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Two Discrete Regions of Interleukin-2 (IL2) Receptor β Independently Mediate IL2 Activation of a PD98059/Rapamycin/ Wortmannin-insensitive Stat5a/b Serine Kinase*

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Many cytokines, hormones, and growth factors activate Janus kinases to tyrosine phosphorylate select members of the Stat transcription factors. For full transcriptional activation, Stat1 and Stat3 also require phosphorylation of a conserved serine residue within a mitogen-activated protein kinase phosphorylation consensus site. On the other hand, two recently identified and highly homologous Stat5a and Stat5b proteins lack this putative mitogen-activated protein kinase phosphorylation site. The present study set out to establish whether Stat5a and Stat5b are under the control of an interleukin-2 (IL2)-activated Stat5 serine kinase. We now report that IL2 stimulated marked phosphorylation of serine and tyrosine residues of both Stat5a and Stat5b in human T lymphocytes and in several IL2-responsive lymphocytic cell lines. No Stat5a/b phosphothreonine was detected. Phosphoamino acid analysis also revealed that Stat5a/b phosphotyrosine levels were maximized within 1–5 min of IL2 stimulation, whereas serine phosphorylation kinetics were slower. Interestingly, IL2-induced serine phosphorylation of Stat5a differed quantitatively and temporally from that of Stat5b with Stat5a serine phosphorylation leveling off after 10 min and the more pronounced Stat5b response continuing to rise for at least 60 min of IL2 stimulation. Furthermore, we identified two discrete domains of IL2 receptor β (IL2Rβ) that could independently restore the ability of a truncated IL2Rβ mutant to mediate Stat5a/b phosphorylation and DNA binding to the γ-activated site of the β-casein gene promoter. These observations demonstrated that there is no strict requirement for one particular IL2Rβ region for Stat5 phosphorylation. Finally, we established that the IL2-activated Stat5a/b serine kinase is insensitive to several selective inhibitors of known IL2-stimulated kinases including MEK1/MEK2 (PD98059), mTOR (rapamycin), and phosphatidylinositol 3-kinase (wortmannin) as determined by phosphoamino acid and DNA binding analysis, thus suggesting that a yet-to-be-identified serine kinase mediates Stat5a/b activation.

Interleukin-2 (IL2)[1] is a key regulator of normal immune function and acts on a variety of lymphoid cell types including T lymphocytes, B lymphocytes, and natural killer cells (1). IL2-induced effects are mediated by heterodimerization of two related transmembrane proteins of the hematopoietin receptor family that are designated IL2 receptor β- and γ-chains (IL2Rβ and ILR2γ) (2–4). In addition to this pair of essential receptor subunits, a third non-conforming protein with a short cytoplasmic domain (TAC or IL2Rα) represents an accessory receptor subunit that can serve as a positive affinity modulator through its regulated expression (5–7). IL2-induced dimerization of IL2Rβ and IL2Rγ results in stimulation of the receptor-associated Janus kinases (JAK) JAK1 and/or JAK3 through intermolecular transphosphorylation (8, 9). Activation of JAKs initiates intracellular signaling cascades that include Src tyrosine kinases, phosphatases, serine-threonine kinases, and a family of transcription factors known as signal transducers and activators of transcription (Stats). Stats act in concert with other transcription factors to control cell growth and differentiation (10, 11).

At present, seven members of the Stat transcription factor family have been identified (10, 12). Stat proteins are characterized by a central DNA-binding motif, a COOH-terminal transactivation domain, a Src homology (SH) domain 2, and an SH3-like domain (12). Current models hold that newly phosphorylated tyrosine residues within activated receptor complexes direct the recruitment of Stats from the cytoplasm via their SH2 domains (13). Subsequently, JAK enzymes catalyze Stat tyrosine phosphorylation, which facilitates dimerization and disengagement of Stats from the receptor complex. Serine phosphorylation of Stat1α and Stat3 was also recently reported to be critical for interferon-induced nuclear translocalization and maximal transcriptional activation (14). Serine-threonine kinases of the mitogen-activated protein kinase (MAPK) family were suggested to perform this function (14, 15). In support of this proposal, mutation of the serine residue of a conserved MAPK consensus phosphorylation site (*X*-Pro-*X*-Ser-Pro) (corresponding to Ser^{727} of human Stat1α) to alanine abolished interferon-γ- and interferon-α-induced serine phosphorylation of Stat1α and Stat3, respectively (15). Moreover, the MAPK

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[1] The abbreviations used are: IL2, interleukin-2; IL2R, IL2 receptor; Stat, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; JAK, Janus kinase; MEK, MAPK kinase; SH, Src homology.

ERK2 reportedly binds to the α-chain of the interferon-α/β receptor and coprecipitates with Stat1α in an interferon-β-inducible manner (16). However, the involvement and significance of MAPKs as general Stat serine kinases are still controversial (17).

In contrast to Stat1 and Stat3, two more recently identified and highly homologous Stat5 proteins do not contain this conserved putative MAPK phosphorylation site (18–22). Stat5 was originally identified as a prolactin-responsive mammary gland factor (20) but has since been found to be regulated by a variety of cytokines including IL2–5, IL7, IL9, IL13, IL15, thrombopoietin, erythropoietin, growth hormone, and granulocyte-macrophage colony-stimulating factor (12, 23). Many of these factors also activate the Ras/MAPK pathway to regulate a number of cellular events including cell growth and differentiation as well as other physiological responses. However, the Ras/MAPK pathway does not appear to be crucial for IL2-induced cell proliferation or Stat5 activation (24, 25). It is therefore possible that Stat5a and Stat5b activities are regulated differently from other Stats and may not be inducibly phosphorylated on serine residues. Using IL2 (a potent activator of Stat5), the present study specifically set out to establish whether Stat5a and Stat5b are under the control of an IL2-activated Stat5 serine kinase. We now report that IL2 markedly induced phosphorylation of both Stat5a and Stat5b on serine and tyrosine but not on threonine residues in several target cell lines tested including normal human T lymphocytes. Moreover, we suggest that these phosphorylation events are mediated independently from several known IL2-activated signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal rabbit antisera were raised against peptides derived from the unique COOH termini of Stat5a and Stat5b as described previously (21) or from R & D Systems (catalog no. PA-ST5A or PA-ST5B). These antibodies recognized mouse, rat, and human forms of Stat5a or Stat5b and were used for immunoprecipitation and immunoblotting.

Cell Culture and Treatment—The rat T cell lymphoma cell line Nb2-11C or human T lymphocytes obtained from normal donors (26) were grown in RPMI 1640 medium containing 10% fetal calf serum (Sigma, catalog no. F 2442), 2 mM L-glutamine, 5 mM HEPES buffer, pH 7.3, and penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively). The T lymphocytes were activated for 72 h with phytohemagglutinin (1 μg/ml) and were subsequently made quiescent by washing and incubating for 24 h in RPMI 1640 medium containing 1% fetal calf serum before exposure to cytokines. Nb2 cells were quieted for 24 h in the above medium except that 10% gelded horse serum was substituted for fetal calf serum. The IL3-dependent murine Ba/F3 cell clones expressing various IL2Rβ mutants were generated and cultured as described previously (27) in RPMI 1640 medium with 10% fetal calf serum supplemented with 1,300 units/ml hygromycin B (Sigma, catalog no. H 3274) and 2% WEHI-3B supernatant as a source of IL3. Cells were stimulated with 100 nM recombinant human IL2 (Hoffmann-La Roche) at 37 °C as indicated in the corresponding figure legends. Cell pellets were frozen at −70 °C.

Solubilization of Membrane Proteins and Immunoprecipitation—Frozen cells were thawed on ice and solubilized in lysis buffer (10^8 cells/ml) containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 200 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin. Cell lysates were rotated end over end at 4 °C for 60 min, and insoluble material was pelleted at 12,000 × *g* for 20 min. Depending on the experiment, supernatants were incubated by rotating end over end for 2 h at 4 °C with either Stat5a or Stat5b antibodies (5 μl/ml). Antibodies were captured by incubation for 30 min with protein A-Sepharose beads (Pharmacia Biotech Inc.). Precipitated material was eluted by boiling in SDS-sample buffer for 4 min and was subjected to 7.5% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membrane (Immobilon, Millipore, catalog no. 1PVH 00010) as described previously (28).

[^{32}P]Orthophosphate Labeling and Phosphoamino Acid Analysis—Nb2-11C, phytohemagglutinin-activated human T lymphocytes, or Ba/F3 cells were metabolically labeled with 0.75 mCi/ml [^{32}P]orthophosphate (DuPont NEN) for 2 h at 37 °C and stimulated with 100 nM IL2 for up to 60 min. Cells used for kinase inhibitor experiments were pre-incubated for 1 h with either Me_2SO as a mock control, 100 μM PD98059 (New England Biolabs, Inc., catalog no. 9900L), 10 nM rapamycin (Calbiochem, catalog no. 553210-Q), or 100 nM wortmannin (Calbiochem, catalog no. 681675-Q). After treatment with IL2 (see figure legends for times), cells were lysed and immunoprecipitated as described above. Proteins were eluted from protein A-Sepharose beads, separated on SDS-PAGE (7.5% polyacrylamide), and transferred to polyvinylidene difluoride membranes. Labeled proteins were visualized by autoradiography and analyzed by phosphoamino acid analysis as described previously (29). Labeled Stat5a and Stat5b proteins were excised from polyvinylidene difluoride membranes and exposed to limited hydrolysis in 6 N HCl at 110 °C for 90 min. Samples were then dried, resuspended in water with phosphoamino acid standards, and spotted onto a thin layer cellulose acetate gel. One-dimensional thin layer electrophoresis was performed at 1500 V for 40 min in buffer containing pyridine:acetic acid:water at a 10:100:1890 ratio. Standards were visualized with ninhydrin, and samples were analyzed by autoradiography. Densitometric quantitation of individual phosphoamino acids were performed using a Molecular Dynamics PhosphorImager:SF. Counts/min volumes were normalized against the background and plotted as arbitrary units.

Electrophoretic Mobility Shift Assay (EMSA)—Ba/F3 cell clones expressing various IL2Rβ mutants and treated as described were pelleted by centrifugation and immediately solubilized in EMSA lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.2% Nonidet P-40, 1 mM orthovanadate, 25 mM sodium fluoride, 200 μM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin. Lysates were incubated on ice for 20 min and clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. For the EMSA (30), 1 μg of ^{32}P-labeled oligonucleotide corresponding to the β-casein gene sequence (5′-AGATTTCTAGGAATTCAATCC-3′) were generated by end labeling and incubated with 10 μg of protein from cellular lysates in 30 μl of binding mixture (50 mM Tris-Cl, pH 7.4, 25 mM $MgCl_2$, 5 mM dithiothreitol, 50% glycerol) at room temperature for 20 min, and samples were pre-incubated with 1 μl of either normal rabbit serum or antisera specific to Stat5a or Stat5b transcription factors as indicated. Polyacrylamide gels (5%) containing 5% glycerol and 0.25 × Tris borate/EDTA were pre-run in 0.25 × Tris borate/EDTA buffer at 4–10 °C for 1.5 h at 270 V. After loading of samples, the gels were run at room temperature for approximately 3 h at 250 V. Gels were dried by heating under vacuum conditions and exposed to x-ray film (X-Omat, Kodak).

RESULTS

IL2 Stimulates Phosphorylation of Tyrosine and Serine Residues of Stat5a and Stat5b in Target Cells—To establish whether Stat5a and Stat5b are under control of an IL2-activated serine kinase, the phosphorylation status of Stat5a and Stat5b was first analyzed in activated human T lymphocytes and the rat Nb2 lymphoma cell line. Cells were metabolically labeled with [^{32}P]orthophosphate and incubated with or without IL2 for 10 min (Fig. 1). Stat5a and Stat5b were individually immunoprecipitated from cell lysates and separated by SDS-PAGE (Fig. 1, *lower panel*). This was followed by phosphoamino acid analysis after acid hydrolysis of the isolated Stat5a and Stat5b proteins (Fig. 1, *upper panel*). As seen in Fig. 1, IL2 stimulated incorporation of phosphate into Stat5a and Stat5b in either cell type, and the accompanying phosphoamino acid analysis specifically established that both Stat5a and Stat5b were inducibly phosphorylated on serine and tyrosine but not on threonine residues (Fig. 1, *upper panel*). This was also the case in the human natural killer cell line YT (data not shown) and the mouse pro-B cell line Ba/F3 that had been stably transfected with human IL2Rβ (see Fig. 3). We therefore conclude that Stat5a and Stat5b are under the control of an IL2-activated serine kinase. Furthermore, similar results were obtained when the same cells (Nb2 and T lymphocytes) were stimulated with either IL7 or IL9 (data not shown) thus suggesting that combined tyrosine and serine phosphorylation of Stat5a and Stat5b represents a general mechanism of cytokine regulation.

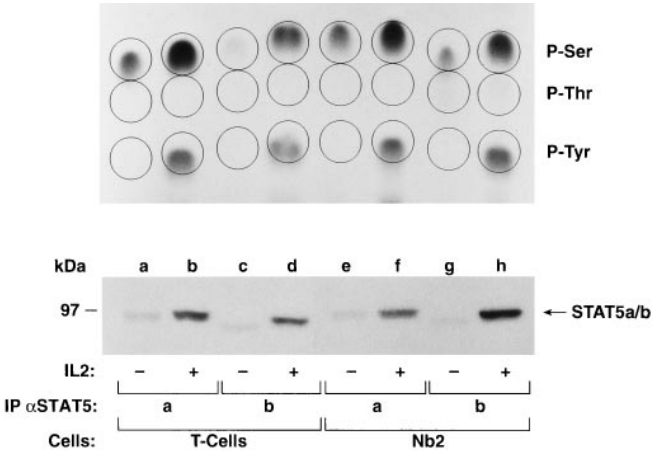

FIG. 1. **IL2-induced serine and tyrosine phosphorylation of Stat5a and Stat5b in human T lymphocytes and in the rat Nb2-11C cell line.** Phytohemagglutinin-activated human T lymphocytes or Nb2-11C cells were metabolically labeled with [^{32}P]orthophosphate and stimulated with (+) or without (−) 100 nM IL2 for 10 min. The *lower panel* shows an autoradiography of Stat5a or Stat5b isolated from clarified cell lysates by immunoprecipitation with specific polyclonal antibodies (*lanes a*, *b*, *e*, and *f*, αStat5a; *lanes c*, *d*, *g*, and *h*, αStat5b). The proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and exposed to film. The *arrow* denotes Stat5a or Stat5b proteins. The molecular mass marker (*kDa*) is indicated on the *left*. The *upper panel* shows the phosphoamino acid analysis of Stat5a or Stat5b proteins excised from the blot shown in the *lower panel* that were subjected to acid hydrolysis and thin-layer electrophoresis. Migrational locations of phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), or phosphotyrosine (*P-Tyr*) are *circled* and indicated on the *right*.

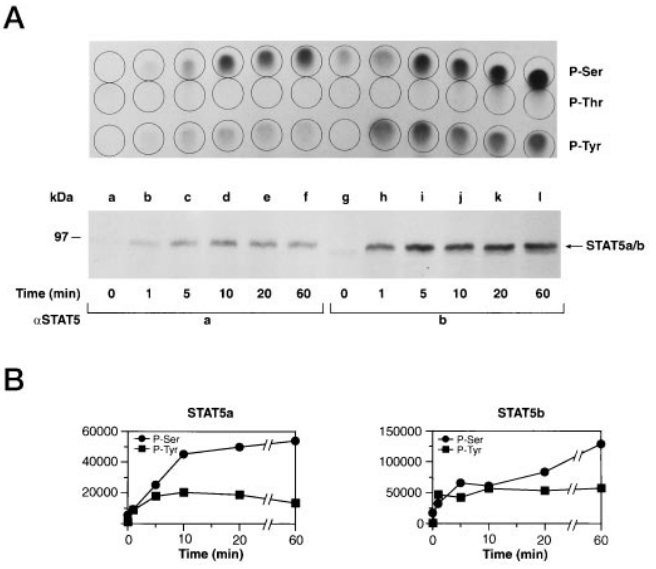

FIG. 2. **IL2-modulated serine and tyrosine phosphorylation kinetics of Stat5a and Stat5b in human T lymphocytes.** Phytohemagglutinin-activated human T lymphocytes were metabolically labeled with [^{32}P]orthophosphate and stimulated with 100 nM IL2 from 0 to 60 min at 37 °C. The *lower section* of *Panel A* shows an autoradiography of Stat5a or Stat5b isolated from cell lysates by immunoprecipitation with anti-Stat5a (*lanes a–f*, αStat5a) or anti-Stat5b (*lanes g–l*, αStat5b) antibodies. The *arrow* denotes Stat5a or Stat5b. The molecular mass marker (*kDa*) is indicated on the *left*. The *upper section* of *Panel A* shows the phosphoamino acid analysis of Stat5a or Stat5b proteins excised from the blot shown in the lower section. Migrational locations of phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), or phosphotyrosine (*P-Tyr*) are circled and indicated on the *right*. *Panel B* displays the densitometric values of Stat5a and Stat5b tyrosine and serine phosphorylation kinetics (shown in *Panel A*, *upper section*). The *ordinate* represents time of IL2 stimulation (min), and the *abscissa* displays counts of [^{32}P]phosphoserine and [^{32}P]phosphotyrosine residues obtained by quantifying phosphoamino acids using a Molecular Dynamics PhosphorImager:SF with counts/min volumes normalized against the background and plotted as arbitrary units. Tyrosine and serine phosphorylation is indicated by *squares* and *circles*, respectively.

IL2-induced Serine Phosphorylation Kinetics of Stat5a Differ from Stat5b—To assess whether IL2-regulated phosphorylation of serine and tyrosine residues of Stat5a and Stat5b differed in extent and kinetics, we compared time courses of Stat5a and Stat5b phosphorylation status during IL2 stimulation of activated human T lymphocytes. Cells were metabolically labeled with [^{32}P]orthophosphate and were then stimulated with IL2 for up to 60 min. Cell pellets were lysed and immunoprecipitated with antibodies to either Stat5a or Stat5b. SDS-PAGE analysis (Fig. 2*A*, *lower panel*) showed that IL2 stimulated general phosphorylation of Stat5a (*lanes a–f*) and Stat5b (*lanes g–l*) within 1 min. Whereas incorporation of phosphate into Stat5a reached maximal levels within 10 min, Stat5b continued to incorporate phosphate during the entire 60-min period (Fig. 2*A*, *lower panel*). This difference in IL2-induced phosphorylation of Stat5a and Stat5b could be resolved further by phosphoamino acid analysis.

The corresponding analysis of individual phosphoamino acids is shown in the *upper panel* of Fig. 2*A*. First, the results demonstrated that IL2 induced rapid serine and tyrosine phosphorylation of both proteins. In contrast, no threonine phosphorylation of Stat5a or Stat5b was detected over the entire time course. The isotope incorporated into serine and tyrosine residues was quantitated by PhosphorImager:SF analysis, and the results are plotted as line diagrams in Fig. 2*B*. The incorporation of phosphate into tyrosine residues of Stat5a and Stat5b was rapid and reached maximal levels within 1–5 min. In contrast, incorporation of phosphate into serine residues was more protracted and differed kinetically between Stat5a and Stat5b. Whereas serine phosphorylation of Stat5a reached a plateau after 10 min, Stat5b phosphoserine continued to accumulate over the entire 60-min period. The continued increase in total Stat5b phosphate content beyond 10 min (Fig. 2*A*, *lower panel*) could be ascribed to increasing levels of phosphoserine (Fig. 2, *A* and *B*).

The data shown in Fig. 2 are also representative of a series of experiments that suggest that the extent of IL2-induced incorporation of phosphate into Stat5b is higher than that of Stat5a in activated human T lymphocytes. Parallel immunoblot analysis showed that the levels of Stat5a and Stat5b proteins in activated human T lymphocytes were comparable (not shown). A similar preferential induction of phosphorylation of Stat5b by IL2 was seen in Nb2 pre-T lymphoma cells (Fig. 1, *lanes e–h*), the human natural killer cell line YT (not shown), and the mouse pro-B cell line Ba/F3 (Fig. 3*B*). Further studies are needed to determine if this differential phosphorylation of Stat5a and Stat5b by IL2 is due to preferential selectivity of IL2 receptor complexes for Stat5b or whether Stat5b contains more phosphorylated serine residues.

JAK3 and JAK1 are presumed to be the IL2-activated Stat5 tyrosine kinases. The temporal relationship between IL2-induced phosphorylation of Stat5a and Stat5b on tyrosine *versus* serine residues with rapid tyrosine phosphorylation and more protracted serine phosphorylation kinetics is consistent with an activation of JAK3/JAK1 before activation of the Stat5 serine kinase. However, serine and tyrosine phosphorylation of Stat5 molecules may occur independently. Since we previously had established that the COOH-terminal region of IL2Rβ was not required for JAK3 activation, we next investigated whether this domain was required for activation of the Stat5a/b serine kinase.

Two Discrete Domains of IL2Rβ Can Independently Mediate Stat5a/b Serine Phosphorylation and DNA Binding Activi-

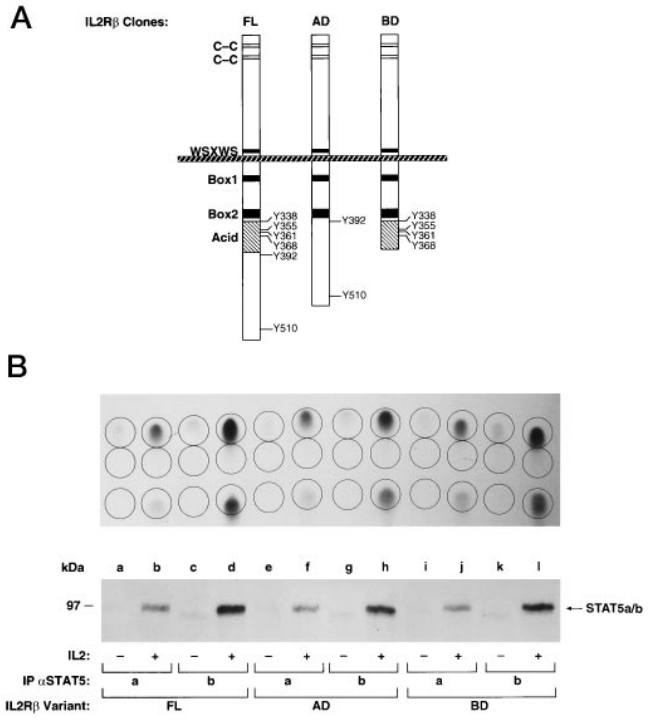

FIG. 3. **IL2 activation of the Stat5a/b serine kinase does not require the Shc binding site (Tyr^{338}), the acid-rich domain, or the COOH terminus of IL2R*β*.** *Panel A* reviews the structure of three human IL2Rβ mutants stably introduced into the murine IL3-dependent Ba/F3 cell line. The full-length (*FL*) receptor was not modified, the AD mutant had an internal deletion corresponding to amino acids Gln^{315}-Pro^{384}, and the BD mutant was truncated at the amino acid position Leu^{385}. The inactive mutant SD, which is truncated at Gln^{314} (27), is not shown. The diagram also depicts the relative position of the homology boxes 1 (*Box1*) and 2 (*Box2*), the acid-rich region (*Acid*) as well as the conserved tyrosine residues. Ba/F3 cells expressing IL2Rβ mutants FL, AD, and BD were metabolically labeled with [^{32}P]orthophosphate and stimulated with (+) or without (−) 100 nM IL2 for 10 min. The *lower section* of *Panel B* represents an autoradiography of Stat5a (*lanes a*, *b*, *e*, *f*, *i*, and *j*, αStat5a) or Stat5b (*lanes c*, *d*, *g*, *h*, *k*, and *l*, αStat5b) isolated from clarified cell lysates by immunoprecipitation with specific polyclonal antibodies and separated by SDS-PAGE. The *arrow* denotes Stat5a or Stat5b. The molecular mass marker (*kDa*) is indicated on the *left*. The upper section of *Panel B* shows the phosphoamino acid analysis of Stat5a or Stat5b that had been excised from the blot shown underneath and subjected to acid hydrolysis and thin-layer electrophoresis. Migrational locations of phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), or phosphotyrosine (*P-Tyr*) are *circled* and indicated on the *right*.

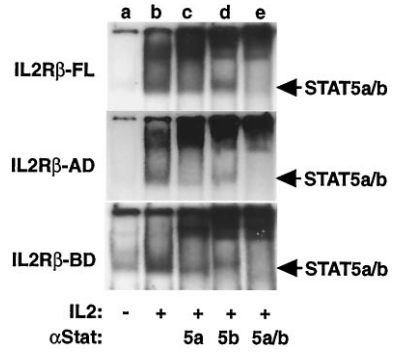

FIG. 4. **Stat5a/b electrophoretic mobility shift assay of IL2R*β* variants probed with an oligonucleotide corresponding to the *β*-casein gene promoter.** IL2Rβ-transfected Ba/F3 cells including FL (*upper panel*), AD (*middle panel*), or BD (*lower panel*) were incubated with medium (−) or 100 nM IL2 (+) for 10 min at 37 °C, and lysates corresponding to 10 μg of protein were incubated with either normal rabbit serum (*lane b*), α-Stat5a (*lane c*), α-Stat5b (*lane d*), or α-Stat5a plus α-Stat5b (*lane e*) in combination with an oligonucleotide probe corresponding to the prolactin response element of the β-casein gene promoter (*lanes a–e*). Migrational locations of Stat5a/b are indicated by the *arrows* on the *right*.

ty—We stably introduced a series of IL2Rβ variants into the murine IL3-dependent lymphoblastoma line Ba/F3 (27). The structures of wild-type (FL) IL2Rβ and mutants AD and BD are reviewed in Fig. 3*A*. Previous analysis had shown that Ba/F3 cells expressing wild-type (FL) receptors or mutant receptor forms devoid of either the acid-rich region (AD) or the COOH terminus (BD) were capable of mediating IL2-induced proliferation and Jak3 activation (9, 27). Stable cell clones expressing these forms of IL2Rβ were metabolically labeled with [^{32}P]orthophosphate and incubated with or without IL2 for 10 min. The cells were then lysed, and Stat5a or Stat5b were immunoprecipitated with appropriate antibodies. Analysis of immunoprecipitated Stat5 proteins by SDS-PAGE (Fig. 3*B*, *lower panel*) showed that IL2-induced Stat5a/b phosphorylation was mediated by FL (*lanes a–d*), AD (*lanes e–h*), and BD (*lanes i–l*) forms of IL2Rβ. On the other hand, a mutant of IL2Rβ (designated SD) that lacks both the acid-rich region and the COOH terminus failed to mediate inducible phosphorylation of Stat5a or Stat5b (not shown). We have previously demonstrated that the SD mutant does not mediate JAK1/JAK3 activation or proliferative signals (9, 27). Specific phosphoamino acid analysis of inducibly phosphorylated Stat5a and Stat5b proteins from IL2-stimulated Ba/F3 cells expressing either FL, AD, or BD receptors revealed that each of these biologically active receptor variants were correspondingly competent to mediate IL2-induced serine and tyrosine phosphorylation (Fig. 3*B*, *upper panel*). The present observation that BD is capable of mediating Stat5a/b phosphorylation (Fig. 3*B*, *lanes i–l*) is consistent with the previously proposed roles of Tyr^{392} and Tyr^{510} of IL2Rβ as essential Stat5 docking sites (9, 31, 32). However, we have noted an approximately 50% lower efficiency of BD to mediate Stat5a/b tyrosine phosphorylation as compared with FL and AD by antiphosphotyrosine immunoblotting of immunoprecipitated Stat5a/b proteins (not shown). It is therefore possible that the improved assay sensitivity by the [^{32}P]orthophosphate-based method explains the failure of these initial reports to detect additional regions of IL2Rβ that can mediate Stat5 phosphorylation. Based upon the present data, we conclude that two non-overlapping regions of IL2Rβ can reconstitute the loss of ability of the truncated SD mutant to mediate Stat5a/b serine and tyrosine phosphorylation. This also demonstrates that neither region is strictly required for the IL2-activated Stat5 serine kinase.

We next investigated the ability of Ba/F3 IL2Rβ variants to induce Stat5a/b DNA binding to an oligonucleotide probe corresponding to the prolactin response element of the β-casein gene promoter (Fig. 4). IL2Rβ clones designated FL (*upper panel*), AD (*middle panel*), and BD (*lower panel*) were treated as described in Fig. 3 and then lysed and clarified by centrifugation and incubated with a ^{32}P-labeled β-casein probe in the absence or presence of specific Stat5a/b antisera. Each IL2Rβ clone that was stimulated with IL2 displayed formation of a similar DNA complex (*lane b*). Moreover, this complex could be partially supershifted with anti-Stat5a (*lane c*) or anti-Stat5b (*lane d*) sera alone or completely when both antibodies were used in combination (*lane e*). Visualization of the BD-mediated Stat5b·DNA complex required a 2-fold longer exposure in the EMSA than complexes induced via AD and FL receptor forms, which is consistent with a somewhat lower efficiency of BD to mediate Stat5 activation as discussed above. Nonetheless, both BD and AD are capable of mediating IL2-induced Stat5a/b DNA binding, which demonstrates that two non-overlapping regions of IL2Rβ are independently competent to activate Stat5a/b.

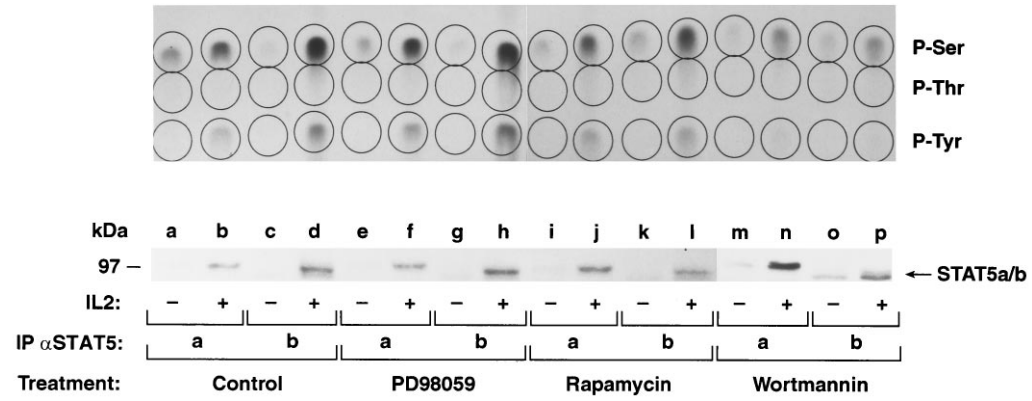

FIG. 5. **IL2-induced activation of the Stat5a/b serine kinase in Ba/F3-AD cells is not blocked by rapamycin, wortmannin, or PD98059.** Ba/F3 cells stably expressing IL2Rβ mutant AD cells were metabolically labeled with [^{32}P]orthophosphate for 2 h, treated for an additional hour with either Me_2SO as a mock control (*lanes a–d*), 100 μM PD90859 (*lanes e–h*), 10 nM rapamycin (*lanes i–l*), or 100 nM wortmannin (*lanes m–p*). Cells were then stimulated with (+) or without (−) 100 nM IL2 for 10 min. The *lower section* shows an autoradiography of Stat5a (*lanes a*, *b*, *e*, *f*, *i*, *j*, *m*, and *n*, αStat5a) or Stat5b (*lanes c*, *d*, *g*, *h*, *k*, *l*, *o*, and *p*, αStat5b) isolated from clarified cell lysates by immunoprecipitation with specific polyclonal antibodies and separated by SDS-PAGE. The *arrow* denotes Stat5a or Stat5b. The molecular mass marker (*kDa*) is indicated on the *left*. The *upper section* shows incorporation of labeled phosphate by phosphoamino acid analysis of Stat5a/b as indicated under "Experimental Procedures." Migrational locations of phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), or phosphotyrosine (*P-Tyr*) are *circled* and indicated on the *right*.

Lastly, our analysis showed that mediation of IL2-induced serine phosphorylation of Stat5a/b was uncompromised in the AD mutant, which is devoid of Tyr^{338}. This tyrosine residue represents a docking site for the adapter protein SHC and has been shown to be critical for the coupling of IL2 receptors to the Ras/MEK/MAPK pathway (33, 34). Taken together with the absence of classical MAPK consensus phosphorylation sites from Stat5a/b, this observation suggests that the Stat5 serine kinase is not MAPK.

IL2-activated Stat5a/b Serine Kinase Is Insensitive to Inhibitors of Known IL2-activated Kinases MEK1/MEK2, PI3K, and mTOR—IL2 is a recognized activator of several kinases including ERK1/2, PI3K, and p70^{S6K} (25, 35–38). To investigate the involvement of these kinases or their downstream counterparts in IL2-induced Stat5a/b serine phosphorylation, we tested the effect of selective inhibitors on IL2-mediated phosphorylation of Stat5a/b. For these studies we used Ba/F3 cells stably expressing the IL2Rβ variant AD, since this receptor mutant is capable of mediating Stat5a/b serine phosphorylation but lacks the SHC/Ras/MAPK coupling site. Ba/F3-AD cells were metabolically labeled with [^{32}P]orthophosphate and treated with inhibitors of MEK1/MEK2 (100 μM PD98059), mTOR (10 nM rapamycin) (37–39), or PI3K (100 nM wortmannin) for 1 h before stimulation with or without IL2 for 10 min. Stat5a and Stat5b proteins were independently immunoprecipitated from the cell lysates and were analyzed for inducible serine phosphorylation (Fig. 5). The phosphoamino acid analysis is shown in the *upper panel*, whereas total incorporated phosphate into Stat5a/b is shown in the *lower panel*. Compared with control samples (Fig. 5, *lanes a–d*), pretreatment with either PD98059 (*lanes e–h*) or rapamycin (*lanes i–l*) had no significant effects on IL2-induced Stat5a or Stat5b serine or tyrosine phosphorylation. Wortmannin pretreatment of cells (*lanes m–p*) was associated with moderate inhibition of IL2-induced incorporation of radiolabeled phosphate into Stat5a/b. However, this was a non-selective inhibition of both serine and tyrosine phosphorylation residues, suggesting that wortmannin may reduce the overall cell labeling efficiency or be generally toxic rather than specifically inhibiting a Stat5a/b serine kinase. From these experiments we conclude that IL2 stimulates Stat5a/b serine phosphorylation via serine kinases other than and independent of MEK1/2, mTor, and PI3K. These include the MEK-dependent serine kinases ERK1/2 and the PI3K- and/or mTOR-activated p70^{S6K}. To substantiate this conclusion, we assessed the effect of these inhibitors on IL2-induced Stat5a/b binding to DNA.

Rapamycin, Wortmannin, or PD98059 Pretreatment Does Not Affect IL2-inducible Stat5a/b DNA Binding—IL2Rβ-AD cells were pre-treated with either Me_2SO-control (Fig. 6, *upper panel*), 100 μM PD98059 (*second panel*), 10 nM rapamycin (*third panel*), or 100 mM wortmannin (*lower panel*) were stimulated without or with 100 nM IL2 for 10 min, and lysates were assayed with a ^{32}P-labeled β-casein promoter probe. EMSA analysis revealed that neither of these inhibitors affected the ability of IL2-induced Stat5a/b to form complexes with the γ-activated site of the β-casein gene promoter (*lanes a* and *b*). Thus, there was good correlation between inducible Stat5a/b serine/tyrosine phosphorylation and DNA binding activity. Moreover, the Stat5a/b complex could be partially supershifted with anti-Stat5a (*lane e*) or anti-Stat5b (*lane f*) sera alone or completely when both antibodies were used in combination (*lane g*), suggesting that the inhibitors also did not affect the relative formation of Stat5 homo- and heterodimers.

DISCUSSION

Temporal Dissociation of Stat5a/b Serine Phosphorylation from Tyrosine Phosphorylation—This study presents direct evidence for the existence of a Stat5a/b serine kinase in human T lymphocytes and lymphoid cell lines that is stimulated by IL2. In serum- or factor-deprived cells, Stat5a and Stat5b existed primarily in an unphosphorylated state but underwent rapid and marked phosphorylation of Stat5a/b on serine and tyrosine residues in response to IL2. No phosphorylation of threonine residues was observed over an expanded time course of 60 min. Tyrosine phosphorylation levels of Stat5a and Stat5b were parallel and peaked within 1–5 min and then remained elevated for at least 60 min of continued IL2 treatment. In contrast, Stat5a and Stat5b serine phosphorylation kinetics were slower, suggesting that the serine kinase is activated after tyrosine kinases.

Consistent with this notion, current models of IL2 receptor signal transduction hold that the tyrosine kinases JAK3/JAK1 are the first intracellular enzymes to be activated and that they directly phosphorylate Stat proteins on tyrosine residues (11). The rapid kinetics of Stat5a/b tyrosine phosphorylation were similar to the time response of IL2-induced JAK3 autophosphorylation observed in human T lymphocytes (9). At present, there is no evidence to suggest that JAKs are also serine kinases, and the temporal dissociation of Stat5a/b serine and

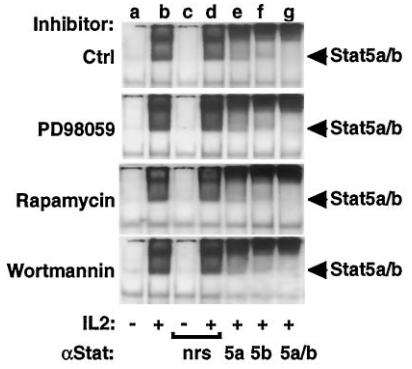

FIG. 6. **Pretreatment of IL2Rβ-AD cells with rapamycin, wortmannin, or PD98059 does not inhibit IL2-induced Stat5a/b DNA binding as judged by EMSA analysis.** IL2Rβ-AD cells were treated as described in Fig. 5 with either Me_2SO as a control (*first panel*), 100 μM PD98059 (*second panel*), 10 nM rapamycin (*third panel*), or 100 mM wortmannin (*fourth panel*) and incubated with medium (−) or 100 nM IL2 (+) for 10 min at 37 °C. Lysates corresponding to 10 μg of protein were incubated in the absence of antibody (*lanes a* and *b*), normal rabbit serum (*lanes c* and *d*), α-Stat5a (*lane e*), α-Stat5b (*lane f*), or α-Stat5a plus α-Stat5b (*lane g*) in combination with an oligonucleotide probe corresponding to the prolactin response element of the β-casein gene promoter (*lanes a–e*). Migrational locations of Stat5a/b are indicated by *arrows* on the *right*.

tyrosine phosphorylation reported here suggests that a distinct Stat5a/b serine kinase is activated subsequent to JAKs. The Stat5a and Stat5b genes are highly homologous (96%). At present, no published work to date has demonstrated functional differences between the two proteins. Interestingly, the kinetics of serine phosphorylation differed between Stat5a and Stat5b in T lymphocytes. Whereas IL2-induced incorporation of phosphate into Stat5a serine residues leveled off after 10 min, Stat5b phosphoserine levels continued to rise during the 60-min period analyzed. Thus, our data suggest that there are differences between IL2-induced Stat5a and Stat5b phosphorylation kinetics. The main implication of this dissimilarity is that Stat5a and Stat5b operate differently and therefore do not simply constitute duplicated gene products with identical functions. Support for the notion that Stat5a and Stat5b are not redundant gene products comes from Stat5a deficient mice, which are unable to lactate (40). Thus, Stat5a deficiency *in vivo* cannot be compensated for by the intact Stat5b gene.

The mechanism underlying the observed prolonged Stat5b serine phosphorylation relative to that of Stat5a is currently unclear. It may be due to phosphorylation of an additional serine residue specific to Stat5b. In support of this possibility, Stat5b undergoes a more marked mobility shift than Stat5a upon IL2 stimulation, and the levels of total incorporated phosphate into Stat5b is higher than that into Stat5a by a factor of at least 2 in each of the cell lines examined including T lymphocytes (Fig. 2), Nb2 cells (Fig. 1), and Ba/F3 cells (Figs. 3 and 5). These cell types expressed levels of Stat5a comparable to those of Stat5b as determined by immunoblotting (not shown). Further studies including targeted mutagenesis of Stat5a/b serine residues are needed to determine the exact phosphorylation sites. The cellular location where Stat5a and Stat5b become serine-phosphorylated also remains to be established. Is the Stat5a/b serine kinase associated with the activated receptor complex or does serine phosphorylation occur in the cytoplasm during the transit of Stat5a/b to the cell nucleus? Our functional analysis of cytoplasmic regions of IL2Rβ revealed that no single receptor region was responsible for mediating IL2-induced Stat5a/b serine phosphorylation. This indirectly supports the concept that the Stat5a/b serine kinase is not associated with IL2Rβ and suggests that Stat5a/b serine phosphorylation occurs at a postreceptor level. Ongoing studies are addressing this issue.

Significance of IL2Rβ Domain-specific Activation of Stat5a/b Serine Phosphorylation—To identify regions of the cytoplasmic domain of IL2Rβ required for activating the IL2-responsive Stat5a/b kinase(s), we analyzed the ability of several mutant forms of this receptor subunit to mediate IL2-induced Stat5a/b serine phosphorylation. Although we could temporally dissociate tyrosine phosphorylation from serine phosphorylation, the two phosphorylation events could not be dissociated based upon different structural requirements of the cytoplasmic domain. Mutant AD, which lacks the acid region (Asp^{315}-Asp^{384}) with the SHC binding site (Tyr^{338}) but contains the remaining COOH-terminal region (Leu^{385}-Val^{525}), was capable of mediating IL2-induced Stat5a/b tyrosine and serine phosphorylation (Fig. 3*B*). Similarly, mutant BD (which contains the acidic domain but lacks the COOH-terminal region (Leu^{385}-Val^{525}) was also capable of mediating IL2-induced Stat5a/b serine and tyrosine phosphorylation. Thus, we specifically conclude that deletion of the COOH-terminal 210 amino acids of IL2Rβ resulted in the combined loss of ability to mediate IL2-induced Stat5a/b tyrosine and serine phosphorylation. This combined loss of function could be reconstituted by fusing either of two distinct cytoplasmic regions back onto the truncated IL2Rβ mutant. Therefore, two large cytoplasmic regions of IL2Rβ can independently reconstitute IL2-induced Stat5a/b serine kinase activation.

The IL2Rβ mutant BD, which lacks the two previously reported Stat5 docking sites Tyr^{392} and Tyr^{510} (9, 31, 32), was nevertheless capable of mediating IL2-induced Stat5a/b serine and tyrosine phosphorylation. However, BD did elicit a somewhat reduced capacity to activate Stat5a/b as compared with FL and AD clones by EMSA (Fig. 4) and antiphosphotyrosine immunoblotting (data not shown). This suggests the presence of one or more suboptimal Stat5 docking sites among the remaining tyrosines (Tyr^{338}, Tyr^{355}, Tyr^{361}, and Tyr^{368}) that were previously not recognized by Stat5 analyses (9, 31, 32). Interestingly, none of these residues within IL2Rβ fits the proposed consensus Stat5 docking sequence Asp-Ala-Tyr(P) (41). It has been suggested that the SHC recruitment motif containing Tyr^{338} may provide a third Stat5 binding site based upon recent results with HT-2 murine T lymphocyte cells (42). However, it is also possible that IL2 (like many other cytokine receptors including erythropoietin (43), epidermal growth factor (44), granulocyte colony-stimulating factor (45), prolactin (46), and IL9 (47)) can mediate Stat5 activation independent of most if not all cytoplasmic receptor tyrosine residues. These reports suggest that signaling proteins such as IRS-1/2 or JAK enzymes may provide alternative binding sites for Stats.

IL2 Receptor-mediated Activation of Serine Kinases ERK1/2, mTOR, or PI3K Is Not Required for IL2-induced Serine Phosphorylation of Stat5a/b—IL2 can activate tyrosine kinases JAK3 and JAK1. Of these, JAK3 is probably the principal mediator of IL2-induced Stat5a/b tyrosine phosphorylation (9). Although the identity of the IL2-stimulated Stat5a/b serine kinase is unknown, IL2 has been shown to activate several candidate serine kinases. IL2 and many other cytokines can activate the MAPKs ERK1/2 via the SHC/Grb2/SOS/Ras/RAF-1/MEK pathway in T lymphocytes (34). However, we detected no activation of Erk1 or Erk2 by IL2 in Ba/F3 cells stably transfected with IL2Rβ (in contrast to IL3, which is a potent inducer of SHC tyrosine phosphorylation and Erk1/2 activation in these cells) as measured by both antiactive MAPK antibodies and a myelin basic protein substrate assay.[2] Despite the ab-

[2] R. A. Kirken, M. G. Malabarba, H. Rui, and W. L. Farrar, unpublished data.

sence of an IL2-stimulated Erk1/2 response in IL2 receptor-expressing Ba/F3 cells, IL2-induced Stat5a/b serine phosphorylation levels were comparable to those of T lymphocytes (Figs. 2 and 3). Deletion of the acid-rich region of IL2Rβ (AD mutant), which couples IL2Rβ to the SHC/ERK1/2 pathway in other cells (33, 34), also did not affect the extent of IL2-induced Stat5a/b serine phosphorylation in Ba/F3 cells. Furthermore, the SHC/ERK1/2 incompetent AD mutant of IL2Rβ also mediated IL2-induced Stat5a/b serine phosphorylation and DNA binding activity to the β-casein probe (Figs. 5 and 6) in the presence of PD98059, a MAPK kinase (MEK1/MEK2) inhibitor. Thus, we conclude from these studies of IL2 receptor signaling in Ba/F3 cells that Erk1 and Erk2 are not IL2-induced Stat5a/b serine kinases and that neither Mek1/2 nor the acid-rich region of IL2Rβ are required for IL2-induced Stat5a/b serine phosphorylation.

Other potential IL2-activated Stat5a/b serine kinases are $p70^{S6K}$, PI3K, and mTOR. Recent evidence has suggested that IL2-induced activation of $p70^{S6K}$ is mediated via p85-p110 PI3K or through the recently identified serine kinase related to PI3K, mTOR (39). Both p85-p110 PI3K and mTOR belong to an increasing family of PI3K homologues that are inactivated by rapamycin or wortmannin (48). However, pretreatment of IL2-responsive Ba/F3 cells with high concentrations of either rapamycin or wortmannin did not significantly affect IL2-induced Stat5a/b serine phosphorylation or binding to the β-casein gene promoter (Figs. 5 and 6). We therefore conclude that the IL2-activated Stat5a/b serine kinase is not critically dependent upon ERK1/2, $p70^{S6K}$, or PI3K/mTOR kinase or its associated pathways. Consistent with this view, a separate study showed that the inhibitor wortmannin did not block IL2-induced Stat5 DNA binding and transcriptional activation in Kit225 cells (25).

In conclusion, we have demonstrated that Stat5a and Stat5b are rapidly phosphorylated on serine and tyrosine residues in response to IL2. Stat5b appeared to be preferentially phosphorylated and displayed more protracted serine phosphorylation kinetics than Stat5a. Activation of the Stat5a/b serine kinase was not critically dependent on the COOH terminus or the acid-rich region of IL2Rβ, since either region could independently reconstitute the ability of an inactive truncated IL2Rβ mutant to mediate IL2-induced Stat5a/b serine phosphorylation. Moreover, disruption of the MAPK pathway by deletion of the Shc recruitment site of IL2Rβ or through pharmacological Mek1/2 inhibition or inhibition of PI3K- and mTor-dependent pathways failed to block IL2-induced Stat5a/b serine phosphorylation. These results provide the incentive to determine the biological role of Stat5a/b serine phosphorylation and also form the basis for a molecular and pharmacological strategy to identify the IL2-activated Stat5a/b serine kinase.

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