washed twice with ice-cold PBS before adding 50 μl of Buffer II (10 mM Tris-HCl, 0.5 mM dithiothreitol, 10 mM β-glycerol phosphate, 0.2 mM EGTA, 5 mM MgCl₂, 350 mM KCl, 25% glycerol and the protease inhibitors aprotinin, chymostatin, antipain, and pepstatin at 10 μg/ml) to lyse the nuclei. After a 15-min incubation on ice, nuclear lysates were centrifuged at 14,000 rpm for 5 min at 4 °C, and this supernatant fraction was classified as nuclear. The protein concentration in each fraction was determined using Bio-Rad bovine gamma globulin as standard protein and Bio-Rad protein assay reagent.

Cell Cycle Analysis—5 × 10⁶ COS cells were washed with PBS buffer and fixed in 80% ethanol on ice for 1 h. After fixation, cells were washed with PBS twice before resuspension in RNase A (100 μg/ml)/PIB (propidium iodide staining buffer: PBS buffer with the addition of 0.12% Triton X-100 and 0.12 mM EDTA) for 45 min at 37 °C. PI solution (5 mg/ml propidium iodide dissolved in PIB) was added at 50 μg/ml, and the reaction was incubated at room temperature in the dark for at least 1 h. The analysis was performed on the day of the assay using a flow cytometer (Becton Dickinson).

![Fig. 1](http://www.jbc.org/) Both wild-type and modified pro-IL-16 undergo normal processing in transfected COS cells. A, schematic representations of wild-type pro-IL-16 protein as well as N-terminal GFP-fused and C-terminal FLAG-tagged pro-IL-16 protein (GFP-80-FLAG). B, Western blot of COS cell lysates following transfection either with wild-type pro-IL-16 cDNA (lane 1) or with (GFP)-pro-IL-16-(FLAG) (lane 2) constructs. The Western blot was probed with monoclonal anti-IL-16 antibody. This antibody recognizes a region in the C-terminal third PDZ domain (i.e., the mature secreted IL-16) of pro-IL-16.

![Fig. 2](http://www.jbc.org/) Confocal images of autofluorescence (green) and immunofluorescence (red) of GFP-80-FLAG-transfected COS cells. A, green fluorescence represents both full-length as well as N-terminal-cleaved pro-IL-16 proteins and shows minimal nuclear and predominant perinuclear localization. B, red fluorescence is limited to cytoplasmic staining, which represents either full-length pro-IL-16 or C-terminal mature IL-16. C, an overlay image of green and red fluorescence shows that the full-length pro-IL-16 protein is located in perinuclear and reticular regions (yellow); the N-terminal profragment of IL-16 targets into the nucleus, and the C-terminal IL-16 remains in the cytoplasm. D, phase contrast image of COS cells shown in A–C. Arrows indicate COS cells that do not express pro-IL-16 protein.

![Fig. 3](http://www.jbc.org/) Schematic diagram of three pro-IL-16 deletions and their expression in transfected COS cells. A, structural alignment of deletions. B, Western blot of lysates of COS cells transfected with the N-terminal FLAG-tagged deletion constructs noted on the top of each lane and designated by the kDa. The blot was probed with anti-FLAG M2 antibody.
RESULTS

COS Cell Expression and Processing of Pro-IL-16 and Its Derivative—Schematic representations of the wild-type pro-IL-16 and a construct of the full-length 80-kDa pro-IL-16 with a GFP-fused N-terminal and a FLAG-tagged C-terminal (GFP80-FLAG) are shown in Fig. 1A. Expression and natural processing of the GFP80-FLAG protein in the transfected COS cells were compared with wild-type pro-IL-16 by Western blot of the transfected COS cell lysate probed with anti-IL-16 antibody (Fig. 1B). The Western analysis demonstrates that pro-IL-16 is processed normally in COS cells, justifying the use of this system to explore the fate of pro-IL-16 after cleavage.

Subcellular Localization of IL-16—Double immunofluorescence confocal microscopic analysis of COS cells transfected with the GFP80-FLAG construct revealed that the GFP green fluorescence localized to both cytoplasm and nucleus (Fig. 2A). In contrast, staining with a rhodamine-labeled anti-FLAG antibody localized red fluorescence in the cytoplasm only (Fig. 2B). The differential distribution of the selectively truncated Pro-IL-16 proteins in transfected COS cells is shown in Fig. 4A and 4B. The 60-kDa N-terminal prodomain of IL-16 is localized in the nucleus, while the C-terminal mature IL-16 (20 kDa) is located in the cytoplasm. The 30-kDa protein of pro-IL-16 remains in the cytoplasm.

Subcellular fractionation confirms the nuclear targeting motif of pro-IL-16. Table I provides a quantitative assessment of the intracellular distribution of GFP and GFP fusion proteins in COS cells transfected with the indicated constructs.

FIG. 4. Differential distribution of the selectively truncated Pro-IL-16 proteins in transfected COS cells. A1 and A2, N-terminal prodomain of IL-16 (60 kDa) is localized in the nucleus. B1 and B2, C-terminal mature IL-16 (20 kDa) locates in the cytoplasm. C1 and C2, the 30-kDa protein of pro-IL-16 remains in the cytoplasm.

FIG. 5. Mutation of the NLS significantly reduced nuclear targeting activity of the N-terminal prodomain of IL-16. A, with nonmutated NLS, the C-terminal-deleted pro-IL-16 locates predominantly in the nucleus (60 kDa NLSW). B, after NLS mutation (60 kDa NLSM, as shown in Fig. 6), the nuclear localization of C-terminal-deleted pro-IL-16 is dramatically reduced.

FIG. 6. The NLS of pro-IL-16 regulates the translocation of the IL-16 prodomain. A, schematic representation of the NLS mutation in the prodomain of IL-16 (GFP60 NLSM). B, immunoblot of cytoplasmic (C) and nuclear (N) fractions of COS cells transfected with constructs of either GFP60 (lanes 1 and 2), GFP60, NLS-mutated GFP60 (lanes 3 and 4), GFP60 NLSM, nuclear-localized GFP (lanes 5 and 6, NLS GFP), or GFP (lanes 7 and 8), respectively. The blot was probed first with anti-GFP polyclonal antibody (upper) and then stripped and reprobed with anti-tubulin monoclonal antibody (bottom). Subcellular fractionation confirms the nuclear targeting motif of pro-IL-16.

TABLE I

| COS cells transfected with construct of | Percentage of expressed protein* in the cytoplasm | Percentage of expressed protein* in the nucleus |
|----------------------------------------|-----------------------------------------------|---------------------------------------------|
| GFP                                    | 99.3                                          | 0.7                                         |
| NLS-GFP                                | 0.1                                           | 99.9                                        |
| GFP60                                  | 46.6                                          | 53.4                                        |
| GFP60-NLSM                             | 77.0                                          | 23.0                                        |

* Proteins were quantified by densitometric analysis of Western blot.
Forty-eight hours after transfection, cells were collected, stained with propidium iodide, and sorted by flow cytometry to identify transfected cells (GFP positive). The gated green fluorescent-positive cells were analyzed for cell cycle profile by relative DNA content according to the red fluorescence of propidium iodide.

Identification of a Nuclear Localization Sequence (NLS)—Knowing the N-terminal region of pro-IL-16 between amino acid 1 and 257 is necessary for nuclear targeting, we next searched the sequence of pro-IL-16 to see whether there were any consensus nuclear-targeting motifs. One basic region that could function as a bipartite nuclear localization sequence (NLS) was found between amino acids 79 and 101 ($^{79}$KKGP-PVAPKPAWFRQSLKGLAN$^{101}$). Similar bipartite motifs have been found in many nuclear proteins and serve as consensus sequences for nuclear targeting (11). To evaluate the nuclear localization properties of this NLS, we performed mutagenesis to change all five basic amino acids into alanines (indicated as underlined) at the both N and C termini of this NLS ($^{79}$AAGPPVAPKPAWFRQSLKGLAN$^{101}$). As shown in Fig. 5, the NLS-mutated prodomain of pro-IL-16 shows reduced nuclear-targeting activity (60 kDa NLSM), when compared with nonmutated protein (60 kDa NLSW).

To determine whether the prodomain of pro-IL-16 localized to the nucleus has an effect on cell growth, we performed cell cycle analysis on transfected COS cells. To facilitate flow cytometric cell cycle analysis in transiently transfected cells, we made new pro-IL-16 wild-type and NLS mutant constructs with an N-terminal-fused GFP reporter (Fig. 6A). As a control for the effects of nuclear GFP, we made a construct that fused an NLS sequence from human T cell leukemia virus type 1 (HTLV-1) tax protein to GFP (NLS-GFP). To investigate nuclear targeting of the NLS mutant of pro-IL-16 and confirm the distribution of expressed proteins, subcellular fractionation was performed following transfection of COS cells with plasmid GFP constructs; NLS-GFP, GFP-60, or GFP-60-NLSM. Immunoblots of the transfected COS cells with anti-GFP antibody as an indication of nuclear fraction free of the construct (Fig. 4C) implies that the nuclear localization signal of pro-IL-16 is located in the N-terminal first 257 amino acid residues of pro-IL-16.

Effects of Nuclear Targeting of IL-16 Prodomain on the Cell Cycle—Forty-eight hours after transfecting COS cells with the above constructs, the cells were collected and stained with propidium iodide and then analyzed by flow cytometry to identify transfected cells (GFP-positive). The cell cycle profile of the transfected COS cells (gated on green fluorescent-positive cells) revealed that overexpression of the wild-type prodomain of...
IL-16 (Fig. 7, GFP60), but not NLS mutant (Fig. 7, GFP60 NLS), induced an accumulation of the cells in G₀/G₁ phase (Table II). Cell cycle analysis of cells transfected with an independent NLS linked to GFP (Fig. 7, NLS GFP) had a similar G₀/G₁ profile to GFP alone transfected cells (Fig. 7, GFP) as well as untransfected cells (not shown). Fluorescence activated cell sorting (FACS) analysis revealed that ~40% of GFP or NLS-GFP transfected cells were in G₀/G₁, whereas ~53% of GFP60-transfected cells remained in G₀/G₁ phase (Table II). There is ~13% G₀/G₁ arrest in COS cells overexpressing GFP60 compared with GFP or NLS-GFP. By contrast, only 41% of GFP60-NLS transfected COS cells were in the G₀/G₁ phase, and COS cells that expressed this mutant protein had a similar cell cycle profile as those cells that expressed GFP or NLS-GFP (Table II). We confirmed these studies by transfection of the same expression vectors in 3T3 cells (data not shown).

DISCUSSION

The autofluorescent (GFP fusion) and immunofluorescent (FLAG tag) localization studies suggest that in transfected COS cells, the N-terminal prodomain of IL-16 translocates into the nucleus following cleavage and release of the C-terminal bioactive IL-16. Using site-directed mutagenesis we were able to identify a putative NLS of pro-IL-16 between amino acids 79 and 101. However, the current studies did not demonstrate whether other sequences in pro-IL-16 might also contribute to the nuclear import. Our cell fractionation results of COS cells transfected with wild-type or NLS-mutated pro-IL-16 constructs correlated with the fluorescent immunohistochemistry results and confirmed the nuclear localization properties of the prodomain of IL-16. The function of pro-IL-16 in the nucleus appears to be cell growth-related as nuclear translocation of the prodomain results in G₀/G₁ arrest.

Our data suggest that the 80 kDa primary pro-IL-16 translocation result could have two distinct functions after caspase-3 processing. The C-terminal segment of pro-IL-16 is a secreted protein that acts as a typical cytokine. The remaining N-terminal prodomain of IL-16 translocates to the nucleus after release of C-terminal polypeptide where it may influence cell cycle regulation. A similar phenomenon has been reported for IL-1α. Pro-IL-1α is released by caspase-1 cleavage and appears to affect cell growth (12). IL-16 and IL-1α share some general similarities. They are both processed by caspase family enzymes. Neither secreted mature cytokine has a secretary leader sequence, and following caspase enzyme processing their remaining prodomains translocate to the nucleus. However, unlike the negative effect we observed with N-terminal prodomain of IL-16, the N-terminal prodomain of IL-1α has been reported to function as a transforming nuclear oncprotein (12).

Pro-IL-16 contains multiple PDZ domains. Proteins with PDZ motifs often function in intracellular signal transduction (13) and in structures at plasma membrane (14, 15). Pro-IL-16 of lymphocyte origin contains one putative cdc2 kinase substrate site (56TPPK59), which lies N-terminal to the nuclear localization sequence. The sequence 56TPPK59 is similar to the consensus sequence that is phosphorylated by the mitotic cdc2 kinase or H1 kinase (16). The cdc2 kinase motif suggests a way that nuclear pro-IL-16 might participate in cell cycle control. Along these lines, several PDZ domain-containing proteins have been found to localize in the nucleus (17, 18) although their functions are not completely understood.

It is unclear how full-length unprocessed pro-IL-16 remains predominantly in the transfected COS cells cytoplasm, whereas the prodomain translocates to the nucleus. Extracellular sequestration mechanisms for regulating entry of proteins into the nucleus have been reported for many transcription factors, such as NF-kB (19), SREBP-1 (sterol regulatory element-binding protein 1, Ref. 20), and certain steroid hormone receptors. In each of these cases the entry of protein into nucleus is a regulated event. In the case of SREBP-1 and -2, nuclear entry is controlled by the proteolytic processing of the C-terminal segment bound to the endoplasmic reticular membrane (20). However, overcoming an existing nuclear exporting signal controls entry of other nuclear proteins. LINK-1, a protein serine/threonine kinase concentrates in the nucleus after deletion of a C-terminal portion of PDZ domain, which contains two nuclear exporting signals (21, 22). The perinuclear localization of pro-IL-16 prior to cleavage suggests an element in the C terminus (perhaps the PDZ domain) may bind to a perinuclear protein. Once cleaved from the third PDZ domain, the released N-terminal prodomain is free to translocate into the nucleus. However, any of the previously noted mechanisms might regulate translocation of the prodomain of IL-16 into the nucleus. The cellular localization and the biological function of pro-IL-16 in human T lymphocytes are currently under investigation.

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