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Activating point mutations in the common β subunit of the human GM-CSF, IL-3 and IL-5 receptors suggest the involvement of β subunit dimerization and cell type-specific molecules in signalling

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We have combined retroviral expression cloning with random mutagenesis to identify two activating point mutations in the common signal-transducing subunit (hβc) of the receptors for human granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5 by virtue of their ability to confer factor independence on the haemopoietic cell line, FDC-P1. One mutation (V449E) is located within the transmembrane domain and, by analogy with a similar mutation in the *neu* oncogene, may act by inducing dimerization of hβc. The other mutation (I374N) lies in the extracellular, membrane-proximal portion of hβc. Neither of these mutants, nor a previously described mutant of hβc (FIΔ, which has a small duplication in the extracellular region), was capable of inducing factor independence in CTLL-2 cells, while only V449E could induce factor independence in BAF-B03 cells. These results imply that the extracellular and transmembrane mutations act by different mechanisms. Furthermore, they imply that the mutants, and hence also wild-type hβc, interact with cell type-specific signalling molecules. Models are presented which illustrate how these mutations may act and predict some of the characteristics of the putative receptor-associated signalling molecules.

Keywords: cytokine receptor superfamily/human GM-CSF receptor common β chain/oncogenic activation/polymerase chain reaction mutagenesis/retroviral expression cloning

Introduction

Granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-3 stimulate the proliferation, differentiation and functional activity of a wide variety of haemopoietic cells, including neutrophils, eosinophils, monocytes and early progenitor cells (reviewed by Metcalf, 1986; Clark and Kamen, 1987). In addition, the capacity of GM-CSF and IL-3 to stimulate the proliferation of eosinophil progenitors is also shared by IL-5 (reviewed by Sanderson, 1992). This functional overlap, as well as the cross-competition for binding to receptors on the surface of human haemopoietic cells (Lopez *et al.*, 1991), has a clear correlate in the structure and composition of the receptors for these three CSFs. The high-affinity receptors for human GM-CSF (hGMR), IL-3 (hIL-3R) and IL-5 (hIL-5R) are composed of ligand-specific α subunits (hGMRα, hIL-3Rα and hIL-5Rα) associated with a common β subunit (hβc). The α subunits bind their cognate factors with low affinity, whereas hβc alone does not bind any of these factors detectably, but is required, in association with the α subunits, to confer high-affinity binding (Gearing *et al.*, 1989; Hayashida *et al.*, 1990; Kitamura *et al.*, 1991a; Tavernier *et al.*, 1991). Moreover, hβc is essential for signal transduction (Kitamura *et al.*, 1991b; Kitamura and Miyajima, 1992; Sakamaki *et al.*, 1992) and, as a shared signal-transducing component, provides a molecular explanation for the overlapping biological characteristics of GM-CSF, IL-3 and IL-5.

Both subunits of the receptors for GM-CSF, IL-3 and IL-5 are members of a large family, often termed the cytokine receptor family (reviewed by Miyajima *et al.*, 1992), that includes the receptors for many haemopoietic growth factors. Receptors of this class are characterized by a conserved structure [including the hallmark WSXWS (Trp-Ser-Xaa-Trp-Ser) motif] in their extracellular domains, which was first predicted by Bazan (1990), and then refined by determination of the structure of the complex between human growth hormone (hGH) and its receptor (hGHR) (de Vos *et al.*, 1992). Unlike the other major class of growth factor receptors, the receptor tyrosine kinases, members of the cytokine receptor family do not appear to possess any intrinsic enzymatic activities that could account for intracellular signalling.

Despite the fact that β subunits and their associated α subunits lack an intrinsic tyrosine kinase activity, it has been thoroughly documented that tyrosine phosphorylation of a number of proteins, including the β subunits themselves, is rapidly induced following ligand binding (Duronio *et al.*, 1992; Sakamaki *et al.*, 1992; Hanazono *et al.*, 1993). At least two distinct regions of the intracellular domain of hβc are involved in the generation of separate intracellular responses (Sakamaki *et al.*, 1992; Sato *et al.*, 1993), which implies that hβc is associated with and utilizes multiple effector molecules. Indeed, phosphorylation is believed to be carried out by one or more receptor-associated tyrosine kinases such as JAK-2 (Silvennoinen *et al.*, 1993), Fes (Hanazono *et al.*, 1993) and Lyn (Torigoe *et al.*, 1992). However, the mechanisms by which the effector molecules are activated are not clear. While it is accepted that signalling is mediated by a complex comprising the ligand and both the α and β subunits, neither the precise stoichiometry nor the role of each subunit in signalling is clear. On one hand, the cytoplasmic portion of the α subunit is required for signalling by the GM-CSF and IL-5 receptors (Sakamaki *et al.*, 1992; Takaki *et al.*, 1993; Polotskaya *et al.*, 1994). On the other hand, studies with chimeric receptors suggest that dimerization of the intracellular portion of the β

subunit may be sufficient to initiate signal transduction (e.g. Sakamaki *et al.*, 1993; Takaki *et al.*, 1994; see Discussion).

Regardless of the precise mechanism by which they function, the GM-CSF, IL-3 and IL-5 receptors transduce proliferative signals, and it would therefore seem likely that their common signalling subunit, hβc, represents a potential target for oncogenic activation. Indeed, the oncogenic potential of several members of the cytokine receptor superfamily has already been demonstrated: the v-*mpl* oncogene of the myeloproliferative leukaemia virus (MPLV) encodes a truncated form of the recently characterized c-MPL receptor for thrombopoietin (Souyri *et al.*, 1990; Vigon *et al.*, 1992; Lok *et al.*, 1994), and a single amino acid substitution in the extracellular domain of the erythropoietin receptor confers factor-independent growth and tumorigenicity on factor-dependent haemopoietic cells (Yoshimura *et al.*, 1990). More recently, a constitutively active mutant of hβc (FIΔ), that arose spontaneously following retroviral transduction, was isolated by virtue of its ability to confer factor independence and tumorigenicity on a murine haemopoietic cell line (D'Andrea *et al.*, 1994).

Here we report the identification of two activating point mutations in hβc that were generated using a polymerase chain reaction (PCR)-based random mutagenesis procedure. By combining this procedure with a retroviral expression cloning system recently developed in our laboratory (Rayner and Gonda, 1994), these mutants were isolated by virtue of their ability to confer factor independence on a factor-dependent haemopoietic cell line. The ability of point mutations to activate hβc supports the possibility that such mutations could contribute to human leukaemia. In addition, the nature and properties of these mutants also have important implications for the mechanism of signalling by the receptors that utilize the hβc subunit.

Results

Generation of a retroviral expression library of point-mutated hβc cDNA constructs

To investigate the oncogenic potential of hβc, we introduced point mutations randomly into a 940 bp segment of the hβc cDNA encoding the 191 membrane-proximal residues of the extracellular domain, the transmembrane domain and the first 92 residues of the cytoplasmic domain (Figure 1A). We identified this segment as a potential target for activating mutations because it encompasses several sequences that have been implicated in signalling and/or activation of cytokine receptors: (i) the membrane-proximal cytoplasmic region essential for signal transduction (D'Andrea *et al.*, 1991; Sakamaki *et al.*, 1992; Sato *et al.*, 1993; Ziegler *et al.*, 1993); (ii) the highly conserved WSXWS sequence that is a hallmark of the cytokine receptor superfamily (Bazan, 1990); (iii) the equivalent extracellular region of c-Mpl remaining in the v-Mpl oncoprotein (Souyri *et al.*, 1990; Vigon *et al.*, 1992); and (iv) the extracellular sequence duplicated in the constitutively active hβc mutant described by D'Andrea *et al.* (1994).

Our approach to identifying activating mutations in hβc was to construct a bank of random point mutants and then

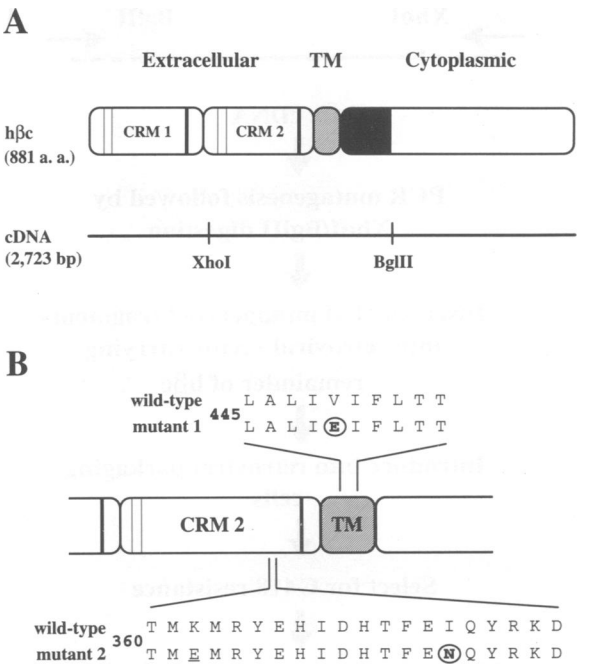

Fig. 1. Location of activating point mutations in the region of the β subunit of the human GM-CSF/IL-3/IL-5 receptors subjected to random mutagenesis. (**A**) Schematic illustration of hβc showing the two cytokine receptor modules (CRMs: Goodall *et al.*, 1993), the conserved cysteine residues (thin vertical lines) and the characteristic WSXWS motifs (thick vertical lines) [see Bazan (1990) for description of these elements]. Also shown are the transmembrane domain (hatched) and the minimal region essential for proliferative signalling in the cytoplasmic domain (Sakamaki *et al.*, 1992) (black shading). The positions in the hβc cDNA of the *Xho*I and *Bgl*II sites that delimit the fragment used for mutagenesis are shown underneath. (**B**) Location of point mutations in activated forms of hβc. The structural features of hβc are illustrated as in (A). The sequences of the regions containing mutations in mutants 1 and 2 (see text) are expanded; the activating mutations are circled and in bold type and the additional mutation in mutant 2 is underlined.

insert these en masse into a retroviral expression vector. The resultant retroviruses were then used to infect a murine factor-dependent haemopoietic cell line (FDC-P1), following which these cells were selected for the ability to grow in the absence of factor. This procedure, which is outlined in Figure 2, is essentially a combination of the PCR mutagenesis technique described by Cadwell and Joyce (1992) and methodology previously developed in this laboratory (Rayner and Gonda, 1994).

As described in Materials and methods, we were able to define PCR conditions, based on those described by Cadwell and Joyce (1992), that resulted in the unbiased, random generation of mutations at the desired rate of ~0.2% (1 in 500 bp). The mutated hβc fragments were inserted directionally into pRUFNeo-hβc (see Materials and methods and Figure 2) from which the segment subjected to PCR mutagenesis had been excised. This resulted in the generation of a library, comprising 1.7×10^5 plasmid clones, of hβc cDNAs bearing point mutations in the targeted segment only ($h\beta c^{mut}$). Using procedures described previously (Rayner and Gonda, 1994), the plasmid DNA was used to generate a retroviral library estimated to contain 2.1×10^4 independent viral producer clones. Assuming that there was no overwhelming bias in the procedure, this should adequately represent all of the possible point mutations in the 940 bp hβc fragment.

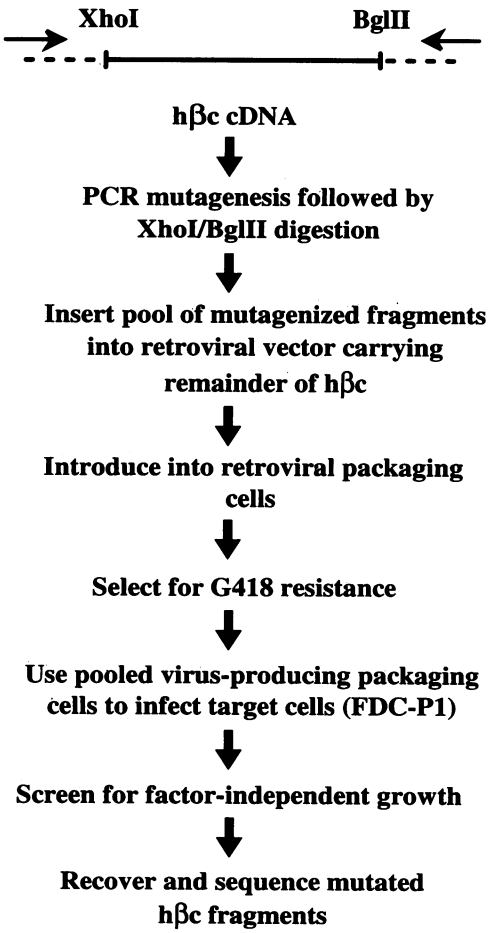

Fig. 2. Outline of the strategy used for generation and expression of hβc mutants. The arrows at the top represent the primers used for PCR amplification/mutagenesis of the segment of hβc shown in Figure 1A; these lie just outside the *BglII* and *XhoI* sites (see Materials and methods). Mutagenized fragments were ligated into the pRUFNeo-hβc plasmid which had been digested with *BglII* and *XhoI*. The resultant pool of plasmids was introduced into Ψ2 retroviral packaging cells as described by Rayner and Gonda (1994). Following G418 selection, the pooled virus-producing Ψ2 cells were used to infect FDC-P1 cells, which were subsequently selected for factor independence. The mutated hβc fragments were recovered from factor-independent FDC-P1 cells by PCR using the same primers as for the original mutagenesis/amplification.

Isolation of factor-independent FDC-P1 cells carrying constitutively active hβc mutants

FDC-P1 cells are dependent on mouse GM-CSF (mGM-CSF) or IL-3 (mIL-3) for growth, and normally die within days when starved of either factor. However, they do proliferate in response to low concentrations of human GM-CSF (hGM-CSF) or IL-3 (hIL-3) if the appropriate α subunit (hGMRα or hIL-3Rα) is co-expressed with hβc (our unpublished results). We therefore reasoned that infection of FDC-P1 cells with a retrovirus encoding a constitutively active form of hβc should induce factor-independent proliferation; this approach has been validated by the recent isolation of a constitutively active hβc mutant, FIΔ, expressed in factor-independent FDC-P1 cells (D'Andrea *et al.*, 1994).

Thus, to isolate factor-independent cells expressing constitutively active hβc point mutants present in the hβc expression library, FDC-P1 cells were infected by co-cultivation with the pool of $h\beta c^{mut}$ virus-producing Ψ2 cells (see Materials and methods). Parallel co-cultivations were also performed with uninfected Ψ2 cells and Ψ2 cells producing wild-type hβc retrovirus. After 1 week in liquid culture in the absence of factor, all four FDC-P1 cell pools infected with the mutant hβc retroviral library contained viable, proliferating cells, while no such cells were present in the control cultures. Factor independence was not a result of autocrine growth factor production as conditioned medium from the factor-independent cell pools did not support the growth of uninfected FDC-P1 cells (data not shown). A total of 31 factor-independent clones was isolated from the factor-independent liquid culture pools by agar plating; each of these was analysed further. Based on infection frequency and the number of independent mutants (detected by sequencing and/or restriction analysis; see also below and Figure 7), we estimate the frequency of factor-independent mutations to be ≥ 1 in 10^5.

Identification of activating mutations in constitutively active hβc mutants

During the course of recovering the mutated region of hβc from the factor-independent clones by PCR, it was discovered that 22/31 clones contained an additional *BglII* restriction site (as shown in Figure 7). Sequencing of one such clone (mutant 1) revealed that this was due to a T to A mutation at nucleotide 1374, which results in a change of the amino acid valine to glutamic acid at residue 449 (V449E) within the transmembrane domain (see Figure 1B). This is strikingly reminiscent of the activating point mutation (V664E) found by Bargmann *et al.* (1986) in the rat *neu* oncogene (see Discussion). Sequence analysis of one other clone that lacked the extra *BglII* site (mutant 2) revealed two point mutations: an A to G substitution at nucleotide 1112 which resulted in the substitution of lysine 362 to glutamic acid (K362E), and a T to A substitution at nucleotide 1149 which resulted in the substitution of isoleucine 374 to asparagine (I374N), both of which lie in the extracellular portion of the targeted region (see Figure 1B).

To confirm that the V449E mutation was indeed capable of activating hβc, and to identify which of the two mutations in mutant 2 was responsible for activation, all three mutations were re-created independently by site-directed mutagenesis. We also constructed two additional mutants, V449Q and V449D, based on other mutations known to activate c-*neu* (Bargmann and Weinberg, 1988; see also below). Following insertion into the pRUFNeo retroviral vector and transfection into Ψ2 packaging cells, these mutants, as well as wild-type hβc, were again introduced into FDC-P1 cells, which were then selected either for G418 resistance or for growth in medium without mGM-CSF. All of the viruses efficiently generated G418-resistant cells, which were subsequently analysed for hβc expression by antibody staining and flow cytometry. In these and subsequent experiments, we also included the previously described activated hβc mutant FIΔ, which contains a 37 amino acid duplication in the membrane-proximal portion of the extracellular domain (D'Andrea *et al.*, 1994). Figure 3 shows that a substantial proportion of FDC-P1 cells infected with each of these viruses, except that carrying the V449D mutant, expressed hβc.

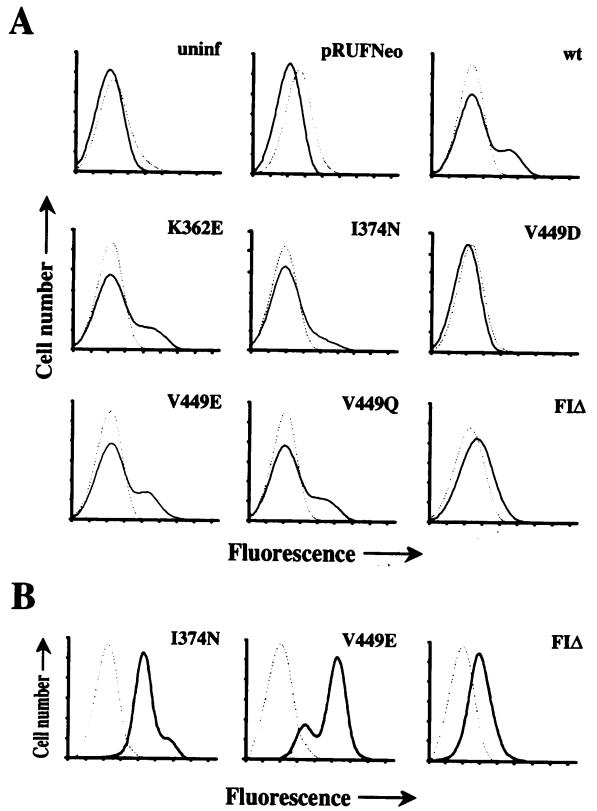

Fig. 3. Flow cytometric analysis of wild-type (wt) and mutant hβc expression on infected FDC-P1 cells. Dotted lines (.....) represent cells stained with an irrelevant control antibody and solid lines (—) indicate staining with anti-hβc antibody. Cell number and fluorescence are in arbitrary units; the latter is plotted on a logarithmic scale. (**A**) Cells selected for resistance to G418; also shown are analyses of uninfected cells and cells infected with the pRUFNeo vector alone. (**B**) Cells selected for growth in the absence of factor.

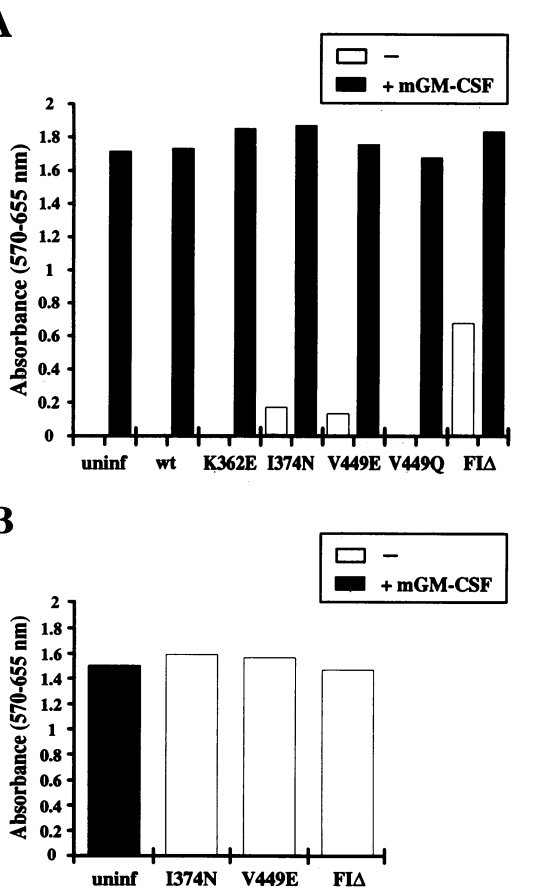

Fig. 4. Proliferation of FDC-P1 cells infected with hβc mutants in the presence and absence of murine GM-CSF. (**A**) Proliferation of cells selected and maintained prior to assay in mGM-CSF plus (except for the uninfected control cells) G418. Proliferation assays were carried out, as described in Materials and methods, in the presence or absence of mGM-CSF for uninfected FDC-P1 cells and cells infected with wild-type hβc (wt) or the indicated hβc mutants. (**B**) Proliferation of FDC-P1 cells infected with activated hβc mutants that were selected prior to assay by growth in factor-free medium. Proliferation of uninfected FDC-P1 cells in the presence of mGM-CSF is shown for comparison.

(Repeated attempts to obtain surface expression of V449D were unsuccessful.) However, only the I374N, V449E and, as expected, FIΔ mutants conferred factor independence on the FDC-P1 cells; the K362E, V449Q or (not surprisingly) the V449D mutants all failed to do so (Figure 4A; the data for the non-expressing V449D mutant is not shown).

Although the degree of proliferation in the absence of factor shown by cells infected with the V449E, I374N and FIΔ mutants was lower than that seen with factor, this probably reflects the fact that only a proportion of each G418-selected population expressed hβc (Figure 3). To eliminate the possibility that the factor-independent populations represented secondary mutations that conferred a selective advantage, we performed colony assays on FDC-P1 cells, immediately after infection, in the presence or absence of mGM-CSF. The results (Table I) show that between 28 and 78% of clonogenic cells infected with viruses encoding V449E, I374N or FIΔ were factor-independent; this proportion is far too high to be due to secondary mutations in a subset of infected cells. When these same G418-resistant populations were subsequently selected for factor independence, the proliferation observed in the absence of factor was comparable with that of uninfected FDC-P1 cells in the presence of mGM-CSF (Figure 4B). The presence of the appropriate hβc cDNA constructs in the factor-independent FDC-P1 cells was further confirmed by recovery of the entire hβc fragment by PCR from genomic DNA, followed by restriction enzyme digestions diagnostic of each mutant (Figure 7B). We therefore concluded that the V449E and I374N substitutions could confer factor independence on FDC-P1 cells and thus could constitutively activate hβc. Note that the latter mutation results in the loss of a *Bst*YI restriction site (as shown in Figure 7); by digesting with *Bst*YI, we subsequently found that all nine of the factor-independent clones that lacked the V449E mutation carried the I374N mutation (data not shown).

Biological activity of hβc mutants expressed in other cell types

While the FDC-P1 cells used in the preceding studies do not express human α subunits, they do express murine GMRα and IL-3Rα subunits. Because signalling by normal GM-CSF, IL-3 and IL-5 receptors requires formation of a complex of α subunits, β subunits and ligand, we wished to investigate whether the ligand-independent mutants could function in the absence of any α subunits. For these studies, we chose the murine IL-2-dependent T-cell line CTLL-2 (Cerottini *et al.*, 1974) because it does not express

Table I. Frequency of factor independence following infection of FDC-P1 cells with hβc mutants[a]

FDC-P1 cells infected with[b]:	Number of colonies[c]			
	mGM-CSF	mGM-CSF + G418	No factor	Percent factor-independent[d]
No virus	550	0	0	0
Wild-type hβc	689	562	0	0
I374N	936	736	573	77.9
V449E	886	565	157	27.8
FIΔ	587	491	305	62.1

[a]Cells were washed and plated in agar-containing medium, as described in Materials and methods, immediately after co-cultivation with Ψ2 cells.
[b]Virus-producing cells were sorted by flow cytometry for those expressing hβc prior to use in this experiment.
[c]Average number of colonies present on duplicate agar plates seeded with 10^3 cells.
[d]Calculated as the percentage of infected, i.e. G418-resistant, colonies that were factor-independent.

any endogenous GM-CSF/IL-3/IL-5 receptor chains but has been shown to proliferate in the presence of human GM-CSF following introduction of both receptor subunits (Kitamura *et al.*, 1991b).

Although infection of CTLL-2 cells by the murine retroviruses used was very inefficient (probably ≤ 0.01% as estimated by G418 resistance; unpublished observations) we were able to isolate CTLL-2 infectants, expressing both wild-type and mutant (I374N, V449E and FIΔ) forms of hβc, by selecting for G418 resistance and maintaining the cells in the presence of IL-2. Moreover, flow cytometric analysis (Figure 5A) showed that a substantial proportion of these cells clearly exhibited cell surface expression of β subunits. Somewhat to our surprise, however, the resulting cell lines failed to proliferate in the absence of IL-2 (Figure 6A). Because of the low frequency of infection, and the consequent possibility that the CTLL-2 populations may have comprised only one or a few clones, we wondered whether they were expressing aberrant, i.e. spontaneously mutated, forms of the original constructs. However, recovery of the hβc cDNAs from these cells by PCR generated products of the expected size and which exhibited the expected restriction enzyme digestion patterns (Figure 7C).

One possible explanation for this result was that the activated hβc mutants function by interacting with an α subunit (see also Discussion) and thus were unable to function in CTLL-2 cells lacking α subunits, but could interact with the GMRα or IL-3Rα subunits in the FDC-P1 cells. To test this possibility, and also to confirm that the CTLL-2 lines we derived could in fact respond to normal GMR-generated signals, we superinfected CTLL-2 cells expressing either wild-type or mutant β subunits with a vector carrying the hGMRα subunit and a puromycin resistance gene (pRUFPuro-hGMRα). We reasoned that if the mutant β subunits interacted with mouse α subunits, then it would be likely that they would also interact with human α subunits.

We were able to derive puromycin-resistant populations from each β subunit-expressing CTLL-2 line (i.e. wild-type, I374N, V449E and FIΔ); as only a proportion of these (12.3–33.2%) expressed the α subunit (data not shown), the latter were purified by preparative flow cytometry. Re-analysis of the sorted cells showed that they expressed both subunits of the human GMR (Figure 5B). Nevertheless, none of the resulting populations could proliferate in the absence of IL-2 (Figure 6B), indicating that the presence of the hGMRα subunit did not enable

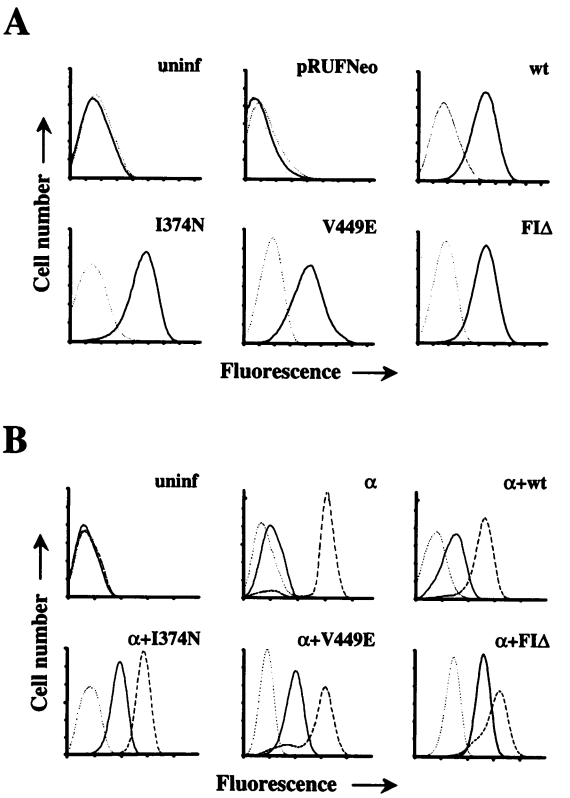

Fig. 5. Flow cytometric analysis of hβc and hGMRα expression on infected CTLL-2 cells. Nomenclature and axes are as in Figure 3. Dotted lines (.....) represent cells stained with an irrelevant control antibody, solid lines (▬) indicate staining with anti-hβc antibody and dashed lines (-------) indicate staining with anti-hGMRα antibody. (**A**) CTLL-2 cells infected with retroviruses encoding the hβc mutants indicated on each histogram were stained with an anti-hβc monoclonal antibody as described in Materials and methods. (**B**) CTLL-2 cells infected with retroviruses encoding the hβc mutants indicated on each histogram were superinfected with a retrovirus encoding hGMRα and stained with anti-hβc and anti-hGMRα monoclonal antibodies. For comparison, analyses of uninfected CTLL-2 cells and cells infected only with the hGMRα virus (α) are also shown.

the constitutive generation of proliferative signals by either hβc mutant.

The possibility remained, though, that despite the PCR analysis (Figure 7C) there were other defects in the β subunits expressed by the CTLL-2 cells or that our CTLL-2 cells could not respond to GMR-generated signals. We therefore transferred CTLL-2 cells co-expressing α and β subunits from medium containing IL-2 to medium con-

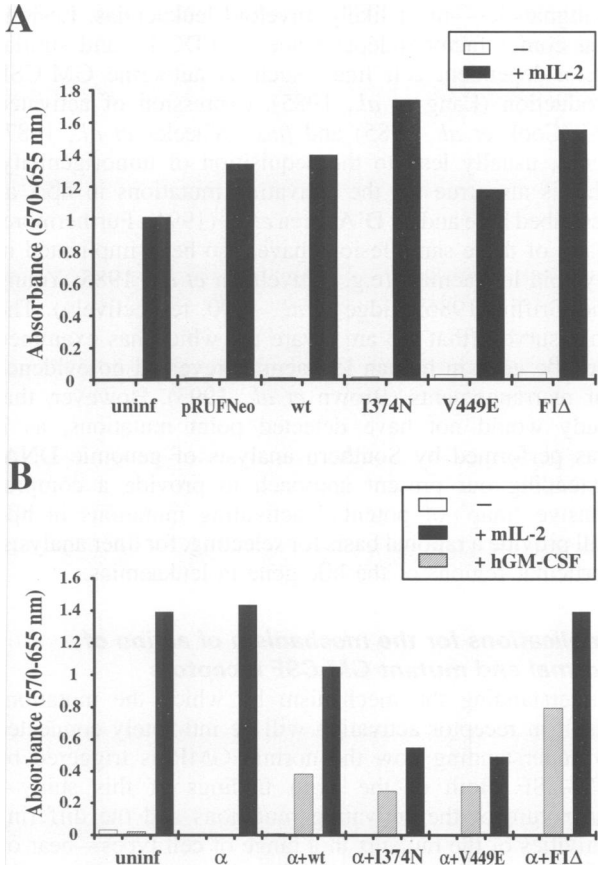

Fig. 6. Proliferation of CTLL-2 cells infected with hβc mutants in the presence and absence of human GM-CSF or mouse IL-2.
(**A**) Proliferation of cells selected and maintained prior to assay in IL-2 plus (except for the uninfected control cells) G418. Proliferation assays were carried out, as described in Materials and methods, in the presence or absence of IL-2 for uninfected CTLL-2 cells (uninf), cells infected with the vector alone (pRUFNeo), wild-type hβc (wt) or the indicated hβc mutants. (**B**) Proliferation of CTLL-2 cells infected with activated hβc mutants and superinfected with a retrovirus encoding hGMRα. Proliferation assays were carried out in the presence of mIL-2, hGM-CSF or in the absence of either factor, as indicated. Proliferation of uninfected CTLL-2 cells (uninf) and cells infected only with the hGMRα (α) virus is shown for comparison.

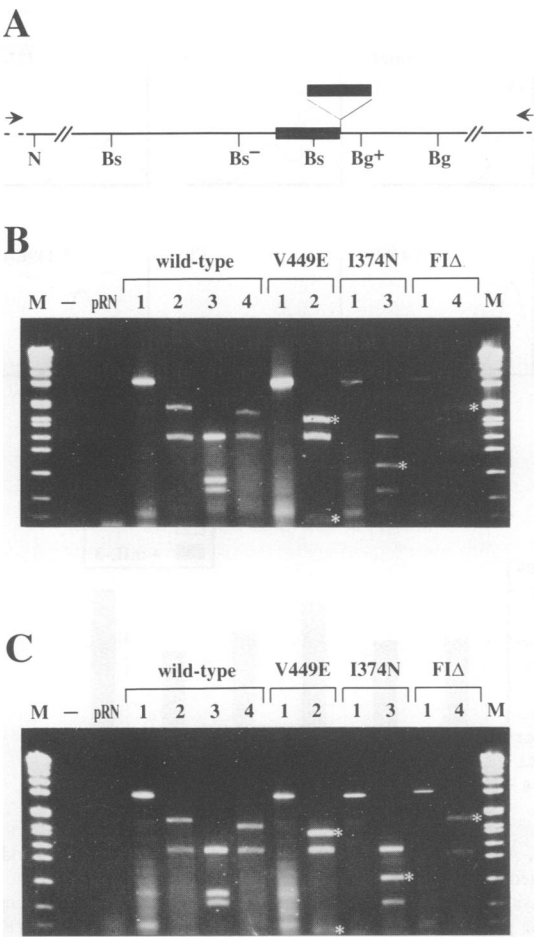

Fig. 7. Analysis of proviral hβc sequences in infected cells by PCR and restriction enzyme digestion. (**A**) Map of hβc cDNA showing *Nco*I (N), *Bst*YI (Bs) and *Bgl*II (Bg) restriction sites used to authenticate each form of hβc, as well as the region duplicated in FIΔ (indicated by boxes). The restriction sites affected by the point mutations are indicated as Bg^+ (gained in V449E) and Bs^- (lost in I374N). Arrows indicate the positions of PCR primers used to amplify hβc fragments from genomic DNA. (**B**) Electrophoretic analysis of PCR products generated from genomic DNA of FDC-P1 cells infected with the indicated hβc mutants; controls are reactions containing no DNA (–) or DNA from cells infected with the vector alone (pRN). Lanes M contain DNA size standards [SPP-1 phage DNA digested with *Eco*RI (Bresatec Ltd, Adelaide, South Australia)]. PCR products were either undigested (lanes 1), digested with *Bgl*II (lanes 2), *Bst*YI (lanes 3) or *Nco*I plus *Bgl*II (lanes 4). Bands in each digest that differ between the mutants and the wild-type are indicated by asterisks in the lanes containing the DNA from the appropriate mutant-infected cells. (**C**) Electrophoretic analysis, as in (B), of PCR products generated from genomic DNA of CTLL-2 cells infected with the indicated hβc mutants.

taining hGM-CSF. Cells expressing the wild-type hβc, and also those expressing the mutant β subunits, continued to grow in hGM-CSF, as shown by proliferation assays (Figure 6B); in fact, growth could be maintained in as little as 1 ng/ml hGM-CSF (data not shown) which would be expected to stimulate only high-affinity receptors. This indicates that the mutant receptors could still interact in an apparently normal manner with ligand and α subunits.

In view of these results, we wondered whether the ability of the mutants to confer factor independence was restricted to FDC-P1 cells. Retroviruses containing the mutant and wild-type forms of hβc were therefore used to infect the IL-3-dependent BAF-B03 subline of the pro-B cell line, Ba/F3. Figure 8A shows that, as before, surface expression of wild-type hβc and each mutant (V449E, I374N and FIΔ) could be readily obtained. However, only V449E could confer factor independence on these cells, as shown by proliferation assays (Figure 8B) and prolonged monitoring of liquid cultures in factor-free medium (data not shown). As seen with the CTLL-2 cells, superinfection of BAF-F03 cells expressing I374N and FIΔ with the hGMRα subunit failed to confer factor independence (data not shown). Nevertheless, hGMRα subunit expression did allow these cells to proliferate in 1 ng/ml hGM-CSF (data not shown).

Tumorigenicity of hβc point mutants

The ability to confer factor independence on haemopoietic cell lines is generally indicative of tumorigenic potential (see Discussion). To test whether the V449E and I374N mutations were capable of conferring tumorigenicity, we injected factor-independent FDC-P1 cells expressing each of the mutant β subunits into syngeneic mice. Uninfected

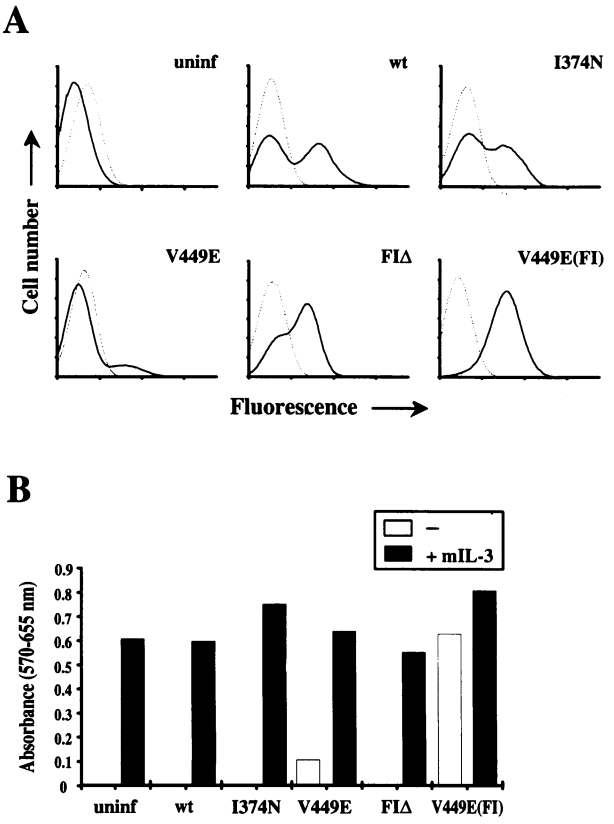

Fig. 8. Analysis of BAF-F03 cells infected with retroviruses encoding activated forms of hβc. (**A**) Flow cytometric analysis of hβc expression. Procedures and nomenclature are as for Figure 3, except that the panel labelled 'V449E(FI)' shows staining of BAF-F03 cells infected with the V449E mutant and selected for factor-independent growth. (**B**) Proliferation of the BAF-F03 cells depicted in (A) in the presence and absence of mouse IL-3.

FDC-P1 cells and FDC-P1 cells expressing wild-type hβc were injected as controls. All four mice injected with V449E, I374N and, as expected (D'Andrea *et al.*, 1994), FIΔ-expressing cells developed tumours at the site of injection within 3 weeks, while none of the control mice had developed tumours by 9 weeks post-inoculation.

Discussion

Oncogenic potential of the GM-CSF receptor

In this report, we have shown that hβc can be activated by point mutations leading to single amino acid substitutions. To date, the only other member of the cytokine receptor family shown to be activated by point mutation is the erythropoietin receptor; in this case activation resulted from the substitution of cysteine residues in the extracelluar domain (Yoshimura *et al.*, 1990; Watowich *et al.*, 1994). It seems quite likely that there may also be other point mutations that can activate hβc. For example, the studies described here have targeted only about one-third of the hβc cDNA, so there may well be potential activating mutations in other parts of the molecule and/or in associated α subunits.

These findings, and the previous report of a structural rearrangement that leads to activation of hβc (i.e. the FIΔ mutant; D'Andrea *et al.*, 1994), raise the possibility that activating mutations in hβc may contribute to human malignancies—most likely, myeloid leukaemias. Lesions that confer factor independence on FDC-P1 and similar factor-dependent cell lines, such as autocrine GM-CSF production (Lang *et al.*, 1985), expression of activated *abl* (Cook *et al.*, 1985) and *fms* (Wheeler *et al.*, 1987) genes, usually lead to the acquisition of tumorigenicity. This is also true for the activating mutations in hβc, as described here and by D'Andrea *et al.* (1994). Furthermore, many of these same lesions have also been implicated in myeloid leukaemias (e.g. Shtivelman *et al.*, 1985; Young and Griffin, 1986; Ridge *et al.*, 1990, respectively). The only survey (that we are aware of) which has examined the hβc gene in human leukaemias revealed no evidence for rearrangements (Brown *et al.*, 1993). However, that study would not have detected point mutations, as it was performed by Southern analysis of genomic DNA. Extending our present approach to provide a comprehensive 'map' of potential activating mutations in hβc will provide a rational basis for selecting, for finer analysis, particular regions of the hβc gene in leukaemias.

Implications for the mechanism of action of normal and mutant GM-CSF receptors

Understanding the mechanism by which the mutations result in receptor activation will be intimately connected to understanding how the normal GMR is triggered by GM-CSF. Both of the main findings of this study—the nature of the activating mutations and the differing activities of the mutants in a range of cell types—bear on this issue. The differing abilities of the mutants to confer factor independence on BAF-F03 cells indicate that they exert their effects by distinct mechanisms, which is consistent with the positions of the mutations within hβc. Furthermore, our results imply that the mutants, and, most likely, the normal receptors, interact with cell-type specific molecules. It is difficult to avoid the conclusion that there are (at least) two such molecules present in FDC-P1 cells that are involved: one, which is required for signalling by the V449E mutant, is also present in BAF-F03 cells but is absent from CTLL-2 cells; and a second which is required for signalling by the I374N and FIΔ mutants but which is absent from both BAF-F03 and CTLL-2 cells. It is unlikely that either of these components are α subunits, as co-expressing the human GMRα subunit (Figure 6) or either the human or murine IL-3Rα subunits (data not shown) with the hβc mutants in CTLL-2 cells did not result in factor-independent growth. Similarly, introduction of the human α subunits failed to allow BAF-F03 cells expressing the I374N or FIΔ mutants to proliferate in the absence of factor (data not shown).

Mechanism of activation by the V449E mutation. At the moment, the transmembrane domain mutation V449E is probably the simplest to interpret since it is analogous to the activating mutation (V664E) found in the rat *neu* oncogene (Bargmann *et al.*, 1986). Several reports have indicated that the *neu* mutation probably acts by inducing constitutive receptor dimerization (Sternberg and Gullick, 1989; Weiner *et al.*, 1989). Since dimerization is believed to be the key step in ligand-induced signalling by all receptor tyrosine kinases (reviewed by Ullrich and Schlessinger, 1990), the mutation has essentially the same effect as ligand binding. By analogy, we propose that the

V449E mutation acts by inducing ligand-independent dimerization of the β subunit and initiating the generation of intracellular signals.

The exact basis for dimerization induced by hydrophilic substitutions in the transmembrane domain is unclear, even though models have been proposed for Neu that invoke particular interactions between residues in the mutant transmembrane domains (Sternberg and Gullick, 1989) or conformational alterations (Brandt-Rauf *et al.*, 1990). Our model for V449E is based on the striking similarity to activating mutations in Neu. However, the sequence surrounding the mutated valine in hβc does not conform to the 'rules' worked out for Neu (Cao *et al.*, 1992), although both hβc and Neu have an alanine four residues N-terminal to the substituted valine. Moreover, not all receptors can be activated by such mutations. Thus it is very difficult to speculate as to why the V449Q and V449D mutations do not activate hβc; structural or modelling data will be necessary to provide further insight into the requirements for activation.

The notion that β subunit dimerization can trigger intracellular signalling is supported by a number of observations pertaining to murine or human β subunits and to other members of the cytokine receptor family. Several members of this family (e.g. the receptors for G-CSF, Epo and human growth hormone) clearly function as homodimers; in these cases ligand binding induces dimerization which, in turn, results in the generation of proliferative signals (Fukunaga *et al.*, 1990; de Vos *et al.*, 1992; Watowich *et al.*, 1994). A second important case is that of the IL-6 receptor which, like the GMR, is heteromeric (Taga *et al.*, 1989) and comprises a ligand binding α subunit plus the signalling subunit, gp130 (which can be viewed as the equivalent of the GMR β subunit). Activation of the IL-6 receptor by ligand results in the formation of a heteromeric complex that contains a gp130 dimer (Murakami *et al.*, 1993) along with two IL-6 receptor α subunits (Ward *et al.*, 1994). With regard to GMR/IL-3R/IL-5R β subunits, it has been shown that a chimera containing the extracellular region of the Epo receptor and the intracellular domain of the murine $\beta_{IL\text{-}3}$ (AIC2A) subunit can mediate Epo-dependent proliferation (at least in Ba/F3 cells; Sakamaki *et al.*, 1993). In addition, other experiments have shown that chimeras comprising the extracellular region of the GMRα subunit (K.Arai, personal communication) or the IL-5Rα subunit (Takaki *et al.*, 1994) and the intracellular region of hβc can transduce proliferative signals in the presence of normal hβc and the appropriate ligand. These data imply that dimerization of the intracellular portion of the β subunit is sufficient to initiate cellular proliferation.

One model that could explain the inability of the V449E mutant (see Figure 6) and the EpoR–$\beta_{IL\text{-}3}$ chimera (Sakamaki *et al.*, 1993) to function in CTLL-2 cells is illustrated in Figure 9A. The model assumes that the normal receptor complex (or at least one form thereof—see below) comprises two α and two β subunits, and proposes: (i) that there is a factor present in, for example, FDC-P1 cells (factor 'Y') which must be associated with hβc homodimers in order to generate proliferative signals but which is absent from CTLL-2 cells; and (ii) that association of α subunit plus ligand can compensate for the lack of factor Y by bringing into the complex another factor ('X', which is present in CTLL-2 cells and probably in other cells also) which overlaps in function with factor Y. Thus, the dimerized β subunit could deliver proliferative signals in cells such as FDC-P1 and BAF-F03 where Y is present, but could only signal in CTLL-2 cells following ligand-induced association with an α subunit.

Possible candidates for molecules X and Y might be non-receptor tyrosine kinases such as members of the *src* family, as the IL-3R and the GMR have been reported to associate with and/or activate the products of the *fes* (Hanazono *et al.*, 1993) and the *src*-related *lyn* (Torigoe *et al.*, 1992) genes. While the JAK-2 kinase is also associated with the IL-3R (Silvennoinen *et al.*, 1993), it is unlikely that X or Y is JAK-2 since the latter is also present in CTLL-2 cells (Mano *et al.*, 1993); it is more likely that JAK kinases are required in addition to X or Y. Alternatively, X and Y may be adaptor molecules that, by analogy with GRB2 (Lowenstein *et al.*, 1992) for example, bring other components of the signal transduction pathway into the receptor complex. A third possibility is that they are members of the STAT family of transcription factors that associate with many members of the cytokine receptor family (reviewed by Darnell *et al.*, 1994).

Mechanism of activation by the I374N and FIΔ mutations. For reasons discussed above—in particular their differing abilities to function in BAF-F03 cells—these mutations are unlikely to act in the same way as V449E, i.e. by formation of hβc homodimers. Moreover, as both are within the extracellular portion of hβc, it appears likely that they may function by interacting with another membrane-spanning molecule. Such a situation would be superficially reminiscent of the case of the IL-2 receptor which has a third component, the γ subunit (Takeshita *et al.*, 1992). However, the analogy is not strictly appropriate because the IL-2R γ subunit appears to be essential for signalling (reviewed by Taniguchi and Minami, 1993). One could, however, postulate that a myeloid-specific 'γ subunit' could associate with an αβ dimer to provide a function which overlaps that of a second β subunit. Functional receptors could then have one of two compositions—$(\alpha\beta)_2$ or αβγ. Thus the I374N and FIΔ mutants may form βγ complexes constitutively, i.e. in the absence of α subunits or ligand, and generate proliferative signals in myeloid (e.g. FDC-P1) cells but not in CTLL-2 or BAF-F03 cells, which represent the T- and B-cell lineages, respectively. Consistent with this, preliminary studies have indicated that FIΔ (and V449E) can induce factor-independent colony formation by myeloid progenitors from murine fetal liver (unpublished observations of M.McCormack and the authors). This model is illustrated in Figure 9B.

It is not clear how the I374N and FIΔ mutations could induce constitutive association with an additional, membrane-spanning receptor component such as a putative γ subunit. However, one plausible explanation comes from examining the structure of the regions involved. Sequence alignment of the extracellular regions of hβc and hGHR (Bazan, 1990; Goodall *et al.*, 1993) suggests that Ile374 lies in the β strand C of the membrane-proximal domain of hβc. Furthermore, predictions based on the structure of hGHR (de Vos *et al.*, 1992; C.J.Bagley and A.Lopez, personal communication) suggest that the side chain of

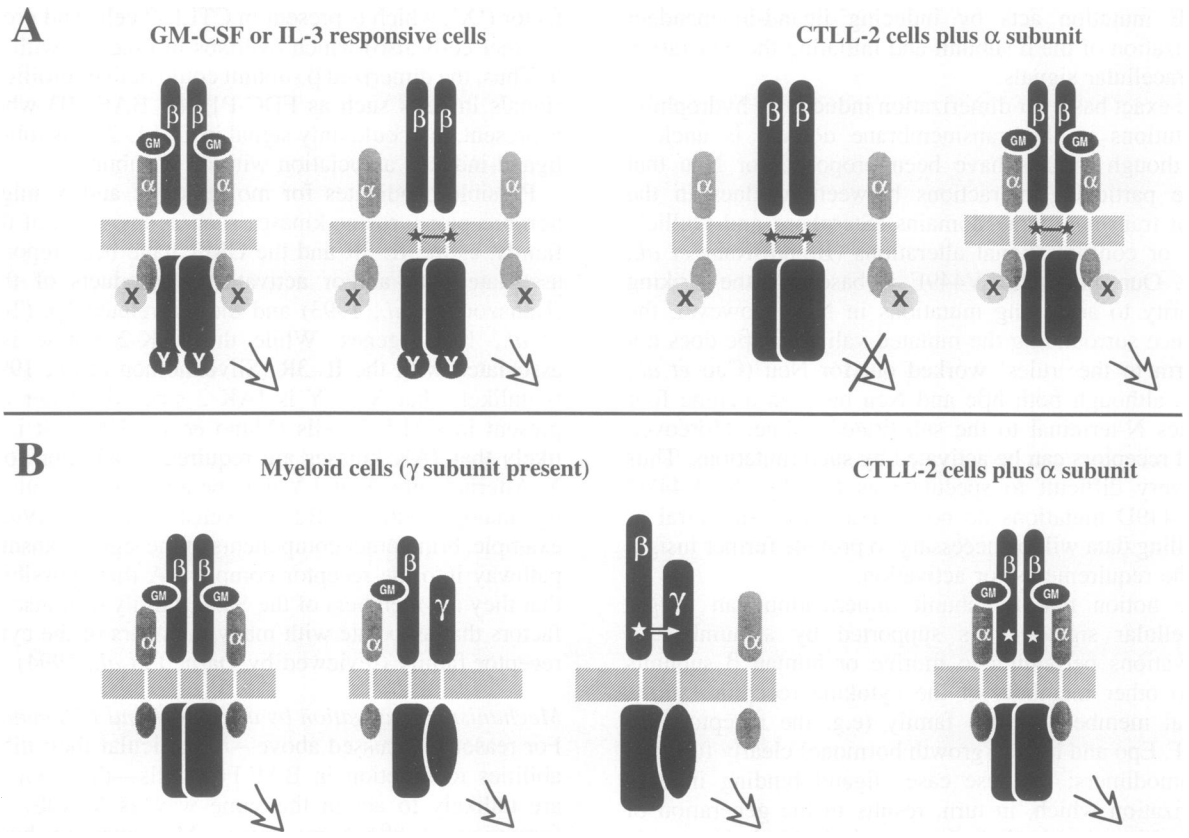

Fig. 9. Models for the mechanism of activation and cell-type specificity of constitutive hβc mutants. In each panel, the cell membrane is represented by the hatched rectangle, with the extracellular domains of the α, β and putative γ subunits shown above the rectangle. The positions of activating mutations are indicated by stars. The ligand indicated is GM-CSF (GM) but the models pertain equally to IL-3 and IL-5. Generation of proliferative signals is indicated by the large arrow at the bottom right of each panel. Both models assume that (one form of) the normal receptor complex contains two α plus two β subunits and that dimerization of the β subunit can trigger proliferative signalling. (**A**) A model pertaining to the V449E mutant which postulates that the transmembrane mutation results in ligand-independent hβc dimerization. This model also invokes the existence of an intracellular signalling molecule, Y, which is specific to IL-3- or GM-CSF-responsive cells (i.e. FDC-P1 or BAF-F03 in this study) and which associates with the β subunit. Molecule X, which can substitute for Y in the receptor complex, associates with the α subunit. Proliferative signalling requires a complex containing either X or Y. See text for further explanation. (**B**) A model pertaining to the I374N and FIΔ mutants which invokes the existence of a third receptor subunit, a 'γ' subunit, which is specific for myeloid cells. The model postulates that the γ subunit can associate with the β subunit of an αβ heterodimer and can contribute to proliferative signalling in concert with the β subunit. Thus, two alternative forms of the normal receptor complex, $(\alpha\beta)_2$ or αβγ, can exist in myeloid cells; the I374N and FIΔ mutations would then function by allowing constitutive association with the γ subunit. In both (A) and (B) the mutant β subunits can form functional $(\alpha\beta)_2$ complexes in CTLL-2 cells when both α subunit and ligand are present.

Ile374 may contact the hydrophobic side chains of valine residues in the conserved RVRVR sequence within the F strand of hβc. Intriguingly, the F strand is part of the region duplicated in the FIΔ mutant; thus, both the duplication in the FIΔ mutant and the hydrophilic substitution in I374N may be affecting a common structure or surface that includes strands F and C—and which, we suggest, may be involved in interacting with the putative γ subunit. A recent report by Grube and Cochrane (1994) supports the notion that strand C may play a regulatory role in interactions with other receptor subunits, as it was found that a peptide corresponding to strand C of the IL-6 receptor (α subunit) can inhibit signalling by IL-6 without affecting ligand binding. We therefore suggest, in a similar vein to D'Andrea *et al.* (1994), that association with ligand plus α subunit, a disruptive amino acid substitution such as I374N in the β strand C or duplication of strand F may all act to unmask an interactive surface involving strand F, allowing association of hβc with the putative γ subunit and triggering the generation of intracellular signals.

Both of the models illustrated in Figure 9 require a degree of functional redundancy between components of the GMR (i.e. between X and Y, and between the β and 'γ' subunits). This redundancy may not be complete, however, because the studies presented here only address proliferation in established cell lines; it is possible that the 'redundant' components may vary in their abilities to generate, say, differentiative signals.

We also wish to point out the potentially general utility of the methodology used to isolate the mutants described here. While a retroviral library of random mutants has already been used by Druker and Roberts (1991), the PCR mutagenesis approach used here is much simpler than the chemical mutagenesis employed by these authors. Because many receptors, including members of both receptor tyrosine kinase and cytokine receptor families, can induce ligand-dependent proliferation of factor-dependent haemopoietic cell lines (e.g. Di Fiore *et al.*, 1990; D'Andrea *et al.*, 1991; Sakamaki *et al.*, 1993; Ziegler *et al.*, 1993), the procedure described here offers the possibility of identifying activating mutations in any of these molecules.

Furthermore, this option may also apply to intracellular molecules that are involved in receptor-mediated signalling. Finally, it should be possible to extend this approach to other types of screens using different cell types, including primary cells, and to devise screens for loss-of-function mutants as well as gain-of-function mutants. This could facilitate the identification of functional domains and structures in molecules involved in other cellular functions such as differentiation, adhesion or gene regulation.

Materials and methods

Cell lines and cDNAs

PA317 (Miller and Buttimore, 1986) and Ψ2 (Mann *et al.*, 1983) retrovirus packaging cell lines were maintained in DME medium supplemented with 10% fetal calf serum (FCS). The IL-3/GM-CSF-dependent mouse myeloid cell line, FDC-P1 (Dexter *et al.*, 1980), and the BAF-B03 subline (Hatakeyama *et al.*, 1989) of the IL-3-dependent mouse pro-B cell line, Ba/F3, were maintained in the same medium, as above, containing 80 units/ml mouse GM-CSF (produced by an engineered yeast strain and kindly supplied by Dr Tracy Wilson, Walter and Eliza Hall Institute, Melbourne) or 300 units/ml mouse IL-3 (produced from a baculovirus vector and kindly supplied by Dr Andrew Hapel, John Curtin School of Medical Research, Canberra), respectively. The IL-2-dependent mouse T-cell line, CTLL-2 (Cerottini *et al.*, 1974), was maintained in RPMI-1640 medium supplemented with 10% FCS and 50 μM 2-mercaptoethanol, in the presence of 100 units/ml bacterially synthesized mouse IL-2 (expressed from the plasmid pTRC11 which was kindly provided by Dr G.Zurawski, DNAX, Palo Alto) or 20% conditioned medium from MLA cells (CTLL medium).

The hβc cDNA (Hayashida *et al.*, 1990) used here was that described by Barry *et al.* (1994). cDNA for hGMRα (Gearing *et al.*, 1989) was kindly provided by Dr Nic Nicola (Walter and Eliza Hall Institute, Melbourne, Australia). The activated FIΔ mutant of hβc has been described previously (D'Andrea *et al.*, 1994).

Site-directed mutagenesis and construction of expression plasmids

Site-directed mutagenesis was carried out on single-stranded DNA with mutagenic oligonucleotides using the pAlter-1 system (Promega) in accordance with the manufacturer's instructions. All mutations were confirmed by DNA sequencing, following which mutant hβc cDNAs were blunt-end ligated between end-filled *Bgl*II and *Xho*I restriction sites of the pRUFNeo retroviral vector. The pRUFNeo retroviral vector was modified from the version described by Rayner and Gonda (1994) by the removal of the *Nco*I restriction site from the multiple cloning site.

The pRUFPuro retroviral expression vector was constructed by replacing the MC1Neo cassette of pRUFNeo with an SV40/puromycin resistance cassette from pBabePuro (Morgenstern and Land, 1990). A vector for expressing hGMRα was constructed by inserting the hGMRα cDNA into the *Xho*I site of pRUFPuro.

To facilitate cloning of hβc PCR fragments (see below), a silent mutation creating a *Xho*I restriction site was introduced into the wild-type hβc cDNA by changing nucleotide 772 (Hayashida *et al.*, 1990; sequence accession number M38275) from G to C. This cDNA was then inserted into pRUFNeo as above to generate pRUFNeo-hβc.

PCR mutagenesis and construction of a point-mutated hβc cDNA library

Random point mutations were introduced into a 940 bp *Xho*I–*Bgl*II segment, bases 770–1710 (Hayashida *et al.*, 1990) of the hβc cDNA, by PCR mutagenesis based on the method described by Cadwell and Joyce (1992). The primers used for amplification were 5′-TGGAGCCCAG-AGGTTTGCTGGGACT-3′ (nucleotides 713–737) and 5′-GGGCCC-ATTGAAGTCAAAGCTGGAA-3′ (nucleotides 1804–1780), defining a 1091 bp fragment. Standard and mutagenic reactions (in a volume of 50 μl) were performed on 5 ng of pRUFNeo-hβc plasmid DNA using 12 μM of each primer, and were cycled in a MiniCycler (MJ Research) for 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min. Reactions were denatured at 95°C for 5 min prior to cycling. Standard reactions contained an equimolar concentration of dNTPs (0.2 mM), 1.5 mM $MgCl_2$ and 2.5 units of *Taq* polymerase (Perkin Elmer), whereas mutagenic reactions contained 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 7 mM $MgCl_2$, 5 units of *Taq* polymerase and varying concentrations of $MnCl_2$. To estimate mutation frequencies, PCR products were purified from agarose gels, subcloned into appropriate pGEM vectors (Promega) and sequenced; the results are shown in Table II. No strong mutational bias was observed under these reaction conditions, although there was a weak bias towards T to N and N to A substitutions similar to that reported by Cadwell and Joyce (1992).

Table II. Effect of Mg^{2+} and Mn^{2+} concentrations on PCR mutation rate

[$MnCl_2$] mM	[$MgCl_2$] mM	Bases sequenced	Mutation rate
0	7.0	2520	1/840
0.1	7.0	3900	1/300
0.25	7.0	3060	1/510
0.5	7.0	2800	1/400
0	1.5	2200	1/2200

A library of hβc fragments containing mutations at the desired frequency (0.2%) was regenerated by performing the mutagenic PCR as above in the presence of 0.25 mM $MnCl_2$. Following digestion with *Xho*I and *Bgl*II, mutant 940 bp fragments were agarose gel-purified and ligated directionally into the pRUFNeo-hβc from which the *Xho*I–*Bgl*II fragment of hβc had been excised. Following transformation of *Escherichia coli* (DH10B), the resultant hβc cDNA point-mutant library (pRUFNeo-hβc^{mut}) of 1.7×10^5 independent plasmid clones was further amplified as described previously (Rayner and Gonda, 1994).

Expression of mutant hβc cDNAs in factor-independent FDC-P1 cells

Retroviral DNA was used to generate a library of retroviruses as described by Rayner and Gonda (1994). Briefly, PA317 amphotropic packaging cells were transfected with pRUFNeo-hβc^{mut} plasmid DNA and virus-containing transient supernatants were filtered and used to infect Ψ2 ecotropic packaging cells. Infected Ψ2 cells were selected in medium containing 400 μg/ml G418, pooled, and used to infect FDC-P1 cells. This was achieved by co-cultivating 5×10^5 FDC-P1 cells with 10^6 irradiated (30 Gy) Ψ2 producer cells for 48 h in each of four 25 cm^2 flasks. The FDC-P1 cells from each flask were harvested, washed and selected for factor-independent growth in liquid culture medium containing G418 (1 mg/ml) without GM-CSF. Plating in soft agar was performed essentially as described by Johnson (1980); GM-CSF (80 units/ml) or G418 (1 mg/ml) was added as required.

Rescue of mutant hβc cDNAs from factor-independent cells

Genomic DNA was isolated from cells using a Proteinase K/SDS procedure essentially as described by Hughes *et al.* (1979). PCR was performed on 100 ng of genomic DNA with *Pfu* DNA polymerase (Stratagene) under conditions recommended by the manufacturer. The primers and cycling parameters used were as described above. PCR products were inserted into pGEM vectors (Promega), and sequenced.

Characterization of activating hβc point mutations

Point mutations generated in hβc by site-directed mutagenesis were tested for their ability to confer factor independence by expression in FDC-P1 cells or CTLL-2 cells. Ψ2 cells were transfected by the calcium phosphate procedure (as described by Rayner and Gonda, 1994) and stable transfectants selected with G418 (400 μg/ml) prior to co-cultivation. FDC-P1 and BAF-F03 cells were infected by co-cultivation and selected for factor-independent growth as before. For CTLL-2 infections, 4×10^6 stably transfected Ψ2 cells were irradiated (30 Gy) and co-cultivated with 10^6 CTLL-2 cells in CTLL medium (see above) for 2 days in 75 cm^2 flasks. CTLL-2 cells were separated from the adherent Ψ2 cells and selected in CTLL medium containing G418 at 750 μg/ml. After several weeks in culture, selected cells were washed and incubated in medium with or without factor.

To generate CTLL-2 cells co-expressing wild-type hGMRα and hβc mutants, Ψ2 cells were transfected with pRUFPuro-hGMRα plasmid DNA and stable transfectants selected with puromycin at 2 μg/ml. CTLL-2 cells expressing hβc mutants were infected by co-cultivation as before and selected in CTLL medium containing puromycin at 2 μg/ml.

Cell sorting and analysis of receptor subunit expression by flow cytometry

Selected cells expressing hGMRα were collected by cell sorting on a FACStar^{PLUS} flow cytometer (Becton-Dickinson). Briefly, cells were washed and resuspended in cold RPMI-1640 medium supplemented with 5% FCS (RPMI-FCS). Cells were incubated with the anti-hGMRα monoclonal antibody 8G6 (Q.Sun and A.Lopez, manuscript in preparation) for 20 min on ice, washed and subsequently incubated with FITC-conjugated anti-mouse IgG (Silenus) for 20 min on ice. After washing and resuspension in cold RPMI-FCS, the cells were sorted, and the positive cell population collected in CTLL medium.

Expression of hβc mutants on the cell surface of infected FDC-P1, BAF-F03 or CTLL-2 cells was confirmed by flow cytometric analysis using an Epics-Profile II analyser (Coulter) by staining, as described above, with the anti-hβc monoclonal antibody 3D7 (Q.Sun and A.Lopez, manuscript in preparation). hGMRα expression was analysed as above by staining with the monoclonal antibody 8G6.

Cell proliferation assays

Infected cells (FDC-P1, BAF-F03 or CTLL-2) were washed twice and triplicate samples of equal cell number (5×10^3) were cultured in a 96-well microtitre plate with or without appropriate growth factor for 72 h. Cell proliferation was measured by the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega).

Tumorigenicity studies

FDC-P1 cells (5×10^6) expressing each of the mutant β subunits (V449E, I374N or FIΔ) were injected subcutaneously into each of four 10-week-old male DBA2 mice; as controls, mice were injected with cells expressing wild-type hβc or uninfected FDC-P1 cells. Tumour formation was monitored at weekly intervals.

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Note added in proof

We note a recent publication describing the activation of the mouse βc subunit by an amino-terminal truncation [J.Hanneman, T.Hara, M.Kawai, A.Miyajima, W.Osterlag and C.Stocking (1995) Sequential mutations in the interleukin-3 (IL-3)/granulocyte-macrophage-colony-stimulating factor/IL-5 receptor β-subunit genes are necessary for the complete conversion to growth autonomy mediated by a truncated βc subunit. *Mol. Cell. Biol.*, **15**, 2402–2412].