Interleukin (IL)-6 is a major regulator of hepatic acute-phase plasma protein (APP) genes. The membrane-proximal 133-amino acid cytoplasmic domain of glycoprotein (gp) 130, containing one copy of the Box3 motif, is sufficient to transmit a productive signal to endogenous APP genes in rat hepatoma H-35 cells. In contrast, a mutant gp130 domain lacking the Box3 motif activates Janus kinases to a normal level but fails to activate signal transducer and activator of transcription 3 and to up-regulate a number of APP genes, including thiostatin, fibrinogen, hemopexin, and haptoglobin. However, in the absence of Box3, gp130 still stimulates the expression of α2-macroglobulin and synergizes with IL-1 to up-regulate α2-acid glycoprotein. The Box3 motif is not required for activation of the SH2-containing protein tyrosine phosphatase 2 or the mitogen-activated protein kinase (MAPK), nor is the immediate induction of transcription element. Signals of SH2 domain interaction and serve as the direct downstream targets of the Janus family of tyrosine kinases (JAKs) (20, 32, 33). Phosphorylated STATs form dimers and acquire DNA binding activity. After translocation from the cytoplasm to the nucleus, STAT dimers bind to specific regulatory elements to transactivate gene expression. Other signaling pathways have been described to have modulatory effects on the JAK/STAT pathway. For example, it has been demonstrated that the mitogen-activated protein kinase (MAPK) targets a specific serine residue at position 727 on STAT3 (34, 35). This serine phosphorylation has been proposed to have an enhancing effect on the transactivation potential of STAT3 (35).

The STAT3-independent Signaling Pathway by Glycoprotein 130 in Hepatic Cells*

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Interleukin (IL)-6 is a pleiotropic cytokine that elicits a wide variety of biologic activities in different cell types (1–3). In liver, IL-6 is a prominent inducer of the acute-phase plasma proteins (APPs) which function to protect the host against the harmful consequences of inflammation and to restore normal homeostasis (3–5). IL-6 acts in concert with other factors such as IL-1, tumor necrosis factor, and glucocorticoids to modulate the expression of APP genes. Based on the requirement of IL-1 for maximal expression, APP genes are classified into type I and type II genes (6). For type I APP genes such as α2-acid glycoprotein (AGP), IL-1 synergizes with IL-6 and glucocorticoids in stimulating gene expression. In contrast, maximal expression of type II APP genes depends on IL-6 and glucocorticoids, and IL-1 inhibits expression of some type II APP genes such as thiostatin (Tst) and fibrinogen (Fb). The IL-6 responsive elements (IL-6RE) of a number of APP gene promoters have been mapped (6–16). These elements are both necessary and sufficient to confer IL-6 responsiveness. With the IL-6RE as binding substrate, IL-6-induced transcription factors have been purified, and the corresponding cDNAs were subsequently cloned (17, 18). These factors were identified as members of the Signal Transducer and Activator of Transcription family, STAT1, STAT3, and STAT5 which are also involved in the signaling of many other hematopoietic cytokines (19, 20).

The receptor for IL-6 consists of a ligand-binding subunit, IL-6 receptor-α, and a signal-transducing subunit, gp130 (21, 22). Upon ligand binding, two of each receptor subunit form a hexameric complex with two IL-6 molecules and initiate downstream signaling events (23, 24). Signal transduction depends on the homodimerization of the cytoplasmic domains of gp130 (25, 26). Comparison of the cytoplasmic regions between gp130 and other IL-6-type cytokine receptors including the receptors for leukemia inhibitory factor (LIF), oncostatin M (OSM), and granulocyte colony-stimulating factor (G-CSF) reveals three conserved sequence motifs, namely Box1, Box2, and Box3 (26–29). The Box3 motif, with a consensus amino acid sequence of YXXQ, occurs in multiple copies in gp130, LIF receptor, and OSM receptor and represents a binding site for STAT1 and STAT3 (30, 31).

The current model of IL-6 signaling suggests that STAT proteins are recruited to the receptor via a phosphotyrosine-SH2 domain interaction and serve as the direct downstream targets of the Janus family of tyrosine kinases (JAKs) (20, 32, 33). Phosphorylated STATs form dimers and acquire DNA binding activity. After translocation from the cytoplasm to the nucleus, STAT dimers bind to specific regulatory elements to transactivate gene expression. Other signaling pathways have been described to have modulatory effects on the JAK/STAT pathway. For example, it has been demonstrated that the mitogen-activated protein kinase (MAPK) targets a specific serine residue at position 727 on STAT3 (34, 35). This serine phosphorylation has been proposed to have an enhancing effect on the transactivation potential of STAT3 (35).
Previously, we characterized the signaling capability of gp130 in hepatoma cells by the transient transfection approach, and we showed that gp130 cytoplasmic domain engages at least two pathways that differ in their requirement for the Box3 motif of gp130 (36, 37). In this experimental system, the Box3-dependent pathway appears to involve STAT3 in the regulation of some APP gene promoters when presented as part of episomal constructs (38). However, a direct assessment of the precise role for Box3 and STAT3 in the control of endogenous APP genes has not yet been possible. Unfortunately, a STAT3 knock-out mouse is not available to address this question as STAT3-deficient embryos die during early stage of development (39). On the other hand, the Box3-independent pathway signals to an artificial element, the hematopoietin receptor response element (HRRE), which corresponds to the core IL-6RE of the rat α2-macroglobulin gene (α2-MG) and to the “distal regulatory element” in the α1-acid glycoprotein (AGP) gene promoter (40). The chromosomal genes regulated by this proposed pathway have not yet been identified. Here we assessed the importance of the Box3 motif in controlling endogenous APP genes by generating stable hepatoma cell lines expressing chimeric G-CSFR-gp130 receptor. We also attempted to elucidate the components of the Box3-independent signaling pathway in these cells. Our results indicate that both Box3-dependent and Box3-independent pathways contribute to the regulation of APP genes. The data also suggest a functional role of Box3 in modulating the duration of MAPK activity.

**EXPERIMENTAL PROCEDURES**

**Receptor Constructs**—The chimeric receptor containing the extracellular domain of human G-CSF receptor and the transmembrane and the first 133 amino acids of the intracellular domain of gp130 (designated G-CSFR-WT-gp130) and the wild-type construct, designated as a tyrosine mutation in the Box3 sequence (designated as G-gp130(133)-M3), have been described previously (36). The chimeric receptor construct containing the full-length 277 residue, wild-type gp130 cytoplasmic domain with C-terminal FLAG epitope, has been described previously (41). The equivalent FLAG epitope (DYKDDDKK) was also introduced at the end of G-gp130(133)-WT, yielding G-gp130(133)-WT-FLAG. Cells and Treatments—Parental rat hepatoma H-35 cells and hepatoma H-35 cells stably expressing chimeric G-CSFR-gp130 receptor were used in these experiments.

**Generation of H-35 Cells Stably Expressing Chimeric G-CSFR-gp130 Receptors**—Previously, by constructing G-CSFR-gp130 chimeric receptors, we have reconstituted signaling events in transiently transfected hepatoma cells, which were similar to those initiated by endogenous gp130 (26). Deletion analysis of gp130 indicated that the membrane-proximal 133 amino acids of gp130 were sufficient to induce transcription of co-transfected chloramphenicol acetyltransferase reporter-
gene constructs through the IL-6RE and some APP gene promoters (26, 36). Within this region, a single tyrosine residue (Tyr-126) in a Box3 sequence (XYYQ) was found to be critical for the regulation. Since the transient transfection approach has its technical limitation in reproducing physiologically normal conditions, we assessed the signaling specificity conferred by the Box3 motif on the regulation of endogenous APP genes in hepatic cells, by generating lines of rat hepatoma H-35 cells stably expressing the chimeric receptor, G-gp130(133)WT, and its Box3 mutant counterpart, G-gp130(133)M3, using the retroviral vector MINV. From several clonal lines of transduced H-35 cells obtained, one representative line of each receptor type was selected. Northern blot analysis indicated equal levels of expression of the messenger RNAs encoding the expected bicistronic receptor-neo transcripts (Fig. 1A). The two G-gp130 lines also expressed comparable G-CSF binding activity on the cell surface as shown by the 125I-G-CSF binding assay (Fig. 1B). The number of binding sites in each case was estimated to be about 1,500 sites per cell. With the latter development of H-35 cell lines transduced with FLAG-tagged receptors, we could also visualize the level of receptor proteins by Western blotting (Fig. 4, bottom).

Role of Box3 in the JAK-STAT Pathway—Signaling by hematopoietin receptors is initiated by members of the JAK family (19, 46). Immunoprecipitation and Western blot analyses showed that JAK1 was induced to a comparable level by G-CSF in cells expressing either the wild-type or the M3 mutant G-gp130 receptor (Fig. 2, upper panel). TYK2 (Fig. 2, lower panel) and JAK2 (data not shown) were likewise stimulated by G-CSF to a similar extent despite a relatively higher basal level in untreated cells. Importantly, the activation of JAKs by G-CSF was similar to that induced by IL-6.

Next, we examined the effects of the Box3 mutation on the activation of STAT proteins which are recognized downstream targets of JAKs. Expression of the chimeric receptors did not noticeably alter the magnitude or the kinetics of STAT activation induced by IL-6 (Fig. 3, upper panel). A strong activation of SIE-binding activities that resolved into three bands was observed 15 min after IL-6 treatment. As shown previously, these bands represented STAT3 homodimer (top band), STAT3/STAT1 heterodimer (middle band), and STAT1 homodimer (bottom band), respectively (47). Only the STAT homodimer was maintained during longer treatment. Upon exposure to G-CSF, G-gp130(133)WT mediated a characteristic pattern of STAT activation similar to IL-6, albeit lower in magnitude, probably reflecting the effect of deleting three of the four Box3 motifs (Fig. 3, lower panel). In contrast, the Box3 mutant receptor failed to stimulate any significant amount of detectable SIE binding activity. These results indicate that although Box3 is not required for JAK activation, it is indispensable for the activation of STATs.

Cooperation of Box3-dependent and Box3-independent Pathways in the Regulation of APP Genes—STAT3 was originally purified as a protein binding to the IL-6RE of the α2-MG gene promoter (also called the acute-phase response factor (APRF)). By transient transfection, we demonstrated that STAT3 is able to transactivate certain APP gene promoters. It has also been shown that Box3 acts as a receptor-binding site for STAT3, thus implicating an essential role for Box3 in the control of APP genes. To test this functional link, we examined the effects of the Box3 mutation on the expression of the endogenous APP genes in the stable G-gp130 cell lines. Northern blot analysis of four representative APP genes, including Tst, α2M, hemopexin, and haptoglobin, showed an up-regulation of RNA levels in response to G-CSF in cells expressing G-gp130(133)WT (Fig. 4A). The M3 mutant cells were unresponsive to similar treatment, and no induction of these APPs was detected. The IL-6
response of the two different cell lines was, however, comparable.

The lower APP stimulation by G-gp130(133)WT than by endogenous IL-6R was attributed to the reduced activation of STAT3 by the chimeric receptor. To confirm in the G-CSFR-gp130 context the relevance of four versus one Box3 elements in determining quantitative level APP induction, we compared APP response in pool cultures of H-35 cells transduced with G-gp130(277) or G-gp130(133), both tagged at the C terminus with FLAG epitope. In separate experiments (not presented), we have established that the FLAG epitope has no appreciable influence on signaling gp130 cytoplasmic domains. The FLAG epitope allowed immunodetection of both receptor proteins and their ligand-induced phosphorylation (Fig. 4B). FLAG antibody reaction with G-gp130(133) yielded an approximately 2-fold higher signal than with G-gp130(277). However, the signal with antiphosphotyrosine antibody was severalfold lower with G-gp130(133), which is in agreement with the reduced number of phosphorylation sites. Treatment of these cells for 24 h with G-CSF resulted in a stimulated production of thiostatin in G-gp130(277) cells that was similar to IL-6 treatment (Fig. 4C, samples 1 and 2). In contrast, G-gp130(133)WT cells responded to G-CSF by a production of Tst that was only 15% that to IL-6 (samples 4 and 5). An equivalently low Tst induction by G-CSF was noted in cells expressing G-gp130(133) without FLAG epitope (Fig. 4A and Fig. 5B). Cells treated with the combination of G-CSF and IL-6 indicated an enhanced response (Fig. 4A).

FIG. 4. Box3-dependent regulation of APP expression. A, cells were treated with cytokines in the presence of dexamethasone for 16 h. Total RNA was prepared, and equal aliquots were analyzed using specific cDNA probes for APP genes (α-FB, α-fibrinogen; TST, thiostatin; HPX, hemopexin; and HP, haptoglobin). Equal loading is demonstrated by ethidium bromide (EtBr) staining of 28 S rRNA band. B, H-35 cells transduced with G-gp130(277)WT-FLAG or G-gp130(133)WT-FLAG were treated for 15 min and receptors immunoprecipitated (i.p.) with anti-FLAG antibodies. The proteins following separation were analyzed on Western blot (w.b.) first with anti-phosphotyrosine (PY) followed by anti-FLAG antibodies. Equal protein loading is demonstrated by the comparable staining of nonspecific protein bands. C, cells were incubated for 24 h in serum-free medium containing IL-6 or G-CSF but in the absence of dexamethasone. Some cultures received in addition 25 μM PD98059. Tst produced by each culture during the subsequent 24-h treatment period was determined by immunoelectrophoresis, normalized to equal cell number in the culture, and expressed relative to the value obtained for IL-6-treated cells in each culture series (= 100%) (mean ± S.D.; n = 3–5).

FIG. 5. Box3-independent regulation of α1-acid glycoprotein and α2-macroglobulin. A, cells were treated with indicated combinations of cytokines in the presence of dexamethasone for 48 h. Equal aliquots of total RNA were analyzed with cDNA probes for AGP or α2-MG. B, cells were treated in two time periods of 24 h each with serum-free medium containing 1 μM dexamethasone and the cytokines as indicated. The amount of α2-MG produced during the second 24-h period was determined by immunoelectrophoresis (left panel). The relative change in concentration of α2-MG and Tst was visualized by Western blotting using equal aliquots of culture medium and reaction with anti-α2-MG and Tst antibodies combined (right panel).
A stimulatory action of G-gp130(133)M3 on the expression of the four APPs in Fig. 4A was not detectable, and an inhibitory effect, if any, was not identifiable due to the low-to-non-detectable basal expression of these APPs. In testing the response of G-gp130(133)M3 cells (Fig. 4C) (or recently established H-35 cells expressing G-gp130(133)M3-FLAG (data not shown)) with the combination of IL-6 and G-CSF, the stimulatory action of IL-6 was reduced rather than enhanced (Fig. 4C, sample 9). This suggests that the signaling retained by G-gp130(133)M3 was opposing, in part, the signaling of the Box3-dependent pathway that is effective on the Tst gene.

Previously, by transient transfection of reporter gene constructs, we identified the distal response element of the rat AGP gene as a genetic target of the Box3-independent gp130 signaling pathway. We have also observed that the rat α2-MG promoter is unique among other APP gene promoters in its responsiveness to some Box3-deficient hematopoietin receptors such as IL-2R. These observations prompted us to examine the influence of the Box3 mutation on the regulation of the endogenous AGP and α2-MG genes. In G-gp130(133)WT cells, G-CSF treatment led to increased RNA expression of both AGP and α2-MG (Fig. 5A). IL-1 synergized with G-CSF in the stimulation of AGP but exerted an inhibitory effect on α2-MG expression. In G-gp130(133)M3 cells, G-CSF alone had no detectable stimulatory effect, but synergized prominently with IL-1 to enhance AGP expression. In contrast, the expression of α2-MG was stimulated by G-CSF alone. Interestingly, the G-CSF effect was not suppressed by IL-1 suggesting IL-1 action was directed toward suppressing the Box3-dependent stimulatory signal of gp130. Treatment with IL-6 yielded similar results in the two stable G-gp130 lines. IL-6 alone stimulated the expression of both AGP and α2-MG. As described previously, IL-1 synergized with IL-6 to up-regulate AGP expression but reduced the IL-6-induced expression of α2-MG. The differential regulation of α2-MG and thioctacin provided evidence that the gp130-derived signals did not act uniformly on type II APPs. The signals triggered by the combination of G-CSF and IL-6 proved to act additively on α2-MG gene in both G-gp130(133)-WT and G-gp130(133)M3 cells (Fig. 5B, left panel), whereas the expression of Tst in the latter cell line was substantially reduced below that of IL-6 alone (Fig. 5B, right panel). Collectively, these results indicated that, in the absence of STAT3 activation, the Box3-independent pathway did contribute to the control of a subset of APP genes as represented by AGP and α2-MG.

**MAPK Activity Is Prolonged in the Box3 Mutant**—We then sought to identify the downstream signaling molecules in the Box3-independent pathway which seem to exert both a stimulatory (α2-MG) and an inhibitory (Tst) effect. G-CSF treatment resulted in the phosphorylation of SHP-2 to similar levels in the two stable G-gp130 cell lines, regardless of the Box3 mutation (Fig. 6, upper panel). Unlike gp130, we did not observe association of SHP-2 with the chimeric receptors, possibly due to reduced affinity of SHP-2 for the chimeric receptor as a result of the cytoplasmic truncation. MAPK has been shown to be the downstream effector molecule of SHP-2 and thus was expected to be activated in a similar fashion. As predicted, G-CSF induced ERK1/2 phosphorylation to a similar level as IL-6 did (Fig. 6, upper panel). No significant difference in the magnitude and duration of ERK1/2 activation through the chimeric receptors between the two G-gp130 cell lines was observed.

In hepatic cells, IL-6 regulates the expression of a set of early response genes including egr-1 and junB, which are the probable targets of MAPK-responsive transcription factors. Therefore, we investigated the regulation of these genes by the chimeric receptors. Northern blot analysis showed that the induction of egr-1 and junB mRNAs after 30 min of G-CSF treatment was not detectably affected by the Box3 mutation (Fig. 6, lower panel). The magnitudes of the responses were comparable between cells expressing the two different chimeric receptors. Unexpectedly, however, time course analysis revealed that G-CSF induced an extended period of MAPK activity in cells expressing the Box3 mutant gp130 (Fig. 7A). While MAPK activity (like phosphorylated STAT3) declined after 10–20 min treatment and reached essentially basal level after 45 min in cells with G-gp130(133)WT, an elevated level of MAPK activity was still maintained in M3 cells after 1 h. Concomitantly, in parallel to the MAPK activity, RNA level of egr-1 was increased for an extended period in M3 cell (Fig. 7B).

**Box3 Mediates a Growth Inhibitory Signal in H-35 Cells**—IL-6 is known to be an important growth factor for cells such as melanoma and plasmacytoma (48, 49). On the other hand, in a few cell lines, for example myeloid leukemic M1 cells, IL-6 exhibits a growth inhibitory effect. These observations suggest that the effects of IL-6 on cell proliferation are determined by cellular context. We examined the influence of IL-6 on the growth of hepatic cells, and we tested whether the growth response would be affected by the Box3 mutation. Thymidine incorporation assays showed that IL-6 inhibited proliferation of H-35 cells (Fig. 8A). The results of G-CSF treatment demonstrated that the membrane-proximal 133 amino acids were sufficient to confer, at least in part, the inhibitory response. The Box3 mutation completely abolished the negative effect of gp130 on cell growth. Cell counts from long term cultures indicated reduced numbers of G-gp130(133)WT cells following

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2 J. Ripperger and G. H. Fey, unpublished observations.
G-CSF treatment but no observable growth inhibition by G-CSF on G-gp130(133)M3 cells (Fig. 8B). Box3-lacking gp130 Induces Distinct Morphologic Changes—MAPK has been described to associate with microtubules and to participate in the control of the cytoskeletal structure (50, 51). As shown in Fig. 9A, treatment of M3 cells (panel 11), but not WT cells (panel 5), for 24 h with G-CSF resulted in distinct changes in cell morphology. The compact single cell monolayers (panels 4 and 10) tended to desegregate, and the cells developed a rounded shape. Notable, the G-CSF-induced changes became apparent only when the cells were maintained in the presence of dexamethasone (compare panels 8 and 11). No significant changes were observed with IL-6 treatment (panels 6 and 12).

To determine whether enhanced ERK activity or loss of STAT3 activation was responsible for the M3 culture phenotype, the cells were treated with G-CSF and dexamethasone either in the presence of the MEK-1 inhibitor PD98059 (to reduce or inhibit ERK activation) or in the presence of IL-6 (to co-activate STAT3) (Fig. 9B). Although we used a concentration of PD98059 that effectively suppressed ERK activation by G-CSF and IL-6 (data not shown), the G-CSF-dependent morphologic change of the M3 cell culture was maintained. In contrast, IL-6 treatment significantly suppressed the G-CSF effect, suggesting that loss of STAT3 rather than the enhanced ERK activation was critical in gaining the M3 phenotype. Analyses of APPs released by M3 cells treated with G-CSF and dexamethasone either in the presence of the MEK-1 inhibitor PD98059 (to reduce or inhibit ERK activation) or in the presence of IL-6 (data not shown), the G-CSF-dependent morphologic change of the M3 cell culture was maintained. In contrast, IL-6 treatment significantly suppressed the G-CSF effect, suggesting that loss of STAT3 rather than the enhanced ERK activation was critical in gaining the M3 phenotype. Analyses of APPs released by M3 cells treated with G-CSF and dexamethasone in the presence of PD98059 revealed that the inhibitory signal of G-gp130(133)M3 on IL-6-stimulated Tst expression was relieved (Fig. 4C, samples 11 and 13). The PD98059-sensitive effect on Tst was similarly observed in the presence of dexamethasone (data not shown), indicating that the ERK-dependent inhibitory process was not influenced by steroid treatment.

**DISCUSSION**

Our earlier investigations focusing on the transcriptional control through isolated APP gene promoter elements have demonstrated that IL-6-type cytokine receptors engage separate signal-transducing pathways and that the Box3-dependent pathway is responsible for regulation of the IL-6RE in the APP gene promoters (36). Together with the observations that the Box3 motif of gp130 is a binding site for STAT3 and that STAT3 exists in protein complexes associated with the IL-6RE, it seems reasonable to assume that STAT3 is the principal regulatory factor controlling induction of those APP genes that contain IL-6REs (17, 18, 33, 52). Data from this report are consistent with this notion, but they also suggest that the
Box3-independent signal appreciably contributes to the regulatory process. Our major findings on the relationship between Box3 and APP gene regulation are as follows: 1) the Box3 motif in gp130 is necessary for maximal expression of most, if not all, APP genes, and this is tightly linked to the activation of STAT3; and 2) APP gene exhibits varying degrees of dependence on the Box3 signal. Some genes including Tst, Fb, and hemopexin have an obligate requirement for STAT3. In contrast, α2-MG expression can be regulated, at least partially, in the absence of detectable active STAT3. 3) As highlighted by our analysis of AGP gene expression, IL-1 exerts a considerable synergistic effect with the gp130 signal independent of Box3; and 4) with removal of a Box3 motif, gp130 signaling toward the ERK pathway is enhanced that, in part, attenuates the effect of the Box3-dependent pathway as observed for Tst expression.

The Box3-derived signal, as manifested by and often equated to STAT3 activation, appears to be critical for the immediate induction of APP genes. Although loss of the Box3 signal eliminated the capability of gp130 to mediate immediate induction of APP genes, during long term treatment (hours to days), the Box3-independent signal seems to be capable of inducing relevant levels of expression of genes like α2-MG. The factors responding to the Box3-independent signal are still poorly understood. Since MAPK activation, in particular that of ERKs, is largely unaffected by the Box3 mutation, the downstream targets of ERKs are appealing candidates for effectors of APP gene regulation in the absence of the Box3 motif. MAPK is known to phosphorylate and modulate the activities of transcription factors such as STAT3, C/EBPβ, CREB, SRF, AP-1, ATF-2, and Elk-1 (35, 53, 54). It is conceivable that the factors other than STATs also act directly on the APP promoters and may explain the modulatory effect attributed to growth factors that engage ERKs (55, 56). However, at present, there is no evidence that any of these non-STAT factors account significantly for IL-6-enhanced expression of type II APPs (57, 58). On the other hand, the transcription factors, whose synthesis is regulated by the Box3-independent signal (Fig. 6), may also contribute indirectly to the APP gene regulation in the later period, in part, through stimulating proliferative activity of the cells. Increased proliferation of the hepatic cells has been noted to lower cytokine regulation of APP genes (56, 59).

The structure/function relationship of gp130 has been assessed in other experimental cell systems including the murine pro-B cell line BAF-B03 (60), the myeloid leukemic cell line M1 (61), and the rat pheochromocytoma cell line PC12 (62). IL-6 displays distinct biological activities in these cells. In BAF/B03 cells, gp130 mediates a proliferative signal stimulating cell growth. Conversely, IL-6 inhibits the growth of M1 and PC12 cells and promotes their differentiation. In response to IL-6, M1 cells are arrested at G0/G1 phase and express macrophage-like morphologic changes and inducible nitric oxide synthase, whereas PDC12 cells develop neurite outgrowth when pre-treated with nerve growth factor. Here we observed that besides the induction of APP genes, IL-6 causes growth inhibition of H-35 cells. This biological response of H-35 cells has not been previously reported. For all of the IL-6 effects described above, the minimal receptor domain required was mapped to the membrane-proximal 133 amino acids in the cytoplasmic region of gp130, in which a single Box3 motif was found to be necessary for generating an effective signal. Inactivation of all functional Box3 sequences, as in the case of G-gp130(133)M3, abol-
ished all of the above biologic activities, except for BAF/B03 cells in which proliferation can be maintained if the cells do not undergo IL-3 starvation. Since in each of these cell systems the mutation of Box3 parallels the loss of STAT3 activity, a pivotal role of STAT3 in regulating diverse cellular functions in different cellular contexts is strongly suggested.

Although IL-6 through wild-type gp130 induces growth inhibition of both M1 and H-35 cells, comparison of the expression profiles of the growth-related genes reveals some major differences. For example, egr-1 and junB are stimulated rapidly in H-35 cells, with a peak at 30 min and returning to basal level by 1 h. In contrast, elevated levels of both mRNAs are maintained for at least 24 h in M1 cells. These observations suggest that the regulation of growth inhibition may be different in M1 cells and H-35 cells. Therefore, H-35 cells can serve as an alternative model for the study of IL-6-mediated growth control.

Inasmuch as cytokine receptors with a single Box3 motif are known, such as those for G-CSF, leptin, and thrombopoietin, receptors containing multiple copies of Box3 seem to be more common. In addition to gp130, the receptors for OSM and IL-10 contain two copies, whereas that for LIF contains three copies of the Box3 motif (26–29, 63). The reason for the presence of multiple copies of the Box3 is not well understood. In the H-35-derived cell line, G-gp130(133)WT, the level of APP gene expression induced by G-CSF is consistently lower than that elicited by G-gp130(277)WT or IL-6 (Fig. 4C). This correlated with the level of STAT3 activation, which is also lower following G-CSF treatment (Fig. 3). It is not likely due to a lower level of expression of the chimeric receptor (Fig. 4B), since the activation levels of JAKs and MAPK stimulated by G-CSF is equal to, if not better than, those induced by IL-6. Thus, we argue that the reduced G-CSF response may be related to the fact that there is only a single Box3 in the chimeric receptor, compared with four copies in the full-length gp130. Conceivably, the presence of multiple Box3 sequences might confer a “dose effect” on the signaling capacity of the receptor affecting some biologic activities. Of course, we cannot exclude the possibility that signaling molecules other than STAT3 might also bind to Box3, and different Box3 motifs may have discrete preferred substrates. However, to date, no other Box3-interacting proteins have been reported.

One of the intriguing findings in our study is that inactivation of the Box3 motif leads to prolonged MAPK activation. This observation has not been reported in similar studies with other cell systems. It has been described that SHP-2 acts upstream of the Ras-Raf-MAPK signaling cascade and that Tyr-118 in the cytoplasmic domain of gp130 is required for the association of SHP-2 to the receptor as well as for the activation of SHP-2 (41, 60). Mutation of the SHP-2 site abrogates the activation of MAPK (60). Notably, the SHP-2-binding site is in close proximity to Tyr-126 of the Box3 motif. We hypothesize that binding of STAT3 to the first Box3 motif would impede SHP-2 association to gp130 by physical exclusion. Thus, tyrosine mutation of Box3 provides an unobstructed access of SHP-2 to gp130 leading to prolonged MAPK activity. If so, it is reasonable to assume that this interference between STAT3 and SHP-2 would not occur with Box3 motifs located more distantly on gp130. This also implies that the multiple copies of Box3 may not be functionally identical. The first Box3 could exert a specific inhibitory effect on SHP-2, thereby regulating the duration of MAPK activation. It is known that the duration of MAPK activation has a determining effect on phenotypic outcome. For example, in PC12 cells, epidermal growth factor stimulates a transient activation of MAPK promoting cell growth. However, sustained MAPK activation by nerve growth factor causes neuronal differentiation (62). Similarly, in F-36P erythroleukemia cells, transient MAPK activity by IL-3 favors cell proliferation, whereas more robust MAPK activation by thrombopoietin elicits megakaryocytic differentiation (64). We observed that the Box3 mutation resulted in prolonged ERK activity, sustained Egr-1 expression, and morphological changes in H-35 cells. Evidently, two processes govern gp130 signal specificity, one determines the activation of specific signaling molecules (qualitative aspect) and the other monitors the length of their activation time (quantitative aspect).

Finally, although STAT3 has been established to be a critical transcription factor for APP gene induction in gp130 signaling, other factors appear to be engaged which contribute, both positively and negatively, to APP gene regulation. The modulatory actions of these factors may occur at several levels, perhaps affecting concentration or activity of the IL-6 receptor subunits and its signal-transducing mechanisms, or modifying transcriptional and post-transcriptional events underlying APP gene expression (65). By eliminating Box3, we remove the STAT3 pathway from gp130 signal transduction and enhance transcriptional responses transmitted by the Box3-independent pathway. Based on the recent observations that prevention of SHP-2 recruitment to gp130 essentially removes the MAPK pathway and simultaneously enhances the effect of the STAT3-dependent pathway on APP gene expression, a significant cross-modulation between the two pathways seems to exist (41). Currently, work is underway to identify effects in addition to the MAPKs and the downstream targets involved in the Box3-independent signaling pathway of gp130 and to understand how these factors cooperate with STAT3 to execute proper control of APP gene expression.

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