Functional Analysis of a Single Chain Chimeric α/β-Granulocyte-Macrophage Colony-stimulating Factor Receptor

IMPORTANCE OF A GLUTAMATE RESIDUE IN THE TRANSMEMBRANE REGION

(Received for publication, March 18, 1999, and in revised form, August 3, 1999)

Sabine Kafert, Susanne Luther, Inga Böll, Katharina Wagner, Arnold Ganser, and Matthias Eder†‡

From the Department of Hematology and Oncology, Hannover Medical School, D-30625 Hannover, Germany

To analyze the function of each subunit of the receptor for granulocyte-macrophage colony-stimulating factor (GM-CSF), GMR, we previously generated a single-chain chimeric receptor by fusion of the extracellular and transmembrane domain from the α-subunit (α-GMR) to the intracellular part of the β-subunit (β-GMR) introducing an additional glutamate residue at the fusion site (α/β-GMR). We demonstrated the capacity of α/β-GMR to bind GM-CSF with low affinity and to induce GM-CSF-dependent activation of tyrosine kinase activity and proliferation in transfected Ba/F3 cells. To further compare the functions of wild type and chimeric receptors, we now report that this α/β-GMR is sufficient to mediate morphological changes, expression of αc- and βc-integrin receptor subunits, and serine-phosphorylation of Akt kinase. To analyze the function of the glutamate residue at the fusion region of α/β-GMR various point mutants changing this amino acid and its position were expressed in Ba/F3 cells. None of these mutants was capable of supporting GM-CSF-dependent proliferation; however, when β-GMR was coexpressed, GM-CSF mediated short and long term proliferation. Interestingly, some mutants but not α/β-GMR can induce proliferation in the presence of an anti-α-GMR antibody. These data demonstrate the significance of a glutamate residue in the transmembrane region of α/β-GMR for ligand-induced receptor activation.

Granzanoocyte-macrophage colony-stimulating factor (GM-CSF) mediates proliferation, survival, differentiation, and functional activities in myeloid cells (1, 2). GM-CSF binds to the GM-CSF receptor (GMR), consisting of a low affinity binding α-subunit (α-GMR) and a high affinity converting β-subunit essential for signal transduction and shared with the receptors for IL-3 and IL-5 (common β, β3-IL/GMR) (3–6). Both receptor chains belong to the cytokine receptor family characterized by common sequence and structural motifs such as the WSXWS motif in the extracellular domain close to the cell membrane (7).

The initial event of ligand-induced cytokine receptor activation are not completely understood. For single chain receptors such as EpoR or granulocyte colony-stimulating factor receptor, ligand-induced receptor dimerization is believed to mediate activation of receptor associated tyrosine kinases of the JAK family (8, 9). However, recent studies on the EpoR demonstrate a ligand-induced conformational change in preformed receptor complexes and suggest that dimerization or oligomerization may be necessary but not sufficient for the initiation of intracellular signal transduction (10). Both models for receptor activation, ligand-induced assembly of receptor chains and ligand-dependent conformational changes in preformed receptor complexes, may also be involved in the activation of heteromeric cytokine receptors such as GMR. Indeed, we have shown the GM-CSF-dependent coimmunoprecipitation of human α-GMR and β-GMR in transfected NIH3T3 cells (11), and Woodcock et al. (12) demonstrated the existence of both preformed and GM-CSF-inducible GMR complexes with different ratios of each in different hematopoietic cells.

The initial event in intracellular signaling of cytokine receptors is believed to be the transphosphorylation and activation of members of the JAK tyrosine kinase family. The kinases may constitutively associate with receptor chains as demonstrated for the interaction of JAK2 and β-GMR (13) or EpoR (14), and the kinases may be brought into a steric conformation enabling activation upon ligand-receptor interaction (10). Alternatively, JAKs may be recruited into ligand-activated receptor complexes (15), for example by ligand-induced unfolding of motifs in receptor subunits involved in receptor-kinase interaction. To analyze the contribution of individual receptor domains of human α-GMR and β-GMR, we previously cloned a chimeric α/β-GMR by fusion of the extracellular and transmembrane sequence of α-GMR to the intracellular part of β-GMR that we found capable of GM-CSF binding with low affinity, receptor internalization, induction of tyrosine kinase activity, and short and long term proliferation of transfected Ba/F3 cells in the absence of wild type human α-GMR and β-GMR (11). This α/β-GMR contains an additional glutamate residue at the fusion region introduced for cloning purposes. In contrast to this construct, several groups reported on slightly different chimeric α/β-GMR and IL-5Rα/β-GMR constructs that were only functional as high affinity receptors in the presence of β-GMR (16, 17). It was concluded from these studies that the cytoplasmic domain of α-GMR, although indispensable for the function of the wild type GMR (18–20), does not harbor nonredundant and necessary sequences for the functions analyzed so far in transfected cells. However, some unique signaling capacities of α-GMR have been reported (21, 22), and ligand-dependent signaling even in the absence of β-GMR such as induction of...
glucose uptake has also been linked to α-GMR (25).

To further compare the functions mediated by the wild type GMR and the chimeric α/β-GMR and to identify potential defects due to the lack of α-GMR sequences, we now extend the characterization of α/β-GMR and demonstrate its capacity to signal for reorganization of the cellular cytoskeleton and morphological changes, for surface expression of α, and β intégrins, and for serine phosphorylation of Akt protein kinase at Ser-473 in a GM-CSF-dependent manner. Moreover, we demonstrate the essential function of a glutamate residue at a specific position at the transition of the transmembrane to the cytoplasmic region for ligand-induced receptor function, providing new insights into initial events of GMR activation.

MATERIALS AND METHODS

Reagents—Restriction enzymes, DNA-modifying enzymes, and DNA molecular weight markers were purchased from MBI Fermentas (Vilnius, Lithuania) or New England Biolabs (Schwalbach, Germany). All PCR primers were synthesized by MWG-BIOTECH (Ebersberg, Germany). Fine chemicals were purchased from Sigma (Deisenhofen, Germany) and were of analytical or molecular biology grade. Agarose for gel electrophoresis was from Life Technologies, Inc.

Pair-Wise Cloning of Human α/β-GMR Mutants—Cloning of the chimeric receptor. The GMR introducing a glutamate residue has been described (11). Nucleotide positions of the β-GMR cDNA insert are given according to Hayashida et al. (3). Site-directed mutagenesis was performed using the QuickChange Mutagenesis Kit (Stratagene) and oligonucleotides encoding the desired mutant sequences as recommended by the manufacturer (see Table II). Successful cloning was confirmed by restriction analysis and by sequencing of the mutated region. All constructs are cloned into pECD3 (Invitrogen).

Transfection of Ba/F3 Cells—The murine cell line Ba/F3 was cultured in RPMI medium supplemented with glutamic acid, antibiotics, 5% fetal calf serum (Biochrome), and 10% WEHI-conditioned medium for the supply of murine IL (mIL)-3. In initial experiments, recombinant mIL-3 (10 ng/ml; Sigma) was found to be as effective as 10% WEHI-CM to induce proliferation, morphological changes, and Akt phosphorylation. For transfection, 1×10^6 cells were washed twice in RPMI 1640 without any supplements and electroporated at 350 V and 950 microfarads in 800 μl of serum-free medium in the presence of 20 μg of expression vector encoding the respective GMR constructs, purified by phenol/chloroform extraction. After 2 days, the cells were washed, split in half, and selected under two conditions in order to control the capacity of the transfected receptor variant to support GM-CSF-dependent proliferation: 1) in the presence of 20 ng/ml recombinant hGM-CSF but without mIL-3 or 2) in the presence of mIL-3 and the antibiotic G418 (Sigma) at a concentration of 1 μg/ml.

RT-PCR Analysis—For analysis of mRNA expression of α/β-GMR variants, total cellular RNA was prepared from transfected cells using TRIZOTM reagent (Stratagene Technologies) according to the instructions of the manufacturer. cDNA was transcribed from 2.5 μg of total cellular RNA in a 50-μl reaction mix containing 100 units of Moloney murine leukemia virus reverse transcriptase (RT) in the appropriate buffer conditions (Stratagene), 40 units of RNase inhibitor (RNase Block, Stratagene), 1 μg of oligo(dT) (Amersham Pharmacia Biotech), 10 μM dithiothreitol, and nucleotides at a concentration of 1 × each (Roche Molecular Biochemicals). Using the primers 5′-GGAGGACAGGACCCAAAGG (sense) and 5′-CCCCAGCTTGTTGACC (antisense), a β-GMR fragment corresponding to nucleotides 2078-2711 was amplified from 10 μl of the RT reaction in a volume of 50 μl containing 50 pmol of both sense and antisense primer, 4 μl of 10× PCR buffer, including 15 mM MgCl2, and 2.5 units of Taq polymerase (Sigma) by 40 cycles of denaturation (95°C), annealing (55°C), and extension (72°C). As a control, tubulin was amplified under the same conditions using the primers 5′-TTCCCCGACCCGTGGTACGATCGTGTTT (sense) and 5′-CATGTCCTGGCCGCTGGCTATGGTATGGTGA (antisense) (where N represents any nucleotide).

FACS Analysis—Surface expression of the α/β-GMR variants was analyzed using an antibody against an extracellular epitope of the human α-GMR subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; GM-CSF 5-20; 2 μg/10^6 cells). The cells were stained with a fluorescein isothiocyanate-labeled rabbit anti-mouse antibody (Dianova). For analysis of integrin expression, specific antibodies and isotype controls were obtained from Pharmingen (integrin α, mouse CD49d, 01270D, isotype control, 34491A; integrin β, mouse CD29, 08351D; isotype control, 54481A). The cells were stained with an R-phycocerythrin-conjugated F(ab’)2 goat anti-rat IgG Fragment (1623/112-116-143; Dianova). All antibodies were used at 10 μg/ml in phosphate-buffered saline.

Immunoblotting—Transfected Ba/F3 cells were starved overnight for mIL-3 and hGM-CSF, respectively, and restimulated with either hGM-CSF or mIL-3 for 5 min. The cells were prepared as described (11). Briefly, stimulation was stopped by the addition of ice cold phosphate-buffered saline, the cells were collected by centrifugation at 500 g, and the pellets were suspended in lysis buffer. After 15–25 min on ice, the lysates were cleared by centrifugation at 4°C, 12,000 × g for 15 min, and the supernatants were stored in aliquots at −80°C or in Lysenov (Vilnius, Lithuania) or New England Biolabs (Schwalbach, Germany). As a loading control, the same lysates were processed simultaneously and probed with a phosphorylation-independent anti-Akt antibody supplied with the kit.

Binding of 125I-Labeled GM-CSF—Transfected Ba/F3 cells were studied for GM-CSF binding as described (11). Briefly, 7.5 × 10^5 cells were incubated for 60 min on ice with 125I-labeled GM-CSF (NEN Life Science Products) at different concentrations between 0.04 and 6.42 nM in the presence or absence of unlabeled GM-CSF (1 μM). However, in some experiments, saturation could not be reached under the conditions tested even if undiluted 125I-GM-CSF was added to the cell samples. After incubation, the samples were centrifuged, resuspended, and layered over a cushion of 0.7 ml of fetal calf serum. After centrifugation, the supernatant was discarded, and the radioactivity was measured in a Berthold MAG 315 γ-counter. Untransformed specific binding data were analyzed by Scatchard plot assuming a single class of binding sites, Kd and Bmax, represent the equilibrium dissociation constant and the density of the binding sites per cell, respectively.

Proliferation Assay—GM-CSF-dependent proliferation of Ba/F3 cells was assayed using the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Inc., Eugene, OR) according to the instructions of the manufacturer. Briefly, the cells were seeded in 96-well tissue culture plates at a density of 10^5 cells per 200 μl in the absence or presence of hGM-CSF ranging from 1 pg/ml to 1 μg/ml. After 48–72 h, the cells were harvested by centrifugation, lysed in the buffer provided with the kit, and stained with the CyQUANT GR dye for 5 min at room temperature. Fluorescence was quantitated using an enzyme-linked immunosorbent assay reader (540 nm).

Prediction of Protein Structure—Predictions of protein structure were done using the SEAP program, utilizing the parameters of Chou and Fasman (24) and Kyte and Doolittle (25) to predict averaged secondary structure and hydrophobicity in protein chains, respectively. For the analysis discussed in this paper, a range of 4 amino acids was used for the averaged prediction.

RESULTS

α/β-GMR Mediates GM-CSF-dependent Changes in the Cell Morphology—In order to compare the functional capacities of the wild type α + β GMR and the chimeric α/β-GMR and to identify potential defects due to the lack of α-GMR sequences in the chimeric receptor, we expressed α-GMR together with β-GMR or α/β-GMR alone in the murine IL-3-dependent cell line Ba/F3. This cell line expresses the murine IL-3 receptor including the murine common β-chain but does not respond to murine GM-CSF, mIL-3, or hGM-CSF. Since human receptors for GM-CSF lack cross-reactivity to murine cytokines, their function can easily be analyzed after expression in these cells.

Growth factor-induced changes in cell morphology of Ba/F3 cells were studied by phase-contrast microscopy every 10 min after starvation and restimulation with mIL-3 and hGM-CSF, respectively. When untransfected, wild type α + β-GMR- or chimeric α/β-GMR-expressing Ba/F3 cells were starved for growth factor overnight, all cells in culture were of a circular shape the next morning. However, 1 h after restimulation with mIL-3 or hGM-CSF for GMR-expressing cells, symmetric and unregular, biconcave shape were present, which increased in

2 C. Urbanke, personal communication.
frequency to up to 50% within 6 h. In wild type α + β-GMR- and in chimeric α/β-GMR-expressing cells, the effects of GM-CSF were more modest as compared with mIL-3 in some experiments. The mIL-3- or hGM-CSF-induced changes in cell morphology could be delayed by preincubation with the PI-3K inhibitor wortmannin and severely diminished or completely blocked by the repeated addition of wortmannin every 60 min without any loss of cell viability (Table I (top) and data not shown).

**GM-CSF-dependent Up-regulation of α4- and β1-Integrin Expression in α/β-GMR-expressing Ba/F3 Cells**—In previous studies, the ability of Ba/F3 cells to adhere to fibronectin has been used to monitor functional activity of these cells (26). Since we noted adhesion of α/β-GMR-expressing Ba/F3 cells to fibronectin, but not to collagen or gelatin, after stimulation with mIL-3 or hGM-CSF in a Ca2+-dependent manner in preliminary experiments, the surface expression of murine α4- and β1-integrin subunits was studied by FACS analysis. When untransfected Ba/F3 cells were starved and restimulated with mIL-3, a slight but reproducible up-regulation of α4- and β1-integrin subunits at the cell surface was first detectable after 1 h and increased further during a time course of 3–6 h (data not shown). Similarly, in α/β-GMR-expressing Ba/F3 cells stimulated for 4 h with either mIL-3 or hGM-CSF, a small but reproducible up-regulation of α4- and more distinct β1-integrin expression was detected in several independent clones of αβ-GMR-transfected cells (Fig. 1). hGM-CSF induced up-regulation of integrin subunits to the same extent as mIL-3.

**IL-3- and GM-CSF-induced Serine Phosphorylation of Akt Protein Kinase**—The phosphorylation of the serine/threonine kinase Akt at the regulatory position, Ser-473, was analyzed in untransfected, wild type α-GMR-, wild type α + β-GMR-, or chimeric α/β-GMR-expressing Ba/F3 cells. Cells were starved overnight and restimulated the next morning with either mIL-3 or hGM-CSF. Akt phosphorylation was analyzed in cellular lysates by immunoblotting with a monoclonal antibody specific for Akt phosphorylated at Ser-473. Akt phosphorylated at Ser-473 is undetectable in untransfected Ba/F3 cells after overnight starvation but is clearly found after 5 min of stimulation with mIL-3 (Fig. 2A). As expected, untransfected cells do not respond to hGM-CSF. In contrast, both wild type α + β-GMR- and α/β-GMR-transfected Ba/F3 cells display Akt phosphorylation at Ser-473 in response to both mIL-3 and hGM-CSF (Fig. 2B). The extent of Akt phosphorylation seems to be lower after stimulation with hGM-CSF as compared with mIL-3 in these experiments. When the cells were pretreated with wortmannin, the level of Akt phosphorylation was considerably lowered (Fig. 2B). Notably, in Ba/F3 transfectants expressing α-GMR alone, GM-CSF did not induce detectable phosphorylation of Akt at Ser-473.

**Cloning and Expression of α/β-GMR Point Mutants**—In contrast to chimeric constructs composed of sequences from α-GMR and β-GMR reported by others (16), α/β-GMR is capable of GM-CSF-dependent signal transduction as a low affinity GMR in the absence of β-GMR. We therefore analyzed the function of the additional glutamate residue introduced at the fusion region of the transmembrane α-GMR sequence to the cytoplasmic sequence of β-GMR by generation of the following point mutations using site-directed mutagenesis (Table II): deletion of the additional glutamate residue (αβ-GMR-DGlu); replacement with glutamine (αβ-GMR-Gln) or glycine (αβ-GMR-Gly), respectively; and positioning of the glutamate residue by 1 (αβ-GMR-Glu−1) or 14 amino acids (αβ-GMR-Glu−14). This generated an L16 (27). α/β-GMR and all variants were transfected into Ba/F3 cells, and clones were selected either in hGM-CSF- or with G418 in the presence of mIL-3. Whereas Ba/F3 cells transfected with the αβ-GMR cDNA could be grown in the presence

### Table I

**Morphological changes of Ba/F3 cells in response to growth factor**

Untransfected Ba/F3 cells (top) and transfected Ba/F3 cells (bottom) were starved overnight and restimulated the next morning with either 10% WEHI supernatant as a source of murine IL-3 (mIL-3) or with hGM-CSF (GM; 20 ng/ml). Wortmannin (Wor; 20 nm final concentration) was given 45 min prior to growth factor stimulation and at the time of stimulation alone (single dose) or every hour (repeated treatment; rep.). Thereafter, each sample was evaluated by phase-contrast microscopy after 1, 2, 3, 4, and 5 h for the presence of cells with marked morphology alterations.

| IL-3 | We | Untransfected Ba/F3 cells |
|------|----|--------------------------|
|      |    | 1 h 2 h 3 h 4 h 5 h     |
|      |    | − − − + + +            |
|      |    | + + + + + −            |
|      |    | + (+) + + + + −       |
|      |    | + (+) (+) (+) + + +   |
|      |    | + (+) (+) (+) (+) + + |

| Transfectant | IL-3/GM | We | Transfected Ba/F3 cells |
|--------------|---------|----|------------------------|
| αβ-GMR       | IL-3    | −  | (+) + + +             |
| αβ-GMR       | IL-3    | −  | (+) + + +             |
| αβ-GMR       | GM      | −  | (+) (+) + +           |
| αβ-GMR       | IL-3    | −  | (+) (+) + +           |
| αβ-GMR       | GM      | −  | (+) (+) + +           |

**Fig. 1. Surface expression of integrin subunits α4 and β1 in Ba/F3 cells.** Ba/F3 cells transfected with αβ-GMR were starved overnight for growth factor and stimulated the next morning with mIL-3 or hGM-CSF (hGM) for 4 h. Surface expression of integrin subunits α4 and β1 was detected by FACS analysis and is shown as absolute number of positive cells graphed versus fluorescence intensity. Increased surface expression after growth factor stimulation is shown in comparison with the unstimulated controls for two representative clones transfected with αβ-GMR. Negative controls with the second step antibody alone and with isotype control antibodies were identical in any conditions (not shown).
of hGM-CSF as reported earlier (11), only drug-resistant and mIL-3-dependent clones could be selected for all α/β-GMR mutants (up to three independent experiments per mutant; data not shown). Expression of the receptor cDNA was confirmed on the mRNA level by RT-PCR, and surface expression was studied by FACS analysis (Fig. 3). While α/β-GMR and α/β-GMRΔGlu were expressed at comparable levels, surface expression of α/β-GMR-Gln, α/β-GMR-Gly, and α/β-GMR-Glu(−14) was more modest. Surface expression of α/β-GMR-Glu(−1) could not be detected by FACS analysis in eight independent RT-PCR positive clones. Nevertheless, the variant turned out to be functionally expressed as confirmed by cotransfection of wild type β-GMR (see below). To analyze GM-CSF binding by α/β-GMR mutants with comparable receptor expression as determined by FACS analysis, the equilibrium binding characteristics of the clones α/β-GMR and of α/β-GMRΔGlu were studied. The estimated values for $K_D$ were 1.5 and 1.2 nM, and the receptor numbers per cell were 4700 and 2000 for α/β-GMR- and α/β-GMRΔGlu-expressing cells, respectively, as derived from Scatchard plot analysis. We also performed iterative fitting by least square regression and obtained comparable values (data not shown).

**Function of α/β-GMR Point Mutants**—For functional analysis, two independently derived Br/F3 clones expressing each of the α/β-GMR mutants were tested for GM-CSF-dependent proliferation in the absence of mIL-3. Except for α/β-GMR transfectants, Br/F3 cells expressing any of the α/β-GMR mutants failed to proliferate in the absence of mIL-3, and all cells died within 48–72 h in the presence of hGM-CSF. However, when Br/F3 cells expressing mutant α/β-GMR constructs were co-transfected with wild type β-GMR and selected with hGM-CSF, double transfectants were obtained for every α/β-GMR mutant. Again, GM-CSF-dependent proliferation was assayed for double transfected cells. In contrast to the single transfectants, all double-transfected Br/F3 cells were capable of signaling for GM-CSF-dependent proliferation (Fig. 4, A–F), whereas Br/F3 cells transfected with β-GMR alone did not grow in the presence of hGM-CSF (data not shown). In addition, all double transfected clones besides α/β-GMR-Glu(−1)-expressing cells could be grown in hGM-CSF for several weeks in a factor-dependent manner. In contrast, α/β-GMR-Glu(−1)-expressing cells tended to transform rapidly to factor-independent growth either in the presence or absence of β-GMR after culture in mIL-3 or hGM-CSF in some experiments (data not shown).

To address the effect of bridging the single chain chimeric α/β-GMR mutants, α/β-GMR- and α/β-GMRΔGlu-transfected Br/F3 cells were seeded without growth factor in the presence of increasing concentrations of an anti-α-GMR antibody (Fig. 5). Interestingly, cross-linking of α/β-GMRΔGlu but not α/β-GMR by an anti-α-GMR antibody was sufficient to induce cell survival and proliferation in a dose-dependent manner. Similarly, α/β-GMR-Gln-transfected Br/F3 cells were also able to grow in the presence of anti-α-GMR antibody (data not shown).

**Prediction of Secondary Protein Structure**—To further analyze the role of the glutamate residue close to the transition of the transmembrane to the cytoplasmic region, we performed prediction of the hydrophobicity and of the secondary structure of this region for α/β-GMR and each of the α/β-GMR mutants in comparison with the wild type subunits (Fig. 6 and data not shown). Introduction of either glutamate (α/β-GMR) or glutam...
Fig. 3. Surface expression of α/β-GMR variants expressed in Ba/F3 cells. Stable transfectants generated with the α/β-GMR point mutants described in Table II were stained for surface expression of the receptor with an antibody against the extracellular part of human α-GMR in comparison with untransfected Ba/F3 cells. The absolute numbers of positive cells are graphed versus fluorescence intensity.
mine \((\alpha/\beta\text{-GMR-Gln})\) markedly changes the hydrophobicity as compared with \(\alpha/\beta\text{-GMR-DGlu}\) (Fig. 6). As expected, the effect of glycine introduction is more modest, while introduction of glutamate at positions 21 and 214 again enhance the hydrophilicity at the respective sites (not shown). Interestingly, the analysis suggests a \(\beta\)-sheet rather than \(\alpha\)-helical conformation of this part of the transmembrane domain for both wild type \(\alpha\)- and \(\beta\)-GMR as well as the chimeric variants.

**DISCUSSION**

This study explores the functional capacities of a low affinity single chain chimeric \(\alpha/\beta\)-GMR in the absence of intracellular \(\alpha\)-GMR sequences when stably expressed in Ba/F3 cells. We have previously shown that the chimeric \(\alpha/\beta\)-GMR is sufficient for GM-CSF-dependent short and long term proliferation of Ba/F3 and 32D cells (Ref. 11 and data not shown) and of inducing GM-CSF-dependent tyrosine phosphorylation of cellular substrates. We show here that \(\alpha/\beta\)-GMR-expressing Ba/F3 cells also display GM-CSF-dependent changes in cell morphology, surface expression of integrin subunits, and serine phosphorylation of the serine/threonine kinase Akt (or protein kinase B), implicated in antiapoptotic signaling (28, 29).

Cytoskeletal function and integrin expression are regulated by IL-3 or GM-CSF in wild type GMR and in \(\alpha/\beta\)-GMR-expressing Ba/F3 cells. Both functions are involved in regulating the interaction of hematopoietic cells with the microenvironment, and defects of both cytoskeletal functions and adhesion are found in Bcr-Abl-expressing cells (30, 31). The biochemical events mediating the effects of IL-3 or GM-CSF on cell morphology and \(\alpha_4\text{- and } \beta_1\text{-integrin expression are currently not exactly characterized, but they are likely to involve small GTPases of the Rac/Rho/Cdc42 family for mediating membrane ruffling, formation of actin stress fibers, lamellipodia, and increased cell motility (32). We show here that IL-3- and GM-CSF-induced changes in cell morphology of Ba/F3 cells require activity of PI-3K and do not necessarily require sequences encoded by the cytoplasmic region of \(\alpha\)-GMR.

The serine/threonine kinase Akt is linked to the apoptotic cell machinery through at least one of its substrates, the proapoptotic BCL-2 family member BAD, which can be phosphorylated at Ser-136 by Akt (29). BAD interacts with 14-3-3 protein isoforms in a phophoserine-dependent manner that appears to prevent the proapoptotic association of BAD with BCL-2 or BCL-X\(_L\) (29). In addition, Akt has been shown to become phosphorylated and activated by IL-3 in hematopoietic...
Phosphorylation of Akt after stimulation with GM-CSF but can currently not be essential for survival of hematopoietic cells under the conditions tested. GM-CSF-dependent effects on cell morphology, integrin surface expression, and Akt phosphorylation in α/β-GMR-transfected Ba/F3 cells occur in the absence of cytoplasmic α-GMR sequences. Therefore, with respect to all of these functions and in addition to survival, proliferation, receptor internalization, and tyrosine phosphorylation (11), the intracellular α-chain can be replaced by sequences of β-GMR in this specific α/β-GMR. Interestingly, human β-GMR has been described to exist as a constitutively preformed homodimer both in vivo (35) and in vitro. Since the intracellular domain of α-GMR cannot be deleted in the wild type GMR without loss of receptor function (18–20), our data suggest a potential role of intracellular α-GMR sequences in the initial positioning or in inducing an appropriate conformational change of β-GMR chains leading to phosphorylation of Jak2 through the wild type GMR. Additionally, sequences from α-GMR may also be involved in terminating GMR signaling.

Point mutations in the transmembrane region of α/β-GMR were found to affect surface expression, GM-CSF binding, and functions such as mitogenic signaling, cytoskeletal changes, and phosphorylation of Akt in Ba/F3 cells expressing the respective GMR chimeras. Whereas deletion of the glutamate residue slightly enhances surface expression as compared with α/β-GMR, replacement by glycine or glutamine at identical positions as well as positioning of the glutamate residue 14 amino acids to the N terminus decreases expression of the respective receptor. Interestingly, surface expression of α/β-GMR-Glu(−1) was not detectable at all by FACS analysis, but GM-CSF-dependent signaling upon coexpression of β-GMR implies its presence at the cell surface. We are currently investigating the mechanism of the reduced surface expression of the α/β-GMR-Glu(−1) variant in comparison with the other receptor chimeras.

The mechanism involved in activation of the single chain α/β-GMR, i.e. GM-CSF-induced homo-oligomerization or association with endogenous signaling molecules such as AIC2B or molecules that substitute for the function of α-GMR sequences, are currently not known. To distinguish between these possibilities, we transfected murine 3T3 fibroblasts with the cDNA encoding α/β-GMR. So far we could not find any biochemical evidence for GM-CSF-dependent receptor function in several independent RT-PCR positive clones. However, since we failed to prove surface expression of α/β-GMR so far, we cannot decide between both mechanisms of receptor activation in the fibroblast model (data not shown). In addition, receptor cross-linking by an anti-α-GMR antibody had no proliferative effect in α/β-GMR transfected Ba/F3 cells. In contrast, α/β-GMR-Glu and α/β-GMR-Gln-transfected Ba/F3 cells, which cannot prolif-
erate in response to hGM-CSF, were able to survive and proliferate in the presence of the tested anti-α-GMR antibody in a dose-dependent manner. Therefore, the glutamate residue in the single chain chimeric α/β-GMR is essential for ligand-dependent receptor function but precludes activation by the cross-linking antibody used in this study. Since the reverse is true for the variants of α/β-GMR-ΔGlu and α/β-GMR-Gln, ligand- and antibody-dependent receptor activation seem to be mutually exclusive.

As shown by the present study, a glutamate residue at a unique position in the transmembrane region at the transition to the cytoplasm is necessary for GM-CSF-dependent signaling of α/β-GMR. Prediction of secondary protein structure cannot explain the unique features of the glutamate residue, since there was no significant difference between the functional α/β-GMR and the nonfunctional α/β-GMR-Gln. These data suggest that the negative charge introduced by the glutamate residue in α/β-GMR as opposed to glutamine is essential for ligand-dependent receptor function of α/β-GMR. Interestingly, there are several observations describing a specific role of glutamate residues within the transmembrane region for constitutive receptor activation. First, mutational screens for activating mutations in β-GMR revealed, among others, an amino acid exchange V449E within the transmembrane domain, whereas V449Q does not activate β-GMR (27). Second, the oncogenic activity of the neu/neu/erb gene product relies on a V664E substitution localized within the transmembrane domain (36, 37). There is experimental evidence for this mutation to enhance constitutive oligomerization of oncogenic neu-V664E in contrast to e-neu (38, 39). When a panel of neu mutations was investigated, it turned out that besides V664E only V664Q and, to a much lower extent, V664D, had transforming capacity, in contrast to V664E or V665E and four other amino acid substitutions at position 664 (Gly, Lys, His, and Tyr) (36). However, when a mutation analogous to neu-V664E was introduced into the epidermal growth factor receptor, activity of the receptor remained fully ligand-dependent (40). In addition, charged amino acids in the transmembrane region are also believed to be essential for the interaction of natural killer cell inhibitory receptor with the immunoreceptor DAP12 (41).

Constitutive receptor oligomerization is believed to be necessary for constitutive receptor activation. For example, the transforming R129C mutation in the EpR α polypeptide leads to constitutive receptor dimerization (42). However, the actual situation is more complex, since dimerization of EpR may be necessary but not sufficient for receptor activation (10, 43). In contrast to constitutively active receptor mutants, the α/β-GMR described remains fully ligand-dependent. Structural data of the transmembrane and the membrane-proximal region are needed to understand the molecular details of receptor interactions determining gain or loss of function of cellular receptors in a ligand-dependent or -independent manner.

Acknowledgments—We acknowledge Claus Urbanke, Medical School Hannover, for carrying out predictions of secondary protein structure and assisting with their interpretation.

REFERENCES

1. Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. G., Harlan, J. M., Klemanoff, S. J., Waltersdorph, A., Wong, G., Clark, S. C., and Vadas, M. A. (1980) J. Clin. Invest. 78, 1220–1228
2. Metcalf, D. (1989) Nature 339, 27–30
3. Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K.-I., Yokota, T., and Miyajima, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9655–9659
4. Kitamura, T., Sato, N., Arai, K., and Miyajima, A. (1991) Cell 66, 1165–1174
5. Tavernier, J., Devos, R., Cornelis, S., Tuytens, T., Van der Heyden, J., Fiers, W., and Plaetinck, G. (1991) Cell 66, 1175–1184
6. Bagley, C. J., Woodcock, J. M., Stomski, F. C., and Lopez, A. F. (1997) Blood 89, 1471–1482
7. Bazan, J. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6934–6938
8. Schnidler, C., and Darnell, J. E. (1995) Annu. Rev. Biochem. 64, 621–651
9. Ihle, J. N. (1995) Nature 377, 591–594
10. Livnah O., Stura, E. A., Wong, G., Clark, S. C., and Vadas, M. A. (1995) Biophys. Biochem. Res. Commun. 208, 368–375
11. Takagi, S., Kanazawa, H., Shibata, M., and Takatsu, K. (1994) Mol. Cell. Biol. 14, 7494–7413
12. Weiss, M., Yokoyama, C., Shikama, Y., Naugle, C., Druker, B., and Sieff, C. A. (1993) Blood 82, 3298–3306
13. Sakamaki, K., Miyajima, I., Kitamura, T., and Miyajima, A. (1992) EMBO J. 11, 3541–3549
14. Ronco, L. V., Doyle, S. E., Raines, M., Park, L. S., and Gasson, J. C. (1995) J. Immunol. 154, 3444–3453
15. Doyle, S. E. and Gasson, J. C. (1998) Blood 92, 867–876
16. Matsuguchi, T., Zhao, Y., Lilly, M. B., and Krafth, A. S. (1997) J. Biol. Chem. 272, 17450–17459
17. Ding, D. X., Rivas, C. I., Heaney, M. L., Raines, M. A., Vera, J. C., and Golde, D. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2537–2541
18. Chou, P. Y., and Fasman, G. D. (1974) Biochemistry 13, 211–222
19. Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
20. Okusa, K., Smith, L., Griffin, J. D., and Foster, R. (1997) Blood 90, 4759–4766
21. Jenkins, B. J., D’Andrea, A. D., and Gonda, T. J. (1995) EMBO J. 14, 4267–4268
22. Hemmings, B. A. (1997) Science 275, 628–630
23. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotch, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
24. Salgia, R., Li, J. L., Ewaniuk, D. S., Pear, W., Psick, E., Burke, S. A., Ernst, T., Sattler, M., Chen, L. B., and Griffin, J. D. (1997) J. Clin. Invest. 100, 46–57
25. Bhatia, R., Wayner, E. A., McIlave, P. G., and Verfaillie, C. M. (1994) J. Clin. Invest. 94, 384–391
26. Nohes, C. D., and Hall, A. (1995) Cell 81, 53–62
27. Songyang, Z., Baltimore, D., Cantley, L. C., Kaplan, D. R., and Franke, T. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11345–11350
28. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. S., Kaplan, D. R., and Greenberg, M. E. (1997) Science 275, 661–665
29. Muto, A., Watanabe, S., Miyajima, A., Yokota, T., and Arai, K. (1996) J. Exp. Med. 183, 1911–1916
30. Bargmann, C. I., and Weinberg, R. A. (1988) EMBO J. 7, 2043–2052
31. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) Cell 45, 649–657
32. Sternberg, M. J., and Gullik, W. J. (1989) Nature 339, 587
33. Weiner, D. B., Liu, J., Cohen, J. A., Williams, V. W., and Greene, M. I. (1989) Nature 339, 230–231
34. Carpenter, C. D., Ingraham, H. A., Cochet, C., Walton, G. M., Lazar, C. S., Sowadski, J. M., Rosenfeld, M. G., and Gill, G. N. (1991) J. Biol. Chem. 266, 570–575
35. Lanier, L. L., Curiel, B. C., Wu, J., Leong, C., and Phillips, J. H. (1998) Nature 391, 703–707
36. Watowich, S. S., Yoshimura, A., Longmore, G. D., Hilen, D. J., Yoshimura, Y., and Lodish, H. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2140–2144
37. Remy, I., Wilson, A., and Michnick, S. W. (1999) Science 283, 990–992
38. Gearing, D. P., King, A., Gough, N. M., and Nicola, N. A. (1989) EMBO J. 8, 3667–3676
39. Stern, D. F., Heffernan, P. A., and Weinberg, R. A. (1986) Mol. Cell. Biol. 6, 1729–1740
40. Noguchi, C. T., Bae, K. S., Chin, K., Wada, Y., Schechter, A. N., and Hankins, W. D. (1991) Blood 78, 2548–2556