HMG-I(Y) Phosphorylation Status as a Nuclear Target Regulated through Insulin Receptor Substrate-1 and the I4R Motif of the Interleukin-4 Receptor*

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Interleukin (IL)-4 is a cytokine produced by T cells, mast cells, and basophils (1, 2). Although originally identified on the basis of its stimulation of B lymphocyte proliferation, the pleiotropic effects of IL-4 are now known to include regulation of proliferation, gene expression, and stable differentiation of both lymphocytic and other hematopoietic cells (2, 3). The target genes crucial for IL-4 induction of proliferation are not known, whereas genes activated in B lymphocytes by IL-4 include CD23, class II major histocompatibility antigens, molecules critical to the regulation of T cell activation by B cells, and the immunoglobulin heavy chain ε locus in its germ line arrangement (3–8). Importantly, the genes regulated by IL-4 exert regulatory influences on immune function for which the precise level of gene expression is critical (9, 10). These varied biological functions of IL-4 are regulated through one or more receptor complexes that include a high affinity binding chain, IL-4Rα, a member of the hematopoietin receptor gene superfamily (11–13). The IL-4Rα chain pairs with an accessory chain, γc, while other forms of IL-4 receptor may use a different accessory chain (13–15). Accordingly, the mechanisms by which IL-4Rα transduces signals to nuclear proteins represent a fundamental issue in understanding the functions of IL-4.

IL-4Rα is widely expressed and contains an extended cytoplasmic tail devoid of intrinsic kinase activity. Non-covalently associated kinases of the Janus kinase family, IL-4 receptor Stat6, and the Leukemia Society of America through its scholar program (to M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: IL, interleukin; IL-4R, IL-4 receptor; Stat, signal transduction and activation of transcription; IRS, insulin receptor substrate; Ge, germ line Cε (region of immunoglobulin heavy chain locus); Pl 3-K, phosphatidylinositol 3-kinase; hu, human; m, mouse; W.T., wild type; PAGE, polyacrylamide gel electrophoresis; PSL, arbitrary density units.

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Stat6 remain unclear. One potential target for Stat6-independent regulation is the non-histone chromosomal protein HMG-I(Y). HMG-I(Y) is a nuclear protein involved in the signal-induced regulation of multiple genes, including virus activation of interferon β, IL-1 activation of E-selectin, induction of IL-2Rα chain and repression of IL-4 transcription in activated T cells, and regulation of the germ line immunoglobulin epsilon (Ge) promoter (30–36). The phosphorylation status of HMG-I(Y) is regulated in response to IL-4, and the phosphorylated form of HMG-I(Y) exhibits a lower affinity for Ge promoter DNA in vitro, suggesting that phosphorylation decreases the repressor effect of HMG-I(Y) at the Ge promoter in B lymphocytes (37). Since HMG-I(Y) phosphorylation status is regulated through a pathway that appeared independent from Stat6 activation but sensitive to the immunosuppressive agent rapamycin (37, 38), we formulated the hypothesis that this phosphorylation is regulated through the I4R motif rather than the membrane-distal phosphotyrosine residues implicated in Stat6 activation. To investigate this hypothesis, the ability of wild-type and mutant forms of the IL-4 receptor to regulate HMG-I(Y) phosphorylation was measured. Because the I4R motif is linked to IRS-1 recruitment and activation of the lipid kinase PI 3-kinase, we also investigated the requirement for these signal transduction elements in IL-4-inducible HMG-I(Y) phosphorylation.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling—Stable transfectants of the IL-3-dependent myeloid progenitor line 32D, bearing wild-type or mutant human IL-4Rα chains with or without transfected rat IRS-1, have been described previously (26, 28). Specific clones used in this study were as follows: wild-type hIL-4R (W.T.; clones 8-2B4 and 8-2B4A6), full-length hIL-4R with Y497F (clones 8-5B6 and 8-5D3), and truncations whose exact ends are at residue 657 (d657), 655 (d557) (5-3WY2), 6-3W3 and 6-3W13, respectively. These cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units ml⁻¹ penicillin, 50 units ml⁻¹ streptomycin, 3 mM l-glutamine, 100 μM 2-mercaptoethanol (RP/10F is complete RPMI 1640 media with 10% fetal bovine serum), and 5% conditioned medium from WEHI-3 cells as a source of IL-3. To prepare conditioned medium, WEHI-3 cells were maintained in Iscove’s medium supplemented with 10% fetal bovine serum, 50 units ml⁻¹ penicillin, 50 units ml⁻¹ streptomycin, and 3 mM l-glutamine. After culture for 4 days in stationary phase, cell debris were removed by centrifugation and sterile filtration. For metabolic labeling experiments, 32D cells (10⁶ cells ml⁻¹) were removed from WEHI-3-conditioned medium and then cultured for 20 h in RPMI 10F. M12 B lymphoma cells transfected with a truncated hIL-2Rβ and the Chim-1 chimera of hIL-4Rα were cultured in the presence or absence of hIL-4 (5 ng ml⁻¹) for 5 min at 22 °C. The reaction was terminated by dilution in ice-cold PBS containing 100 μM NaN₃, 50 mM Hepes, pH 7.5, 0.15 M NaCl, 0.5% Nonidet P-40, followed by immunoprecipitation with a rat monoclonal antibody (clone 5C11) raised against the amino terminus of HMG-I(Y), and 5% sodium dodecyl sulfate (SDS)-PAGE. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes, cut into pieces, and scanned on a Fuji BAS 1000 phosphorimager. The phosphorylation status of HMG-I(Y) was determined by the Bradford method (Bio-Rad).

Analysis of Nuclear Proteins—After labeling as described above, cells were harvested, rinsed twice with ice-cold phosphate-buffered saline, and then P-40 in 0.5% Nonidet P-40 in 10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.1 mM EGTA, 0.5 mM sodium orthovanadate (RSB) supplemented with 20 μM leupeptin, 10 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, and 1 mM sodium fluoride. Nuclei were pelleted after lysis of the cells, and nuclear extracts were prepared as described previously (37) except for supplementation with protease and phosphatase inhibitors as above. Resolution of basic-dimensional electrophoretic on one-dimensional gels followed by SDS-PAGE was performed as described previously (37, 40). For selected experiments, the two-dimensional gels were silver-stained to detect relative protein levels, then exposed to autoradiographic film and phosphorimaging plates, whereas in other experiments aliquots of the nuclear proteins were silver-stained separately.

To quantify incorporation of 32P into phosphorylated HMG-I(Y) using a Fuji BAS 1000 phosphorimager, the density units (“PSLs”) incorporated into HMG-I(Y) in each gel were adjusted by subtracting plate background. This incorporation into HMG-I(Y) was normalized by dividing HMG-I(Y)-specific PSLs by the net PSLs in an internal reference spot that was consistently resolved from other labeled proteins. The efficiency of the hIL-4R transfected into a given 2D clone relative to the total hIL-4Rs defined as follows: ((normalized HMG-I(Y)PSLs in HMG-I(Y)transfected) / (normalized PSLs in HMG-I(Y)mouse IL-4-treated) - (normalized PSLs in HMG-I(Y)mouse IL-4-treated)). For gel shift analyses, 32D cells were cultured in RPMI 1640 medium with 10% fetal bovine serum in the absence of IL-3 for 0 or 2 h and then stimulated with IL-4 for 0.5 h. Electrophoretic mobility shift analyses were performed using a double-stranded oligonucleotide representing the Stat6 binding site spanning nucleotides −122 to −104 of the Ge promoter after preparation of whole cell or nuclear extracts, as described previously (38, 41).

Immunoprecipitation and Immunoblotting—Analyses of phosphoryrosine-containing proteins were performed as described previously (28). After pretreatment with genistein as appropriate, cells deprived of serum for 17 h at 37 °C were cultured in the presence or absence of hIL-4 (5 ng ml⁻¹) for 5 min at 22 °C. The reaction was terminated by dilution in ice-cold PBS containing 100 μM NaN₃, 50 mM Hepes, pH 7.5, 0.15 M NaCl, 0.5% Nonidet P-40, with 50 mM NaF, 10 mM NaPPi, and protease inhibitors (28), followed by immunoprecipitation of proteins in the soluble fraction using a polyclonal rabbit antiserum against rat IRS-1 (generous gift of L. M. Wang and J. Pierce, LCMRB, National Institutes of Health). Precipitates were washed in lysis buffer, dissolved in SDS sample buffer, and separated by SDS-PAGE. Resolved proteins were transferred to polyvinylidene difluoride membranes and probed with the 4G1 monoclonal antiphosphotyrosine antibody or rabbit anti-IRS-1. Bound antibodies were detected using enhanced chemiluminescence (Amersham Corp.).

RESULTS
The I4R Motif Is Essential for IL-4-induced HMG-I(Y) Phosphorylation—At least two spatially distinct motifs have been identified within the cytoplasmic domain of the IL-4 receptor α chain as follows: an I4R region originally linked to control of proliferation but not gene expression or Stat6 phosphorylation (20, 28), and dominant Stat6 docking sites distal to amino acid 557 (19, 22, 42). More recent evidence indicates that lymphocyte proliferation is impaired by Stat6 gene disruption (23–25), whereas HMG-I(Y) phosphorylation status has been correlated with proliferation of cells (43, 44). These observations are consistent with a role for either or both of the IL-4-Rα motifs in regulation of HMG-I(Y) phosphorylation status. To determine which of these IL-4-Rα motifs, the I4R or Stat6 docking motifs, mediates IL-4 regulation of HMG-I(Y) phosphorylation, the ability of wild-type and mutant forms of the human IL-4Rα (hIL-4R) to confer regulated HMG-I(Y) phosphorylation was analyzed using transfectants derived from the myeloid progenitor line 32D.

To measure IL-4 regulation of HMG-I(Y) phosphorylation, metabolic labeling experiments and two-dimensional acid-urea/SDS-PAGE analyses were performed using a panel of 32D transfectants all of which also expressed rat IRS-1. Because of
IRS-1 and IL-4R I4R Motif Regulate HMG-I(Y) Phosphorylation

![Diagram](image)

**Fig. 1.** IL-4-inducible phosphorylation of HMG-I(Y) in the 32D cell line requires an intact I4R motif. *A,* a Y497F substitution in human IL-4Rα abolishes huIL-4-inducible HMG-I(Y) phosphorylation. Stably transfected variants of the IL-3-dependent mouse myeloid progenitor cell line 32D were used for metabolic labeling experiments. Each clone expressed rat IRS-1 and either a wild-type IL-4R genitor cell line 32D were used for metabolic labeling experiments. Stably transfected variants of the IL-3-dependent mouse myeloid pro-

Masses of radiolabeled nuclear proteins derived from equal cell num-

huiIL-4 or mIL-4 as indicated. Cells cultured in 32PO4 were then cultured for 4 hi n

Function of the I4R motif has previously been shown to require a tyrosine at position 497 (Tyr-1) (28). Accordingly, to test if I4R function is required for IL-4 to regulate HMG-I(Y) phosphorylation, parallel labeling experiments were performed using cells transfected with a full-length human IL-4Ra bearing the Tyr → Phe substitution that blocks IRS-1 recruitment and inactivates the I4R motif (28). This Y497F (Y1F) substitution completely eliminated the ability of huIL-4 to induce increases in HMG-I(Y) phosphorylation in each of the two clones tested (8-5B6 and 8-5D3). In sharp contrast, the endogenous wild-type mouse IL-4R in these clones was fully competent to increase HMG-I(Y) phosphorylation (Fig. 1A, right-hand panels; Fig. 1C). This finding indicates that the failure to increase HMG-I(Y) labeling is specific for the mutant human receptor and contradicts the alternative possibility that the huIL-4R Y497F mutant leads to clones in which the basal rate of HMG-

The above data provide evidence that an intact I4R is essential for regulation of IL-4-inducible increases in HMG-I(Y) phosphorylation.

The known potential for clone-to-clone and inter-experimental variation, we employed a widely used strategy to control for these technical issues. Human and mouse IL-4 exhibit species-specific binding to their respective receptors. Thus, the effect of human IL-4 receptors expressed on a given 32D clone can be compared with the effect of the endogenous mouse IL-4 receptors, and huIL-4R expressed on different clones also can be compared. As shown in Fig. 1A, IL-4 increases the phosphor-

the first dimension followed by 15% SDS-PAGE. The acid-urea dimension ran from right to left on the horizontal axis, and SDS-PAGE was from top to bottom. The HMG-I and Y spots are indicated by double arrows on the autoradiographs. B, constant HMG-I(Y) levels among experimental samples. Representative results of silver-stained two-dimensional acid-urea/SDS gels used to resolve the nuclear ex-

Bob Spots co-migrating with the core histones (H2a, H2b, H3, and H4) demonstrated no significant phosphorylation, whereas histone H1 mig-

the ordinate plots PSLs, arbitrary units of 32P incorporation into HMG-I(Y). The phosphorylation status of other basic nuclear proteins resolved in these two-dimensional gels also was sensitive to IL-4 and de-

Human IL-4 receptors that terminate at
The basis for the apparent increase in potency of the transfected huIL-4Ra relative to the endogenous wild-type mouse receptor on each clone is not clear. However, analysis of the data without normalization to an internal reference standard did not affect the findings.

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FIG. 2. I4R-dependent regulation of HMG-I(Y) phosphorylation is quantitatively independent of nuclear Stat6 induction. A, minimal Stat6 induction by a receptor fully competent to signal HMG-I(Y) phosphorylation. The autoradiographic results of a representative gel mobility shift analysis using stable transfectants of the 32D cell line are shown. After growth in IL-3, the indicated clones were incubated in media alone (lanes 1 and 4) or media supplemented with mouse (lanes 2 and 5) or human IL-4 (lanes 3 and 6) for 0.5 h. Following preparation of whole cell extracts, assays of Stat binding activity were performed using a 32P-labeled oligonucleotide probe bearing the n4 Stat binding site from the mouse Ge promoter (38). These data were obtained using clones 8–2B4 (WT, lanes 1–3) and 3–9W5 (d557, lanes 4–6). B, the results of metabolic labeling experiments were quantified as described under "Materials and Methods" after phosphorimaging the electrophoretograms generated by two-dimensional acid-urea/SDS-PAGE. For each individual gel, normalized phosphorylation was calculated as the incorporation of phosphate into HMG-I(Y) divided by the labeling of an internal reference protein. Normalized phosphorylation induced by huIL-4, reflecting the transfected human receptor, was then divided by that induced by mIL-4, reflecting the endogenous wild-type mouse receptor. This latter value was defined as the relative efficiency, which is zero if phosphate on HMG-I(Y) was no greater than basal phosphate incorporation. As an internal control, mouse IL-4 induced significant increases in HMG-I(Y) phosphorylation over basal levels in each clone. Each bar represents the data from at least three independent experiments and two individual clones of 32D cells transfected with IRS-1 and the indicated receptor. The human receptors tested were wild-type (WT), full-length Y497F, and truncations whose end points are at residue 557 (d557) or 557 (d657). Schematic diagrams to the left show the approximate positions of Tyr-1 to Tyr-4 (residues 497, 575, 603, and 631, respectively) in the huIL-4Ra molecule.

FIG. 3. The IRS-1 is essential for IL-4 induction of HMG-I(Y) phosphorylation. A, metabolic labeling and quantitation of HMG-I(Y) phosphorylation were performed as described under "Materials and Methods," using 32D cells stably transfected with the d557 construct, alone or together with IRS-1, as indicated. Fold induction is defined as radioactivity incorporated into HMG-I(Y) after IL-4 treatment divided by that incorporated in untreated cells. The results (mean ± S.E.) for two independent clones for each construct are shown. Each data point represents the mean from three independent experiments. Open bars represent results after huIL-4 treatment, and filled bars show results for mIL-4R activation through the endogenous receptor. B, pooled results of the above experiments after normalization to an internal reference protein as described under "Materials and Methods" and Fig. 2B. Cells were cultured in media alone (open bars), human IL-4 (hatched bars), or mouse IL-4 (filled bars) as indicated.

The IRS-1 Adaptor Protein Is Required for IL-4-Inducible HMG-I(Y) Phosphorylation—Although 32D cells express functional γc chains and a normal level of mIL-4Ra (about 1000 receptors/cell), mIL-4 does not induce proliferation unless expression of the adaptor protein IRS-1 is conferred by transfection (26). Expression of IRS-1 in 32D cells significantly increases their mitogenic response to IL-4 and potentiates tyrosine phosphorylation of downstream targets such as IRS-1 and IRS-2 (4PS) in response to mIL-4 (27). Moreover, the role of IRS-1 and its phosphorylation are dependent on a functional I4R motif (28). These findings prompted us to hypothesize that IRS-1 is a component of the signal transduction pathway linking IL-4 with HMG-I(Y) phosphorylation status. The experiments presented above (Figs. 1 and 2) were performed using...
clones that express IRS-1. To investigate the requirement for IRS-1 in this pathway, we compared the ability of IL-4 to regulate HMG-I(Y) phosphorylation in 32D cells co-expressing huIL-4Rα and IRS-1 to its effect on clones expressing huIL-4Rα alone. The results (Fig. 3A) show that regulated phosphorylation occurred only when the IRS-1 protein was present. Because the phosphorylation of other nuclear proteins increased in IL-4-treated IRS-1− cells, we normalized phosphorylation of HMG-I(Y) to that of the internal reference protein. These normalized results (Fig. 3B) show that IL-4 induced a 3-fold increase in the incorporation of phosphate into HMG-I(Y) only if IRS-1 was also present. Accordingly, we conclude that IRS-1 is a component of the signaling pathway that links the IL-4Rα chain with HMG-I(Y) phosphorylation.

Wortmannin Inhibits IL-4-induced HMG-I(Y) Phosphorylation—The immunosuppressant rapamycin, which leads to selective inhibition of pp70 S6 kinase, blocks IL-4-induced HMG-I(Y) phosphorylation and reduces Ge transcription activity in the human B cell line JY (37). One molecule important in growth factor regulation of pp70 S6 kinase activity is the lipid kinase PI 3-K, which is involved in platelet-derived growth factor and insulin receptor signaling to pp70 S6 kinase (45). Tyrosine-phosphorylated IRS-1 and IRS-2 (4PS) induced by IL-4 interact with the regulatory subunit (p85) of PI 3-kinase (PI 3-K) and recruit increased PI 3-K activity to the IL-4 receptor (27). In addition, expression of IRS-1 in 32D cells enhanced the stimulation of PI 3-kinase and pp70 S6 kinase by insulin, insulin-like growth factor-1, or IL-4 (46). In light of the relationship of PI 3-K to IL-4 receptor signaling and pp70 S6 kinase activity, we used wortmannin as a selective inhibitor of PI 3-kinase (47, 48) to investigate the possible role of PI 3-K in the regulation of HMG-I(Y) phosphorylation. The inhibitor concentration was chosen in accordance with a prior determination of the dose-response relationship for inhibition of PI 3-K activity (47). This wortmannin treatment led to 95% inhibition of inducible phosphorylation of HMG-I(Y) in addition to a decrease in overall phosphorylation of HMG-I(Y) and several other basic nuclear proteins (Fig. 4A). This decrease represented a decrease in specific activity rather than a decrease in protein levels in wortmannin- and IL-4-treated cells (Fig. 4B). Consistent with its biochemical characterization as an irreversible inhibitor of PI 3-K (50), the inhibition of IL-4-inducible phosphorylation was not reversed during an overnight washout (Fig. 4A). In agreement with our previous results with the JY B cell line (37), rapamycin almost completely inhibited IL-4-inducible HMG-I(Y) phosphorylation, whereas in contrast the tyrosine kinase inhibitor genistein led to only partial inhibition (Fig. 4C). To evaluate whether these results reflected general or nonspecific toxicity of the wortmannin treatment, and to investigate if this pathway impacts Stat6 binding activity, 32D cells were subjected to overnight inhibition with wortmannin, with 100 nM wortmannin for 2 h prior to stimulation with IL-4. Prior to IL-4 stimulation, samples labeled washout were cultured 20 h in media alone after the wortmannin pretreatment. Similar results were obtained for huIL-4R(d557). B, results of silver staining of the samples shown in A. C, wortmannin and rapamycin block HMG-I(Y) phosphorylation. Quantitation of the inhibitory effect of wortmannin was performed as described in Fig. 1. Cells were separately treated with 100 nM rapamycin, 75 μM genistein, or 100 nM wortmannin (as in Ref. 47) for 2 h prior to addition of 32PPO4 and IL-4. Quantification of radiolabeled HMG-I(Y) after IL-4 treatment was performed as described in Fig. 1. Relative activity was calculated as 32P incorporated in the presence of IL-4 and an inhibitor divided by incorporation in IL-4 alone (no inhibitor). Similar results were obtained when the data were normalized to an internal reference protein as described under "Materials and Methods" and Fig. 2B. The results represent pooled data from two clones expressing each construct and the mean of three independent experiments.

![Image](https://example.com/image.png)

**Fig. 4.** Wortmannin inhibits IL-4-induced HMG-I(Y) phosphorylation. A, 32D-derived clones expressing huIL-4R (W.T.) and rat IRS-1 were subjected to metabolic labeling and two-dimensional acid-urea/SDS-PAGE as in Fig. 1A. Where indicated, cells were incubated with 100 nM wortmannin for 2 h prior to stimulation with IL-4. Prior to IL-4 stimulation, samples labeled washout were cultured 20 h in media alone after the wortmannin pretreatment. Similar results were obtained for huIL-4R(d557). B, results of silver staining of the samples shown in A. C, wortmannin and rapamycin block HMG-I(Y) phosphorylation. Quantitation of the inhibitory effect of wortmannin was performed as described in Fig. 1. Cells were separately treated with 100 nM rapamycin, 75 μM genistein, or 100 nM wortmannin (as in Ref. 47) for 2 h prior to addition of 32PPO4 and IL-4. Quantification of radiolabeled HMG-I(Y) after IL-4 treatment was performed as described in Fig. 1. Relative activity was calculated as 32P incorporated in the presence of IL-4 and an inhibitor divided by incorporation in IL-4 alone (no inhibitor). Similar results were obtained when the data were normalized to an internal reference protein as described under "Materials and Methods" and Fig. 2B. The results represent pooled data from two clones expressing each construct and the mean of three independent experiments. •, huIL4; ■, mIL4.
followed by re-treatment and measurement of Stat6 induction by IL-4. As shown in Fig. 5A, wortmannin treatment for 20 h (lane 6) or 2 h (lane 7) did not affect induction of Stat-like proteins by IL-4. Moreover, cell recovery and the frequency of apoptotic cells were not affected by wortmannin during these incubation periods (data not shown). Taken together, these data indicate that a PI 3-kinase inhibitor and a pp70 S6 kinase inhibitor block IL-4 induction of HMG-I(Y) phosphorylation while leaving the Jak-Stat signal transduction pathway intact.

It was surprising that genistein, which is thought to block tyrosine kinase-mediated activation of Stat6 in response to IL-4, had only a partial effect on HMG-I(Y) phosphorylation (37, 49). Data presented above indicate that this regulated phosphorylation is associated with IRS-1 recruitment to the IL-4R, a process that enhances tyrosine phosphorylation of IRS-1 (26–28). To test whether genistein would block the ability of IL-4 to induce IRS-1 phosphorylation, immunoblots were used to determine the level of IL-4-induced IRS-1 tyrosine phosphorylation after inhibition by genistein (Fig. 5B). Although the high expression of IRS-1 in these cells leads to a significant level of basal IRS-1 phosphorylation, IL-4 induced an increase in IRS-1 phosphorylation, and this increase was not significantly inhibited by genistein. These results demonstrate that the pathways leading to IRS-1 and HMG-I(Y) phosphorylation both remain active under conditions that potentially block Stat6 induction. Taken together, our findings indicate that the Tyr-1–IRS pathway is of critical importance in signaling the phosphorylation of a nuclear protein involved in transcriptional regulation.

**DISCUSSION**

IL-4 exerts its disparate actions on hematopoietic cells through a cell surface receptor whose individual domains represent potential checkpoints for regulation of signal transduction. Integration of the signaling pathways initiated at these domains provides the basis for the biologic specificity of IL-4. One signaling pathway initiates at a region termed the I4R motif due to homology of its function in the context of insulin and IL-4 receptor signaling (28). Although it is clear that the I4R motif is crucial for the regulation of proliferative responses to IL-4 and an emerging body of evidence suggests its involvement in gene expression, there has been no evidence that the recruitment of IRS proteins to the I4R regulates proteins involved in gene transcription. The data presented in this study demonstrate that the I4R initiates signals that regulate the phosphorylation status of a set of nuclear proteins that includes HMG-I(Y), a known transcriptional component (30–36). Consistent with the known ability of tyrosine phosphorylation of Tyr-1 in the I4R motif to recruit the adaptor molecule IRS-1 (28), IRS-1 expression is required for IL-4-inducible phosphorylation of HMG-I(Y).

Moreover, the recruitment and activation of PI 3-kinase through IRS-1 (26) are inhibited by wortmannin, which also inhibits HMG-I(Y) phosphorylation. Taken together, these data indicate that the phosphorylation of Tyr-1 in the I4R initiates a signal transduction pathway that culminates in increased phosphorylation of HMG-I(Y) and other as yet unidentified nuclear proteins.

This pathway, connecting IL-4Rs at the cell surface to nuclear proteins such as HMG-I(Y), is likely to involve the pp70 S6 kinase at an intermediate step. Activation of pp70 S6 kinase is crucial in cell cycle progression (51, 52), participates in the regulation of gene expression (53), and may contribute to an anti-apoptotic effect of IL-4 (54). However, very few nuclear proteins other than the transcription factor CREM have been identified downstream from this enzyme (53). IL-4 treatment of 32D cells increases pp70 S6 kinase activity, and the degree of activation is enhanced in IRS-1-expressing cells (46). Moreover, this serine/threonine kinase is regulated by phosphorylation events that include a PI-3 kinase-dependent pathway (45, 47).

We have previously shown that the immunosuppressant rapamycin, an inhibitor whose known intracellular target is pp70 S6 kinase, blocks IL-4-inducible phosphorylation of HMG-I(Y) in a cell cycle-independent manner and decreases induction of Gs RNA by IL-4 (37). The present data confirm in 32D cells that rapamycin is an inhibitor of regulated HMG-I(Y) phosphorylation and indicate that the pathway leading to this regulation involves IRS-1 and PI 3-kinase. Preliminary studies further support a biochemical linkage in which HMG-I(Y) lies downstream from pp70 S6 kinase. Taken together, the present findings suggest a model in which phosphorylation of Tyr-1 in the I4R motif leads to increased phosphorylation of HMG-I(Y) through recruitment of IRS-1 followed by enhancement of pp70 S6 kinase activity through PI 3-kinase.

These data further substantiate that this signal transduction pathway is not modulated by the activation state of Stat6.

* D. Z. Wang and M. Boothby, unpublished observations.
A truncated huIL-4Rα that contains Tyr-1 but none of the distal tyrosines, d557, is capable of triggering only minimal Stat6 induction (Fig. 2A), yet IL-4 binding to d557 induces quantitatively normal HMG-I(Y) phosphorylation (Figs. 2B and 3B). Moreover, the presence of the IRS-1 adaptor protein is not required for full activation, nuclear translocation, or DNA binding activity of a Stat6-like mobility shift complex (42), whereas regulation of HMG-I(Y) phosphorylation requires the presence of IRS-1. Signaling through the IL-4Rα mutant Y1F (clone 8–5D3) is unable to induce HMG-I(Y) phosphorylation yet is competent to induce Stat6 (42). Finally, we have exploited the observation that a chimeric receptor (IL-2Rβ/IL-4Rα) is competent to signal the partial induction of CD23 (Chim-1 in Ref. 29) but causes no detectable Stat 6 activation in a B lymphoma cell line. This chimera, containing only the IL-4 region of the IL-4R, was sufficient to confer inducible HMG-I(Y) phosphorylation in response to receptor engagement, whereas the truncated IL-2Rβ used in the chimera was incapable of signaling such increases.7 This observation suggests that the inducible phosphorylation could contribute to modulation of the equilibrium binding of HMG-I(Y) to DNA after IL-4-NF-AT (63). Since it is well established that HMG-I(Y) phosphorylation can either enhance or inhibit the transcriptional activity of a variety of proteins (55, 56). However, Ras, Raf, and mitogen-activated protein kinase-like pathways are unlikely that mitogen-activated protein kinase-like pathways contribute substantially to regulation of IL-4 function through Stat6. However, the region surrounding Tyr-1 enhances downstream gene expression triggered by the Tyr-2, Tyr-3, and Tyr-4 residues (amino acid residues 575, 603, and 631, respectively) of the human IL-4Rα intracytoplasmic tail at comparable levels of Stat6 binding activity in transfected M12 B cells.8 These findings linking cytokine-regulated phosphorylation of a nuclear protein involved in the regulation of gene transcription provide a potential Stat6-independent mechanism by which transcriptional activity can be regulated.

A second level of integration might occur through combinatorial interactions. The Ge Stat6 binding motif is insufficient to confer IL-4 inductibility to certain minimal promoters, and this defect may be related mechanistically to a requirement for C/EBP or NF-κB/Rel sites (38, 61). Moreover, IL-4-inducible gene expression and proliferation are impaired in normal mouse lymphoid cells with altered NF-κB/Rel signal transduction pathways (62). Although IL-4 does not appear to regulate C/EBP or NF-κB/Rel proteins directly, HMG-I(Y) is able to recruit NF-κB/Rel dimers through its ability to interact directly with p50 or c-Rel or to bend DNA (35). HMG-I(Y) can also increase recruitment of transcriptional activators such as c-Rel (35) or interfere with DNA binding of an activator such as NF-AT (63). Since it is well established that HMG-I(Y) phosphorylation decreases its affinity for DNA (37, 44), a decrease in the equilibrium binding of HMG-I(Y) to DNA after IL-4-inducible phosphorylation could contribute to modulation of Stat6 function on the basis of cooperating transcription factors.

Although this latter mechanism remains to be proven as a consequence of IL-4-inducible HMG-I(Y) phosphorylation, these findings provide the first Stat-independent linkage of IRS-1, the IL4 motif, and tyrosine 497 (Tyr-1) to regulated modification of a transcriptional component.

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IRS-1 and IL-4R I4R Motif Regulate HMG-I(Y) Phosphorylation

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