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Characterization of the Interleukin-4 Nuclear Activated Factor/STAT and Its Activation Independent of the Insulin Receptor Substrate Proteins*

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The activation of a latent DNA binding factor by interleukin-4 (IL-4), the IL-4 nuclear activated factor (IL-4 NAF), occurs within minutes of IL-4 binding to its receptor. Molecular characterization of IL-4 NAF by ultraviolet light cross-linking experiments revealed a single protein of 120–130 kDa in contact with the DNA target site. Glycerol gradient sedimentation analysis indicated a molecular mass of IL-4 NAF consistent with a monomer that is capable of binding DNA. The IL-4 NAF target site is a palindromic sequence that is also recognized by the interferon-induced transcription factor, p91/STAT1α. However, IL-4 NAF and p91/STAT1α display distinguishable DNA binding specificities that may generate one level of specificity in the expression of target genes. Previous studies suggested the involvement of the insulin receptor substrate-1 (IRS-1) in the IL-4 signal transduction pathway. Although IRS-1 is involved in the stimulation of mitogenesis, our results demonstrate that activation of IL-4 NAF is independent of IRS-signaling proteins. The results of this study indicate that IL-4 stimulates bifurcating signal pathways that can direct mitogenesis via the IRS-signaling proteins and specific gene expression via the IL-4 NAF.

Cells respond to extracellular stimuli with distinct biological changes that can dictate proliferation, differentiation, or death. Within minutes of extracellular stimulation, signals are transmitted from the cell surface to the nucleus culminating in an alteration of specific gene expression. Interleukin-4 (IL-4)[1] is a polypeptide cytokine that initially was identified by its potent effect on B lymphocyte proliferation (Ref. 1, and for review, see Ref. 2). It is now known that the physiological effects of IL-4 are not restricted to B cells but in fact are elicited in a variety of cell types. Activated T lymphocytes and mast cells produce and secrete IL-4, which can function in an autocrine manner or can stimulate other cells of both hematopoietic and nonhematopoietic lineages.

The actions of IL-4 appear to be dependent on the stimulation of specific gene expression. IL-4 induces the transcription of genes involved in immune recognition, including the immunoglobulin E receptor (FcεRII) (3, 4) and the major histocompatibility complex class II genes (for review, see Refs. 5 and 6). Furthermore, IL-4 stimulates the transcriptional activation of immunoglobulin constant region genes IgE and IgG_1 and thereby promotes Ig class switching (for review, see Ref. 7). Some of the transcriptional effects of IL-4 are either analogous or antagonistic in comparison with the effects of another cytokine, interferon-γ (IFN-γ). We have found that IL-4 may exert these effects by activating a DNA binding factor that recognizes the same DNA sequence that is recognized by an IFN-γ-induced factor (8).

Recent studies in the IFN-γ system have identified a receptor to nucleus signal pathway that involves the activation of a latent DNA binding factor composed of a 91-kDa protein (for review, see Refs. 9 and 10). This protein is a member of the newly emerging family of latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation and have been termed signal transducers and activators of transcription (STAT) (10). The 91-kDa protein that is tyrosine phosphorylated in response to IFN-γ has been designated STAT1α. Following phosphorylation of STAT1α, it translocates to the nucleus and binds as a dimer to the IFN-γ-activated site (GAS), a palindromic DNA sequence in the promoter of genes transcriptionally responsive to IFN-γ (11).

A GAS-binding factor distinct from STAT1α is activated by tyrosine phosphorylation following stimulation with IL-4 that we termed the IL-4 nuclear activated factor (IL-4 NAF) (8, 12, 13). Recently, a gene encoding an IL-4-induced STAT (STAT6) has been cloned that may serve as the component of IL-4 NAF activity (14). It remains to be determined how transcription factors such as STAT1α and STAT6 bind to the same DNA target site but elicit differential gene transcription. In this report, we characterize the nature of the DNA binding component of IL-4 NAF and in addition compare the GAS-binding specificities of IL-4 NAF with the IFN-γ-activated STAT1α.

Previous studies have indicated the involvement of a common signaling molecule in the response to either IL-4 or insulin (for review, see Ref. 15). A prominent tyrosine phosphorylated protein originally called the IL-4 phosphorylated substrate (4PS) appears following IL-4 stimulation of hematopoietic cells. Recently the gene encoding this protein has been cloned and found to be related to the insulin receptor substrate-1 (IRS-1); it has been designated as IRS-2.[2] The IRS-signaling proteins contain multiple sites of tyrosine phosphorylation that can act as binding sites for signaling molecules that have Src homology

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[1] The abbreviations used are: IL, interleukin; IFN-γ, interferon-γ; STAT, signal transducer and activator of transcription; GAS, IFN-γ-activated site; IL-4 NAF, IL-4 nuclear activated factor; 4PS, IL-4 phosphorylated substrate; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein.

[2] M. White, unpublished results.

2 domains such as the growth factor receptor-bound protein 2 (Grb2) and phosphatidylinositol 3-kinase (for review, see Ref. 16). In this manner, the IRS-signaling proteins act to amplify the signals initiated at the receptor. The mitogenic response of cells to insulin or IL-4 appears to require IRS molecules (17). A cell line that lacks IRS-signaling proteins and does not respond mitogenically to IL-4 can be reverted to a responsive phenotype by complementation with the IRS-1 gene. In this study we test the involvement of IRS-1 in the activation of IL-4 NAF and the stimulation of DNA synthesis by IL-4.

EXPERIMENTAL PROCEDURES

Cell Culture—CCL2 and S3 HeLa cell lines (ATCC, Rockville, MD) were maintained as monolayers in Dulbecco's modified Eagle's medium containing 8% fetal bovine serum or as suspension in minimal essential medium containing 5% calf serum. The IL-3-dependent murine myeloid progenitor cell line, FDC-P1, was a gift of Dr. Steven Anderson (University of Colorado at Denver). The 32D and 32D-derived lines were described previously (17). They were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 5% WEHI-3 IL-3 conditioned media. Stable 32D transfectant cells expressing IRS-1 were maintained in selective media with 5 mM histidinol (Sigma) (17).

Nuclear Extracts and Gel Shift Analysis—Cells were stimulated with cytokine for 15 min. All IL-3-dependent murine myeloid progenitor cell lines were placed in serum-free media in the absence of IL-3 for 4 h prior to stimulation. Human and murine recombinant IL-4 were a gift from Schering-Plough Research Institute (Kenilworth, NJ), and human recombinant IFN-γ was a gift from Hoffmann-LaRoche Inc. (Nutley, NJ). IL-3 was obtained as conditioned media from WEHI-3 cell supernatants. Nuclear extracts were prepared as described previously, and electrophoretic mobility shift analyses were performed with a double-stranded oligonucleotide representing the GAS site from the FcγRI gene (−33 to −14), 5′ GTATTTCCCAGAAAAGGAAC 3′ (8).

Partial Purification—HeLa cells in suspension culture (Cell Culture Center, Minneapolis, MN) were stimulated for 15 min with IL-4, and nuclear extracts were prepared as described previously (8). Nuclear protein was chromatographed on P-11 phosphocellulose (Whatman Bio-Systems, UK) or SP Sepharose (Pharmacia Biotech Inc.). Bound proteins were eluted with increasing concentrations of KCl in 20 mM Hepes, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride.

Photoaffinity Cross-linking—5-Bromodeoxyuridine was incorporated in the oligonucleotide 5′-GATCTATTTCCCAGAAAAGGAACAT-3′ by Klenow polymerase and the primer 5′-ATGTTCC-3′. The 5-bromodeoxyuridine-substituted double-stranded oligonucleotide was subsequently end-labeled with T4 polynucleotide kinase and [γ^{32}P]ATP for use in a preparative gel shift with partially purified material. Ultraviolet light cross-linking of protein-DNA complexes was performed in the gel for 5 min at 254 nm with a UV Stratalinker (Stratagene, La Jolla, CA). Gel slices representing the specific protein-DNA complexes or corresponding regions of the gel with control extract were excised and eluted at 65 °C in SDS sample buffer before analysis on a SDS-polyacrylamide gel.

Glycerol Gradient Sedimentation—Partially purified IL-4 NAF was concentrated in Microcon-30 concentrators (Amicon, Beverly, MA) and layered onto 4-ml 25–50% glycerol gradients (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.001% Nonidet P-40). Centrifugation was performed in a SW60 rotor at 40,000 rpm at 4 °C for 44 h. Molecular size standards were centrifuged on a separate gradient (apoferritin, 443 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; and carbonic anhydrase, 29 kDa). Fractions of 150 μl were collected and assayed for IL-4 NAF DNA binding activity. Sedimentation of protein markers was analyzed by Coomassie Blue staining of SDS-polyacrylamide gels.

Proliferation Assay—Duplicate samples of murine myeloid cells (4×10^5 cells/ml) were starved in serum-free RPMI 1640 media in 24-well plates for 6 h prior to treatment with IL-4 for 16 h. Subsequently, cells were pulse-labeled for 5 h with [^{3}H]thymidine (3 μCi/well) and lysed, and trichloroacetic acid-precipitable material was collected onto nitrocellulose filters and quantified by liquid scintillation.

MAP Kinase Activation Assay—Cells were placed in serum-free media for 4 h prior to stimulation with either murine IL-4, human insulin (Boehringer Mannheim), IL-3-conditioned media, or unconditioned media. MAPK gel mobility assays were performed by lysing the cells in 50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin. Following electrophoresis, proteins were transferred to Immobilon (Millipore, Bedford, MA), and immunoblotted with antibody that recognizes ERK-1 and ERK-2 (K-23, Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were detected by enhanced chemiluminescense (DuPont NEN). *In vitro* kinase assays were performed essentially as described previously (18, 19). MAPK was immunoprecipitated with anti-ERK-1 (C-16, Santa Cruz Biotechnology), and immunoprecipitates were used in an *in vitro* kinase assay with 2 μg of myelin basic protein (MBP) (Sigma) and 10 μCi of [γ^{32}P]ATP. ^{32}P-labeled MBP was analyzed following electrophoresis and quantitated with a Betaimager (Betascope 603, Betagen, Waltham, MA).

RESULTS

Characterization of IL-4 NAF—We previously described the activation of a latent DNA binding factor by IL-4 termed IL-4 NAF (8). Our initial studies were performed in hematopoietic cell types, such as the human monocytic cell line THP-1. However, IL-4 is also known to stimulate proliferation and to alter specific gene expression in nonhematopoietic cell lineages (20, 21). For this reason, we sought to determine whether IL-4 could signal the activation of IL-4 NAF in nonhematopoietic cells that express the IL-4 receptor such as the human epithelial HeLa cell line (22) (Fig. 1*A*). Nuclear extracts were prepared from untreated and IL-4-treated HeLa cells and were analyzed for IL-4 NAF activity by a gel shift assay with an oligonucleotide representing the FcγRI GAS target site (8). IL-4 stimulation of HeLa cells resulted in the appearance of the IL-4 NAF complex (*lane 2*). DNA binding specificity was demonstrated with a 50-fold excess of unlabeled oligonucleotide that competed with radiolabeled oligonucleotide for IL-4 NAF binding (*lane 3*). Migration of the IL-4 NAF complex in the gel was similar with extracts from HeLa or THP-1 cells (data not shown). Therefore, both hematopoietic and nonhematopoietic cells appear to respond to IL-4 by signaling the activation of a similar latent DNA binding factor.

The molecular composition of IL-4 NAF is distinct from the IFN-γ-induced STAT1α factor that binds to the GAS site. We previously demonstrated that STAT1α is not a component of the IL-4 NAF complex (8). To further characterize this complex, IL-4 NAF was partially purified from HeLa cells by standard chromatography methods. Photoaffinity cross-linking was performed with fractions from a P-11 phosphocellulose column to identify the molecular weight of the IL-4 NAF DNA binding component (Fig. 1*B*). A bromodeoxyuridine-substituted probe was used in a preparative gel shift analysis with a partially purified IL-4 NAF fraction that eluted at 300 mM KCl or a control fraction that eluted at 400 mM KCl that did not contain IL-4 NAF. Proteins contacting the FcγRI GAS oligonucleotide were cross-linked to the DNA by exposing the gel to UV light. After autoradiography, gel slices representing the IL-4 NAF complex or an adjacent control lane corresponding to a DNA binding reaction with a chromatography fraction that did not contain IL-4 NAF were excised and analyzed by a SDS-polyacrylamide gel. The protein contacting the GAS is visible by virtue of its covalent attachment to the radiolabeled DNA. The control gel slice did not reveal any protein cross-linked to the DNA (*U*). The IL-4 NAF complex contained a protein that specifically cross-linked to the DNA (*B*). Relative mobility of the DNA-protein complex predicts a molecular size of the IL-4 NAF DNA binding component to be 120–130 kDa, taking into account the molecular weight of the cross-linked oligonucleotide. These results suggest that the DNA binding component of IL-4 NAF is a single protein subunit.

It is possible that IL-4 NAF is a multimeric protein complex and the UV cross-linked protein is the subunit associated with DNA. To determine the native molecular weight of IL-4 NAF, glycerol gradient sedimentation analysis was performed (Fig. 1*C*). Partially purified IL-4 NAF from a SP Sepharose column

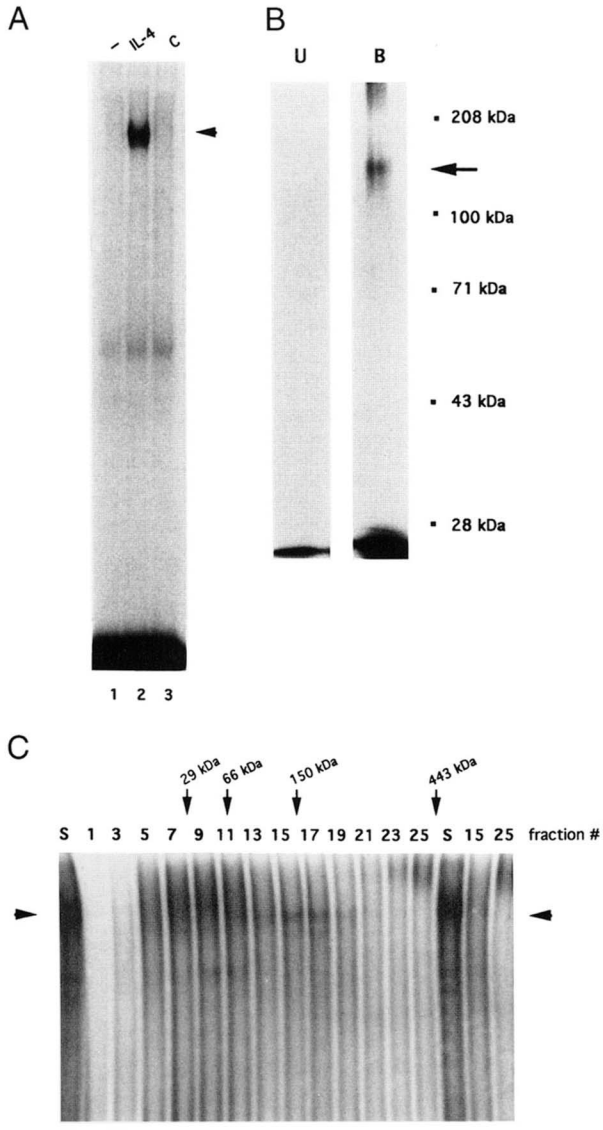

FIG. 1. **Characterization of IL-4 NAF in HeLa cells.** *A*, gel mobility shift analysis of IL-4-treated HeLa cells. Nuclear extracts were prepared from HeLa cells either untreated (*lane 1*) or treated for 15 min with IL-4 (20 ng/ml) (*lanes 2* and *3*). Gel shift analysis was performed as described previously with an end-labeled double-stranded oligonucleotide representing the GAS site from the FcγRI gene. Migration of IL-4 NAF (*lane 2*) is depicted by an *arrow*. To demonstrate binding specificity a 50-fold molar excess of unlabeled oligonucleotide was included in the binding reaction (*lane 3*). *B*, photoaffinity cross-linking of IL-4 NAF. Proteins chromatographed on P-11 phosphocellulose were fractionated as material that bound IL-4 NAF and eluted at 300 mM KCl (*B*) or as unbound material that eluted at 400 mM KCl (*U*). A preparative gel shift assay was performed with a bromodeoxyuridine-substituted FcγRI GAS probe. The gel was exposed to UV light and autoradiographed. Gel slices were excised and analyzed on a 9% SDS-polyacrylamide gel. The position of protein standards is shown (in kDa), and migration of the IL-4 NAF DNA binding component cross-linked to DNA is depicted by an *arrow*. *C*, glycerol gradient sedimentation analysis. Partially purified IL-4 NAF or molecular weight standards were layered onto 25–50% glycerol gradients for sedimentation analysis. Every other collected fraction from the IL-4 NAF gradient was analyzed by a gel shift assay. A sample of IL-4 NAF activity prior to sedimentation is shown as a control (*S*). The peak migration of protein standards, as determined by Coomassie Blue staining, is shown *above* the *lanes*. A competition analysis with a 50-fold molar excess of unlabeled GAS oligonucleotide is shown with fraction *15* and *25* in the *rightmost lanes* to demonstrate binding specificity.

or molecular weight standards were centrifuged through 25–50% glycerol gradients. The sedimentation coefficients of protein standards with known molecular mass were estimated by their position in the gradient and detected by SDS-polyacrylamide electrophoresis followed by staining with Coomassie Blue. IL-4 NAF activity was tested by a gel shift assay and found predominantly between fractions 15 and 17. This peak fraction represents a protein with a relative size of 140–150 kDa. A slower migrating protein-DNA complex in fraction 25 was not specific since it did not compete for binding with unlabeled specific oligonucleotide (*rightmost lane*). The mass of the IL-4 NAF complex predicted by this sedimentation analysis is similar to the size of the DNA binding component identified by UV cross-linking. It appears that IL-4 NAF predominantly exists as a monomer under these experimental conditions. However, it is possible that IL-4 NAF exists as a dimer and dissociates during glycerol gradient sedimentation.

IL-4 NAF DNA Binding Specificity—The promoters of several IL-4 responsive genes contain a GAA palindromic sequence similar to the GAS element that competes for IL-4 NAF binding (8). The number of nucleotides separating the GAA inverted repeat sequence in these promoters varies with the most common spacing being 3 or 4 nucleotides. To determine whether the DNA binding specificity of IL-4 NAF was dependent upon this spacing, a binding study was performed with double-stranded oligonucleotides representing the FcγRI GAS site with two, three, four, or five nucleotides between the inverted repeat (Fig. 2*A*). These oligonucleotides were analyzed for their ability to compete for IL-4 NAF binding by a gel shift assay (Fig. 2*B*). Nuclear extracts from IL-4-treated HeLa cells were incubated with a 100- or 200-fold molar excess of unlabeled variant spacer oligonucleotides prior to the addition of the radiolabeled probe. The DNA sequence with three nucleotides (*lanes 5* and *6*) or four nucleotides (*lanes 7* and *8*) between the inverted repeats competed efficiently for IL-4 NAF binding. However, the DNA sequence with two nucleotides (*lanes 3* and *4*) or five nucleotides (*lanes 9* and *10*) between the repeats did not compete effectively. Therefore, nucleotide spacing between the inverted repeat may define the specificity of IL-4 NAF binding to GAS-like elements.

To determine if the DNA binding specificity of STAT1α was distinct from IL-4 NAF, nuclear extracts from IFN-γ-treated cells were subjected to a similar analysis (Fig. 2*C*). We found that STAT1α bound most efficiently to the DNA with three nucleotides between the inverted repeat (*lanes 5* and *6*) and with less efficiency to the DNA with two nucleotides between the repeats (*lanes 3* and *4*). The DNA sequence with four or five nucleotides between the repeats did not compete efficiently (*lanes 7–10*). Therefore, the DNA binding specificities of STAT1α and IL-4 NAF are distinct. This finding would predict that IFN-γ-induced STAT1α and IL-4-induced IL-4 NAF can recognize both overlapping and distinct subsets of genes.

IL-4 NAF Activation Independent of IRS-signaling Proteins—IL-4 induces a signaling pathway that involves the tyrosine phosphorylation of 4PS/IRS-2, a cellular protein antigenically related to IRS-1,[2] in IL-3-dependent murine myeloid cell lines (23, 24). In these murine myeloid cells, IL-4 stimulates the tyrosine phosphorylation of 4PS/IRS-2 and mitogenesis. 32D cells are a specific murine myeloid lineage that lack IRS-signaling proteins and are not responsive to IL-4-induced mitogenesis (17). However, following transfection and overexpression of the IRS-1 gene in 32D cells, IL-4 signaling events such as the tyrosine phosphorylation of IRS-1 and mitogenesis are restored. To determine if the activation of IL-4 NAF is dependent upon IRS-signaling proteins, these IRS-deficient cells were used as a model system (Fig. 3). 32D cells that lack IRS molecules (*lanes 1–3* and *7–9*) and 32D cells transfected with the IRS-1 gene (*lanes 4–6* and *10–12*) were analyzed for their ability to signal the activation of IL-4 NAF by IL-4. In

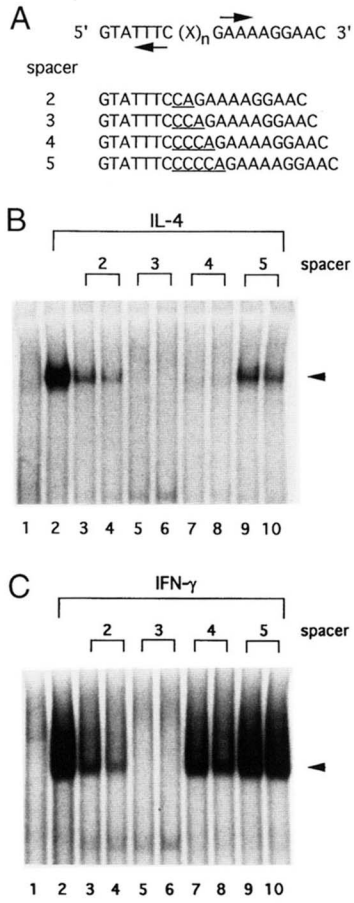

FIG. 2. **Analysis of IL-4 NAF DNA binding specificity.** *A*, single-stranded sequences representing the FcγRI GAS site (−33 to −14) with two, three, four, or five nucleotides (*underlined*) between the GAA palindrome (*arrows*) are shown. *B*, nuclear extracts from HeLa cells untreated (*lane 1*) or IL-4-treated (20 ng/ml) for 15 min (*lanes 2–10*) were used in a competitive gel shift analysis. A 100- or 200-fold molar excess of unlabeled double-stranded oligonucleotide was included in the binding reaction before incubation with the native FcγRI radiolabeled GAS probe. The spacer designations refer to nucleotide number between the inverted repeat (shown in Fig. 2*A*). Migration of the IL-4 NAF complex is shown by an *arrow*. *C*, nuclear extracts from untreated (*lane 1*) or 15 min IFN-γ treated (1000 units/ml) HeLa cells (*lanes 2–10*) were analyzed in a competitive gel shift assay with a 100- or 200-fold molar excess of the double-stranded oligonucleotides described in *A*.

addition, all cell lines were stimulated with IL-3 as a control. Nuclear extracts from untreated, IL-3-treated, or IL-4-treated cells were analyzed by a gel shift assay using an oligonucleotide representing the FcγRI GAS site. All of the cells responded to IL-3 with the activation of a latent GAS binding factor (25). Interestingly, IL-4 NAF activation occurred in all of the cell lines, even those deficient in IRS-signaling proteins. The overexpression of IRS-1 did not appear to alter the level of IL-4 NAF activation (*lanes 6* and *12*). Therefore, the IRS proteins are not required for signal activation of IL-4 NAF. The IL-4 NAF activation represents an independent signal pathway to gene expression.

Since the 32D myeloid cells that lack IRS-signaling proteins responded to IL-4 with the activation of IL-4 NAF, the ability of IL-4 to signal mitogenesis in these cells was analyzed. IRS-deficient cells (32D), IRS-1-expressing cells (32D+IRS-1), and a positive control cell line containing 4PS/IRS-2[1] (FDC-P1) were untreated or treated with IL-4, and DNA synthesis was measured by [^{3}H]thymidine incorporation (Fig. 4*A*). In FDC-P1 cells, IL-4 stimulated DNA synthesis by increasing [^{3}H]thymidine incorporation 20-fold over unstimulated cells. 32D cells did not undergo a substantial increase in DNA synthesis, even

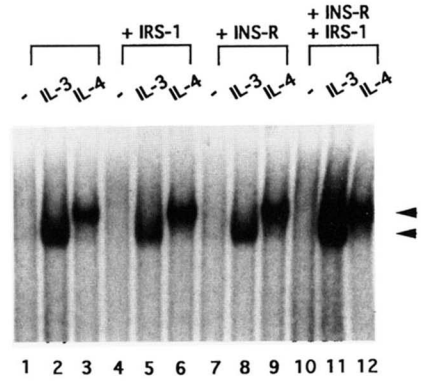

FIG. 3. **Activation of IL-4 NAF is independent of IRS-signaling proteins.** Murine myeloid cell lines were untreated (−), treated with IL-3, or treated with IL-4 (200 ng/ml) for 15 min. Nuclear extracts were prepared from 32D cells (*lanes 1–3*), 32D+IRS-1 cells (*lanes 4–6*), 32D+INS-R (*lanes 7–9*), or 32D+INS-R/IRS-1 (*lanes 10–12*) and analyzed by a gel shift assay with a FcγRI GAS probe. The migration of protein-DNA complexes is depicted by *arrows*.

though IL-4 NAF was activated. IRS-1-expressing 32D cells stimulated DNA synthesis as efficiently as FDC-P1 cells. Therefore in the absence of IRS-signaling proteins, IL-4 NAF activation alone is not sufficient to signal mitogenesis.

Many signaling pathways that control cellular proliferation or differentiation initiate a cascade of events that activate the MAPKs (also known as extracellular signal regulated kinases) (for review, see Refs. 26 and 27). For this reason, we analyzed the activation of two well characterized MAPKs of 42 (p42) and 44 kDa (p44) following IL-4 stimulation (Fig. 4*B*). Latent MAPK is unphosphorylated, and activation requires tyrosine and threonine phosphorylation by a dual specificity kinase. The phosphorylated MAPK migrates more slowly than unphosphorylated MAPK during electrophoresis in SDS-polyacrylamide gels and can be visualized by immunoblot assays (28). This assay was employed to detect activated MAPK following treatment of 32D+INS-R/IRS-1 cells with IL-3, IL-4, or insulin for either 10 min or 2 h. IL-3 stimulation resulted in a sustained activation of MAPK as phosphorylated levels of MAPK were still high at 2 h (*lanes 1* and *6*). Insulin activated MAPK but produced only a short-lived response (*lane 3 versus 8*). This assay did not detect IL-4 activation of MAPK. An alternative method to evaluate MAPK activation was performed with an *in vitro* kinase assay using MBP as a substrate molecule (Fig. 4*C*). Radiolabeled MBP was quantified, and results demonstrated that IL-3 and insulin reproducibly stimulated activation of MAPK, whereas IL-4 did not.

DISCUSSION

Binding of IL-4 to specific cell surface receptors stimulates the activation of a latent DNA binding factor, IL-4 NAF (8, 12, 13). Activation of IL-4 NAF occurs in both hematopoietic and nonhematopoietic cell lineages and likely mediates specific effects on gene expression. To characterize this transcription factor, we have used UV cross-linking experiments to determine the molecular size of its DNA binding component. A single protein of molecular size 120–130 kDa was identified by this technique (Fig. 1*B*). Recently a gene encoding an IL-4-induced GAS-binding factor was cloned from a hematopoietic line (14). The cDNA of this gene predicts a 94-kDa protein with significant homology to a family of transcription factors known as STATs and has been termed IL-4 STAT or STAT6 (10, 14). It remains to be determined whether IL-4 NAF and STAT6 are identical; however, the activities indicate that they represent the same factor. The reason for the greater molecular mass detected by our biochemical studies could reflect protein mod-

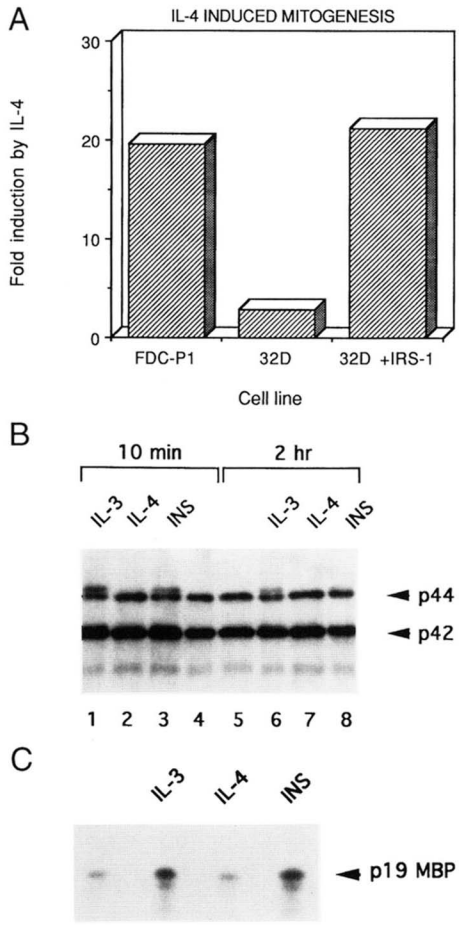

FIG. 4. *A*, IL-4-induced DNA synthesis. FDC-P1, 32D, and 32D+IRS-1 cells were starved for 6 h before treatment with IL-4 (100 ng/ml) or media for 16 h. DNA synthesis was measured by incubation of the cells with [^{3}H]thymidine for 5 h. [^{3}H]Thymidine incorporation was calculated as a -fold induction of cpm in IL-4-stimulated cells over unstimulated cells and is depicted as a bar graph for each cell line. *B*, activation of MAPK. 32D+INS-R/IRS-1 cells were stimulated with either IL-3 (*lanes 1* and *6*), IL-4 (200 ng/ml) (*lanes 2* and *7*), insulin (250 ng/ml) (*lanes 3* and *8*), or unconditioned media (*lanes 4* and *5*) for either 10 min (*lanes 1–4*) or 2 h (*lanes 5–8*). 25 μg of protein lysate were electrophoresed and immunoblotted with antibody that recognizes MAPK. The mobilities of p44 MAPK and p42 MAPK are indicated by *arrows*. *C*, *in vitro* MAPK assay. 32D+INS-R/IRS-1 cells were stimulated as in *B* for 15 min, lysed, and immunoprecipitated with antibody to MAPK. MAPK immunoprecipitates were used in an *in vitro* kinase assay with MBP as substrate. Phosphorylated MBP is shown by an *arrow*.

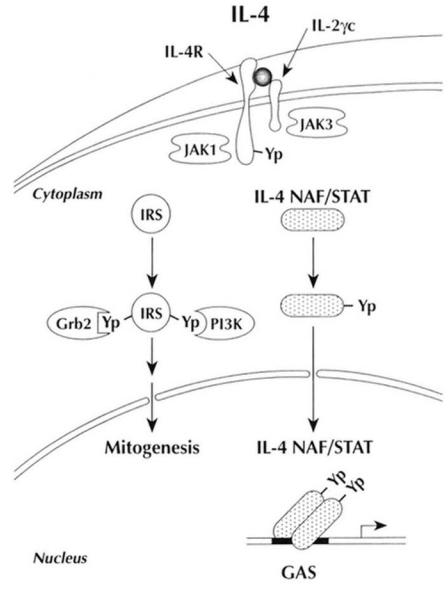

FIG. 5. **Illustrative model of the IL-4 signal transduction pathway.** Bifurcating IL-4 signals emanate from a ligand-bound IL-4 receptor. IL-4 binding activates JAK1 and JAK3 and the subsequent tyrosine phosphorylation of IL-4 NAF/STAT. Activated IL-4 NAF/STAT translocates to the nucleus and binds to a specific target site (*GAS*) in the promoters of regulated genes. IL-4 also stimulates the tyrosine phosphorylation of IRS proteins and their association with signaling molecules that include phosphatidylinositol 3-kinase and Grb2 (39) that may be involved in mitogenesis.

ification and awaits further study.

Recent studies indicate that the STAT family members STAT1α and STAT6 bind to their target DNA sites as dimers (11, 14). Since the IL-4 NAF target site has an inverted repeat sequence (palindrome), the IL-4 NAF may bind the site as a dimer. To determine if the molecular mass of IL-4 NAF corresponded to a dimer, a glycerol gradient sedimentation analysis was performed. The results of this study indicated that the native molecular weight of IL-4 NAF was similar to that of the DNA binding component, and suggested that it exists as a monomer in solution (Fig. 1*C*). It is possible that IL-4 NAF exists as a dimer in solution, but it dissociates to monomers during glycerol gradient sedimentation. If this is the case, our results would suggest that an IL-4 NAF monomer can bind to DNA or that the monomers can interact to form dimers by association with the DNA target.

Distinct members of the STAT family of transcription factors are activated by different cytokines and growth factors, and although the STATs appear to recognize a similar GAA palindromic sequence, the cytokines elicit different biological responses and gene expression. To understand the specificity of gene activation induced by distinct extracellular ligands, we analyzed the DNA binding specificites of STAT1α and IL-4 NAF. The GAA inverted repeats found in various promoters differ in the number of nucleotides that separate the repeat (8). For this reason, we tested inverted repeat sequences separated by two, three, four, or five nucleotides for their ability to be recognized by STAT1α or IL-4 NAF (Fig. 2). IL-4 NAF was found to bind preferentially to inverted repeats separated by three or four nucleotides, whereas STAT1α predominantly binds to an inverted repeat separated by three nucleotides. Inverted repeats with a spacing of three or four nucleotides are common in the promoters of the IL-4-responsive genes FcϵRIIb and Cγ1 (8). Our finding that the DNA binding properties of IL-4 NAF and STAT1α are distinct suggests that nucleotide spacing can generate one level of specificity in the expression of target genes by each factor. The ability of transcription factors to discriminate between similar repeat sequences separated by different distance is best exemplified with retinoid receptors (29). In this system, DNA binding specificity is determined by the number of nucleotides between direct repeat sequences. Our studies indicate that a similar mechanism is utilized by the DNA binding factors that recognize the GAA inverted repeat of the GAS.

The activation of IL-4 NAF requires tyrosine phosphorylation and occurs within minutes of IL-4 binding to specific cell surface receptors (8, 12, 13). The IL-4 receptor possesses a single transmembrane domain, but it does not possess intrinsic kinase activity (2, 15). Recent studies have demonstrated activation of the Janus kinase (JAK) family of cytoplasmic protein tyrosine kinases, JAK1 and JAK3, following IL-4 treatment (30, 31). In addition, dimerization of the IL-4 receptor with the IL-2 receptor γ chain is believed to initiate signal activation (32, 33). Several observations have suggested the involvement of the IRS signaling system in the IL-4 signal pathway. Fol-

lowing IL-4 stimulation, 4PS/IRS-2 is tyrosine-phosphorylated and associates with the p85 regulatory subunit of phosphatidylinositol 3-kinase believed to be involved in mediating a mitogenic response (23, 24). In addition, murine myeloid 32D cells that lack IRS signaling proteins are not responsive mitogenically to IL-4 (17). For these reasons, we sought to determine if IRS-signaling proteins are required for activation of IL-4 NAF. Our results demonstrate that activation of IL-4 NAF is independent of IRS molecules (Fig. 3). While the IRS molecules clearly play a role in the ability of IL-4 to fully induce mitogenesis (Fig. 4*A*; Ref. 17), it was not required for signal activation of the transcription factor IL-4 NAF. Although a small increase in mitogenesis was induced by IL-4 in the absence of IRS molecules (~10% of control), activation of IL-4 NAF does not appear to be sufficient to stimulate proliferation.

Many extracellular stimuli that trigger cellular proliferation activate the MAPK pathway (for review, see Refs. 26 and 27). In fact, constitutive activation of MAPK kinase, the regulator of MAPK, is sufficient for cellular transformation and tumorigenesis (34, 35). Conversely, negative interfering mutants of MAPK kinase inhibit proliferation and revert transformed cells (34). We examined the effects of IL-3, IL-4, and insulin on MAPK in the murine myeloid cells that express IRS-1 and found that IL-4 does not activate MAPK, in accordance with other observations (Fig. 4, *B* and *C*) (36, 37). Insulin and IL-3 were used as positive controls for the activation of MAPK (19, 36, 38). Insulin activated MAPK for a short (10 min) duration, but IL-3 treatment produced a sustained activation of MAPK (>2 h) (Fig. 4*B*). IL-3-induced mitogenesis may depend on prolonged activation of MAPK.

The results of this study suggest that bifurcating pathways emanate from a ligand bound IL-4 receptor to produce specific biological responses such as proliferation and differentiation (Fig. 5). IL-4 signals a direct receptor to nucleus pathway that entails the activation of a latent DNA binding factor, IL-4 NAF, which functions to regulate specific gene expression necessary for differentiation. A second signal pathway involves the activation of IRS-signaling proteins and their association with specific targets such as phosphatidylinositol 3-kinase that function to signal mitogenesis. It is possible that signals from both pathways intersect at a common point to produce a mitogenic response. In addition, unidentified pathways may be stimulated following activation of this complex signaling network that contribute to the pluripotent effects of IL-4 on various cell types.

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Note Added in Proof—Subsequent to this study a specific antibody that recognizes STAT6 became available (Santa Cruz Biotechnology). This antibody recognizes the IL-4 NAF complex in a gel mobility assay indicating that STAT6 is a component of IL-4 NAF.

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