STAT3 Is a Serine Kinase Target in T Lymphocytes

INTERLEUKIN 2 AND T CELL ANTIGEN RECEPTOR SIGNALS CONVERGE UPON SERINE 727*

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Interleukin 2 (IL-2) induces tyrosine phosphorylation of STATs 3 and 5 (signal transducer and activator of transcription). We now show that IL-2 regulation of STAT3 proteins in T cells is a complex response involving activation of two forms of STAT3: 90-kDa STAT3a and an 83-kDa carboxyl-terminal truncated STAT3b. The phosphorylation of STAT proteins on serine residues is also required for competent STAT transcription. A critical serine phosphorylation site in STAT3a is at position 727. In this study we have produced an antiserum specific for STAT3a proteins phosphorylated on serine 727 and used this to monitor the phosphorylation of this residue during T lymphocyte activation. Our results show that phosphorylation of STAT3a on serine 727 is not constitutive in quiescent T cells but can be induced by the cytokine IL-2. Interestingly, triggering of the T cell antigen receptor complex or activation of protein kinase C with phorbol esters also induces phosphorylation of serine 727 but without simultaneously inducing STAT3 tyrosine phosphorylation or DNA binding. Hence, the present results show that STAT3 serine phosphorylation can be regulated independently of the tyrosine phosphorylation of this molecule. IL-2 and T cell antigen receptor complex induction of STAT3a serine 727 phosphorylation is dependent on the activity of the MEK/ERK pathway. Previous studies have identified H-7-sensitive kinase pathways that regulate STAT3 DNA binding. We show that H-7-sensitive pathways regulate STAT3 DNA binding in T cells. Nevertheless, we show that H-7-sensitive kinases do not regulate STAT3 tyrosine phosphorylation or phosphorylation of serine 727. These results thus show that STAT3 proteins are targets for multiple kinase pathways in T cells and can integrate signals from both cytokine receptors and antigen receptors.

The cytokine interleukin-2 (IL-2)† controls G1-S phase progression, T cell clonal expansion, and functional differentiation (1). High affinity IL-2 receptors trigger activation of tyrosine kinases notably the Janus kinases (JAKs) 1 and 2 (2, 3) and p56lck (4). IL-2 also regulates the activity of p21ras (5), the kinases Raf-1 (6), and ERK (7, 8), phosphatidylinositol 3-kinase (9–11), and p70 S6 kinase (12–14).

IL-2-activated transcription factors include members of the STAT (signal transducer and activator of transcription) family: STAT3 and STAT5 (15–19). STAT activation involves tyrosine phosphorylation which allows for Src homology (SH) 2 domain-mediated homodimerization or heterodimerization. Activated and tyrosine-phosphorylated STATs then translocate to the nucleus to form sequence-specific DNA binding complexes and enable cytokine-mediated gene transcription (reviewed in Ref. 20). Serine kinases are critical for STAT activation and can regulate STAT DNA binding or transcriptional activity (21–26). In reconstitution experiments, it has been recently shown that for STAT1 to elicit IFN-γ-mediated antigrowth activity, the serine phosphorylation site (Ser-727) is required (27). We have also recently shown that IL-2 regulation of STAT5 is mediated by both tyrosine and serine/threonine kinase pathways (28). STAT5 serine phosphorylation in T cells is regulated by an as yet uncharacterized kinase whose activity is required for STAT5 transcriptional activity.

IL-2 induces the tyrosine phosphorylation and DNA binding of STAT3. The phosphorylation of serine 727 in the STAT3 carboxyl terminus is important for STAT3 transcriptional function, but neither has there been any characterization of STAT3 serine phosphorylation nor any study of the identity of STAT3 serine kinases in T cells. Recent debates have focused particularly on the role of MEK/ERK kinase pathways in STAT activation (29). However, the identity of the STAT3 serine 727 kinase has not been fully resolved in any cellular system. This site forms an in vitro substrate for the MAP kinase ERKs which raised the possibility that STAT3 integrates signals from the MAP kinases. This hypothesis has not been fully explored in vivo, in particular, for certain cell systems phosphorylation of serine 727 is constitutive and in others, subject to regulation by extracellular stimuli (25). Herein we present evidence that IL-2 induces tyrosine phosphorylation and DNA binding of two different forms of STAT3: 90-kDa STAT3a and 83-kDa STAT3b. IL-2 also induces serine phosphorylation of serine 727 in STAT3a via a MEK/ERK pathway. The present data also show that STAT3 tyrosine and serine phosphorylation can be independently regulated: activation of MAP kinases by phorbol esters or T cell antigen receptor cross-linking induces phosphorylation of STAT3a on serine 727 without concurrently inducing STAT3 DNA binding or tyrosine phosphorylation. The phosphorylation of serine 727 is not obligatory for DNA binding or tyrosine phosphorylation of STAT3. Nevertheless, we find a H-7-sensitive kinase pathway that does not regulate STAT3 tyrosine phosphorylation or phosphorylation of serine 727 in STAT3a is able to modulate STAT3 DNA binding. These data collectively indicate the role of multiple T cell signaling pathways in mediating differential control over STAT activation.

MATERIALS AND METHODS

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† The abbreviations used are: IL-2, interleukin 2; IFN, interferon; MAP, mitogen-activated protein; MEK, MAP kinase kinase; PDBu, phorbol 12,13-dibutyrate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; SIEM, sis-inducible element mutant; TCR, T cell antigen receptor complex.

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receptor expressing human peripheral blood-derived T lymphoblasts were generated and maintained as described previously (31). Cells were quiesced by washing three times in RPMI 1640, and replacing in RPMI 1640 with 10% serum in the absence of IL-2 for 48–72 h. The inhibitors H-7, 1–5-(o-quinolinylsulfonyl)-2-naphthylpiperazinone (Sigma), and PD90860 (a gift of L. O'Neill, Trinity College, Dublin) were preincubated with cells in RPMI 1640 for 30 min prior to cytokine stimulation. Recombinant IL-2 (1 µl), interferon α (1000 units/ml), or phorbol 12,13-dibutyrat (50 ng/ml) was incubated at indicated times. Interferon α (IFNa) was a gift from I. Kerr. Phorbol 12,13-dibutyrat (PDBu) was from Calbiochem.

Affinity Purification of DNA and Proteins—Whole cell extracts were prepared by lysis of approximately 10–20 x 10^6 cells/ml in a lysis buffer comprised of 50 mM Tris-Cl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.1 mM EDTA, 10 mM sodium fluoride, 10% glycerol, 1 mM Na3VO3, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM protease inhibitors, 1 mM leupeptin, 1 µg/ml chymostatin. The oligonucleotide sequence used was derived from the high-affinity SHC of the c-fos gene (SIEM67) GTCGACATTTCCGGAATTC. DNA-binding proteins were isolated from extracts of approximately 15 x 10^6 cells in the above buffer by incubation at 4 °C for 1 h with 1 µg of double-stranded, 5'-biotinylated oligonucleotide coupled to 30 µl of a 50% suspension of streptavidinagarose (Sigma). For affinity purification of proteins binding to the phosphopeptide IFN-α Y440F, the phosphoantibody peptide TSGFYD-KPPD was coupled to Aff-Gel 10 (Bio-Rad) at 5 mg/ml, and 30 µl were used to purify binding proteins from 1 ml of cell lysate. Binding was at 4 °C for 1 h, and complexes were washed twice in lysis buffer prior to elution by boiling in reducing sample buffer. Proteins were separated by SDS-PAGE on 7.5% acrylamide, 0.2% bisacrylamide gels and 15% acrylamide, 0.075% methylene diamine gels, and detected by Western blotting using antibodies raised to the consensus tyrosine phosphorylation motif YPYDVPDYA (Santa Cruz, CA, and a gift from I. Kerr, respectively). For analysis of STAT3 tyrosine phosphorylation by Western blot analysis, antibodies raised to the consensus tyrosine phosphorylation motif were used (residues 701–709); either using a tyrosine phosphopeptide (α-Py STAT3) or the equivalent non-phosphorylated peptide (α-STAT3 control). Both antibodies were from New England Biolabs.

Generation of Phosphoantibodies—The peptide sequence corresponding to human STAT3 at site 717–736, TCSNTVIDLPMSPRALDSLMQ, was synthesized with serine 727 as a N-terminal (residues 1–175) raised anti-STAT3 N-T fusion protein, carboxyl-terminal (residues 750–769) raised anti-STAT3 C-T peptide, and carboxyl-terminal (residues 688–727) raised anti-STAT3 C-T fusion protein (Transduction Laboratories, Santa Cruz, CA, and a gift from I. Kerr, respectively). For analysis of STAT3 tyrosine phosphorylation by Western blot analysis, antibodies raised to the consensus tyrosine phosphorylation motif were used (residues 701–709); either using a tyrosine phosphopeptide (α-Py STAT3) or the equivalent non-phosphorylated peptide (α-STAT3 control). Both antibodies were from New England Biolabs.

RESULTS

IL-2 Induces DNA Binding of STAT3α and STAT3β in T Cells—Biotinylated oligonucleotides comprising the high-affinity mutant sial-inducible element (SIEM) was used to affinity purify STAT proteins from cell extracts of quiescent or IL-2-stimulated human peripheral blood-derived T lymphoblasts.

The data in Fig. 1A show that IL-2 induces DNA binding of STAT3α and STAT3β in peripheral blood-derived T cells. IL-2 induced DNA binding of STAT3α and STAT3β in the absence of a COOH-terminal fusion protein comprising residues 688–727 of STAT3alpha and STAT3beta, being a carboxyl-terminal truncated splice variant of a single STAT3 gene (32, 33). To further characterize the 90- and 83-kDa IL-2-induced STAT3 proteins, termed hereafter as STAT3α and STAT3β, respectively, antisera raised against different domains of STAT3 were used in Western blot analyses of the STAT3 proteins in total T cell lysates and in DNA binding complexes (Fig. 1B). Additionally, a tyrosine phosphopeptide comprising the tyrosine-phosphorylation site (Y440) of the γ-interferon receptor α-chain was used to generate an affinity matrix for the purification of STAT3 from cell extracts. The data in Fig. 1B show that antisera raised against an NH2-terminal STAT3 fusion protein and antisera generated against a fusion protein comprising residues 688–727 of STAT3 recognize two STAT3 proteins in T cell lysates and in the Tyr-440 peptide complexes. They also recognize both IL-2-induced STAT3 proteins in SIEM DNA binding complexes. In contrast, antisera raised against a COOH-terminal STAT3 peptide comprising residues 750–769 recognize only the 90-
kDa STAT3 and not the 83-kDa protein (Fig. 1B). The 83-kDa STAT3β protein in T cells thus corresponds to the recently described carboxyl-terminal truncated spliced variant of STAT3 (32, 33). Activated and tyrosine phosphorylated STAT proteins were readily detected in IL-2 activated T cells using anti-phosphotyrosine antibodies raised to the tyrosine phosphorylation site of EGFR. Biotinylated antibodies raised to residues (750–769) of STAT3. Proteins were prepared from IL-2 activated T cells using anti-phosphotyrosine antibodies raised to the tyrosine phosphorylation site of STAT3 (Tyr-705) (Fig. 2).

IL-2 Induces Phosphorylation of Serine 727 in STAT3α—There was a discernable reduction in the electrophoretic mobility of STAT3α but not STAT3β isolated from IL-2-activated Kit225 cells and in IL-2-activated peripheral blood T lymphoblasts (Fig. 3A and data not shown). Both forms of STAT3α (termed s, slow, and f, fast; for the different electrophoretic mobility shift properties) can equally bind DNA upon IL-2 activation and are both tyrosine-phosphorylated. The ability of STAT3α to undergo an electrophoretic mobility shift in response to IL-2 could reflect IL-2-regulated serine phosphorylation of STAT3. There is a serine phosphorylation site at residue 727 in STAT3α that is required for transcriptional activation (25). Generation of phosphorylation state-specific antibodies has been described previously (34) and had been shown to be successful in the analysis of site-specific phosphorylations of a variety of proteins (35–37), including transcription factors (38). Thus to circumvent radiolabeling techniques and to explore whether IL-2 is inducing the phosphorylation of STAT3α on serine 727, we generated rabbit antisera selectively reactive to a phosphopeptide corresponding to the serine 727 site in STAT3α. The selectivity of this antisera for the phosphorylated serine 727 STAT3 peptide compared with the nonphosphorylated peptide is shown by ELISA (Fig. 3B).

The phosphoserine 727 STAT3 antisera could weakly immunoprecipitate STAT3α proteins from quiescent peripheral blood-derived T cells but levels of STAT3α immunoprecipitable with the phosphoserine 727 STAT3 antisera were increased markedly in cells lysates isolated from IL-2 stimulated T cells (Fig. 4A). The carboxyl-terminal truncated STAT3β protein lacking the serine 727 site could not be immunoprecipitated with the phosphoserine 727 STAT3 antisera from either quiescent or IL-2-activated cells. On Western blot analysis, IL-2 induced STAT3α DNA bound proteins were also reactive with the phosphoserine 727 STAT3 antisera (Fig. 4B). The reactivity of the phosphoserine 727 STAT3 antisera with STAT3α proteins isolated from IL-2-activated T cells could be blocked by competition with the phosphorylated serine 727 STAT3 peptide but not the nonphosphorylated peptide (data not shown).

STAT3 proteins, affinity purified using the IFN-γR Tyr-440 tyrosine phosphopeptide matrix were similarly analyzed for immunoreactivity with the phosphoserine 727 STAT3 antisera. There was a low level reactivity of phosphoserine 727 STAT3 antisera with STAT3 proteins isolated from quiescent cells but this was markedly increased upon IL-2 stimulation (Fig. 4C). No reactivity of phosphoserine 727 STAT3 antisera with STAT3β could be detected. These data collectively show that IL-2 induces serine phosphorylation of STAT3α on serine 727 in peripheral blood-derived T lymphoblasts.

Serine 727 in STAT3α Is Regulated by the MEK/ERK Pathway in T Cells—Xenopus MAP kinase can phosphorylate STAT1 in vitro at the carboxyl terminus consensus MAPK phosphorylation site (25). The equivalent in vitro ERK site in STAT3 is serine 727. To explore directly the role of the MEK/ERK pathway in STAT3 regulation by IL-2 we used an inhibitor of the ERK stimulatory kinase, MAP kinase kinase (MEK), PD098059. The specificity of PD098059 as a MEK inhibitor has been previously described (39, 40). To monitor the effectiveness of STAT3 Regulation in T Cells
of the MEK inhibitor in T cells, we examined the phosphorylation of ERK induced by IL-2 in T cells pretreated with PD098059. As an additional means to monitor ERK activation in IL-2-treated cells, hyperphosphorylation of ERK substrates were also analyzed. Cellular substrates for ERK in T cells, as in many cells, include the p21<sup>ras</sup> effector molecule Raf-1, and the guanine nucleotide exchange protein Sos (41, 42). These proteins are both upstream regulators of the MAP kinases, but are phosphorylated by the MAP kinases through feedback signaling mechanisms. Hyperphosphorylated ERK, Raf-1, and Sos have reduced electrophoretic mobility when analyzed by SDS-PAGE. The data in Fig. 5A show that IL-2 regulated hyperphosphorylation of ERK, Sos, and Raf-1 are inhibited by the MEK inhibitor PD098059, demonstrating that this inhibitor is effective at blocking the MEK/ERK pathway in T cells. Analysis of STAT3<sup>a</sup> and STAT3<sup>b</sup> in DNA bound complexes (Fig. 5B) shows that IL-2 induced changes in STAT3<sup>a</sup> electrophoretic mobility were inhibited by the MEK inhibitor PD098059, however, this inhibitor did not prevent IL-2 induced DNA binding of STAT3.<br><br>To examine directly the effects of PD098059 on IL-2-induced phosphorylation of serine 727 of STAT3<sup>a</sup> we used the phospho-serine 727 STAT3 antibodies. The data in Fig. 5C shows a marked increase in reactivity of the phospho-serine 727 STAT3<sup>a</sup> antisera with STAT3<sup>a</sup>, isolated from IL-2-activated T cells. This increased reactivity was inhibited in STAT3<sup>a</sup> proteins isolated from IL-2-treated T cells that had been preincubated with the MEK inhibitor PD098059. Hence, inhibition of the
MEK/ERK pathway prevents the IL-2-induced phosphorylation of serine 727 in STAT3α.

The Induction of STAT3α Phosphorylation on Serine 727 Does Not Require Tyrosine Phosphorylation of STAT3α—The phorbol ester PDBu stimulates protein kinase C and activates the ERK/MAP kinase pathway independently of IL-2 receptor activation. If STAT3α is regulated by MEK/ERK then one possibility is that PDBu, which activates ERK via a protein kinase C/Raf-1 pathway independent of IL-2, could also induce the phosphorylation of serine 727 in STAT3α. This is assuming that serine phosphorylation of STAT proteins can proceed independently of their tyrosine phosphorylation. The data in Fig. 6A show that PDBu did not induce changes in the electrophoretic mobility of STAT5 but similar to IL-2, PDBu induced changes in the electrophoretic mobility of STAT3α but not STAT3β. Moreover, immunoprecipitation and Western blot analyses of STAT3 proteins showed that PDBu induced STAT3α reactivity with the phosphoserine 727 STAT3 antisera (Fig. 6B, panels a and b). PDBu did not induce DNA binding of STAT3 probably because of the failure of phorbol esters to induce STAT3 tyrosine phosphorylation (Fig. 6C and data not shown). The PDBu data thus show that phosphorylation of STAT3α on serine 727 can occur independently of DNA binding or tyrosine phosphorylation.

Phorbol esters are pharmacological activators of the ERK pathway and to examine whether a more physiological signal for ERK activation can induce phosphorylation of STAT3 proteins in the absence of STAT3 tyrosine phosphorylation, we examined the effects of triggering the T cell antigen receptor complex on STAT3. Previous studies have shown that triggering of the T cell antigen receptor (TCR) complex does not induce tyrosine phosphorylation of STAT3 (31). However, the data in Fig. 6D show that STAT3α proteins isolated from TCR-activated T cells are reactive with the phosphoserine 727 STAT3 antisera compared with the weak reactivity seen in STAT3α proteins isolated from quiescent T cells. These results show that STAT3α can be phosphorylated on serine 727 in response to triggering the TCR complex. This effect is prevented by pretreating T cells with the MEK inhibitor PD098059; consistent with a model in which TCR regulation of the phosphorylation of serine 727 being mediated by the MEK/ERK pathway (Fig. 6D).

IL-2 induced STAT3 but Not STAT5 DNA Binding Is Regulated by a H-7 Serine Kinase Pathway—The data above show that IL-2 regulates a MEK-sensitive serine kinase that is acting on serine 727 in the COOH terminus of STAT3α. The activity of this MEK-dependent pathway is not necessary or sufficient for IL-2-induced STAT3α DNA binding (Fig. 6D).

**Fig. 6. Effects of PDBu and the T cell antigen receptor agonist UCHT-1 on STAT3.** A, Kit225 cells were untreated (lane 1), stimulated with IL-2 (lane 2), or PDBu (lane 3) for 10 min. Whole cell extracts were prepared and acetone-precipitated proteins were subjected to SDS-PAGE and Western blot using STAT5 and STAT3 antibodies. B, panel a, Kit225 cells were prepared as above followed by Western analysis using phosphoserine 727 STAT3 (lanes 1–3) or STAT3 antibodies (lanes 4–6). B, panel b, Kit225 cells were treated as above and whole cell extracts were prepared, followed by immunoprecipitation using phosphoserine 727 STAT3 antibodies. Proteins were separated by SDS-PAGE and detected by Western analysis using STAT3 antibodies. C, Kit225 were treated as above and DNA-binding proteins capable of binding to the SIEM-oligonucleotide were affinity purified and subjected to SDS-PAGE and Western blot using STAT5 and STAT3 antibodies (as indicated). D, quiescent T lymphoblasts were untreated or stimulated with IL-2 (1 nM) (lane 2) or UCHT-1 (1 μg/ml) (lanes 3 and 4) for 10 min following a 30-min preincubation in the absence (lanes 1–3) or presence (lane 4) of MEK inhibitor PD098059 (50 μM) (lane 4). Whole cell extracts were prepared and STAT proteins were affinity purified using agarose-conjugated tyrosine phosphopeptides. Proteins were separated by SDS-PAGE followed by Western blot using phosphoserine 727 STAT3 antibodies. Positions of molecular weight markers on the left are indicated in kDa.
in Fig. 7A show that pretreatment of T cells with the MEK inhibitor does not abrogate IL-2 induced STAT3α/β tyrosine phosphorylation (Fig. 7A, lane 2 versus lane 3). Thus, although the MEK pathway can control STAT3α phosphorylation on serine 727, its activity is not required for IL-2 induced DNA binding of STAT3α or STAT3β or tyrosine phosphorylation. In certain cells, cytokine induction of STAT3 DNA binding can be regulated by a serine kinase pathway sensitive to the serine/threonine kinase inhibitor H-7 (24). H-7 does not block the activation of the MEK/ERK pathway in T cells (28) but the role of H-7-sensitive kinases in IL-2/STAT3 regulation in T cells had not been examined. We considered the possibility that STAT3 could be regulated by two different serine kinases. We therefore examined the effect of H-7 pretreatment of T cells on IL-2 regulation of STAT3. The data in Fig. 7B shows that IL-2-induced STAT3α/β DNA binding was inhibited in T cells pretreated with H-7. H-7 did not inhibit IL-2-induced tyrosine phosphorylation of STAT3α/β (Fig. 7A, lane 2 versus 7). The effect of H-7 on STAT3 DNA binding showed selectivity since this inhibitor did not prevent IL-2 induced tyrosine phosphorylation of STAT3 or STAT5 (Fig. 7A). Nor was DNA binding of STAT5 proteins affected by H-7 (Fig. 7B) although as described previously, H-7 inhibits the generation of the serine-phosphorylated STAT5 p2 form as shown by the effect of H-7 on IL-2-induced electrophoretic mobility shift of STAT5 proteins. (28) (Fig. 7B).

The inhibitor H-7 also did not prevent the formation of hyperphosphorylated STAT3α in IL-2-activated cells in contrast to the effects of the MEK inhibitor PD098059 (Fig. 7A lane 7 versus lane 3, respectively). Concurrently, STAT3α proteins from IL-2 activated, H-7-pretreated T cells were also reactive with phosphoserine 727 STAT3 antisera (Fig. 7C), demonstrating that H-7 in T cells does not inhibit IL-2-induced phosphorylation of serine 727 in STAT3α. Thus, H-7 inhibits IL-2 induction of STAT3 DNA bound complexes without preventing tyrosine phosphorylation or phosphorylation of serine 727 in STAT3α.

**DISCUSSION**

The present study reveals that IL-2 activates DNA binding of two forms of STAT3 in T cells: 90-kDa STAT3α and an 83-kDa carboxyl-terminal truncated STAT3β. We show moreover that STAT3 proteins are both tyrosine- and serine-phosphorylated in IL-2-activated T cells. Notably, we developed a specific antibody reactive with phosphorylated serine 727 STAT3 antisera (Fig. 7C), demonstrating that serine 727 phosphorylation is key for STAT3 transcriptional activity (25). The present results also reveal that phosphorylation of serine 727 in STAT3α is not only regulated by IL-2 but can be regulated by the TCR or phorbol esters (Fig. 8). IL-2 activation of STAT3 concurrently induces phosphorylation on serine 727 and STAT3 tyrosine phosphorylation. However, STAT3 phosphorylation on serine 727 in response to TCR ligation or phorbol esters occurs without concomitant induction of tyrosine phosphorylation or DNA binding of STAT3. Accordingly, the serine and tyrosine phosphorylation pathways that target the STATs can be controlled independently by extracellular stimuli. A continual challenge in T cell biology is to understand the mechanisms that regulate the immune specificity of T cell responses. Phosphorylation of serine 727 in STAT3α is required for maximal STAT3 transcriptional activity (25). The ability of T cells to respond to TCR triggering by inducing phosphorylation of STAT3 on a residue that is key for STAT3 transcriptional activity reveals that in T cells there is “crosstalk” regulation of the STATs by members of the antigen receptor family. This allows the transcriptional
activity of STAT3α in T cells to be linked to the immune activation status of the cells; a link which may facilitate the immune specificity of T cell responses.

The inhibitor H-7 has been one tool with which to explore STAT serine kinases in a variety of cell systems, particularly, the regulation of STAT3 by H-7-sensitive kinases has been described. Because of these previous experiments with H-7 we were prompted to see whether H-7 could regulate the phosphorylation of serine 727 in STAT3α. The present study shows that IL-2-induced STAT3 DNA binding can be regulated by a H-7-sensitive pathway. Nevertheless, the H-7-sensitive pathway does not regulate STAT3α serine 727 phosphorylation. The H-7 target is unknown but is intriguing because of the effects of H-7 on STAT3 DNA binding. The simplest interpretation of the H-7 data is that STAT3α integrates signals from two serine kinase pathways in T cells: a MEK-regulated pathway and a H-7-sensitive pathway. These pathways are independent as H-7 does not prevent IL-2 activation of the MEK/ERK2 pathway (28) or the phosphorylation of serine 727 in STAT3α. H-7, however, does inhibit IL-2-activated STAT3 DNA binding in T cells. Conversely, the MEK/ERK2 pathway regulates phosphorylation of serine 727 in STAT3α but is not necessary for IL-2 induced STAT3 DNA binding. The regulation of both STAT3α and STAT3β DNA binding indicates that the H-7-sensitive pathway must regulate a site outside the variant carboxyl termini of the two forms of STAT3. It is possible that through a H-7-sensitive kinase, phosphorylation at, or close to, the DNA-binding domain increases DNA binding affinity of STAT3 proteins. Nevertheless, it is also possible that the H-7 effect on STAT3 proteins is indirect and mediated through association with other transcription factors or co-factors; H-7-inhibited phosphorylation of these associated factors could contribute to a loss in STAT3 DNA binding affinity.

We have shown previously that IL-2 activation of STAT5 involves the convergent action of both tyrosine and serine/threonine kinases (28). The STAT5 serine kinase is H-7 sensitive and not regulated by MEK and cannot be stimulated by activators of the MAP kinase cascade (28). Herein we show that the STAT3 727 serine kinase is sensitive to a MEK inhibitor and can be stimulated by receptors that activate the MEK cascade independently of STAT5 tyrosine phosphorylation. The involvement of the MEK pathway clearly distinguishes IL-2 regulation of STAT3α and STAT5 and shows how a single cytokine working in the same cell can use distinct serine kinase pathways to regulate different STATs. There has been considerable debate as to the identity of STAT serine kinases. The present results show that regarding the identity of STAT serine kinases may reflect that cytokines can target STATs involving multiple and functionally different serine kinase pathways.

The MEK/ERK pathway is known to have an essential role in the activation responses of mature T cells and in thymocyte differentiation: the transition of thymocytes from double negative to double positive cells requires MEK/ERK2 activity as essential and not regulated by MEK and cannot be stimulated by cytokines working in the same cell can use distinct serine kinase pathways to regulate different STATs. There has been considerable debate as to the identity of STAT serine kinases. The present results show that regarding the identity of STAT serine kinases may reflect that cytokines can target STATs involving multiple and functionally different serine kinase pathways.

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