Transmembrane Domain of gp130 Contributes to Intracellular Signal Transduction in Hepatic Cells*

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Interleukin-6 (IL-6) induces the expression of acute phase plasma protein genes in hepatic cells through the action of gp130, the signal-transducing subunit of the IL-6 receptor. To identify whether the transmembrane domain of gp130 is required for signaling function, cytoplasmic forms of gp130 were constructed that consisted of the tetramerizing N-terminal domain of Bcr linked to the transmembrane and cytoplasmic domains of gp130 (Bcr/gp130) or just to the cytoplasmic domain of gp130 (Bcr/gp130ΔTM). The expression and function of both constructs were determined in transiently transfected COS-1 and HepG2 cells. Bcr/gp130 is capable of interacting with JAK1, JAK2, and TYK2; is constitutively active; and induces gene expression through IL-6-responsive elements. In contrast, Bcr/gp130ΔTM, while expressed at a higher level than Bcr/gp130 and still able to interact with JAK1, is ineffective in recruiting the endogenous signal transduction pathways for inducing gene expression. However, Bcr/gp130ΔTM initiates partial signaling in the presence of overexpressed JAK1 and TYK2, but not JAK2. The data suggest that the transmembrane domain of gp130 is necessary for signal transduction and determines the interaction with members of the Janus kinase family.

Structure/function analyses of gp130, the common signal-transducing receptor subunit of IL-6-type cytokines, have identified subregions in the intracellular domain that are required for signal transduction (1, 2). Box 1 and Box 2 elements determine the association and activation of members of the JAK (Janus kinase) family. Studies on cell lines deficient in specific JAK isoforms suggested that, upon IL-6 binding and receptor subunit oligomerization including dimerization of gp130 (3, 4), the signaling process is initiated by JAK1 and is fully executed by JAK2 and TYK2 (5). Four Box 3 sequence motifs within the cytoplasmic gp130 domain provide tyrosine phosphorylation sites that serve as a docking element for STAT (signal transducer and activator of transcription) and STAT3 (6–8). The STAT proteins become phosphorylated, likely by the gp130-associated JAK proteins; dimerize with each other; and display DNA-binding activity. Following nuclear translocation, the STAT complexes presumably bind to regulatory elements of IL-6-responsive genes and contribute to the induction of transcription (9–13).

The gp130-specific signaling process is reproducible with chimeric constructs in which the intracellular and transmembrane domains of gp130 have been recombined with subunits of other hematopoietin receptors. This suggests that signaling is primarily controlled by the juxtamembrane and distal cytoplasmic domain structures (9, 14). The transmembrane domain generally is assumed to serve as a membrane anchor for the receptor subunits, but otherwise may not contribute to receptor signaling. To assess whether the transmembrane domain per se is not required for signal transduction, we generated cytoplasmically localized, signal-transducing gp130 molecules. Here we report the application of fusion proteins between the tetramerizing N-terminal peptide of Bcr (15) and the cytoplasmic domain of gp130 and document the relevance of the transmembrane domain for engaging JAK isoforms and for gene induction.

MATERIALS AND METHODS

DNA Constructs—The chimeric gp130 constructs are depicted in Fig. 1A. To generate Bcr/gp130, the EcoRI-NotI fragment of pDC-G-CSFR-gp130 (14), encoding residues 561–874 of gp130, was linked to the 3′-end of the EcoRI-BalI fragment of pCD-p190Bcr/Abl (16), encoding the tetramerizing first exon sequence of Bcr (residues 1–66). The same gp130 fragment was also ligated to the 3′-end of the 152-base pair upstream segment of the rat STAT1 cDNA (18) in frame with the initiation methionine codon, yielding gp130cyto. Bcr/gp130ΔTM was constructed as Bcr/gp130, except that the gp130 segment from residues 594 to 874, encoding the two last residues of the transmembrane domain and the entire cytoplasmic domain, was generated by polymerase chain reaction. FLAG epitope-tagged constructs were produced by adding an oligonucleotide encoding the FLAG epitope (DYKDDDDK) to the carboxyl terminus of gp130. All fusion constructs were inserted into the pCD expression vector (17). The p190Bcr/Abl cDNA was inserted as an EcoRI fragment into pSV-Sport1. Previously described were the expression vectors for human gp130 (18); rat STAT1, STAT3, and STAT5 (9, 12, 19); and STAT3Δ55C (20); Prk5-JAK1 (21); pEFBos-JAK2 (22); pDC-TYK2 (9); and pHIX5xIL-6RE-CAT, containing five tandem copies of the IL-6-responsive element of the rat hemopexin gene in pCAT (23) and the internal transfection marker, pIE-MUP (14).

Cells and Transfection—COS-1 and HepG2 cells were maintained as described (9, 24). COS-1 cells were transfected with 5 μg of DNA/ml by the DEAE-dextran method (25), and HepG2 cells with 20–23 μg of DNA/ml by the calcium phosphate method (26). For EMSA and Western blot analysis, cells were cultured for 8 h in serum-free medium and then treated for 5 min to 24 h with 100 ng/ml of G-CSF (Immunex Corp.). For CAT gene regulation, the cytokine treatment lasted 24 h. To determine CAT activity within the linear range of the enzyme reaction, aliquots of serially diluted cell extracts were used. The values were normalized to the cotransfected marker, MUP, and calculated relative to the control-treated cell cultures in each experimental series. The means ± S.D. of at least three independently performed transfection experiments are shown.

EMSA, Immunoprecipitations, and Immunofluorescence—Whole cell lysates were prepared as described previously (27), and the DNA-binding activity was analyzed by EMSA using 32P-labeled double-stranded m67SIE for STAT1 and STAT3 (27) and TB2 for STAT5 (9, 28). For immunoprecipitation, transfected cells were lysed in 1% Non-

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1 The abbreviations used are: IL-6, interleukin-6; IL-6R, interleukin-6 receptor; IL-6RE, interleukin-6-responsive element; TM, transmembrane; EMSA, electrophoretic mobility shift assay; G-CSF, granulocyte colony-stimulating factor; G-CSFR, granulocyte colony-stimulating factor receptor; CAT, chloramphenicol acetyltransferase.
idet P-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM EGTA. The cleared cell extracts were reacted with monoclonal antibody against Abl (Pharmingen) or against FLAG (M2; Eastman Kodak Co.). Immune complexes were collected by binding to protein G-Sepharose (Pharmacia Biotech Inc.) and analyzed by either one- or two-dimensional polyacrylamide gel electrophoresis (Bio-Rad). Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) and reacted, depending upon the experimental settings, with anti-phosphotyrosine (PY20), anti-JAK1, or anti-TYK2 antibody from Transduction Laboratories; anti-phosphotyrosine STAT3 antibody from New England Biolabs Inc.; or anti-STAT3 (C-20), anti-JAK2 (C-20), anti-SHP-2 (C-19), anti-FLAG, or anti-Bcr (N-14) antibody from Santa Cruz Biotechnology Inc. The immune complexes were visualized by the enhanced chemiluminescence reaction (Amersham Corp.). For immunofluorescent detection of anti-Bcr antibody-reactive proteins, transfected cells were cultured on coverslips, fixed with cold methanol, and reacted with anti-Bcr antibody followed by fluorescein-conjugated rabbit anti-mouse immunoglobulin. Cells were photographed on a Zeiss fluorescence microscope.

Column Fractionation—COS-1 cells, transfected with Bcr/gp130-FLAG or Bcr/gp130ΔTM-FLAG, were lysed (5 × 10⁷ cells/ml) in the same buffer as used for immunoprecipitation. After centrifugation at 100,000 × g for 1 h, 200 μl of the supernatant fraction were applied onto a Sephacryl S-300 column (6 × 300 mm; Pharmacia Biotech Inc.) and chromatographed in lysis buffer at a flow rate of 1.7 ml/h. The eluate was collected in 280-μl fractions. Aliquots (30 μl) from alternate fractions were analyzed for anti-FLAG antibody-reactive proteins by Western blotting. The column was calibrated with blue dextran (exclusion volume indicator), dimeric and monomeric bovine serum albumin, and ovalbumin.

RESULTS AND DISCUSSION

Expression of Bcr/gp130—To determine the role of the transmembrane domain in gp130 signaling, we designed two cytoplasmic fusion proteins (Bcr/gp130 and Bcr/gp130ΔTM) that differ from each other by the presence of the transmembrane domain (Fig. 1A). FLAG epitope-tagged versions were also prepared to facilitate immunodetection and immunoprecipitation. The proteins were predicted to tetramerize through the N-terminal Bcr peptide (15), thereby bringing cytoplasmic gp130 domains into close proximity of each other. This complex formation is similar to what is assumed to occur in ligand-activated G-CSFR-gp130 (14) or IL-6R (1–4).

Within the range of experimental variation, transient transfection of the Bcr/gp130 constructs into HepG2 and COS-1 cells...
indicated comparable expression of the respective mRNAs (Fig. 1B). The synthesis of the proteins with the expected sizes of 42,500 Da for Bcr/gp130 and 39,000 Da for Bcr/gp130ΔTM was detected (Fig. 1C). One or two additional, smaller sized proteins were also visible in Bcr/gp130ΔTM-transfected cells that may represent proteolytic degradation products. Although expression of the Bcr/gp130 proteins was somewhat variable among individual transfection experiments in both cell types, the immunodetectable level of Bcr/gp130ΔTM (with or without FLAG) was consistently severalfold higher than that of Bcr/gp130. Transfection of gp130cyto-FLAG, which lacks the N-terminal Bcr extension, yielded undetectable to trace amounts of accumulated protein (Fig. 1C), despite a mRNA level (Fig. 1B) and protein synthesis (data not shown) that were equivalent to the Bcr-modified constructs. This suggests that the cytoplasmic gp130 protein is rapidly turned over and that the Bcr domain enhances the stability of the fusion protein. Immunocytochemical staining demonstrated the predominant cytoplasmic localization of the Bcr/gp130 proteins (Fig. 1D).

Oligomerization of Bcr/gp130 proteins was determined by size fractionation (Fig. 2A) and by co-immunoprecipitation with p190Bcr/Abl (Fig. 2B). Gel filtration of extracts from COS-1 cells expressing Bcr/gp130-FLAG or Bcr/gp130ΔTM-FLAG (Fig. 2A) revealed that the major fractions of the fusion proteins eluted with apparent sizes of 160 and 140 kDa, respectively, in agreement with the sizes expected for tetrameric complexes. Identical results were obtained with the Bcr/gp130 constructs without the FLAG epitope (data not shown). The Bcr-specific interaction was identified by the association of Bcr/gp130 with coexpressed p190Bcr/Abl and co-immunoprecipitation with Abl antibody (Fig. 2B).

Interaction of Bcr/gp130 with JAK Proteins—The tyrosine phosphorylation of the gp130 cytoplasmic domain is an indicator for the immediate action of gp130-containing receptor complexes (6–8). However, this phosphorylation is reported to be transient. Nevertheless, we expected that signaling-competent Bcr/gp130 proteins might show detectable tyrosine phosphorylation. We immunoprecipitated Bcr/gp130-FLAG from the cell extract, but failed to detect a reaction with anti-phosphoryso-rosine antibodies on two-dimensional immunoblots (Fig. 3A). In the presence of overexpressed JAK1, however, a fraction of Bcr/gp130 reacted with anti-phosphotyrosine antibodies, demonstrating that Bcr/gp130 can serve as substrate for this kinase.

To determine the influence of the transmembrane domain on phosphorylation by JAK isoforms, FLAG-tagged Bcr/gp130 and Bcr/gp130ΔTM were tested under comparable transfection conditions in HepG2 cells in the presence of increasing doses of JAK1, JAK2, or TYK2 (Fig. 3B). Despite the low expression of Bcr/gp130, its phosphorylation by each kinase was apparent. The action of JAK1 appeared to be most prominent, followed by that of TYK2 and JAK2. In contrast, the relatively abundant Bcr/gp130ΔTM construct showed a much lower phosphorylation in the presence of TYK2 and only a trace in the presence of JAK2. Phosphorylation of Bcr/gp130ΔTM by JAK2 could only be observed clearly in cells that expressed the kinase several-fold above that seen in Fig. 3B (data not shown).

The results in Fig. 3 (A and B) illustrate that both Bcr/gp130 constructs are accessible to overexpressed JAK proteins. Since, however, signaling of the normal gp130 protein is believed to be mediated by JAK proteins physically associated with the intracellular domain of gp130 (6), we needed to demonstrate that such an interaction of JAK proteins with the cytoplasmic Bcr/gp130 construct was possible. To do so, we overexpressed in COS-1 cells FLAG-tagged Bcr/gp130 or Bcr/gp130ΔTM in the presence of JAK1, JAK2, or TYK2 (Fig. 3C). The Bcr/gp130 proteins were then immunoprecipitated and analyzed by immunoblotting for coprecipitated JAK proteins. Moreover, the kinase-mediated phosphorylation of tyrosine 759 of gp130, which serves as a binding site for the cytoplasmic protein-tyrosine phosphatase SHP-2 (see Fig. 1A and Ref. 6), should be recognized by detection of COS-1 cell-derived SHP-2 in the immunoprecipitates. As shown in Fig. 3C, a prominent association of JAK1 and TYK2 with Bcr/gp130 was seen. Surprisingly, repeated experiments yielded only a minor signal for JAK2. Nevertheless, each of the kinases promoted recovery of SHP-2 with Bcr/gp130 that was significantly above the association seen in the cells not transfected with JAK proteins. An equivalent series of transfection experiments carried out with Bcr/gp130ΔTM showed that only JAK1 was detectably associated with the protein (Fig. 3C). The ratios of JAK1 to FLAG signal seen with the Bcr/gp130 and Bcr/gp130ΔTM complexes also indicated that the latter construct was less effective in retaining the kinase. The restricted interaction of the kinases with Bcr/gp130ΔTM was similarly reflected in the detection of co-immunoprecipitated SHP-2.

Involvement of STAT Proteins—Since activation of DNA binding by STAT1, STAT3, and, to a lesser extent, STAT5 is characteristic for gp130-mediated signaling and is particularly prominent after treatment of cells for a few minutes with IL-6-type cytokines (1, 9, 19), we determined whether Bcr/gp130 expression had similar effects on STAT proteins in
Role of the Transmembrane Domain

FIG. 3. Association of Bcr/gp130 with JAK proteins. A, extracts of HepG2 cells transfected with Bcr/gp130-FLAG (15 μg/ml) and JAK1 (5 μg/ml) as indicated were immunoprecipitated with anti-FLAG antibody and separated on two-dimensional polyacrylamide gel. The proteins transferred to membranes were reacted first with anti-phosphotyrosine antibody (anti-PY) and, following stripping of the membrane, with anti-FLAG antibody. Only the gel area of Bcr/gp130-FLAG proteins is reproduced. B, HepG2 cells were transfected with Bcr/gp130-FLAG or Bcr/gp130ΔTM-FLAG (15 μg/ml) together with increasing doses of JAK proteins (0, 0.2, 1, and 5 μg/ml) as indicated. Equal aliquots of total cell lysates were separated in two sets by one-dimensional polyacrylamide gel electrophoresis. After electrotransfer of the proteins, the membrane from one set of samples was reacted with anti-FLAG antibodies (left panel). The membrane from the other set was divided into two sections: the sections below the 80-kDa marker were reacted with anti-phosphotyrosine antibody (note the endogenous phosphotyrosine protein at 60 kDa) and, following stripping of the membrane, were reacted with anti-phosphotyrosine antibody (note the endogenous phosphotyrosine protein at ~60 kDa and phosphotyrosine (PY)-Bcr/gp130 below), and the sections above the 80-kDa marker were reacted with the indicated anti-kinase antibodies. C, COS-1 cells were transfected with Bcr/gp130-FLAG or Bcr/gp130ΔTM-FLAG (3 μg/ml) in combination with expression vectors for the JAK proteins (2 μg/ml) and JAK1 and TYK2 gave similar results. Although JAK2, even at a relatively low dose and in the absence of Bcr/gp130, activated coexpressed STAT1 and STAT3 (lanes 6 and 7), its action was enhanced in the presence of Bcr/gp130 (lanes 9 and 10). Most prominent was the combined action of Bcr/gp130 and JAK2 on STAT5B (lane 15). None of the JAK effects approached that of G-CSFR-gp130, which activated, within 15 min of G-CSF treatment, endogenous STAT1 and STAT3 or cotransfected STAT proteins (lanes 11–13 and 18). For proper comparison, however, we needed to consider that if Bcr/gp130 functioned as a constitutively active factor, it should produce an effect on STAT proteins that was more similar to that elicited by continuous treatment of cells with cytokines engaging gp130. Indeed, HepG2 cells, when transfected with G-CSFR-gp130 and treated with G-CSF for 24 h, showed a DNA-binding activity of STAT3 that was slightly above control levels and was comparable to that of Bcr/gp130-transfected cells (Fig. 4B, upper panel). Furthermore, a minor elevated tyrosine phosphorylation of endogenous STAT3 was detectable in both transfected cell types (Fig. 4B, lower panel).

Bcr/gp130 Induces Gene Expression—The gene-inducing action of the Bcr/gp130 constructs was determined by transfecting HepG2 cells with increasing amounts of expression vector for Bcr/gp130 together with the IL-6RE-containing CAT reporter gene construct (Fig. 5A). Both Bcr/gp130 and Bcr/gp130-FLAG mediated a dose-dependent increase in CAT activity and were, at 5 μg/ml, almost as effective as the ligand-induced response of endogenous IL-6R or transfected G-CSFR-gp130. This result also showed that the addition of the FLAG epitope just carboxyl-terminal to Box 3d only slightly reduced the signaling function of Bcr/gp130. HepG2 cells, which were similarly transfected with G-CSFR-gp130 or with full-length human gp130, but not subjected to any cytokine treatment, did not produce an increase in CAT reporter gene expression (Fig. 5A). This demonstrated that simply overexpressing gp130 with the transmembrane domain, but present in presumably monomeric form, was not sufficient for gene induction. The engagement of STAT3 by Bcr/gp130 for signaling was apparent by the modestly enhanced gene induction with overexpressed STAT3 and by the drastic reduction in the presence of coexpressed dominant-negative STAT3Δ55C (Fig. 5B). In contrast, Bcr/gp130ΔTM was inactive in gene induction (Fig. 5A), even at the highest concentrations tested. Moreover, over-
expressed STAT3 was also unable to restore signaling leading to gene induction (Fig. 5B).

The effect of Bcr/gp130 was lower than that of ligand-activated G-CSFR-gp130 (Fig. 5A), which may, in part, be due to the limited access of Bcr/gp130 to the signaling molecules, i.e., JAK proteins. Therefore, we co-introduced JAK expression vectors at a dose that alone was essentially ineffective in inducing the IL-6RE reporter gene (Fig. 5C). As shown previously (19), at higher concentrations, overexpressed JAK proteins are capable of inducing cytokine-responsive gene constructs independently of the action of IL-6-type cytokine receptors. Combinations of Bcr/gp130 with JAK1, JAK2, or TYK2 increased 2–4-fold the gene-inducing activity of submaximal doses of Bcr/gp130 (Fig. 5C). Interestingly, JAK1 and TYK2, but not JAK2, also cooperated with Bcr/gp130ΔTM and produced a 5-fold elevated CAT expression. This gene induction by Bcr/gp130ΔTM also demonstrated that the deletion of the transmembrane domains did not generate a strictly inactive protein. Taken together, these results suggest that the transmembrane domain of gp130 is necessary for the assembly of a signaling-competent complex of the gp130 cytoplasmic domains. Moreover, this region appears to determine, in part, the interaction with and/or the activation of JAK isoforms. The JAK-specific gene induction mediated by the Bcr/gp130 constructs followed qualitatively the pattern of phosphorylation of the Bcr/gp130 proteins by the JAK proteins as noted in Fig. 3B. The results obtained with Bcr/gp130ΔTM and shown in Figs. 3C and 5C also suggest that TYK2, unlike JAK2, may mediate gene induction without being physically associated with gp130.

The Bcr/gp130 construct is a constitutively active cytoplasmic protein that shows IL-6R signaling capability. As such, the chimeric protein might also act as an oncogenic factor in cells that respond to IL-6 and related cytokines by enhanced proliferation (1). However, our attempts to introduce an IL-3-independent growth of Ba/F3 cells by stable expression of Bcr/gp130 were negative.2 One possibility is that, due to the cytoplasmic localization, Bcr/gp130 does not have adequate access to the signal transduction pathways that require action at the plasma membrane site (1, 2). This may also explain the less effective gene induction by Bcr/gp130 relative to G-CSFR-gp130. Yet, the chimeric Bcr/gp130 construct is sufficiently active to induce gene expression with the specificity of Box 3-containing hematopoietin receptors (9, 14, 24). This particular feature has provided us with an experimental tool to determine a functional role for the transmembrane domains. Considering that the receptors for IL-6-type cytokines are predicted to act as oligomeric complexes in which the transmembrane domains of at least two signal-transducing subunits are involved, a functional contribution may be exerted by each of the transmembrane domains. It is also conceivable that the transmembrane domains of IL-6Ra or IL-11R similarly contribute to signaling. Soluble IL-6Ra or IL-11R, lacking the transmembrane and intracellular domains, mediates ligand-dependent signaling through binding to membrane gp130 (29–31). Therefore, no specific functional role has been attributed to the deleted receptor domains. However, comparative studies have indicated a much lower signaling efficiency through the soluble receptors...

2 H. Kim and H. Baumann, unpublished data.
than through the membrane receptors (31, 32), suggesting a supportive function for the transmembrane/cytoplasmic domain of the ligand-binding subunits. Given that the transmembrane domain assumes an α-helical structure (33), we assume that the transmembrane domain may be necessary for aligning or transmitting conformational changes to the cytoplasmic regions of gp130, thereby facilitating the binding or functional recruitment of signaling intermediates like JAK or STAT proteins.

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