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Stat5 and Sp1 Regulate Transcription of the Cyclin D2 Gene in Response to IL-2

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The IL-2R promotes rapid expansion of activated T cells through signals mediated by the adaptor protein Shc and the transcription factor Stat5. By the IL-2R. IL-2-responsive induction of a luciferase reporter gene containing 1624 bp of the cyclin D2 promoter/enhancer was studied in the murine CD8+ T cell line CTLL2. Reporter gene deletion analysis and EMSAs indicate an IL-2-regulated enhancer element flanks nucleotide −1204 and binds a complex of at least three proteins. The enhancer element is bound constitutively by Stat5 and an unknown factor(s) and inducibly by Stat5 in response to IL-2. The Stat5 binding site was essential for IL-2-mediated reporter gene activity, and maximum induction required the adjacent Sp1 binding site. Receptor mutagenesis studies in the pro-B cell line BA/FG (a derivative of the BA/F3 cell line) demonstrated a correlation between Stat5 activity and cyclin D2 mRNA levels when the Stat5 signal was isolated, disrupted, and then rescued. Further, a dominant-negative form of Stat5 lacking the trans-activation domain inhibited induction of cyclin D2 mRNA. We propose that the IL-2R regulates the cyclin D2 gene in part through formation of an enhancer complex containing Stat5 and Sp1. The Journal of Immunology, 2001, 166: 1723–1729.

The high affinity IL-2R complex includes three proteins (3). IL-2Rα regulates ligand receptor affinity, and IL-2β and the common γ-chain (γc)3 initiate intracellular signaling. The tyrosine kinases Janus kinase 1 (Jak1) and Jak3 associate with the membrane proximal regions of IL-2Rβ and γc, respectively, and undergo catalytic activation upon ligand-induced heterodimerization of IL-2Rβ and γc (4–6). Activation of Jak1 and Jak3 leads to phosphorylation of at least three tyrosine residues on IL-2Rβ (Y338, Y392, and Y510) (7). Primed by the phosphorylation events, the receptor complex generates two major proliferative signals. One signal is mediated by the adaptor protein Shc (7, 8), which binds to phosphorylated Y338, undergoes tyrosine phosphorylation, and activates two downstream pathways. The Grb2/Sos complex is recruited to Shc (9–11), and Sos activates the Ras-mitogen-activated protein kinase pathway up-regulating genes such as c-fos and c-jun (12, 13). Additionally, the phosphatidylinositol-3 kinase (PI3K) signaling pathway is activated through recruitment of the adaptor protein GAB2 (14–17). While no genes have been linked directly to the PI3K pathway, regulation of cyclin D3 and p27kip1 expression, pRB phosphorylation, and E2F activity is attributed to PI3K activity in response to IL-2 in T cells (18, 19).

A second proliferative signal activated by the IL-2R involves the transcription factor Stat5 (20, 21). Stat5 is recruited to phosphorylated Y510 (7), becomes tyrosine phosphorylated, homodimerizes with other Stat molecules, and directly translocates to the nucleus (22). Expression of Stat5 mutants that lack trans-activation potential impairs the IL-2-activated proliferative response in lymphocytes (23, 24), and Stat5-deficient mice have compromised T cell proliferative responses (25). Growth-related target genes of Stat5 in the context of the IL-2R include c-myc, bcl-x, bcl2 (23), IL-2Rα (26, 27), and pim-1 (28). Other target genes of Stat5 that have been identified downstream of other cytokine receptors include cyclin D1 (29) and p21cip1 (30).

The D-type cyclins (D1, D2, and D3) are among the first regulatory proteins to appear in G1 in response to mitogens. Cyclin D2 and D3 mRNA levels increase in early G1 in primary T cells stimulated by IL-2 (31) or stimulated by PHA and 12-O-tetradecanoylphorbol-13-acetate (32). Cyclin D2 and D3 protein expression is up-regulated in primary T cells stimulated by anti-CD3, but not in T cells derived from Stat5-deficient mice (35). Several mechanisms for cyclin regulation that may be relevant to IL-2 signaling have been identified. In an erythroleukemia cell line, IL-3-activated Stat5 regulates the transcriptional activity of cyclin D1 at a specific gene enhancer region (29). In colon carcinoma and breast cancer cell lines, serum-activated PI3K controls mRNA translation of D-type cyclins (33). Finally, c-Myc has been implicated in the transcriptional induction of cyclin D2 in Rat1 and NIH-3T3 cells (34).

We have studied the transcriptional regulation of the cyclin D2 gene in T cells. We report evidence of an enhancer element in the cyclin D2 promoter/enhancer that binds Stat5, specificity protein
(Sp1), and an unknown factor(s). Maximal enhancer activity requires both the Stat5 and Sp1 binding sites, suggesting functional cooperation between these factors. Additionally, receptor mutagenesis studies indicate the endogenous cyclin D2 gene is regulated by the Stat5 pathway downstream of IL-2 in the absence of Shc-mediated signaling. Thus, this work reveals a direct pathway from the IL-2R to a key cell cycle regulatory gene via a mechanism involving the transcription factors Stat5 and Sp1.

Materials and Methods

**Plasmid construction**

**D2-Luc.** Part (1624 bp) of the human cyclin D2 promoter/enhancer immediately upstream of the translation start site (provided by Dov Shiffman (35)) was introduced into the luciferase vector PGL-3 (Promega, Madison, WI) to generate the plasmid D2-Luc.

1. **–1303, –1294, –444 D2-Luc.** Deletions from the 5' end of the 1624-bp promoter/enhancer were completed using convenient restriction enzyme sites for StuI, SmaI, and PvuII to produce 1303-, 1294-, and 444-bp fragments of the cyclin D2 gene in PGL-3 basic, respectively.

2. **–1303 D2-Luc mutated Sp1.** The Sp1 site mutation was created by PCR-based site-directed mutagenesis of a 97-bp region between the StuI and SmaI sites of D2-Luc. The 97-bp fragment was introduced into –1303 D2-Luc between the same restriction sites.

3. **–1303 D2-Luc mutated Stat5.** The Stat5 site mutation was introduced by splice overlap extension (SOE) PCR. The mutated, SOE-amplified region was digested with KpnI and AflII and then introduced into –1303 D2-Luc between the same sites.

4. **(–1227 to –1168)Luc.** A 60-bp region of the human cyclin D2 gene from –1227 to –1168 was amplified by PCR and cloned into the PGL-3 promoter vector between the SacI and XhoI sites.

**Other plasmids.** Mutants of IL-2Rβ and Stat5 have been described previously (23). The DNA sequences of all plasmid regions subjected to restriction enzyme- or PCR-based mutagenesis were confirmed by standard methods.

**Cell culture and transfections**

The murine IL-2-dependent T cell line CTLL-2 was obtained from American Type Culture Collection (Manassas, VA) and was maintained in RPMI (Life Technologies, Gaithersburg, MD) with 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, and 25 mM 2-ME. Cells were passaged with 50 U/ml human IL-2 (Chiron, Emeryville, CA). The experiments cells were stimulated with 100 U/ml IL-2.

The murine IL-3-dependent pro-B cell line BA/F3 was obtained from Immunix (Seattle, WA) and maintained in RPMI with 10% WEHI3-conditioned medium as a source of murine IL-3, 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. BA/F3 cells were maintained on 100 ng/ml recombinant human G-CSF (Amgen, Thousand Oaks, CA) in the absence of IL-3. Introduction of the gpl30 cytoplasmic receptor chain allows BA/F3 cells to grow through SHP-2 and Stat3 rather than Stat5 (37).

To generate stable BA/F3 transfectants, linearized plasmids were introduced into cells by electroporation, and transfectants were selected for resistance to G418 (Life Technologies) in 96-well plates at limiting dilution to isolate independent subclones. Receptor expression was assessed by flow cytometry with an Ab to human IL-2R (PharMingen, San Diego, CA). Stat5 expression was assessed by Western blot with Abs to Stat5 and the FLAG epitope tag (Transduction Laboratories, Lexington, KY) and Sigma (St. Louis, MO). Subclones with comparable IL-2Rβ and Stat5 expression were chosen for further analyses.

**Northern blots**

BA/F3 cells were washed three times in PBS and resuspended at 1 × 10⁶ cells/ml. Cells were deprived of cytokine for 8 h, then harvested either unstimulated or at serial time points after cytokine stimulation. Cells were washed with buffer H (20 mM HEPES (pH 7.9), 1 mM EDTA, 2 mM magnesium chloride, 1 mM sodium o-vanadate, 20 mM sodium fluoride, 1 mM DTT, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1 μg/ml leupeptin) and lysed at 10⁶ cells/ml in buffer H plus 0.2% Nonidet P-40 at 0°C. Nuclei were pelleted by centrifugation, and protein was extracted with buffer K (buffer H plus 0.42 M sodium chloride and 20% (v/v) glycerol). Nuclear extracts were boiled in sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Nitrocellulose membranes were blocked with TTBS (0.1 M, pH 7.5; Tris base, 0.9% sodium chloride, and 0.05% Tween 20) containing 5% powdered skim milk (Carnation, Glendale, CA) and probed with polyclonal Abs to cyclin D2 or cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then washed with TTBS, probed with goat anti-rabbit Ab (Life Technologies), and washed again with TTBS. Bound Ab was detected by ECL (Amersham, Arlington Heights, IL).

**EMSA**

Nuclear extracts were prepared as described for Western analysis. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA) and normalized across time points. DNA oligonucleotides were annealed, radiolabeled by an end-filling T4 polymerase reaction, and purified with a Microspin G-25 column (Pharmacia, Piscataway, NJ). EMSA probes longer than 30 bp were synthesized by PCR using Taq and (α-32P)ATP for radiolabeling and were purified with a Microspin G-25 column. DNA probe (one part at 5000 cpm/μl) and nuclear extract (one part) were mixed with two parts EMSA buffer (50 mM potassium chloride, 15 mM HEPES (pH 7.9), 15% glycerol, 1 mM DTT, and 0.1 mg/ml poly(dI-dC) and incubated at room temperature for 30 min. In competition reactions, unlabeled probe was added in at least a 10/1 molar excess over radiolabeled probe. In supershifting experiments, 2.2 μg of Sp1 or Stat5 Ab was added per 10 μl of EMSA reaction (Santa Cruz Biotechnology). Reaction mixtures were electrophoresed on a nonreducing 0.25%–Tris-buffered Na-acrylamide gel and visualized by autoradiography. The probe sequences (sense strands) used in this study include: –1227 to –1168, CAC TCG CCC CCT CCC CCT CCC GGG CCA TTT CCT AGA, and –1168 to –1130, ATT CGA TCG GGG CGG GGC GAG C. The probe sequences (sense strands) used in this study include: –1227 to –1168, CAC TCG CCC CCT CCC CCT CCC GGG CCA TTT CCT AGA, and –1168 to –1130, ATT CGA TCG GGG CGG GGC GAG C. The probe sequences (sense strands) used in this study include: –1227 to –1168, CAC TCG CCC CCT CCC CCT CCC GGG CCA TTT CCT AGA, and –1168 to –1130, ATT CGA TCG GGG CGG GGC GAG C.

**Luciferase reporter gene assays**

CTLL2 cells were pelleted, resuspended at 1.25 × 10⁶ cells/ml in PBS with 10 mM MgCl₂, and incubated for 10 min at room temperature with 100 μg of circular plasmid DNA. Transfection was conducted by electroporation at 250 V and 960 μF, and the transfected cells were left at room temperature for an additional 10 min before being incubated overnight in complete medium plus IL-2. Cells were washed three times with PBS, split into six groups, and incubated 4 h in complete medium with IL-2. Three groups were left unstimulated, and three groups were stimulated with IL-2 for 5 h. At 5 h, 50 μl of cell culture was mixed with 450 μl of 1× Promega cell culture lysis reagent on ice. Fifteen microliters of lysate was mixed with 75 μl of Promega luciferase substrate reagent, and luciferase activity was measured with a United Technologies Packard Minaxi Tri-Carb 4000 series liquid scintillation counter (Downers Grove, IL). Means were calculated for the three unstimulated and the three stimulated replicates, and fold induction was calculated by dividing the mean for the stimulated cells by the mean for the unstimulated cells.
Results

Identification of an IL-2-responsive enhancer region in the cyclin D2 gene

Experiments to identify an IL-2-responsive enhancer region in the cyclin D2 gene were performed in the murine CD8+ T cell line CTLL2. CTLL2 cells demonstrate robust Ag-independent growth in response to exogenous IL-2. IL-2 induced cyclin D2 mRNA and protein as assessed by Northern and Western analyses (Fig. 1). The short delay in cyclin D2 protein appearance relative to mRNA appearance indicates that cyclin D2 expression in response to IL-2 is largely regulated at the mRNA level.

We studied the transcriptional regulation of the cyclin D2 gene in response to IL-2 using a luciferase reporter gene containing 1624 bp of the cyclin D2 promoter/enhancer (referred to as D2-Luc). The 1624-bp fragment represents the region immediately upstream of the translational start site in the cyclin D2 gene. D2-Luc was transiently transfected into CTLL2 cells. Transfected cells were deprived of IL-2 for 4 h and then were either left unstimulated or were stimulated with IL-2 for an additional 5 h. All reporter gene assays used triplicate samples and were repeated 10–30 times to assure statistical significance. D2-Luc was induced 2.7-fold in CTLL2 cells in response to IL-2 (Fig. 2). The level of induction was slightly lower than that observed for the endogenous cyclin D2 gene by Northern analysis, indicating that either additional enhancer sites exist outside of the 1624-bp cyclin D2 gene fragment studied here or proper chromatin structure found only in the endogenous gene is required for full induction.

Deletion mutants of D2-Luc were evaluated to identify the IL-2-responsive region(s) within the 1624-bp promoter/enhancer. The region between −1624 and −1303 was dispensable for induction by IL-2 (Fig. 2). Deletion of the −1624 to −1204 region resulted in a decrease in fold induction to 2.0, and deletion to the −1168 site diminished fold induction to 1.6. Thus, the regions between −1303 to −1204 and −1204 to −1168 appear to contain important regulatory sites for induction of D2-Luc. The region downstream of −444 bp contains binding sites for basal transcriptional machinery (35) and possibly enhancer elements, but was not investigated further in this study.

Stat5 and Sp1 binding sites flank the −1204 enhancer region

EMSAs were used to analyze a broad region surrounding nucleotide −1204 for IL-2-inducible binding of proteins to DNA. We made 10 overlapping 60-bp DNA probes spanning the region from −1307 to −848. The DNA probes were mixed with nuclear extracts from unstimulated and IL-2-stimulated CTLL2 cells. Several probes showed constitutive protein binding, and one probe showed diminished protein binding with IL-2 stimulation. The EMSA probe spanning nucleotides −1227 to −1168 showed protein binding changes in response to IL-2 (Fig. 3A). The probe spans nucleotide −1204 and thus contains a portion of the functionally important regions defined by the D2-Luc reporter gene (Fig. 3A). Before IL-2 stimulation, two bands were clearly observed with the −1227 to −1168 probe (bands 1 and 2 at time zero). After stimulation, a third and a fourth band appeared (bands 3 and 4), and the original bands 1 and 2 diminished. Changes in protein-DNA complexes represented by the four bands occurred within 30 min and persisted for at least 8 h (data not shown).

The TRANSFAC program (38) identified a number of putative transcription factor binding sites within the 60-bp region, including sites for Sp1 and Stat5. Ab supershifting and cold competition studies confirmed that the transcription factors Sp1 and Stat5 bind to the −1227 to −1168 EMSA probe. An unlabeled oligonucleotide encoding a consensus Sp1 site competed with the −1227 to −1168 probe to eliminate bands 1 and 3. An unlabeled oligonucleotide encoding a defined Stat5 binding site (FCR) competed with the −1227 to −1168 probe to eliminate bands 3 and 4 (Fig. 3A). Similarly, addition of an anti-Sp1 Ab to the EMSA binding reaction eliminated bands 1 and 3, and anti-Stat5 Abs eliminated band 4. Thus, band 1 is attributed to Sp1 binding, and band 4 is attributed to Stat5 binding. Band 3 required both Sp1 and Stat5 binding. An unidentified protein or protein complex is represented by band 2. Point mutations confirmed the locations of the binding sites for Sp1, Stat5, and the unknown factor(s) within the −1227 to −1168 probe. Mutation of −1217 through −1214 bp (CTCC to AGAA) abrogated binding of Sp1 and the unknown factor(s), as evidenced by elimination of bands 1, 2, and 3 (Fig. 3B). Substitution of the highly conserved AA at −1192 and −1191 to CC abrogated Stat5 binding to the −1227 to −1168 probe as evidenced by elimination of bands 3 and 4. The relative locations of the Sp1 and Stat5 binding sites are well conserved between the human and rat cyclin D2 genes, further suggesting that this region may be functionally important (Fig. 3C).

The binding sites for Sp1 and the unknown factor(s) were further studied using smaller DNA probes. A probe of nucleotides −1227 to −1208 containing the Sp1 site showed two constitutive bands (Fig. 4). The upper band corresponds to Sp1, as determined
by supershifting Ab and cold competition EMSA reactions. The same Sp1 site base substitution used previously (−1217 to −1214 from CTCC to AGAA) eliminated both bands; therefore, the lower band in the −1227 to −1208 probe appears to represent the same factor(s) responsible for band 2 observed with the longer −1227 to −1168 probe. Putative binding sites for AP-2, myeloid zinc finger 1 (MZF1), and early growth response 1 (Egr-1) lie within the −1227 to −1208 region, but addition of unlabeled oligonucleotides encoding consensus binding sites for each of these three proteins failed to competitively eliminate the lower band. Point mutations throughout the −1227 to −1208 region failed to uncouple binding of Sp1 vs the unknown factor(s) (data not shown). EMSA reactions were performed with a 2-fold titration series of DNA probe to achieve limiting concentrations. The upper and lower bands disappeared at the same probe concentration (data not shown). Therefore, Sp1 and the unknown factor(s) bind to the same site with relatively equal affinity.

We conclude that Sp1, Stat5, and an unknown factor(s) bind to the −1227 to −1168 probe flanking the −1204 enhancer site. The dependence of EMSA band 3 on the presence of both Sp1 and Stat5 is consistent with the formation of a complex containing constitutively bound Sp1 and inducibly bound Stat5. Analysis of point mutants and smaller probes indicates that Sp1 and Stat5 bind DNA independently of each other. The unknown factor(s) may also form an inducible complex with Stat5, which would account for the reduction in band 2 upon IL-2 stimulation. Alternatively, the unknown factor(s) may be inducibly removed from the DNA, which would also diminish band 2.

Contribution of the Stat5 and Sp1 Sites to transcriptional activity

Mutational analysis of the −1303 D2-Luc reporter gene was used to determine the importance of the Stat5 and Sp1 sites to transcriptional activity. IL-2-induced reporter gene activity was reduced to a fold induction of 1.2 after mutation of the Stat5 site (AA to CC at nucleotides −1192 and −1191; Fig. 5A). Inducible reporter gene activity was reduced by ~50% after mutation of the

**FIGURE 3.** In vitro characterization of nuclear proteins binding the −1227 to −1168 enhancer region of the cyclin D2 gene in IL-2-stimulated CTLL2 cells. A, EMSA analysis of a 60-bp DNA probe representing the −1227 to −1168 region of the human cyclin D2 gene. Arrows indicate four bands of interest. Bands 1 and 2 were present in unstimulated cells and diminished with IL-2 stimulation. Bands 3 and 4 appeared in response to IL-2. Competition EMSA reactions using unlabeled oligonucleotides corresponding to consensus Sp1 and Stat5 sites (cold Sp1 and cold FCR) demonstrate that bands 1 and 3 involve Sp1, and bands 3 and 4 involve Stat5. Ab supershifting EMSA reactions (anti-Sp1 and anti-Stat5) confirm these conclusions. The dependence of band 3 on the presence of Sp1 and Stat5 indicates the formation of a complex of constitutively bound Sp1 and inducibly bound Stat5. An unknown factor(s) accounts for band 2. B, EMSA reactions using mutated versions of the −1227 to −1168 DNA probe (underlined) confirm the binding sites for Stat5, Sp1, and the unknown factor(s). C, Sequence comparison between the human and rat cyclin D2 genes in the Sp1 and Stat5 regions. The Stat5 site is conserved exactly, while two possible Sp1 sites exist in the rat gene.

**FIGURE 4.** EMSA analysis of a DNA probe (−1227 to −1208) centered around the Sp1 binding site. CTLL2 cells were stimulated with IL-2 for 0, 2, and 5 h. Two constitutive bands are seen. The upper band involves Sp1, as demonstrated by adding an unlabeled probe corresponding to a consensus Sp1 site or by adding anti-Sp1 Ab. The lower band corresponds to the unknown factor seen with the longer probe in Fig. 3. Mutation of the Sp1 site (CTCC to AGAA) eliminates both bands.
was essential for transcriptional activity, the adjacent Sp1 site was required independent of the Stat5 binding site. While the Stat5 site reporter gene activity.

A 1303 D2-Luc with 22 mutant Sp1 site (CTCC to AGAA at nucleotides 22). The 60-bp enhancer region from 1227 to 1168 was placed upstream of the minimal SV40 promoter in the vector PGL-3 (Fig. 3B). Luciferase induction was approximately equal to that measured with the mutant Sp1 site as described in Fig. 3B.

Sp1 site (CTCC to AGAA at nucleotides 1217 to 1214). Fold induction measured with the mutated Sp1 site was approximately equal to that measured with the −1204 D2-Luc reporter gene that lacks this region (refer to Fig. 2). We conclude that Stat5 is essential for IL-2-mediated induction of D2-Luc, and the Sp1 binding site enhances transcriptional induction.

To test whether the Stat5/Sp1 enhancer region is sufficient for gene induction, we cloned the −1227 to 1168 region into the vector PGL-3 promoter immediately upstream of the minimal SV40 promoter (1227–1168 Luc). Luciferase induction was approximately equal to that observed with D2-Luc reporter gene that was overexpressing a FLAG epitope-tagged version of wild-type Stat5a. Expression of the endogenous cyclin D2 gene correlated with Stat5 activity when the Stat5 site was essential for IL-2-mediated induction of D2-Luc, and the Sp1 binding site enhances transcriptional induction.

The Stat5 pathway regulates expression of the endogenous cyclin D2 gene

Mutants of IL-2Rβ and Stat5 were introduced into the lymphoid cell line BA/FG to investigate the role of Stat5 in the regulation of the endogenous cyclin D2 gene. BA/FG cells are a derivative of the IL-3-dependent pro-B cell line BA/F3 (23). They express a chimeric G-CSF/gp130 receptor and so proliferate in response to G-CSF through a Stat5-independent signaling mechanism. Like BA/F3 cells, BA/FG cells constitutively express the γc subunit of the IL-2R and can be made responsive to IL-2 by introduction of IL-2Rβ.

To test whether Stat5 mediates cyclin D2 induction, we introduced an IL-2Rβ mutant capable of activating Stat5 but not the Shc, Ras/mitogen activated protein (MAP) kinase, or PI3K pathways. The mutant receptor contained a distal portion of the IL-2R β-chain with a single Stat5-activating tyrosine residue (Y310) attached to a truncated form of IL-2Rβ lacking the Shc binding site at Y338 and all other cytoplasmic tyrosines (Fig. 6A). IL-2Rβ Δ325 + Y310, Δ325 + Y310 induced cyclin D2 mRNA as effectively as the endogenous IL-3R (Fig. 6B). Stat5 activity was normal as assessed by EMSA (Fig. 6C). Next, we introduced a derivative of IL-2Rβ Δ325 + Y310 containing a leucine to arginine substitution at residue 511 (Δ325 + Y310 RSL) that impairs the Stat5 binding site (23). Δ325 + Y310 RSL only weakly induced cyclin D2 mRNA in response to IL-2 (Fig. 6B) consistent with markedly reduced Stat5 activity (Fig. 6C). Finally, we rescued Stat5 activity by overexpressing a FLAG epitope-tagged version of wild-type Stat5a with Δ325 + Y310 RSL. Stat5a overexpression overcomes the affinity barrier between Stat5 and Δ325 + Y310 RSL (23). Cyclin D2 mRNA induction was restored (Fig. 6B) with normal Stat5 activity (Fig. 6C). Cyclin D2 mRNA induction correlated with Stat5 activity when the Stat5 signal was isolated, disrupted, and then restored, indicating that Stat5 regulates the endogenous cyclin D2 gene.

The trans-activation domain (TAD) of Stat5 mediates cyclin D2 induction

Stat5 Δ713 is a naturally occurring isoform of Stat5a that lacks the TAD, but is capable of receptor-mediated tyrosine phosphorylation, nuclear translocation, and DNA binding (39). BA/FG clones stably coexpressing a FLAG epitope-tagged version of Stat5Δ713 and IL-2Rβ Δ325 + Y310 were generated to test the requirement for Stat5 in the induction of cyclin D2 mRNA. Stat5 Δ713 exerts no adverse selective pressure on BA/FG cells maintained on G-CSF, because proliferative signaling occurs through SHP-2 and Stat3. Activation of Stat5 Δ713 by IL-2Rβ Δ325 + Y310 failed to induce cyclin D2 mRNA (Fig. 6B). The inducible DNA binding activity of Stat5 Δ713 was high, as confirmed by EMSA with and without a supershifting Ab to the FLAG epitope tag (Fig. 6C). Thus, Stat5 mediates cyclin D2 induction through its TAD.

Discussion

We report on the transcriptional regulation of the cyclin D2 gene by the IL-2R in T cells. IL-2-responsive transcriptional activity is dependent on the formation of an enhancer complex at −1220 to −1191 bp containing Stat5, Sp1, and an unknown factor(s). Stat5 binds inducibly in response to IL-2, and the other factors bind constitutively. In a cyclin D2/luciferase reporter gene, the Stat5 site was essential for IL-2-induced transcriptional activity, and maximum induction required the adjacent Sp1 binding site. In the lymphoid cell line BA/FG, we isolated, disrupted, and then rescued the Stat5 signal by introducing mutated versions of the IL-2Rβ subunit and wild-type murine Stat5a. Expression of the endogenous cyclin D2 gene correlated with...
Stat5 activity. Furthermore, a version of Stat5a lacking the TAD inhibited cyclin D2 induction, demonstrating that the trans-activation function of Stat5 is required.

The functional interaction between Stat5 and Sp1 is consistent with several examples of mitogen-activated transcriptional regulation. Stat family members often act with other factors to promote trans-activation potential (40, 41). In the IL-2Rα gene, Stat5 inducibly binds an enhancer element containing constitutively bound Elf-1 (26, 27), and cooperativity between factors is required for maximal transcriptional activity. Similarly, Stat3 interacts directly with c-Jun to induce transcription of the α2-macroglobulin gene in response to IL-6 (42, 43). Mutants of Stat3 that fail to interact with c-Jun result in decreased gene induction. Finally, Stat5 has been shown to interact with the cofactor Nmi to enhance association with CBP (CBP (CREB (cAMP responsive element binding) binding protein/p300 and promote IL-2-mediated transcription (44). Based on the above results, it has been proposed that Stats interact with other factors to synergistically increase transcription through the formation of enhanceosomes, combinations of proteins that promote gene expression through the recruitment of cofactors and through electrostatic and interfacial surface interactions with the basal transcriptional machinery (45).

A proposed model for Sp1 function predicts strong Sp1 binding to consensus sites in promoters of constitutively expressed housekeeping genes and weaker binding and cooperation with signal-responsive transcriptional activators in mitogen-induced genes (46). Consistent with this model, Sp1 binds to a nonconsensus site on the cyclin D2 gene and depends on adjacent Stat5 binding for transcriptional activity. Cholesterol-induced transcription of the gene encoding the low density lipoprotein receptor has been used as a model system to investigate the role of Sp1 in signal-responsive genes (46, 47). Sp1 acts synergistically with sterol-responsive element binding protein that recruits the cofactor CBP to the enhancer complex. It is proposed that Sp1 acts as a bridge between sterol-responsive element binding protein/CBP and the TATA binding protein-associated factors to enhance transcription. Similarly, on the cyclin D2 promoter Sp1 may link Stat5 and associated cofactors such as Nmi/CBP to the basal transcriptional machinery.

Stat factors interact with Sp1 in at least two other cases. In rat Nb2 T cells, IL-2 stimulation induces Sp1 protein accumulation and binding to an Sp1 consensus oligonucleotide (48). Stat3 and Stat5 participation in the Sp1-DNA complex was detected by supershifting Ab EMSA reactions, but the contributions of these factors to transcriptional activity were not evaluated. In a second study induction of the gene encoding intercellular adhesion molecule-1 in response to IFN-γ was shown to require cooperative interactions between constitutively bound Sp1 and inducibly bound Stat1 on an enhancer element (49). In this case Stat1 and Sp1 appeared to interact directly, as they could be coimmunoprecipitated from IFN-γ-stimulated cells.

Our results establish a direct and immediate link between Stat5 activation and cyclin D2 gene transcription, but expression of cyclin D2 mRNA in response to IL-2 is a delayed-early event (31) (J. J. Moon, unpublished observations). Therefore, it is likely that other essential activators or cofactors are synthesized upon IL-2 stimulation that regulate cyclin D2 gene induction. Consistent with the delayed-early kinetics, c-Myc has been implicated in the regulation of cyclin D2 transcription in nonlymphoid cell lines. It has been proposed that c-Myc regulates cyclin D2 transcription by competitively binding to Max and displacing a transcriptionally repressive Mad-Max complex on an E box at nucleotide −1594 (34). This model has not been confirmed using physiological levels of c-Myc, however. In fact, we show that the E box region of the cyclin D2 promoter Sp1 appeared to interact directly, as they could be coimmunoprecipitated from IFN-γ-stimulated cells. Nevertheless, it remains possible that the E box and the Stat5/Sp1 enhancer complex work in concert to control endogenous cyclin D2 mRNA levels with delayed-early kinetics.

It is not known whether IL-2 has a direct effect on Sp1 activity in T cells. IL-2 does not alter DNA binding (Fig. 4) or Sp1 protein...
levels (data not shown) in CTLL2 cells. As discussed above, this is consistent with several examples of mitogen-induced genes where Sp1 contributes to transcription. On the other hand, Sp1 phosphorylation is modulated during the cell cycle (50) and in response to cytokines such as Neu differentiation factor (51). Our results do not exclude the possibility that IL-2 similarly regulates the phosphorylation state of Sp1 within the enhancer complex, thereby enhancing overall trans-activation potential.

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References