gp130 Transducing Receptor Cross-linking Is Sufficient to Induce Interleukin-6 Type Responses

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gp130 transducing receptor is involved in the formation of high affinity receptors for the cytokines of the interleukin-6 (IL-6) family. Recruitment of gp130 by IL-6 associated to its receptor leads to the dimerization of the transducing component. In the present study we did characterize the B-S12 monoclonal antibody raised against gp130 and able to elicit IL-6 type biological activities. B-S12 antibody triggered strongly the proliferation of TF1 and XG1 hematopoietic cell lines and was able to increase the synthesis of acute phase proteins in HepG2 hepatoma cell line. B-S12 also behaved as a synergistic factor with granulocyte-macrophage colony-stimulating factor for both proliferation and differentiation of CD34-positive hematopoietic cell progenitors. By using a symmetric enzyme-linked immunosorbent assay, allowing the detection of dimeric forms of soluble gp130, we found that addition of B-S12 to gp130 led to its dimerization. Analysis of the tyrosine phosphorylation events in gp130 and Jak kinase family members revealed that B-S12 quickly induced the phosphorylation of gp130 in a neural derived cell line, and that Jak1 and Jak2 were also recruited. In conclusion, we show that gp130 cross-linking with the B-S12 monoclonal antibody was sufficient to generate functional IL-6 type responses in hematopoietic, neural, and hepatic cells.

gp130 transducing receptor is involved in the formation of high affinity receptors for the cytokines of the interleukin-6 (IL-6) family. Recruitment of gp130 by IL-6 associated to its receptor leads to the dimerization of the transducing subunit referred to as gp130/gp190 (33, 36). In the present study we have analyzed the agonistic properties of the B-S12 antibody in its multimeric receptor (22), which can be produced either as a transmembrane protein or as a truncated soluble product (23). Both forms of IL-6 receptor can associate IL-6 to induce gp130 dimerization and downstream signaling events (1, 24). More recently, an IL-11 binding chain was isolated and a similar activation process for gp130 is suspected (25, 26). Regarding to the receptors for LIF, OSM, and CT-1, gp130 will associate to a second transducing subunit referred to as gp190/LIF receptor (14, 27, 28). For CNTF a third additional component or a CNTF receptor was identified and is required to reinforce the interaction of CNTF with the transducing receptor complex composed of gp130 and gp190/LIF receptor (29, 30).

Dimerization of the transducing subunits initiates intracellular signaling by activating members of a family of cytoplasmic receptor-associated tyrosine kinases, referred to as Jak (Janus kinase) (for review, see Ref. 31). Both gp130 and gp190/LIF receptor can associate Jak1, Jak2, and Tyk2 (32). The information is next relayed by a family of transcription factors known as STATs (signal transducers and activators of transcription), which are activated in the cytoplasm before translocation to the nucleus (31). IL-6-related cytokines will preferentially activate STAT1 and STAT3 (33–35).

Induction of gp130 dimerization or its heterodimerization with LIF receptor was well analyzed in the case of IL-6 and CNTF/LIF receptors, respectively (24, 30). Studies regarding the downstream activation processes mediated through the gp130/gp130 or gp130/gp190 pathways were also performed by constructing chimeric receptors composed in their external parts of G-CSF receptor, epidermal growth factor, or TRKc and by the intracellular domains of gp130 or gp190 (33, 36). In the present study we have analyzed the agonistic properties of the B-S12 antibody directed against gp130, and we show that cross-linking of gp130 transducing subunit was sufficient to elicit IL-6 type responses.

MATERIALS AND METHODS

Cells and Reagents—The SK-N-MC neuroblastoma cell line (ATCC, Rockville, MD), and the HepG2 cell line (ATCC) were routinely grown in RPMI culture medium supplemented with 10% fetal calf serum. For the multifactor-dependent TF1 cell line (37) and the XG1 myeloma cell line (38), the culture medium was supplemented, respectively, with 1 ng/ml

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GM-CSF and 1 ng/ml IL-6. Purified human recombinant LIF (10^6 units/mg) produced in Chinese hamster ovary cell line, Escherichia coli recombinant GM-CSF (10^6 units/mg), and human soluble IL-6 receptor were kindly donated by Drs. K. Turner and M. Stahl (Genetics Institute, Boston, MA). IL-6 (10^4 units/mg) and OSM (10^5 units/mg) were purchased from Peprotech (Canton, MA). Soluble human gp130 was bought from R&D Systems (Minneapolis, MN). Purified B-S12 monoclonal antibody (IgG1) obtained as described previously was kindly provided by Dr. J. John Wijdenes from Diaclone (Besançon, France). B-S12 and control IgG1 Fab fragments were generated by using immobilized papain from Pierce, following the manufacturer’s instructions. Fab fragments were then separated from Fc fragments on a protein A column.

Bioassays—Serial dilutions of antibodies or cytokines were added to 15 x 10^3 TF1 or XG1 cells in triplicate in the assay (39). The cells were then incubated for 72 h before being pulsed with 0.5 μCi of [3H]Tdr for the last 4 h of the culture. HepG2 hepatoma cell line was plated in triplicate in 96-well plates at a concentration of 5 x 10^3 cells/well in 200 μl of RPMI medium containing 10% fetal calf serum, 10^{-4} M dexamethasone, and serial dilutions of IL-6 or antibodies (40). After 48 h, the supernatants were harvested and the haptoglobin content determined by ELISA as described previously (41). Hematopoietic progenitor cells were purified from bone marrow from informed donors by using the CD34-positive cell selection system from Applied Immune Science (Menlo Park, CA) and following the manufacturer’s instructions. Purity of the cells was controlled on a FACScan by using an anti-CD34 phycoerythrin-labeled antibody (Becton Dickinson, Mountain View, CA) and was routinely >97% CD34-positive cells. One hundred CD34-positive purified cells were seeded in 24-well plates in triplicate in Iscove’s medium containing 20% fetal calf serum, 0.8% methylcellulose, 5 x 10^{-5} M β-mercaptoethanol, and the combined bincation of cytokines or antibodies. After a 14-day culture period, the colony number was scored.

Soluble gp130 Symmetric ELISA—Detection of the dimeric form of soluble gp130 was achieved by coating the ELISA plates with the B-T12 monoclonal antibody at a concentration of 10 μg/ml in 100 mM carbonate buffer, pH 9.6. After washing and a saturation step, 2 μl soluble gp130 supplemented with 2 μl IL-6 plus 2 μl soluble IL-6 receptor, or 6 μl B-S12 anti-gp130 antibody were added to the wells for an overnight incubation at 4°C. Biotinylated B-T12 mAb was used as tracer antibody at a final concentration of 1 μg/ml. After a 6-h incubation at 37°C, streptavidin peroxidase (Dako, Glostrup, Denmark) was added at a 1/20,000 dilution for an additional 1-h incubation step. ABTS was used as substrate and the reading performed at 405 nm.

Tyrosine Phosphorylation Analysis—Cells were stimulated with OSM or antibodies before being lysed in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100 and proteinase inhibitors. After pelleting insoluble material and protein standardization, the supernatants were immunoprecipitated in the presence of B-T2 antibody raised against gp130, anti-jak1 antibody (Upstate Biotechnology, Inc. (UBI), Lake Placid, NY), or anti-jak2 antibody (UBI). The complexes were then isolated with beads coupled to protein A, submitted to SDS-polyacrylamide gel electrophoresis, and transferred onto an immobilon membrane (Millipore, Bedford, MA). The membranes were incubated in the presence of 4G10 anti-phosphotyrosine mAb, followed by an incubation with a goat anti-mouse immunoglobulin with polyclonal antibody labeled with peroxidase (Tago, Camarillo, CA). The reaction was visualized on a x-ray film with ECL reagent (Amersham, Les Ulis, France) following the manufacturer’s instructions.

RESULTS AND DISCUSSION

B-S12 Antibody Triggered the Proliferative Response of Hematopoietic CD34 Cells and Associated Protein Transcription in HepG2 Hepatoma Cell Line—We recently characterized a set of new monoclonal antibodies raised against the gp130 transducing protein. During the time course of the antibody screening, we did identify the B-S12 mAb, which, in contrast to most other identified antibodies, displayed agonistic properties in several bioassays. The stimulatory properties of the B-S12 antibody were first analyzed by using two human hematopoietic cell lines, XG1 and TF1, respectively, known to be sensitive to the cytokines activating the gp130 transduction pathway and both gp130 and gp190/LIF receptor systems (38, 39). B-S12 mAb was found to strongly trigger the proliferation of TF1 erythro-leukemia cell line and XG1 multiple myeloma cell lines, whereas a control IgG1 did not support the proliferation of these cells (Fig. 1, A and B). This result indicates that the B-S12 antibody can bypass the cytokine requirement to activate the gp130 pathway, and that recruitment of an α binding component was not necessary to generate a functional response. A series of reports had shown that the integrity of two proximal motifs in the intracellular part of gp130, and known as box 1 and 2, were
sufficient to generate a proliferative response (33, 36, 42). On the other hand, it has been demonstrated that regulation of gene transcription in hepatoma or neuroblastoma cell lines was under the dependence of an additional gp130 motif called box 3, and implicating the recruitment of STAT3 shuttle transducing protein (33, 42). In line with these observations, we have tried to further characterize the functional properties of the B-S12 antibody in order to know, if in addition to its ability to stimulate cell proliferation, the antibody could also increase gene transcription. The experiments were performed in HepG2 hepatoma cell line where the induction of haptoglobin secretion was monitored. Fig. 1C shows that B-S12 antibody was able to up-regulate the induction of haptoglobin secretion, whereas an IgG1 control antibody was without effect. Nevertheless, B-S12 was about a thousandfold less potent than IL-6 used as positive control. This result shows that gp130 activation pathway by the B-S12 antibody led not only to a proliferative response, as observed in the hematopoietic cell lines, but also to an increase in gene transcription in hepatic cells.

**B-S12 Antibody Induced Both Proliferation and Differentiation of CD34 Hematopoietic Progenitors—** Several IL-6-related cytokines were reported to display synergistic activities on both proliferation and differentiation processes of hematopoietic cells. To examine the potential role of the B-S12 anti-gp130 antibody on the expansion of hematopoietic cell progenitors, its effect was tested in combination with GM-CSF on purified human CD34-positive cells by using a methylcellulose clonal assay. B-S12 antibody alone, like the IgG1 isotype control did not induce colony formation (Table I). Addition of 1 ng/ml GM-CSF to the culture stimulated the generation of 11 ± 1.4 colonies after a 2-week culture period. To assess the synergistic effect of IL-6 type cytokines in the culture system, LIF was preferred to IL-6 because this latest cytokine only displayed weak effects in human CD34 cell culture and the adjuvance of soluble IL-6 receptor was usually required to observe a response (43). Addition of LIF to GM-CSF doubled the score of colonies in the cultures in agreement with the published studies (10, 44). Similar results were obtained by introducing B-S12 antibody in combination with GM-CSF in the CD34-positive cell cultures. Moreover, the average size of the observed colonies generated in the presence of B-S12 and GM-CSF was twice larger than those detected in the presence of GM-CSF alone.3 These experiments show that B-S12 antibody, like most of the cytokines belonging to the IL-6 family, synergistically promoted potent expansion of human hematopoietic progenitor cells in vitro.

**B-S12 Antibody-induced Dimerization of the gp130 Receptor Transducing Component.**—Fab fragments were generated from the B-S12 antibody and tested in the proliferative TF1 assay to determine whether the observed agonistic properties of the antibody were dependent on the presence of the two functional sites expressed by the immunoglobulin. Fig. 2A shows that B-S12-derived Fab fragments failed to trigger the proliferation of the TF1 cell line. Cross-linking of the Fab fragments by using a second antibody recognizing the mouse immunoglobulins did not allow the restoration of a functional signal, indicating that a very precise configuration of the antibody was likely required to bring the proliferative information. In addition, coating of Fab fragments to a plastic dish did not restore the signal (data not shown). The lost of biological properties of the Fab fragments indicates that B-S12 might induce a dimerization of gp130 transducing protein to generate a biological response. To assess this hypothesis, induction of gp130 dimerization was analyzed in a symmetric ELISA where the same B-T12 anti-gp130 mAb was used as control. This result shows that gp130 activation pathway by the B-S12 antibody led not only to a proliferative response, as observed in the hematopoietic cell lines, but also to an increase in gene transcription in hepatic cells.

**Table I**

| Culture conditions | Scored colonies |
|--------------------|-----------------|
| Medium alone       | 1 ± 0           |
| GM-CSF (1 ng/ml)   | 11 ± 1.4        |
| LIF (50 ng/ml)     | 0.6 ± 0.9       |
| GM-CSF + LIF       | 21 ± 1          |
| B-S12 (25 μg/ml)   | 0 ± 0           |
| IgG1 (25 μg/ml)    | 0.3 ± 0.4       |
| GM-CSF + B-S12     | 18.3 ± 1.8      |
| GM-CSF + IgG1      | 11.5 ± 0.5      |

3 In the present study, the number of granulocyte-macrophage colony-forming units, blast cell colonies, and mixed colonies has been calculated; the phenotypic detail of the generated colonies will be presented in another study.
Their tyrosine phosphorylation level was analyzed as described in an increased optical density value will be a reflection of the addition of 2 nM soluble IL-6 receptor and IL-6 to 2 nM soluble gp130. Neuronal tissue and, in addition, gp130 tyrosine phosphorylation of the transducing protein. The experimental approach was readily detectable in this cell line. Fig. 3 A, B-S12 mAb-induced tyrosine phosphorylation of Jak1, Jak2, and gp130 receptor component. In panel A, SK-N-MC neuroblastoma cells were incubated for 20 min in the presence of increasing concentrations of B-S12 mAb or with 50 ng/ml OSM. After cell lysis, gp130 was immunoprecipitated, run on SDS-polyacrylamide gel electrophoresis, blotted on a membrane, and its tyrosine phosphorylation content analyzed by using an anti-Tyr(P) mAb. In B, SK-N-MC cells were stimulated for 10 min with 50 ng/ml OSM or with 50 μg/ml antibodies for the indicated periods of time. After cell lysis, the samples were treated as described in A. In C, resting or B-S12 activated SK-N-MC cells (50 μg/ml B-S12 mAbs, for 20 min) were lysed, and Jak1 (lanes 1) and Jak2 (lanes 2) signaling proteins immunoprecipitated. Their tyrosine phosphorylation level was analyzed as described in A.

Coating and tracer antibody. In these conditions the detection of an increased optical density value will be a reflection of the dimerization of the soluble form of gp130. As shown in Fig. 2B, addition of 2 nM soluble IL-6 receptor and IL-6 to 2 nM soluble gp130 conducted to an increased signal in the symmetric gp130 ELISA. This result sustains the notion that IL-6, IL-6 receptor, and gp130 can reassociate together in solution to generate a trimeric or an hexameric complex as reported before (45, 46). In a similar manner introduction of the B-S12 antibody, instead of IL-6 and its soluble receptor, led to a dimerization of the soluble form of gp130 transducing protein. Interestingly B-S12 antibody failed to inhibit IL-6-driven proliferation of the TF1 cell line and did not interfere with the binding of radiolabeled IL-6 to its high affinity receptor (data not shown). These results indicate that the antibody and the cytokine recruited gp130 through different functional sites.

 gp130 and Jak Family Members Were Tyrosine-phosphorylated in Response to B-S12 Stimulation—Receptor activation by the IL-6 family of cytokines results in the tyrosine phosphorylation of the transducing receptor subunits and downstream regulatory proteins (32, 33). Functional activation of gp130 transducer by B-S12 antibody was assessed by analyzing tyrosine phosphorylation of the transducing protein. The experiments were performed by using the SK-N-MC neuroblastoma cell line, since IL-6-related cytokines displayed many effects in neuronal tissue and, in addition, gp130 tyrosine phosphorylation was readily detectable in this cell line. Fig. 3A shows that the recruitment of gp130, and its tyrosine phosphorylation by the B-S12 antibody was dose-dependent. After a 5-min interaction with the agonistic antibody tyrosine phosphorylation of gp130 could already be detected (Fig. 3B). The observed signal peaked after a 20-min contact, but remained weaker than the one observed by incubating the SK-N-MC cell line in the presence of OSM. A control antibody added in the culture for 20 min did not activate gp130 tyrosine phosphorylation underlying the specificity of the detected signal. Jak1, Jak2, and Tyk2 kinases have been shown to be implicated in the activation of gp130 and gp190/LIF receptor (32–34). This led us to investigate the involvement of Jak-Tyk kinases in signaling initiated by the B-S12 monoclonal antibody. Tyrosine phosphorylation of Jak1 and Jak2 was observed after stimulation by the agonistic B-S12 antibody (Fig. 3C). In contrast we did not detect Tyk2 activation after treating the cells with B-S12 antibody, or OSM, even if Tyk2 protein could be detected by immunoblotting in the cell lysate (data not shown). A similar pattern of activation was described previously for another neuronal derived cell line, and some tissue variations in the Jak-Tyk recruitment by the IL-6 type of cytokines were reported (32). These results show that B-S12 antibody was able to activate gp130 pathway in neuronal cells as well, by recruiting Jak1 and Jak2 kinases and inducing tyrosine phosphorylation of the transducing protein.

CONCLUSION

Receptor-induced dimerization is a common mechanism required for signaling in both cytokine receptors and receptor tyrosine kinases (for review, see Ref. 47). Activation processes of cytokine receptors leading to the formation of functional dimers were analyzed by using different approaches, such as introduction of disulfide bonds between transducing subunits or by generating chimeric receptors composed of the intracellular parts of the β and γ IL-2 receptor chains and the extracellular region of c-kit, GM-CSF receptor, or G-CSF receptor (48–51). Biological properties of the IL-6 cytokine family mediated through an homodimerization of gp130 transducing protein or its heterodimerization with LIF receptor were analyzed by using chimeric receptors substituted in their external parts by G-CSF receptor, epidermal growth factor receptor, or TRK-C corresponding regions (24, 30). The obtained results have reinforced the notion that homo- or heterodimerization of gp130 and gp190 lead to an activation of gene transcription mediated through the Stat3/box 3 distal motif interaction, but little is known about the regulation of the proliferative signal provided by dimerization of the signaling receptor proteins (42, 33). In the present study we have reported that dimerization of gp130 was sufficient to elicit IL-6 type responses. These results sustain the idea that the major contribution of the α IL-6 receptor subunit is to increase IL-6/gp130 interaction and gp130 dimerization at the cell surface. In addition, neutralizing antibodies directed against IL-6 or IL-6 receptor were not able to inhibit the responses mediated through B-S12 activation, indicating a direct effect of the agonistic antibody on gp130 recruitment (results not shown). We also show that B-S12-induced dimerization of gp130 triggered both cell proliferation and gene transcription, and that functional responses could be observed in hematopoietic, hepatic, and neural derived cell lines. Tyrosine phosphorylation analysis of gp190/LIF receptor performed after B-S12 cell stimulation did not reveal a significant increased activation of this receptor subunit, indicating that the observed responses were only under the dependence of gp130 pathway (data not shown). Interestingly a study performed by using bispecific chimeric antibodies able to recognize two of three receptor subunits involved in the formation of high affinity IL-2 receptor has shown that cross-linking of transducing receptor subunits in these conditions could lead to a functional response (52). Trans-
gp130 Dimerization-induced Functional Responses

position of this approach to the gp130/gp190 system, by generating bispecific antibodies able to recognize both transducing components, could be of interest in some situations such as expansion of hematopoietic progenitors, where activation of gp130 by B-S12 antibody already led to a efficient response.

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