RAPID COMMUNICATION

A Mutation of the Common Receptor Subunit for Interleukin-3 (IL-3), Granulocyte-Macrophage Colony-Stimulating Factor, and IL-5 That Leads to Ligand Independence and Tumorigenicity

By Richard D'Andrea, John Rayner, Paul Moretti, Angel Lopez, Gregory J. Goodall, Thomas J. Gonda, and Matthew Vadas

The cytokines interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor bind with high affinity to a receptor complex that contains a ligand-specific α-chain and a common β-chain, hfeC. We report here the isolation of a mutant form of hfeC, from growth factor-independent cells, that arose spontaneously after infection of a murine factor-dependent hematopoietic cell line (FDC-P1) with a retroviral hfe expression construct. Analysis of this hfe mutation shows that a small (37 amino acid) duplication of extracellular sequence that includes two conserved sequence motifs is sufficient to confer ligand-independent growth on these cells and lead to tumorigenicity. Because this is a conserved region in the cytokine receptor superfamily, our results suggest that the large family of cytokine receptors has the capacity to become oncogenically active. © 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Construction of retrovirus expressing hfeC. The construction of a full-length hfeC cDNA has been described elsewhere. The full-length coding sequence was excised from a pcDNA1hfeC subclone and ligated into pRuFlNeo. Generation of factor-independent cells. Retroviral DNA was used to transfect an ecotropic packaging cell line, pW7, and virus from G418-resistant cells was used to infect the murine myeloid cell line, FDC-P1, by cocultivation. Cells were selected in G418 and, after several washes, incubated in medium with or without growth factor. After an extended time in culture (1 to 2 weeks), FDC-P1 cells from a wild-type hfeC infection were clearly growing in the absence of any added growth factor. Parallel cocultivations with hfeC producing hIL3Rα or pRuFlNeo retroviruses showed no growth on FDC-P1 cells in the absence of factor. In subsequent experiments, we have not generated factor-independent cells from similar cocultivations with hfeC retrovirus, implying that this mutant was generated spontaneously from a rare rearrangement during infection.

Isolation of genomic DNA and Southern analysis. Genomic DNA was isolated from cells using a Proteinase K/SDS procedure and analyzed using a standard Southern protocol. To detect hfeC sequences, a 32P-labeled probe was prepared by random oligo-extension (Multiprime Labelling System; Amersham, Arlington Heights, IL) of the hfe coding fragment excised and purified from a pcDNA1 subclone.

Polymerase chain reaction (PCR) and nucleotide sequencing. PCR was performed on 100 ng of genomic DNA using standard protocols. The primers used for amplification were 5' TGGATCCCTCTGTGCGTGAATTCGACG 3' and 5' TGAATTCATAAGAGCTCAGTGAG 3'. Reactions were performed in a Perkin Elmer Thermocycler (Perkin Elmer Cetus, Norwalk, CT) and the cycling parameters were 30 cycles of 94°C for 1 minute, 57°C for 2 minutes, and 72°C for 3 minutes, with a final 10-minute extension in cycle 30. Reactions were denatured at 95°C for 5 minutes before cycling. PCR products were cloned into pGEM2 (Promega, Madison, WI) for DNA sequencing. Sequencing was performed using T7 Polymerase (Sequenase; US Biochemical Corp [USB], Cleveland, OH) as per the manufacturer's protocols.

Proliferation assays. FDC-P1 cells were infected as described and G418-resistant cells were maintained in the presence of growth factor (80 U/mL of murine GM-CSF). To assay proliferation, cells were washed and divided equally into medium with or without growth factor for 12 hours. Samples containing equivalent numbers...
Fig 1. Generation and characterization of factor-independent cells. (A) Diagrammatic representation of the hβc retroviral construct. The vector used is pRUF NLNeo. Only relevant restriction sites are shown. (B) Southern analysis of human IL-3-dependent cells (FDC-P1 cells expressing hIL-3Ra and hβc) and the factor-independent hβc FDC-P1 cell line. Filters were probed with an hβc coding fragment. The wild-type hβc fragments are 4.4 kb in the Xba I digest and 3.4 kb and 1.0 kb in the Xba I/Bgl II digest. Clearly visible is a larger hβc fragment in the factor-independent FDC-P1 cells. (C) PCR from genomic DNA with internal hβc primers. The product generated spans the transmembrane domain and includes a small segment of cytoplasmic sequence. Lane a, markers, φX174 Hae III (Pharmacia, P-L Biochemicals Inc, Milwaukee, WI). Lane b, PCR using genomic DNA from factor-independent FDC-P1 cells as template. Lane c, PCR using genomic DNA from human IL-3-dependent FDC-P1 cells as in (B) as template. PCR products spanning the N-terminal and C-terminal regions of hβc were identical in both samples (data not shown).

of cells (4 × 10^7) were incubated in the presence of [3H] thymidine and DNA synthesis measured using a standard protocol described previously.21

In vitro mutagenesis. For site-directed mutagenesis, the fragment to be mutagenized was cloned into pALTER (Promega) and single-stranded DNA prepared after infection of transformed Escherichia coli with helper phage (R408). DNA was prepared and mutagenesis performed with a mutagenic oligonucleotide following the protocols provided by the manufacturer.

RESULTS

Isolation and characterization of factor-independent cells.
In the course of our studies using FDC-P1 cells infected
with an hβc retrovirus, a cell line arose spontaneously that grew in the absence of mouse GM-CSF and IL-3 (see Materials and Methods and Fig IA). To determine possible causes for this, we first examined cell supernatants for the presence of factors that could support the growth of FDC-P1 cells. Conditioned medium derived from the factor-independent cells did not support growth of untransfected cells, indicating that factor independence was not caused by autocrine production of growth factor. Secondly, we tested these cells for overexpression of hβc. However, immunostaining with the hβc-specific monoclonal antibody, 5F9, indicated that these cells were expressing similar levels of hβc to control human IL-3–dependent cells (data not shown). We characterized these cells further to determine whether there had been any alteration in the hβc cDNA on infection and generation of factor independence. Southern analysis indicated that two retroviral integrants were present in a factor-independent cell line (Fig 1B, HindIII digest). Digestion with a restriction enzyme that excises the proviral DNA (Fig 1B, Xba I and Xba IIIBgl I digests) indicated the presence of a larger than

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\text{Fig 1.} \quad \text{(Cont'd) (D) Sequence of the 0.8-kb hβc PCR product from factor-independent FDC-P1 cells showing the 111-bp tandem duplication in the extracellular domain. Only the sequence between the primers is shown. The transmembrane domain is indicated (underlined). The duplication leads to an in frame insertion resulting in a 37 amino acid duplicated segment (shaded) in the membrane proximal region of the molecule. This PCR product also contains a single amino acid substitution (boxed). Two copies of the conserved WSXWS motif are also underlined.}
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normal internal fragment in one copy of the provirus. PCR amplification of DNA derived from the factor-independent cells using internal hβc primers indicated that there was an alteration giving rise to a larger internal hβc PCR product (Fig 1C, lane b), consistent with the Southern analysis. The altered PCR product spanned the transmembrane domain and membrane proximal segments of the receptor. Nucleotide sequencing of cloned PCR products generated from these cells indicated that the larger hβc product contained both a single point mutation (resulting in alteration of threonine to lysine) and a tandem duplication of 37 amino acids in the extracellular portion of the receptor, proximal to the transmembrane domain (Fig 1D). We have reconstructed an hβc retrovirus containing this altered fragment. A full-length hβc receptor sequence was reconstructed in the retroviral vector, pRUFNLNeo, using restriction fragments from hβc subclones and the 0.8-kb PCR fragment (see Fig 1C). Cells were transfected with the resultant plasmid, pRUFNLβcFLΔ, and the G418-resistant producer cells used to introduce retrovirus into FDC-P1 cells. Cells infected with this retrovirus were capable of growth in the absence of added growth factor. Cells infected with control virus (either vector alone or hβc retrovirus) gave no growth in the absence of added growth factor (Fig 2). To show that the duplication alone could confer factor-independent growth, we cloned the reconstructed hβc into pALTER (Promega) and restored the altered lysine residue to threonine by site-directed mutagenesis. FDC-P1 cells selected for G418 resistance after infection with this retrovirus (pRUFNLβcFL370) were again factor-independent, indicating that the point mutation was not involved in generating factor independence in these cells. Cells infected with mutant and control retrovirus were also plated in agar with and without growth factor. Only cells infected with pRUFNLβcFLΔ formed G418-resistant colonies in agar in the absence of added factor (data not shown), consistent with expression of the mutant hβc being sufficient to confer factor-independent growth on FDC-P1 cells. Clearly, factor independence in the original cell line is not conferred as a result of retroviral integration or sequence alteration in another region of hβc. We can also rule out a requirement for the normal hβc product, derived from the second retroviral insertion, in the factor-independent phenotype of the original cell line. We interpret these results as indicating that the alteration in receptor sequence has led to constitutive activation of the hβc subunit.

**Tumorigenicity.** Although members of the cytokine receptor superfamily have not yet been associated clinically with oncogenesis, an activated form of the murine erythropoietin receptor (mEpoR) has been reported and shown to have tumourigenic potential.\(^{23,24}\) We therefore tested the tumourigenic potential of the mutant hβc molecule by injecting factor-independent and control FDC-P1 cells into syngeneic mice (Table 1). Control cells, infected with the pRUFNLNeo vector or with a wild-type hβc retrovirus, were not tumorigenic. However, FDC-P1 cells, infected with pRUFβcFLΔ (a retrovirus expressing the mutant form of hβc), generated solid tumors with a short latency in all mice injected. This result shows the tumorigenic capacity of activated hβc and emphasizes the oncogenic potential of the large family of cytokine receptors.

**DISCUSSION**

We have shown that a small (37 amino acid) duplication of extracellular hβc sequence is sufficient to confer ligand-independent growth on FDC-P1 cells and leads to tumourigenicity. We believe that the sequence defined by this duplication may be important in signalling because the altered receptor structure must in some way mimic a ligand-induced signalling event. Structural predictions suggest that the seg-

**Table 1. Tumourigenicity of Transfected FDC-P1 Cells**

<table>
<thead>
<tr>
<th>Cells Injected</th>
<th>Tumors Observed/No. of Mice Injected</th>
</tr>
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<tbody>
<tr>
<td>HBBS</td>
<td>0/3</td>
</tr>
<tr>
<td>FDC-P1 (pRUFNeo)</td>
<td>0/4</td>
</tr>
<tr>
<td>FDC-P1 (pRUFhβc)</td>
<td>0/4</td>
</tr>
<tr>
<td>FDC-P1 (pRUFhβcFL370)</td>
<td>5/5</td>
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FDC-P1 cells were infected with wild-type and mutant hβc retrovirus (indicated in parentheses) and selected for G418 resistance. To assess tumourigenicity, cells were injected into syngeneic DBA2 male mice (8 weeks old). Cells (5 × 10⁵ per mouse) were washed in serum-free medium and resuspended in Hanks' Balanced Salt Solution (HBBS; 1 × 10⁵ cells/mL) and 0.5 mL was injected into mice subcutaneously at a single site. Mice were observed for 2 to 3 months for signs of palpable or visible tumors at the site of injection. Tumourigenic cells gave rise to visible masses with a short latency after injection (2 to 3 weeks). Mice with solid tumors were killed and autopsies were performed to assess the extent of tumor formation. Representative mice injected with nontumorigenic cells were also killed and postmortems were performed to ensure that tumors had not developed.

**Fig 2.** Proliferation of retrovirally infected FDC-P1 cells measured by [³H] thymidine incorporation.\(^{21}\) (a) Cells expressing hβc containing the duplicated segment and with the single amino acid alteration restored to wild-type; (b) original factor-independent cells; (c) cells expressing hβc from a retrovirus containing the recovered PCR fragment (see Fig 1C); (d) cells infected with the pRUFNLNeo vector; (e) cells infected with a wild-type hβc retrovirus (Fig 1A). Presence or absence of growth factor (GM-CSF) is indicated.
Fig 3. Possible role of the duplicated segment in hpc activation. (A) Alignment of receptor amino acid sequences with the duplicated segment from the mutant hpc. The predicted location of h-strands E' and F' (see Bazan9) is shown with light grey shading. Amino acids shown to be important in association of IL-6Ra with gp1309 are underlined. Conserved sequence motifs discussed in the text are boxed. Only the cytokine receptor segment of v-mpl is shown. Sequences have already been described.9,39,59,77,98,99 (B) Model for formation of a functional hpc receptor complex. Constitutive activation may involve the formation of an exposed site capable of mediating dimerization through association with an equivalent region of a second similar hpc molecule. We speculate that, in the absence of ligand, the membrane proximal region of hpc is masked and hence prevented from association and dimer formation. In the presence of ligand, an intermediate complex may be the a-chain hpc heterodimer. Association with the a-chain ligand complex may relieve masking and allow hpc dimerization. Whether a-chain remains associated with the active complex is not known. In mutant hpc, the duplicated segment may permit the membrane proximal domains of mutant hpc monomers to interact in the absence of ligand or a-chain.
ment duplicated comprises two $\beta$-strands (E' and F') and two connecting loops, E'-F' and F'-G'. It also includes the WSXWS motif and an adjacent conserved basic region (consensus YXXXV/RX, see Patchy) corresponding to the predicted $\beta$-strand F' (see Fig 3A). The following mechanisms of activation are possible: (1) activation occurring through association of mutant h$\beta$c with murine receptor $\alpha$-chains expressed on these cells; (2) activation through an altered tertiary structure that mimics a ligand-induced event; and (3) the duplicated segment allows h$\beta$c dimer formation in the absence of ligand and hence plays a direct role in activation of the mutant receptor. The duplicated segment may form an exposed site mediating interaction of two h$\beta$c molecules. In the normal receptor, the single copy of this site maybe involved in dimer formation, but only in the presence of specific $\alpha$-chain and bound ligand (Fig 3B). 

Indirect support for this third model (3) above) comes from recent studies with gp130, the signalling subunit for IL-6, CNTF, and LIF. Based on the fact that receptor complexes for IL-3, IL-5, and GM-CSF share a signalling subunit, as do those for IL-6, Oncostatin M, CNTF, and LIF, it has been suggested that h$\beta$c may form a dimer in a similar fashion to gp130. The observation that the signalling subunits, h$\beta$c and gp130, have homology in their intracellular domains further suggests that there are similarities in the mechanisms mediating signalling. Additional support for this model comes from a study with a chimeric receptor containing the extracellular domain of the mEpoR and the intracellular domain of a murine h$\beta$c homologue, AIC2A. This chimera mediates Epo-dependent proliferation consistent with the activated form of AIC2A being a dimer.

Several studies on other receptors within this superfamily suggest the importance of this conserved membrane proximal segment in signal transduction. The role of the WSXWS motif has been extensively studied, although results are conflicting. Whereas mutations in this sequence have been reported to abolish ligand binding, mutations in the WSXWS motif of mEpoR suggest a role in signalling. A comprehensive mutagenesis of the IL-6 receptor $\alpha$-chain (IL-6R$\alpha$) identified seven mutations that appear to specifically abolish signalling by preventing association with a signalling subunit, gp130. All except one of these lie in strands E' and F' and the adjoining loop (underlined in Fig 3A), implying that this region is important in receptor subunit association. These observations, together with the recent evidence that IL-6R$\alpha$ complexes with a gp130 dimer, suggest that there could be a region in IL-6R$\alpha$ that includes the conserved $\beta$-strand F' (and perhaps the WSXWS motif) that is important in interaction with one of the gp130 molecules. Perhaps in h$\beta$c this region is important in dimer formation.

The h$\beta$c receptor contains two of the characteristic cytokine receptor modules and we speculate that the N-terminal receptor structure masks a membrane proximal site involved in dimerization, thereby preventing association. Other cloned cytokine receptor subunits, LIFR$\beta$ and MPL, resemble the h$\beta$c structure in that they have a repeated cytokine receptor module. Truncation from the N-terminus may be expected to activate these receptors. Although we have not yet tested N-terminal truncations of h$\beta$c, the oncogene v-mpl represents a truncation of this type. It is notable that the extracellular region remaining in the v-mpl product includes the conserved segment duplicated in the h$\beta$c mutant (see Fig 3A), consistent with some of these sequences being essential for signalling and perhaps contributing to formation of a dimeric complex.

In conclusion, the activated form of h$\beta$c suggests that a membrane proximal extracellular region of cytokine receptor subunits has an important role in signalling and we suggest that this region may be critical for dimerization (Fig 3B). Duplication of this segment may allow h$\beta$c association in the absence of ligand and hence constitutively activate the receptor. Indeed, mEpoR is activated by forced dimerization through a mutation that introduces a cysteine residue. This constitutive EpoR mutation and the h$\beta$c mutant described here suggest that mutations leading to inappropriate receptor subunit association could be a possible mode of oncogenic activation for several cytokine receptor family members.

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REFERENCES

7. Tavernier J, Devos R, Cornelis S, Tuybens T, Van der Heyden J, Fiers W, Plaittack G: A human high affinity interleukin-5 receptor (ILSR) is composed of an IL-5-specific chain and a $\beta$ chain shared with the receptor from GM-CSF. Cell 66:1175, 1991
10. Sakamaki K, Miyajima I, Kitamura T, Miyajima A: Critical cytoplasmic domains of the common $\beta$ subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. EMBO J 11:3541, 1992


24. Goodall GJ, Bagley CJ, Vadas MA, Lopez AF: A model for the interaction of the GM-CSF, IL-3 and IL-5 receptors with their ligands. Growth Factors 8:87, 1993


33. Gearing DP, Thut CJ, VandenBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman D, Beckmann MP: Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J 10:2839, 1991


A mutation of the common receptor subunit for interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor, and IL-5 that leads to ligand independence and tumorigenicity

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