A Novel Sequence in the Coiled-coil Domain of Stat3 Essential for Its Nuclear Translocation*

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Stat3 is activated by cytokines and growth factors via specific tyrosine phosphorylation, dimerization, and nuclear translocation. However, the mechanism involved in its nuclear translocation is unclear. In this study, by systematic deletion and site-directed mutagenesis we identified Arg-214/215 in the α-helix 2 region of the coiled-coil domain of Stat3 as a novel sequence element essential for its nuclear translocation, stimulated by epidermal growth factor as well as by interleukin-6. Furthermore, we identified Arg-414/417 in the DNA binding domain as also required for the nuclear localization of Stat3. This sequence element corresponds to Lys-410/413 of Stat1, a reported sequence for Stat1 nuclear translocation. On the other hand, Leu-411 of Stat3, corresponding to Leu-407 of Stat1, a necessary residue for Stat1 nuclear transport, is not essential for Stat3 nuclear import. The mutant of Arg-214/215 or Arg-414/417 was shown to be tyrosyl-phosphorylated normally but failed to enter the nucleus in response to epidermal growth factor or interleukin-6. The defect, however, can be rescued by the wild-type Stat3 but cannot be compensated by these two mutants. Mutations on Arg-414/417, but not Arg-214/215, destroy the DNA binding activity of Stat3. Our data for the first time identified a sequence element located in the coiled-coil domain that is involved in the ligand-induced nuclear translocation of Stat3. This novel sequence together with a conserved sequence element in the DNA binding domain coordinates to mediate the nuclear translocation of Stat3.

In eukaryotic cells, certain latent transcription factors are primarily located in the cytoplasm under unstimulated conditions. Upon an extracellular stimulation, however, they are rapidly translocated into the nucleus, where they bind to the regulatory elements of the target genes and regulate their expression. Some of these factors are subsequently exported to the cytoplasm. The nuclear import and export of the transcription factors therefore function as an important biological switch for the control of gene expression and cellular responses. In general, proteins to be imported contain a specific target sequence designated NLS, a nuclear localization signal, consisting of either a stretch of basic amino acids or two basic stretches separated by a spacer ranging from 10 to 37 amino acids (1, 2). These proteins bind to a cytoplasmic receptor composed of importin α and importin β. Importin α belongs to a divergent family of proteins that serves as adaptors between the substrates and importin β (3, 4). Importin β docks the NLS-containing protein and the importin α complex at the nuclear pore complex, which is a specialized structure of the nuclear envelope (5–8). The complex is translocated into the nucleus through the nuclear pore complex in an energy-dependent manner (9).

STATs are a family of latent cytoplasmic transcription factors that were named by virtue of their novel and unique dual functions as signaling molecules in the cytoplasm and as transcription factors after nuclear translocation. Stat proteins are primarily located in the cytoplasm. Upon cytokine stimulation, Stat proteins are recruited to the cytokine receptors and phosphorylated by the receptor-associated tyrosine kinases, Janus kinases, on a single tyrosine residue at the C termini. Stat proteins form homo- or heterodimers via reciprocal interactions between the SH2 domains and the phosphotyrosine and translocate into the nucleus, where they bind to DNA and regulate transcription of their target genes (10, 11).

Seven known mammalian Stat proteins, denoted by Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6, have been identified. They are activated by various cytokines and growth factors and play important roles in diverse cellular processes such as the antiviral protection, immune responses, cell growth, and apoptosis by regulating expression of numerous genes (12–14). The crystal structures of Stat proteins (15–17) revealed several conserved functional domains including an N-terminal domain (ND), a coiled-coil domain (CC), a DNA binding domain (DB), and a linker domain (LK), followed by an SH2 domain and a C-terminal transactivation domain (CT). Although the nuclear translocation is a key control for the activities, no classical NLS was found in Stat proteins, and mechanisms of their nuclear import were unclear for a long time. Recently, a few reports, mainly focused on Stat1, appeared to address this issue. An arginine/lysine-rich element in the DNA binding domain of Stat1 is reported to be important for the interferon-induced nuclear import of Stat1 and Stat2 (18, 19). In addition, leucine 407 of Stat1, which is also located in the DNA binding domain, has been demonstrated to be required for its nuclear import (20). Furthermore, importin α (NPI-1) was shown to interact with Stat1 homodimer and Stat1/Stat2 heterodimer and to be involved in their nuclear import (20–22). On the other hand, a region (465VVVT469) in Stat5b was reported to be involved in its nuclear translocation stimulated by growth hormone (23).

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‡ The abbreviations used are: NLS, nuclear localization signal; STATs, signal transducers and activators of transcription; EGF, epidermal growth factor; IL-6, interleukin-6; ND, N-terminal domain; CC, coiled-coil domain; DB, DNA binding domain; LK, linker domain; CT, C-terminal transactivation domain; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; LMB, leptomycin B; EPO, erythropoietin; PBS, phosphate-buffered saline; SIF, serum-induced factor.
Stat3 was originally cloned as an acute-phase response factor activated by IL-6 in mouse liver and also by homology to Stat1 (24, 25). In addition to Stat1 and Stat2, Stat3 plays a broader role in a variety of biological responses, such as cell growth, transformation, survival, and early embryonic development (28–32). Although the dimerization of Stat3 was reported to be required and sufficient for its nuclear translocation (33), the sequences and the mechanism of nuclear translocation of Stat3 in response to cytokines and growth factors were largely unknown. During the previous study in identification of domains involving the cellular localization of Stat3 and its interacting protein, GRIM-19, we surprisingly observed that the truncation mutant of Stat3 containing the N-terminal domain and the coiled-coil domain was constitutively localized in the nucleus of MCF-7 cells (34). In this study we attempted to further identify the specific sequences for Stat3 nuclear localization. By systematic deletion and numerous site-directed mutagenesis, we found two sequence clusters located in the coiled-coil domain and the DNA binding domain of Stat3 to be required for EGF-induced nuclear translocation. We also provide evidence showing that Stat proteins may be imported to the nucleus via overlapping and distinct sequence elements.

**EXPERIMENTAL PROCEDURES**

**Constructions of Expression Plasmids**—The murine Stat3 and its deletion mutants, ST3-ND.4H, ST3-DB.LK, ST3-SH2.CT, ST3-ND, and ST3–4H were cloned into pXJ40-FLAG as described previously (35). Deletion mutants ST3-ND.3H1–290, ST3-ND.2H1–290, ST3-ND.1H1–199 were generated by PCR using primers containing the HindIII site at 5' and the Kpn1 site at 3'. The murine Stat3 expression plasmid as a template. The respective PCR product was cloned into pXJ40-FLAG. Structured Stat3 and green fluorescent protein (GFP) fusion proteins were constructed by PCR using primers with the HindIII site at 5' and the Kpn1 site at 3' and inserted into pEGFP-N1 (Clontech). ST3-ND.4HΔ1H and ST3-ND.4HΔH2 were generated by PCR using primers with the HindIII site at 5’ and the Kpn1 site at 3’ and ST3-3H1 and ST3-ΔH2 as templates (35) and cloned into pXJ40-FLAG. Point mutations were prepared using primers containing the appropriate mutations and the full-length Stat3 as a template. The long template PCR kit with Pfu DNA polymerase (Promega) was used followed by Dpn1 digestion and subsequent transformation. The full-length Stat3 and the mutants R214/R215A and R414A/R417A were cloned into both pXJ40-FLAG and pXJ40-Myc (36). The mutagenesis was confirmed by sequencing on ABI PRISM 3700 DNA Analyzer using BigDye (version 3.0) (Applied Biosystems).

**Reagents and Antibodies**—EGF and IL-6 were purchased from Upstate Biotechnology and R&D Systems, respectively. Leptomycin B (LMB) and anti-FLAG antibody (M2) were purchased from Sigma. Anti-Stat3 (N terminus) was obtained from BD Biosciences. Anti-Stat3 (C terminus) and anti-Myc antibodies were purchased from Santa Cruz Biotechnology, and anti-phospho-Tyr705 was obtained from Cell Signaling.

**Cell Culture and DNA Transfection**—COS-1 and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium, and MCF-7 cells were grown in RPMI supplemented with 10% fetal bovine serum purchased at 4°C and sequentially washed with PBS, PBS containing 0.1% paraformaldehyde, permeabilized with 0.1% Triton X-100, and washed with PBS in the absence of Triton X-100.

**Immunoprecipitation and Immunoblotting**—Transfected cells were washed with cold PBS and lysed in radio immunoprecipitation assay buffer. The lysates containing 1 mg of total proteins were subjected to immunoprecipitation / immunoblotting as described previously (37). The precipitates were washed, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane followed by immunoblot analysis with the respective antibodies.

**Cell Fractionation and Electrophoretic Mobility Shift Assay (EMSA)**—The whole cell extracts were prepared from 60-mm dishes. Cells were lysed with buffer E (20 mM HEPES (pH 7.0), 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 10% glycerol, 1 mM dithiothreitol) containing 0.4% KCl, 0.4% Triton X-100, and protease inhibitors including 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 mM benzamidine, and 50 μg/ml phenylmethylsulfonyl fluoride. The extract was sonicated, clarified by centrifugation, and quantitated by using the Bio-Rad protein assay kit (38). EMSAs were performed as described previously (37). Cytoplasmic and nuclear extracts were prepared as follows. Transfected COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum for 24 h before stimulation with 100 ng/ml EGF for 15 min. Cells were rinsed with PBS before lysis in buffer A (10 mM HEPES-KOH (pH 7.0), 1.5 mM MgCl2, 0.2 mM EDTA, 5 mM NaF, 0.1 mM EGTA, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). Cell lysates were incubated on ice for 10 min followed by 10 s of vortexing and 10 s of centrifugation at high speed in a microcentrifuge. The supernatant was collected as cytoplasmic extract, and the pellet was washed twice with buffer A and dissolved in buffer B (20 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA (pH 8), 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and incubated on ice for 20 min. After a final 2-min spin, the supernatant (nuclear extract) was collected. The cytoplasmic or the nuclear extract containing 12 μg of proteins were resolved on 7.5% SDS-PAGE and subjected to Western blot analysis.

**RESULTS**

**The Mutant Containing the N-Terminal Section of Stat3 Expresses Constitutively in the Nucleus**—Generally, latent Stat3 proteins primarily localize in the cytoplasm and translocate into the nucleus after cytokine or growth factor stimulation. However, low levels of a ligand-independent constitutive nuclear expression of Stat1 and Stat3 were also reported in COS-1 and NIH 3T3 cells (39, 40). In our previous studies, to map the interaction domain of Stat3 with its interacting protein, GRIM-19, we found that the mutant of Stat3 containing the N-terminal portion of Stat3 is constitutively localized in the nucleus in a breast cancer cell line, MCF-7 (34). In this study, first we tried to confirm this observation in MCF-7 cells as well as in other cell types. Three large FLAG-tagged truncation mutants covering the N-terminal, the middle, or the C-terminal section of Stat3 protein were transfected into MCF-7 cells, and their cellular localizations were examined. The wild-type Stat3 is located in both the cytoplasm and the nucleus in unstimulated cells, as observed by indirect fluorescence. In sharp contrast, the mutant containing the N-terminal region (ST3-ND.4H) exclusively localized in the nucleus, whereas the mutant consisting of the middle (ST3-DB.LK) or the C-terminal section (ST3-SH2.CT) mainly expressed in the cytoplasm (Fig. 1B). A similar observation was also made in COS-1 cells (data not shown). These results suggested that the N-terminal domains of Stat3 contain a sequence for the nuclear localization.

**α-Helix 2 of the Coiled-coil Domain of Stat3 Harbors a Nuclear Localization Signal**—The N-terminal region of Stat3 contains two functional domains: the N-domain (ND) and the coiled-coil domain (CC). The CC domain is composed of four antiparallel α helices, of which two are long (α1 and α2) and two are short (α3 and α4) helices (15). To further delineate the region that is responsible for the nuclear import of Stat3, we constructed a series of truncation mutants by sequentially deleting the α-helices of the CC domain from the C terminus of...
The cellular localization of these mutants was examined in MCF-7 cells. The results showed that mutants ST3-ND.3H1-198 (lacking α-helix 4) and ST3-ND.2H1-260 (lacking α-helices 3 and 4) expressed in the nucleus. However, further deletion of α-helix 2 (ST3-ND.1H1-198) and α-helix 1 (ST3-ND) resulted in a loss of the exclusive nuclear localization, whereas ST3–4H, containing only the CC domain, predominantly localized in the nucleus (Fig. 2B).

These results suggest an NLS exists in the CC domain. To confirm these results and to demonstrate that the NLS in the CC domain can promote nuclear import of a heterogeneous protein, the N-terminal fragments were linked to a GFP, and their cellular localization was examined. GFP alone expressed in the cytoplasm. However, the GFP fusion containing either the CC domain alone (ST3–4H-GFP) or together with ND (ST3-ND.4H-GFP) accumulated in the nucleus, but the GFP fusion containing ND alone (ST3-ND-GFP) localized mainly in the cytoplasm (Fig. 2C).

To further map the NLS within the CC domain, α-helix 1 or 2 was removed individually from the mutant ST3-ND.4H (Fig. 2A). The results indicated that the mutant lacking α-helix 1 (ND.4H-ΔH1) accumulated in the nucleus, but the mutant deleting α-helix 2 (ND.4H-ΔH2) localized in the cytoplasm (Fig. 2D). Together, these data demonstrated that the α-helix 2 contains a potential NLS for nuclear localization.

Identification of Arg-214/215 of Stat3 as a Novel Element for the EGF-induced Nuclear Translocation—To further define the sequence(s) important in the CC domain for Stat3 nuclear localization, we searched for the basic amino acid residues in the CC domain. Six stretches that contain double or triple basic amino acids (Arg-152/Lys-153/Arg-154, Lys-161/Lys-163, Lys-177/Lys-180, Arg-197/Lys-199, Arg-214/Arg-215, and Lys-244/Arg-245/Arg-246) were found and chosen for site-directed mutagenesis (Fig. 3A). These basic residues were replaced by alanine in the full-length Stat3. These point mutants were named according to the position of the mutations which are listed in the lower panel of Fig. 3B. COS-1 cells express low levels of endogenous Stat3 and respond to EGF strongly. We transfected these mutants to COS-1 cells and evaluated their cellular localizations in the cells either untreated or stimulated with EGF. Strikingly, in contrast to the wild-type Stat3 that was mostly distributed in the cytoplasm in the untreated cells and translocated into the nucleus upon EGF stimulation, a double mutant R214A/R215A with mutations in α-helix 2 failed to be translocated from the cytoplasm to the nucleus after EGF treatment (Fig. 3B). The rest mutants, on the other hand, behaved like the wild-type Stat3. This result indicated that residues Arg-214/215 are essential for the EGF-induced nuclear translocation of Stat3.

Arg-414/417 of Stat3 in the DB Domain Is Also Required for Stat3 Nuclear Translocation—The NLS of Stat1 and Stat5b was reported to be located in the DB domain (18–20, 23), in which Lys-410/413 and Leu-407 were defined to be the critical residues for Stat1 nuclear translocation. We therefore also searched basic residues in the DB domain of Stat3 and made five double or triple mutants (Arg-335/Lys-340, Lys-348/Arg-350/Lys-354, Lys-363/Lys-365/Lys-370, Arg-379/Arg-382/Lys-383) in the full-length Stat3. In addition, a single mutant with a mutation in Leu-411 corresponding to Leu-407 in Stat1 was also prepared. A similar experiment was performed with these mutants. We found that mutations in Arg-414/417, corresponding to Lys-410/413 of Stat1, lost its nuclear translocation induced by EGF, whereas the other mutants, including L411A, were able to be translocated into the nucleus (Fig. 3B). Together, these data suggest that two elements, Arg-214/215 in the CC domain and Arg-414/417 in the DB domain, are required for Stat3 nuclear translocation in response to EGF.

Lack of nuclear accumulation of the mutants R214A/R215A and R414A/R417A could be due to effective nuclear export. To exclude this possibility, we pretreated cells with the nuclear export inhibitor, LMB, which inhibits Stat1 nuclear export by binding to the chromosome region maintenance-1/exportin1 shuttling receptor (41). The results showed that although the wild-type Stat3 accumulated highly in the nucleus after EGF stimulation in the presence of LMB, both mutants remained in the cytoplasm in the absence or presence of LMB (Fig. 3C). These data suggest that lack of nuclear accumulation of these mutants is not due to constitutive nuclear export.
Cell Fractionation Assay of the Nuclear Translocation of Stat3 Mutants—To further confirm our observations in a quantitative manner, we tested the cellular localization of the mutant and the wild-type Stat3 by cellular fractionation followed by immunoblotting analysis. Small amounts of the proteins encoded by the wild-type and the mutant Stat3 with point mutations were detectable in the nuclear portion in untreated cells, which may represent a constitutive basal nuclear import. Upon EGF treatment, the wild-type Stat3 accumulated in the nuclear fraction with a concomitant decrease in the cytoplasmic fraction. In contrast, a majority of the two point mutants remained in the cytoplasm, no increase was observed for the mutant R214A/R215A, and only a slight increase was detected for the mutant R414A/R417A in the nucleus. This increase was in a comparable range with that of Y705F, a mutant Stat3 that cannot be Tyr-phosphorylated and translocated into the nucleus (Fig. 4, top panels). As controls, protein poly(ADP-ribose) polymerase (42) was detected only in the nucleus, and tubulin was detected only in the cytoplasm; both maintained a constant expression level in the absence or presence of EGF.

Arg-214/215 and Arg-414/417 Are Also Essential for the IL-6-induced Nuclear Import—IL-6 type cytokines belong to a major cytokine family that stimulates Stat3 activity. To exclude the possibility that the two sequences for nuclear transport is ligand-specific, we analyzed the nuclear translocation of Stat3 in response to IL-6. We cotransfected COS-1 cells, which do not express IL-6 receptors, with an Myc-tagged Stat3 and a chimeric Epo-gp130 receptor consisting of the extracellular domain of the murine erythropoietin (Epo) receptor and the transmembrane and intracellular domains of the human gp130 receptor tagged with the FLAG epitope at its C terminus (Epo-gp130-FLAG) (43). The cellular localization of Stat3 was tested with or without Epo treatment. The results showed that both the wild-type and the mutant Stat3 with point mutations localized primarily in the cytoplasm in the untreated cells. However, when the cells were treated with Epo, only the wild-type Stat3 was translocated into the nucleus, whereas the mutants remained in the cytoplasm (Fig. 5). Expression of the Epo-gp130 was also examined. Epo-gp130 was observed mainly in the cytoplasm with characteristics of endocytic vesicles and Golgi apparatus in both unstimulated and Epo-stimulated cells. This may represent a constitutive endocytosis of Epo-gp130 receptor chimeras, which was reported previously for the wild-type gp130 that undergoes constitutive internalization
independent of IL-6 (44). These data suggested that similar NLS elements were utilized for the Stat3 nuclear translocation in the EGF and IL-6 signaling.

**Effects of the Mutations on Stat3 Activities**—We next examined the Tyr phosphorylation status of these mutants in response to EGF with an anti-phospho-Tyr-705 antibody. The two point mutants were not phosphorylated in the unstimulated cells but phosphorylated on Tyr-705 in the EGF-stimulated cells in a comparable level with that of the wild-type Stat3 (Fig. 6A). These results suggest that these mutations did not affect the Tyr phosphorylation and imply that their deficiency in the nuclear translocation is not due to an impaired Tyr phosphorylation.

We further analyzed the DNA binding activity of the mutants using whole cell lysates by EMSA using the high affinity binding site of Stat3, high affinity serum-inducible element, as a probe (27, 45). Three complexes were observed in the whole cell lysates derived from the wild-type Stat3-transfected cells after EGF stimulation (Fig. 6B, lane 9). SIF-A and SIF-C contain homodimers of the transfected

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**Fig. 3. Identification of Arg-214/215 and Arg-414/417 of Stat3 as required elements for the EGF-induced nuclear translocation.** A, diagram indicating the positions of the stretches containing the basic amino acids in the α-helices 1 and 2 of the coiled-coil domain and the DB. ND represents N-terminal domain. The numbers indicate the amino acid residues. The mutants were prepared by replacing the respective double or triple basic amino acid residues by alanine in the full-length Stat3. The names of the point mutants are listed in the lower panel of B. B, the FLAG-tagged wild type Stat3 and the point mutants shown in A were transfected into COS-1 cells. Cells were either left untreated (−EGF) or treated with EGF (100 ng/ml) for 15 min. The cellular localization of the wtStat3 and the mutants R214A/R215A and R414A/R417A was examined in the absence or presence of EGF treatment. The mutants R152/K153/R154A containing point mutations in the α-helix 1 region and K363/365/370A-containing mutations in the DNA binding domain are displayed as representatives of the rest of the mutants. The nuclear translocation of all the point mutants in response to EGF is summarized. C, effect of LMB on the nuclear translocation of Stat3. FLAG-tagged wild-type Stat3 and mutant R214A/R215A and R414A/R417A were transfected into COS-1 cells. Cells were pretreated with LMB (10 nM) for 30 min followed by EGF stimulation for 30 min. The cellular localization of Stat proteins was examined with anti-FLAG antibody.
Stat3 and the endogenous Stat1, respectively, and SIF-B is a heterodimer of the transfected Stat3 and the endogenous Stat1 (46). The identity of the complexes was confirmed by supershift assay using anti-Stat1 or -Stat3 antibody (Fig. 6, lanes 10–11). R214A/R215A homodimer bound strongly to DNA, and the complex can be supershifted by Stat3 antibody (lanes 12 and 14), whereas R414A/R417A failed to bind DNA (lane 15). The complex observed in lane 15 represented the endogenous Stat1, confirmed by super-shifted Stat1 antibody (lane 16). Collectively, our data demonstrated that the two mutants can be Tyr-phosphorylated properly.

Wild-type Stat3 Rescues R214A/R215A and R414A/R417A in the Nuclear Translocation—Because of the normal Tyr phosphorylation, we wondered whether the wild-type Stat3 could form dimers with the mutants and, therefore, rescue their defect in the nuclear translocation. To this end, we constructed the Myc-tagged mutant R214A/R215A and R414A/R417A and transfected them together with the FLAG-tagged wild-type Stat3 into COS-1 cells. In the untreated cells, both mutants and the wild-type Stat3 localized in the cytoplasm. After EGF stimulation, however, both mutant proteins were detectable in the nucleus by anti-Myc antibody (Fig. 7). Lack of nuclear staining was observed for both mutants after EGF stimulation (Fig. 7, bottom panel).

Human liver hepatoma cell line HepG2 expresses a substantial amount of endogenous Stat3 that is strongly activated by IL-6 stimulation. We transfected the FLAG-tagged-R214A/R215A or -R414A/R417A in these cells and found that the endogenous Stat3 can rescue the defect of the mutants in the nuclear translocation induced by IL-6 (data not shown). These results demonstrated that wild-type Stat3, either the transfected or the endogenous, both are able to rescue the loss-function mutants in the nuclear translocation. These data also suggest that in order for an effective nuclear transport to occur, two NLS sequences are required and must be located in one molecule of the dimer.

In summary, we have identified two sequence elements for Stat3 nuclear translocation. Arg-214/215 and Arg-414/417 are required for the IL-6-induced nuclear translocation of Stat3. Arg-214/215 is unique for Stat3, and Arg-414/417 is common for Stat1 and Stat3. Unlike Stat1, Leu-411 of Stat3 is not necessary for its nuclear import. The positions of the amino acid residues that are involved in the NLS function are indicated in the three-dimensional structure of the Stat3β homodimer bound to DNA (Fig. 8).
Nuclear Translocation of Stat3

Fig. 6. Effects of the mutations on Tyr phosphorylation and the DNA binding activity. A, COS-1 cells were transfected with the vector, wild-type Stat3, mutant R214A/R215A, or R414A/R417A, as indicated. The cells were either left uninduced or induced with EGF, and the whole cell lysates were prepared. Cell lysates were resolved by 7.5% SDS-PAGE, and blotted with anti-phospho-Tyr-705 Stat3 antibody (upper panel). The blot was stripped and re-probed with anti-FLAG antibody (lower panel). B, EMSA was performed with 25 μg of total proteins using affinity binding site of Stat3, high affinity serum-inducible element, as a probe as described under "Experimental Procedures." FP (lane 1) indicates free probe. The supershift assay was performed by incubating anti-Stat1 (ST1) or anti-Stat3 (ST3) antibody with the cell extracts in the binding reaction.

DISCUSSION

Arg-214/215 Is a Novel Element for the Stat3 Nuclear Translocation—Stat proteins transduce the signals of cytokines and growth factors from their cognate receptors on the cell surface to the nucleus. This process involves sequential events including the specific Tyr phosphorylation, dimerization, and the nuclear translocation, which is a key control for their transcriptional activity. Previous studies have suggested a potential role of the DNA binding domain of Stat proteins, namely Stat1 and Stat5b, in their nuclear accumulation. Specific residues in this domain were identified. However, the mechanism involved in the nuclear transport of Stat3 is largely unknown. For example, does Stat3 use a NLS similar to that of Stat1? If it does not, what is the sequence required for its nuclear localization? Do the NLSs of Stat proteins only localize in the DNA binding domain? We have attempted to address these questions in this report. By systematic truncation and deletion mutations, we first identified a region, α-helix 2 in the coiled-coil domain, harboring a sequence for its nuclear accumulation (Figs. 1 and 2). Consistent with this finding, we subsequently defined Arg-214/215 in the α-helix 2 of Stat3 to be necessary for the EGF- and IL-6-inducible nuclear translocation (Fig. 3–5). This is the first time that an NLS element is identified outside the DNA binding domain of Stat proteins. On the other hand, we have identified Arg-414/417 in the DNA binding domain of Stat3, which is in the corresponding position of Lys-410/413 in Stat1 protein previously reported for the interferon-γ-induced nuclear translocation (18, 19) and is also essential for the ligand-induced nuclear translocation of Stat3. Our data therefore demonstrate that two sequence clusters are required for the wild-type Stat3 nuclear translocation stimulated by growth factors and cytokines. Interestingly, truncation mutants (ST3-ND4H and ST3-4H) containing the coiled-coil domain are constitutively localized in the nucleus in both MCF-7 and COS-1 cells without ligand stimulation. Mutations in Arg-214/215, however, destroy such nuclear localization (Fig. 1 and data not shown). In contrast, the truncation mutant containing the DNA binding and linker domains (ST3-DB.LK) is localized in the cytoplasm, although it contains Arg-414/417 sequence element. These results suggest that Arg-214/215 has an NLS function that is sufficient to allow the truncation mutants to accumulate in the nucleus, whereas Arg-414/417 lacks this ability. The reason for this is unknown. One possibility is that this sequence is masked in the truncation mutant. Alternatively, the truncation mutant may be imported to the nucleus but exported to the cytoplasm efficiently. Three nuclear export signal elements have been reported in Stat3 recently (47), and two of them (404–414 and 524–535) are located in this truncation mutant, which supports the later hypothesis.

Stat Proteins Utilize Different NLS for Their Nuclear Translocation—The overall structures of Stat1 and Stat3 are very similar (15, 16). It was thus postulated that the members of the Stat family use the same pathway for nuclear import. However, some differences in control of the nuclear transport among Stat proteins were observed. First, when similar point mutations of Stat3 (Arg-214/215) in the coiled-coil domain were introduced to the corresponding amino acid residues in Stat1 (Arg-210/Lys-211), it did not affect its nuclear import induced by interferon-γ.2 This is consistent with the previous report indicating that a single mutation in either Arg-210 or Lys-211 of Stat1 did not affect its nuclear translocation (21). In agreement with the finding, the truncation mutant of Stat1 containing the ND and the CC domain is located in the cytoplasm but not in the nucleus in MCF-7 cells (data not shown). Second, although Leu-407 is the key element for the interferon-inducible nuclear translocation of Stat1, our results showed that its corresponding residue in Stat3, Leu-411, is not necessary for the nuclear translocation. Third, a region (466VVVI469) in Stat5b DNA binding domain was reported to be involved in the growth hormone-induced nuclear translocation, but mutation of VVV in Stat3 has no effect on its nuclear translocation (23). These data suggest that different members of Stat family may utilize distinct sets of NLS for their nuclear translocation.

Possible Mechanism for Stat3 Nuclear Import—Both Arg-214/215 and Arg-414/417 are different from the conventional NLS that consists of either a single or a bipartite basic amino acid stretches, generally longer than two residues, identified in many proteins (48). Therefore, how these elements are involved in its nuclear import is currently unknown. It has been reported that Leu-407 is involved in a direct binding of the importin-α5, and a heterodimer of Stat1 containing a monomer of the wild-type Stat1 and a L407A mutant is sufficient for the binding and the subsequent nuclear import to occur (20). On the other hand, Lys-410/413 of Stat1 is also reported to be bound directly by importin-α5. However, only the Stat1 dimer that has both intact Lys-410/413 elements in each monomer is able to bind to two importin-α5 molecules, which facilitates its nuclear translocation (22). Our preliminary results reveal that Stat3 is able to associate with certain subtypes of importin α, suggesting that Stat3 may also use importin α-mediated nu-

J. Ma and X. Cao, unpublished observation.
clear import pathway. Our results in this report indicate that two NLS elements must exist in one molecule of the dimeric Stat3 (Fig. 7), suggesting that both NLS elements must be in close proximity or in a specific conformation for effective binding of the importins.

It has been documented that the dimer formation of Stat proteins (except Stat2 that cannot form homodimer) is necessary and sufficient for the nuclear translocation. The apparent role of Tyr phosphorylation is to form dimer, inducing a conformational change that is sufficient to unmask the latent STAT nuclear localization sequence for subsequent nuclear translocation (33). Our data support this hypothesis. The deletion mutants containing the coiled-coil domain of Stat3 display a constitutive nuclear localization (Fig. 1 and Fig. 2). This could be due to deletion of the other domains that normally mask the NLS elements located in the coiled-coil domain. Therefore, exposure of the NLS, which may be masked in the latent status, is a key event for the nuclear translocation. On the other hand, in this study we also demonstrate that although the mutants R214A/R215A and R414A/R417A defect in the nuclear import, they are Tyr-phosphorylated and form dimers properly after EGF stimulation (Fig. 6 and data not shown). The results suggest that the dimer formation itself is necessary but not sufficient to trigger nuclear translocation. Specific res-

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3 J. Ma and X. Cao, unpublished data.
ides are required for the nuclear translocation either by direct binding to the transport proteins or maintaining the proper conformation of Stat3 dimer.

Interestingly, we observed that although R414A/R417A lost its ability for DNA binding, similar to the case reported in Stat1, the mutant R214A/R215A somehow displayed DNA binding activity stronger than the wild-type Stat3, and the migration in the EMSA was faster (Fig. 6B). This could be due to a conformational change caused by replacing two basic amino acids in the coiled-coil domain. Besides, the SIF-B (the heterodimer of R214A/R215A and endogenous Stat1) was not detectable. A few possibilities are considered. First, the mutant loses its ability to dimerize with Stat1. Second, the migration of SIF-A and SIF-B is too close to be separated. Third, the DNA loses its ability to dimerize with Stat1. Indeed, the intensity of SIF-A and SIF-B is too close to be separated. A few possibilities are considered. First, the mutant loses its ability to dimerize with Stat1. Second, the migration of SIF-A and SIF-B is too close to be separated. Third, the DNA loses its ability to dimerize with Stat1. A few possibilities are considered. First, the mutant

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A Novel Sequence in the Coiled-coil Domain of Stat3 Essential for Its Nuclear Translocation

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