BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 22–27 (1998)
ARTICLE NO. RC988900

A Selective Switch-on System for Self-Renewal of Embryonic Stem Cells Using Chimeric Cytokine Receptors

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Received May 29, 1998

Propagation of embryonic stem (ES) cells with an undifferentiated pluripotential phenotype depends on leukemia inhibitory factor (LIF). The LIF receptor complex is composed of a heterodimer of LIF receptor α (LIFRα) and gp130. To activate LIFR signaling pathways independently from endogenous ones, we constructed chimeric receptors by linking the extracellular domain of human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α or β (hGMRα or β) to the transmembrane and cytoplasmic regions of either mouse LIFRα or gp130. hGMRα-mLIFR/ hGMRβ-mgp130 or hGMRα-mgp130/ hGMRβ-mgp130, but not hGMRα-mLIFR/ hGMRβ-mLIFR, preserved the self-renewal activity in A3 ES cells. All of these chimeric receptors were phosphorylated after hGM-CSF stimulation without phosphorylation of endogenous gp130. Phosphorylation of the signal transducer and activator of transcription 3 through chimeric receptors correlated with the undifferentiated phenotype. Therefore, these chimeric receptors prove useful to analyze mechanisms of the self-renewal of ES cells. © 1998 Academic Press

Murine embryonic stem (ES) cells are isolated from the inner cell mass of preimplanted embryos. The undifferentiated pluripotential phenotype of ES cells is maintained in the presence of leukemia inhibitory factor (LIF) (1,2) and withdrawal of LIF leads to the differentiation. This phenomenon suggests a close correlation between the undifferentiated phenotype and the LIF signaling pathways in ES cells.

The LIF receptor complex (LIFR) is composed of an LIF-binding subunit, LIFRα (sometimes known as LIFRβ), and the glycoprotein gp130 (3). gp130 is an essential component of functional receptors for interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), IL-11 and cardiotrophin-1 (CT-1). LIFRα is also shared with receptors for CNTF, OSM, CT-1 (4-9). In accordance with these structural similarities, the biological actions of LIF and related cytokines, such as IL-6, IL-11, OSM, CT-1, and CNTF largely overlap. The self-renewal of ES cells is maintained by not only LIF but IL-11 or soluble IL-6 receptor/IL-6 (9). Common activities of LIF family cytokines have been attributed to the sharing of gp130. Despite a high degree of sequence similarity between gp130 and LIFRα, distinct contributions of gp130 and LIFRα cytoplasmic domains to LIF-induced signal transduction have to be examined in a precise fashion (10).

To analyze self-renewal mechanism of ES cells, we expressed chimeric receptors that comprised the extracellular domain of hGMR and the transmembrane and cytoplasmic regions of either hGMR, mouse LIFRα (mLIFRα) or mouse gp130 (mgp130). GM-CSF functions in a species-specific manner (11). This approach facilitated hGM-CSF-dependent activation of chimeric receptors, independently of endogenous receptor chains. hGMRα-mLIFR/ hGMRβ-mgp130 (L/G) or hGMRα-mgp130/ hGMRβ-mgp130 (G/G), but not hGMRα/hGMRβ (W/W) or hGMRα-mLIFR/ hGMRβ-mLIFR (L/L), preserved the self-renewal activity in A3 ES cells. Activation of the signal transducer and activator of transcription 3 (STAT3) through chimeric receptors correlated with the undifferentiated phenotype.

MATERIALS AND METHODS

Cell culture and cytokines. The ES cell line A3.1, derived from the parental line A3, was passsaged in ES cell medium (Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, 10 mM non-essential amino acids, 200 mM L-glutamine, 0.03 mM adenosine, 0.03 mM cytidine, 0.03 mM guanosine, 0.03 mM uridine, 0.01 mM

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0006-291X/98 $25.00

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thymidine, 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 mg/ml streptomycin and 10 ng/ml LIF) in the absence of feeder cells. Recombinant human LIF(hLIF) and human GM-CSF (hGM-CSF) were kindly provided by AMGEN. To maintain parental or transfected ES cells, 20 ng/ml concentration of hLIF or 10 ng/ml concentration of hGM-CSF was used.

DNA constructs. The cDNA of mLIFRα and mgp130 were cloned from ES cell line A3.1 by RT-PCR. The PCR products were inserted into pSP72 (Promega) at PvuII site and nucleotide sequences were confirmed using the 373A DNA sequencing system (PERKIN ELMER). hGMRα and hGMRβ cDNA (pCEV4-hGMRα and pME18S-KH97) were kindly provided by Dr. A. Miyajima (the University of Tokyo). All these cDNA were transferred into pCAG at XhoI site, which was provided from Dr. J. Miyazaki (Osaka University) (12). Finally, all constructs were driven from the CAG promoter in ES cells. The chimeric receptor constructions, which have the extracellular domain of hGMRα or β linking to the transmembrane and cytoplasmic regions of either mLIFRα or mgp130, were generated by overlap extension, using PCR (13). In short, complementary oligonucleotide primers and PCR were used to generate two DNA fragments with overlapping ends. In the case of hGMRα-mgp130, the following two components were first generated by PCR; the extracellular domain fragment of hGMRα with the first 9 bp of the transmembrane domain of mgp130 and the transmembrane/intracellular domains of mgp130 with the last 9 bp of the extracellular domain of hGMRα. These fragments were combined in a subsequent fusion reaction in which the overlapping ends were annealed, allowing the 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The resulting fusion product was further amplified by PCR. This PCR product was inserted into pSP72 and nucleotide sequences were confirmed, using the 373A DNA sequencing system. Subsequently, the following three fragments were prepared; first part of pCAG with first part of hGMRα, chimeric fragment from the latter part of hGMRα and first part of the transmembrane/intracellular mgp130, and the latter part of transmembrane/intracellular mgp130 + the latter part of pCAG. These three fragments were ligated, resulting in completion of the chimeric receptor under control of the CAG promoter. To establish stable transfectants, cDNA were subcloned into pMKIT neo or pKIK hygB containing a neomycin or hygromycin resistance gene.

Establishments of stable transfectants. Stable transfectants of ES cell lines were obtained by electroporation of 20 μg of linearized expression plasmids of wild-type hGMRα/hGMRβ (W/W), hGMRα-mLIFR/hGMRβ-mgp130 (L/G), hGMRα-mgp130/hGMRβ-mgp130 (G/G) or hGMRα-mLIFR/hGMRβ-mLIFR (L/L), essentially described (14). Expression of receptors was confirmed by flow cytometry with FACScan (Becton Dickinson), using monoclonal antibodies directed against the α and β subunits of hGMR (Santa Cruz Biotechnology, Inc.).

ES cell assays. The extent to which cytokine-mediated signaling prevents ES cell differentiation was determined based on morphology, alkaline phosphatase staining and expression of SSEA-1. 2 X 10^4 transfectants were plated on gelatin-coated 6-well multiwell plates (Falcon) in ES cell medium containing hLIF, hGM-CSF or no factor. Five days after inoculation, morphology of the ES cells were observed. Simultaneously, cultured transfectants were scraped off using trypsin-EDTA solution and cytospins were done. Cells were stained for alkaline phosphatase activity using Sigma Diagnostic Kit No. 86 (Sigma). The ratio of alkaline phosphatase-positive cells was scored from a total of randomly chosen 300 cells. The expression of SSEA-1 was examined using anti-SSEA-1 monoclonal antibody KM380 (Kyowa) and flow cytometry.

Analysis of tyrosine phosphorylated proteins. After factor depletion for 12h, the cells were resuspended in the same medium followed by stimulation with or without hLIF (200 ng/ml; Amgen) or hGM-CSF (100 ng/ml) at 37°C for the indicated period of time. After harvesting in ice-cold PBS containing 1 mM Na_3V0_4, cells were lysed at a density of 1 X10^7 cells/ml in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 4 mg/ml aprotinin, 4 mg/ml leupeptin, 1 mM Na_3VO_4). The supernatant was incubated for 1 hour at 4°C with anti-phosphotyrosine monoclonal antibody PY-20 (Transduction Lab.), anti-hGMR α monoclonal antibody S-20, anti-hGMR β antibody S-20, or anti-mouse STAT3 monoclonal antibody C-20 (Santa Cruz). The protein-antibody complex were collected with protein G-Sepharose (Pharmacia). The immunoprecipitated pellets were suspended in Laemmli's sample buffer and subjected to SDS-PAGE and then Western blotting using mouse anti-phosphotyrosine monoclonal antibody 4G10 (UBI), rabbit anti-mgp130 antibody M-20 (Santa Cruz), mouse anti-hGMRα monoclonal antibody S-50 (Santa Cruz), rabbit anti-hGMRβ antibody N-20 (Santa Cruz), or mouse anti-STAT-3 monoclonal antibody Clone 84 (Transduction Lab.). The blots were developed using Chemiluminescence System (NEB Life Science), according to the manufacturer's instructions.

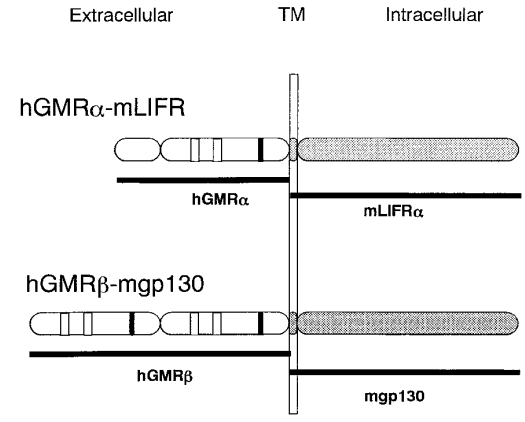

FIG. 1. Construction of chimeric receptors. Chimeric receptor was constructed by linking the extracellular domain of hGM-CSFRα or hGM-CSFRβ to the transmembrane and cytoplasmic regions of mLIFRα or mgp130, respectively. Both transgenes were driven by CAG promoter.

RESULTS

Constuction and expression of wild-type hGMR or chimeric receptors in A3.1 ES cells. We constructed chimeric receptors that consisted of the extracellular domain of hGMRα or β fused with the transmembrane and intracellular domains of mgp130 or mLIFRα. These chimeric receptors and wild-type hGMR were regulated by the CAG promoter (Fig. 1). The following combination of the chimeric or wild-type receptors was co-transfected into A3 ES cells by electroporation; wild-type hGMRα/hGMRβ (W/W), hGMRα-mLIFR/hGMRβ-mgp130 (L/G), hGMRα-mgp130/hGMRβ-mgp130 (G/G), hGMRα-mLIFR/hGMRβ-mLIFR (L/L). The expression level of transfected receptors was confirmed by flow cytometry analysis with an anti-hGMRα antibody or an anti-hGMRβ antibody (data not shown), the epitope of which exists in the extracellular domain. We thus obtained 4 W/W, 4 L/G, 3 G/G and 3 L/L clones. We present here results obtained from a representative clone for each combination as essentially the same results were observed for the different clones. Simultaneously, the proliferation activities through these chime-

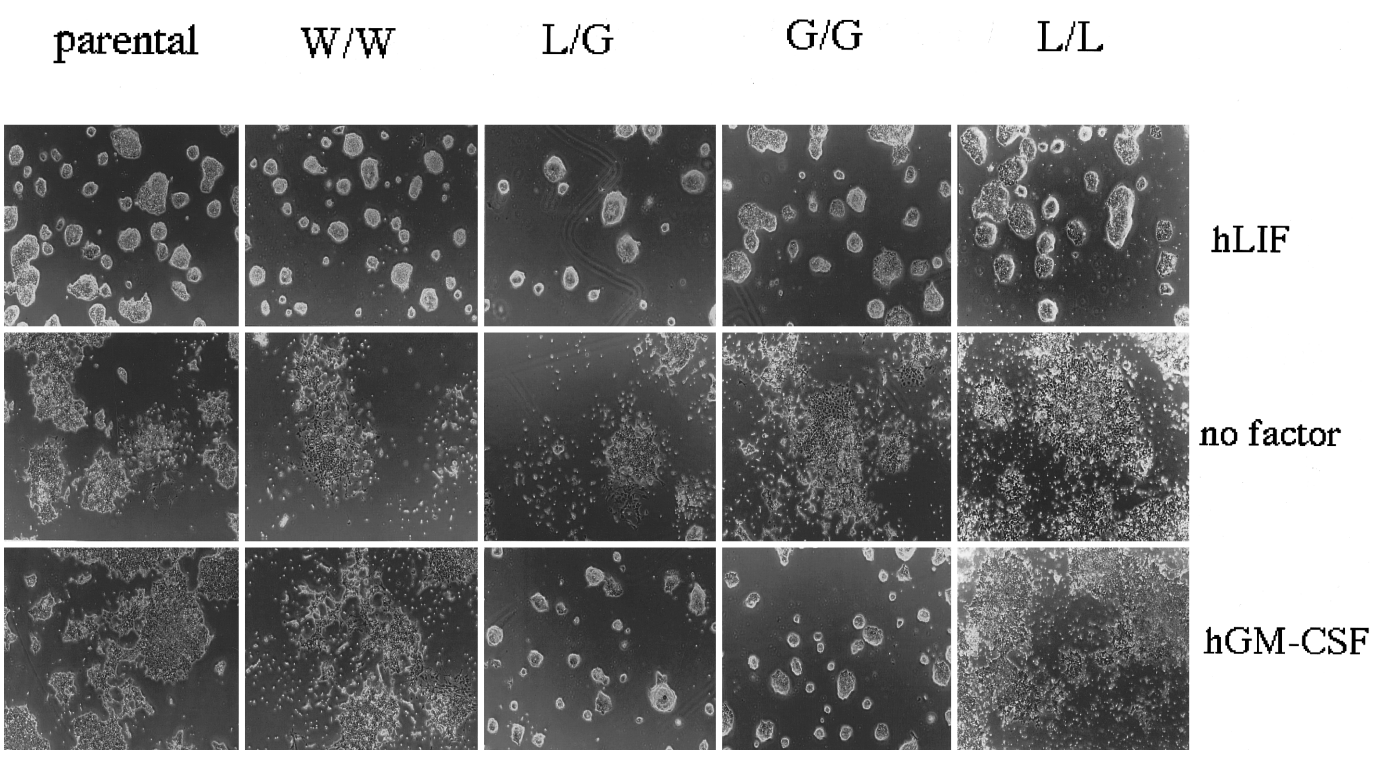

FIG. 2. Morphology of ES cells transfected with chimeric receptors. Morphology of ES cells transfected with wild-type hGMRα/hGMRβ (W/W), hGMRα-mLIFR/hGMRβ-mgp130 (L/G), hGMRα-mgp130/hGMRβ-mgp130 (G/G), hGMRα-mLIFR/hGMRβ-mLIFR (L/L), was examined in case of exposure or not exposure of hLIF or hGM-CSF for 7 days. The undifferentiated state was determined by the maintenance of compact colony formation.

ric receptors were examined in the mouse pro-B cell line, Ba/F3 cells by MTT assay. W/W, L/G or G/G construct transduced the proliferation signal in response to hGM-CSF, whereas L/L construct did not (data not shown).

hGM-CSF does not induce self-renewal of ES cells expressing wild-type hGMR. LIF induces growth arrest and macrophage differentiation in M1 cells. Interestingly, M1 cells expressing W/W show the same phenotypic change in response to hGM-CSF as to LIF (15). To examine whether W/W also transduces the self-renewal signal in ES cells in response to hGM-CSF stimulation, we examined the morphology, the level of alkaline phosphatase and the SSEA-1 expression in ES cells expressing W/W. hLIF maintained the phenotype of compact colonies while W/W ES cells became large, flattened, differentiated phenotype in the presence of 20 ng/ml hGM-CSF (Fig. 2). This concentration of hGM-CSF could support the growth signal of Ba/F3, expressing W/W. Simultaneously, W/W ES cells lost the high level of alkaline phosphatase after switching from hLIF to hGM-CSF stimulation (data not shown). The SSEA-1, which is expressed in undifferentiated ES cells, also could not be maintained in W/W ES cells by hGM-CSF (Fig. 3). These results indicate that wild-type hGMR could not compensate for the signal through LIFR in ES cells.

Heterodimeric or gp130 homodimeric chimeric receptor transduces self-renewal signal but LIFRα homodimeric chimeric receptor does not. Three combinations of chimeric receptors were examined for their potential to transduce the self-renewal signal; L/G, G/G and L/L (Fig. 2). The L/G or G/G ES cells formed compact colonies, a morphological undifferentiated state, in the presence of 20 ng/ml hGM-CSF as well as in the presence of hLIF, whereas L/L ES cells did not. We also examined the undifferentiated characteristics in these ES cells regarding the following indicators, the level of alkaline phosphatase and the expression of SSEA-1. Both of these were maintained in L/G and G/G ES cells, but not in L/L. L/L ES cells differentiated into large, flattened, differentiated phenotype and lost both the high level of the alkaline phosphatase and the expression of SSEA-1. Thus, L/G or G/G but not L/L compensated for the signal through wild type LIFR.

Chimeric receptors are phosphorylated independently of endogenous LIFR. Phosphorylation of receptors is required for signal transduction following ligand binding. In the case of hGMR, tyrosine phosphorylation in the GMRβ chain but not in GMRα occurs, since cytoplasmic region of hGMRα does not contain tyrosine residues. In the case of LIFR, both subunits of LIFRα and gp130 become phosphorylated after LIF stimula-

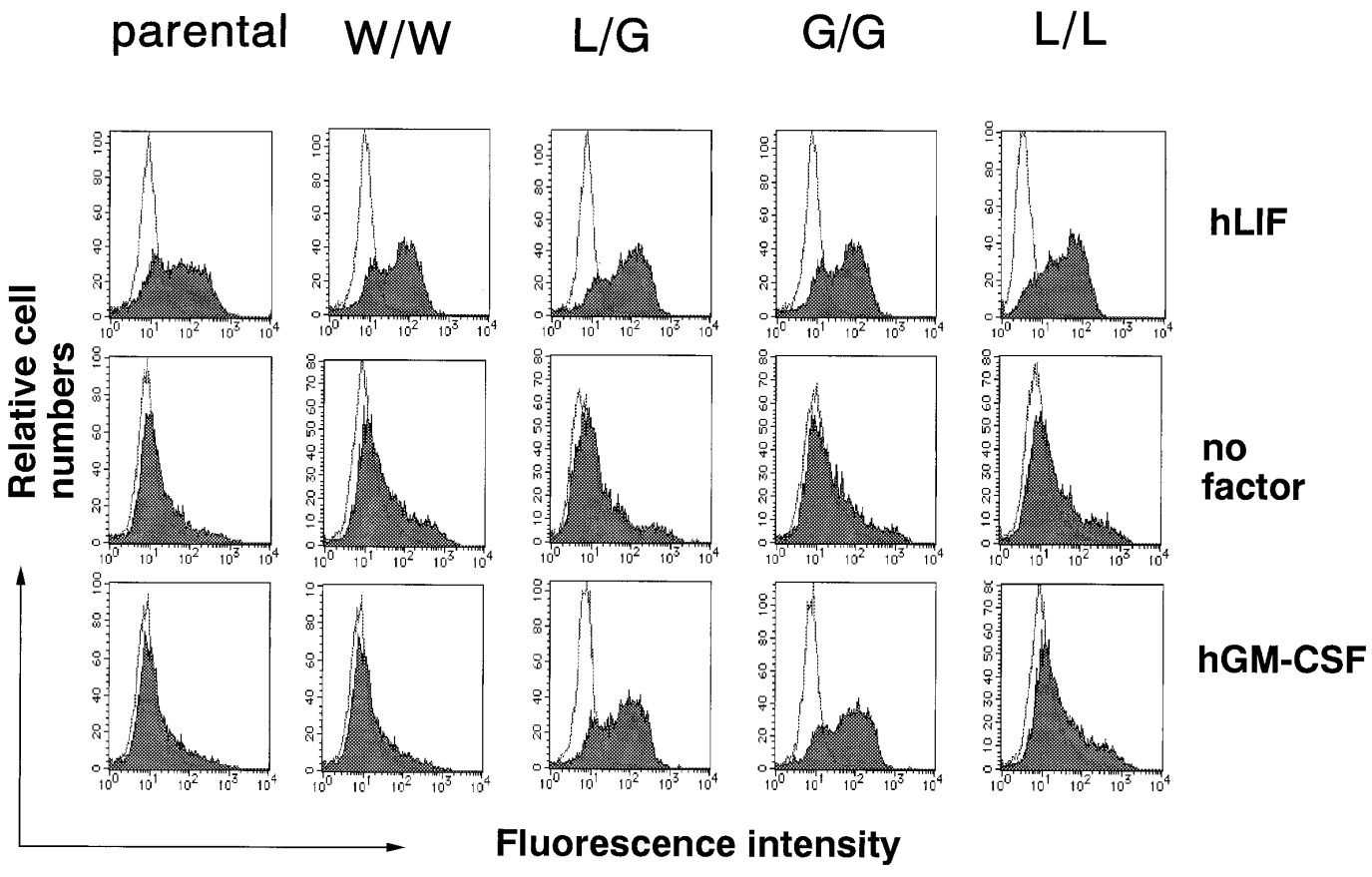

FIG. 3. SSEA-1 expression of ES Cells Transfected with Chimeric Receptors. The expression of SSEA-1 was examined by flow cytometry. The parental, W/W, L/G, G/G or L/L ES cells were maintained for 7 days in the presence of the indicated factor.

tion. Therefore, we asked whether the transfected wild-type hGMR or chimeric receptors could be phosphorylated in response to hGM-CSF. Simultaneously, phosphorylation of the corresponding endogenous receptors were examined. In W/W ES cells, tyrosine phosphorylation of wild-type hGMRβ was evident after hGM-CSF stimulation but not of wild-type hGMRα (Fig. 4). Thus, the transfected receptors themselves were activated although the self-renewal signal was not transduced. In L/G, G/G or L/L ES cells, hGM-CSF phosphorylated the transfected chimeric receptors, but not endogenous gp130. Conversely, hLIF stimulation phosphorylated only endogenous gp130, not transfected ones. These data showed that all of the transfected receptors were phosphorylated independently of endogenous LIFR. However, as shown in Fig. 2, L/L did not support self-renewal signal of ES cells, though they were phosphorylated , a situation similar to that of W/W. These data indicate that the phosphorylation status of the receptors does not correlate with their self-renewal capacities.

STAT3 phosphorylation correlates with self-renewal of ES cells. The transcription factor STAT3, which is phosphorylated after LIF stimulation, was identified as one component of transcription factor complexes binding LIF-responsive element in ES cells. To determine whether STAT3 phosphorylation correlates with self-renewal activity, we examined the tyrosine phosphorylation of STAT3 in parental, W/W, L/G, G/G or L/L ES cells in the presence of hLIF or hGM-CSF (Fig. 5). The tyrosine phosphorylation of STAT3 was observed in the ES cells in response to hLIF. However, among ES cells expressing with wild-type or chimeric receptors, only L/G or G/G, which manifested the undifferentiated phenotype in response to hGM-CSF, induced the phosphorylation of STAT3, therefore STAT3 phosphorylation correlates with the self-renewal of ES cells.

DISCUSSION

LIF plays a key role in the maintenance of the pluripotential phenotype and proliferation of ES cells *in vitro* (1, 2). To determine the signal transduction pathway after LIF stimulation, precise analysis of LIFR had to be done precisely. For this purpose, we established the system using the chimeric receptor to activate the LIF signal transduction pathway, independently of the endogenous receptor.

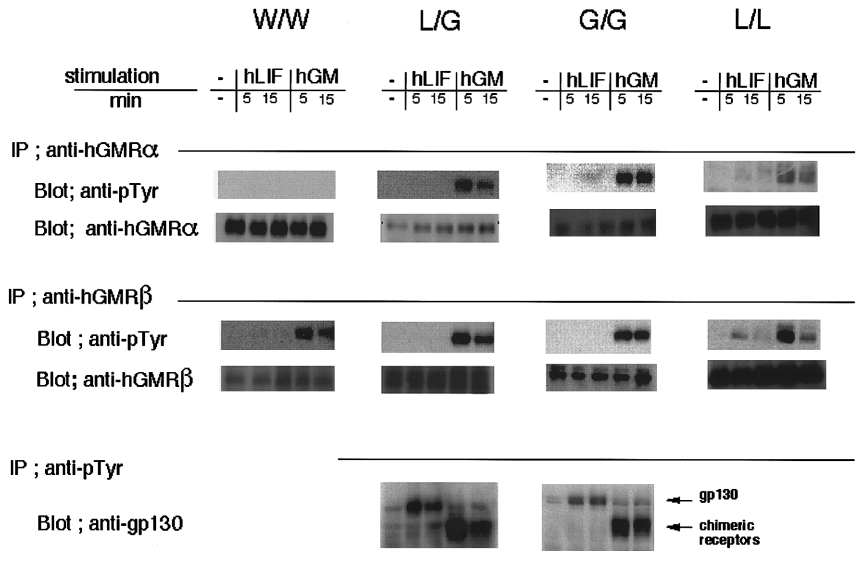

FIG. 4. Induction of tyrosine phosphorylation of receptors in response to hGM-CSF. After 12 hr factor depletion, ES cells were stimulated with the indicated factor for 5 min or 15 min. Cell lysates were immunoprecipitated with antibody against phosphotyrosine, hGMRα, or hGMRβ, and immunoblotted with antibody against phosphotyrosine, hGMRα, hGMRβ, or gp130.

In this paper, we reported that L/G or G/G but not W/W or L/L could transduce the self-renewal signal, although W/W, L/G or G/G but not L/L could transduce the proliferation signal in the MTT assay using Ba/F3 cells (data not shown). In Western blotting analysis, all of these transduced receptor proved to be phosphorylated in response to hGM-CSF stimulation, despite STAT3 phosphorylation was observed only in the case of L/G or G/G. The self-renewal capacity through these receptors was correlated with the phosphorylation of STAT3.

Recently, it has been reported that M1 cells transfected with W/W can be induced to growth arrest and macrophage differentiation in response to GM-CSF as well as LIF (15). Thus, it became necessary to investigate whether wild-type hGMR could transduce the self-renewal signal in ES cells or not in response to hGM-CSF. Unlike the case in M1 cells, hGM-CSF could not maintain the undifferentiated phenotype, despite phosphorylation of the transduced hGMRβ chain. This means that a specific signal through LIFR is needed for the self-renewal of ES cells.

LIF uses receptors consisting of a heterodimeric complex of gp130 and LIFRα. Despite a high degree of sequence similarity between gp130 and LIFRα, distinct contributions of gp130 and LIFRα cytoplasmic domains remains to be examined. In our experiments, L/L chimeric receptors were phosphorylated in response to

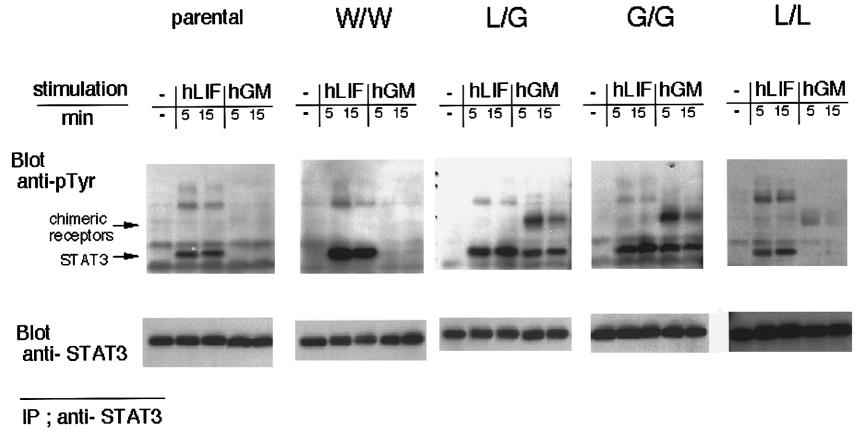

FIG. 5. Tyrosine phosphorylation of STAT3 in response to hGM-CSF. Cell lysates were prepared from parental, W/W, L/G, G/G, or L/L ES cells, after the indicated stimulation. After being immunoprecipitated with anti-STAT3 monoclonal antibody, membranes were immunoblotted with anti-phosphotyrosine antibody or a different anti-STAT3 antibody.

hGM-CSF stimulation without affecting the endogenous LIFR. This implies that LIFRα did not transduce the self-renewal signal and the particular signal through gp130 is required to maintain undifferentiated ES cells. The phosphorylation of STAT3 was closely correlated with the self-renewal of ES cells. Therefore, the capability of STAT3 phosphorylation through receptor stimulation, not receptor phosphorylation itself, is a key point whether self-renewal occurs or not.

Starr *et al.* also examined the self-renewal activity in ES cells using cytokine chimeric receptors (16). They used chimeric receptors by linking the extracellular domain of granulocyte colony-stimulating factor receptor (G-CSFR) to the transmembrane and cytoplasmic regions of either LIFRα or gp130. Although G-CSFR-gp130 receptor homodimer mediated a G-CSF-induced signal in M1 cells, Ba/F3 cells and ES cells, G-CSGR-LIFRα receptor homodimer was only functional in ES cells. The discrepancy between our results and theirs might be due to the use of 1) different cells and 2) different extracellular domains. To evaluate the undifferentiated state, we examined the morphology, the expression of alkaline phosphatase and the SSEA-1 expression; Starr *et al.* examined the morphology and the chemical selection using hprt under *hck* promoter.

As mentioned above, the specific contribution of STAT3 toward the self-renewal of ES cells deserves much. In our system, the phosphorylation of STAT3 was correlated with the undifferentiated phenotypes of ES cells. STAT3 activation was observed only on L/G or G/G ES cells, not on W/W or L/L. On the contrary, in Starr's experiment, STAT3 was activated through G-CSFR-gp130 and G-CSFR-LIFRα. Albeit the discrepancy of the signal through LIFRα, the close correlation of STAT3 activation and the self-renewal was observed in either case. Recently, Behrmann *et al.* also examined STAT3 activation using chimeric receptors composed of the interleukin-5 receptor and LIFRα in COS-7 cells (17). According to their report, STAT3 itself was not activated through homodimerization of LIFRα cytoplasmic tails. This report and our result imply that homodimerization of LIFRα could not transduce the signal effectively.

Starr *et al.* also revealed STAT5 activation through G-CSFR-gp130 and G-CSFR-LIFRα (16). In our system, STAT5 was activated in L/G and G/G ES cells in response to hGM-CSF. Simultaneously, STAT5 was also activated in W/W ES cells, which manifested differentiated phenotype in the presence of hGM-CSF (data not shown).

Regardless, the activation of STAT3 activation closely correlated with the self-renewal of ES cells, suggesting that STAT3 has an important role for undifferentiated state of ES cells. Boeuf *et al.* who uses a dominant-negative STAT3 also suggested such a correlation (18). Therefore, it would be interesting to analyze how STAT3 contributes to the regulation of self-renewal in ES cells. For this purpose, our chimeric cytokine receptor system proves useful to analyze mechanisms of the self-renewal in ES cells.

ACKNOWLEDGMENTS

We thank Drs. Rhyuichi Nishinakamura, Tatsutoshi Nakahata, Ken-ichi Arai, George Morstyn, Flederic Fletcher, and Ian McNiece for the helpful discussions and criticisms on the manuscript. We also thank Dr. Flederic Fletcher for preparing oligonucleotides. The Department of Stem Cell Regulation is supported by Amgen Corporation, Japan.

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