Uncoupling of Proliferation and Stat5 Activation in Thymic Stromal Lymphopoietin-Mediated Signal Transduction

Deborah E. Isaksen, Heinz Baumann, Baohua Zhou, Sebastien Nivollet, Andrew G. Farr, Steven D. Levin and Steven F. Ziegler

*J Immunol* 2002; 168:3288-3294; doi: 10.4049/jimmunol.168.7.3288
http://www.jimmunol.org/content/168/7/3288

References
This article cites 36 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/168/7/3288.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Uncoupling of Proliferation and Stat5 Activation in Thymic Stromal Lymphopoietin-Mediated Signal Transduction

Deborah E. Isaksen, Heinz Baumann, Baohua Zhou, Sebastien Nivollet, Andrew G. Farr, and Steven F. Ziegler

Thymic stromal lymphopoietin (TSLP) is a cytokine that facilitates B lymphocyte differentiation and costimulates T cells. Previous studies have demonstrated that a functional TSLP receptor complex is a heterodimer consisting of the TSLP receptor and the IL-7R α-chain. TSLP-mediated signaling is unique among members of the cytokine receptor family in that activation of the transcription factor Stat5 occurs without detectable Janus kinase activation. Using a variety of biological systems we demonstrate here that TSLP-mediated Stat5 activation can be uncoupled from proliferation. We also show that the single tyrosine residue in the cytoplasmic domain of the TSLP receptor is critical for TSLP-mediated proliferation, but is dispensable for Stat5 activation. Our data demonstrate that TSLP-mediated Stat5 activation is insufficient for cell proliferation and identifies residues within the TSLP receptor complex required to mediate these downstream events. 


Materials and Methods

Cell culture

HepG2 cells (9) and Phoenix amphotropic retroviral producer cells (10) were maintained in DMEM supplemented with 10% FCS and antibiotics. BafM7R, NAG8/7, and IxN/2B (11) cells were maintained in RPMI 1640 supplemented with 10% FCS, antibiotics, 1 mM sodium pyruvate, 20 μg/ml 2-ME, and, unless otherwise specified, 1 ng/ml murine IL-7 or 20 ng/ml murine TSLP. IL-7 and TSLP were obtained as previously described (4).

RT-PCR

Total RNA from IxN/2B cells was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). First-strand cDNA was made using random primers and a CDNA synthesis kit (Stratagene, La Jolla, CA). Overlapping fragments of TSLPR and IL-7Rα were PCR-amplified and sequenced using gene-specific primers.
Flow cytometry

Abs used for flow cytometry and purchased from CalTag Laboratories (Bur-lingame, CA) included anti-huCD2-PE, anti-huCD5-FITC, anti-mouse IgG1-PE, anti-mouse IgG2b-TriColor, and streptavidin-PE. Mouse IgG1 anti-huGM-CSFRα and mouse IgG2b anti-huGM-CSFRβ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A biotinylated anti-TSLPR Ab (2B29) was obtained from A. G. Farr (manuscript in preparation). Flow cytometry was performed on 5 × 10^6 cells using a FACScan flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA).

Proliferation assay

Cells were plated in triplicate to 96-well flat-bottom plates (1.5 × 10^4 BaF/7 cells/well or 2 × 10^5 NAG8/7 cells/well) and cultured for 62 h with the indicated quantity of cytokine and with or without PPI (obtained from 1 M NaNO_3, Pfi, Groten, CT). PPI was used over a range of concentrations as indicated. Proliferation was measured by [3H]thymidine incorporation following addition of [3H]thymidine to the culture medium for the final 15 h.

At the time BaF7 cells were seeded into the 96-well plate, they were also seeded into a flask and grown in the presence of TSLP. These parallel cultures were analyzed for surface expression of GMαR and β by flow cytometry on the same day. Incorporation was measured. BaF/7 data are expressed as the mean cpm from triplicate wells minus the mean cpm obtained from wells with medium alone. GM-CSF values were normalized to account for the percentage of cells that did not express both chimeric receptor chains and thus would be able to respond to 20 ng/ml TSLP but not 100 ng/ml GM-CSF.

Immunoprecipitations and Western blots

Cells were washed four times with PBS and cytokine-starved for 5–7 h at 37°C. BaF7/7 cells were also serum-starved. When appropriate, PPI was added 30 min before stimulation at 20 μM. Before proceeding further with BaF7/7 cells, flow cytometry was used to confirm that >90% of the cells expressed both huGMαR and β chimeric receptors. Cells at 1 × 10^6 were stimulated at 37°C for 20 min with or without GM-CSF (100 ng/ml), TSLP (100 ng/ml), or IL-7 (25 ng/ml). To stop the stimulation, cold PBS plus 1 mM NaNO_3, Pfi, were added. Cell lysates were generated using TNT buffer (25 mM Tris-HCl (pH 8), 150 mM NaCl, and 1% Triton X-100) plus protease inhibitors as previously described (36).

Clarified lysates at 1 × 10^6 cells/ml were either loaded onto a 6 or 10% SDS-PAGE gel following the addition of SDS loading buffer or were subjected to immunoprecipitation with an anti-Stat5 antisera as previously described (36). Processing of the Western blots with an anti-phospho-tyrosine Ab (4G10) or anti-Stat5 Ab was also as previously described (36). Blots were developed using chemiluminescent detection and were stripped for reprobing with the Western Blot Recycling kit (Chemicon, Temecula, CA). Chemiluminescent detection using the phospho-Stat5 Ab (Sigma-Aldrich, St. Louis, MO) on BaF7/7 whole cell lysates required the SuperSignal West Femto Maximum Sensitivity Substrate (Fierce, Rockford, IL).

Construction of chimeric receptors

The huGM-CSFRβ extracellular domain (with the GM-CSFRα signal sequence for improved expression) and the huGM-CSFRα extracellular domain (12) were subcloned as NotI-BamHI fragments into pcDNA3.1 Neo− (Invitrogen, Carlsbad, CA). The BamHI site was introduced at the 3′ end of the GMαRβ extracellular domain by primer-mediated site-directed mutagenesis using Pfu polymerase (Stratagene). This resulted in a single conversion from a substitution of a glutamic acid to an aspartic acid at the membrane-proximal end of the GMαRβ extracellular domain. In a similar manner, either a BamHI or an EcoRI site was introduced at the 3′ end of the GMαRα extracellular domain, which resulted in an aspartic acid to glutamic acid substitution one or two residues, respectively, upstream of the predicted start of the transmembrane domain.

GMαRβIL-7Rα chimeric receptors were generated by inserting in-frame the IL-7Rα transmembrane domain plus cytoplasmic domain (mutated or wild type) as a BamHI-EcoRI cassette into the previously generated pcDNA3.1 Neo− GMαRβ expression vector. The BamHI site upstream of the IL-7Rα transmembrane domain is naturally occurring. Site-specific mutations of the IL-7Rα cytoplasmic domain were performed by primer-mediated site-directed mutagenesis using Pfu polymerase. The GMαRFIL7Rα chimeric receptors were generated in a similar manner. Each TSLPR transmembrane domain plus cytoplasmic domain construct (mutated or wild type) was PCR-amplified and then inserted in-frame as a BamHI-EcoRI or EcoRI-NolI cassette into the previously generated pcDNA3.1 Neo− GMαRα vector. The sequence of each construct was confirmed by DNA sequence analysis using the Prism Big-Dye terminator cycle sequencing kit (PE Applied Biosystems, Norwalk, CT).

HepG2 transfection and CAT analysis

HepG2 cells were transfected by the calcium phosphate method using 20 μg/ml total plasmid DNA and 2–3 × 10^6 cells (13). Cells were cotransfected with expression vectors for the internal transfection control mouse major urinary protein (MUP), rat Stat5b (14), GMαRFIL7Rα, and GMαRFIL7Rα constructs as described above, and pSG5RR−chloramphenicol acetyl transferase (CAT), which contains eight tandem copies of the 27-bp human IL-7Rα receptor response element in pCAT (15). At an overnight recovery period, cultures were released from the plate with trypsin and divided into six-well culture plates. After an additional 24 h, subcultures were treated for 24 h with serum-free medium containing 100 ng/ml huGM-CSF or were left unstimulated. Medium was then collected and subjected to immunoelectrophoresis to quantitate expression of the cotransfected control MUP plasmid. CAT activity for each culture was determined and normalized to the amount of MUP expression (defined as 1.0). Data are presented as the mean of three or more trials.

Retroviral constructs, production, and infection

The pMl2 and pMI.5 retroviral vectors contain the internal ribosome entry site from encephalomyocarditis virus upstream of a CAT encoding the extracellular domain and transmembrane domain of huCD2 or huCD5, respectively (16). Subcloning of the chimeric GMαRFIL7Rα or GMαRFIL7Rα receptors as NotI-EcoRI or EcoRI-SalI fragments upstream of the internal ribosome entry site results in transcription of a bicistronic mRNA that directs translation of the chimeric receptor and the truncated huCD2 or huCD5. Wild-type versions of the chimeric receptors were subcloned into pMl2, while the mutated versions were subcloned into pMI.5.

Retroviral vectors were packaged in Phoenix amphotropic cells (10). Twenty-four hours before transfection, 1.75 × 10^6 cells were plated in a 60-mm tissue culture dish. The calcium phosphate mammalian cell transfection kit (5 Prime→3 Prime, Boulder, CO) was used to transfect 12 μg DNA/dish. Ten hours post-transfection the culture medium was replaced with 4 ml fresh medium. Retroviral supernatants were collected 48 h post-transfection and passed through a 0.45-μm pore size filter syringe. Retroviral supernatants were either used immediately or were stored at −70°C.

The generation of BaF/7 cells expressing both the GMαRFIL7Rα and GMαRFIL7Rα receptor chains was a two-step process. First, cells that expressed GMαRFIL7Rα wild-type (wt) and huCD2 were generated. After enrichment for GMαR or huCD2 expression (see below), these cells were used as the recipients for the second round of infection with retrovirus encoding the GMαRFIL7Rα wild-type or mutated receptor. For the infection, 1.5 × 10^6 cells (in 100 μl) were placed in a single well of a 24-well plate, and 1 ml of the filtered, retroviral supernatant and 8 μg/ml polybrene (hexadimethrine bromide; Sigma-Aldrich) were added. The plate was centrifuged (1100 × g) for 90 min at room temperature. After completion of the spin, 1 ml culture medium, IL-7, and additional polybrene (8 μg/ml) final) were gently added. Cells were cultured at 32°C overnight. Twenty-four hours postinfection cells were pelleted, washed, and cultured at 37°C in BaF3 culture medium plus IL-7 or TSLP.

Magnetic bead selection

Two days after the retroviral infection and periodically while the cell lines were being maintained, cells were positively selected for expression of the chimeric receptors or the linked huCD2 or huCD5 markers using mouse IgG2a–pan Mouse Dynabeads as directed by the manufacturer (Dynal Biotech, Oslo, Norway). Abs used for the selection included murine mAbs to huGM-CSFRα and -β (Santa Cruz Biotechnology) and huCD2 and -5 (CalTag Laboratories).

Analysis of cis expression

Total cellular RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Fifteen micrograms of total RNA was separated on a 1% agarose/2.2 M formaldehyde gel, transferred to Transfer-IT membranes (CPG, Lincoln Park, NJ), and cross-linked by UV irradiation. Blots were hybridized as described previously (4), except that washing steps were conducted at 65°C.
Results

IxN/2B cells activate Stat5 but do not proliferate in response to TSLP

IxN/2B is an IL-7-dependent, bone marrow-derived, pre-B cell line that binds TSLP with high affinity (2). However, unlike IL-7, TSLP failed to support the proliferation of these cells (Fig. 1a). This result was surprising in light of the finding that IxN/2B cells expressed the two chains of the TSLP receptor, TSLPR and IL-7Rα, on their cell surface at levels comparable to the NAG8/7 cell line, which does proliferate in response to TSLP (Fig. 1b and data not shown; Ref. 36). Moreover, analysis of cDNAs encoding TSLPR and IL-7Rα from these cells revealed no sequence abnormalities (data not shown).

To determine whether TSLPR signal transduction was intact in IxN/2B cells, we assayed for Stat5 activation following TSLP stimulation. As shown in Fig. 1c, treatment of IxN/2B cells with either TSLP or IL-7 resulted in tyrosine phosphorylation of Stat5, and the levels of phosphorylation were comparable to those seen in the TSLP- and IL-7-responsive NAG8/7 cells. Thus, despite normal Stat5 activation, TSLP failed to drive the proliferation of IxN/2B cells. These data suggest that Stat5 activation is insufficient for TSLP-mediated proliferation, a finding that is novel within the cytokine receptor family.

Inhibition of Src family kinases blocks TSLP-mediated proliferation but not Stat5 activation

Engagement of the TSLP receptor results in Stat5 activation in the absence of Jak activation. We began a mutational analysis of the receptors for erythropoietin, IL-4, IL-5, and IL-6 has identified a membrane-proximal domain, rich in hydrophobic amino acids and proline residues, that has been termed the Box1 domain, and this domain is critical for Jak and Stat activation and the induction of proliferation (18–23). In fact, mutations to the Box1 domain of the erythropoietin or gp130 receptors prevent the physical association of Jak2 with the receptor (19, 24, 25).

To uncover sequence domains responsible for TSLP-mediated Stat5 activation and/or proliferation, we began a mutational analysis of TSLPR and IL-7Rα. Mutational analysis of the receptors for TSLP and IL-7Rα. Mutation of the receptors for TSLP and IL-7Rα was engineered into the Box1 region of both receptor subunits. The incorporation of [3H]thymidine was measured in NAG8/7 cells cultured in the presence of either IL-7 or TSLP with the indicated concentration of PP1. Results shown are representative of four different experiments. PP1 has no effect on Stat5 activation mediated by IL-7 or TSLP. NAG8/7 cells were stimulated in the presence or the absence of PP1, and immunoprecipitated Stat5 was blotted with an anti-Stat5 antibody. The arrow indicates the position of the tyrosine-phosphorylated Stat5. Reprobing of the same blot with the anti-Stat5 antisera revealed that all lanes had equivalent amounts of Stat5 (data not shown). The results shown are representative of three separate experiments.

Stat5 activation and cell proliferation require the Box1 domain of both TSLPR and IL-7Rα

To uncover sequence domains responsible for TSLP-mediated Stat5 activation and/or proliferation, we began a mutational analysis of TSLPR and IL-7Rα. Mutational analysis of the receptors for erythropoietin, IL-4, IL-5, and IL-6 has identified a membrane-proximal domain, rich in hydrophobic amino acids and proline residues, that has been termed the Box1 domain, and this domain is critical for Jak and Stat activation and the induction of proliferation (18–23). In fact, mutations to the Box1 domain of the erythropoietin or gp130 receptors prevent the physical association of Jak2 with the receptor (19, 24, 25).

As described above, TSLP stimulation activates Stat5 without detectable Jak activation (4, 36). In an effort to determine whether the Box1 domains of TSLPR and IL-7Rα are involved in TSLP-mediated Stat5 activation and proliferation, point mutations were introduced into the Box1 region of both receptor subunits. The incorporation of [3H]thymidine was measured in NAG8/7 cells cultured in the presence of either IL-7 or TSLP. Incorporation of [3H]thymidine was measured in NAG8/7 cells cultured in the presence of either IL-7 or TSLP with the indicated concentration of PP1. Results shown are representative of four different experiments. PP1 has no effect on Stat5 activation mediated by IL-7 or TSLP. NAG8/7 cells were stimulated in the presence or the absence of PP1, and immunoprecipitated Stat5 was blotted with an anti-Stat5 antibody. The arrow indicates the position of the tyrosine-phosphorylated Stat5. Reprobing of the same blot with the anti-Stat5 antisera revealed that all lanes had equivalent amounts of Stat5 (data not shown). The results shown are representative of three separate experiments.

FIGURE 1. IxN/2B cells activate Stat5 but do not proliferate in response to TSLP. a, IxN/2B cells proliferated in response to IL-7 but not TSLP. b, Cell surface expression of endogenous TSLPR on TSLP- and IL-7-responsive NAG8/7 cells and on IL-7-responsive IxN/2B cells. Cells were stained with a biotinylated Ab to TSLPR and PE-conjugated streptavidin and were analyzed for fluorescence intensity by flow cytometry. Control cells are IxN/2B cells stained with a biotinylated, isotype-matched, control Ab. c, Cells were starved of cytokine for 4 h, then stimulated with either IL-7 or TSLP. Cell lysates were immunoprecipitated using anti-Stat5 antisera. Shown is an anti-phosphotyrosine blot of the immunoprecipitated material. Reprobing with anti-Stat5 antisera showed equal loading in each lane (data not shown).

FIGURE 2. Src family kinase inhibitor blocks TSLP-mediated proliferation but not Stat5 activation. a, PP1 inhibits the proliferation of NAG8/7 cells in response to both IL-7 and TSLP. Incorporation of [3H]thymidine was measured in NAG8/7 cells cultured in the presence of either IL-7 or TSLP with the indicated concentration of PP1. Results shown are representative of four different experiments. b, PP1 has no effect on Stat5 activation mediated by IL-7 or TSLP. NAG8/7 cells were stimulated in the presence or the absence of PP1, and immunoprecipitated Stat5 was blotted with an anti-phosphotyrosine Ab. The arrow indicates the position of the tyrosine-phosphorylated Stat5. Reprobing of the same blot with the anti-Stat5 antisera revealed that all lanes had equivalent amounts of Stat5 (data not shown). The results shown are representative of three separate experiments.
mutated receptor chains were then tested for their ability to activate Stat5. For these experiments we used a receptor reconstitution system that has previously been used to study Stat activation by a number of cytokine receptors, including the TSLP receptor (4). Briefly, this system involves cotransfection of the HepG2 human hepatoma cell line with cDNAs encoding Stat5b, cytokine receptor subunits, and a Stat5-responsive reporter gene (15). The relative ability of the transfected cells to activate Stat5 upon treatment with the appropriate cytokine is measured as CAT activity. Previous studies using this system showed that TSLP-induced CAT activity required the addition of Stat5a or Stat5b and that the endogenous expression of Stat1 and Stat3 by the HepG2 cells was insufficient for TSLP signaling (4).

Chimeric receptors consisting of the huGM-CSFRα extracellular domain fused in-frame to the murine TSLP transmembrane and cytoplasmic domain (herein referred to as GMRα/TSLPR) and the huGM-CSFRα extracellular domain fused in-frame to the murine IL-7Rα transmembrane and cytoplasmic domain (GMRβ/IL-7R) were used in these experiments. The use of chimeric receptors allowed us to extend the mutagenesis studies to cell lines that naturally express the TSLP receptor subunits. Treatment of HepG2 cells expressing GMRα/TSLPR and GMRβ/IL-7R with huGM-CSF led to activation of the Stat5-responsive promoter and CAT expression. CAT activity was dependent upon the addition of both GMRα- and GMRβ-containing receptor chains, GM-CSF, and cDNA encoding Stat5b (Fig. 3 and data not shown) (4). Similar results were obtained using native receptor subunits (data not shown).

When the first two conserved proline residues of the Box1 domain of either TSLPR or IL-7Rα were changed to serine residues, cytokine-induced Stat5 activation was abolished or severely inhibited (Fig. 3). Although disruption of the Box1 domain of either receptor subunit blocked TSLP-mediated Stat5 activation, IL-7-mediated Stat5 activation occurred despite disruption of the Box1 domain of IL-7Rα. That is, Stat5 was activated when GMRβ/IL-7Rbox1 was paired with a wild-type GMRαγ (Fig. 3b). This result highlights yet another difference between the IL-7 and TSLP signaling pathways: TSLP-mediated Stat5 activation requires an intact Box1 domain in both receptor chains, while IL-7-mediated Stat5 activation occurs without an intact IL-7Rα Box1 domain.

Sequence conservation among type 1 cytokine receptor chains, including TSLPR, is also maintained carboxy-terminal to the Box1 domain (Fig. 3a). Indeed, a highly conserved tryptophan (W) residue within this region in the erythropoietin and gp130 receptors is crucial for Jak-Stat activation (18, 26). Using the CAT assay to test the requirement for the conserved tryptophan of TSLPR (mutation W34R), we found that this residue was required for TSLP-mediated Stat5 activation (Fig. 3b).

Next, the Box1 and W34R mutations were tested for their ability to support cytokine-dependent proliferation in the murine, IL-3-responsive, pro-B cell line Ba/F3. For these experiments we used a Ba/F3 subline (BafM7R) that had been stably transfected with the IL-7Rα-chain. This cell line naturally expresses TSLP and γc and, consequently, BafM7R cells respond to TSLP, IL-7, or IL-3 (2). Retrovirus-mediated infection was used to generate BafM7R cells that expressed the wild-type or mutant chimeric receptor chains GMRα/IL-7R or GMRβ/IL-7R (Fig. 2). That is, Stat5 was activated when GMRα/IL-7Rbox1 was paired with GMRβ containing receptor chains, GM-CSF, and cDNA encoding Stat5b (Fig. 3 and data not shown). The utilization of the murine endogenous TSLP receptor complex was engaged, the cells did not proliferate, indicating that the TSLP signaling pathway was intact. The Box1 domain is boxed. Residues that are conserved in at least four of the seven sequences are shaded. The two underlined proline residues in TSLPR or IL-7Rα were changed to serine residues in the Box1 mutations described in the text. The underlined tryptophan residue in TSLPR was changed to an arginine residue in the W34R mutation. HepG2 cells were transiently cotransfected with a Stat-responsive CAT reporter plasmid, Stat5b cDNA, and cDNA encoding wild-type or mutated chimeric receptor chains as indicated. Subcultures were treated with medium alone or GM-CSF and analyzed for CAT activity. For each trial, the CAT activity detected using wild-type receptor pairs upon GM-CSF stimulation was set at 100% and was used to calculate the relative activity detected from cultures expressing the mutated receptors. Data are presented as the mean ± SD of three to six trials.

The murine endogenous TSLP receptor complex was engaged, the cells did proliferate, indicating that the TSLP signaling pathway was intact. The murine endogenous TSLP receptor complex was engaged, the cells did proliferate, indicating that the TSLP signaling pathway was intact. The murine endogenous TSLP receptor complex was engaged, the cells did proliferate, indicating that the TSLP signaling pathway was intact. The murine endogenous TSLP receptor complex was engaged, the cells did proliferate, indicating that the TSLP signaling pathway was intact. The murine endogenous TSLP receptor complex was engaged, the cells did proliferate, indicating that the TSLP signaling pathway was intact. The murine endogenous TSLP receptor complex was engaged, the cells did proliferate, indicating that the TSLP signaling pathway was intact.

Single cytoplasmic tyrosine of TSLPR is not required but is sufficient for Stat5 activation

For several cytokine receptors, the recruitment of Stat5 is dependent upon phosphorylation of tyrosine residues within the cytoplasmic domain of the receptors (2). The TSLPR subunit contains only one cytoplasmic tyrosine residue (Y103) that resides three amino acids away from the carboxyl terminus. To test the requirement of this residue for TSLP-mediated Stat5 activation, Y103 was replaced with a phenylalanine (mutation Y103F). As shown in Table II, normal levels of Stat5-mediated CAT activity were detected using the GMRα/Y103F and GMRβ/IL-7Rwt receptor pair. In addition, experiments combining a carboxyl-terminally truncated GMRβ/IL-7R construct (GMRβ/IL-7R125cyt) that lacked all four cytoplasmic tyrosine residues with GMRα/TSLPRwt activated Stat5 (Table II). However, when GMRβ/IL-7R125cyt was paired with GMRα/Y103F, a receptor combination that has no
cytoplasmic tyrosine residues, Stat5 activation was blocked (Table II). Thus, the four cytoplasmic tyrosines of IL-7Rα were not required when the cytoplasmic tyrosine of TSLPR was present and vice versa. However, a receptor complex lacking all cytoplasmic tyrosine residues on both chains was unable to activate Stat5.

### Cytoplasmic tyrosine residue of TSLPR is required for proliferation

To determine whether Y103 was required for cell proliferation, we used retroviral transduction to generate BafM7R cells expressing GMRα/Y103F and GMRβ/IL-7Rwt (yf/wt cells). These cells were unable to proliferate in response to GM-CSF (Fig. 4a), even though we demonstrated that this receptor combination was capable of mediating Stat5 activation in HepG2 cells (Table II). To confirm that Stat5 activation was occurring in yf/wt-expressing BafM7R cells, lysates from these cells were tested for the presence of activated Stat5 following GM-CSF stimulation. As shown in Fig. 4b, tyrosine-phosphorylated Stat5 was detected in yf/wt cells following GM-CSF treatment at levels comparable to those seen following TSLP stimulation. The requirement of Y103 for proliferation, but not Stat5 activation, correlates with results obtained using the IxN/2B cell line (Fig. 1) and NAG8/7 cells treated with PP1 (Fig. 2).

To further investigate Stat5 activation in yf/wt cells we examined induction of the cis gene by GM-CSF and TSLP. Previous studies have shown that transcription of the cis gene is rapidly induced following TSLPR engagement (4). For these experiments, yf/wt and wt/wt cells were stimulated with either TSLP or GM-CSF for varying times and analyzed for cis gene expression. As shown in Fig. 5, TSLP treatment of both lines resulted in up-regulation of the cis gene. However, only in wt/wt cells, and not in yf/wt cells, was cis induced in response to GM-CSF treatment. To confirm these results we have used a reporter gene consisting of 305 bp of the cis gene promoter fused to luciferase, as was used previously to analyze TSLP activation of Stat5 (4). Consistent with the Northern blot analysis, we found that TSLP was capable of inducing luciferase activity in both cell lines following transfection of the reporter, while GM-CSF treatment only resulted in induction of luciferase activity in wt/wt cells (data not shown).

### Table I. An intact Box1 region in TSLPR or IL-7Ra is required for cell proliferation

<table>
<thead>
<tr>
<th>Expression Constructs</th>
<th>[3H]Thymidine Incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM-CSF</td>
</tr>
<tr>
<td>GMRα/IL-7R</td>
<td>93,585</td>
</tr>
<tr>
<td>wt</td>
<td>0</td>
</tr>
<tr>
<td>Box1</td>
<td>0</td>
</tr>
<tr>
<td>W34R</td>
<td>0</td>
</tr>
<tr>
<td>wt</td>
<td>124</td>
</tr>
</tbody>
</table>

* Data from a representative experimental series (repeated at least three times) are presented as the mean cpm from triplicate wells minus those obtained from wells without added cytokine. GM-CSF values are normalized to account for the percentage of cells that did not express both receptor chains, by flow cytometric analysis, and thus would be able to respond to TSLP but not GM-CSF.

### Table II. The sole cytoplasmic tyrosine of TSLPR is sufficient but not required for Stat5 activation

<table>
<thead>
<tr>
<th>GMRα/TS LPR Construct</th>
<th>C-Terminal Residues</th>
<th>GMRβ/IL-7R Construct</th>
<th>Stat5 Activation (CAT activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>DSGYMTL*</td>
<td>wt</td>
<td>41.4 ± 4.6</td>
</tr>
<tr>
<td>wt</td>
<td>DSGYMTL*</td>
<td>125cst</td>
<td>45.0 ± 8.2</td>
</tr>
<tr>
<td>Y103F</td>
<td>DSGFMETL*</td>
<td>wt</td>
<td>46.2 ± 5.4</td>
</tr>
<tr>
<td>Y103F</td>
<td>DSGFMETL*</td>
<td>125cst</td>
<td>3.2 ± 1.7</td>
</tr>
</tbody>
</table>

* The asterisk denotes the carboxyl-terminal end of the protein.

* The 125cst construct lacks the 70 carboxyl-terminal residues of GMRβ/IL-7R including all four cytoplasmic tyrosine residues.

* CAT activity is presented as the fold stimulation following GM-CSF treatment with 1 being the amount of acetylated chloramphenicol detected without added cytokine (mean of three to eight trials ± SE).

* Value of p < 0.005 using an unpaired t test.

**FIGURE 4.** The single cytoplasmic tyrosine of TSLPR is required for cell proliferation but not Stat5 activation. *a*, BafM7R cells stably expressing GMRα/IL-7Rwt alone, GMRα/TSLPRwt and GMRβ/IL-7wt (wt/wt), or GMRα/Y103F and GMRβ/IL-7wt (yf/wt) were tested for their ability to proliferate in the presence of increasing amounts of GM-CSF or, as a control, TSLP. The mean [3H]thymidine uptake from triplicate wells was normalized to account for the percentage of cells that expressed the chimeric receptors, as evidenced by flow cytometry as shown. The percentages listed within the bar graph represent the amount of [3H]thymidine incorporated into the cells following stimulation with 100 ng/ml GM-CSF as a percentage of that seen when stimulated with TSLP (mean of three to six trials ± SD). *b*, Whole cell lysates from GM-CSF- or TSLP-stimulated wt/wt and yf/wt BafM7R cells were size-separated, and the resulting Western blot was blotted with an anti-phosphotyrosine Stat5a antisem. Re-probing of the blot with anti-Stat5a antisem revealed total Stat5 protein levels. Both antisera were capable of recognizing Stat5a and Stat5b.

**FIGURE 5.** Substitution of tyrosine 103 to phenylalanine in TSLPR prevents cis mRNA induction. BafM7R cells expressing GMRβ/IL-7R with either GMRα/TS LPR (wt/wt) or Y103F (yf/wt) were starved of cytokine for 6 h, after which time the cells were cultured in the absence or the presence of GM-CSF or TSLP for the indicated times. RNA was prepared and analyzed by Northern blotting using a probe for cis. The blot was then stripped and reprobed for GAPDH to control for sample loading. The position of cis mRNA is indicated.
Discussion

Unlike all other known members of the cytokine receptor family, engagement of the TSLP receptor complex leads to activation of a Stat protein [(Stat5)] in the absence of Jak involvement. Although the precise identity of the kinase required for TSLP-mediated Stat5 activation is not known, our previous work has suggested the involvement of Tec family kinases (4). To further define the signal transduction pathway used by the TSLP receptor, we have used targeted mutagenesis of TSLPR and IL-7Rα to identify sequence elements crucial for TSLP-mediated Stat5 activation and cell proliferation. This approach allowed us to define regions of each receptor subunit required for both processes as well as a single mutation in TSLPR that abolishes TSLP-mediated cell proliferation but had no affect on Stat5 phosphorylation. This mutation, along with the IxN/2B cell line and PP1-treated NAGB/7 cells, provides further evidence for the uncoupling of Stat5 activation and cell proliferation resulting from TSLP receptor engagement.

Similar to other cytokine receptors, the membrane-proximal Box1 domain of TSLP and IL-7Rα was required for Stat5 activation as well as proliferation. This result in itself is not surprising given that Box1 domains in other cytokine receptors act as Jak binding domains and as such are required for Stat activation and proliferation (18, 19, 25, 26). However, unlike other cytokine receptors, TSLP receptor engagement does not activate any of the four known Jak, and overexpression of kinase-inactive versions of Jak1, Jak2, or Jak3 has no inhibitory effect on TSLP-mediated Stat5 activation (Refs. 4 and 36 and P. A. Trobridge and S. D. Levin, unpublished observations).

Thus, the question remains as to why mutations to the Box1 domain of either TSLPR or IL-7Rα prevent Stat5 activation. These domains, because they are located adjacent to the transmembrane domain, may be required for proper dimerization or folding of the receptor subunits. Conversely, they may be required for docking of signaling proteins, other than Jak family kinases, such as a Tec family kinase. Indeed, overexpression of a kinase-inactive version of Tec was able to partially inhibit TSLP-mediated Stat5 activation (4). Moreover, overexpression of Bmx, another member of the Tec kinase family, in COS cells induced Stat5 activation without activation of endogenous Jaks (27).

One interesting byproduct of these studies is the finding that mutations to the Box1 domain of IL-7Rα do not prevent IL-7-mediated Stat5 activation (Fig. 3). A similar observation was made for IL-2Rβ by Higuchi et al. (28). In their study mutations in IL-2Rβ that abrogated Jak1 binding did not prevent IL-2-mediated Jak3 or Stat5 activation. It is therefore likely that the Box1 domain in IL-7Rα, and therefore Jak1 binding, are dispensable for IL-7 signaling. These findings are in contrast to those for mice deficient in Jak1, in which thymocytes have a defect in γ-coupled cytokine signaling (including IL-7 and IL-2) (29). However, thymocytes from these mice also have diminished responses to phorbol ester and calcium ionophore as well as to gpl30-coupled cytokines and therefore may have a general survival deficit (29).

A common theme of the data presented in this study is that TSLP-mediated Stat5 activation can be uncoupled from cell proliferation. We have demonstrated, using three independent systems, that Stat5 is tyrosine-phosphorylated under conditions that inhibit a TSLP-mediated proliferative response. The finding that the Src family kinase inhibitor PP1 can inhibit TSLP proliferation, but not Stat5 activation, suggests that the signaling pathway downstream of the TSLP receptor complex splits, with a Src family kinase involved in proliferative responses and a member of a different tyrosine kinase family responsible for Stat5 phosphorylation. This finding is consistent with our previous data showing that overexpression of Csk (a negative regulator of Src family kinases) did not affect TSLP-mediated Stat5 activation (4). The lack of a proliferative response to TSLP in IxN/2B cells, despite the presence of normal TSLPR and IL-7Rα on the cell surface (Fig. 1) and the ability to bind TSLP with high affinity (2), also supports the idea of distinct signaling pathways regulating Stat5 and proliferation. As shown in Fig. 1, while these cells did not proliferate in response to TSLP, TSLP treatment did lead to Stat5 phosphorylation. Thus, it would appear that IxN/2B cells have a defect in the pathway that leads to TSLP-mediated cell proliferation, while the pathway that leads to Stat5 activation is intact. The nature of this defect remains to be determined, but, given the data presented here, it may be due to a defect in or altered expression of a Src family kinase.

The final demonstration of the uncoupling of Stat5 activation and cell proliferation comes from the analysis of cells that express a mutated TSLPR cytoplasmic domain. This mutant, Y103F, was expressed as part of a chimeric receptor complex (GMα/IL-7Rα and GMRβ/IL-7Rα) in BalM7R cells (y/wt BalM7R cells). This cell line, when stimulated with GM-CSF, activated Stat5 but did not proliferate, yet endogenous TSLP responses were intact (Fig. 4). Interestingly, the y/wt BalM7R cells also did not up-regulate the Stat5-inducible gene cis following GM-CSF treatment. This finding was extended using a reporter plasmid that contained a portion of the cis gene promoter. This is in contrast to the data presented in Table I, showing that the TSLPR Y103F mutant was capable of activating the HRRE-CAT reporter. A possible explanation for this result comes from an examination of the promoters used in these reporter plasmids. The HRRE-CAT reporter has eight copies of a Stat5 binding site (15). The cis reporter contains the sequences −100 to −404 from the human cis promoter (30). In addition to four Stat5 binding sites, this region contains three binding sites for Sp-1, which has been shown to cooperate with Stat5 in the regulation of other genes, including cyclin D2 (31). It is possible that the Y103F mutation in TSLPR results in the inability to activate Sp-1.

There are many reports of cytokine receptor mutations that block both Stat activation and proliferation (18, 23, 26, 32–34), including the Box1 mutations described in this work. There are also reports of cytokine receptor mutations that eliminate Stat activation without affecting cell proliferation, including erythropoietin- and IL-2-induced Stat5 activation (18), and thrombopoietin-induced Stat1, -3, and -5 activation (35). To our knowledge this is the first report of cytokine receptor-mediated Stat5 activation without corresponding cell proliferation. This unique feature of TSLP receptor signaling may be a consequence of the apparently novel mechanism used by this receptor in the activation of Stat5. In receptor systems that use Janus family kinases for Stat5 activation, signals that result in Stat activation may be sufficient for proliferation. Whatever the mechanism of TSLP-mediated Stat5 activation, it is clearly different at several levels from Jak-activating receptors such as the IL-7R. This is also illustrated by the fact that TSLP activates only Stat5a and Stat5b, whereas IL-7 can activate other Stats as well (4). These differences between the IL-7 and TSLP signaling pathways may explain in part the differences seen in the response of B cell precursors to each cytokine. IL-7 treatment of fetal liver or bone marrow results in the outgrowth of B220+ IgM− pre-B cells. In contrast, TSLP treatment of the same populations results in the production of B220+ IgM+ early B cells (36).
Acknowledgments
We are grateful to Dr. Brad H. Nelson (Virginia Mason Research Center) for the GM-CSF-Rα and β-plasmids, to Erin Kinzie for assistance with the CAT assay system, to Dr. Michael Bevan (University of Washington) for the pM1.2 and pM3.5 expression vectors, and to Drs. Deborah Kasprwiczk and Brad H. Nelson for critical reading of the manuscript.

References