# Prointerleukin-16 Contains a Functional CcN Motif that Regulates Nuclear Localization†

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ABSTRACT: The immunomodulatory cytokine interleukin-16 (IL-16) represents the secreted C-terminus of a larger precursor, pro-IL-16. Following cleavage by caspase 3, the residual N-terminal domain translocates into the nucleus, inducing $G_0/G_1$ cell cycle arrest. We have previously identified a classical bipartite nuclear localization sequence (NLS) in the N-terminal domain of pro-IL-16. We now show that N-terminal to the NLS domain of pro-IL-16 are protein kinase CK2 substrate and cdc2 kinase substrate sites which, along with the NLS, constitute a dual phosphorylation-regulated CcN motif which regulates nuclear localization of pro-IL-16. In addition, we demonstrate that mutation of either site is associated with impairment of the N-terminal domain's ability to induce $G_0/G_1$ cell cycle arrest. This is the first description of a functional CcN motif in a cytokine precursor.

Interleukin-16 (IL-16)[1] was the first described T cell chemoattractant factor (*1*, *2*). It has since been shown that IL-16 also functions as a primer of T cell proliferation (*3*), a modulator of inflammatory and immune responses (*4*−*7*), a stimulus of B cell differentiation (*8*), and an inhibitor of HIV replication (*9*−*11*). These diverse functions are attributed to mature IL-16, the 121-amino acid secreted C-terminal peptide which is cleaved by caspase 3 from the 631-amino acid precursor protein, pro-IL-16.

Following cleavage by caspase 3, the residual N-terminal domain has a very high degree of interspecies homology (*12*−*14*) and contains a number of consensus motifs, including two PDZ domains associated with intracellular functions. Using fluorescence immunochemistry, we previously demonstrated that pro-IL-16 is localized in the perinuclear cytoplasm (*15*). Following cleavage by caspase 3, the N-terminal domain translocates into the nucleus utilizing a classical bipartite basic nuclear localization sequence (NLS) (*15*). Most recently, further examination of the amino acid sequence of the N-terminal domain identified a potential protein kinase CK2 substrate site and a possible cdc2 kinase substrate site.

The combination of a protein kinase CK2 substrate site, a cdc2 kinase substrate site, and NLS constitutes a CcN motif (*16*), which has not previously been identified in a cytokine precursor. A CcN motif is a nuclear localization signal regulated by dual phosphorylation. Phosphorylation of the protein kinase CK2 substrate site increases the rate of NLS-dependent nuclear import, and phosphorylation of the cdc2 kinase substrate site inhibits nuclear transport. Phosphorylation of the protein kinase CK2 substrate site increases the rate of nuclear import by increasing the protein's affinity for importin 58, the smaller subunit of the NLS−receptor complex (*17*, *18*). The larger subunit, importin 97, targets the complex to the nuclear pore complex (NPC) through its affinity for NPC components such as nucleoporins (*19*−*23*). Subsequent translocation into the nucleus is mediated by the GTP-binding protein Ran/TC4 (*24*) and interacting factor p10/NTF2 (*25*−*28*). Conversely, phosphorylation of the cdc2 kinase substrate site is believed to inhibit nuclear transport by increasing the protein's affinity for a cytoplasmic retention factor (*16*). Both cdc2 protein kinase and protein kinase CK2 are known to be involved in the cell cycle. Numerous proteins that contain confirmed or putative CcN motifs have been identified, including SV40 T antigen, interferon-induced factor 16, murine stress-inducible protein 1 (mST1), nucleoplasmin, SWI5, lamins, and p53 (*16*, *29*−*33*). Notably, all of the proteins play a role in cell cycle regulation and/or cell proliferation.

The nuclear location, the presence of potential cell cycle-related kinase substrate sites, and the presence of a potential CcN motif suggest that the N-terminal domain of pro-IL-16 may play a role in regulating the cell cycle, consistent with our previous report that nuclear targeting of the N-terminal domain of pro-IL-16 is associated with an increased level of $G_0/G_1$ cell cycle arrest (*15*). Mutation of the NLS prevents both nuclear translocation of the N-terminal domain and $G_0/G_1$ cell cycle arrest (*15*). Only one other cytokine has been shown to have a biologically active precursor molecule, interleukin-1 (IL-1). In contrast to expression of pro-IL-16 which induces cell cycle arrest, expression of pro-IL-1α in

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[1] Abbreviations: cdc2m, mutant cdc2 kinase substrate site; CK2, protein kinase CK2; CKIIm, mutant casein kinase II substrate site; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HIV, human immunodeficiency virus; IL-16, interleukin-16; KCl, potassium chloride; MgCl, magnesium chloride; mST1, murine stress-inducible protein-1; NLS, nuclear localization sequence; NLSm, mutant nuclear localization sequence; NPC, nuclear pore complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SV40, simian virus 40; WT, wild type.

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glomerular mesangial cells results in cell transformation and uncontrolled growth (*34*).

This study demonstrates that there are functional protein kinase CK2 and cdc2 kinase substrate sites in pro-IL-16. We show that mutation of the protein kinase CK2 substrate site is associated with a decreased level of accumulation of the N-terminal domain of pro-IL-16 in the nucleus. In contrast, mutation of the cdc2 kinase substrate site is associated with an increased level of the N-terminal domain in the nucleus. The data suggest that the protein kinase CK2 substrate site, the cdc2 kinase substrate site, and the NLS on the N-terminal domain of pro-IL-16 constitute a functional CcN motif. Mutation of any of the three sites impairs the ability of the N-terminal domain to induce $G_0/G_1$ cell cycle arrest. This report represents the first description of a functional CcN motif in association with a cytokine.

## MATERIALS AND METHODS

*GST cDNA Constructs.* A pXM−GFP−N-terminal domain of pro-IL-16 fusion construct was prepared as described previously (*15*). Using this construct as a template, recombination PCR was performed. The 5′-primer encoded a *Bam*HI site, and the 3′-primer encoded an *Eco*RI site. The 3′-primer was directed just downstream from the nuclear localization sequence. The resulting PCR product encoded a fragment of the N-terminal domain of pro-IL-16 which included the putative CcN motif. The PCR product was subcloned into the pGEX vector using the 5′-*Bam*HI and 3′-*Eco*RI sites in the vector (Amersham Pharmacia Biotech). PCR mutagenesis was performed to create a single-amino acid substitution. The threonine in the putative cdc2 protein kinase substrate site was mutated to alanine, or the serine in the potential protein kinase CK2 substrate site was mutated to alanine. The PCR product was cloned back into the pGEX vector.

*GST Protein Purification*. The DH5α *Escherichia coli* strain (GibcoBRL) was transformed with the plasmid of interest. Following large-volume overnight culture, the *E. coli* were harvested, resuspended in 1× PBS, sonicated, and incubated in the presence of 20% Triton-X for 1 h at 4 °C. Following centrifugation, the supernatant was passed through a 1 mL column of glutathione−Sepharose 4B beads (Amersham Pharmacia Biotech). The column was then washed and the protein eluted from the column. The eluate was concentrated using a Centricon filter (Amicon).

*In Vitro Protein Kinase CK2 Kinase Assay*. Thirty micrograms of product from the GST fusion protein purification step was incubated at 30 °C for 5 min in the presence of 1× protein kinase CK2 buffer (New England Biolabs), 200 μM ATP (Sigma), 200 μCi/μmol [$^{32}$P]ATP (New England Nucleus), and 500 units of recombinant protein kinase CK2 (New England Biolabs). The reaction was terminated by adding 1× SDS sample buffer (0.06 M Tris-HCl, 2% SDS, 5% 2-β-mercaptoethanol, 0.001% bromophenol blue, and 0.1% glycerol) and loading the sample on an SDS gel.

*In Vitro cdc2 Protein Kinase Assay.* Forty micrograms of product from the GST fusion protein purification step was incubated at 30 °C for 15 min in the presence of 1× cdc2 buffer (New England Biolabs), 100 μM ATP (Sigma), 100 μCi/μmol [$^{32}$P]ATP (NEN), and 20 units of recombinant cdc2 protein kinase (New England Biolabs). The reaction was terminated by adding 1× SDS sample buffer (0.06 M Tris-HCl, 2% SDS, 5% 2-β-mercaptoethanol, 0.001% bromophenol blue, and 0.1% glycerol) and loading the sample on an SDS gel.

*Protein Kinase Assay Using Cell Lysates as the Kinase Source.* COS cells were harvested from culture and washed with 1× PBS. The cells were then resuspended in lysis buffer (25 mM Tris, 250 mM NaCl, 1.5 mM EDTA, 1 mM PMSF, 0.5% NP-40, and 0.5 μg/mL protease inhibitors, including chymotrypsin, antipain, leupeptin, aprotinin, and pepstatin) and incubated on ice for 15 min. Following centrifugation, the supernatant was collected as the whole cell lysate. Forty micrograms of product from the GST fusion protein purification step was incubated at 30 °C for 15 min in the presence of buffer (100 mM Tris, 20 mM $MgCl_2$, 100 mM NaCl, and 50 mM KCl), 12 μg of whole cell lysate, 200 μM ATP (Sigma), and 200 μCi/μmol [$^{32}$P]ATP (NEN). The reaction was terminated by adding 1× SDS sample buffer (0.06 M Tris-HCl, 2% SDS, 5% 2-β-mercaptoethanol, 0.001% bromophenol blue, and 0.1% glycerol) and loading the sample on an SDS gel.

*GFP cDNA Constructs.* A pXM−GFP−N-terminal domain of pro-IL-16 fusion construct was prepared as described previously (*15*). PCR mutagenesis was performed using this construct as a template. Outside primers encoded a 5′-*Kpn* site and a 3′-*Eco*RI site. Inside primers encoded selected point mutations. The serine in the putative protein kinase CK2 substrate site was mutated to alanine, and the threonine in the putative cdc2 kinase substrate site was mutated to glycine. The PCR product was cloned back into the pXM vector.

*Transfections.* For the cell fractionation and cell cycle analysis studies, COS cell transfections were performed and cells were collected as previously described (*35*). Briefly, 10 μg of plasmid was added to 5 million cells in a total volume of 0.8 mL of serum-free growth medium. The sample underwent electroporation using 300 mV. Following electroporation, the mixture was incubated at room temperature for 10 min. The sample was then added to 10 mL of growth medium containing 10% fetal bovine serum and antibiotics and allowed to incubate at 37 °C in the presence of 5% $CO_2$.

*Cell Fractionation and Cell Cycle Analysis.* Cell fractionation and cell cycle analysis were performed as previously described (*15*), 48 h after transfection. Briefly, cells were harvested, suspended in a low-salt buffer (0.02 M Tris-HCl, 0.0005 M DTT, 1 mM PMSF, 0.01 M β-glycerol phosphate, 0.3 M sucrose, 0.2 mM EGTA, 0.005 M MgCl, 0.01 M KCl, and protease inhibitors), and lysed with 0.5% NP-40. The lysate was centrifuged, and the supernatant containing cytoplasmic proteins was collected. The remaining nuclear pellet was resuspended in a high-salt buffer (0.01 M Tris-HCl, 0.0005 M DTT, 1 mM PMSF, 0.01 M β-glycerol phosphate, 0.2 mM EGTA, 0.005 M MgCl, 0.35 M KCl, 25% glycerol, and protease inhibitors), and the nuclei were lysed. The lysate was centrifuged and the supernatant containing nuclear proteins collected. For cell cycle analysis, cells were harvested and incubated in a 35% ethanol solution for 1 h at 4 °C. Following fixation, the cells were washed with 1× PBS and resuspended in a 1× PBS solution containing 0.25 mg/mL RNAse and 50 μg/mL propidium

38 Q P N A S L N E E E G T Q
51 G H P D G T P P K L D T A
64 N G T P K V Y K S A D S S
77 T V K K G P P V A P K P A
90 W F R Q S L K G L R N R

FIGURE 1: N-Terminal domain of pro-IL-16 containing a potential CcN motif. The sequence of amino acids surrounding the putative CcN motif of the N-terminal domain of Pro-IL-16 is shown. Identified are a potential protein kinase CK2 substrate site (box with dashed line), a potential cdc2 kinase substrate site (box with solid line), and a bipartite nuclear localization sequence (box with dash−dot line).

iodide for 1 h at 4 °C. The cell cycle was then analyzed using FACS (Becton-Dickinson).

*Immunoblotting.* Western blot analysis was performed as described elsewhere (*35*). The polyclonal rabbit anti-N-terminal domain of the pro-IL-16 antibody (provided by the Kornfeld lab) was used as the primary antibody at a concentration of 1 $\mu$g/mL; the polyclonal rabbit anti-phosphothreonine antibody (Cell Signaling Technology) was used as the primary antibody at a dilution of 1:1000, and the polyclonal rabbit anti-GFP antibody (Santa Cruz Biotechnology) was used as the primary antibody at a concentration of 1 $\mu$g/mL. The goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) was used as the secondary antibody at a dilution of 1:4000.

## RESULTS

*Identification of a CcN Motif.* After confirming the presence of a NLS on the N-terminal domain, we examined the amino acid sequence of pro-IL-16. Knowing that NLS-dependent nuclear transport is often regulated by phosphorylation, we sought kinase substrate sites that may function as regulatory signals. A potential protein kinase CK2 substrate site was identified between amino acids 42 and 46 ($^{42}$SLNEE$^{46}$). In addition, between amino acids 56 and 59, a possible cdc2 protein kinase substrate site was found ($^{56}$TPPK$^{59}$). These sites are upstream from the bipartite nuclear localization sequence which extends from amino acid 79 to 101 ($^{79}$KKGPPVAPKPAWFRQSLKGLRNR$^{101}$). These potential consensus sequences are in proximity, increasing the potential that they are functionally linked (Figure 1).

*Protein Kinase CK2 and cdc2 Protein Kinase Phosphorylate the N-Terminal Domain of Pro-IL-16 in Vitro*. To determine whether the putative protein kinase CK2 and cdc2 protein kinase substrate sites are phosphorylated by protein kinase CK2 and cdc2 protein kinase, respectively, we performed in vitro kinase assays. A 15 kDa CcN motif-containing protein fragment of the 60 kDa N-terminal domain of pro-IL-16 was linked to GST to serve as a kinase substrate. This construct focused all potential phosphorylation events on the CcN motif elements and eliminated irrelevant N- and C-terminal sequences of pro-IL-16.

A wild-type [GST−NT(short)−WT] construct and an identical construct with a serine to alanine substitution in the putative protein kinase CK2 substrate site [GST−NT-(short)−CK2m] were subjected to phosphorylation by catalytically active recombinant protein kinase CK2 (Figure 2A). The wild-type protein fusion construct was phosphorylated by protein kinase CK2. When the protein kinase CK2 substrate site was mutated, the level of phosphorylation was markedly reduced (Figure 2B). Thus, we confirm that the N-terminal ($^{42}$SLNEE$^{46}$) sequence of pro-IL-16 is in fact a site for protein kinase CK2 phosphorylation. Western blots were performed on the kinase assay samples (Figure 2B). The GST construct alone was not phosphorylated by CKII (data not shown).

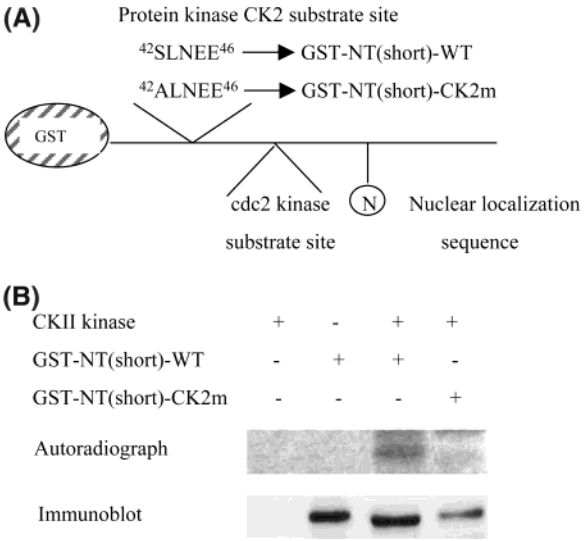



FIGURE 2: N-Terminal domain of pro-IL-16 containing a protein kinase CK2 substrate site. (A) Schematic representation of the GST fusion proteins used in the in vitro kinase assay. The protein is a 15 kDa fragment of the N-terminal domain of pro-IL-16 that includes the putative CcN motif. The constructs included the wild type [GST−NT(short)−WT] and a protein kinase CK2 substrate site mutation [GST−NT(short)−CK2m]. (B) Autoradiograph of the kinase assay (top panel) showing that the wild-type substrate was phosphorylated by protein kinase CK2 (lane 3) and single-amino acid replacement (Ser → Ala) abolished the phosphorylation (lane 4). The corresponding immunoblot (bottom panel) shows unequal loading of the protein constructs. Densitometry was utilized to correct for unequal loading. The results confirm that the level of phosphorylation seen using the wild-type protein construct is decreased when the protein kinase CK2 substrate site-mutated protein construct is used.

Table 1: Normalization of Autoradiograph Bands Using Densitometry[a]

| | | | | | | |
|---|---|---|---|---|---|---|
| protein kinase CK2 | + | + | + | − | − | − |
| Cdc2 kinase | − | − | − | + | + | + |
| GST−NT(short)−WT | − | + | − | − | + | − |
| GST−NT(short)−CK2m | − | − | + | − | − | − |
| GST−NT(short)−cdc2M | − | − | − | − | − | + |
| autoradiograph counts/ immunoblot counts | <0.1 | 0.30 | 0.20 | <0.1 | 0.45 | 0.20 |

[a] Densitometry was performed to normalize for the variability in GST fusion protein purification. The counts obtained from each autoradiograph band were divided by the counts obtained from the corresponding band on the immunoblot.

Variability of GST protein purification was normalized using densitometry; the counts obtained from each autoradiograph band were divided by the counts obtained from the corresponding band on the immunoblot. The results confirmed that the level of phosphorylation is reduced when the protein kinase CK2 substrate site is mutated (0.30 for the wild type vs 0.20 for the mutated protein kinase CK2 substrate site) (Table 1).

The same wild-type [GST−NT(short)−WT] construct and an identical construct with a threonine to alanine substitution in its putative cdc2 protein kinase substrate site [GST−NT-

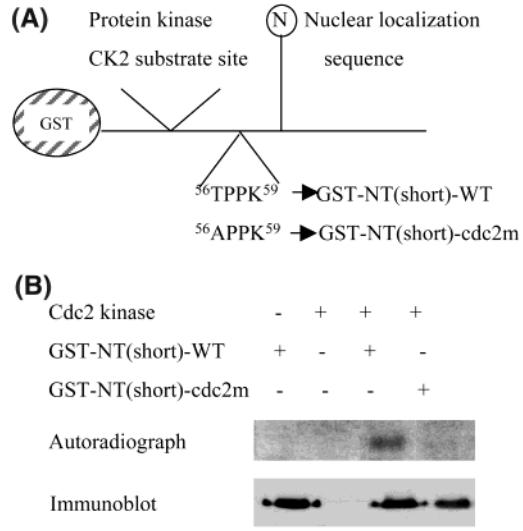



FIGURE 3: N-Terminal domain of pro-IL-16 containing a cdc2 kinase substrate site. (A) Schematic representation of the GST fusion proteins used in the in vitro kinase assay. The protein is a 15 kDa fragment of the N-terminal domain of pro-IL-16 that includes the putative CcN motif. The constructs included both the wild type [GST−NT(short)−WT] and a cdc2 kinase substrate site mutation [GST−NT(short)−cdc2m]. (B) Autoradiograph of the kinase assay (top panel) showing that the wild-type substrate was phosphorylated by cdc2 kinase (lane 3) and the single-amino acid replacement (Thr → Ala) abolished the phosphorylation (lane 4). The corresponding immunoblot (bottom panel) shows equal loading of the protein constructs.

(short)−cdc2m] were subjected to phosphorylation by catalytically active recombinant cdc2 protein kinase (Figure 3A). The assay demonstrated phosphorylation of the wild-type protein construct by cdc2 kinase. The extent of phosphorylation was diminished when the cdc2 kinase substrate site-mutated protein construct was used as a substrate, confirming that cdc2 kinase phosphorylates the N-terminal domain of pro-IL-16 at the identified substrate site (Figure 3B). An immunoblot was performed which demonstrated equal loading. Of note, GST alone is not phosphorylated by cdc2 protein kinase (data not shown). Densitometry confirmed that the level of phosphorylation was reduced when the cdc2 kinase substrate site was mutated (0.45 for the wild type vs 0.20 for the mutated CKII substrate site) (Table 1).

*The N-Terminal Domain of Pro-IL-16 Is Phosphorylated in Vivo*. To determine whether pro-IL-16 is phosphorylated in vivo, the wild-type N-terminal domain of pro-IL-16 (pXM−GFP−NT−pro-IL-16-WT) was overexpressed in COS cells. Following whole cell lysis, immunoblotting was performed using the primary antibody directed against phosphothreonine residues. Immunoblotting revealed that the N-terminal domain of pro-IL-16 is phosphorylated in vivo (Figure 4A). Using the primary antibody directed against GFP, immunoblotting was repeated with the same lysates to confirm the identity of the phosphoprotein (Figure 4B). Coomassie blue staining verified equal protein loading (data not shown). To further support the observation that the N-terminal domain of pro-IL-16 is phosphorylated in vivo, we performed a kinase assay using whole cell extracts as the kinase source and the fusion protein, GST−NT(short)−WT, as the substrate. Results demonstrated phosphorylation of the N-terminal domain of pro-IL-16 (data not shown).

*Protein Kinase CK2-Mediated Phosphorylation Promotes Nuclear Translocation*. Protein kinase CK2-mediated phosphorylation of the protein kinase CK2 substrate site in a CcN motif typically increases the rate of nuclear import, likely by increasing the protein's affinity for the nuclear pore complex. We hypothesized that if the protein kinase CK2 substrate site on the N-terminal domain of pro-IL-16 is functioning as part of a CcN motif, then mutation of that site would result in a decreased level of accumulation of the N-terminal domain in the nucleus. Using a COS cell expression system, we tested this hypothesis. COS cells were transfected with plasmids encoding the entire N-terminal domain of pro-IL-16, with either a normal protein kinase CK2 substrate site (pXM−GFP−NT−pro-IL-16-WT), a mutant protein kinase CK2 substrate site (pXM−GFP−NT−pro-IL-16-CK2m), or a mutant nuclear localization sequence (pXM−GFP−NT−pro-IL-16-NLSm) (Figure 5A).

Nuclear fractionation revealed that mutation of the protein kinase CK2 substrate site significantly reduced the amount of N-terminal domain that translocated into the nucleus compared to the wild type. The reduction was similar to that seen with mutation of the nuclear localization sequence. Reciprocal changes were not detected in the cytoplasmic extracts, likely because the quantity of protein in the cytoplasmic extracts was approximately 15−20-fold greater than that in the nuclear extracts. To prevent cytoplasmic contamination of the nuclear extracts, immunoblotting was performed using the anti-tubulin antibody as the primary antibody. The absence of tubulin in the nuclear fractions

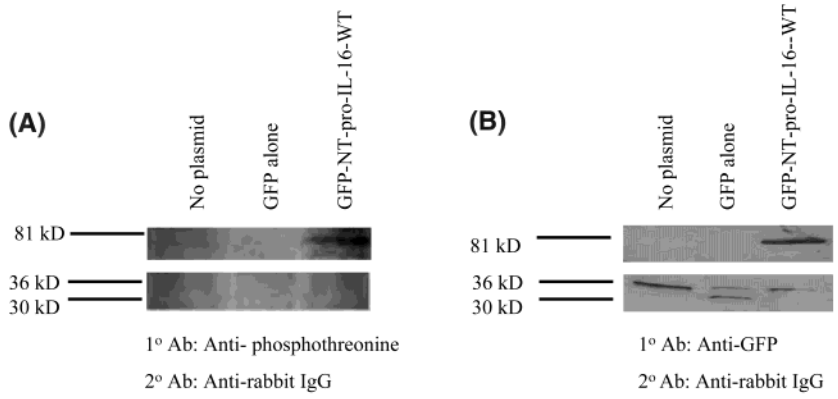
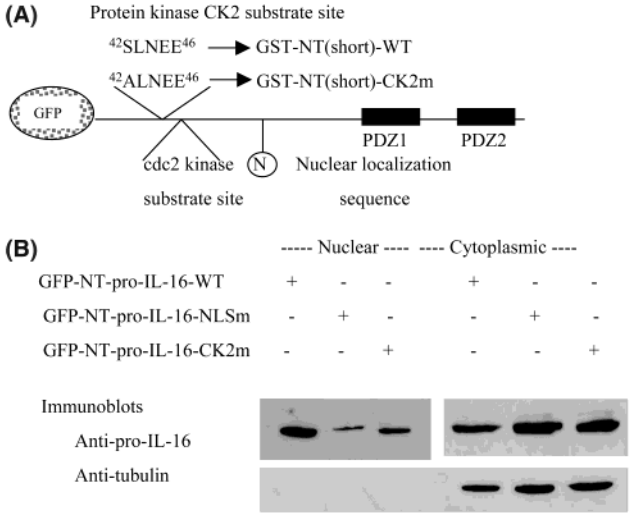



FIGURE 4: N-Terminal domain of pro-IL-16 phosphorylated in vivo. (A) Immunoblot of whole cell lysates using the polyclonal anti-phosphothreonine antibody as the primary antibody. The GFP−N-terminal domain of pro-IL-16 fusion protein is phosphorylated in vivo (top). GFP alone is not (bottom). (B) Immunoblot of the same whole cell lysates using polyclonal anti-GFP as the primary antibody confirming the identity and anticipated size of the GFP−N-terminal domain of pro-IL-16 fusion protein (top) and GFP alone (bottom). The band seen in all three lanes is nonspecific.



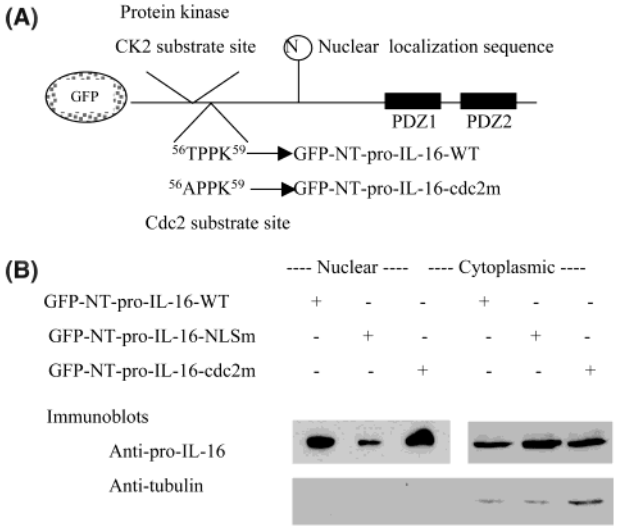

(C)

| | GFP-NT-pro-IL-16-WT | GFP-NT-pro-IL-16-NLSm | GFP-NT-pro-IL-16-CK2m |
|---|---|---|---|
| Nuclear fraction relative to WT | 1.0 | 0.3 | 0.45 |
| +/- Standard deviation | N/A | 0.15 | 0.2 |

FIGURE 5: Mutation of the protein kinase CK2 substrate site associated with a decreased level of the N-terminal domain of pro-IL-16 in the nucleus. (A) Schematic diagram of the GFP−N-terminal domain of pro-IL-16 fusion proteins. (B) Immunoblots showing the nuclear and cytoplasmic fractions containing the wild-type N-terminal domain of pro-IL-16 (GFP−NT−pro-IL-16-WT) (lanes 1 and 4), the N-terminal domain with the nuclear localization sequence mutated (GFP−NT−pro-IL-16-NLSm) (lanes 2 and 5), and N-terminal domain with the protein kinase CK2 substrate site mutated (GFP−NT−pro-IL-16-CK2m) (lanes 3 and 6). (C) Densitometry results quantifying the relative differences of the amount of the N-terminal domain in the nucleus, expressed as a fraction (counts of fusion protein in question divided by counts of the wild-type fusion protein).

confirms that cytoplasmic contamination did not occur (Figure 5B). Coomassie blue staining of the gel demonstrated equal loading. Densitometric analysis revealed a 2.2-fold reduction in the amount of N-terminal domain in the nucleus when the protein kinase CK2 substrate site was mutated (counts of CK2m/counts of WT = 0.45 ± 0.2, $N = 2$) and a 3.3-fold reduction when the nuclear localization sequence was mutated (counts of NLSm/counts of WT = 0.30 ± 0.15, $N = 5$) (Figure 5C).

*Mutation of the cdc2 Kinase Substrate Site of Pro-IL-16 Increases the Rate of Nuclear Translocation.* Phosphorylation of the cdc2 kinase substrate site in a CcN motif typically decreases the rate of nuclear localization sequence-dependent nuclear import, likely by increasing the protein's affinity for a cytoplasmic retention factor. We hypothesized that mutation of the cdc2 kinase substrate site would result in an inability of the protein to bind the putative cytoplasmic retention factor, thereby increasing the level of accumulation of the N-terminal domain of pro-IL-16 in the nucleus. COS cells were transfected with plasmids encoding the entire N-terminal domain of pro-IL-16. The plasmids encoded either the wild type (pXM−GFP−NT−pro-IL-16-WT), a mutant nuclear localization sequence (pXM−GFP−NT−pro-IL-16-NLSm), or a mutant cdc2 kinase substrate site (pXM−GFP−NT−pro-IL-16-cdc2m) (Figure 6A).




(C)

| | GFP-NT-pro-IL-16-WT | GFP-NT-pro-IL-16-NLSm | GFP-NT-pro-IL-16-cdc2m |
|---|---|---|---|
| Nuclear fraction relative to WT | 1.0 | 0.3 | 1.5 |
| +/- Standard deviation | N/A | 0.15 | 0.3 |

FIGURE 6: Mutation of the cdc2 kinase substrate site is associated with an increased level of the N-terminal domain of pro-IL-16 in the nucleus. (A) Schematic diagram of the GFP−N-terminal domain of pro-IL-16 fusion proteins. (B) Immunoblots showing the nuclear fraction containing the wild-type N-terminal domain of pro-IL-16 (GFP−NT−pro-IL-16-WT) (lanes 1 and 4), the N-terminal domain with the nuclear localization sequence mutated (GFP−NT−pro-IL-16-NLSm) (lanes 2 and 5), and the N-terminal domain with the cdc2 kinase substrate site mutated (GFP−NT−pro-IL-16-cdc2m) (lanes 3 and 6). (C) Densitometry results quantifying the relative differences of the amount of the N-terminal domain in the nucleus, expressed as a fraction (counts of the fusion protein in question divided by the counts of the wild-type fusion protein).

Nuclear fractionation revealed that mutation of the cdc2 kinase substrate site resulted in an increase in the amount of N-terminal domain detected in the nucleus compared to that with the wild type. Reciprocal changes in the cytoplasmic fractions were neither seen nor expected. The absence of tubulin in the nuclear fractions confirms that cytoplasmic contamination did not occur (Figure 6B). Coomassie blue staining of the gel demonstrated equal loading. Densitometry was employed to quantitate the change and revealed a 1.5-fold increase in the amount of N-terminal domain in the nucleus (counts of cdc2m/counts of WT = 1.5 ± 0.3, $N = 4$) (Figure 6C).

*Mutation of either the Protein Kinase CK2 Substrate Site or the cdc2 Kinase Substrate Site Impairs the Enhanced $G_0/G_1$ Cell Cycle Arrest Associated with the Wild-Type N-Terminal Domain of IL-16.* Forty-eight hours after transfection of COS cells with plasmids encoding GFP alone (pXM−GFP), GFP fused to the wild-type N-terminal domain (pXM−GFP−NT−pro-IL-16-WT), GFP fused to the nuclear localization sequence-mutated N-terminal domain (pXM−GFP−NT−pro-IL-16-NLSm), GFP fused to the protein kinase CK2 substrate site-mutated N-terminal domain (pXM−GFP−NT−pro-IL-16-CK2m), or GFP fused to the cdc2 kinase substrate site-mutated N-terminal domain (pXM−GFP−NT−pro-IL-16-cdc2m), the cells were collected, stained

Table 2: Cell Cycle Analysis[a]

| sample | fraction of mutant NT−pro-IL-16 relative to wild-type NT−pro-IL-16 in the nucleus | percent change of cells arrested in $G_0/G_1$ after transfection with NT−pro-IL-16 compared to transfection with vector alone |
|---|---|---|
| GFP−NT−pro-IL-16-WT | 1.0 | 25.0 ± 3.6% |
| GFP−NT−pro-IL-16-NLSm | 0.3 ± 0.13 | −2.4 ± 3.5% |
| GFP−NT−pro-IL-16-cdc2m | 1.5 ± 0.33 | 10.0 ± 3.8% |
| GFP−NT−pro-IL-16-CK2m | 0.4 ± 0.21 | 2.9 ± 4.2% |

[a] COS cells were suspended in a 35% ethanol solution for 1 h at 4 °C and then resuspended in a 1× PBS solution containing 0.25 mg/mL RNAs and 50 μg/mL propidium iodide for 1 h at 4 °C. The cell cycle was then analyzed using flow cytometry.

with propidium iodide, and analyzed for cell cycle progression by flow cytometry. Only cells expressing GFP were analyzed.

Compared to the percentage of cells transfected with GFP alone, the percentage of cells arrested in the $G_0/G_1$ phase of the cell cycle increased 25% when they were transfected with the wild-type N-terminal domain of pro-IL-16. In contrast, when the cells were transfected with the N-terminal domain that had its protein kinase CK2 substrate site or nuclear localization sequence mutated, there was no increase in the level of $G_0/G_1$ cell cycle arrest, confirming that impaired translocation to the nucleus eliminated the nuclear effects of pro-IL-16 on the cell cycle. However, when the cdc2 kinase substrate site-mutated N-terminal domain was transfected, which resulted in more pro-IL-16 in the nucleus, there was an only 10% increase in the percentage of cells arrested in the $G_0/G_1$ phase of the cell cycle (Table 2), suggesting an additional role for this site in the nucleus.

## DISCUSSION

We have previously shown that the N-terminal domain of pro-IL-16 translocates into the nucleus, and this translocation can be prevented by mutation of a bipartite nuclear localization sequence (*15*). The current study reveals that the bipartite nuclear localization sequence does not act alone. Rather, it is part of a CcN motif, a nuclear localization signal regulated by dual phosphorylation that consists of a protein kinase CK2 substrate site, a cdc2 kinase substrate site, and a nuclear localization sequence. From our nuclear localization experiments, it can be ascertained that disruption of any element will alter nuclear localization. When the protein kinase CK2 substrate site or nuclear localization sequence is mutated, the level of nuclear translocation is diminished. When the cdc2 kinase substrate site is mutated, nuclear translocation is enhanced.

Phosphorylation of the protein kinase CK2 substrate site increases the rate of nuclear import by increasing the protein's affinity for importin 58, the smaller subunit of the NLS−receptor complex (*17*, *18*). Importin 97, the larger subunit, then targets the complex to the nuclear pore complex (NPC) through its affinity for NPC components such as nucleoporins (*19*−*23*). Subsequent translocation into the nucleus is mediated by the GTP-binding protein Ran/TC4 (*24*) and interacting factor p10/NTF2 (*25*−*28*). Phosphorylation of the cdc2 kinase substrate site is believed to inhibit nuclear transport by increasing the protein's affinity for a cytoplasmic retention factor (*16*). Our data support the hypothesis that the nuclear localization of pro-IL-16 is similarly regulated by dual phosphorylation, although the mechanism has not been clearly defined.

This work on pro-IL-16 represents the first report in which a cytokine or cytokine precursor is shown to contain a functional CcN motif. The SV40 T antigen was the first CcN motif-containing protein identified (*16*). Interferon-induced factor 16 and murine stress-inducible protein 1 (mST1) are two of the few additional proteins that have been definitively shown to contain CcN motifs (*29*, *30*). Many proteins have been identified that contain putative CcN motifs, however. They include other interferon-inducible factors, nucleoplasmin, SWI5, lamins, and p53 (*29*−*33*). For our studies, it is important to note that all of the proteins with real or putative CcN motifs have functions related to the cell cycle.

Human p53 is of particular interest as a potential CcN motif-containing inhibitor of cell proliferation. Nuclear accumulation of p53 is NLS-dependent, and both cdc2 protein kinase and protein kinase CK2 kinase phosphorylate p53 (*36*−*39*). However, it has not been shown that phosphorylation of either site influences nuclear localization of p53. Further, the protein kinase CK2 substrate site (Ser386) lies C-terminal to the cdc2 kinase substrate site (Ser315), making the existence of a functional CcN motif questionable.

Consistent with a cell cycle effect, we have previously shown that the function of the N-terminal domain of pro-IL-16 in the nucleus is directly related to cell proliferation since nuclear translocation of the N-terminal domain results in an increased level of $G_0/G_1$ cell cycle arrest (*15*). The presence of a functional CcN motif is further indirect evidence of a role in regulation of the cell cycle. In addition, both cdc2 protein kinase and protein kinase CK2 are known to be directly involved in the cell cycle. Constitutively expressed cdc2 protein kinase associates with cyclically expressed cyclin B (or cyclin A) in a rate-limiting step (*40*). The resulting complex resembles the invertebrate mitosis promoting factor (MPF) (*41*). MPF phosphorylates histone-1 (H1) protein, disrupting its association with chromatin (*40*, *42*, *43*). It is hypothesized that this provides DNA topoisomerase II access to chromatin thereby promoting chromatin condensation and advancement to the mitotic phase of the cell cycle (*40*, *44*). Cdc2 protein kinase phosphorylates lamins, impairing their ability to polymerize, leading to nuclear membrane breakdown (*40*). After telophase, phosphatases return allowing the nuclear membrane to repair itself (*40*). Protein kinase CK2 is a ubiquitous protein kinase that phosphorylates more than 160 described substrates. Among its many actions, protein kinase CK2 changes the activity of proteins involved in DNA replication and DNA repair (topoisomerase II and DNA ligase), transcription factors, and growth factors (p53 and p21) (*40*, *45*).

Pro-IL-16 is the second cytokine precursor molecule with known bioactivity. Pro-IL-1α was the first cytokine precursor molecule shown to have an intracellular function. Pro-IL-1α is the precursor protein of IL-1α, an inflammatory and immunomodulatory cytokine. It is cleaved by a calcium-activated neutral protease, calpain. Following cleavage, the C-terminal peptide is secreted from the cell as IL-1α. Studies using rat glomerular mesangial cells and human promyelocytic leukemia HL-60 cells have demonstrated that the pro piece undergoes NLS-mediated translocation into the nucleus where it functions as a transformation oncoprotein (*34*). The mechanism for its growth effect has not been reported;

however, myristoylation and serine 90 phosphorylation appear to be required.

The current study shows that alteration of any element of the CcN motif impairs the ability of the N-terminal domain of pro-IL-16 to induce the increased level of $G_0/G_1$ cell cycle arrest. Because mutation of the protein kinase CK2 substrate site or nuclear localization sequence results in a decreased amount of the N-terminal domain localizing in the nucleus, it is not surprising that these mutations are associated with a diminished level of $G_0/G_1$ cell cycle arrest. It is interesting, however, that mutation of the cdc2 kinase substrate site results in a significant increase in the amount of N-terminal domain in the nucleus but a smaller increase in the level of $G_0/G_1$ cell cycle arrest compared to that with the wild-type N-terminal domain. This suggests that although the N-terminal domain of pro-IL-16 reaches the nucleus in a larger amount, it is less functional. It is feasible that phosphorylation of the N-terminal domain by cdc2 protein kinase is important not only to nuclear localization but also to the cell cycle-related function of pro-IL-16. If the function of the N-terminal domain of pro-IL-16 is in fact partially dependent on cdc2 protein kinase, this would be the first description of this action.

These findings suggest a dual functional response to synthesis and secretion of IL-16 in T cells. The mature protein is secreted and interacts with CD4, inducing a refractory state to antigenic stimulation as well as stimulation by certain chemokines (*46*, *47*). In addition, following cleavage, pro-IL-16 translocates to the nucleus where it helps to regulate cell cycle progression. The combined effect would be regionally suppressed CD4+ T cell activation and proliferation.

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