Analysis of γc-Family Cytokine Target Genes

IDENTIFICATION OF DUAL-SPECIFICITY PHOSPHATASE 5 (DUSP5) AS A REGULATOR OF MITOGEN-ACTIVATED PROTEIN KINASE ACTIVITY IN INTERLEUKIN-2 SIGNALING*

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The common cytokine receptor γ chain (γc) is essential for normal immune development and function. Mutations in γc result in X-linked severe combined immunodeficiency (XSCID) (1), a disease in which affected individuals are highly susceptible to infections resulting from the defective development of T and NK cells and nonfunctional B cells. γc is a component of the receptors for multiple cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (2). These cytokines have both redundant and distinctive actions on lymphocytes. IL-2 is a growth and survival factor for activated T lymphocytes and is also essential for activation-induced cell death (AICD) and the prevention of T cell anergy, processes involved in the maintenance of peripheral tolerance (3). IL-4 is also a T-cell growth factor and is necessary for the development of Th2 cells (4), which regulate humoral immune responses, whereas IL-7 is required for the development and growth of T lymphocytes but can also support the growth of peripheral T lymphocytes (5–7). IL-15 has overlapping functions with IL-2, but, unlike IL-2, IL-15 is essential for NK-cell differentiation and is more important than IL-2 in the generation of CD8+ memory T cells (8). Although transgenic expression of IL-9 causes thymomas, suggesting that IL-9 can regulate lymphocyte growth (9), IL-9 knock-out mice do not manifest abnormalities in T-cell development or function but exhibit defective mast cell proliferation and mucus production (10). Based on in vitro studies, IL-21 has potential roles for T-cell, B-cell, and NK-cell biology (11).

Only limited information is available regarding the genes that are activated by γc-dependent cytokines. To investigate the basis for overlapping and distinctive actions of γc-dependent cytokines, we used the “Lymphochip” microarray, a specialized cDNA array enriched for genes expressed in lymphocytes (12), to identify genes regulated by IL-2, IL-4, IL-7, and IL-15 in peripheral blood T lymphocytes. IL-2, IL-7, and IL-15 regulated most genes in a similar manner, whereas the pattern seen with IL-4 was more distinctive. Although most γc-dependent genes are redundantly regulated by various stimuli, certain cytokine-specific patterns of gene expression were noted. One gene induced by IL-2, IL-7, and IL-15 but not IL-4 was dual-specificity phosphatase 5 (DUSP5) (13, 14). IL-2 is known to activate several signaling pathways, including Ras-MAP kinase, Jak-STAT, and phosphoinositol 3-kinase/Akt/p70 S6 kinase pathways (15), and we now provide evidence for a role for DUSP5 as part of a negative feedback loop that controls IL-2-induced MAPK activity in T cells. This is the first demonstration of a role for DUSP5 in T-cell biology and identifies a mechanism by which IL-2-mediated MAPK activity can be controlled.

MATERIALS AND METHODS

Cell Cultures—Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Amersham Biosciences) density gradient centrifugation, stimulated for 18 h with phytohemagglutinin (PHA, Roche Molecular Biochemicals, 2 μg/ml), and expanded for 2 weeks in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 2 mM glutamine (complete RPMI medium) supplemented with PHA (500 ng/ml) and IL-2 (50 units/ml). The cells were washed twice and resuspended in complete medium without PHA or IL-2 for 3 days. The purity of T cells was evaluated by flow cytometry, and only cultures with >95% CD3+ T cells were used for further

Interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 form a family of cytokines based on their sharing the common cytokine receptor γ chain, γc, which is mutated in X-linked severe combined immunodeficiency (XSCID). As a step toward further elucidating the mechanism of action of these cytokines in T-cell biology, we compared the gene expression profiles of IL-2, IL-4, IL-7, IL-15 in T cells using cDNA microarrays. IL-2, IL-7, and IL-15 each induced a highly similar set of genes, whereas IL-4 induced distinct genes correlating with differential STAT protein activation by this cytokine. One gene induced by IL-2, IL-7, and IL-15 but not IL-4 was dual-specificity phosphatase 5 (DUSP5). In IL-2-dependent CTLL-2 cells, we show that IL-2-induced ERK-1/2 activity was inhibited by wild type DUSP5 but markedly increased by an inactive form of DUSP5, suggesting a negative feedback role for DUSP5 in IL-2 signaling. Our findings provide insights into the shared versus distinct actions of different members of the γc family of cytokines. Moreover, we have identified a DUSP5-dependent negative regulatory pathway for MAPK activity in T cells.

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**DUSP5 Regulates MAPK Activity in IL-2 Signaling**

**Western Blotting, Immunoprecipitation, and Kinase Assays**—For Western blotting, cells were harvested, washed with phosphate-buffered saline, and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM Na3VO4, 5 mM NaF, 10 μM each of leupeptin and aprotinin, and 1 mM 4-2-aminooethylbenzenesulfonyl fluoride) and centrifuged at 14,000 × g at 4 °C for 15 min. 15 μg of protein per lane was Western blotted. For immunoprecipitations, 150 μg of protein lysate was used. ERK-1/2 kinase activity was determined using Elk-1 as a substrate and the p44/42 MAP kinase assay kit (Cell Signaling). We used antibodies to phosphorylated ERK-1/2, phosphorylated MEK1, ERK-1/2, phosphorylated Stats (all from Cell Signaling), MEK1 (Transduction Laboratories), Myc-epitope (9E10), IL-2Rβ phosphotyrosine antibody (PY99) (all from Santa Cruz Biotechnology), and anti-β-actin (AC-15, Sigma). DUSP5 anti-serum was a gift from Dr. Jack E. Dixon.

**RESULTS**

**IL-2, IL-7, and IL-15 Induce a Highly Similar Set of Genes,** whereas IL-2 Induces Both Overlapping and Distinct Genes—Using cDNA microarrays, we identified 137 genes that were induced at least 2-fold by IL-2, IL-4, IL-7, or IL-15 in preactivated T lymphocytes (genes induced or repressed at least 2-fold are in Figs. 1–3 and will be made available at llmpp.nih.gov/cytokines). A one-sample t test was performed on the log ratios to evaluate the significance of the observed fold changes. We observed that 99% of IL-2-regulated (five experiments), 100% of IL-7-regulated (four experiments), 74% of IL-4-regulated (three experiments), and 54% of IL-15-regulated (three experiments) genes were differentially expressed to a degree that achieved statistical significance (two-sided p < 0.05). We hypothesize that the lower significance for the IL-4 and IL-15 groups results in large part from the lower power associated with having only three experiments for each of these cytokines. The induced genes (Figs. 1 and 2) included many genes previously known to be regulated by IL-2, such as those encoding the IL-2Rα chain, cyclin D2 (15), SOCS1 (19), CIS1 (20), and Pim-1 (21), indicating the validity of our analysis. IL-4Ra was also induced by IL-4, as reported previously (22). We also identified 34 repressed genes (Fig. 3). Although the use of 2-fold induction criteria is common, smaller changes in gene expression levels can also be biologically significant, and certain known IL-2-induced genes were not identified by the 2-fold criteria. We therefore searched the microarray data for genes whose expression was induced or repressed by more than 40% (based on microarray analysis) in at least any 8 of the 15 experiments. 95 additional genes fulfilled these less stringent criteria (data not shown but will be made available at llmpp.nih.gov/cytokines). This group included c-myc (15) and Bcl-XL (23), genes whose expression levels are known to be regulated by IL-2. Thus, it is important to evaluate genes whose mRNA levels are changed less than 2-fold as well.

The gene expression data were analyzed using average linkage hierarchical clustering (the Pearson correlation coefficient was used as the distance metric) (18), yielding dendrograms that group experiments and genes based on the degree of similarity of their expression patterns (see Fig. 1). We separately analyzed genes that were induced by IL-2, IL-4, IL-7, or IL-15 (Fig. 1A), and the expression profiles seen with IL-2, IL-7, and IL-15 were very similar (see for example Fig. 1, B–D). Some genes were induced in a similar fashion by all four cytokines (Fig. 1B). A number of genes were strongly induced by IL-2, IL-7, and IL-15 but not induced or only weakly induced by IL-4 (Fig. 1C). A third set of genes was preferentially induced by IL-4 (Fig. 1D).

To determine whether the differences between the gene expression profiles for IL-2 and IL-4 represented differences in the kinetics of gene expression between the cytokines rather than induction of different genes, we studied gene expression 0.5, 2, 4, 6, and 8 h after cytokine treatment (Fig. 1, E and F). In the four panels on the left, multiple experiments are shown at the 4-h time point for IL-2, IL-7, IL-15, and IL-4, whereas in
the three panels on the right data are shown for two time courses for IL-2 and one for IL-4. Most genes showed increased expression in a cytokine-restricted manner between 0.5 and 2 h, and this increase was sustained for at least 8 h. For example, leukemia inhibitory factor (LIF) and biliary glycoprotein were induced by IL-2 at 30 min but were not induced by IL-4 at any time point (second and third rows from the top in Fig. 1E).

\(\gamma\)-Dependent Cytokines Induce a Group of Genes That Are Highly Expressed in Proliferating Cell Lines but Are Expressed at a Low Level in CLL Cells—Most of the genes we identified have not been previously linked to cytokine responses and many are functionally uncharacterized. One strategy for finding clues to the functions mediated by these genes is to define expression “signatures” characterizing cellular processes. One such expression signature is defined by a set of genes whose expression correlates with cell proliferation in that they are highly expressed in proliferating cell lines (Fig. 2A, third panel from the left, lanes A–G) but are expressed at low level in CLL cells (lanes H–L) which are relatively quiescent in their growth properties (16). ~20% of the genes induced by IL-2, IL-4, IL-7, and IL-15 fulfilled these criteria.

Most Genes Induced by IL-2, IL-4, IL-7, or IL-15 Are Induced by Multiple Stimuli—We sought to identify a set of genes that could distinguish a \(\gamma\)-dependent cytokine response from other activation events, and we compared genes induced or repressed by IL-2, IL-4, IL-7, and IL-15 with those regulated in PBMCs (>70% T cells) by PI and in B cells after antigen receptor

**Fig. 1.** Hierarchical clustering of genes induced by IL-2, IL-4, IL-7, and IL-15. The color intensity reflects the magnitude of induction (red squares) or repression (green squares). Gray squares indicate missing or excluded data. Asterisks indicate genes whose identity was verified by re-sequencing. A, array dendrogram obtained by hierarchical clustering of expression data from 137 induced genes in a total of 15 array experiments comprising four different cytokines. Samples stimulated for 4 h by IL-2, IL-7, and IL-15 cluster together (black branches), indicating a similar gene expression response, whereas the samples induced by IL-4 form a separate group (blue branches). B, hierarchical clustering of genes similarly induced at least 2-fold by IL-2, IL-4, IL-7, and IL-15. Each column represents data from one experiment, and each row represents the measurements for a given gene across all experiments. C, hierarchical clustering of genes induced more potently by IL-2, IL-7, and IL-15 than by IL-4. Genes in this cluster were induced more than 2-fold by IL-2, IL-7, and IL-15, and their expression was at least two times higher in IL-2, IL-7, and IL-15-stimulated cultures than in IL-4-stimulated cultures. D, hierarchical clustering of genes preferentially induced by IL-4. Genes in this cluster were induced at least 2-fold by IL-4, and their expression was at least two times higher in IL-4 stimulated cultures than in cultures stimulated by IL-2, IL-7, or IL-15. E, kinetics of IL-2- and IL-4-induced gene expression. Gene expression data for highly induced genes after 4 h of stimulation with IL-2 (five experiments), IL-7 (four experiments), IL-15 (three experiments), and IL-4 (three experiments) and corresponding measurements in three time course experiments (two IL-2 experiments and one IL-4 experiment) at 0, 0.5, 2, 4, 6, and 8 h. The genes were ranked by their average up-regulation in response to IL-2, IL-7, and IL-15. Data from the 20 most induced genes are shown. F, genes induced more than 2-fold by IL-4 that, in addition, were at least 2-fold more highly induced by IL-4 than by IL-2, IL-7, or IL-15. In panels B–D the “gene tree” on the left indicates the degree of similarity in gene expression across all the experiments. The blue circles correspond to genes whose expression was also studied by Northern blotting in Fig. 3.
Fig. 2. Analysis of expression patterns of cytokine-induced genes in PI-stimulated PBMCs (PBMC Act.), B cells after anti-IgM crosslinking (B-cell Act.), proliferating cell lines, and CLL cells. A, a set of genes induced by IL-2, IL-4, IL-7, or IL-15 that were 3-fold more expressed in proliferating cell lines than in CLL cells. B, cytokine-inducible genes that are induced 2-fold by PI stimulation of PBMCs and BCR.
cross-linking (Fig. 2). We found that 73% of the genes induced by the cytokines were also induced in PI-stimulated PBMCs (Fig. 2, A–C, first column of panels from the left). The induction typically occurred within 1 h, minimizing the possibility that PI-dependent cytokine production was responsible for the PI effect. BCR stimulation of B cells induced less overlapping (41%) gene expression profiles (Fig. 2, A and B versus C and D, compare first and second panels from the left), consistent with the use of nonshared signaling pathways or lineage-specific differences in expression. 23% of the γ-dependent genes showed a more restricted expression pattern, as they were not up-regulated by either PI treatment of PBMCs or BCR cross-linking of B cells.

γ-Dependent Cytokines Repress Certain Genes, Some of Which Are Highly Expressed in CLL Cells—We also identified 34 genes that were consistently repressed by IL-2, IL-4, IL-7, and/or IL-15 (Fig. 3), most of which were also repressed in PBMCs treated with PI or B cells treated with anti-IgM. Analogous to many of the activated genes being poorly expressed in CLL cells, many of the repressed genes were more highly expressed in CLL cells than in highly proliferating cell lines (Fig. 3, third panel from the left, lanes H–L versus A–G), suggesting a correlation between the expression of these genes and establishing or maintaining a more quiescent state.

Confirmation of Microarray Results by Northern Blotting—We confirmed the induction or repression of select genes (those encoding IL-2R, TRAIL, MAPKAPK3, DUSP5, Mal, IL-4Rα, and TSC-22R; blue circles in Figs. 1–3) by Northern blot analysis (Fig. 4). IL-2Rα, TRAIL, and DUSP5 were more potently induced by IL-2, IL-7, and IL-15 than by IL-4. Mal and IL-4Rα were most strongly induced by IL-4. MAPKAPK3 was induced by all four cytokines, and TSC-22R was reppressed by all of the cytokines.

**DUSP5 Regulates IL-2-Dependent Phosphorylation and Catalytic Activity of ERK-1/2**—One of the genes induced by IL-2, IL-7, and IL-15 but not by IL-4 was that encoding DUSP5, a dual-specificity phosphatase originally cloned from mammary epithelial (13) and liver (14) cell lines. DUSP5 is also known as hVH-3 (14) and is a dual-specificity phosphatase induced by serum stimulation and heat shock (13). DUSP5 can hydrolyze proteins at both phosphothreonine/threonine residues, and recombinant DUSP5 can decrease the catalytic activity of purified ERK-1 protein in vitro (13, 14); but its previously has not been evaluated, and it has not previously been shown to be expressed in T cells.

Because IL-2 can activate ERK-1/2 as well as induce DUSP5 expression, we hypothesized that DUSP5 induction might be part of a negative feedback loop controlling IL-2-induced MAPK activity. We first confirmed that IL-2 could induce ERK-1/2 phosphorylation in the T cells (Fig. 5A, top panel, lanes 1–3, see arrow). IL-15 also induced phosphorylation of ERK-1/2, but neither IL-4 nor IL-7 shared this property (Fig. 5A, top panel, lanes 10–12 versus 4–6 and 7–9). In the same experiments IL-2, IL-7, and IL-15 induced phosphorylation of Stat5 (Fig. 5A, middle panel, lanes 1–3, 7–9, and 10–12), and IL-4 induced phosphorylation of Stat6 (Fig. 5A, bottom panel, lanes 4–6), demonstrating the responsiveness of the cells to all of these cytokines. Using real time PCR, we confirmed the induction of DUSP5 mRNA in preactivated PBMCs within 30 min of stimulation with IL-2, with a subsequent decline (Fig. 5B). Similarly, an antiserum raised against a DUSP5-GST fusion protein Western blotted an IL-2-inducible band of a molecular weight appropriate for DUSP5 (Fig. 5C, upper panel), whereas a control anti-serum could not detect (data not shown), suggesting that IL-2 stimulation also increases DUSP5 protein levels.

We next investigated whether DUSP5 could regulate IL-2-induced ERK-1/2 phosphorylation using an IL-2 receptor reconstitution system (24) in 293T cells. In this setting, ERK-1/2 phosphorylation was induced by IL-2 (Fig. 5D, lane 2 versus lane 1), and this phosphorylation was inhibited when the cells were treated with DUSP5 GST fusion protein.
were also transfected with wild type DUSP5 (Fig. 5D, lanes 3 and 4). Transfection of an inactive (C263S) mutant of DUSP5 did not affect ERK-1/2 phosphorylation (Fig. 5D, compare lane 6 to lane 2), which is consistent with the fact that 293T cells express very low levels of endogenous DUSP5 (data not shown). As expected, 293T cells transfected with constitutively active MEK1 showed increased ERK-1/2 activity that was independent of IL-2 (Fig. 5D, lanes 7 and 8).

To further evaluate the possible role of DUSP5 in the regulation of IL-2-induced MAPK activity, we stably transfected IL-2-dependent CTLL-2 cells with wild type and inactive DUSP5. CTLL-2 is a murine T cell line that has been widely used to study IL-2 biology and signaling (see, for example, Refs. 25 and 26). We studied two clones that constitutively express...
wild type DUSP5 (WT1, WT2) and three clones that constitutively express an inactive mutant of DUSP5 (M1, M2, M3), as demonstrated by Western blotting with anti-Myc epitope antibody (Fig. 6A, top panel, lanes 1–20). The clones expressing the inactive form of DUSP5 showed markedly increased phosphorylation of ERK-1/2 (doublet band, Fig. 6A, second panel from top, lanes 9–20) in response to IL-2 when compared with parental CTLL-2 cells (lanes 21–24) or clones expressing wild type DUSP5 (lanes 1–8). Similarly, as evaluated by an in vitro kinase assay, clones expressing inactive DUSP5 exhibited higher IL-2-induced ERK-1/2 kinase activity than did the parental cells (Fig. 6B, lanes 7–15 versus 16–19), whereas clones expressing wild type DUSP5 showed decreased activity (Fig. 6B, lanes 1–6). As ERK-1/2 protein levels were not affected by DUSP5 expression (Fig. 6A, third panel from the top, lanes 1–24), these results indicate that DUSP5 negatively regulates the activity of ERK-1 and ERK-2. We also studied the effect of DUSP5 on IL-2-induced phosphorylation of MEK1 and Stat5 (Fig. 6A, fourth and fifth panels from the top) and IL-2Rβ (Fig. 6C), but no changes were observed. Thus, DUSP5 appears to selectively regulate ERK-1 and ERK-2 activity in IL-2 signaling.

**DISCUSSION**

In this study, we investigated genes regulated by IL-2, IL-4, IL-7, and IL-15. Although some data regarding genes regulated by these cytokines have been previously generated, comparative data on the effects of these cytokines on large numbers of genes have not been available. Genes induced by IL-2 have been most extensively studied, and IL-2 is known to induce a number of genes including, for example, those encoding c-Myc (15), c-Fos (15), c-Jun (15), IL-2Rα chain (15), Fim-1 (21), Bcl-2 (15), and the SOCS family proteins SOCS1 (19) and CIS1 (20). Previous studies have sought to identify IL-2-regulated genes in a more systematic way (27–29) but have revealed largely nonoverlapping sets of IL-2-induced transcripts, suggesting that many other IL-2-regulated genes remained to be identified. In our microarray analysis 137 genes appeared to be induced, and 34 genes appeared to repressed by IL-2, IL-4, IL-7, or IL-15. A significant number of these genes (20%) are related to cell proliferation based on their high level expression in proliferating cell lines but low expression in relatively quiescent CLL cells (16), in accord with the known mitogenic function of these cytokines on activated T cells.

Hierarchical clustering of these genes revealed that the induced genes fell into two major groups, i.e. those regulated preferentially by IL-2, IL-7, and IL-15, and those regulated by IL-4. It was noteworthy that IL-2, IL-7, and IL-15 induced almost identical gene expression patterns in T lymphocytes, at least at the doses we used. This supports the concept that multiple cytokines can similarly provide survival/growth signals and that the specificity of cytokine action is largely determined by cell type or developmental stage specific expression of cytokine receptor(s) and the availability of ligand(s) (30–32). However, it is possible that cell type-dependent differences also exist. For example, it has been suggested that IL-15 uses different receptor or signaling pathways depending on cell type (33, 34), which could also explain why IL-2 and IL-15 induce similar gene responses and proliferation in T cells, whereas only IL-15 is essential for NK cell differentiation.

Although IL-4 induced a set of genes overlapping those induced by IL-2, IL-7, and IL-15, there were also many differences. Most genes that were induced by IL-2, IL-7, or IL-15 were not induced by IL-4 or were induced at only a very low level. However, some genes (e.g. those encoding SOCS-1, CIS1, and Bcl-2) were induced in a similar fashion by all of the cytokines, whereas others (e.g. those encoding IL-4Rα and Mal) were more strongly induced by IL-4. The basis for this more distinctive pattern for IL-4 may be explained by the fact that...
IL-4 activates primarily Stat6, whereas the other cytokines preferentially activate Stat3, Stat5a, and Stat5b (35). For example, the promoter for IL-4Rα, which is regulated by IL-4, contains Stat6 binding sites (36), whereas the IL-2Rα promoter, which is regulated by IL-2, contains binding sites for Stat5a and Stat5b (37–39). It will be important to determine whether genes induced preferentially by IL-4, such as Mal, contribute to functions unique to IL-4 such as the induction of Th2 differentiation among T lymphocytes.

Most of the genes that we found to be induced by γc-dependent cytokines were also induced by the combination of PMA plus ionomycin (74%), and many were induced after BCR stimulation of B lymphocytes (57%). One of these genes, the IL-2Rα gene is regulated by at least five positive regulatory regions (PRRs) (15, 37), and suggests that DUSP5 regulation of MAPK is a general theme in T lymphocyte activation and signaling.

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