RAPID COMMUNICATION

A Low-Affinity Human Granulocyte-Macrophage Colony-Stimulating Factor/ Murine Erythropoietin Hybrid Receptor Functions in Murine Cell Lines

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To identify domains in hematopoietic growth factor receptors that are important for signal transduction, a hybrid receptor (GMER) was constructed by splicing the DNA of the entire extracellular and transmembrane domains of the human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α2 subunit (GMR) to the cytoplasmic domain of the murine erythropoietin receptor (mEpoR). The hybrid receptor was introduced into the interleukin-3 factor-dependent murine hematopoietic cell line Ba/F3. Cells that expressed high receptor numbers were selected by cell sorting using phycoerythrin-labeled human GM-CSF. Immunoprecipitation of GMER from Ba/F3 cells showed a band with an Mr of 105,000 daltons. Human GM-CSF binding to Ba/F3 cells that expressed GMER showed a kd of 3.0 nmol/L and 475 binding sites/cell, while the same cells that expressed GMR had 300 sites/cell and a kd of 3.5 nmol/L.

The proliferative response to GM-CSF of Ba/F3 cells that expressed GMER showed ½ maximal cell growth (as measured by 3H-thymidine incorporation) at a GM-CSF concentration of 2.5 × 10⁻⁸ mol/L. When cultured in human GM-CSF, Ba/F3-GMER cells expressed cell surface glycoporphin. Similar results were obtained with Ba/F3 cells transfected with the mEpoR and cultured in erythropoietin. Expression of GMR plus the human GM-CSF receptor β chain in the same cell line also resulted in human GM-CSF stimulated proliferation: however, cell surface glycoporphin was not detected. These data show that a low-affinity GM-CSF/Epo hybrid receptor can promote GM-CSF-dependent proliferation and can induce the expression of glycoporphin, an erythroid-specific protein.

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for 90 minutes at 37°C. Cells were then plated on ice for 10 minutes, and electroporated at 980 μF and 250 V with the time constant set by the apparatus (Bio-Rad, Richmond, CA). The transfected cells were allowed to recover for 3 to 4 days in the presence of ml-3, and were then selected for neomycin resistance in G418, and the ability to grow in hGM-CSF. Three to 4 weeks later, surviving cells were subjected to fluorescence activated cell sorting (FACS; Becton-Dickinson, Rutherford, NJ) with phycoerythrin-labeled GM-CSF essentially according to the directions of the manufacturer (R & D Systems). Cells that expressed high levels of GMER or GMR (top 5% to 7%) were collected and resorted three to four more times to isolate cells with the highest receptor expression.

**Immunoprecipitation.** Ba/F3 wt and Ba/F3-GMER cells were grown in RPMI methionine-free media, supplemented with 2% FCS and grown in RPMI methionine-free media, supplemented with 2% FCS and were then selected for neomycin resistance in G418, and the ability to grow in hGM-CSF. Three to 4 weeks later, surviving cells were subjected to fluorescence activated cell sorting (FACS; Becton-Dickinson, Rutherford, NJ) with phycoerythrin-labeled GM-CSF essentially according to the directions of the manufacturer (R & D Systems). Cells that expressed high levels of GMER or GMR (top 5% to 7%) were collected and resorted three to four more times to isolate cells with the highest receptor expression.

**GM-CSF binding to cells.** Radioiodinated GM-CSF was purchased from New England Nuclear. The 125I-GM-CSF had a specific activity of 4.9 × 10^16 cpm/mol. Before the binding of 125I-GM-CSF, all factor-dependent cells were cultured for 24 hours or more in GM-CSF-free RPMI media containing 10% Wehi-conditioned media, followed by 6 hours in RPMI containing 10% FCS. The cells (1 × 10^6) were washed and then resuspended in binding media RPMI with 4% FCS, 30 mmol/L Hepes pH 7.4, 0.04% mmol/L sodium azide containing the indicated concentration of radiolabeled GM-CSF at 4°C. The cells were then centrifuged through 100% FCS with 0.04% sodium azide at 4°C and the cell pellets counted in a gamma counter (Packard Instrument Company, Meriden, CT). Nonspecific binding, which was never more than 25% of total binding, was determined by counting the pellet from cells that were incubated in 150-fold excess, unlabeled GM-CSF for 30 minutes before the addition of labeled factor.

**Thymidine incorporation.** Ba/F3 wild-type cells and cells that expressed GMER or GMR + β were washed three times and plated at 3 × 10^6 cells/well in the appropriate media for the indicated time in microtiter wells containing 160 μL of media. Three hours and 15 minutes before harvesting, 1 μCi of 3H thymidine (New England Nuclear) was added to each of the triplicate samples and incubated at 37°C. Cells were harvested on filter paper by an automated harvester (Skatron, Tranby, Norway) and counted in a β counter (Wallac, Gaithersburg, MD).

**Assay of cell surface glycoporphin.** Ba/F3 cells that expressed GMER, GMR + β, and mEpoR as well as wild-type cells were maintained as previously mentioned. For each analysis, the cells were washed and then resuspended for 2 hours in PBS containing 6% goat serum, 0.02% sodium azide, and a 1/60 dilution of rabbit antimurine-glycoporphin serum or control serum. The cells were washed and resuspended in 6% goat serum and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit (Fab2) was added to a final concentration of 20 μg/mL (Organon Tecknika, Durham, NC). The cells were washed and fixed in 2% paraformaldehyde in PBS, and cell surface immunofluorescence was measured by FACS.

**RESULTS**

**Expression of GMER.** Immunoprecipitation was performed to document the expression of GMER in Ba/F3 cells. Autoradiography of an SDS polyacrylamide gel is shown in Fig 1. A unique protein with a molecular weight of 105,000 daltons is shown in lane 1 when the Ba/F3-GMER cell lysate was immunoprecipitated with anti-c-terminus mEpoR antisera. It is not present in the Ba/F3-GMER cell lysate immunoprecipitated with control serum (lane 2) or in the Ba/F3 wt cell lysate immunoprecipitated with the anti-receptor antisera (lane 3). The size of the unique immunoprecipitated
band is similar to that predicted for a protein comprising the glycosylated extracellular domain of the GMR and the larger cytoplasmic domain of the mEpoR (GMR portion = 37,500, mEpoR cytoplasmic tail = 24,500, glycosylation = 40,000).

**GM-CSF binding to GMER.** The expression of cell surface GMER was characterized by the binding of $^{125}$I-GM-CSF to Ba/F3 cells. The results are shown as a Scatchard plot (Fig 2). These data are from one of four similar experiments. Ba/F3-GMER were observed to have a $k_d$ for hGM-CSF of $3.0 \times 10^{-9}$ mol/L (range 3.0 to 7.5 $\times 10^{-9}$ mol/L) and the transfected receptor was expressed at 475 sites/cell (range 450 to 1,100 sites/cell). When the GMR receptor was expressed in the same cells, the $k_d$ was $3.5 \times 10^{-9}$ mol/L (range 3.0 to 6 $\times 10^{-9}$ mol/L) and there were 300 receptor sites/cell (range 60 to 300 sites/cell). Thus, both GMER and GMR bind GM-CSF with identical low affinities. Because both cell lines were subjected to selection by FACS, the differences in the number of sites per cell cannot be interpreted as suggesting a regulatory role for the cytoplasmic domain in cell surface receptor expression.

**Growth characteristics of cells that express GMER.** To determine whether the GMER expressed in murine Ba/F3 cells could transduce a GM-CSF-dependent proliferation signal, a growth curve was performed (Fig 3A). In this experiment (representative of four experiments), Ba/F3 cells that express GMER or GMR + $\beta$ were grown in the presence of hGM-CSF ($2.0 \times 10^{-7}$ mol/L or $3.3 \times 10^{-10}$ mol/L, respectively). As controls, Ba/F3-GMER were cultured in mIL-3 ($5.5 \times 10^{-11}$ mol/L) and Ba/F3 wt were cultured in hGM-CSF ($2.0 \times 10^{-7}$ mol/L). At the indicated times, the cells were harvested after a 3.25-hour incubation with thymidine. The zero time point is the time that the cells were plated. Ba/F3-GMER and Ba/F3-GMR + $\beta$ proliferated when cultured in the presence of hGM-CSF. Both had a doubling time of approximately 13 hours, which was identical to the growth rate of Ba/F3-GMER in mIL-3. As expected, the wild-type cells did not incorporate thymidine when incubated in hGM-CSF.

A dose response of these cells to GM-CSF was then performed to see whether the observed proliferation corresponded to receptor occupancy. The cells were cultured in the presence of the indicated concentrations of GM-CSF and thymidine incorporation was measured 24 hours after the cells were plated. As shown in Fig 3B, Ba/F3-GMER and Ba/F3-GMR + $\beta$ responded to hGM-CSF in a dose-dependent manner. In Ba/F3-GMER $\frac{\beta}{2}$ maximal stimulation (range 2.5 to $40 \times 10^{-8}$ mol/L) occurred at a GM-CSF concentration of $2.5 \times 10^{-8}$ mol/L. In contrast, Ba/F3 cells expressing GMR
+ β had ½ maximal growth at 1 × 10^{-12} mol/L GM-CSF (range 1 to 10 × 10^{-12} mol/L), consistent with the presence of high-affinity GM-CSF binding on these cells. The dose response of Ba/F3-GMER to mIL-3 was identical to the response of Ba/F3-GMR + β to hGM-CSF, as Ba/F3 cells express both the α and β subunits of the murine IL-3R. Similar growth characteristics were observed in NSF-78 cells transfected with GMER (data not shown). The data show that the proliferative response to GM-CSF in cells that express GMER occurs over a concentration of GM-CSF that correlates with occupancy of the low-affinity hybrid receptor and is not a result of a decreased sensitivity of these cells to growth factors.

**Induction of cell surface glycophorin by GMER.** In addition to proliferative signaling, we wanted to determine whether the GMER could transmit a signal specific for its cytoplasmic tail. We postulated that the cytoplasmic region of the GMER might confer an erythroid-specific response because it consists of the EpoR tail. Glycophorins are a set of cell surface erythroid-specific membrane proteins that increase during erythroid differentiation. Rabbit polyclonal antibody against glycophorin, reactive against all the murine forms of this molecule, was used to assay cell surface expression of this protein in Ba/F3 cells that expressed either GMER, mEpoR or GMR + β. As shown in panel B of Fig 4, Ba/F3-GMER grown in GM-CSF expressed cell surface glycophorin. Ba/F3 cells transfected with mEpoR and grown in erythropoietin demonstrated similar glycophorin expression (Fig 4C). Because the β chain of the GM-CSF receptor is thought to be important for high-affinity “physiological” signal transduction, Ba/F3-GMR + β cells were tested in a similar manner. In sharp contrast, no detectable cell surface glycophorin was observed when these cells were grown in GM-CSF (Fig 4A). These data show that in addition to stimulating proliferation, the binding of GM-CSF to GMER also results in the transmission of specific differentiation signals through the erythropoietin receptor cytoplasmic domain.

**DISCUSSION**

To identify domains in the GM-CSF or erythropoietin receptors that are involved in signal transduction, we developed a hybrid GM-CSF/erythropoietin receptor. Such hybrid molecules have been used to characterize domains of other cell surface receptors. The transfected HGF hybrid receptor GMER has been expressed in a variety of cell lines, including DA-3, Ba/F3, NSF-78, and FDCP-1. Binding studies show that GMER, like GMR, binds GM-CSF with low affinity. Murine IL-3-dependent cell lines transfected with this hybrid proliferate in a dose-dependent manner in the presence of hGM-CSF. This demonstrates that the GM-CSF cytoplasmic tail can be substituted by the murine EpoR tail, without abrogating the proliferation signals elicited by GM-CSF. However, the concentration of GM-CSF required for ½ maximal proliferation is somewhat higher than that expected from the kd for GM-CSF of the hybrid receptor. This suggests that receptor signalling may be compromised. If homodimerization of the epoR proves relevant for signal transduction and is dependent on the extracellular region, as suggested by studies on the highly homologous human growth hormone receptor, then it is possible that the substituted GM-CSF extracellular domain does not dimerize as efficiently. A postreceptor signalling defect is unlikely because Ba/F3-mEpoR have an appropriate proliferative response to erythropoietin.

In contrast to this study, Mori et al18 found that a hybrid receptor which consisted essentially of the extracellular and transmembrane domains of the IL-2 receptor β, and the cytoplasmic region of the mEpoR, failed to support IL-2-dependent proliferation in a Ba/F3 subclone. One explanation is that the maximal concentration of IL-2 they tested (1.0 × 10^{-9} mol/L) was lower than the concentration of hGM-CSF required to give stimulation in our system. Another possibility is that in addition to the mEpoR tail, the extracellular and/or transmembrane regions of the GM-CSF receptor may also be important for ligand induced proliferation by GMER. Interactions between the GM-CSF receptor portion of GMER with either other GM-CSF receptor subunits or with other accessory molecules may be responsible.

To determine if the mEpoR segment of GMER has a specific role in the signal transduction pathways that leads to differentiation, the expression of cell surface glycophorin on Ba/F3 cells was examined. Murine glycophorins are a set of integral membrane proteins and, like their human counter-
parts, are essentially restricted to erythroid cells. The pro-
erythroblast is the earliest cell on which glycophorin has been
detected.19 Expression of GMER in Ba/F3 exposed to hGM-
CSF induced cell surface glycophorin, as measured by im-
munofluorescence. The expression of mEpoR in these cells
also resulted in immunologically detectable glycophorin lev-
els. However, GMR alone (not shown) or in combination
with common β was unable to induce an increase in surface
glycophorin expression. This experiment indicates that the
cyttoplasmic domain of the Epo receptor is sufficient to stim-
ulate the expression of this lineage-specific protein even when
stimulated by GM-CSF, and implies that the cyttoplasmic
domains of growth factor receptors do not merely act per-
missively for cell growth and survival, but can directly induce
cellular differentiation; this is consistent with studies with c-
fms20 and nonhematopoietic receptors such as the EGF re-
ceptor.21 or a hybrid NGF-EGF receptor.22 However, despite
their expression of glycophorin, Ba/F3 cells containing the
GMER do not express β globin message (assayed by RNase
protection) and do not terminally differentiate into eryth-
rocytes (data not shown). Additional signals such as those
transmitted by the mEpoR transmembrane domain may be
required. Alternately, a component of the differentiation po-
tential of Ba/F3 cells could be defective. The latter is possible,
because unlike other pre-B cell lines made in a similar fashion,
the parental Ba cell line does not differentiate to mature B
cells in vivo.23

In conclusion, a hybrid GM-CSF/erythropoietin receptor
has been shown to confer both proliferative and erythroid
specific differentiative signals in response to GM-CSF in mu-
rine cells. Further analysis of this system may allow the de-
tection of specific receptor domains that control proliferation
and differentiation.

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