The Carboxyl-terminal Region of STAT3 Controls Gene Induction by the Mouse Haptoglobin Promoter

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Haptoglobin (HP) is one of the major acute phase plasma proteins in the mouse, and its synthesis is additively induced by interleukin (IL)-6 and glucocorticoids. STAT3 serves as the mediator of the IL-6 receptor signal and appears to contribute to the transcriptional induction of acute phase protein genes. The carboxyl-terminal region of STAT3, consisting of an acidic domain and containing a serine phosphorylation site, has been proposed to contribute to the induction process. To assess the role of STAT3 in the transcriptional control of the HP promoter, we applied two mutant forms of STAT3: one with a deletion of the carboxyl-terminal 55 amino acid residues, STAT3Δ55C, and the other with a substitution of serine 727 to alanine, STAT3SA. Like the wild-type STAT3, both mutant STAT3 forms are activated by the signal-transducing subunit of the IL-6 receptor, gp130, or by co-transfected IL-3 receptor. Ectopic expression and activation of wild-type STAT3 or STAT3SA in HepG2 hepatoma cells similarly enhance transcription through the IL-6-response element of the HP promoter. This enhancement is specific for STAT3 and cannot be reproduced by STAT1 or STAT5. In contrast, STAT3Δ55C inhibits IL-6-induced transcriptional activation. Interestingly, whereas receptor-activated STAT3 also enhances stimulation of the haptoglobin promoter by dexamethasone through the glucocorticoid receptor, activated STAT3Δ55C reduces the regulation below the level achieved by the glucocorticoid receptor alone. This transdominant action by STAT3Δ55C is dependent on a functional IL-6-responsive element. The data suggest that the carboxyl-terminal domain, but not its serine phosphorylation site of STAT3, is required for transcription as part of the hematopoietin receptor signaling as well as for cooperation with other transcription factors such as the glucocorticoid receptor.

Acute phase response is elicited by the various forms of inflammatory condition, tissue injury, and infection (1). The recruitment of the systemic defense mechanisms is initiated by many cell types at the site of local inflammation and includes macrophages, mast cells, fibroblasts, and endothelial cells and involves the production, release, and distribution of several cytokines. A major component of the systemic acute phase response is the enhanced production of several plasma proteins in the liver that are collectively called acute phase plasma proteins (APPs) (1). In most vertebrate species, haptoglobin (HP) is one of the core sets of APPs, and its synthesis is generally increased severalfold. In the mouse, the increase is 30-fold and exceptionally high (2). HP functions as a hemoglobin-binding protein and is most effective in clearing free hemoglobin from the circulation (3). IL-6 is the major inducer of the hepatic production of most APPs, whereas IL-1 and tumor necrosis factor-α act only on a subset of APPs (4). Glucocorticoids or dexamethasone enhance the production of many APPs through yet-to-be-defined mechanisms. The mouse HP gene is an example of APPs that are strongly induced by both IL-6 and dexamethasone (2).

Signaling by the IL-6 receptor is mediated by the signal-transducing common subunit gp130 through Janus kinases associated with the cytoplasmic domain containing the Box-1 and Box-2 motifs (5). Following phosphorylation on the tyrosine residue in the Box-3 motifs of gp130, STAT3 is recruited to the gp130 and is activated by receptor-associated Janus kinases (JAKs) through phosphorylation (6). Activated STAT3 dimerizes, translocates to the nucleus, and binds to specific DNA sequences (7). The interaction of STAT3 with specific cis-acting elements has been correlated with enhanced transcription of the responsive target genes including APPs (8, 9). Recent analyses have suggested that additional modifications of STAT3 protein alter its function as a transcriptional activator. Phosphorylation on serine 727 has been shown to enhance DNA binding affinity and activation of transcription (10, 11). Deletion of the carboxyl-terminal region, as found in the STAT3β form, rendered the protein inactive as an activator of transcription and as a regulator of differentiation of the myeloid cell line M1 in response to IL-6 (12–14). Although characterization of STAT3 has indicated structural domains relevant for gene induction, the action of critical STAT3 mutant forms, in the context of an IL-6-sensitive gene promoter, has not been described. The promoter of the mouse HP gene has been selected as a model to define the relevance of the carboxyl-terminal peptide and the serine 727 phosphorylation site for regulation by IL-6R and other hematopoietin receptors in hepatic cells.

Previously, we have found that the 237-bp HP promoter contains cis-acting elements for mediating induction by IL-6 and dexamethasone, and includes two C/EBP binding sites ("A" element at positions −213 to −198 and "C" element at −137 to −123), the IL-6 response element (IL-6RE) ("B" element at positions −165 to −152), and the PEA-3 binding site (at positions −110 to −87) (see Fig. 6A; Ref. 2). Although no glucocorticoid receptor (GR) binding site could be identified in the...
HepG2 cells were transfected by the modified calcium phosphate transfection procedure, and the cDNA forms were cloned into the human cytomegalovirus pLA2.2C promoter region to produce the expression vector. All of the mutations were verified by DNA sequence analysis. 

**EXPERIMENTAL PROCEDURES**

**Cells and Cytokines—** COS-1 and HepG2 cells (15) were maintained in minimal essential medium supplemented with 5% and 10% fetal calf serum, respectively. Mouse hepatoma cells, Hepa-1 (16), were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were treated with serum-free medium containing 100 ng/ml IL-6, (Genetics Institute), IL-3 (Sandoz), or GCSF (Immunex Corp). Where necessary, 1 μM dexamethasone was used.

**DNA Constructs—** Expression vector encoding the human GCSFR-gp130 chimeric receptor (GCSFR-gp130(133)) (17), IL-3 receptor (α and β) (18), rat glucocorticoid receptor (pRSVGR) (20), and rat STAT3 (9) have been described. Plasmid pmHP(237)-CAT contains the promoter of the mouse HP (human HP) gene (19), MAPK kinase substrate site, and STAT3 with a truncation of the acidic carboxyl-terminal fragment as observed in STAT3Δ55C (12, 13) but lacking the latter’s additional 7 residues encoded by the extra exon sequence. We verified the expression of the STAT3 proteins by transiently transfecting COS-1 cells (Fig. 1A). Western blot analysis (bottom part, mA) demonstrated that each STAT form was produced in approximately equal amounts, yielding levels that exceeded severalfold that of the endogenous STAT3 (Fig. 1A, Ctrl). The major portion of the immuno-detectable STAT3 proteins migrated with the expected sizes. However, a minor fraction of STAT3wt and STAT3SA migrated with an approximately 5000 smaller molecular size. These forms probably represent proteolytically modified STAT3 proteins (see similar forms detected in HepG2 cell extracts; Fig. 2A).

The activation of the transfected STAT3 proteins by gp130 signals, was demonstrated by the co-transfected chimeric receptor, GCSFR-gp130(133). This receptor consists of the extracellular domain of the human GCSFR fused to the transmembrane and the cytoplasmic domain of 133 residues of human gp130. The chimeric receptor is considered to undergo a GCSF-dependent dimerization that also includes the cytoplasmic gp130 domains. An analogous dimerization process has been predicted for the bona fide ligand-activated IL-6R (27, 28).

Expression of the chimeric receptor provides the advantage that signaling function of gp130 can be analyzed specifically in transfected cells and independently of the endogenous gp130 (17, 29). Extracts from cells treated with or without GCSF indicated that each STAT form was activated by the receptor and yielded comparable SIE-binding activity (Fig. 1A, upper panel). As shown previously (9) in COS-1 cells not transfected with any STAT expression vectors, GCSFR-gp130 activated detectable SIE-binding activity of endogenous STAT3 proteins consisting primarily of STAT1. In the presence of overproduced STAT3, however, the predicted SIE binding activities characteristic for STAT3 homodimers (SIF-A complexes, Ref. 23) were obtained. Only a minor fraction of heterodimers between STAT3 and endogenous STAT1 (SIF-B complex) appeared as an additional band below the STAT3 homodimer complex. As predicted, the SIE complexes formed with wild-type STAT3 and STAT3SA were recognized by the C-20 anti-STAT3 antibody, yielding a supershifted EMSA pattern (Fig. 1A, upper panel, lanes 7 and 8). The STAT3Δ55C-containing complex was not recognized by C-20 antibody (lane 9) but showed a characteristic slower mobility probably due to the loss of the acidic C-terminal fragment that bears the epitope for the C-20 antibody (see smaller size on Western blot in Fig. 1A).

The reaction with anti-phosphotyrosine STAT3 (Fig. 1A, lower panel) illustrated that the phosphorylation at tyrosine 705 in each STAT3 form was correlated with the DNA binding activity of the protein.

To show in our experimental system that overexpressed STAT3Δ55C forms heterodimers with STAT1 or STAT3 (see also Ref. 13) we transfected varying amounts of expression vector for STAT3Δ55C, STAT3, and STAT3SA into COS-1 cells. DNA binding activity of the STAT proteins was activated through GCSFR-gp130 and determined by EMSA. Dose-dependent formation of heterodimers between STAT3Δ55C and STAT3SA was dependent on the presence of the GR and requires the cytokine response region at positions – 184 to – 106 of the HP promoter (2). Here we report the specificity with which STAT3 contributes to transcription through the IL-6RE of the HP gene and the relevance of the IL-6RE and STAT3 in mediating induction by IL-6 and dexamethasone.

**RESULTS**

**Wild-type STAT3, STAT3SA, and STAT3Δ55C Show the Same DNA Binding Activity—** To characterize the role of STAT3 in the regulation of mouse HP gene elements, we constructed two key mutant forms of STAT3: STAT3SA with a mutation of the serine 725 to alanine at its carboxyl-terminal kinase substrate site, and STAT3Δ55C with a truncation of the acidic carboxyl-terminal fragment as observed in STAT3Δ55C (12, 13) but lacking the latter’s additional 7 residues encoded by the extra exon sequence. We verified the expression of the STAT3 proteins by transiently transfecting COS-1 cells (Fig. 1A). Western blot analysis (bottom part, mA) demonstrated that each STAT form was produced in approximately equal amounts, yielding levels that exceeded severalfold that of the endogenous STAT3 (Fig. 1A, Ctrl). The major portion of the immuno-detectable STAT3 proteins migrated with the expected sizes. However, a minor fraction of STAT3wt and STAT3SA migrated with an approximately 5000 smaller molecular size. These forms probably represent proteolytically modified STAT3 proteins (see similar forms detected in HepG2 cell extracts; Fig. 2A).

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To show in our experimental system that overexpressed STAT3Δ55C forms heterodimers with STAT1 or STAT3 (see also Ref. 13) we transfected varying amounts of expression vector for STAT3Δ55C, STAT3, and STAT3SA into COS-1 cells. DNA binding activity of the STAT proteins was activated through GCSFR-gp130 and determined by EMSA. Dose-dependent formation of heterodimers between STAT3Δ55C and STAT3SA was dependent on the presence of the GR and requires the cytokine response region at positions – 184 to – 106 of the HP promoter (2).
Signal Transduction of STAT3

STAT3, COS-1 cells were transfected with expression vectors for STAT3, bound complexes, SIF-A, -B, and -C, containing rat STAT1 and STAT3. These were subjected to EMSA. Whole cell extract of IL-6-treated rat hepatoma 55C were transfected into COS-1 cells together with GCSFR-gp130. STAT3 function as an inducer of transcription through the HP promoter.

IL-6 Response through the HP Promoter Is Enhanced by STAT3—To identify the role of STAT proteins in the IL-6 regulation of the mouse HP promoter, we needed to test the IL-6-responsive mHP(237)-CAT construct in hepatic cells, because COS-1 cells failed to produce a liver-like regulation of that construct (data not shown). Since no mouse hepatoma cells with IL-6-regulatable endogenous HP gene expression have been identified, we selected HepG2 cells as the experimental system for their prominent IL-6 responsiveness (2, 9, 17). These cells were transfected with pHP(237)-CAT alone or together with expression vectors for the various STAT isoforms to determine by Western blot analysis that the expression of the mHP(237)-CAT construct was increased 2–5-fold. In contrast, STAT3 was expressed at the same level as wild-type STAT3 and bound to SIE with apparently comparable affinity, we determined whether the point mutation had indeed modified the substrate behavior of STAT3 for MAPK. COS cell extracts containing overexpressed STAT3 or STAT3SA were reacted in vitro with MAPK and [\(^{32}\)P]ATP. Immunoprecipitation and gel electrophoresis showed that wild-type STAT3, but not STAT3SA, was prominently labeled (data not shown), indicating that the serine to alanine mutation had effectively removed a principle phosphorylation site in STAT3.

Taken together, these results confirmed the expected biochemical properties of the two mutant STAT3 forms and also indicated that these proteins were essentially indistinguishable in their activation by the gp130 signal, complex formation, and binding to DNA substrates. Hence, these mutant forms fulfilled the preconditions for their application in probing STAT3 function as an inducer of transcription through the HP promoter.

The quantitation of CAT activities (Fig. 2B) revealed that the expression of the mHP(237)-CAT construct was increased 2–5-fold by the action of GCSFR-gp130 and endogenous IL-6R. The cytokine (e.g. IL-6) response was further enhanced by co-treatment with dexamethasone. Co-expression of STAT1 or STAT5B was ineffective in modulating the regulation. STAT3, as well as STAT3SA, however, enhanced induction in GCSF and IL-6-treated cells by 2–3-fold. Nonetheless, the transfection efficiency was sufficient to verify the presence of the overexpressed STAT proteins by Western blotting and activation of their DNA binding activity through receptor signals by EMSA (Fig. 2A). By comparing the Western blot and EMSA signals for STAT3 in STAT3-transfected cells with that in control or non-STAT3 transfected cells, and by taking into consideration the low transfection efficiency, we estimated that the amount of ectopically expressed STAT3 proteins exceeded the level of endogenous STAT3 by at least 10-fold. Although the low level production of GCSFR-gp130 rendered detection of the receptor protein in the transfected culture difficult, we could detect only a relative low percentage of transfected HepG2 cells in the culture (see “Experimental Procedures”) and lower protein expression than in COS-1 cells (data not shown). Nonetheless, the transfection efficiency was sufficient to verify the presence of the overexpressed STAT proteins by Western blotting and activation of their DNA binding activity through receptor signals by EMSA (Fig. 2A). By comparing the Western blot and EMSA signals for STAT3 in STAT3-transfected cells with that in control or non-STAT3 transfected cells, and by taking into consideration the low transfection efficiency, we estimated that the amount of ectopically expressed STAT3 proteins exceeded the level of endogenous STAT3 by at least 10-fold. Although the low level production of GCSFR-gp130 rendered detection of the receptor protein in the transfected culture difficult, we could determine by Western blot analysis that the expression of the mHP(237)-CAT was relatively comparable among separately transfected cell cultures (Fig. 2A, bottom part).

The influence of the STAT3 forms on mHP-CAT gene regul-
Signal Transduction of STAT3

STAT3 Action Involves the IL-6RE—The B element located at positions −165 to −152 of the HP promoter is conserved among HP genes from rodents and primates and has been identified as an IL-6-responsive element (IL-6RE) (33). Since, for unknown reasons, a binding of the isolated HP IL-6RE sequence to STAT3 could not be detected by the standard EMSA conditions as used in Fig. 1, we verified by functional cell assay that this element was the target of the regulation by STAT3. Four tandem copies of the core sequence of the mouse HP IL-6RE (see sequence in Fig. 6A) inserted it into a heterologous promoter-CAT construct, and this construct was transfected into HepG2 cells, along with STAT3 forms (Fig. 2C, lower panel). The results revealed that the element was responsive to IL-6 treatment and that both STAT3 and STAT3SA similarly enhanced the induction. As noted for the HP promoter construct, STAT3Δ55C was inhibitory.

STAT3-specific Action Determined by IL-3R Action—The signaling functions of the overexpressed STAT3 forms, as shown in Fig. 2C, appear to be superimposed on those exerted by the endogenous signal-transducing components, including the resident STAT proteins that are utilized by IL-6R or GCSFR-gp130 (Fig. 2A). A more direct demonstration of the signaling action of the transfected STAT3 forms was possible through activation by co-transfected IL-3R. IL-3R lacks a Box-3 sequence motif in its cytoplasmic domains and, therefore, is ineffective in recruiting and activating endogenous STAT3, but it is able to activate STAT3 proteins when overexpressed (Fig. 3A; Ref. 30). As shown in Fig. 3B, the IL-3R did not detectably induce the HP promoter or HP-IL-6RE construct. However, in combination with either wild-type STAT3 or STAT3SA, a strong induction was obtained. In the same experimental systems, STAT3Δ55C was ineffective.

STAT proteins (10 μg/ml), and empty expression vector to a total of 20 μg/ml. In this experiment, rat STAT1β instead of STAT1α was used for easier detection of the transfected protein in the presence of the relatively abundant endogenous STAT1. After 1 day of recovery, cells were divided in 6-well plates. The cells were treated with IL-6 (Vector Control) or GCSF for 15 min. One part of the collected cells was dissolved directly in SDS sample buffer for Western blot analysis of GCSFR-gp130 (only cytokine-treated cells), and the remaining part was used for preparing whole cell extract. Aliquots containing 5 and 30 μg of protein were used for EMSA and Western analysis, respectively. For EMSA, SIE (all lanes except last lane) or TB2 (last lane with STAT3B) served as binding substrate. For STAT protein Western analyses, extracts were electrophoresed in quadruplicate, and proteins on the nitrocellulose membrane were reacted with the indicated antibodies. For detecting CAT gene regulation, HepG2 cells were transfected with a similar combination of plasmids as in A, consisting of the reporter mHP(237)-CAT (15 μg/ml) and expression vector for GCSFR-gp130 (1 μg/ml) and indicated STAT proteins (5 μg/ml). In this case, rat STAT1α was used. After 1 day of recovery, the cell cultures were divided and treated for 24 h with the indicated cytokine or dexamethasone. The thin layer pattern of one reaction is shown. The fold stimulation measured in this experimental series is indicated above the autoradiogram. C, HepG2 cells were transfected with a 15 μg/ml of mHP(237)-CAT (upper panel) or mHP(IL-6RE)-CAT (lower panel) and the increasing amounts of expression vector for STAT3, STAT3SA, or STAT3Δ55C as marked. The DNA amount in each transfection was adjusted to a total of 25 μg/ml with pUC13 DNA. Cells were treated with IL-6 for 24 h, and CAT activities were determined. All values are expressed relative to control culture without IL-6 treatment and STAT3 transfection.

FIG. 2. Effect of STAT proteins on gene induction through the HP promoter. A, HepG2 cells were transfected with a combination of expression vectors for GCSFR-gp130(133) (5 μg/ml), the indicated lation through the endogenous IL-6R (Fig. 2C, upper panel) or GCSFR-gp130 (data not shown) was dependent on the dose of co-transfected STAT3 expression vectors. Generally, a maximal enhancement of the cytokine signal by wild-type STAT3 and STAT3SA was attained with 1–5 μg/ml transfected expression vector. With increasing amounts of STAT3Δ55C expression vector, the cytokine receptor-mediated induction of the reporter gene declined, reaching basal level in some instances.

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This negative interference of STAT3 wild-type, attenuated gene regulation below the level of combination of IL-6 and dexamethasone, STAT3D (Fig. 4 right panel), thus excluding the possibility that the GR acted indirectly through the STAT proteins interacting with the IL-6RE. To provide evidence that dexamethasone treatment is capable of regulating not only episomal plasmid constructs but also the chromosomal mouse HP gene (the HP gene in HepG2 cells does not respond to dexamethasone; Ref. 33), we turned to the mouse hepatoma Hepa-1 cell line. Previous studies have shown that Hepa-1 cells respond to dexamethasone by increased production of haptoglobin (34), although the same cells failed to respond to any IL-6-type cytokines. Hence, these cells were of limited value as an experimental system for characterizing the cytokine response elements of the HP gene. As shown in Fig. 4D, a 2-h treatment of Hepa-1 cells led to a prominent increase of HP mRNA, and this action was largely maintained in cells with cycloheximide-inhibited protein synthesis. We conclude from these results that dexamethasone, via the GR, induced HP gene transcription. The transfection experiments in HepG2 cells suggested that the 237-bp promoter, but not its IL-6RE, is the target of the GR.

Functional Cooperativity between STAT3 and GR—To assess the specific contribution of wild-type and mutant STAT3 to the action of GR on HP promoter, we determine the STAT3 dose dependence of the regulation by utilizing the IL-3R-mediated STAT activation process introduced in Fig. 3. Maximal induction of the reporter gene construct was attained with 1.5 μg/ml of wild type STAT3 expression vector (Fig. 5A). When using a submaximal concentration (0.5 μg/ml) of wild-type STAT3 and an increasing dose of STAT3Δ55C expression vector, we detected an enhancing effect of a low dose of STAT3Δ55C in IL-3- as well as IL-3 plus dexamethasone-treated cells (Fig. 5B). At a higher dose of STAT3Δ55C, the transdominant inhibitory action of the STAT protein prevailed. This dual effect of STAT3Δ55C on HP promoter activity in IL-3- and dexamethasone-treated cells was even more pronounced in the presence of co-expressed GR (Fig. 5C). We interpret these results to mean that at low dose and approximately equal expression, levels of wild type and STAT3Δ55C heterodimer between the two STAT proteins are formed (see Fig. 1C) and that these, like the wild type STAT3 homodimer, mediate transactivation. At high STAT3Δ55C concentration, STAT3Δ55C homodimer predominates (Fig. 1C) and acts as a competitive inhibitor. The data also suggest that a cooperative action between GR and STAT3 is established that in part would explain the additive induction. The carboxy-terminal portion of STAT3 is probably involved in that cooperativity, and if absent as in STAT3Δ55C dimers, an active inhibition is obtained.

The IL-6RE Is Critical for HP Gene Induction by IL-6 and Dexamethasone—Previous analysis of the mouse HP promoter (2) indicated a contributing role of C/EBP isoforms and PEA-3 for determining expression levels. The present results propose a role of STAT3 in mediating IL-6 induction, most probably through the IL-6RE (Figs. 2C and 3B). To prove the functional relevance of the IL-6RE in the 237-bp promoter context, we mutated the core sequence of this IL-6RE at three positions (MB) as indicated in Fig. 6A. Wild-type and MB constructs were introduced into HepG2 cells (Fig. 6B, upper panel). The mutation had two striking effects: 1) basal expression was reduced ~10-fold; and 2) stimulation by both IL-6 and dexamethasone had been abolished. The mutation was outside of the two principle C/EBP binding sites (Fig. 6A; Ref. 2); therefore, we could still demonstrate transactivation by overexpressed C/EBPβ (data not shown). These results suggest that

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**Fig. 3. IL-3R-mediated STAT3 action.** A, HepG2 cells were transfected with expression vector for IL-3Rα and IL-3Rβ (3 μg/ml each) and STAT3 forms or empty vector as indicated (14 μg/ml). Cell cultures were divided. Extracts from control and IL-3-treated cells were used for EMSA with SIE as probe. Extracts of IL-6-treated HepG2 cells served as markers. B, HepG2 cells were transfected with mHP(237)-CAT (15 μg/ml) or mHP(IL-6RE)-CAT (15 μg/ml) and expression vectors for the indicated STAT3 forms (5 μg/ml) and for IL-3Rα and IL-3Rβ (1 μg/ml each). Similar CAT gene regulations as shown in this experimental series were observed in two additional independent experiments.
the IL-6RE sequence plays a critical role in determining basal expression and in mediating the induction by dexamethasone and IL-6.

The influence of MB mutation in the HP promoter on the specific transactivating function of STAT3 and GR was determined in HepG2 cells overexpressing either transcription factor (Fig. 6B, lower panel). STAT3 was activated by co-expressed IL-3R, but no effect on the HP-CAT construct was detected. In contrast, GR produced a dexamethasone-mediated induction of the MB construct. The GR action was severalfold lower than seen with wild-type HP promoter (Fig. 4B) and was also not influenced by co-expressed and IL-3R-activated wild-type STAT3 or STAT3Δ55C (data not shown). The data support the model that STAT3 through interaction with IL-6RE establishes a functional cooperativity with GR and that the C-terminal portion of STAT3 is necessary for communication with this transcriptional machinery.

**DISCUSSION**

Acute phase regulation of hepatic HP gene expression is primarily mediated by IL-6-type cytokines (35). However, additional regulatory factors need to be considered, such as growth factors and endocrine hormones, including glucocorticoids to explain the full acute phase induction (2, 4, 34). Several signaling pathways with distinct transcription-controlling factors have been proposed for IL-6R (36), some of which may act on the HP gene (2, 37, 38). The results of our study document an active role of STAT3 in mediating hematopoietin receptor signal, in particular that of gp130 as part of the IL-6R complex to the mouse HP gene promoter in hepatic cells. We approached the characterization by using two mutant STAT3 proteins that had been predicted to act as specific inhibitors and applied these to dissect the IL-6R signal mechanisms.

**Fig. 4.** The effect of STAT3Δ55C on dexamethasone regulation through the HP promoter. A, HepG2 cells were transfected with mHP(237)-CAT (15 μg/ml, left) or mHP(IL-6RE)-CAT (15 μg/ml, right) and together with expression vectors for GR (2 μg/ml), STAT3, or STAT3Δ55C (5 μg/ml) as indicated at the top. Subcultures were treated with dexamethasone and IL-6 for 24 h. B, HepG2 cells were transfected with mHP(237)-CAT (15 μg/ml) and increasing amounts of GR. Subcultures were treated with serum-free medium alone (control) or medium containing dexamethasone (Dex), IL-6, or the combination of dexamethasone and IL-6. C, as in B, HepG2 cells were transfected with mHP(237)-CAT without (left) or with GR (2 μg/ml) (right) and increasing amounts of expression vectors for wild type STAT3 (closed symbols) or STAT3Δ55C (open symbols). Subcultures were treated with dexamethasone (Dex; round symbols) or IL-6 plus dexamethasone (IL-6 + Dex; square symbols). The CAT activities were calculated relative to the untreated controls in each experimental series. D, mouse Hepa-1 cells were maintained for 16 h in serum-free medium and then were treated for 2 h with medium alone (Control) or medium containing dexamethasone (Dex). Pretreatment with cycloheximide (10 μg/ml) (CHX) was for 10 min prior to the addition of dexamethasone. Total RNA was subjected to Northern analysis using mouse HP cDNA as probe. The upper panel shows the autoradiogram after 48-h exposure, and the lower panel shows the ethidium bromide-stained pattern of 18 S rRNA.
tion, we assume that receptor-mediated activation of MAPK or any protein kinase C isoforms that might act upon serine 727 (11, 39) does not play a critical role in HP gene induction. This assumption is supported in part by our earlier observation that treatment of hepatic cells with reagents that activate the MAPK pathway or protein kinase C independently of the hematopoietin receptor signal, e.g. by insulin or phorbol ester, could neither mimic nor enhance the action of IL-6 on APP genes or transfected reporter constructs (40, 41). In fact, insulin treatment that activates MAPK (42) and increases serine phosphorylation of STAT3 (43) reduces the IL-6 induction of APP genes (40, 41). We conclude that phosphorylation at serine 727 may not be an important modification for achieving signaling toward APP genes, but we cannot rule out that phosphorylation at other sites influences transcription-inducing activity or cellular turnover of STAT3 (10, 43).

The second mutant STAT3, STAT3\textsubscript{D55C}, has been predicted (based on the precedents of STAT1\textsubscript{b} (10) or STAT5B\textsubscript{D40C} (44)) to be inactive as an inducer of transcription and to act either as a transdominant or competition inhibitor of wild-type STAT3. The mode of action differs from the dominant negative action of the tyrosine 705 mutant STAT3 that, by virtue of being unable to dimerize, acts as a terminator of the signaling at the receptor level (45). STAT3\textsubscript{D55C} has an essentially wild-type function in respect to recruitment to the receptor, activation by receptor-associated kinases, and DNA binding. Evidently, STAT3\textsubscript{D55C} terminates signaling by failing to stimulate transcription in cis. This implies that the carboxyl-terminal acidic fragment may have stimulatory properties analogous to the acidic domains of other transcription factors such as GCN4 or VP16, which in-

Fig. 5. STAT3-specific action on GR function. HepG2 cells were transfected with mHP(237)-CAT (15 μg/ml), IL-3R\textsubscript{a}, and IL-3R\textsubscript{b} (1 μg/ml each) and the expression vector for GR and STAT protein listed at the bottom of each graph. Cells were treated with medium alone (Control), dexamethasone (Dex), IL-3, or IL-3 plus dexamethasone.

Fig. 6. IL-6RE is necessary to mediate IL-6 and dexamethasone response. A, the schematic drawing shows the relative locations of the known elements (elements A, B, and C) within the mouse HP promoter with the positions of the regions identified by the DNase I protection assay (2, 31) listed below and the proposed interaction of transcription factors shown above. The sequence of the wild type (wt) and mutant (MB) IL-6RE is given below. B, HepG2 cells were transfected with mHP(237)-CAT or mHP(237)MB-CAT (15 μg/ml) alone (upper panel) or together with expression vector for IL-3R\textsubscript{a} and IL-3R\textsubscript{b} and STAT3 or GR (lower panel). Subcultures were treated for 24 h with IL-6 or IL-3 and dexamethasone as indicated below each panel. Normalized CAT activities were calculated relative to the control culture with mHP(237)wt-CAT in each series (equal to 1.0).
teract with general transcription factors TFIIB and TFIID (46). STAT3*55C was modeled after STAT3β (12) but differs by not having an extra 7-amino acid extension. In agreement with the previous report (13), STAT3*55C is unlike STAT3β in two important properties: 1) STAT3*55C does not display appreciable DNA binding activity in cells not treated with inducing cytokines, e.g. IL-6 or IL-3; and 2) STAT3*55C shows normal DNA binding activity but fails to induce transcription. The fact that carboxy-terminal-truncated STAT3 forms naturally occur demands consideration of their potential biological relevance.

The inhibitory activity of STAT3*55C was readily detectable on the co-transfected HP gene construct. A similar action is also expected to occur on all endogenous IL-6-responsive APP genes that are also sensitive to STAT3. Due to the technical limitation of the transient transfection system, such an inhibition could not be determined in the HepG2 cells. We attempted by retroviral vector transduction to generate HepG2 cells stably overexpressing STAT3*55C. Although many clonal lines were obtained, none yielded a STAT3*55C expression level that was sufficient to eliminate the action of the endogenous STAT3. However, lines were identified in which the IL-6 induction of HP was reduced by 50% relative to the parental line. Our data confirm a competitive inhibitory action of STAT3*55C in the context of an APP gene promoter (Fig. 5). Furthermore, we show an inhibitory role for STAT3*55C in the case of GR-enhanced transcription through the 237-bp HP promoter (Figs. 4C and 5). This function of STAT3*55C needs to be activated by IL-6 treatment, suggesting that STAT3*55C may interact with the IL-6RE of the HP promoter and thereby attenuate transcription-inducing action of the GR. Thus, the carboxy-terminal peptide of STAT3β, by its deletion, appears to determine the inhibitor phenotype. We have not been able to detect a direct binding of the glucocorticoid receptor with the HP promoter sequence or an interaction of the glucocorticoid receptor with STAT3β. We cannot, therefore, exclude the possibility that the glucocorticoid receptor acts at a site (or sites) distant from the DNA (32). The results with Hep-1 cells (Fig. 4D) at least rule out an indirect mechanism that depends on new protein synthesis.

Combining all information, the following working model for regulation through the HP promoter is proposed. As shown in Fig. 6A, the promoter contains functionally defined interaction and/or response elements that include two sites (sites A and C) for C/EBPβ that flank the single STAT response element (B or IL-6RE) and a more proximally located PEA-3 site that is characteristic to the mouse HP gene (2). A site for the interaction of the GR within the dexamethasone-responsive BC-containing segment (2) has not been found. IL-6RE is sensitive to STAT3 but appears also to be a target of a factor(s) necessary for establishing basal expression and executing the dexamethasone response (Fig. 6B). IL-6 treatment has two effects. One effect is the immediate activation of STAT3 that interacts with the IL-6RE or associated proteins or replaces the factors already bound (31). The other effect is enhancing the production and nuclear concentration of C/EBPβ (37, 38, 47). C/EBPβ binds to the HP promoter, stimulates HP transcription (2), and appears to enhance the effects of STAT3 and GR (5). Although STAT3 alone is sufficient to induce transcription through the isolated IL-6RE (Fig. 3B), to obtain an appreciable magnitude of induction that is equal to that of STAT3 through the HP promoter, an oligomerization of the IL-6RE is required. Thus, we speculate that the combination of STAT3 and C/EBPβ, as predicted for the HP promoter, may be as effective as a complex of several STAT3s, as predicted for the IL-6RE oligomer.
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