

21. A biostatistician (E.J.O.) randomized each patient to either the active or placebo treatment group in blocks of six, stratified by functional class (28) severity.
22. The placebo consisted of 1.0-ml doses of 0.1 M acetic acid subjected to membrane filtration.
23. Three investigators (D.C., C.L., and K.L.S.) obtained the randomization and prepared medication but did not have access to clinical data. No unblinding occurred.
24. Conventional instruments were used to measure RA activity (16). Assistive devices were permitted for walk times. The clinical investigator cared for the patients during the trial and was responsible for safety monitoring. Laboratory safety assessment was performed immediately before randomization and at 2, 4, 8, and 12 weeks thereafter. The assessment comprised a complete blood count, differential and platelet count, liver and renal function tests, prothrombin and partial thromboplastin times, urinalysis, and ESR. HLA typing was performed for alleles of the A, B, C, and DR/DQ loci (36). Serum immunoglobulin M (IgM) rheumatoid factor titers were determined by nephelometry and IgG antibody titers to native type II collagen (expressed as $-\log_2$) by enzyme-linked immunosorbent assay (37) immediately before and at the end of collagen or placebo administration.
25. Before unblinding, decisions were made concerning the analysis of five subjects (8%) that failed to complete the study. One was noncompliant and withdrew for personal reasons on day 40 after only a baseline examination. This patient was excluded from analysis and had been randomized to collagen. Four discontinued their study medication before the end of the 3-month treatment because of worsening arthritis. They were assigned the worst score in the sample for the remainder of the study and included in the analyses. All four had been randomized to placebo. One protocol violation occurred with a patient who increased the daily dose of prednisone from 5 mg to 10 mg just before month 2. Because the patient continued to do poorly and the 10-mg dose was consistent with eligibility requirements, the patient was included in the analyses; the patient had been randomized to collagen. No steroid injections or other problems with compliance occurred.
26. Comparisons between collagen- and placebo-treated patients were performed with the Wilcoxon rank-sum test for continuous measures (such as the number of swollen joints), the Fisher's exact test for dichotomous measures (such as narcotic usage), and the χ^2 trend test for functional class and patient and physician assessments. All measured end points such as the number of swollen joints were compared with entry values before testing; qualitative measures, such as patient and physician assessments and functional class, are presented and analyzed without adjustment for baseline responses. The Student's paired *t* test was used to assess whether changes in the collagen group represented significant improvements over baseline values. Reported *P* values are two-sided.
27. Complete resolution is a more rigorous extension of RA remission criteria (17), reformulated because of the magnitude of improvement in some patients in the initial trial, and is defined by the following conditions: no swollen or tender joints, no morning stiffness or afternoon fatigue, absent arthritis on physician and patient appraisals, functional class I status, and normal ESR (<28 mm/hour) while off prednisone.
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29. Rather than assigning the placebo patients who withdrew from the trial the worst observed value (25), they were given the value from their last visit. Because one of the four dropped out before the 1-month follow-up, that patient was removed from all analyses, reducing the sample size to 28 collagen and 30 placebo patients. By this analysis, the number of tender joints, joint-tenderness index, walk time, patient assessment of severe or very severe disease, and analgesic use was

- significantly ($P \leq 0.05$) improved in the collagen group compared with the placebo group.
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 38. Supported by NIH grants MO1 RR01032 and AG00294 and by a grant from AutoImmune Inc. We thank E. Milford and C. B. Carpenter for HLA typing. In accordance with disclosure guidelines of the Harvard Medical School, D.A.H. and H.L.W. have a financial interest in AutoImmune Inc.

9 June 1993; accepted 23 August 1993

Tyrosine Phosphorylation of DNA Binding Proteins by Multiple Cytokines

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Interferon- α (IFN- α) and IFN- γ regulate gene expression by tyrosine phosphorylation of several transcription factors that have the 91-kilodalton (p91) protein of interferon-stimulated gene factor-3 (ISGF-3) as a common component. Interferon-activated protein complexes bind enhancers present in the promoters of early response genes such as the high-affinity Fc γ receptor gene (Fc γ RI). Treatment of human peripheral blood monocytes or basophils with interleukin-3 (IL-3), IL-5, IL-10, or granulocyte-macrophage colony-stimulating factor (GM-CSF) activated DNA binding proteins that recognized the IFN- γ response region (GRR) located in the promoter of the Fc γ RI gene. Although tyrosine phosphorylation was required for the assembly of each of these GRR binding complexes, only those formed as a result of treatment with IFN- γ or IL-10 contained p91. Instead, complexes activated by IL-3 or GM-CSF contained a tyrosine-phosphorylated protein of 80 kilodaltons. Induction of Fc γ RI RNA occurred only with IFN- γ and IL-10, whereas pretreatment of cells with GM-CSF or IL-3 inhibited IFN- γ induction of Fc γ RI RNA. Thus, several cytokines other than interferons can activate putative transcription factors by tyrosine phosphorylation.

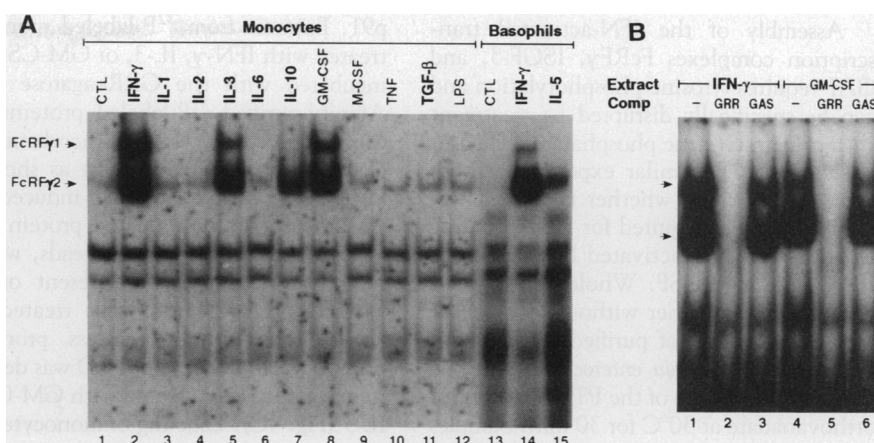
Nuclear or whole-cell extracts prepared from human monocytes incubated with either IFN- γ or IFN- α contain a protein or proteins (FcRF γ) that specifically recognize the GRR in the promoter of the high-affinity immunoglobulin G Fc receptor gene (1-3). Within the FcRF γ complex is a 91-kD tyrosine-phosphorylated protein that is a component of the ISGF-3 transcription complex, which causes IFN- α -stimulated expression of early response genes (2-4). Because the peripheral blood monocyte is a critical target cell for IFN- α , IFN- γ , and other cytokines, experiments were done to determine whether any cytokines other than the interferons might induce the formation of FcRF γ . Whole-cell extracts were prepared from monocytes incubated with various cytokines for 15 min at 37°C and analyzed by electrophoretic mobility-shift assays (EMSA) with a ³²P-labeled oligonucleotide corresponding to the GRR (Fig. 1A) (5). Untreated cells showed no forma-

tion of FcRF γ , whereas extracts prepared from monocytes treated with IL-3 or GM-CSF contained GRR binding complexes that migrated with a mobility different than that of the FcRF γ (Fig. 1A) complex observed after IFN- γ activation. In contrast, IL-10 activated the formation of a GRR binding complex with a mobility similar to that of FcRF γ . Other cytokines that have effects on monocytes—IL-1, IL-2, IL-6, tumor necrosis factor (TNF), monocyte colony-stimulating factor (M-CSF), and lipopolysaccharide—showed no formation of GRR binding complexes.

Binding of FcRF γ and the complexes activated by GM-CSF treatment of monocytes was inhibited by addition of excess unlabeled GRR (Fig. 1B), but not by addition of an unlabeled oligonucleotide corresponding to the IFN- γ activation sequence (GAS) within the promoter of the guanylate-binding protein gene (Fig. 1B) (6). The complexes induced by treatment of monocytes with IL-3 and IL-10 showed similar binding specificities (7). When the GAS oligonucleotide was used as a probe, only

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Fig. 1. Activation of GRR binding complexes by IFN- γ , IL-3, IL-5, IL-10, and GM-CSF. **(A)** Electrophoretic mobility-shift assays with a 32 P-labeled GRR probe and whole-cell extracts. Human monocytes and basophils were purified by leukaphoresis of normal volunteers followed by Ficoll-Hypaque sedimentation and counter-current centrifugal elutriation (1). Monocytes were then incubated with recombinant human IFN- γ (10 ng/ml; Genentech), recombinant human IL-1 (100 ng/ml; Otsuka), recombinant human IL-2 (1 μ g/ml; Chiron), recombinant human IL-3 (50 ng/ml; Amgen), recombinant human IL-5 (50 ng/ml; Genzyme), recombinant human IL-6 (100 U/ml; gift of G. Tosato), recombinant human IL-10 (50 ng/ml; Schering-Plough), recombinant human GM-CSF (30 ng/ml; Schering-Plough), recombinant human M-CSF (100 ng/ml; Genetics Institute), recombinant human TNF- α (10 ng/ml; Genentech), recombinant human TGF- β (10 ng/ml; Genzyme), or lipopolysaccharide (0.25 μ g/ml; Sigma) for 15 min at 37°C. Cell extracts were assayed for GRR binding proteins by EMSA with a 32 P-labeled oligonucleotide probe [5'-AGCATGTTTCAAGGATTTGAGATGATTTCCAGAAAAG-3' and its complement (1)] corresponding to the GRR of the human high-affinity Fc γ receptor gene. The arrows indicating the FcRF γ complexes are intended



only for lane 2. Lanes 5, 7, 8, and 15 represent the shifted complexes activated by the respective cytokine. **(B)** IFN- γ - (lanes 1 to 3) and GM-CSF-induced (lanes 4 to 6) GRR binding complexes formed in the presence of excess unlabeled GRR (lanes 2 and 5) or GAS (lanes 3 and 6) (6).

extracts prepared from cells treated with IFN- γ or IL-10 showed the IFN- γ activation factor (GAF) transcription complex (7). The IFN- γ -induced GAF complex contains tyrosine-phosphorylated p91 (8).

Because the GM-CSF, IL-3, and the IL-5 receptor use a common β subunit for transmembrane signal transduction (9), we determined whether IL-5 might also activate the formation of a complex that bound to the GRR. Peripheral blood basophils were treated with either IFN- γ or IL-5 (Fig. 1A). IL-5 treatment resulted in formation of a GRR binding complex that migrated more slowly than FcRF γ (Fig. 1A). Unlabeled GRR, but not the GAS oligonucleotide, also competed for binding of the IL-5-inducible shift complex (7).

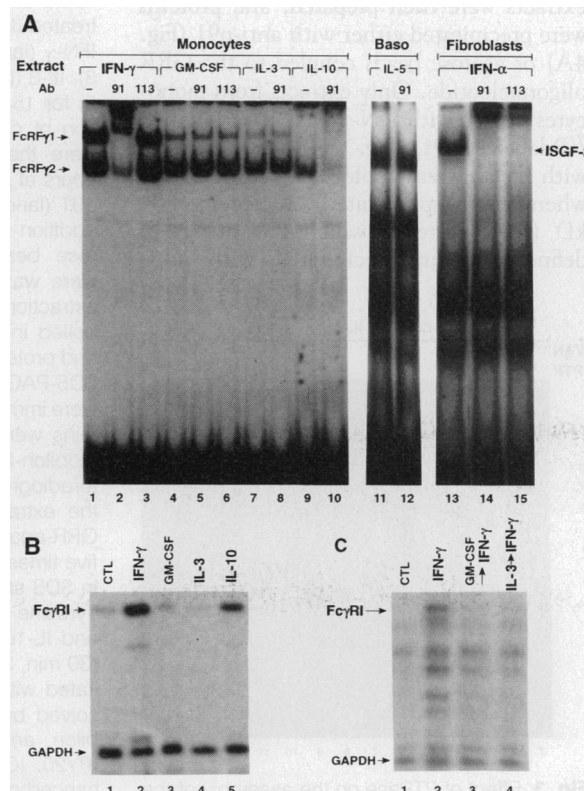
The ISGF-3, GAF, and FcRF γ transcription complexes contain the tyrosine-phosphorylated protein p91 (2-4, 8, 10, 11). To determine whether the complexes induced by GM-CSF, IL-3, IL-5, or IL-10 also contained p91, we did EMSAs after treatment of extracts with an antibody to p91 (anti-p91) (Fig. 2A). Whereas the GRR binding complex present in extracts prepared from IFN- γ - or IL-10-treated monocytes was supershifted, those complexes activated by treatment with the other cytokines were unchanged and therefore were not recognized by anti-p91. An antibody to the 113-kD component of ISGF-3 did not form supershifted complexes with any of the GRR binding proteins.

IFN- γ treatment of monocytes resulted in increased concentrations of Fc γ RI RNA (1). Ribonuclease protection assays were done to determine whether any of the other cytokines that activated the formation of GRR binding complexes might induce Fc γ RI RNA (Fig. 2B). Other than IFN- γ , only IL-10 increased expression of Fc γ RI RNA.

Therefore, only those cytokines that activate GRR binding complexes that contain p91 are capable of enhancing Fc γ RI RNA. Because GM-CSF and IL-3 did not induce Fc γ RI RNA, we pretreated monocytes with these

cytokines to determine whether they might modulate IFN- γ -induced Fc γ RI RNA. Incubation of cells with IL-3 or GM-CSF before IFN- γ treatment resulted in marked inhibition of Fc γ RI RNA induction (Fig. 2C).

Fig. 2. Composition of the cytokine-induced DNA binding complexes. **(A)** Presence of p91 in extracts prepared from monocytes treated with IFN- γ or IL-10. Extracts prepared from monocytes or basophils treated with IFN- γ (lanes 1 to 3), GM-CSF (lanes 4 to 6), IL-3 (lanes 7 and 8), IL-10 (lanes 9 and 10), or IL-5 (lanes 11 and 12) were incubated for 1 hour at 4°C either with antiserum to the 39 COOH-terminal amino acids unique to p91 (3) (lanes 2, 5, 8, 10, 12, and 14) or antiserum to the 113-kD protein of ISGF-3 (lanes 3, 6, and 15). After incubation with the antisera (diluted 1:100), EMSAs were done with the GRR probe. To demonstrate the specificity of the antisera, we also incubated a nuclear extract prepared from IFN- α -treated human diploid fibroblasts with the antibody (lanes 13 to 15). These extracts were then assayed for ISGF-3 formation with a 32 P-labeled oligonucleotide corresponding to the IFN-stimulated response element (ISRE) of the ISG15 gene (11). **(B)** IFN- γ - and IL-10-induced expression of the Fc γ RI RNA. Monocytes were incubated without or with the indicated cytokines for 1 hour. Total RNA was extracted and hybridized with 32 P antisense RNA probes corresponding to Fc γ RI and to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (an internal control) as described (1). The protected bands corresponding to Fc γ RI and GAPDH RNAs are indicated in the figure. Lane 1, control; lane 2, IFN- γ ; lane 3, GM-CSF; lane 4, IL-3; and lane 5, IL-10. **(C)** IL-3 and GMCSF inhibit IFN- γ -induced Fc γ RI RNA expression. Monocytes were untreated (lane 1), treated with IFN- γ alone for 1 hour (lane 2), or treated with either GMCSF (lane 3) or IL-3 (lane 4) for 30 min before the addition of IFN- γ for 1 hour. Total RNA was prepared and analyzed for Fc γ RI and GAPDH RNA by ribonuclease protection assay.



Assembly of the IFN-activated transcription complexes FcRF γ , ISGF-3, and GAF requires tyrosine phosphorylation and can be specifically disrupted by treatment with protein tyrosine phosphatase (PTPase) (4, 6, 11, 12). Similar experiments were done to determine whether tyrosine phosphorylation was required for DNA binding by the complexes activated by IL-3, IL-5, IL-10, and GM-CSF. Whole cell extracts were incubated either without (at 4°C) or with the addition of purified recombinant PTPase from *Yersinia enterocolitica*, in the presence or absence of the PTPase inhibitor orthovanadate at 30°C for 30 min. Samples were then assayed for the presence of GRR binding complexes (Fig. 3). The integrity of each complex was sensitive to PTPase regardless of the inducing cytokine. Vanadate inhibited the effects of the PTPase, confirming the specificity of the phosphatase toward tyrosine (Fig. 3).

To further define which proteins within the DNA binding complexes were selectively tyrosine-phosphorylated in response to cytokine treatment, we incubated monocytes with [³²P]orthophosphate for 3 hours and then with either IFN- γ , GM-CSF, IL-3, or IL-10 for 15 min. Whole cell extracts were then prepared, and proteins were precipitated either with anti-p91 (Fig. 4A) or agarose beads coupled to the GRR oligonucleotide. Only extracts from monocytes treated with IFN- γ or IL-10 contained ³²P-labeled p91 (Fig. 4A) (p91 migrates with an apparent molecular size of 97 kD when it is phosphorylated). A protein of 84 kD (the nature of which has not been defined) immunoprecipitated with anti-

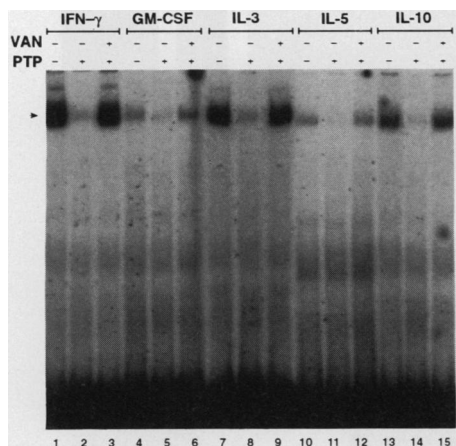


Fig. 3. Effect of PTPase on the assembly of the GRR binding complexes. Extracts prepared from monocytes were incubated (4°C) without (lanes 1, 4, 7, 10, and 13) or with purified recombinant PTP (1 μ g) at 30°C for 30 min alone (lanes 2, 5, 8, 11, and 14) or in the presence of 1 mM orthovanadate (lanes 3, 6, 9, 12, and 15). The reaction mixtures were then assayed for the presence of GRR binding complexes.

p91. Extracts from ³²P-labeled monocytes treated with IFN- γ , IL-3, or GM-CSF were incubated with the GRR-agarose beads. Affinity-purified ³²P-labeled proteins were then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Fig. 4A. IL-3 and GM-CSF induced association of a phosphorylated protein of 80 kD with the GRR-agarose beads, whereas phosphorylated p91 was present only in extracts prepared from cells treated with IFN- γ . Occasionally, a less prominent phosphorylated band of 97 kD was detected in extracts of cells treated with GM-CSF or IL-3 (Fig. 4C). Labeling of monocytes with

[³⁵S]methionine and [³⁵S]cysteine revealed that treatment with the same cytokines induced the binding of labeled proteins to the GRR of the same molecular sizes as those shown to be phosphorylated in this experiment (7).

For confirmation that the IFN- γ - or IL-10-induced phosphorylation of p91 was on tyrosine, immunoprecipitates from IFN- γ - or IL-10-treated monocytes were resolved by SDS-PAGE, transferred to Immobilon membranes, and then probed with antibody to phosphotyrosine (Fig. 4B). Treatment of monocytes with either IFN- γ - or IL-10-induced tyrosine phosphorylation

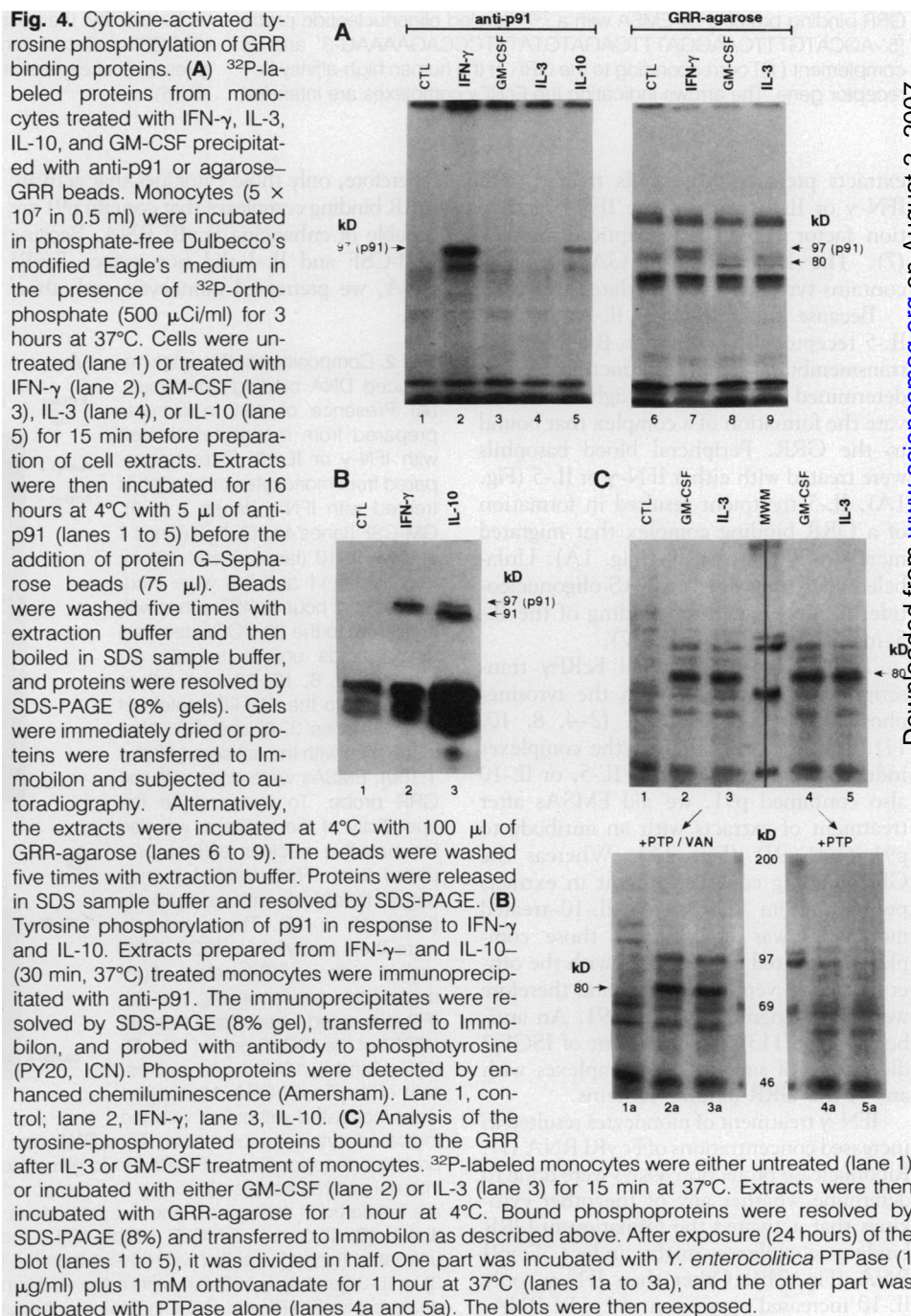


Fig. 4. Cytokine-activated tyrosine phosphorylation of GRR binding proteins. (A) ³²P-labeled proteins from monocytes treated with IFN- γ , IL-3, IL-10, and GM-CSF precipitated with anti-p91 or agarose-GRR beads. Monocytes (5×10^7 in 0.5 ml) were incubated in phosphate-free Dulbecco's modified Eagle's medium in the presence of ³²P-orthophosphate (500 μ Ci/ml) for 3 hours at 37°C. Cells were untreated (lane 1) or treated with IFN- γ (lane 2), GM-CSF (lane 3), IL-3 (lane 4), or IL-10 (lane 5) for 15 min before preparation of cell extracts. Extracts were then incubated for 16 hours at 4°C with 5 μ l of anti-p91 (lanes 1 to 5) before the addition of protein G-Sepharose beads (75 μ l). Beads were washed five times with extraction buffer and then boiled in SDS sample buffer, and proteins were resolved by SDS-PAGE (8% gels). Gels were immediately dried or proteins were transferred to Immobilon and subjected to autoradiography. Alternatively,

the extracts were incubated at 4°C with 100 μ l of GRR-agarose (lanes 6 to 9). The beads were washed five times with extraction buffer. Proteins were released in SDS sample buffer and resolved by SDS-PAGE. (B) Tyrosine phosphorylation of p91 in response to IFN- γ and IL-10. Extracts prepared from IFN- γ - and IL-10- (30 min, 37°C) treated monocytes were immunoprecipitated with anti-p91. The immunoprecipitates were resolved by SDS-PAGE (8% gel), transferred to Immobilon, and probed with antibody to phosphotyrosine (PY20, ICN). Phosphoproteins were detected by enhanced chemiluminescence (Amersham). Lane 1, control; lane 2, IFN- γ ; lane 3, IL-10. (C) Analysis of the tyrosine-phosphorylated proteins bound to the GRR after IL-3 or GM-CSF treatment of monocytes. ³²P-labeled monocytes were either untreated (lane 1) or incubated with either GM-CSF (lane 2) or IL-3 (lane 3) for 15 min at 37°C. Extracts were then incubated with GRR-agarose for 1 hour at 4°C. Bound phosphoproteins were resolved by SDS-PAGE (8%) and transferred to Immobilon as described above. After exposure (24 hours) of the blot (lanes 1 to 5), it was divided in half. One part was incubated with *Y. enterocolitica* PTPase (1 μ g/ml) plus 1 mM orthovanadate for 1 hour at 37°C (lanes 1a to 3a), and the other part was incubated with PTPase alone (lanes 4a and 5a). The blots were then reexposed.

of p91. However, the IL-10 immunoprecipitate also contained a tyrosine phosphorylated protein of 91 kD. This protein did not react with an antibody that recognized both p91 and its 84-kD spliced variant (7).

³²P-labeled phosphoproteins bound to GRR-agarose were visualized by autoradiography and then incubated with *Y. enterocolitica* PTPase (Fig. 4C) in the absence or presence of orthovanadate. *Yersinia* PTPase selectively hydrolyzed the ³²P-labeled IL-3- and GM-CSF-induced 80-kD phosphoprotein, whereas incubation of identical samples in the presence of the PTPase and orthovanadate had no effect. A weaker band of 97 kD was also sensitive to PTPase treatment. These results indicated that the proteins that associated with the GRR were tyrosine phosphorylated as a result of treatment with either IL-3 or GM-CSF.

Although it seems likely that IL-3, IL-5, IL-10, or GM-CSF induce the expression of sets of early response genes that are responsible for their biological actions, no well-defined enhancers have been identified in the promoters of cellular genes that are rapidly activated by these cytokines. IL-10 and IFN- γ activated the formation of a GRR binding complex that contained p91. The presence of p91 in these complexes appears to be important for the ultimate expression of the Fc γ RI gene, because only IFN- γ and IL-10 treatment resulted in increased expression of the RNA. The complexes activated by IL-3, IL-5, and GM-CSF do not contain either p91 or the 113-kD protein of ISGF-3 (13–15), but rather a prominent phosphoprotein of 80 kD. It appears that at least by their relative migration in SDS-PAGE, none of these proteins are identical to the 84-kD component of ISGF-3 that lacks the COOH-terminal 39 amino acids of p91 (15). The assembly of these phosphoproteins into DNA binding complexes appears to be a general mechanism by which growth factors can modulate gene expression through activation of putative transcription factors by tyrosine phosphorylation.

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5. Cells (10⁷) were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in 200 μ l of ice-cold extraction buffer [1 mM MgCl₂, 20 mM Hepes (pH 7.0), 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 0.1% Triton X-100, 200 μ M phenylmethylsulfonyl fluoride, and 20% glycerol]. The suspension was gently vortexed for 10 s and allowed to incubate at 4°C for 10 min. The mixture was centrifuged at 18,000g for 10 min at 4°C, and the supernatant was transferred to a new tube. Protein concentrations were determined and normalized by the addition of extraction buffer.
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2 July 1993; accepted 30 August 1993

Induction by EGF and Interferon- γ of Tyrosine Phosphorylated DNA Binding Proteins in Mouse Liver Nuclei

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Intraperitoneal injection of epidermal growth factor (EGF) into mice resulted in the appearance in liver nuclei of three tyrosine phosphorylated proteins (84, 91, and 92 kilodaltons) within minutes after administration of EGF. Administration of interferon- γ (IFN- γ) resulted in the appearance in liver nuclei of two tyrosine phosphorylated proteins (84 and 91 kilodaltons). The 84- and 91-kilodalton proteins detected after either EGF or IFN- γ administration were identified as the IFN- γ activation factors (GAF). Furthermore, gel shift analysis revealed that these GAF proteins, detected after either EGF or IFN- γ administration, specifically bound to the sis-inducible element of the *c-fos* promoter. Thus, GAF proteins participate in nuclear signaling in both IFN- γ and EGF pathways.

Epidermal growth factor elicits a wide range of physiological responses from a variety of cell types (1–3). All of these responses are believed to be mediated by the membrane-spanning receptor for EGF (EGFR). Ligand binding by the EGFR activates its intrinsic tyrosine kinase activity and results in many cellular alterations, including the generation of signals that activate transcription (4–6) and the tyrosine phosphorylation of cytoplasmic proteins (7–10). We have previously reported that the injection of EGF into mice leads to increased tyrosine phosphorylation of a number of proteins in all organs examined (11, 12).

To detect signaling proteins that might be involved in transcriptional regulation, we isolated liver nuclei and examined them for the presence of tyrosine phosphorylated proteins before and after the administration of EGF (Fig. 1A). A tyrosine phosphorylated protein of approximately 92 kD was readily apparent in nuclei 6 and 20 min after the administration of EGF. The extent of tyrosine phosphorylation of the 92-kD protein was diminished by 60 min and was detectable only in trace amounts in nuclei from control mice. The 92-kD protein could not be extracted from nuclei with

1.0% Triton X-100, indicating that the protein was inside the nucleus and not a cytoplasmic contaminant or a protein associated with the outer nuclear membrane (13). However, more than 90% of this protein could be extracted with 0.2 M NaCl (Fig. 1B). Because the 0.2 M NaCl extraction procedure partially purified the 92-kD protein from the nucleus, the material extracted in this way was used in all subsequent protein characterizations. EGF also induced the appearance of a cytoplasmic 92-kD tyrosine phosphorylated protein (13).

Treatment of responsive cells with interferon- α (IFN- α) induces the formation of a tyrosine phosphorylated complex consisting of three protein subunits (IFN-stimulated gene factor-3 α , ISGF-3 α) that is translocated into the nucleus and forms, with the addition of a fourth subunit (ISGF-3 γ), a DNA binding complex specific for the IFN-stimulated response element (ISRE) present in promoters of IFN- α -responsive genes (14–18). Two of the components of the ISGF-3 α complex are a 91-kD protein and an 84-kD protein, the latter being identical to the 91-kD species but lacking the COOH-terminal 36 amino acids. Treatment of responsive cells with IFN- γ induces the tyrosine phosphorylation of an IFN- γ activation factor (GAF), a DNA binding protein that binds to a sequence (IFN- γ activation site, GAS) required for the tran-

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