STAT4 Requires the N-terminal Domain for Efficient Phosphorylation

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STAT4 (signal transducer and activator of transcription-4) mediates biological effects in response to interleukin-12 (IL-12). STAT4 has multiple domains that have distinct functions in signaling and gene activation. To characterize the role of the STAT4 N-terminal domain in mediating STAT4 biological function, we have generated STAT4-deficient transgenic mice that express human full-length STAT4 or an N-terminal deletion mutant (ΔN-STAT4) lacking the N-terminal 51 amino acids. Whereas full-length STAT4 rescued IL-12 responsiveness, T lymphocytes expressing the STAT4 N-terminal mutant failed to proliferate in response to IL-12 and had limited Th1 cell development as evidenced by minimal interferon-γ production. Deletion of the N-terminal domain resulted in failure of STAT4 to be phosphorylated following IL-12 stimulation despite similar phosphorylation of JAK2 and TYK2 in full-length STAT4 and ΔN-STAT4 transgenic T cells. We demonstrate that the lack of phosphorylation in cultured cells is due to reduced efficiency of phosphorylation of ΔN-STAT4 by Janus kinases. These data support a new model wherein the N-terminal domain is required to mediate the phosphorylation of STAT4 in addition to the previously documented role in gene transactivation.

Interleukin (IL)-12 is a heterodimeric cytokine that is composed of p35 and p40 chains. It is predominantly secreted from antigen-presenting cells, including macrophages and dendritic cells. IL-12 initiates the differentiation of CD4 T cells into Th1 subsets, which are critical for cell-mediated immunity and immunity to bacteria, viruses, and intracellular parasites (1). Interferon (IFN)-γ produced by Th1 cells enhances the activity of cytotoxic T lymphocytes and natural killer cells (1). In addition, IL-12 promotes the proliferation of T cells in response to pathogenic stimulation. Thus, IL-12 is an important regulator in innate and adaptive immunity.

IL-12 mediates its biological function by binding to IL-12 receptors (IL-12Rs) and transmitting signals via the JAK-STAT pathway. IL-12Rβ1 and IL-12Rβ2 belong to the gp130 cytokine receptor superfamily. The IL-12Rs do not contain intrinsic kinase activity, but instead physically associate with members of the Janus family of protein-tyrosine kinases (JAKs); IL-12Rβ1 pre-associates with TYK2, and IL-12Rβ2 with JAK2 (2). The binding of IL-12 to its receptor activates the JAKs that phosphorylate the tyrosine residues located in the cytoplasmic region of IL-12Rβ2. Subsequently, the phosphorylated receptors recruit STAT4 through interaction of the STAT4 SH2 domain with the phosphotyrosine at position 800 in the IL-12Rβ2 chain (3, 4). STAT4 then becomes phosphorylated at Tyr973 (5), dimerizes, and translocates into the nucleus to activate gene transcription.

STAT4 is a critical mediator of IL-12-stimulated gene regulation. STAT4-deficient mice have greatly decreased Th1 cell differentiation and lack many other biological functions mediated by IL-12 signaling (6, 7). STAT proteins have several functional domains, including a central DNA-binding domain, an SH2 domain, and a C-terminal transactivation domain (8). Based on the crystal structures of STAT1 and STAT3 (9, 10), STAT proteins also have a coiled-coil domain that may mediate protein-protein interaction (11).

An N-terminal domain has also been identified in STAT proteins that, although dispensable for dimerization, is required for the tetramerization that mediates cooperative association with tandem STAT4 DNA-binding sites (12). The crystal structure of the N-terminal domain of STAT4, comprising the first 124 amino acids, has revealed that it is composed of eight α-helices that assemble into a hook-like structure (13). Recent studies using retroviral expression in primary T cells and cell lines suggest that the STAT4 N-terminal domain may also be important for STAT4 phosphorylation (14). However, IL-12-stimulated STAT4 activation was not extensively examined.

To delineate the function of the N-terminal domain of STAT4 in vivo and to determine which biological functions may require the N-terminal domain, we generated transgenic mice that express full-length STAT4 or a STAT4 mutant (ΔN-STAT4) lacking the N-terminal 51 amino acids, with a deletion of the regions from the N terminus through the fifth α-helix. These mice were mated with STAT4-deficient mice, so transgenic STAT4 is expressed in the absence of endogenous STAT4. Whereas transgenic expression of full-length STAT4 rescued IL-12-stimulated proliferation, Th1 generation, and CD25 induction on the STAT4−/− background, transgenic expression of ΔN-STAT4 did not rescue any of these functions. This corre-
lates with a lack of IL-12-induced phosphorylation of ΔN-STAT4, despite normal activation of JAK2 and TYK2. Further analysis demonstrated that the STAT4 N-terminal domain is required for efficient phosphorylation by JAK2, but is not required for interaction with phosphorylated peptide from the IL-12Rβ2 chain. These results demonstrate a critical role for the N-terminal domain in proximal events leading to the IL-12-stimulated phosphorylation of STAT4.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—STAT4−/− mice were previously described (6) and backcrossed eight generations to the C57BL/6 genetic background. The cDNAs for human full-length STAT4 and an N-terminal deletion mutant (ΔN-STAT4) lacking the N-terminal 51 amino acids were cloned into a vector containing the CD2 locus control region (15, 16), and transgenic mice were generated at the Indiana University Transgenic Facility. Transgenic mice produced on the C3H genetic background were backcrossed to C57BL/6 and two generations to STAT4-deficient C57BL/6 mice to generate STAT4-deficient transgenic mice. Control wild-type mice (C57BL/6) were purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN).

Analysis of Transgenic Mice—Transgenic mice were analyzed by Southern blotting and Western blotting. The genomic DNA and total RNA were extracted from the tails and spleens, respectively. The probes used in Southern and Northern blotting were the cDNAs from human full-length STAT4 labeled with [α-32P]dCTP using the random decamer reagent (Amersham, Austin, TX). Alternatively, transgenic mice were typed by PCR using upstream (GAGCTTATAGGCGTATGGAATG) and downstream (TTGTGTCACCTGATGGGTGTT) primers. Western blotting was carried out on total protein extracted from spleens using anti-STAT4 polyclonal antibody specific for the C-terminal portion of STAT4 (Santa Cruz Biotechnology, Santa Cruz, CA).

Proliferation Assay—Cells isolated from spleens and lymph nodes were activated for 72 h with plate-bound anti-CD3 antibody to activate T cells, and to make them IL-12-responsive. Cells were then plated in triplicate at 5 × 105 cells/well in a round-bottomed 96-well plate in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) and stimulated with various doses of IL-12 (Genetics Institute, Cambridge, MA) ranging from 0 to 1 ng/ml or with IL-2 (8 ng/ml) for 72 h. Cells were pulsed for the last 16–18 h with 3H-thymidine (1 μCi/well). The incorporation of [3H]thymidine was measured with a liquid scintillation counter.

CD25 Expression by Flow Cytometry—Splenocytes isolated from mice were activated with plate-bound anti-CD3 antibody at 2 μg/ml for 3 days. Cells purified through Histopaque-1077 (Sigma) at 1 × 106 cells/ml were incubated with IL-2 (2 ng/ml) or with IL-12 (8 ng/ml) and anti-IL-12 antibody (2 μg/ml) to eliminate IL-2-induced CD25 expression or left unstimulated (anti-IL-2 antibody alone). Following overnight incubation, cells were stained with anti-CD25 antibody conjugated with fluorescein isothiocyanate (BD Biosciences). CD25 expression on the activated cell population was analyzed by flow cytometry (FACScan, BD Biosciences). The mean fluorescence intensity was evaluated by the CellQuest program (BD Biosciences).

IFN-γ Production by Th1 Cells—CD4+ T cells were isolated by positive selection using MiniMacs beads (Miltenyi Biotech, Auburn, CA) and differentiated into Th1 cells as described (17). Differentiated Th1 cells were restimulated with plate-bound anti-CD3 antibody (2 μg/ml), with or without IL-12 (2 ng/ml) or with IL-12 (2 ng/ml) and IL-18 (25 ng/ml) for 24 h. The cultures were collected for enzyme-linked immunosorbent assay to measure the level of IFN-γ production (18).

Protein Phosphorylation Analysis by Immunoprecipitation and Immunoblotting—Cells isolated from spleens and lymph nodes were activated with plate-bound anti-CD3 antibody at 2 μg/ml for 3 days. Ten million cells purified through Histopaque-1077 were incubated with or without IL-12 at 2 ng/ml for 20 min at 37°C in a 5% CO2 incubator. In the case of STAT4 phosphorylation, the phosphatase inhibitor sodium orthovanadate at various doses was included. The cells were washed three times with phosphate-buffered saline and lysed with protein lysis buffer (19). Total protein extracts (1 mg) were immunoprecipitated individually with anti-JAK2 polyclonal antibody (Upstate Biotechnology, Inc.), anti-TYK2 polyclonal antibody (Santa Cruz Biotechnology). Phosphorylated proteins were detected with anti-phosphotyrosine antibody PY99 (Santa Cruz Biotechnology) or 4G10 (Upstate Biotechnology, Inc.) and visualized with a chemiluminescence kit (ECL, Amer sham Biosciences). STAT4 phosphorylation was detected using anti-phospho-STAT4 antibody (Zymed Laboratories, Inc., South San Francisco, CA), followed by striping and reprobing the membranes with polyclonal antibody against the C terminus of STAT4 (Santa Cruz Biotechnology).

STAT4 Analysis in COS-7 Cells—COS-7 cells grown on 6-well plates were transiently transfected using FuGENE reagents (Roche Applied Science) with 1 μg of STAT4 or ΔN-STAT4 plasmid in the presence of transfection reagents. The transfection reagents were added to the cells for 6–8 h. After incubation for 48 h at 37°C in a 5% CO2 incubator, cells were lysed to extract the total protein. The protein extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-STAT4 antibody. The membranes were stripped and reprobed with polyclonal antibody against the C terminus of STAT4. For DNA binding assays, extracts were prepared from COS-7 cells transfected with STAT4 or ΔN-STAT4 and JAK2-expressing plasmids. Cells were activated with plate-bound anti-CD3 antibody at 2 μg/ml, with or without IL-12 (25 ng/ml) and IL-18 (25 ng/ml) for 24 h. Total cell extracts were made 48 h later, and luciferase levels were analyzed with a luciferase assay kit (Promega, Madison WI). Luciferase activities were corrected for transfection efficiency by measuring β-galactosidase levels (Galacto-Light system, Applied Biosystems, Foster City, CA) and protein concentration.

Peptide Binding Assay—The biotinylated phosphopeptide DLP-ThDGYip**LPSNDD and the identical non-phosphorylated peptide were synthesized (Genemed Biotechnologies, Inc., San Francisco, CA) for the in vitro binding assay. Increasing amounts of total protein extracts (100, 200, and 400 μg) from pooled spleens and lymph nodes were incubated with the biotinylated phosphopeptide (100 nm) overnight at 4°C in binding buffer B (20 mM Hepes, 15 mM NaCl, 0.5 mM dithiothreitol, and 0.5% Igepal) at 2 μg/ml. The complex of peptide-bound STAT4 was precipitated by immobilized streptavidin (Upstate Biotechnology, Inc.). The precipitation of STAT4 with the biotinylated peptides was confirmed by Western blotting with polyclonal antibody against the C terminus of STAT4.

RESULTS

Generation of Transgenic Mice—Mice positive for a transgene of human full-length STAT4 (CD2:STAT4) or an N-terminal deletion mutant (CD2:ΔN-STAT4) lacking the first 51 amino acids (Fig. 1A) were generated on the C57BL/6 STAT4−/− background as described under “Experimental Procedures.” The presence of transgenes in mice was tested by Southern analysis of tail genomic DNA (Fig. 1B) or by PCR. Expression of transgenic STAT4 was confirmed by Northern and Western analyses (Fig. 1B). Two forms of ΔN-STAT4 were observed in some Western analyses (Fig. 1B). This may reflect increased degradation of the ΔN-STAT4 form and correlated with relatively low expression despite high STAT4 mRNA levels (Fig. 1B). Four founder lines were initially characterized and found to have similar phenotypes. Two CD2:ΔN-STAT4 founder lines (1078 and 1106) were selected and used for detailed analysis.

CD2-ΔN-STAT4 Cells Do Not Respond to IL-12—As previously demonstrated, STAT4-deficient activated T cells do not proliferate in response to IL-12 (6, 7, 20). To determine whether the N-terminal portion of STAT4 is required for proliferation of T cells in response to IL-12, we activated spleen cells with

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CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. Wild-type and CD2:STAT4 cells demonstrated IL-12-induced CD25 expression, whereas there was no antibody to CD25. 

induction of CD25 expression in STAT4-deficient cells (Fig. 3). The diminished induction in CD2:STAT4 cells correlated with lower levels of STAT4-expressed in these cells (Fig. 1). Importantly, CD25 expression was not induced by IL-12 in CD2:ΔN-STAT4 transgenic mice (Fig. 3). Induction of CD25 expression by IL-2 was similar in all genotypes, demonstrating that the CD25 locus is still cytokine-responsive in STAT4-deficient and CD2:ΔN-STAT4 transgenic mice.

STAT4 activation following IL-12 stimulation contributes to the development of Th1 cells and is important for the production of IFN-γ, the hallmark cytokine of Th1 cells (24). We next examined whether CD4 T cells from STAT4 transgenic mice could be polarized into Th1 cells. Purified CD4 T cells were stimulated with plate-bound anti-CD3 antibody, anti-CD28 antibody, IL-12, and anti-IL-4 antibody for 6 days. Cells were then restimulated with anti-CD3 antibody alone, with IL-12 alone, or with IL-12 in combination with IL-18. Wild-type and CD2:STAT4 cells produced significant levels of IFN-γ following each of the stimuli (Fig. 4) (25), and diminished induction in CD2:STAT4 cells correlated with lower levels of STAT4 expression in these cells (Fig. 1). As previously described, STAT4-deficient cells are greatly or completely impaired in their ability to produce IFN-γ following anti-CD3 antibody or IL-12 stimulation, respectively (6, 7). Th1 cultures from the two founder lines (1078 and 1106) of CD2:ΔN-STAT4 transgenic mice produced low levels of IFN-γ following anti-CD3 antibody stimulation and undetectable amounts of IFN-γ following either IL-12 or IL-12 plus IL-18 stimulation (Fig. 4), a phenotype similar to that seen in STAT4-deficient mice. To confirm that Th1 differentiation (and not simply IFN-γ production) was impaired in CD2:ΔN-STAT4 mice, we also examined the expression of Th1-restricted genes, including IL-12Rβ2, lympho-
The N-terminal Domain Is Required for STAT4 Phosphorylation—Since ΔN-STAT4 was not sufficient to mediate any IL-12-stimulated functions, we next examined the phosphorylation of STAT4 following IL-12 stimulation to confirm normal STAT4 activity, the integrity of the IL-12-activated JAKs JAK2 and TYK2 (Fig. 6A and 6B) in wild-type, CD2:STAT4, and CD2:ΔN-STAT4 transgenic cells. However, even with treatment of the phosphatase inhibitor, STAT4 from CD2:ΔN-STAT4 transgenic cells did not become detectably phosphorylated.

We have previously demonstrated that JAK2 and TYK2 activation is normal in STAT4-deficient cells (19). To determine whether the lack of STAT4 phosphorylation observed in CD2:ΔN-STAT4 transgenic mice resulted from deficient JAK kinase activity, the integrity of the IL-12-activated JAKs JAK2 and TYK2 was evaluated upon IL-12 stimulation. IL-12 induced comparable phosphorylation of JAK2 (Fig. 6A) and TYK2 (Fig. 6B) in wild-type, CD2:STAT4, and CD2:ΔN-STAT4 transgenic cells.

Binding of STAT4 to Phosphopeptide Derived from IL-12Rβ2—To begin to determine the nature of the defect in ΔN-STAT4 activation, we first examined the ability of full-length and mutant STAT4 to interact with the IL-12R. The tyrosine at position 800 in the IL-12Rβ2 protein mediates the recruitment of STAT4 to the receptor complex. Furthermore, the binding of STAT4 to this region is specific and occurs only when this tyrosine is phosphorylated (Fig. 7B) (3, 4).

To determine whether ΔN-STAT4 interacts with the IL-12Rβ2 phosphopeptide, we performed a phosphopeptide binding assay using total cell extracts from CD2:STAT4 and CD2:ΔN-STAT4 mice. Direct interaction of ΔN-STAT4 with the phosphopeptide derived from the IL-12Rβ2 subunit was readily observed to a level similar to that of full-length STAT4 with increasing amounts of cell extract (Fig. 7C).

Thus, ΔN-STAT4 is not sufficient to mediate Th1 differentiation. The N-terminal Domain Is Required for STAT4 Phosphorylation—Since ΔN-STAT4 was not sufficient to mediate any IL-12-stimulated functions, we next examined the phosphorylation of STAT4 following IL-12 stimulation to confirm normal activation of ΔN-STAT4. IL-12 induced tyrosine phosphorylation of STAT4 in wild-type and CD2:ΔN-STAT4 cells (Fig. 5). In contrast, IL-12 did not induce tyrosine phosphorylation of STAT4 in CD2:ΔN-STAT4 transgenic mice. To increase the sensitivity of phosphotyrosine analysis, sodium orthovanadate, a phosphatase inhibitor, was included during IL-12 stimulation in the presence or absence of IL-12 for 30 min following preincubation in the presence of increasing doses (0.1, 0.5, and 1.0 mM) of sodium orthovanadate (NaVan) for 60 min. Total cell extracts from wild-type (25 μg), CD2:STAT4 (100 μg), and CD2:ΔN-STAT4 (200 μg) mice were resolved by SDS-PAGE, and anti-phospho-STAT4 antibody (p-Stat4) was used for Western analysis. The blots were stripped and reprobed with anti-STAT4 antibody as a control. IB, immunoblot.
are capable of interacting with the IL-12R.

The STAT4 N-terminal Domain Mediates Efficient Phosphorylation—To further characterize the inability of N-STAT4 to be phosphorylated, we transfected COS-7 cells with plasmids expressing full-length STAT4 or ΔN-STAT4 and cotransfected with increasing amounts of JAK2 plasmid DNA. As shown in Fig. 8A, no phospho-STAT4 was detected in the absence of transfected JAK2. However, JAK2 dose-dependent phosphorylation of STAT4 and ΔN-STAT4 was observed when the JAK2 plasmid was cotransfected. The level of phosphorylation was determined by the ratio of phosphorylated STAT4 to total STAT4 protein using densitometry, and the results of this analysis are shown in Fig. 8B. More full-length STAT4 was phosphorylated with lower concentrations of JAK2 compared with ΔN-STAT4, although equal phosphorylation of both forms of STAT4 was detected upon the expression of high levels of JAK2 (Fig. 8B).

To confirm these results, we also performed an in vitro kinase assay with recombinant full-length STAT4 and ΔN-STAT4. Recombinant proteins were incubated with a JAK2 enzyme-agarose complex, and phospho-STAT4 was detected by Western analysis. As shown in Fig. 8C, phosphorylation of ΔN-STAT4 protein was detectable, but in a much lower level compared with full-length STAT4 proteins. Thus, ΔN-STAT4 is phosphorylated by JAK2 less efficiently compared with full-length STAT4.

The N-terminal Domain Is Not Required for Transactivation from a STAT4-responsive Element—Knowing that high levels of JAK2 expression can lead to equivalent activation of full-length STAT4 and ΔN-STAT4, we wanted to test whether both forms would still be capable of binding DNA and activating transcription. We first tested the ability of full-length STAT4 and ΔN-STAT4 to bind DNA by incubating cell extracts from COS-7 cells, which had been transfected with either full-length STAT4 or ΔN-STAT4 in the presence or absence of JAK2 expression, with biotinylated oligonucleotides containing STAT4-binding sites (Fig. 9A). Complexes were precipitated with streptavidin-agarose and analyzed by Western blotting. Both full-length STAT4 and ΔN-STAT4 were capable of binding to DNA (Fig. 9B). We then tested whether full-length or mutant STAT4 would activate a reporter containing two copies of the IFN regulatory factor-1 promoter STAT4/cAMP-responsive element-binding site known to mediate STAT4 transactivation (26). Plasmids encoding full-length STAT4 or ΔN-STAT4 and the reporter plasmid were transfected into COS-7 cells in the absence or presence of a JAK2 plasmid. Full-length STAT4 and ΔN-STAT4 were capable of equally inducing luciferase activity from the reporter plasmid (Fig. 9C) (12). Thus, defects in ΔN-STAT4 function are restricted to proximal events during STAT4 phosphorylation.

DISCUSSION

The N-terminal domain of STAT proteins may have several functions based on published reports. To address the importance of the N-terminal domain in STAT4 function, we have generated STAT4(ΔN)-transgenic mice expressing ΔN-STAT4, which lacks the N-terminal 51 amino acids. These mice allowed us to characterize the role of the N-terminal domain by examining a wide array of biological assays. Transgenic expression of full-length STAT4 (but not ΔN-STAT4) on a STAT4(ΔN)-background rescued IL-12-stimulated biological functions, including proliferation and Th1 differentiation. This was due to the lack of ΔN-STAT4 phosphorylation in transgenic T cells follow-
ing IL-12 stimulation. We have demonstrated that the lack of phosphorylation in cultured cells was due to reduced efficiency of phosphorylation of ΔN-STAT4 by JAKs. These data support a model wherein the N-terminal domain is required to mediate the biological function of STAT4.

In our study using several different systems examining STAT4 phosphorylation in primary transgenic cells (Fig. 5) and in transfected COS-7 cells and in a cell-free reaction (Fig. 8), the ΔN-STAT4 proteins demonstrated decreased tyrosine phosphorylation. Transfection data indicated that the mutant ΔN-STAT4 protein was capable of serving as a substrate, albeit less efficiently, for JAK2. Thus, this deficiency may be overcome when JAK2 is overexpressed (12). It is also possible that the N-terminal domain may mediate interactions with the IL-12Rβ2 chain. The N-terminal region of STAT2 has been shown to mediate interactions with the IFNα receptor 2 (27). However, the interaction of STAT2 with the IFNα receptor is not required for activation (28), and we have shown that transgenic ΔN-STAT4 interacted normally with a phosphopeptide from the IL-12Rβ2 chain. Thus, the Δ1–51 mutation of STAT4 affects the efficiency of phosphorylation by JAKs rather than being required for interactions of STAT4 with the receptor.

The precise role of the N-terminal domain of STAT proteins in phosphorylation events is still unclear. We have demonstrated that the ΔN-STAT4 proteins from cells of the transgenic mice were not activated upon IL-12 stimulation as evidenced by undetectable tyrosine phosphorylation (Fig. 5). Specific mutation of Trp75 or double mutation of Lys84 and Arg85 demonstrated a similar role for the STAT4 N-terminal domain in tyrosine phosphorylation (14). Mutation of Trp77, which is conserved in all STAT protein N-terminal domains (13), results in a lack of STAT4 phosphorylation following IFN-α stimulation in U3A cells and a lack of nuclear STAT4 in IL-12-stimulated primary T cells (14). Similarly, deletion of the N-terminal 59 amino acids of STAT2 completely abolishes tyrosine phosphorylation following IFN-α stimulation in U6A cells by a mechanism that is distinct from IFN receptor interaction (28, 29). In contrast, the N-terminal domain of all STAT proteins does not seem to be required for activation.
STAT1 lacking the N-terminal 61 amino acids displays constitutive tyrosine phosphorylation (30). In addition, truncated STAT1 lacking the N-terminal 131 amino acids undergoes successful tyrosine phosphorylation in vitro (31), and deletion of 136 amino acids from the N terminus of STAT5 does not affect IL-3-stimulated tyrosine phosphorylation (32). These results suggest that the N-terminal domain may have distinct functions in different STAT proteins. The N-terminal domain is required for normal activation of STAT4 and STAT2. In contrast, the N-terminal domain of STAT1 mediates targeting of a STAT phosphatase (30) as well as cellular distribution (33).

Importantly, the requirement for the N-terminal domain in STAT dimer-dimer interactions leading to stabilization of the protein-DNA complex by cooperative DNA binding has been well documented. Deletion of 88 amino acids from the N terminus of STAT4 does not affect its binding to a single binding site, but abolishes the cooperative binding of two STAT4 dimers to tandem low affinity sites (12). Similarly, truncated STAT1 lacking the N-terminal 44 residues binds to a single high affinity site, but not to a pair of low affinity sites (12). Mutation of the invariant Trp to alanine in STAT1 or STAT5 eliminates tetramer formation (13, 34). The precise nature of the dimerization interface of the STAT proteins is unclear since several examples of mutations that would be predicted to disrupt dimer formation have little effect. Recent analyses of potential dimer interfaces suggest that the interaction may be distinct from that originally predicted (35).

Since the STAT N-terminal domain was crystallized separately from the rest of the STAT protein, it is still unclear how it may interact with other STAT domains. Intramolecular interactions have been shown to be important for receptor interaction and the subsequent activation of STAT3 (36), and this may be important for other STAT proteins as well. Our results suggest that the N-terminal domain of STAT4 may contribute to inter- or intramolecular interactions, which affects its ability to serve as a substrate for JAKs. The potential structural requirements for the N-terminal domain in inter- or intramolecular interactions suggest that distinct mutations could result in proteins that lack activation or that have constitutive activation. Further analysis will be required to determine how this domain mediates distinct STAT protein functions.

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