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*J Immunol* 2008; 180:222-229; doi: 10.4049/jimmunol.180.1.222
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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Nuclear Export of NF90 to Stabilize IL-2 mRNA Is Mediated by AKT-Dependent Phosphorylation at Ser647 in Response to CD28 Costimulation

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IL-2 is one of the most important cytokines required for T cell-mediated immune responses. Costimulation of CD28 in T cells up-regulates IL-2 mRNA levels via transcription activation and mRNA stabilization. Upon T cell activation, NF90, an AU-rich element (ARE)-binding protein, translocates from the nucleus into the cytoplasm, where it binds to the ARE-containing 3′ untranslated regions of IL-2 mRNA and slows down degradation of IL-2 mRNA. The translocation of NF90 is mediated through a nuclear export signal at its N terminus, but how it is triggered is still unclear. Phosphorylation of ARE-binding proteins has been reported as a signal transduction pathway to stabilize ARE-containing transcripts. In this study, we demonstrate that AKT phosphorylates NF90 on Ser647 upon CD28 costimulation. This phosphorylation is necessary for nuclear export of NF90 and IL-2 mRNA stabilization by this protein, because a mutation at Ser647 abolished both functions. We observed that treatment of cells with CD28 costimulation induced distinct increase in phosphorylation of AKT and NF90 at Ser647 concomitantly. Phosphorylation at Ser647 of NF90 up-regulated IL-2 production in response to CD28 costimulation. In vivo and in vitro data support a model in which CD28 costimulation activates AKT to phosphorylate NF90 at Ser647 and phosphorylation triggers NF90 to relocate to the cytoplasm and stabilize IL-2 mRNA. The Journal of Immunology, 2008, 180: 222–229.

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Received for publication April 30, 2007. Accepted for publication September 28, 2007.

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1 This work was supported by the National 973 Program of China (2004CB518605), 863 Projects of China (2006AA020501), the Project of the Shanghai Municipal Science and Technology Commission (03dz0406), and the National Natural Science Foundation of China (30025001).

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3 Abbreviations used in this paper: ARE, AU-rich element; AKT-CA, constitutively active AKT; AUBP, AU-binding protein; CHO, Chinese hamster ovary; CsA, cyclo-

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Phosphorylation of NF90 was analyzed using an in vitro kinase assay. Akt kinase (Cell Signaling Technology) incubated with either purified NF90(-500–702) or controls in the presence of 2 μM kinase buffer, 1 μM of [γ-32P]ATP (10 μCi/ml) for 30 min at 37°C. Proteins were eluted with SDS loading buffer, separated by SDS-PAGE (12%), and analyzed by autoradiography.

Real-time RT-PCR

Total RNA was isolated using TRIzol (Invitrogen Life Technologies) and reverse transcribed into cDNA. The level of IL-2 mRNA was determined by real-time PCR following normalization to GAPDH, using the ∆∆CT method for relative quantitation with IL-2 mRNA levels. PCR was performed on an iCyler iQTM (Bio-Rad) using SYBR Green Premix (Takara Shuzo) as a dsDNA-specific binding dye and continuous fluorescence monitoring. For detection of GAPDH, the sense primers 5'-GAAGTGTGAAAG GTCCGAGTC-3' and antisense primer 5'-GAAGATGGTAGGATGGGAT TTC-3' were used; for detection of IL-2, the primers 5'-TACAACTGGA GCATTACTG-3' (sense) and 5'-GTTTCAGACCTTATTGATC-3' (antisense) were used.

Preparation of cytoplasmic and nuclear extracts

Twenty million cells were disrupted in 0.5 ml of buffer A (0.2% Nonidet P-40, 50 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 1 mM DTT, 8 mM aprotinin, 2 mM leupeptin, and 1 mM PMSF) and centrifuged at 6500 rpm at 4°C, and the supernatant was collected as the cytoplasmic fraction. The nuclear pellet was washed twice with lysis buffer and resuspended in 0.3 ml of buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 25% glycerol, 1.5 mM MgCl2, 1 mM DTT, 8 ng/ml aprotinin, 2 ng/ml leupeptin, and 1 mM PMSF). After incubation on ice for 30 min, the solution was centrifuged at 12,000 rpm for 30 min, and the supernatant was collected as the nuclear extract.

IL-2 radioimmunoassay

After transfection, cells were stimulated for 24 h with anti-CD3 (clone UCHT-1) and anti-CD28 (clone CD28.2) Abs (Sigma-Aldrich) at final concentrations of 1 and 0.5 μg/ml, respectively. CsA was added to the culture, and supernatants were collected at indicated times and assayed by radioimmunoassay for IL-2 secretion.

Results

NF90-Ser467 phosphorylation is required for its nuclear export

NF90 has been reported to play an important role in IL-2 mRNA stabilization. Upon T cell activation, NF90 accumulates in the cytoplasm and binds to AREs located in the 3′ UTR of IL-2 mRNA. Translocation of NF90 is mediated through an N-terminal NES, but how it is triggered is still unclear. Phosphorylation has been shown to regulate nuclear export of some proteins, including NF-AT (41, 42). NF90 moves into the cytoplasmic compartment during mitosis and is highly phosphorylated (43). Therefore, we asked whether nuclear export of NF90 in response to CD28 costimulation is regulated through phosphorylation. The first experiment was to analyze the coding sequence of NF90 for possible phosphorylation sites by kinases downstream of CD28. Interestingly, we found Ser467 located in its RGG box to be one possible phosphorylation site by AKT family. Ser467 was embedded in a specific sequence RXXRXXKS, and this conserved sequence is generally found in AKT substrates, such as glycogen synthase kinase 3β (GSK3β) and BRF1 (Fig. 1, A and B), suggesting that NF90 is most likely to be a substrate of AKT kinase.

Nuclear export is sufficient for IL-2 mRNA stabilization by NF90. To examine whether NF90-Ser467 is required for nuclear export, we constructed NF90-Ser467A. If Ser467 indeed could be phosphorylated upon CD28 costimulation, the mutant would be unable to be phosphorylated. We determined the subcellular distribution of wild-type and mutant NF90 after stimulation (Fig. 1D). In nonstimulated Jurkat cells, more Myc-NF90 was detected in the nuclear fraction than in the cytoplasm. After anti-CD28 and anti-CD3 Ab treatment, Myc-NF90 was much more abundant in the
cytoplasmic fraction. Nuclear translocation was not detected in cells transfected with Myc-NF90-Ser647A. This is consistent with results from microscopy analysis (Fig. 1C). Jurkat cells were transiently transfected with either wild-type or mutant NF90, as indicated. A major portion of GFP-NF90 migrated into the cytoplasm after treatment with anti-CD28 and anti-CD3 Abs, which was not found in cells transfected with GFP-NF90-Ser647A. These results supported our hypothesis that nuclear export of NF90 was regulated through phosphorylation.

**NF90 and AKT protein interaction**

To confirm the possible enzyme-substrate relation between NF90 and AKT, their interaction was first examined in mammalian cells. We constructed HA-AKT and Myc-NF90, and transfected these plasmids into 293T. Forty-eight hours after transient transfection, cell lysates were prepared and incubated with Myc or HA mAb and agarose beads. Following IP, HA or Myc mAb was used for Western blot analysis. The results are shown in Fig. 2A. When Myc-NF90 and HA-AKT were cotransfected, but not control vectors, AKT was detected in the fraction precipitated with the Myc mAb, and Myc-NF90 was detected in the fraction precipitated with the HA mAb. This indicates that the exogenously expressed Myc-NF90 and HA-AKT could interact with each other.

To further verify whether NF90 and AKT interact under physiological conditions in T cells, Jurkat cell lysates were incubated with AKT mAb. As shown in Fig. 2B (left panel), NF90 was...
detected in the endogenous IP fraction by AKT Ab, but not by the control mouse IgG. The reciprocal experiment in Jurkat cells demonstrated the same results (Fig. 2, right panel). Therefore, our results indicate that AKT and NF90 also interact in vivo.

AKT locates in the cytoplasm before cell activation and translocates into nucleus following activation via phosphorylation. Activated AKT phosphorylates and activates other transcriptional factors. Because NF90 contains a conserved nuclear localization signal sequence, we examined whether activated AKT colocalized with NF90. Twenty-four hours after cotransfection with pEGFP-NF90 and pDsRed-AKT constructs into HeLa cells, we incubated the cells in serum-free medium overnight. The cells were then cultured in medium with 10% serum for 45 min and fixed for cofocal microscopy. Indeed, we found that AKT was in nucleus and colocalized with NF90 (Fig. 2C). We conclude from the above experiments that NF90 and AKT interact in vivo and colocalize in the nucleus.

**AKT phosphorylates NF90 at Ser647**

To further confirm NF90 as a substrate of AKT, we performed an in vitro kinase assay. First, we expressed wild-type NF90 and NF90-Ser647A from E. coli with His tag and purified the proteins with Ni-NTA His affinity Resin (Novagen). Purified NF90 and NF90-Ser647A proteins were incubated with AKT kinase and γ-32P-labeled ATP. As shown in Fig. 3A, AKT-phosphorylated wild-type NF90 and the degree of phosphorylation were dependent on the protein levels of AKT and NF90. However, AKT did not phosphorylate NF90-Ser647A, although the amount of AKT protein was 5 times that of wild-type NF90.

An in vivo phosphorylation experiment was performed with Chinese hamster ovary (CHO) cells because it had higher transfection efficiency than 293T cells. First, constitutively active AKT (AKT-CA) or inactive AKT were cotransfected with Myc-NF90 or Myc-NF90-Ser647A together with AKT. Transfection and cell stimulation were performed same as in A. LY294002 (5 and 10 μM) was added at the same time with CsA. Results shown were the average of three independent experiments (n = 6).

**FIGURE 3.** AKT phosphorylates NF90 in vivo and in vitro. A and B, E. coli-expressed NF90 and NF90-Ser647A (different doses), GST (negative control), and/or GSK3β (positive control) were incubated with AKT kinase at different doses, 1 μl of γ-32P-labeled ATP (10 μCi/ml), and kinase buffer for 30 min. The reaction mixture was separated on SDS-PAGE and subjected to autoradiography. The amount of corresponding proteins was indicated with Coomassie blue staining. C, Phosphorylation of NF90 in CHO cells. CHO cells were transfected with expression constructs, as shown in the figure. Thirty-six hours after transfection, Myc Ab was used to pull down tagged protein in the cell lysates. Left panel, Shows the amount of NF90 precipitated. Right panel, Phosphorylation of NF90 in the pull-down fractions was detected with specific phosphorylation Ab for AKT substrates.

**FIGURE 4.** AKT phosphorylates NF90 to stabilize IL-2 mRNA. A, Myc-NF90, Myc-NF90-Ser647A (defective for phosphorylation), or empty Myc vector was transfected into Jurkat cells. Eighteen hours after transfection, PMA and Ca2+ ionophore A23187 were added and cultured for 5 h to increase IL-2 transcription. Following CsA addition, RNA was collected every 30 min and quantified using real-time RT-PCR. B, RNA stability was measured as in A, except that the cells were transfected with the plasmids Myc-NF90 or Myc-NF90-Ser647A together with AKT. C, Transfection and cell stimulation were performed same as in A. Results shown were the average of three independent experiments (n = 6).
Myc-NF90-Ser\textsuperscript{647}A into CHO cells. After pulling down NF90 protein with Myc Ab, we detected phosphorylation of NF90 at Ser\textsuperscript{647} with Ab specific for AKT substrate phosphorylation. This Ab specifically recognizes phosphorylated serine within RXXRXS, the consensus sequence in AKT substrates. As shown in Fig. 3C, phosphorylation at Ser\textsuperscript{647} can only be detected in the precipitated fraction when wild-type NF90 and AKT-CA were cotransfected. In precipitated fractions with NF90 and inactive AKT cotransfection or NF90-Ser\textsuperscript{647}A and AKT-CA cotransfection, we did not detect the phosphorylation. Thus, the above experiments confirmed that AKT phosphorylates NF90 in vitro and in vivo, and NF90 indeed is a substrate of AKT.

**Phosphorylation at Ser\textsuperscript{647} by AKT is critical for NF90 to stabilize IL-2 mRNA**

Previous studies showed that NF90 could bind to the AREs located within IL-2 mRNA 3' UTR, and attenuate the decay of IL-2 mRNA (16). We have found that Ser\textsuperscript{647} is important for NF90 nuclear export and could be phosphorylated by AKT. Therefore, we hypothesized that the phosphorylation of Ser\textsuperscript{647} may be critical for NF90 to stabilize IL-2 mRNA. Myc-NF90, Myc-NF90-Ser\textsuperscript{647}A (defective for phosphorylation), or the empty Myc vector was transfected into Jurkat T cells. Eighteen hours after transfection, PMA and Ca\textsuperscript{2+} ionophore A23187 were added to the culture for 5 h to increase IL-2 transcription. We used CsA to specifically block IL-2 transcription. RNA was collected from the cells every 30 min after CsA addition and was quantified with real-time RT-PCR. The result showed that IL-2 mRNA was degraded rapidly in cells transfected with empty Myc vector and its t\textsubscript{1/2} is \(\approx 30\) min (Fig. 4A). However, introduction of Myc-NF90, but not Myc-NF90-Ser\textsuperscript{647}A, increased the t\textsubscript{1/2} of IL-2 mRNA to \(\approx 60\) min. To further confirm the effect of Ser\textsuperscript{647} phosphorylation, we mutated serine at the 647 position to glutamate (NF90-Ser\textsuperscript{647}E) to mimic the phosphorylation state. We found that NF90-Ser\textsuperscript{647}E stabilized IL-2 mRNA with the same potential as the wild-type NF90, possibly due to constitutive AKT activation in Jurkat cells. Therefore, the phosphorylation at Ser\textsuperscript{647} is critical for NF90 to stabilize IL-2 mRNA.

We have shown that AKT phosphorylated NF90 at Ser\textsuperscript{647}, and this phosphorylation was closely related with its ability to stabilize IL-2 mRNA. The question was then whether AKT modulates IL-2 mRNA stability through phosphorylating NF90 at Ser\textsuperscript{647}. First, we examined IL-2 mRNA stability in Jurkat cells cotransfected with AKT and NF90 or NF90-Ser\textsuperscript{647}A, and found that IL-2 mRNA was much more stable in cells cotransfected with AKT and NF90 than cells cotransfected with AKT and NF90-Ser\textsuperscript{647}A, or transfected with NF90 alone (Fig. 4B). Second, we monitored the ability of NF90 or NF90-Ser\textsuperscript{647}A to stabilize IL-2 mRNA when AKT activity was inhibited. Similar to the previous experiment, Jurkat cells were first transfected with NF90 or NF90-Ser\textsuperscript{647}A, Following stimulation with PMA and A23187 for 5 h, we added CsA to end transcription. At the same time, LY294002, an AKT inhibitor, was also added to the culture. IL-2 mRNA was quantified, as previously described. As shown in Fig. 4C, the stabilization of IL-2 mRNA by NF90 is sensitive to LY294002 treatment, and in the presence of a larger dose of the inhibitor, IL-2 mRNA was more unstable. As predicted, LY294002 did not alter the effect of NF90-Ser\textsuperscript{647}A on IL-2 mRNA stability. Taken together, our experiments indicate that expressing AKT by cotransfection promoted the stabilization of IL-2 mRNA mediated by NF90, and inhibiting AKT activity abrogated the ability of NF90, but not NF90-Ser\textsuperscript{647}A, to stabilize IL-2 mRNA. Therefore, we conclude that AKT modulates IL-2 mRNA stability through phosphorylation of NF90-Ser\textsuperscript{647}.

**CD28 costimulation induces NF90-Ser\textsuperscript{647} phosphorylation by AKT**

IL-2 expression level has been reported to be rapidly elevated upon CD28 costimulation due to increased transcription and posttranscriptional stabilization. In Jurkat cells, AKT activated by CD28 signaling promotes IL-2 transcription, whereas PMA did not activate AKT kinase (44). Based on our previous results, we hypothesized that CD28 costimulation-activated AKT could phosphorylate NF90 to stabilize IL-2 mRNA. To test this hypothesis, we stimulated Jurkat T cells with anti-CD28 and anti-CD3 or together with LY294002 for 1 h. After using NF90 mAb to IP endogenous NF90, we examined phosphorylation of NF90-Ser\textsuperscript{647} with AKT substrate phosphorylation Ab. At the same time, the phosphorylation state of AKT was also determined using Ab to phosphorylated AKT. As shown in Fig. 5A, CD28 costimulation induced phosphorylation of both AKT and NF90-Ser\textsuperscript{647}. If AKT were inhibited with LY294002 following the stimulation, phosphorylation of NF90 at Ser\textsuperscript{647} would also diminish. The physiological relevance of these results was confirmed with human primary T cells (Fig. 5B). Therefore, by activating AKT, CD28 costimulation induced NF90-Ser\textsuperscript{647} phosphorylation.

**NF90-Ser\textsuperscript{647} phosphorylation regulates IL-2 production in response to CD28 costimulation**

To further study the effect of NF90-Ser\textsuperscript{647} phosphorylation in CD28 costimulation-induced T cell activation, we transfected Jurkat cells with NF90 or NF90-Ser\textsuperscript{647}A, stimulated the cells with anti-CD3 and anti-CD28, and examined the production of IL-2 protein by a radioimmunooactivity assay. Empty vector was used as a negative control. In the experiment, anti-CD3 and anti-CD28

\[ \text{CD3/CD28} \quad \text{LY294002} \quad \text{IP: Anti-NF90} \quad \text{Anti-NF90} \quad \text{WB: anti-Substrate-\textsuperscript{AKT}} \]

\[ \text{AKT Antibody} \]
were added to the cell culture 18 h after transfection, and the stimulation lasted for 48 h. The culture medium was collected every several hours for quantifying IL-2 protein (Fig. 6A). In Fig. 6B, CsA was added to block IL-2 transcription after 24 h of stimulation. As shown in Fig. 6B, during the time period before CsA addition, the amount of IL-2 in cell medium was almost the same for cells transfected with NF90 or NF90-Ser647A, which was higher than that transfected with empty vector \((n = 6)\). This indicates that NF90 increases IL-2 transcription and is consistent with previous reports \((35, 36)\). Moreover, this promotion does not depend on phosphorylation at Ser647. Furthermore, 3 h after transcription ended by CsA, there was an obvious difference in levels of IL-2 in culture medium of cells transfected with NF90 or NF90-Ser647A. The amount of IL-2 was much higher in culture medium of cells transfected with NF90 than in those transfected with NF90-Ser647A or empty vector. This indicates that phosphorylation of NF90 at Ser647 plays an important role in the posttranscriptional regulation of IL-2 mRNA induced by CD28 costimulation and is consistent with the data presented above. Of interest is the result that cells transfected with NF90-Ser647A secreted less IL-2 than those transfected with the empty vector. NF90-Ser647A may mediate blocking of endogenous NF90 export to stabilize IL-2 mRNA. In conclusion, we show that phosphorylation of NF90 at Ser647 is a key step in IL-2 production in response to CD28 costimulation.

**Discussion**

Our data demonstrated that NF90 is involved in the up-regulation of IL-2 expression by CD28 costimulation. CD28 promotes AKT phosphorylation of NF90 at Ser647, which results in nuclear export of NF90 and IL-2 mRNA stabilization. AKT is an important component of the PI3K signaling pathway and is activated in T cells stimulated by CD28 Ab \((45, 46)\). Phosphorylation of AKT activates downstream substrates, which efficiently stabilize ARE-containing mRNAs. A consensus sequence for AKT substrates is found in the NF90 coding sequence, with Ser647 being the conserved phosphorylation site. The in vitro and in vivo kinase assay experiments proved that AKT indeed could phosphorylate NF90 at Ser647 (Fig. 3).

The stability of IL-2 mRNA is controlled by the presence of ARE in the 3’ UTR. The current model is that constitutive ARE-binding proteins, such as tristetraprolin, recruit a multicomponent exosome that degrades the associated mRNA molecule. The signals that interfere with exosome recruitment and induce mRNA stabilization are not well understood. Phosphorylation or competition with nonexosome-recruiting ARE-binding proteins is linked to the stabilization of some transcripts. Based on the demonstration that NF90 could bind and stabilize IL-2 mRNA and that phosphorylation of AKT substrates also stabilizes some ARE-containing mRNAs, we hypothesized that phosphorylation of NF90-Ser647 by AKT might affect its function on IL-2 mRNA stabilization. The difference in stability of IL-2 mRNA between the wild-type and the mutant NF90 depended on phosphorylation of NF90-Ser647, indicating that phosphorylation of NF90-Ser647 is indeed crucial for its ability to stabilize IL-2 mRNA. However, the half-life of IL-2 mRNA in cells transfected with NF90-Ser647E was similar to or even higher than cells transfected with wild-type NF90, which could be explained by Jurkat cells lacking PTEN, an inhibitor of PI3K. In this study, we used PMA to activate the Jurkat cells and induce IL-2 production because PMA does not influence AKT phosphorylation in Jurkat cells (data not shown). This result is consistent with previously reported mechanisms of regulation for many other AUBPs. For example, TTP is phosphorylated at Ser52 and Ser178 by MAPK2 and p38. This phosphorylation results in its binding with 14-3-3. TTP loses its mRNA degradation capacity once it binds to 14-3-3 (47–50). After T cell stimulation by CD28, TTP is phosphorylated by kinases and binds to 14-3-3 (51). This stabilizes the ARE-containing mRNAs such as IL-2 mRNA. BRF1, which contains the same zinc finger protein binding domain as TTP, can be phosphorylated at Ser92 by AKT and stabilizes IL-3...
mRNA (46). We also found that phosphorylation of Ser647 was required for NF90 nuclear export, which may explain how phosphorylation of NF90-Ser647 may affect IL-2 mRNA stability (Fig. 1, C and D). Phosphorylation has been shown to regulate nuclear export of some proteins, including NF-AT (41, 42). The protein conformation might be affected upon phosphorylation, and nuclear export of NF90 mediated through the NES sequence located in its N terminus could be facilitated by the conformation change.

In T cells, the expression of IL-2 increases rapidly upon CD28 costimulus due to increased transcription and mRNA stabilization. Even though PMA is another activator of IL-2 production, PMA did not change AKT activity in Jurkat cells (44). We detected significant increase of NF90 phosphorylation in response to CD28 costimulation, which was accompanied by increased AKT phosphorylation. Once AKT was inhibited, phosphorylation of NF90-Ser647 decreased (Fig. 5). This demonstrates the existence of a phosphorylation cascade from CD28 costimulation to AKT and then to NF90. Moreover, we found that both NF90 and NF90-Ser647A also increased IL-2 protein levels 24 h after stimulation with anti-CD3 and anti-CD28. This phenomenon lasted until the addition of CsA to stop IL-2 transcription. The above observation indicates that NF90 itself could increase IL-2 transcription, which was not dependent on Ser647 phosphorylation. NF90 was originally purified as a protein binding to the same regulatory sequence of IL-2 gene as with other NF-AT family members. However, NF90 does not contain the specific sequences for DNA binding. When Jurkat cell lysate was blocked with NF90 Ab in the in vitro transcription assay, the transcription of the reporter gene containing the IL-2 transcription-regulatory elements showed significant down-regulation (35, 36). NF90, NF45, Ku protein, and DNA-dependent protein kinase have been found in a large complex associated with IL-2 gene regulatory region in human bronchial endothelial cells (52). This complex activated IL-2 transcription in response to immune-stimulating signals. We also observed that NF90 and NF90-Ser647A affected CD28 costimulation-induced IL-2 expression differently after CsA addition. This is consistent with our results shown in Fig. 4. Because NF90 could stabilize IL-2 mRNA, IL-2 protein levels continued to increase and remained high even after transcription was interrupted by CsA. Meanwhile, NF90-Ser647A cells secreted less IL-2 than cells transfected with the vector, indicating the inability of NF90-Ser647A to stabilize IL-2 mRNA. This might be attributed to the inhibition of nuclear export of endogenous wild-type NF90 by exogenous NF90-Ser647A, thus hampering the ability of endogenous NF90 to stabilize IL-2 mRNA.

Based on our results, we propose a mechanism of IL-2 mRNA stabilization. CD28 signaling activates AKT to phosphorylate NF90 at Ser647. This phosphorylation might change the protein conformation and facilitate the nuclear export of NF90 mediated through the NES sequence located in its N terminus. Phosphorylated NF90 relocates to and accumulates in the cytoplasm and stabilizes IL-2 mRNA. Nevertheless, further studies are also needed to examine whether there are other sites on NF90 that can also be regulated by CD28 signaling and to better understand how the phosphorylation of NF90-Ser647 stabilizes IL-2 mRNA.

Acknowledgments

We thank Alison Wool for editorial assistance in the preparation of this manuscript. We thank Dr. Glen Barber (University of Miami) for supplying NF90 cDNA and Dr. Brian A. Hemmings from Friedrich Miescher Institute for AKT wild-type plasmid and AKT mutant version. Dr. Zhongzhou Yang from Dr. Hemmings’ group also gives us help.

Disclosures

The authors have no financial conflict of interest.

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