Communication

STAT3 and STAT5B Are Targets of Two Different Signal Pathways Activated by Hematopoietin Receptors and Control Transcription via Separate Cytokine Response Elements*

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Transient transfection of expression vectors for various members of the hematopoietin receptor family and STAT proteins into COS-1 cells indicated that each receptor was capable of stimulating the DNA binding activity of STAT1, STAT3, and STAT5B. However, gp130 preferentially activated STAT1 and STAT3. Activation of STAT5B differed from that of the other two in that the box 3 sequence motif in the cytoplasmic domain of gp130 was not required. Moreover, STAT5B and STAT3 enhanced gene transcription via separate regulatory elements. This study has identified two potential signal transduction pathways by which hematopoietin receptors, including the interleukin-6 receptor, control transcription of acute phase plasma protein genes in hepatic cells.

The transcriptional regulation of acute phase plasma protein genes in hepatic cells by IL-6 has been correlated with the activation of DNA binding properties of STAT3 by the signaling activity of gp130 (1–5). The suggested function of STAT3 as a transcription factor was supported by the finding that overexpression of STAT3 and its activation by cytokine receptor and Janus kinases resulted in enhanced transcription via IL-6-responsive gene elements (6). However, the model proposing a principal role for STAT3 as a mediator of acute phase response (3, 7) needed to be refined because: 1) gp130-dependent transcription via certain elements, such as the HRRE, was found to be independent of STAT3 (8); and 2) the acute phase response of the liver included activation of not only STAT3 but also of other members of the STAT protein family, such as STAT5B (9,2). The study of the transcription control mechanisms by specific STAT isoforms has been difficult primarily due to the lack of adequate experimental assay systems. Recently, we have developed techniques to reconstitute the function of hepatic and non-hepatic hematopoietin receptors in transiently transfected hepatoma cells (10). We could define the cytoplasmic domains of the signal transducing receptor subunits required for the induction of transcription through specific regulatory elements (8, 11). This cell assay system was used to characterize the specificity of STAT protein activation by hematopoietin receptors. Two distinct signaling pathways were identified: one specified by the box 3-dependent activation of STAT3 and transcriptional stimulation via an IL-6RE, and the other specified by the box 3-independent activation of STAT5B and transcriptional stimulation via HRRE.

EXPERIMENTAL PROCEDURES

Cells—HepG2 and COS-1 cells were cultured as described (10). Hormonal treatments were carried out in serum-free medium containing 1 µM dexamethasone alone or with 100 ng/ml recombinant human IL-2 (Cetus Corp.), IL-3 (Sandoz), IL-4, G-CSF (Immunex Corp.), GH (Genentech), IL-6 (Genetics Institute), or ovine PRL (National Hormone and Pituitary Program, lot ATP 10677C).

Expression Vectors and CAT Reporter Gene Constructs—Expression vectors for the following receptors have been described previously (10–12): human IL-3Rα (13), IL-3Rβ (14), IL-2Rβ (15), IL-2Rγ (16), IL-4R (17), chimeric human G-CSF-R-gp130 (27), G-CSF-R-gp130 (11) (and its box 3 mutant (M3, Y125A) (8), rabbit GHR (18), and pigeon PRLR (19). Expression vectors for rat STAT1, STAT3 (6), and STAT5B2 consisted of the plasmid SV-Sport1 (Life Technologies, Inc.) containing the cDNAs inserted into the polylinker region. Plasmid EF-BOS-j AK2 (20) was provided by Dr. D. M. Wojchowski (Pennsylvania State University, University Park, PA). The CAT reporter gene constructs pHRRE-CAT and pIL-6RE-CAT have been described (10).

Cell Transfection and Analysis—COS-1 cells were transfected with plasmid DNA by the DEAE-dextran method (21) and HepG2 cells by the calcium phosphate method (22). The cell cultures were subdivided. Subcultures of COS cells were maintained for 16 h in serum-free medium prior to the activation of STAT proteins by treatment with cytokines for 15 min. DNA binding activities of STAT proteins in whole cell extracts were determined by EMSA (23). Double standard glucocorticoids for the high affinity SIEm67 (23), the mammary gland factor binding site of the rat β-casein gene (PRL response element, or PRE; Ref. 24), and TB-2, a duplicated IL-6RE sequence (TB-1) of the rat α₂-macroglobulin gene (2), served as EMSA substrates. The STAT1, STAT3 and STAT5B-containing complexes with PRE and TB-2 were identified by supershift assay using 2 µg of monoclonal anti-STAT1 (Transduction Laboratories), 0.5 µg of rabbit anti-STAT3 (Santa Cruz Biotechnology), or 0.5 µl of antisera against STAT5 (gentamicin gift of Dr. H. Wakao, DNAx). Aliquots (5 µl) of whole cell extracts were electrophoresed on a 6% SDS-polyacrylamide gel, and the proteins were transferred to Immobilon membrane (Millipore). The membranes were reacted with either rabbit anti-STAT3 or anti-STAT5 (Transduction Laboratory) and then processed for chemiluminescent reactions (Amer-

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1 The abbreviations used are: IL, interleukin; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GAS, γ-activating site; G-CSF, granulocyte colony-stimulatory factor; GH, growth hormone; HRRE, hematopoietin receptor response element; PRE, prolactin response element; PRL, prolactin; -R, receptor; RE, response element; SIE, sis-inducible element; STAT, signal transducer and activator of transcription.

2 J. Ripperger, unpublished data.
Activation of Multiple STAT Proteins by gp130—To identify the STAT proteins activated by gp130, we employed three oligonucleotides as diagnostic substrates: the high affinity SIE (23), the PRE of the β-casein gene (24), and TB-2 (2). Each probe yielded a specific EMSA pattern of IL-6-regulated DNA binding proteins in H-35 cells (Fig. 1). Both STAT1 and STAT3 bound to the SIE, giving rise to the complexes SIF-A, -B, and -C (23). The β-casein element was recognized primarily by the STAT1 homodimers (co-migrating with SIF-C). The STAT proteins contributing to the slow mobility TB-2 complex could not be deduced from the relative position of the untreated control cultures in each experimental series (defined as = 1.0).

RESULTS AND DISCUSSION

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Activation of STATs in COS-1 cells and activation by gp130 and IL-3R—A, expression vectors for G-CSFR-gp130 (277) (1 μg/ml) and either for STAT5B or STAT3 (concentrations indicated) were transfected into COS-1 cells. Whole cell extracts of duplicate subcultures (control and G-CSF-treated) containing 5 μg of protein was analyzed by Western blotting for STAT protein expression. Blots were processed simultaneously for chemiluminescent reaction. B, COS cells were transfected with expression vectors for G-CSFR-gp130 (277), IL-3Rα and IL-3Rβ (1 μg/ml each), and STAT1, STAT3, or STAT5B (2.5 μg/ml) as indicated at the top. Trisulfate subcultures were treated as control or with IL-3 (3) or G-CSF (6) as shown at the bottom. Identical amounts of whole cell extracts were reacted with SIE, β-casein element, and TB-2, and processed for EMSA.

Both receptor types were capable of transducing the signal to each STAT isoform. The comparison also indicated that IL-3R prefered STAT5B, whereas gp130 prefered STAT1 and STAT3, consistent with the action of native IL-6R in H-35 cells (Fig. 1). The EMSA patterns as well as antibody supershift assays (not shown) did not provide any evidence for appreciable formation of heterodimers between STAT1 and STAT5B or between STAT3 and STAT5B. TB-2 contains two IL-6REs in a 20-base pair span and was thus predicted to bind two STAT dimers. Surprisingly, this probe did not produce detectable amounts of mixed tetrameric complexes containing one homodimer each of STAT1 and STAT5B, as judged from the absence of complexes with intermediate mobilities on EMSA (Fig. 2B) or reacting to both anti-STAT1 and anti-STAT5 (not shown). We hypothesized that either two different STAT dimers were incompatible in simultaneous binding to TB-2, or that STAT5B binds as a preformed tetramer. The results obtained with pairwise combinations of STATs indicated a certain degree of competition among STATs for activation by the receptors that was correlated with the preferred usage of the particular STAT by the receptor.

Activation of STAT5B by gp130 Is Independent of the Box 3 Motif—Since gp130 activated STAT5B (Fig. 2B), we asked whether this process, like the activation of STAT1 and STAT3 (8, 11), was dependent on a functional box 3 motif. Regulatory features for which the box 3 is important include nuclear localization of STAT proteins (8) and the interaction of STAT5B with a box 3-containing cytokine receptor (12). However, the activation of STAT5B by gp130 was independent of the box 3 motif.
Regulation of Transcription by STAT Factors

Functional screening of various regulatory elements indicated that all elements containing a core sequence related to the γ-activating site (GAS) including the IL-4RE/GAS of the FcyRIγ gene, β-casein PRE, the IL-6RE of the α2-macroglobulin gene, and, foremost, HRRE, were responsive to STAT5B. Transfection of GHR and PRLR together with the HRRE-CAT construct and increasing amounts of STAT5B expression vector established a dose-dependent increase of CAT gene transcription (Fig. 5). The enhancing effect of STAT5B was noted on both the basal and receptor-mediated transcription. The change in transcriptional regulation via HRRE was STAT5B-specific because STAT3 (Fig. 5), STAT1, or STAT6 (data not shown; Ref. 6) proved to be ineffective.

In HepG2 cells, both GHR and PRLR were able to control transcription, in part via STAT5B. This finding differs from similar experiments with STAT5A in COS cells reported by Guilleux et al. (29), who observed that PRLR but not GHR or erythropoietin receptor mediated enhanced gene transcription via the β-casein element. Both studies concur in that the magnitude of STAT5 activation by the receptor did not correlate well with the magnitude of transcriptional induction. For instance, in Fig. 5, neither GHR nor PRLR reconstituted an increase in transcription of comparable magnitude with that by the endogenous IL-6R.

To confirm the role of STAT5B in hematopoietin receptor signaling leading to gene transcription, we extended the analyses to other receptor combinations. We focused on those hematopoietin receptors that had shown low action in HepG2 cells and for which no autocrine pathway or response to serum factors was detectable. Moreover, for some of these receptors, the activation of STAT5 had been already reported (30–32). We achieved transcription-enhancing effects via HRRE and related GAS sequences with c-Mpl, and box 3-deleted constructs of G-CSFR-LIFR(140), G-CSFR-gp130(40), and G-CSFR(27). The most prominent effect of STAT5B on transcription was, however, obtained with IL-2R and IL-4R (Fig. 6). STAT5B enhanced the signal of the transfected receptor, resulting in a transcription of the reporter gene that equaled or even exceeded the effect of the endogenous IL-6R. Moreover, when this optimal experimental receptor system was used in combination with the IL-6RE-CAT construct, the results illustrated the clear difference in the regulatory actions of STAT5B and STAT3. STAT5B enhanced transcription via HRRE but not via IL-6RE, and STAT3 was ineffective via HRRE (Fig. 5) but enhanced transcription via IL-6RE (Fig. 6). What still needs to be determined is the extent to which STAT3 and STAT5, or functionally redundant factors, contribute to the regulation of...
Regulation of Transcription by STAT Factors

Fig. 5. Stimulation of transcription by STAT5B. HepG2 cells were transfected with pHRE-CAT (10 μg/ml) and expression vectors for GHR and PRLR (1 μg/ml each) and increasing amounts of STAT5B or STAT3 as noted. Subcultures were treated without cytokines (control) or with PRL, GH, or IL-6. The change in CAT activity was calculated relative to control cells without STAT supplementation. Values represent means of two separate experimental series.

Fig. 6. Transcription control by IL-2R and IL-4R via STAT5B. HepG2 cells were transfected with either pHRE CAT or pIL-GRE-CAT (10 μg/ml), expression vectors for IL-2Rβ, IL-2Rγ and IL-4R (1 μg/ml each) and either increasing amounts of STAT5B (noted in left panel) or STAT3 or STAT5B (5 μg/ml) (right panel). Subcultures were treated with IL-2, IL-4, or IL-6, and the change in CAT activity was calculated relative to control cultures. Similar regulatory effects were measured in three additional experiments.

genes by IL-6 in hepatoma cells.

This study demonstrates that hematopoietin receptors use two distinguishable pathways to regulate gene transcription. The following model is proposed. The basic signaling pathway probably exerted by most if not all hematopoietin receptors requires minimally the box 1 motif. Box 1 engages one of the JAK family members (33), which recruits primarily STAT5 and much less the other STATs. Upon phosphorylation, the dimeric or oligomeric STAT5 interacts with GAS/PRE/TB-2-related sequences. If the receptor bears a box 3 or related sequence, STAT3 or STAT5B (5) probably exerts by most if not all hematopoietin receptors under such conditions STAT1 or STAT6 can interfere with or modulate the action of the other STAT proteins by competition for binding to the same DNA sequences. Unquestionably, the transfection experiments are crude reconstitutions of the normal regulatory systems operative in cells and may yield artifically exaggerated responses. However, these experiments have emphasized the importance of (a) the relative concentrations of receptors, kinases, and STATs and (b) the sequence specificity of the binding of STATs to DNA response elements. A most notable effect also is that excess amounts of JAKs lead to ligand-independent activation of STATs and transcription, suggesting that under such conditions alternative pathways that are generally unnoticed have become predominant. Moreover, every transcription assay is performed in cells that contribute a complex combination of endogenous factors, some of which may significantly influence the observed regulatory events.

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