Interleukin 4-inducible Phosphorylation of HMG-I(Y) Is Inhibited by Rapamycin*

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The non-histone chromosomal protein HMG-I(Y) participates in repression of transcription directed by a promoter which confers interleukin 4 (IL-4)-inducible activation in transfected B cell lines. Metabolic labeling, phosphoamino acid analyses, and in vitro phosphorylation studies demonstrate that IL-4 induces serine phosphorylation of HMG-I(Y) in B lymphocytes. Phosphopeptide mapping shows that the predominant site of phosphorylation contains a casein kinase II consensus motif. The immunosuppressive agent rapamycin has been shown preferentially to inhibit IgE production by IL-4-treated human B cells. It is shown here that rapamycin inhibits both activation of the human germ line e promoter by IL-4 and IL-4-inducible phosphorylation of HMG-I(Y). These findings demonstrate a rapamycin-sensitive pathway that transduces signals from the IL-4 receptor to nuclear factors that regulate inducible transcription. The affinity of normal nuclear HMG-I(Y) for DNA is increased by dephosphorylation in vitro, whereas in vivo kinase reactions using casein kinase II decrease recombinant HMG-I(Y) binding to DNA. These data further suggest a novel mechanism in which phosphorylation triggered by IL-4 or other cytokines could regulate the effects of HMG-I(Y) on gene transcription.

Immunoglobulin heavy chain isotype switching is regulated by cytokines. The transcriptional activity of promoters located upstream from the heavy chain switch recombination targets is controlled by cytokines as part of isotype switching (1, 2). Deletion of germ line promoter regions from mouse DNA by homologous recombination inactivates isotype switching involving the mutant heavy chain isotype, whereas insertion of a constitutive promoter allowed normal lymphokine dependence to be bypassed (3–5). These data support the involvement of germ line promoters in regulation of immunoglobulin isotype switching. In the case of immunoglobulin E (IgE), the e heavy chain immunoglobulin germ line promoter is activated by interleukin 4 (IL-4) in treatment of human B cells or treatment of mouse B cells by IL-4 plus the polyclonal activator lipopolysaccharide (6–8). The immunosuppressive drug rapamycin inhibits IL-4-dependent IgE synthesis by human B cells more, and at a lower concentration, than that of certain other Ig isotypes (9, 10). However, relatively little is known about the signal transduction pathways that connect the IL-4 receptor to nuclear events that mediate IL-4-dependent gene transcription.

We have previously shown that the induction of germ line RNA reflects activation of transcription, and a DNA fragment from the Gε promoter is sufficient to confer IL-4-inducible activation on a heterologous reporter gene (11). The non-histone chromosomal protein HMG-I(Y) is an alternatively spliced 10–12-kDa high mobility group (HMG) protein that binds to the minor groove of a-T clusters but has some sequence preferences among such a-T regions (12–14). HMG-I(Y) participates in repression of basal activity and increases the inducibility of the Gε and IL-4 promoters (15, 16). It also contributes to regulation of the promoters for interferon-β promoter by virus (17, 18), E-selectin by interleukin 1 (19, 20), and of the IL-2Rα (CD25) promoter by T cell activation (21). Because each of these promoters is inducible, these data raise the question whether HMG-I(Y) is subject to regulation in response to signals from cell surface receptors.

HMG-I(Y) was originally identified as a protein whose phosphorylation is associated with cellular transformation and proliferation (22, 23), whereas IL-4 is able to inhibit the proliferation of leukemic B cells (24). HMG-I(Y) is phosphorylated by cdc2 kinase, and this threonine phosphorylation leads to decreased DNA binding activity of HMG-I(Y) (25, 26). These observations raised the possibility that post-translational modification of HMG-I(Y) may be regulated by IL-4. Thus, IL-4-inducible phosphorylation of HMG-I(Y) could contribute to activation of the Gε promoter through phosphorylation-induced derepression.

To investigate whether IL-4 regulates the phosphorylation status of HMG-I(Y), we have performed metabolic labeling experiments with the IL-4-responsive human B lymphoblastoid cell line JY (27). These experiments demonstrate that IL-4 induces increased phosphorylation of HMG-I(Y), as well as increased phosphorylation of other (unidentified) nuclear proteins. Phosphoamino acid and phosphopeptide mapping analyses localize IL-4-inducible phosphorylation of HMG-I(Y) to carboxyl-terminal serine residues containing a casein kinase II consensus substrate motif. In vitro phosphatase treatment of nuclear extracts and casein kinase II phosphorylation of HMG-I show that phosphorylation is associated with decreased binding activity of HMG-I(Y). We also find that rapamycin partially inhibits Gε promoter induction and causes a profound block of the IL-4-inducible phosphorylation of HMG-I(Y). In contrast, the tyrosine kinase inhibitor genistein has little effect on IL-4-inducible HMG-I(Y) phosphorylation. These inhibitor results suggest that IL-4-inducible phosphorylation of HMG-I(Y) occurs through a pathway independent of STAT protein activation (28, 29). The results also suggest a model of Gε promoter regulation in which IL-4-inducible phosphorylation

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‡ The abbreviations used are: IL-4, interleukin 4; IgE, immunoglobulin E; HMG, high mobility group; PKC, protein kinase C; PI, phosphatidylinositol; TBP, TATA box-binding protein.

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contributes to promoter activation through altered activity of HMG-I(Y).

MATERIALS AND METHODS

Cell Culture—J Y cells (gift of Dr. L. Glimcher) were cultured in Hapes-buffered RPMI 1640 supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Life Technologies, Inc.), 100 μM 2-mercaptoethanol, 3 mM glutamine, and 10% heat-inactivated fetal calf serum. Rapamycin (generous gift of Dr. S. Sehgal, Wyeth-Ayerst, Princeton, NJ) was prepared as a 100 μM stock in absolute ethanol stored at −20°C. One stock was aliquoted and diluted just before use at −20°C. Rapamycin was added to culture solutions for immediate use at 1:1000 in cell cultures. Genistein (Life Technologies, Inc.) was prepared as a stock in Me2SO and diluted to less than 1 part Me2SO carrier/1000 cells per culture, a concentration at which the Me2SO had no effect. Purified recombinant human (hu) IL-4 (R & D Systems, Minneapolis, MN) and generous gifts from Dr. M. Widmer, Immunex, Seattle, WA and Dr. J. DeVries, DNAX, Palo Alto, CA) was used at 5 ng/ml unless otherwise indicated.

Metabolic Labeling Experiments—J Y cells (105/ml) were cultured overnight in RPMI 1640 medium with 10% fetal bovine serum, rinsed twice with serum-free medium, and then resuspended in phosphate-free medium containing 10% dialyzed fetal bovine serum (Life Technologies, Inc.). Cells were labeled for 3 or 4 h with 40 μCi/ml [32P]orthophosphate (DuPont NEN), then washed for the indicated times with hul-L at 5 ng/ml. Total time in label was the same for all samples. For rapamycin treatment, cells were treated with the indicated concentrations of rapamycin for 120 min prior to stimulation with IL-4.

Nuclear Extracts and HMG-I(Y) Analyses—Cells either were cultured in medium with or in medium without exposure to 5 ng/ml human IL-4, harvested, washed, and chilled to 4°C for subsequent steps. Nuclear proteins for two-dimensional gel analyses were salt-extracted from nuclei as described previously (11, 30, 31), precipitated with 50% trichloroacetic acid, pelleted, washed twice with acetone, then dissolved in acid-urea gel buffer (0.9 M acetic acid, 0.12 M 2-mercaptoethanol, and 15% sucrose), containing 4 M urea to avoid variable resolubilization of HMG-I(Y) (23, 32).

Nuclear extracts for phosphorylation analysis were prepared by Nonidet P-40 lysis and high salt extraction of nuclei from J Y cells. Lysis and extraction were performed in the presence of protease inhibitors but not phosphatase inhibitors, as described previously (33, 34). Because HMG-I(Y) is present both in free form and in heteromeric complexes with other nuclear proteins (15, 17–21), in some experiments nuclear extracts were warmed for 3 min at 55 °C to dissociate all complexes, leaving only monomeric HMG-I(Y). Nondenaturing electrophoretic mobility shift assays were performed as described previously (11, 31). In the analyses shown, a 140-base pair 5′fl of the mouse Gs promoter (18–122) was used, as described in Materials and Methods. To verify that the mobility shift complex consisted only of HMG-I(Y), binding inhibition experiments with anti- sera specific for HMG-I(Y) (15, 36) were performed and showed blockade of the nucleoprotein complex. In addition, two double-stranded DNA probes containing the da-T tract at the human Gs promoter initiation sites were used to show that human HMG-I(Y) also binds the human Gs promoter. An oligonucleotide with the sequence CCACCGCACAACAAA-CAACGGTACT (residues 69-104 of Gs) was used, as well as a 145-base pair fragment of DNA spanning residues 52–196 (numbered as in (7)) generated by PCR with the germ line genomic clone pBS-HE1.37 (generous gift of D. Corti and D. Vercelli). Mobility shift data and two-dimensional gel analyses of phosphorylation were quantitated using a Fuji BAS 1000 phosphorimager with the MacBAS software package.

In Vitro Phosphorylation of HMG-I(Y)—Pure casein kinase II (Pro-Mega), and baculovirus-derived κ isoform of protein kinase C (PKC κ, generous gift of J. Exton), were used in 50-μl reactions. Reactions were performed in 25 mM Tris·Cl, pH 7.4, 10 mM MgCl2, 200 mM NaCl, 0.1 mM ATP, 1 μg of HMG-I(Y), and 10–20 units of casein kinase II incubated for 1.5–60 min at 37°C or in 25 mM Tris·Cl, pH 7.5, 2 mM MgCl2, 0.5 mM EGTA, 1 mM dithiothreitol, 50 μM ATP, 15 μg of HMG-I(Y), and PKC κ incubated for 5 min at 30°C (7). After incubation, analytical reaction samples were added to SDS loading buffer, phosphorylated on 15% SDS-PAGE, and transferred to nitrocellulose membranes for autoradiography and subsequent phosphopeptide analysis.

Phosphoamid Acid and Phosphopeptide Analyses—Radiolabeled phosphorylated HMG-I(Y) was transferred from two-dimensional gels onto polyvinylidene difluoride membranes (Millipore) for phosphoamid acid analyses or onto nitrocellulose filters (Schleicher and Schuell) for phosphopeptide mapping (38–41). Filter-bound HMG-I(Y) was subjected to partial acid hydrolysis, and a mixture of the solubilized hydrolysate with unlabeled phosphoamid acid markers (Sigma) was applied to dye-free thin-layer cellulose plates (EM Sciences) for two-dimensional electrophoresis. Phosphoamid acid markers were resolved by electrophoresis at 900 V for 30 min in pH 1.9 buffer in the first dimension, followed by ascending chromatography in pyridine:butanol:acetic acid:H2O (10:15:3:12), as described. Refs. 38 and 39. Most samples were subjected to electrophoresis in pH 8.9 buffer for better assignment of the site of phosphorylation, tryptic digests also were electrophoresed in pH 1.9 and pH 3.5 buffer systems.

RNA Analyses—J Y cells were plated in six-well tissue culture dishes at 2 × 105 cells/ml in 3-m1 portions. Rapamycin was added to cultures 30 min before treatment with IL-4; ethanolic carrier was added to untreated control cultures; comparable results were obtained after overnight pretreatment with rapamycin. Cells were harvested, rinsed twice with phosphate-buffered saline, lysed in the presence of 3 mM LiCl, 6 M urea, and processed according to (42). Total cellular RNAs were resolved under denaturing conditions on formaldehyde-agarose gels, transferred to Nytran Plus filters (Schleicher and Schuell) and probed with an 822-base fragment of genomic DNA from the human Cε locus (generous gift of D. Vercelli) as described (8). Autoradiographic signals were quantitated using a Fuji BAS 1000 phosphorimager and the MacBAS software package.

RESULTS

IL-4-inducible Phosphorylation of HMG-I(Y) —HMG-I(Y) contributes to repression of the Gs promoter and affects the activity of other inducible promoters as well (15–21). To investigate whether the phosphorylation status of HMG-I(Y) is regulated by IL-4, metabolic labeling experiments were performed using J Y, a human cell line in which the Gs promoter is highly responsive to IL-4 treatment (7, 27). The HMG of non-histone chromosomal proteins is defined by their characteristic rapid mobility during electrophoresis on acid-urea gels. Total nuclear proteins labeled by [32P] were therefore analyzed using a two-dimensional acid urea SDS-PAGE system (23, 32). Fig. 1 shows that HMG-I(Y) phosphorylation increased in IL-4-treated cells as compared with controls. These metabolic labeling studies first detected significantly increased phosphorylation at 1 h, with a progressive increase between 1- and 3-h IL-4 treatment (Fig. 1A). Kinetic analyses of Gs induction by IL-4 show a 2 h lag before any detectable accumulation of Ge RNA, with the first significant increase occurring at 2–4 h and a continued progressive increase between 4 and 16 h (Ref. 27 and Fig. 1B). We conclude that IL-4 induces increased phosphorylation of HMG-I(Y) and that increased phosphorylation precedes increased promoter activity.

Localization of the Phosphorylated Residues Within HMG-I(Y)—Growth factor treatment or other stimulation of cells can lead to gain or loss of phosphates from multiple sites within the same nuclear protein or transcription factor, with different functional outcomes for each site (40, 41, 43). However, cyto-kine-inducible changes in the phosphorylation status of HMG-I(Y) have not been reported. Since HMG-I(Y) contains no tyrosine residues, IL-4-inducible phosphorylation is predicted to be regulated by serine/threonine kinases and phosphatases. To investigate whether IL-4 induces phosphorylation on the threonine residues adjacent to the DNA binding domains, as for cdc2 kinase, we first performed phosphoamid acid analyses on metabolically labeled HMG-I(Y). To imitate the conditions of Gs promoter induction, unsynchronized J Y cells were treated with IL-4 for 3 h. These analyses demonstrated that HMG-I(Y)
in IL-4-treated B cells is exclusively phosphorylated on serine residues (Fig. 2). The same pattern was observed in phosphoamino acid analyses of HMG-I(Y) metabolically labeled in control cells not treated with IL-4 (data not shown). In addition, IL-4-inducible phosphorylation of HMG-I(Y) is not limited to human cells or to tumor cells, as a 4–5-fold increase occurs after IL-4 treatment of the growth factor-dependent mouse T cell line CTLL (data not shown).

To localize the site(s) of these phosphoserine residues in HMG-I(Y), metabolically labeled protein was subjected to protease digestion with trypsin or chymotrypsin, and two-dimensional phosphopeptide analyses were performed. The site of phosphorylation was localized to a single highly acidic peptide in IL-4-treated cells (Fig. 3). The same tryptic peptide was observed in control cells. Digestion with V8 protease digestion also generated a single highly acidic peptide (data not shown). In addition, the 32P-labeled chymotryptic and tryptic peptide had almost identical mobility in two-dimensional mapping and were highly acidic (Fig. 3). The data are most consistent with serine phosphorylation sites in the C-terminal tryptic and chymotryptic fragment, which differ only by two amino acids.

The site of basal and IL-4-inducible phosphorylation contains a consensus sequence for phosphorylation by casein kinase II [(S/t)-X-(E/d)]. To confirm the assignment of IL-4-inducible labeling to the carboxyl terminus, and to facilitate analyses of the functional consequences of this phosphorylation, in vitro kinase reactions were performed using a bacterially produced form of HMG-I(Y) with additional residues at the N terminus and purified kinases. As seen in Fig. 4A, casein kinase II efficiently transferred phosphates to HMG-I(Y). IL-4 can cause protein kinase C activation (44, 45), and the zeta (ζ) isoform of protein kinase C can be activated by lipid products of PI 3-kinase (37). PKC ζ might therefore provide a direct link between PI 3-kinase activation and HMG-I(Y) phosphorylation in response to IL-4. Therefore, PKC ζ was tested in addition to casein kinase II. PKC ζ was far less efficient than casein kinase II in transferring phosphates to HMG-I(Y). In quantitative experiments, the transfer of phosphate from ATP to HMG-I(Y) is catalyzed by casein kinase II at least 50% as efficiently as transfer to casein (data not shown). Moreover, addition of 1 mol of phosphate/mol of HMG-I(Y) can be achieved with adequate enzyme concentrations and incubation times (20 units for 1 h.

FIG. 1. Onset of IL-4-inducible phosphorylation of HMG-I(Y) precedes Ge RNA induction. A, the human B lymphoblastoid cell line JY, in which IL-4 induction of germ line RNA expression starts within 4 h after treatment (7), was cultured in carrier-free [32P]orthophosphate. Pure recombinant human IL-4 was present in cultures for the indicated time; all cultures were of equal length with equal cell numbers. Cells were harvested, and two-dimensional acid-urea/SDS-polyacrylamide gels were performed on 0.4 M salt-extracted nuclear proteins. Equal masses of radiolabeled nuclear proteins, derived from equal cell numbers, were resolved on 15% polyacrylamide acid-urea tube gels in the first dimension, then resolved by electrophoresis on 15% SDS-PAGE, a system previously shown to resolve HMG-I and Y from other HMG proteins and histones (13, 23, 32). The acid-urea dimension ran from right to left on the horizontal axis, and the SDS-PAGE was from top to bottom. Control experiments with Western blotting showed that the recovery of HMG-I(Y) was equivalent among samples in experiments. The HMG-I(Y) spot is marked by an arrowhead on each autoradiograph. B, a Northern blot analysis of total cellular RNAs from JY cultured for the indicated period with recombinant human IL-4 at 5 ng/ml. The autoradiograph shows filters probed with a human Cε fragment (8); control hybridizations with GAPDH and ethidium bromide staining showed equal loading of RNAs, including the lanes showing no signal with the Cε probe.

FIG. 2. IL-4-inducible phosphorylation of HMG-I(Y) is on serine. Preparative two-dimensional acid urea/SDS-PAGE gels were performed on salt-extracted nuclei isolated from JY cells metabolically labeled with [32P] during a 3-h IL-4 stimulation. The proteins were electrophoretically transferred to PVP (Immobilon) filters, and the 32P-labeled HMG-I(Y) spot was excised from the blot after autoradiography. Filter-bound HMG-I(Y) was then subjected to acid hydrolysis and two-dimensional resolution of phosphoamino acids. The 32P-labeled spots were localized by autoradiography and correlated with ninhydrin-stained internal control samples of phosphoserine, phosphothreonine, and phosphotyrosine, whose positions are indicated.
with 100 pmol of HMG-I(Y). We conclude that HMG-I(Y) is an efficient substrate for casein kinase II but not PKCζ.

To compare the substrate specificity of the IL-4-inducible kinase activity to that of casein kinase II, the one-dimensional tryptic phosphopeptide pattern of HMG-I(Y) phosphorylated in vitro by casein kinase II was compared with that of nuclear HMG-I(Y) metabolically labeled in IL-4-treated JY cells. As seen in Fig. 4B, casein kinase II and the IL-4-inducible kinase generated the same profile. The amino-terminal extension of bacterially produced HMG-I(Y) would alter the mobility of the other peptide with a net negative charge in both tryptic and chymotryptic digests. We conclude that in the JY cell line, both basal and IL-4-inducible phosphorylation occurs on carboxyl-terminal serines of the HMG-I(Y) molecule and is catalyzed by a kinase with a casein kinase II-like specificity.

Rapamycin Inhibits IL-4-inducible HMG-I(Y) Phosphorylation—

IL-4-dependent production of reaginic (IgE class) antibodies in intact rats (9), and IL-4-dependent IgE production by human B cells (10), are inhibited by rapamycin, an antagonist of a signal transduction pathway associated with PI 3-kinases and related molecules (46–49). Since IL-4 activates receptor-associated PI 3-kinase (50, 51), we investigated whether IL-4-inducible phosphorylation of HMG-I(Y) is inhibited by rapamycin. Fig. 5A shows the results of metabolic labeling experiments with JY cells after analysis of the nuclear proteins on two-dimensional electrophoresis.
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**Fig. 5.** Rapamycin inhibits the stimulation of HMG-I(Y) phosphorylation by HMG-I(Y). A, metabolic labelings were performed as in Fig. 1, using a 3-h culture in IL-4 in each case. Where indicated, cells were cultured in the stated concentration of rapamycin for 30 min before the addition of IL-4. (The direction of the acid-urea electrophoresis was from left to right in all but the unstimulated control). The position of HMG-I(Y) is again marked by an arrow on the autoradiograph. B, samples from the same experiment as in Fig. 1 are shown, and methods are as described in the legend to Fig. 1. Samples treated with genistein were cultured overnight in the presence of 75 μM genistein to evaluate the effect at saturating concentrations of this agent, as in Ref. 28. Dimensions of the acid-urea/SDS-PAGE. The only labeled protein whose identity we have investigated by Western blotting is HMG-I(Y), and its position is indicated by an arrow on each panel. As indicated in Fig. 1A, the labeling of this HMG-I(Y) spot is markedly increased in IL-4-treated cells (upper right-hand panel) as compared with controls (upper left-hand panel). However, little label was incorporated into the HMG-I(Y) spot if the cells had been treated with rapamycin at concentrations in the range of 2–10 nM. This result indicates that the regulatory proteins responsible for IL-4-inducible HMG-I(Y) phosphorylation can be inhibited by rapamycin. The results also indicate that there may be other IL-4-inducible increases in nuclear protein phosphorylation that are inhibited by treatment of JY cells with rapamycin. Rapamycin acts in the nanomolar range. Fig. 5A also shows that in dose response experiments, significant inhibition of nuclear HMG-I(Y) phosphate incorporation by rapamycin occurred by 0.5 nM, with essentially maximal inhibition by 2 nM. Thus, rapamycin inhibition of HMG-I(Y) phosphorylation occurs in a range comparable with that reported for inhibition of growth factor-inducible activity of signal transduction-related kinases (46–49).

The initiation of certain IL-4 receptor signal transduction pathways can depend directly or indirectly on tyrosine kinases. Overnight treatment of a human monocytic cell line with high concentrations of the tyrosine kinase inhibitor genistein blocked the induction of a tyrosine-phosphorylated nuclear protein by IL-4 (IL-4 NAF; Ref. 28). To investigate if the conditions of tyrosine kinase inhibition which blocked IL-4 NAF induction would prevent IL-4-inducible phosphorylation of HMG-I(Y), metabolic labeling experiments were performed with JY cells, and the labeled nuclear proteins were again resolved on two-dimensional acid-urea/SDS-PAGE. As shown in Fig. 5B, genistein inhibition under the conditions used to analyze IL-4 NAF did not prevent the dramatic increase in HMG-I(Y) labeling induced by IL-4. Our interpretation is that the IL-4 can induce signals that lead to increased serine phosphorylation of HMG-I(Y) despite inhibition of cellular responses by genistein.

**Phosphorylation of HMG-I(Y) Decreases Its DNA Binding Activity—**To assess whether IL-4-inducible phosphorylation at the carboxyl-terminal serines of HMG-I(Y) influences its DNA binding activity, we performed mobility shift assays with a radiolabeled fragment of the mouse Gβ promoter bound to the Gβ promoter known to be an HMG-I(Y) binding site associated both with repression of basal activity and with promoter inducibility (Fig. 6) (15). Because the vast majority of both steady state and IL-4-inducible phosphorylation of HMG-I(Y) occurs at the same site within the protein, treatment of nuclear extracts with phosphatases will affect the degree of phosphorylation only of this one site. Therefore, nuclear extracts were incubated in phosphatase buffer only (Fig. 6A, lanes 1 and 4) or in buffer together with a phosphatase active against phosphoserine/threonine (lanes 2 and 5), then used in DNA binding assays. As seen in Fig. 6A, these experiments demonstrated that dephosphorylation increased the DNA binding activity of normal cellular HMG-I(Y). Inclusion of a phosphatase inhibitor prior to the incubation blocked the increase in DNA binding activity (lanes 3 and 6), contradicting the possibility that increased activity is due to proteolytic digestion or some modification other than dephosphorylation.

Because casein kinase II mimics the phosphorylation of HMG-I(Y) induced in response to IL-4 stimulation of JY cells, and stoichiometric addition of phosphate can be achieved, the effect of in vitro phosphorylation also could be assayed. As seen in Fig. 6B, preparations of HMG-I(Y) phosphorylated in vitro with casein kinase II bound to the Gβ promoter probe 3–5-fold less efficiently than purified recombinant material before phosphorylation. We conclude that phosphorylation of the predominant phosphoamino acid site decreases HMG-I(Y) binding ac-
Rapamycin Inhibits IL-4-inducible HMG-I(Y) Phosphorylation

Fig. 6. Phosphorylation decreases the binding of HMG-I(Y) to DNA. A, mobility shift experiments were performed using nondenaturing PAGE and a radiolabeled germ line e promoter DNA probe, of which a representative autoradiogram is shown. Nuclear extracts were prepared under conditions that dissociate the multimeric complex NF-BRE, so that all immunoreactive HMG-I(Y) is in the complex of HMG-I(Y)-probe. The positions of this complex and of free radiolabeled probe DNA are indicated. Nuclear extracts were either subjected to a mock-incubation in phosphatase buffer prior to addition to a binding reaction (lanes 1 and 4) or incubated in phosphatase buffer with the indicated phosphatase (lanes 2, 3, 5, and 6; P.A.P. is potato acid phosphatase). These incubations either contained no phosphatase inhibitors, or the inhibitors sodium fluoride and sodium vanadate (+ phosphatase inhibs). These incubations either contained no phosphatase inhibitors, or the inhibitors sodium fluoride and sodium vanadate (+ phosphatase inhibs). B, an autoradiograph from a mobility shift experiment using a radiolabeled germ line e promoter DNA probe is shown. The assays included 15 ng (lanes 1 and 3) or 30 ng (lanes 2 and 4) purified recombinant HMG-I(Y). In lanes 1 and 2, purified protein not reacted with casein kinase II was used; in lanes 3 and 4, HMG-I(Y) kinased by casein kinase II in the presence of unlabeled ATP was used. A parallel reaction gave an estimated stoichiometry of phosphate transfer as 1.03 mol of PO4/mol of HMG-I(Y), and use of the cold phosphorylated HMG-I(Y) in a second casein kinase II reaction using [32P]ATP gave an estimated stoichiometry of 0.2 mol of PO4/mol of HMG-I(Y). Thus, we estimate that the cold phosphorylation reaction labeled 80–100% of a single serine residue. Products from a labeling reaction were used directly for standard HMG-I(Y) mobility shift assays. C, a scheme of the germ line e promoter, including the sequence in the region to which HMG-I(Y) binds. The promoter region is represented by an open rectangle. A series of vertical hash marks denotes the multiple cap sites previously identified in nuclease protection experiments. The upstream-most cap site has been assigned +1. A series of circles below the gene represents constitutive binding activities and transcription factors whose binding site contributes to Ge promoter function, whereas a stippled circle represents the inducible factor NF-IL4 (28, 29, 55, 59). The + and − symbols denote the role of each binding site. An expansion shows the sequence around the HMG-I(Y) binding site previously mapped at +3 to +9 of the mouse promoter (15), with the dA-T residues underlined and the site in boldface. The sequence at the same position in the human Ge promoter (7) is shown below. Recombinant HMG-I(Y) binds to this human sequence with a binding affinity of approximately 25% that of the mouse sequence.

activity and by extension that IL-4-inducible phosphorylation of HMG-I(Y) decreases its binding activity.

Rapamycin Inhibits Ge Promoter Activation by IL-4—HMG-I(Y) contributes to repression of the Ge promoter and to promoter inducibility (15). Because rapamycin inhibits the IL-4-inducible phosphorylation of HMG-I(Y), it should negate any effect of this post-translational modification on normal Ge promoter function. The induction of germ line e RNAs by IL-4 is regulated by increased transcription (11). Therefore, to quantitatively evaluate the effect of rapamycin on IL-4-inducible Ge promoter activity, we performed Northern blot analyses on total cellular RNAs. Cells treated with IL-4 after rapamycin pretreatment were compared with cells treated only with IL-4 and with untreated cells. These experiments show that although the Ge
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Fig. 7. Rapamycin inhibits the induction of Ge mRNA by interleukin 4. Equal portions of the human B cell line JY were cultured in no IL-4 (lane 1) or for 4 h in 5 nM of pure recombinant human IL-4 (lanes 2–6). After culture, total cellular RNA was prepared, and equal 10-μg portions were analyzed by Northern blots and hybridization with labeled e constant region exons. The cells had been pre-cultured in either 0.1% ethanolic carrier (lane 2); overnight in 7.5 μM genistein (lane 3), an inhibitor of tyrosine phosphorylation; or for 1 h in cycloheximide at 50 μM (lane 4). 1 mM rapamycin (lane 5), 10 mM rapamycin (lane 6). Lane 7 is a control of 1 μg of total cellular RNA from the human myeloma cell line U266, indicating the small difference in mobility of mature (VDJ-Ge) RNA from the germ line e RNA. Control hybridizations with a GAPDH probe, and ethidium bromide gel staining, demonstrated comparable loading of samples. Measurement of signals in this experiment with a PhosphorImager indicated 10 levels of Ge mRNA in the rapamycin-treated samples of 48% (1 nM) and 25% (10 nM) relative to the IL-4-treated sample.

promoter retained some IL-4 inducibility, rapamycin inhibited 50% of the induction by IL-4 at doses in the nM range (Fig. 7). Thus, although rapamycin inhibition of promoter induction by IL-4 clearly is incomplete, we conclude that the inhibition of Ge promoter activity by rapamycin can be correlated with its ability to block IL-4-inducible phosphorylation of a transcriptional repressor.

DISCUSSION

In addition to its control of IgE production, interleukin 4 regulates lymphocyte proliferation and plays a pivotal role in determining the helper function of T cells (52, 53). Despite these important immunoregulatory properties, relatively little is known about signal transduction mechanisms linking IL-4 receptor engagement to the induction of IL-4-dependent genes. Recent studies suggest that receptor-associated protein kinases, including Jak3 phosphorylate proteins such as NF-IL4, which may include Stat6 (Stat IL-4), a new member of a family of transcription factors termed STAT (signal transduction and activation of transcription) proteins (28, 29, 54–58). In the present study, we present evidence of a different pathway stimulated by IL-4. The central findings of this study are (i) IL-4 treatment leads to increased phosphorylation of HMG-I(Y), a protein involved in regulation of an IL-4-dependent promoter and of other inducible promoters; (ii) this increased phosphorylation is refractory to high doses of an inhibitor of tyrosine kinase pathways but sensitive to the immunosuppressive rapamycin; and (iii) phosphorylation can affect functional properties of HMG-I(Y), suggesting a novel mechanism in which HMG-I(Y) function at inducible promoters such as the germ line epsilon (Ge) promoter may be modulated by IL-4-inducible phosphorylation.

IL-4 receptor engagement leads to increased serine phosphorylation of HMG-I(Y), an exclusively nuclear protein involved in the regulation of several inducible promoters (15–21). Phosphorylation is potently inhibited by the immunosuppressive rapamycin, yet is minimally affected by the tyrosine kinase inhibitor genistein at a dose sufficient to inhibit NF-IL4 induction (28) and germ line e RNA accumulation in response to IL-4. Thus, the result is not confined to the JY cell line or to B cells, as we have used the mouse T cell line CTLL in similar metabolic labeling experiments and find IL-4-inducible HMG-I(Y) phosphorylation. In contrast, NF-IL4 induction is inhibited by genistein but resistant to rapamycin, suggesting that IL-4-inducible HMG-I(Y) phosphorylation occurs independent of tyrosine phosphorylation of the factor IL-4 NAF or Stat6 (28, 29, 55). Thus, the properties of this phosphorylation suggest a signal transduction pathway distinct from that required for activation of STAT factors such as STF-IL4, NF-IL4, or STAT IL4 (28, 29, 55). Since rapamycin inhibits IgE synthesis more, and at a lower concentration, than that of certain other Ig isotypes (10), and completely blocks HMG-I(Y) phosphorylation under conditions which decrease RNA levels only to half of control levels, promoter regulation by HMG-I(Y) can only be one among several factors which determine germ line e transcription. IL-4-inducible, tyrosine-phosphorylated nuclear proteins bind at −122 to −104 (NF-IL4 or IL-4 NAF; Refs. 28, 55, and 59) and near the HMG-I(Y)/NF-BRE site at +3 to +9 (STF-IL4; Ref. 56). Mutations at the IL-4 NAF/NF-BRE site show that this sequence also both acts as a repressor element for basal transcription and is involved in inducibility (59, 60).

Taken together, these observations provide evidence of a new signal transduction pathway linking the IL-4 receptor to the nucleus and indicate that full germ line e promoter activation by IL-4 requires derepression through cooperation of rapamycin-dependent serine/threonine kinases with the more direct protein tyrosine kinase pathway.

Independence of IL-4-inducible phosphorylation of HMG-I(Y) from genistein inhibition raises the question which pathway transduces a signal from the IL-4 receptor to HMG-I(Y). This observation is consistent with the ability of an IL-4 receptor mutant devoid of intracytoplasmic tyrosine residues to signal proliferation in a growth factor-dependent cell line (61). Rapamycin inhibition has been implicated in signal transduction pathways that use PI 3-kinase or related molecules (49, 62–64), and IL-4 receptor engagement leads to recruitment of PI 3-kinase as well as molecules such as the insulin receptor substrate 1 (IRS-1) and its homologue 4PS (50, 51). Thus, these early signal transduction molecules may play a role in the genistein-independent regulation of HMG-I(Y) phosphorylation status.

The present data on rapamycin inhibition implicate a specific pathway, downstream from these membrane-associated signal transduction proteins and capable of regulating the phosphorylation status of nuclear proteins. While MAP kinase activation through Ras and Raf is one major signal transduction pathway in which serine/threonine kinases stimulated by growth factors regulate nuclear transciption factors (43, 65), MAP kinase activation by other cytokines is largely refractory to rapamycin (46–48), and IL-4 does not activate Ras, Raf, or MAP kinases (51, 66, 67). Thus, it is unlikely that these pathways account for the HMG-I(Y) phosphorylation reported here. A serine/threonine kinase implicated in later steps in signal transduction, pp70 S6 kinase, is the only growth factor-stimulated signal transduction protein kinase known to be potently inhibited by rapamycin (46–49). Both IL-4 and the cytokine IL-2 lead to rapamycin-sensitive HMG-I(Y) phosphorylation in the cytokine-responsive cell line CTLL. Thus, it is likely that the pathway regulating HMG-I(Y) phosphorylation involves a shared component of the cytokine receptor family. The IL-2

2 D.-Z. Wang, A. L. Cherrington, B. M. Famakin-Mosuro, and M. Boothby, submitted for publication.

3 D.-Z. Wang, P. Ray, and M. Boothby, unpublished observations.
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D.-Z. Wang and M. Boothby, unpublished observations.

receptor shares many signal transduction molecules with the IL-4 receptor (57, 58, 68) and is known to activate the serine/threonine kinase pp70S6 kinase; this activation is inhibited by rapamycin with similar dose-response characteristics to those we observe for inhibition of IL-4-stimulated HMG-I(Y) phosphorylation (46–49). Thus, the present data raise the possibility that pp70 S6 kinase is activated by IL-4.

Moreover, these data suggest that casein kinase II-like activities may be regulated through pp70 S6 kinase in response to IL-4. Fractionation of nucleus and cytoplasm indicates that the phosphorylation of HMG-I(Y) occurs in the nucleus, and there is evidence suggesting that pp70/85 S6 kinase activity may be present in nuclei and regulate the phosphorylation status and activity of transcription factors (32, 69). However, the known substrate specificity of pp70 S6 kinase does not include the casein kinase II consensus site of HMG-I(Y) or any other sequence of HMG-I(Y). These observations raise the question whether some other pathway accounts for this IL-4-inducible phosphorylation. Casein kinase II is regulated by growth factors, including insulin, which shares signal transduction mechanisms with IL-4 (50, 70–72), effects regulatory phosphorylations of nuclear transcription factors (41, 43), and binds directly to the rapamycin binding rotamase FKBP25 (35). However, FKBP25-rapamycin complexes did not inhibit casein kinase II in vitro, and casein kinase II is not reported to be inhibited by rapamycin. Since there is no evidence of rapamycin-inhibiting casein kinase II-like activities, the present data raise the possibility that an indirect pathway relays signals from the IL-4 receptor to casein kinase II-like activities.

The data also suggest that IL-4-inducible phosphorylation of HMG-I(Y) may regulate promoter activity. HMG-I(Y) can either decrease (15, 16) or increase (17–21) promoter activity. Antisense HMG-I(Y) RNA increased basal germ line e promoter activity and inducibility, with relatively little effect on inducible activity. These data are most consistent with a role in basal repression, which is somehow attenuated after B cell stimulation (15). One mechanism for this effect may be interference with the universal transcription factor TATA-binding protein (TBP). TBP is recruited to pol II promoters by the integrated action of promoter- and enhancer-binding transcription factors. Factors necessary to achieve TBP loading at the Ge promoter, or increased loading in response to IL-4, appear to include BSAP (11), NF-IL-4 (59, 60), and Sp1 and p50 NF-κB (59).

The decrease in binding activity after phosphorylation of HMG-I(Y) may therefore decrease its interference with TBP binding to the minor groove of dA-T-rich sequences such as the TATA box, a sequence preference shared by TBP and HMG-I(Y).

However, it is not certain that this mechanism alone would be sufficient to account for the role of HMG-I(Y) in IL-4-stimulated promoter activity. The fraction of total nuclear HMG-I(Y) which actually undergoes phosphorylation in response to IL-4 cannot be determined. HMG-I(Y) is a protein present in nuclei at high prevalence, and we cannot quantify the effective specific activity of [32P]phosphate donors within the J Y cells during labeling, and thus it is not clear what percentage of HMG-I(Y) molecules are phosphorylated during these labeling experiments or during IL-4 stimulation. Moreover, the expected decrease in binding activity if a large fraction of nuclear HMG-I(Y) does undergo IL-4-inducible phosphorylation could be counterbalanced by improved recovery of phosphorylated HMG-I(Y) under the low salt extraction conditions needed for mobility shift experiments. Assays comparing the DNA binding activity of HMG-I(Y) extracted from control and IL-4-treated J Y cells have shown modest decreases in HMG-I(Y) binding activity in IL-4-treated cells. This result is consistent with a model in which IL-4 treatment decreases the ability of a repressor, HMG-I(Y), to act on the Ge promoter and consequently contributes to increased transcription of the promoter, but is variable and not uniformly observed.

An alternative mechanism is suggested by dephosphorylation reactions with total nuclear extracts which demonstrate decreased inclusion of HMG-I(Y) in an IL-4-inducible multimeric complex termed NF-BRE (11, 15). Moreover, rapamycin treatment of cells prior to IL-4 stimulation decreases the formation of NF-BRE. Thus, phosphorylation may alter the association of HMG-I(Y) with proteins that are involved in promoter regulation. In this alternative model, Ge promoter activation could be enhanced if phosphorylation altered the ability of NF-IL-4 (which presumably includes Stat 6), NF-κB p50, or BSAP to load at Ge promoter chromatin in vivo or to recruit TBP (11, 59, 60). Such alterations in protein-protein interactions, while at present speculative, could also suggest a role for HMG-I(Y) phosphorylation in the regulation of other inducible promoters at which HMG-I(Y) has been shown to play a role (16–21). These considerations notwithstanding, the Northern blot experiments which demonstrate that rapamycin decreases Ge RNA levels induced by IL-4, taken together with the phosphorylation data, suggest that the rapamycin-sensitive phosphorylation induced by IL-4 may serve a novel regulatory role through decreased repression of the germ line e promoter.

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