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Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity

Fabrice Gouilleux, Caroline Pallard[1], Isabelle Dusanter-Fourt[1], Hiroshi Wakao[2], Lars-Arne Haldosen[3], Gunnar Norstedt[3], David Levy[4] and Bernd Groner[5]

Institute for Experimental Cancer Research, Tumor Biology Center, Breisacher Strasse 117, D-79106 Freiburg, Germany, [1]ICGM-U363, Hopital Cochin, 27 rue du Faubourg St Jacques, 75014 Paris, France, [2]DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304-1104, USA, [3]Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F60, Novum, S-141 86 Huddinge, Sweden and [4]Department of Pathology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA

[5]Corresponding author

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The molecular components which mediate cytokine signaling from the cell membrane to the nucleus were studied. Upon the interaction of cytokines with their receptors, members of the janus kinase (Jak) family of cytoplasmic protein tyrosine kinases and of the signal transducers and activators of transcription (Stat) family of transcription factors are activated through tyrosine phosphorylation. It has been suggested that the Stat proteins are substrates of the Jak protein tyrosine kinases. MGF-Stat5 is a member of the Stat family which has been found to confer the prolactin response. MGF-Stat5 can be phosphorylated and activated in its DNA binding activity by Jak2. The activation of MGF-Stat5 is not restricted to prolactin. Erythropoietin (EPO) and growth hormone (GH) stimulate the DNA binding activity of MGF-Stat5 in COS cells transfected with vectors encoding EPO receptor and MGF-Stat5 or vectors encoding GH receptor and MGF-Stat5. The activation of DNA binding by prolactin, EPO and GH requires the phosphorylation of tyrosine residue 694 of MGF-Stat5. The transcriptional induction of a β-casein promoter luciferase construct in transiently transfected COS cells is specific for the prolactin activation of MGF-Stat5; it is not observed in EPO- and GH-treated cells. In the UT7 human hematopoietic cell line, EPO and granulocyte-macrophage colony stimulating factor activate the DNA binding activity of a factor closely related to MGF-Stat5 with respect to its immunological reactivity, DNA binding specificity and molecular weight. These results suggest that MGF-Stat5 regulates physiological processes in mammary epithelial cells, as well as in hematopoietic cells. Its DNA binding activity and transactivation potential are differentially regulated in a cytokine- and promoter-specific manner.

Key words: cytokine receptor signaling/Jak kinases/promoter transactivation/Stat factors/tyrosine phosphorylation

Introduction

Protein tyrosine phosphorylation couples the signals originating from the interaction of the cytokine receptors with their specific ligands to the molecules conferring the transcriptional response. Shortly after ligand binding, tyrosine phosphorylation is detected in the cytokine receptors, which activates members of the janus kinase (Jak) family of protein tyrosine kinases and the signal transducers and activators of transcription (Stat) family of transcription factors (Darnell *et al.*, 1994; Wilks and Harpur, 1994). Individual cytokine receptors are linked to specific members of the Jak and Stat families. Jak1 and Jak2 are activated in response to interferon (IFN) γ, Jak1 and Tyk2 in response to IFNα/β, and Jak1 and Jak3 in response to interleukin (IL) 2 and IL-4 (Velazquez *et al.*, 1992; Müller *et al.*, 1993; Watling *et al.*, 1993; Johnston *et al.*, 1994; Witthuhn *et al.*, 1994). The Stat proteins are thought to be substrates of the Jak tyrosine kinases.

So far six members of this family have been cloned. p91-Stat1 constitutes the IFNγ activated transcription factor (GAF; Shuai *et al.*, 1992); p91-Stat1 and p113-Stat2, together with the p48 subunit, constitute the IFNα/β responsive transcription factor (ISGF-3; Fu *et al.*, 1992; Schindler *et al.*, 1992a). Acute-phase response factor APRF-Stat3, is activated in response to IL-6 (Akira *et al.*, 1994; Zhong *et al.*, 1994), MGF-Stat5 in response to prolactin (Wakao *et al.*, 1994) and IL-4-Stat6 by IL-4 (Hou *et al.*, 1994). The cytokine responsible for the activation of Stat4 is not yet known (Yamamoto *et al.*, 1994).

Phosphorylation on tyrosine confers DNA binding activity to the Stat proteins and results in the activation of transcription (Fu, 1992; Schindler *et al.*, 1992b; Shuai *et al.*, 1993; Gouilleux *et al.*, 1994; Yuan *et al.*, 1994). Phosphorylation of tyrosine residues in related sequence contexts has been observed in Stat1, Stat4 and Stat5 (Shuai *et al.*, 1993; Gouilleux *et al.*, 1994; Yamamoto *et al.*, 1994). This raises the possibility that these proteins are the substrates of a common protein tyrosine kinase.

MGF-Stat 5 was identified originally in mammary gland cells of lactating animals (Schmitt-Ney *et al.*, 1991; Wakao *et al.*, 1992). MGF-Stat5 activity in mice is strongly increased at the end of pregnancy and is maintained at high levels by the suckling of the pups (Schmitt-Ney *et al.*, 1992). This effect is mediated by the high levels of circulating prolactin present in suckled animals. MGF-Stat5 can be induced in cultured mammary epithelial cells by prolactin, and its DNA binding activity is absolutely required for the lactogenic hormone induction of the transcription of the β-casein gene (Schmitt-Ney *et al.*, 1991; Standke *et al.*, 1994). MGF-Stat5 also confers the prolactin response to a β-casein gene promoter construct when it is introduced together with a prolactin receptor

gene into non-mammary cells (Gouilleux *et al.*, 1994; Wakao *et al.*, 1994).

MGF-Stat5 binding sites are conserved in the promoter sequences of the milk protein genes in different species, and are also found in the promoters of, for example, interferon response factor 1 (IRF-1), FcγRI, ICAM, FcεRIIb and α_2-macroglobulin genes (Yoshimura and Oka, 1989). Prolactin regulation of the transcription of the early response gene IRF-1 in the rat T-lymphoma cell line Nb2, and of the acute phase response gene α_2-macroglobulin in the uterus and the ovary, has been observed (Yu-Lee *et al.*, 1990; Gaddykurten and Richards, 1991; Gu *et al.*, 1992). MGF-Stat5 mRNA is expressed in different tissues (Wakao *et al.*, 1994). These observations suggest that the regulatory role of MGF-Stat5 is not restricted to the milk protein genes in the mammary gland.

We reported recently that MGF-Stat5 can be phosphorylated by Jak2 (Gouilleux *et al.*, 1994). Jak2 is a kinase which is associated with and activated by the prolactin receptor (Dusanter-Fourt *et al.*, 1994; Rui *et al.*, 1994). However, Jak2 activation is not only mediated by the prolactin receptor; it is also effected by erythropoietin (EPO), growth hormone (GH), G-CSF, IL-5, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, IL-6 and IFNγ receptors (Argetsinger *et al.*, 1993; Müller *et al.*, 1993; Silvennoinen *et al.*, 1993; Watling *et al.*, 1993; Witthuhn *et al.*, 1993; Ihle *et al.*, 1994; Narazaki *et al.*, 1994; Quelle *et al.*, 1994). These observations raised the question as to whether Jak2 activation by these receptors would also result in the activation of MGF-Stat5. The activation of Stat proteins apart from Stat1 has been reported for EPO, GH, IL-3, IL-5 and GM-CSF (Larner *et al.*, 1993; Finbloom *et al.*, 1994; Wood *et al.*, 1995). We have investigated protein DNA complexes induced by different cytokines in transfected COS cells and in human hematopoietic cells. MGF-Stat5 DNA binding activity is induced by (i) EPO in COS cells transfected with MGF-Stat5 and an EPO receptor gene and (ii) GH in cells transfected with MGF-Stat5 and a GH receptor gene. This induction requires the phosphorylation of tyrosine 694. In contrast to the induction of MGF-Stat5 DNA binding activity common to prolactin, EPO and GH, only prolactin was able to induce the transcription of a β-casein promoter luciferase construct. In the hematopoietic cell lines UT7 and U937, EPO and GM-CSF induced the formation of a protein DNA complex very closely related to MGF-Stat5.

Results

MGF-Stat5 is activated in response to EPO and GH in transfected COS cells

The binding of prolactin to its receptor results in the activation of the cytoplasmic tyrosine-specific protein kinase Jak2. The same observation was made after the binding of EPO and GH to their receptors (Argetsinger *et al.*, 1993; Witthuhn *et al.*, 1993). Since Jak2 phosphorylation has been shown to confer DNA binding activity to MGF-Stat5, we examined the consequences of EPO and GH receptor activation for MGF-Stat5.

COS cells were transfected with vectors directing the expression of MGF-Stat5 and the EPO receptor. Nuclear extracts were prepared from EPO-treated transfected cells.

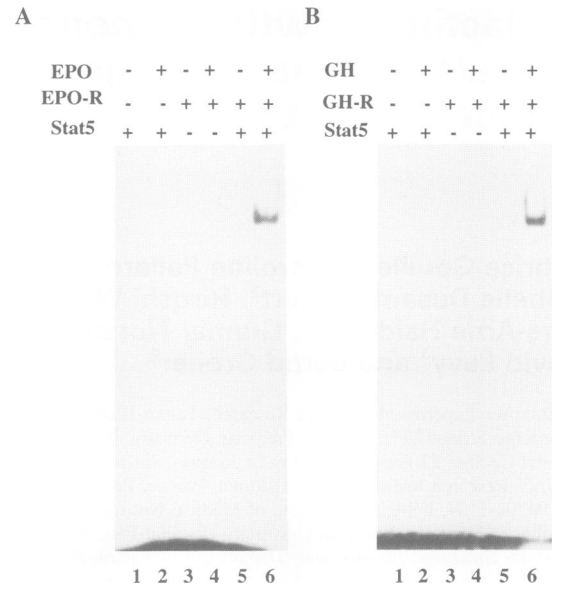

Fig. 1. (**A**) EPO induces MGF-Stat5 DNA binding activity. COS cells were transfected with expression vectors encoding the EPO receptor and MGF-Stat5 as indicated in the figure. Nuclear extracts were prepared and analyzed in bandshift assays using the β-casein promoter MGF-Stat5 binding site as a probe. Cells from which the extracts used in lanes 3–6 were derived were treated with mouse EPO (10 U/ml) for 1 h. (**B**) GH induces MGF-Stat5 DNA binding activity. COS cells were transfected with expression vectors encoding the GH receptor and MGF-Stat5 as indicated in the figure. Nuclear extracts were prepared and analyzed in bandshift assays using the β-casein promoter MGF-Stat5 binding site as a probe. Cells from which the extracts used in lanes 3–6 were derived were treated with bovine GH (100 nM) for 1 h.

MGF-Stat5 DNA complexes were visualized in bandshift assays (Figure 1A). Dependent on the expression of the EPO receptor, EPO causes the activation of MGF-Stat5 (lane 6). No complex was observed in the absence of the transfected receptor (lane 2), MGF-Stat5 (lane 4) or EPO treatment (lane 5). A similar experiment was performed with GH. GH receptor was co-transfected with MGF-Stat5 into COS cells and the cells were treated with GH for 1 h. Nuclear extracts were prepared from cells cultured in the absence or presence of GH and analyzed in bandshift assays (Figure 1B). As in the case of EPO, the DNA binding activity of MGF-Stat 5 is activated in response to GH (lane 6).

MGF-Stat5 DNA binding induced by EPO and GH requires phosphorylation of the tyrosine residue at position 694

Prolactin induction of the DNA binding activity of MGF-Stat5 has been shown to be accompanied by phosphorylation of its tyrosine residue at position 694 (Gouilleux *et al.*, 1994). We investigated if the activation by EPO and GH has the same requirement. COS cells were transfected with vectors directing the expression of MGF-Stat5 [tagged with an influenza virus epitope (E-Stat5) which can be recognized by the monoclonal antibody 12CA5], the EPO receptor, the GH receptor or the prolactin receptor. A mutated E-Stat5 was constructed in which the tyrosine at position 694 was changed into a phenylalanine residue. This mutant version of MGF-Stat5 was also co-transfected into COS cells with the prolactin, GH or EPO

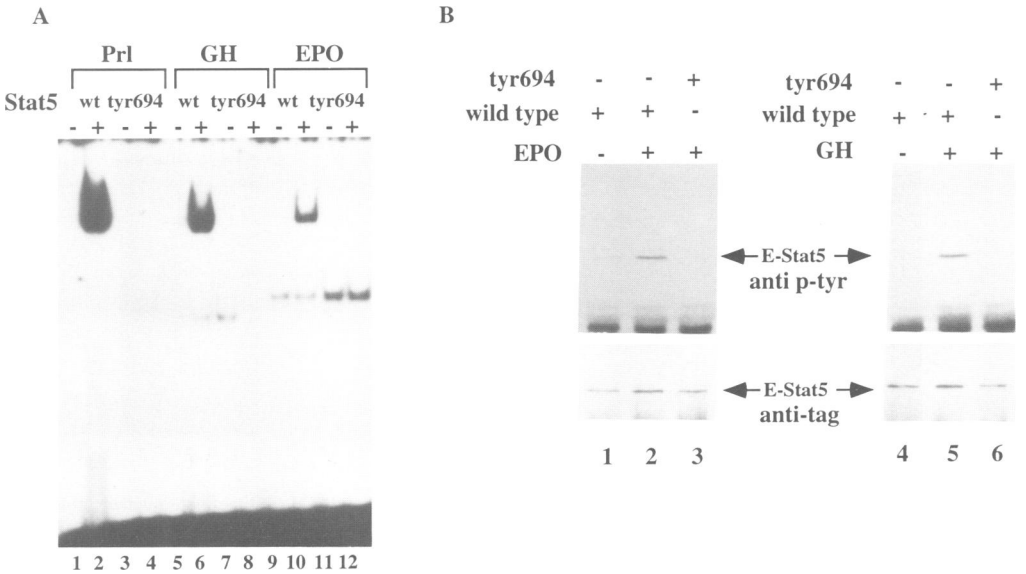

Fig. 2. (**A**) Prolactin, EPO and GH do not activate DNA binding of MGF-Stat5 mutant Tyr694. The epitope-tagged version of MGF-Stat5 (pE-MGF; lanes 1, 2, 5, 6, 9 and 10), or the epitope-tagged mutant version (pE-MGFtyr694, in which the tyrosine at position 694 has been replaced by a phenylalanine; lanes 3, 4, 7, 8, 11 and 12) were expressed in COS cells. Prolactin receptor (lanes 1–4), EPO receptor (lanes 5–8) and GH receptor (lanes 9–12) were co-transfected. After treatment of the cells with prolactin (lanes 2 and 4), EPO (lanes 6 and 8) and GH (lanes 10 and 12), nuclear extracts were prepared and analyzed in bandshift assays. (**B**) MGF-Stat5 mutant Tyr694 is not tyrosine phosphorylated after treatment with EPO and GH. Wild-type E-MGF-Stat5 (lanes 1, 2, 4 and 5) or mutant Tyr694 (lanes 3 and 6) were immunoprecipitated with the monoclonal antibody 12CA5 specific for the influenza tag (E, epitope). Immunoprecipitates were analyzed by Western blotting with the phosphotyrosine-specific antibody 4G10 (upper panel). The membrane was reprobed with the epitope E-specific antibody 12CA5 (lower panel).

receptors. Nuclear extracts were prepared from cytokine-treated cells and bandshift experiments were carried out (Figure 2A). The DNA binding activity of the wild-type protein was induced by prolactin, GH and EPO (lanes 2, 6 and 10). No induction of DNA binding activity was observed when the mutant Tyr694 was analyzed (lanes 4, 8 and 12).

Immunoprecipitations of E-Stat5 from transfected COS cells were carried out with the monoclonal antibody 12CA5. The immunoprecipitates were analyzed in Western blot experiments with the anti-phosphotyrosine-specific antibody 4G10 (Figure 2B, upper panel) or the tag-specific antibody 12CA5. A tyrosine-phosphorylated protein of ~100 kDa was detected in COS cells transfected with the wild-type protein and treated with EPO (lane 2) or GH (lane 5). The appearance of this protein is dependent on the cytokine treatment of the cells (lanes 1 and 4). Mutant protein Tyr694 was not tyrosine phosphorylated in response to EPO or GH (lanes 3 and 6). Reprobing the membrane with the monoclonal antibody 12CA5 showed that the wild-type and the mutant protein were expressed at similar levels in the transfected cells. EPO and GH appear to induce the DNA binding activity of MGF-Stat5 via a similar mechanism to prolactin, requiring the phosphorylation of Tyr694.

Prolactin but not EPO and GH induction of MGF-Stat5 results in the transcriptional activation of a β-casein promoter luciferase construct in COS cells

The mechanisms involved in the activation of MGF-Stat5 DNA binding by prolactin, EPO and GH are probably very similar. The receptors associate with Jak2, and Tyr694 in MGF-Stat5 is essential. We investigated if the different cytokines can be distinguished by their effect on gene-specific transcription. The region −344 to −1 of the β-casein gene promoter is sufficient to confer the lactogenic hormone response in mammary epithelial cells (Doppler *et al.*, 1989; Schmitt-Ney *et al.*, 1991) and the MGF-Stat5-mediated prolactin regulation in transfected COS cells (Gouilleux *et al.*, 1994).

We compared the transcriptional activity of a β-casein promoter−luciferase fusion gene upon activation of MGF-Stat5 with prolactin, EPO and GH. The receptors for prolactin, GH and EPO were co-expressed in COS cells with MGF-Stat5 and a β-casein promoter−luciferase construct. The transfected cells were treated with the respective cytokines. Extracts were prepared and luciferase activities were determined (Figure 3). Prolactin treatment results in a 20- to 30-fold induction of luciferase activity (lane 4). This induction is dependent on the expression of MGF-Stat5 (lane 2). EPO and GH (lanes 8 and 12) are unable to cause the induction of luciferase activity. These results indicate that MGF-Stat5 activation is not sufficient to confer EPO or GH response to the β-casein gene promoter.

EPO and GM-CSF activate an MGF-Stat5-like DNA binding activity in human hematopoietic cell lines

EPO and GM-CSF binding to their receptors has been shown to activate Jak2 and DNA binding proteins of the Stat family distinct from Stat1 (Larner *et al.*, 1993; Finbloom *et al.*, 1994; Quelle *et al.*, 1994). We examined whether EPO and GM-CSF could activate a factor related to MGF-Stat5 in hematopoietic cells. We used the human multipotential hematopoietic cell line UT7 which is strictly dependent on EPO and GM-CSF for growth (Komatsu *et al.*, 1991). We also analysed GM-CSF activation in the

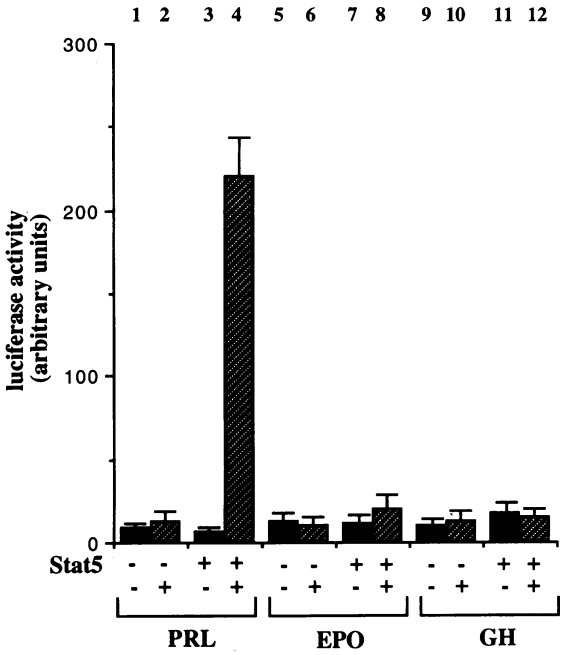

Fig. 3. β-Casein gene promoter activity is induced by prolactin but not by EPO or GH in transfected COS cells. COS cells were transfected with the (−344 to −1) β-casein promoter luciferase construct, pZZ1 (lanes 1–12), prolactin receptor cDNA (lanes 1–4), EPO receptor cDNA (lanes 5–8), GH receptor cDNA (lanes 9–12) and MGF-Stat5 cDNA (lanes 3, 4, 7, 8, 11 and 12). Cells used in lanes 2 and 4 were induced with prolactin, those in lanes 6 and 8 with EPO and those in lanes 10 and 12 with GH 1 day after transfection for 12 h. All transfections included plasmid pCH110. β-Galactosidase assays were carried out to monitor the transfection efficiency. Luciferase activities were determined and normalized with the β-galactosidase activities. Values represent the means of four different experiments.

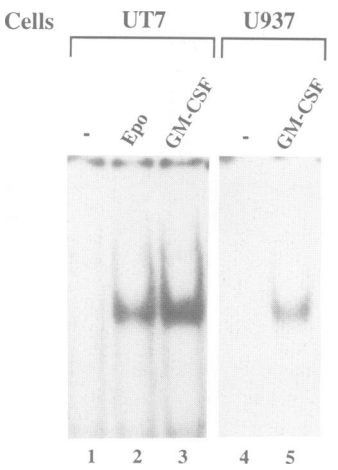

Fig. 4. EPO and GM-CSF activate nuclear proteins which bind to the MGF-Stat5 site in the β-casein gene promoter. UT7 and U937 cells were treated with EPO (10 U/ml, lane 2) or GM-CSF (5 ng/ml, lanes 3 and 5) for 1 h. Nuclear extracts were prepared and analyzed in bandshift assays with a labeled DNA probe representing the MGF-Stat5 binding site in the bovine β-casein gene promoter.

human promonocytic cell line U937. In this cell line GM-CSF activates the DNA binding of differentiation-induced factor (DIF), a factor related to MGF-Stat5 (Barahmand-Pour *et al.*, 1995).

UT7 and U937 cells were treated with EPO or GM-CSF for 1 h and nuclear extracts were prepared. Specific protein−DNA complexes were visualized in bandshift assays (Figure 4). The MGF-Stat5 binding site present in the bovine β-casein gene promoter was used as a radioactive probe (Wakao *et al.*, 1992). EPO and GM-CSF caused the formation of protein complexes with the MGF-Stat5 binding sequence with identical mobilities when extracts from UT7 cells were employed (lanes 2 and 3). The same complex was formed with extracts from U937 cells upon treatment with GM-CSF (lane 5).

The proteins present in the complexes induced by EPO and GM-CSF were characterized further. Antisera specific for individual members of the Stat family (Stat1, Stat3 and Stat5) were employed. An MGF-Stat5-specific antiserum was produced in chickens. The specificity of the antisera was demonstrated in bandshift experiments. Nuclear extracts were obtained from COS cells transfected with MGF-Stat5 and prolactin receptor cDNA, and treated with prolactin. Specific MGF-Stat5 DNA complexes were formed and incubated with the Stat1-, Stat3- and Stat5-specific antisera. The appearance of slower migrating complexes (supershifts) was monitored (Figure 5A). The MGF-Stat5 DNA complex is supershifted by antiserum raised against MGF-Stat5 (lane 5) and by antibodies directed against the N-terminal end (30N) of Stat3 (lane 4). Preimmune serum (lane 6), Stat1 (lane 2) or Stat3 antisera directed against the C-terminal end (30C) of Stat3 (lane 3) did not react with the complex. The antiserum against the C-terminal region of Stat3 (30C) is specific for Stat3 and does not recognize MGF-Stat5. The antiserum raised against the N-terminal region of Stat3 (30N) is known to react with different members of the Stat family; it recognizes MGF-Stat5 as well as Stat1 (data not shown). The antiserum used against MGF-Stat5 does not cross-react with Stat1, Stat3 or Stat6. This was tested in extracts of (i) COS cells induced with IFNγ, (ii) HepG2 and U937 cells induced with IL-6, and (iii) HC11 cells induced with IL-4 (G.Standke and F.Gouilleux, unpublished results).

We examined the presence of MGF-Stat5, or an immunologically cross-reacting factor, in the complexes induced by EPO and GM-CSF in UT7 and U937 cells (Figure 5B). The complexes obtained from EPO- and GM-CSF-induced cells were incubated with Stat-specific antisera and analyzed in bandshift experiments. Complexes activated by EPO (lane 5), GM-CSF in UT7 cells (lane 9) and GM-CSF in U937 cells (lane 12) were supershifted by Stat5 antiserum, but not by Stat1 or Stat3 (30C) antisera. The Stat3 (30N) antiserum also recognizes MGF-Stat5 (Figure 5A, lane 4). This explains the observation of a supershift with Stat3 (30N) antiserum in the EPO-induced complex shown in Figure 5A (lane 4). These results indicate that the EPO and GM-CSF induced a Stat factor immunologically related to MGF-Stat5 (EPO-Stf and GM-CSF-Stf).

MGF-Stat5 and the EPO- and GM-CSF-induced Stat factors exhibit identical DNA binding specificities

We characterized the DNA binding specificities of the EPO- and GM-CSF-induced Stat factors. These experiments were performed with nuclear extracts from (i) UT7 cells treated with EPO or GM-CSF and (ii) COS cells

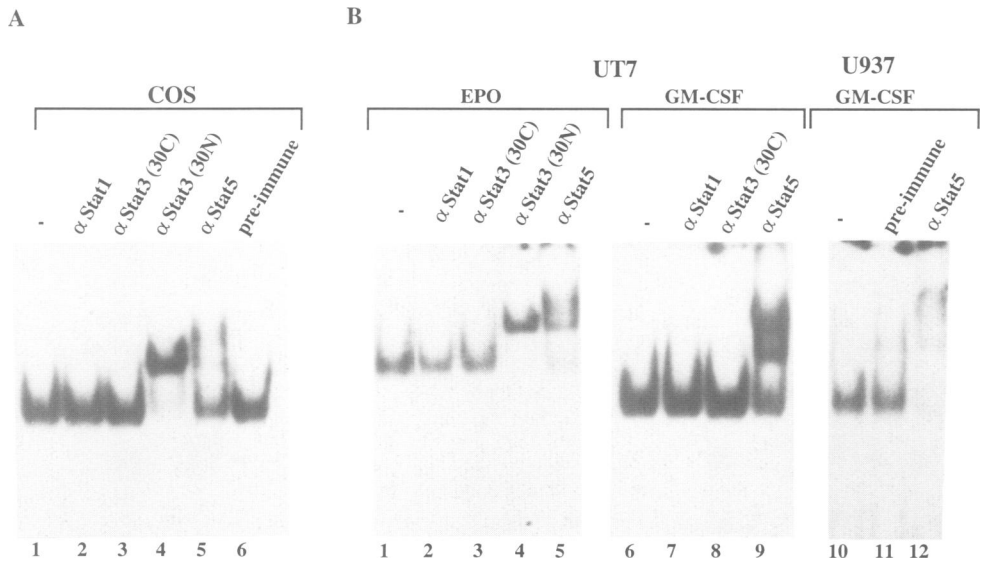

Fig. 5. EPO and GM-CSF activate a factor immunologically related to MGF-Stat5 in UT7 and U937 cells. (**A**) Specificity of the anti-MGF-Stat5 antiserum. MGF-Stat5 and the prolactin receptor were expressed in COS cells. The cells were induced with prolactin and nuclear extracts prepared. The extracts were incubated with Stat1 (lane 2), Stat3 (30C) (lane 3), Stat3 (30N) (lane 4), Stat5 (lane 5)-specific antisera, or preimmune serum (lane 6). The extracts were assayed for binding to the β-casein probe in bandshift experiments. (**B**) Analysis of the EPO- and GM-CSF-induced DNA binding complex by antibody supershift assays. Nuclear extracts prepared from UT7 and U937 cells induced by EPO and GM-CSF were incubated with specific antisera for Stat1 (lanes 2 and 7), Stat3 (30C) (lanes 3 and 8), Stat3 (30N) (lane 4), Stat5 (lanes 5, 9 and 12) or preimmune serum. The extracts were assayed for binding to the β-casein probe in bandshift experiments.

transfected with MGF-Stat5. The MGF-Stat5 binding site in the β-casein promoter was used as a radioactive probe. Various IFNγ activation site (GAS) elements were introduced as oligonucleotide competitors (Figure 6). Complex formation of EPO-induced Stat factor or GM-CSF-induced Stat factor with the β-casein promoter probe was competed by the GAS elements occurring in the FcγRI (lane 8) and the IRF-1 (lane 9) gene promoters. Competition was also observed with the acute phase response element (APRE) of the α_2-macroglobulin gene promoter (lane 4) and the prolactin response element (PRE; lane 2), homologous to the probe used.

The Ly-6E oligonucleotide showed a weak competition for the three different complexes (lane 5). When the Ly-6E oligonucleotide was used as a probe, no binding of MGF-Stat5 was detected (data not shown). No competition was observed with GAS elements from the guanylate binding protein gene promoter (lane 6), the sis inducible element (SIE) from the c-fos gene promoter (lane 7) or the interferon stimulated response element (ISRE) from the interferon α stimulated gene promoter, ISG-15 (lane 10). These results show that MGF-Stat5 and the EPO- and GM-CSF-induced Stat factors have identical DNA binding specificities towards different GAS elements.

The sequences from the α_2-macroglobulin, FcγRI, IRF-1 and β-casein gene promoters, which efficiently compete MGF-Stat5 and EPO- or GM-CSF-induced Stat factor, contain a common palindromic element 5′-TTCNNN-GAA-3′. We examined the effects of exchanging one or two nucleotides in this DNA sequence element on the binding specificity of the EPO- and GM-CSF-induced Stat factor and of MGF-Stat5. The wild-type sequence and the mutated oligonucleotides shown in Figure 7A were used as competitors in bandshift assays. EPO- and GM-CSF-induced Stat factors from nuclear extracts of UT7 cells were compared with MGF-Stat5 from transfected COS cells (Figure 7B). The complexes induced by all three cytokines exhibited identical sensitivity towards competition with the mutated oligonucleotides. No competition was observed with the oligonucleotide B12.13 containing changes of the two As at positions 12 and 13 (lane 4), and the oligonucleotide B5.6 containing changes of the two Ts at positions 5 and 6 (lane 8). Intermediate levels of competition were observed with oligonucleotides B11 and B7 which contained changes of G at position 11 (lane 4) and C at position 7 (lane 7). Oligonucleotide B11 competes more efficiently than B7. The change of nucleotide G into an A in oligonucleotide B11 creates a new palindromic sequence with two bases between TTC and GAA. The intermediate competition efficiency of oligonucleotide B11 suggests that the spacing between the motifs TTC and GAA might be important for DNA binding specificity.

These results indicate that changes in the nucleotides affecting the palindromic character of the binding sequence interfere with the recognition of the EPO- and GM-CSF-induced Stat factors and MGF-Stat5 in an identical fashion. Mutations of nucleotides not affecting the palindromic core sequence do not or only very weakly interfere with complex formation.

EPO- and GM-CSF-induced Stat factors and MGF-Stat5 have identical molecular weights

The antibody recognition specificity and the DNA binding specificity of the EPO- and GM-CSF-induced Stat factors and MGF-Stat5 indicate that the complexes induced by these three cytokines contain very closely related proteins. The partial purification and determination of the molecular weight of the proteins contained in these complexes further confirmed this notion. Nuclear extracts from UT7 cells treated with EPO or GM-CSF were incubated with Sepharose beads coupled to multimerized MGF-Stat5

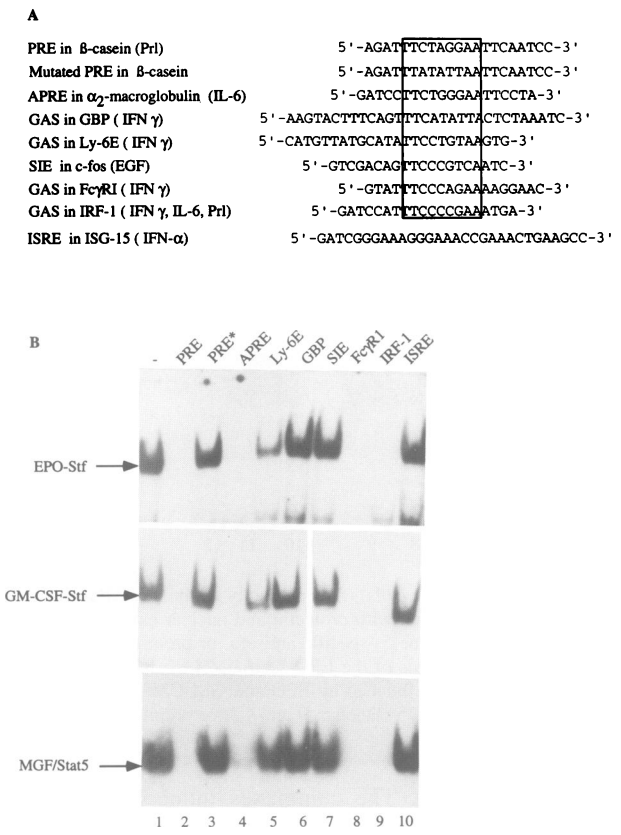

Fig. 6. EPO-induced Stat factor, GM-CSF-induced Stat factor and MGF-Stat5 bind to a subset of GAS elements. (**A**) Sequence of oligonucleotides corresponding to different binding sites of Stat proteins. (**B**) EPO-induced Stat factor and GM-CSF-induced Stat factor (Stf) containing nuclear extracts from UT7 cells, and MGF-Stat5 containing nuclear extracts from transfected COS cells, were analyzed in bandshift assays with the β-casein promoter probe. The indicated oligonucleotides were used in competition assays in a 100-fold molar excess.

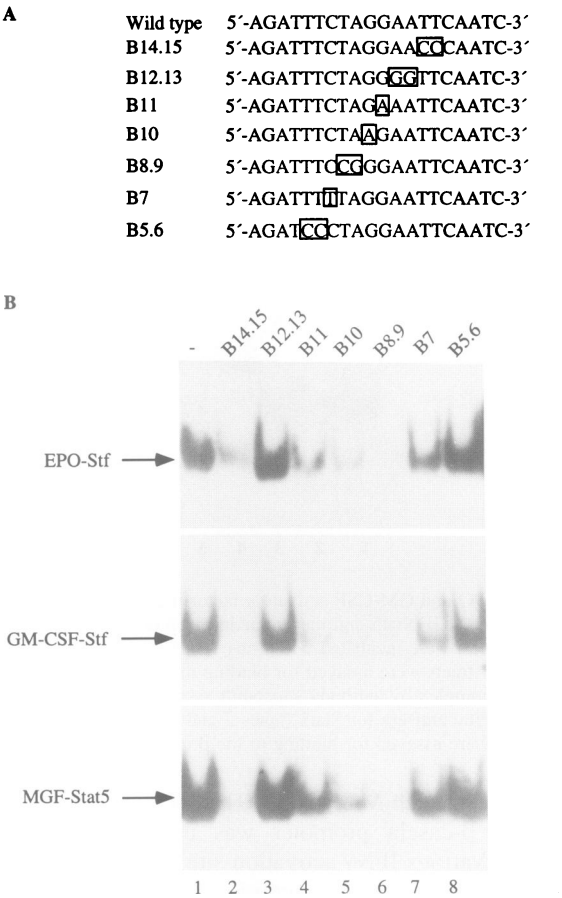

Fig. 7. EPO-induced Stat factor, GM-CSF-induced Stat factor and MGF-Stat5 have identical DNA binding specificities. (**A**) MGF-Stat5 binding sites and mutant variants from the β-casein gene promoter were synthesized. (**B**) EPO-induced Stat factor and GM-CSF-induced Stat factor from UT7 cells, and MGF-Stat5 from transfected COS cells, were introduced in bandshift assays with the β-casein promoter probe. A 100-fold molar excess of competing non-radioactive oligonucleotides was included as indicated.

binding sites. Bound proteins were eluted, analyzed by SDS−PAGE, blotted onto filters and developed with MGF-Stat5-specific antiserum (Figure 8A). The antiserum recognized a protein of 92 kDa present in EPO- and GM-CSF-treated UT7 cells (lanes 2 and 3). This protein was not detected when nuclear extracts from untreated cells were analyzed (lane 1). A nuclear protein isolated from prolactin-treated COS cells transfected with MGF-Stat5 and the prolactin receptor exhibited an identical molecular weight (results not shown). Analysis of the EPO- or GM-CSF-induced proteins showed that these proteins are tyrosine phosphorylated (Figure 8B, lanes 2 and 3). These results indicate that the Stat factors activated by EPO and GM-CSF, and MGF-Stat5 are identical or very closely related.

Discussion

MGF-Stat5 was identified initially as a transcription factor which confers the prolactin response to milk protein gene promoters. Here we show that the activation of MGF-Stat5 is not restricted to the action of prolactin in mammary epithelial cells. This activation can be effected by EPO and GH in transfected COS cells. We have shown previously that the activation of MGF-Stat5 by prolactin is dependent upon the presence of a tyrosine residue at position 694. The same requirement was found for the activation by GH and EPO; this suggests that a protein tyrosine kinase commonly regulated by all three cytokine receptors is responsible, possibly Jak2. Jak2 is activated by these three cytokines (Argetsinger *et al.*, 1993; Witthuhn *et al.*, 1993) and is able to activate MGF-Stat5 DNA binding *in vitro* (Gouilleux *et al.*, 1994).

The results obtained in transfected COS cells are supplemented by those collected from the UT7 and U937 human hematopoietic cell lines. Three lines of evidence suggest that EPO and GM-CSF cause the activation of proteins which are identical or very closely highly related to MGF-Stat5: (i) EPO-induced Stat factor and GM-CSF-induced Stat factor are recognized by an antiserum which reacts with MGF-Stat5, but not with Stat1 or Stat3; (ii) the factors have a DNA binding specificity indistinguishable from MGF-Stat5 when different GAS elements and mutated binding sites are compared in oligonucleotide competition experiments; and (iii) EPO-induced Stat factor and GM-CSF-induced Stat factor have identical molecular weights to MGF-Stat5.

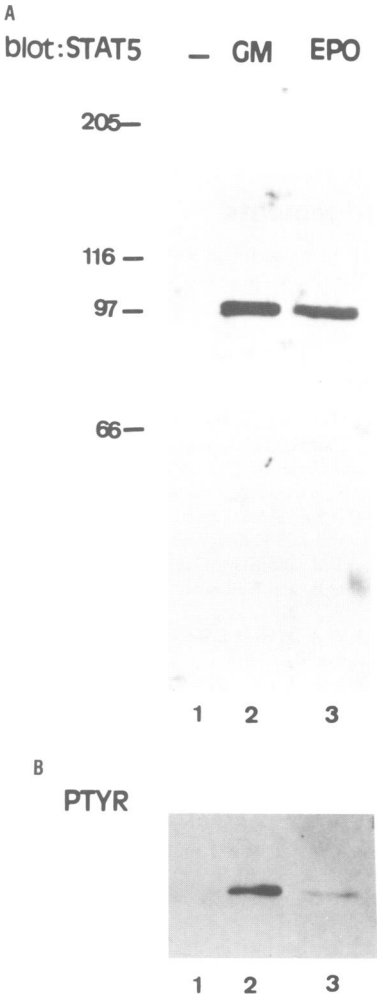

Fig. 8. EPO-induced Stat factor and GM-CSF-induced Stat factor have identical molecular weights to MGF-Stat5. Nuclear extracts from untreated (lane 1), EPO-treated (lane 2) and GM-CSF treated (lane 3) UT7 cells were incubated with the multimerized β-casein oligonucleotides coupled to Sepharose beads. Bound proteins were eluted and analyzed by Western blotting with an MGF-Stat5-specific antiserum (**A**) and a phosphotyrosine-specific antibody (**B**).

A number of additional observations consistent with the data reported here suggest that MGF-Stat5 plays a role in the transduction of signals generated by different cytokines. The GAS elements found in the FcγRI and IRF-1 gene promoters are efficient competitors for factor binding (Figure 6). This is consistent with the observation that EPO and GM-CSF activate Stat proteins in different cell lines that bind to the GAS element of the FcγRI gene promoter (Larner *et al.*, 1993; Finbloom *et al.*, 1994). These factors are probably the same factors that we observed in UT7 cells. They recognize the palindromic sequence 5′-TTCNNNGAA-3′. DIF is activated during the differentiation of myeloid cells by phorbol esters, IFNγ, CSF-1 and GM-CSF (Eilers *et al.*, 1994). It also reacts with the MGF-Stat5-specific antiserum and binds to the GAS sequence of the IFP53 gene promoter (Barahmand-Pour *et al.*, 1995). This GAS element contains the same palindromic sequence. GH activates a factor related to MGF-Stat5 in rat liver. This factor binds to a GAS-like element in the serine protease inhibitor gene promoter which also comprises this palindromic sequence (Wood *et al.*, 1995).

These results suggest that the EPO-induced Stat factor and the GM-CSF-induced Stat factor in UT7 cells are the human homologs of MGF-Stat5. Formally, however, it is possible that they represent new members of the Stat family, closely related to MGF-Stat5 and with a more restricted DNA recognition specificity than described for Stat1 and Stat3 (Pearse *et al.*, 1993; Zhong *et al.*, 1994). It is possible that additional cytokines could utilize MGF-Stat5 signaling. The IL-3 and IL-5 receptors share the same β-chain with the GM-CSF receptor. Jak2 is associated with this β-chain and is activated after binding of the cytokines (Miyajima *et al.*, 1992; Silvennoinen *et al.*, 1993; Ihle *et al.*, 1994; Quelle *et al.*, 1994). This may indicate that MGF-Stat5 may also be activated by IL-3 and IL-5.

The activation of MGF-Stat5 by different cytokines in the same cell line raises the question of the specificity of biological action. EPO and GM-CSF, for example, both induce the same factor in the multipotential hematopoietic cell line UT7. Both cytokines induce the proliferation of UT7 cells, but the cytokines have distinct effects on the differentiation of UT7 cells (Komatsu *et al.*, 1991; Hermine *et al.*, 1992). It is reasonable to suggest that MGF-Stat5 activation might be important for the common effect, proliferation, via the regulation of an overlapping subset of target genes induced by EPO and GM-CSF. Differentiation could be affected by other factors, not commonly regulated by EPO and GM-CSF. Other scenarios can be envisaged. EPO and GM-CSF might activate MGF-Stat5 in a distinct fashion. Identical DNA binding capabilities might be conferred by phosphorylation of Tyr694, but different transactivation potentials could result from additional cytokine-specific phosphorylation events. These phosphorylations could involve additional tyrosine residues or be conferred by serine threonine-specific kinases. The phosphorylation of ATF2 by e-Jun N-terminal protein kinase (JNK) is an example of the post-translational regulation of a transactivation domain (Gupta *et al.*, 1995). Differential regulation of the DNA binding potential and the transactivation potential of MGF-Stat5 by different cytokines could explain why transactivation of the β-casein gene promoter by MGF-Stat5 in transfected COS cells occurs in response to prolactin, but not in response to EPO and GH. The events leading to differential regulation of gene transcription by different cytokines might not all converge at the level of MGF-Stat5. They might involve interacting factors, possibly expressed in a cell type-specific manner and differentially regulated by cytokines (Standke *et al.*, 1994).

Comparison of the C-terminal sequences of different Stat factors revealed that this region is most variable. Transcriptional induction by p91-Stat1α, but not p84-Stat1β, the splicing variant, has been observed (Schindler *et al.*, 1992a; Shuai *et al.*, 1993). Stat1β lacks 38 amino acids at the C-terminus, which might represent the transactivation domain of Stat1α (Shuai *et al.*, 1993). Two similar polypeptides with molecular weights of 92 and 84 kDa have been obtained by affinity purification of MGF-Stat5 (Wakao *et al.*, 1994); they could represent splicing variants with distinct transactivation domains. The activation of closely related factors with identical DNA binding

specificities and different transactivation potentials could be a mechanism to show how specific gene responses to cytokines are achieved.

Materials and methods

Cell culture

UT7 cells were cultured in A-medium, 10% FCS and GM-CSF at 2.5 ng/ml or EPO at 1–2 U/ml. UT7 cells were growth factor-deprived overnight in Iscove medium supplemented with 1% BSA and 100 μg/ml transferrin before induction for 1 h with EPO at 10 U/ml and GM-CSF at 5 ng/ml. U937 cells were maintained in RPMI, 10% FCS and 2 mM glutamine. GM-CSF was added at 5 ng/ml for 1 h in the induction experiments. COS7 cells were maintained in DMEM containing 10% FCS, 2 mM glutamine and 50 μg/ml gentamycin. Depending on the experiments, COS cells were induced with 5 ng/ml ovine prolactin, 10 U/ml mouse EPO (Boehringer Mannheim) or 100 nM bovine GH.

Plasmids and DNA transfections

pXM-MGF(Stat5) or the epitope-tagged construct pE-MGF (Stat5), the corresponding mutant constructs pE-MGFtyr694, pZZ1 and the β-casein promoter construct have been described previously (Gouilleux *et al.*, 1994). Transfection experiments were performed using the calcium phosphate precipitation technique. 5 μg pXM-MGF, pE-MGF or pE-MGFtyr694 and 5 μg prolactin receptor, EPO receptor (D'Andrea *et al.*, 1989) or GH receptor (Matthews *et al.*, 1989) expression vectors were used for the transfections. In addition, 0.5 μg plasmid pCH110 encoding the β-galactosidase gene under the control of the SV40 promoter were included in each transfection as internal controls. In the case of the reporter gene assays, 2 μg β-casein promoter construct pZZ1 were used. The DNA was adjusted to 20 μg with salmon sperm carrier DNA.

Luciferase and β-galactosidase assays

Luciferase and β-galactosidase assays were performed as described previously (Gouilleux *et al.*, 1991) and quantitated in the luminometer 953 (Berthold). Luciferase activities and the amounts of nuclear extracts used in bandshift experiments were corrected for the β-galactosidase values obtained in each experiment.

Preparation of nuclear extracts and EMSAs

Nuclear and cytosolic extracts were prepared as described previously (Standke *et al.*, 1994) with the following modifications. Nuclei were extracted with a hypertonic buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 0.1% Triton, 20% glycerol, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin and 5 μg/ml aprotinin for 20 min at 4°C. The extracts were centrifuged at 14 000 r.p.m. for 5 min. Extracts were frozen in liquid nitrogen and stored at −70°C. Cytosolic and nuclear extracts were used for bandshift experiments or immunoprecipitations. The protocol for EMSAs has been described previously (Wakao *et al.*, 1992). In all bandshift experiments the MGF binding site of the bovine β-casein promoter was used as a probe (5′-AGATTTCTAGGAATTCAAATC-3′). This oligonucleotide was end-labeled with polynucleotide kinase to a specific activity of 8000 c.p.m./fmol.

Antibodies, immunoprecipitation and Western blot analysis

Anti-Stat1 and anti-phosphotyrosine (4G10) antibodies were purchased from UBI. Anti-Stat3 (30N and 30C) antibodies were a gift from D.Levy (New York University Medical Center). Anti-Stat5 antibody was produced in chickens. This antibody was directed against a GST−Stat5 fusion protein containing the N-terminal part of Stat5 (amino acids 6–160). For immunoprecipitations, cell extracts were incubated with 5 μl ascites fluid containing the monoclonal antibody 12CA5 raised against the influenza virus epitope (Babco) for 3 h at 4°C. Immunocomplexes were isolated on protein A-coupled Sepharose. Proteins were resolved on 8% SDS−polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Amicon) and incubated with the monoclonal antibody directed against phosphotyrosine (4G10; UBI). Immunoreactive bands were visualized using an epichemiluminescence Western blotting system (Amersham) according to the manufacturer's protocol.

Oligonucleotide affinity purification of MGF-Stat5

Nuclear extracts were incubated with Sepharose beads coupled to multimerized oligonucleotides containing MGF-Stat5 binding sites from the β-casein gene promoter. The binding reactions were performed for 45 min at 4°C in the presence of 30 μg poly(dI−dC) and 30 μg poly(dA−dT) in the binding buffer containing 100 mM NaCl. After three washes with the binding buffer containing 180 mM NaCl, proteins bound to the Sepharose beads were eluted with the SDS loading buffer, separated by SDS−PAGE, blotted onto polyvinylidene difluoride membrane and visualized with an MGF-specific antibody.

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