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Critical cytoplasmic domains of the common β subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation

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The high-affinity receptors for human granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and interleukin 5 (IL-5) are composed of two distinct subunits, α and β_c. The α subunits are specific for each cytokine, whereas the β subunit (β_c) is shared by the three receptors and is an essential component of signal transduction. We have made a series of mutant β_c cDNAs that delete various regions of the cytoplasmic domain and examined the function of these mutants by coexpressing them with the α subunit of the human GM-CSF receptor (hGMR) in an IL-3-dependent mouse pro-B cell line BaF3. Two domains in the membrane-proximal portion of β_c were found to be important for transducing the hGM-CSF-mediated growth signals: one domain between Arg^{456} and Phe^{487} appears to be essential for proliferation, and the second domain between Val^{518} and Asp^{544} enhances the response to GM-CSF, but is not absolutely required for proliferation. The region between Val^{518} and Leu^{626} was responsible for major tyrosine phosphorylation of 95 and 60 kDa proteins. Thus, β_c-mediated major tyrosine phosphorylation of these proteins was apparently separated from proliferation. However, the β_{517} mutant lacking residues downstream of Val^{518} transmitted a herbimycin-sensitive proliferation signal, suggesting that β_{517} still activates a tyrosine kinase(s). We also evaluated the role of the cytoplasmic domain of the GMR α subunit and the results suggest that it is involved in the hGM-CSF-mediated signal transduction, but is not essential. GM-CSF-induced tyrosine phosphorylation of β_c and the amino acid sequences surrounding the tyrosine residues as well as phosphorylation of deletion mutants suggest that Tyr^{750} is most likely the site for tyrosine phosphorylation.

Key words: colony-stimulating factor/cytokine/cytokine receptor/hemopoiesis/tyrosine kinase

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) act on a wide variety of hemopoietic cells to stimulate their proliferation and differentiation (Metcalf, 1986; Schrader, 1986; Clark and Kamen, 1987; Arai *et al.*, 1990; Gasson, 1991). Interleukin 5 (IL-5) is a lineage-restricted hemopoietic growth factor that acts mainly on eosinophils and some B cells (Takatsu *et al.*, 1988). The high-affinity receptors for these cytokines consist of two distinct subunits, α and β (Hayashida *et al.*, 1990; Devos *et al.*, 1991; Kitamura *et al.*, 1991a,b; Takaki *et al.*, 1991; Tavernier *et al.*, 1991; Hara and Miyajima, 1992). The α subunits (GMRα, IL-3Rα and IL-5Rα) are cytokine-specific binding proteins, and each α subunit alone binds its specific ligand with low affinity (Gearing *et al.*, 1989; Takaki *et al.*, 1990; Devos *et al.*, 1991; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991; Hara and Miyajima, 1992; Murata *et al.*, 1992). In contrast, the β subunit does not bind any cytokine by itself, but forms high-affinity receptors with α subunits. Human GM-CSF (hGM-CSF), IL-3 (hIL-3) and IL-5 (hIL-5) receptors have only one type of β subunit (common β, or β_c) which is shared by the three receptors (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991). Therefore, these human cytokines often compete with each other for binding to their receptors (Gesner *et al.*, 1988; Elliot *et al.*, 1989; Park *et al.*, 1989; Budel *et al.*, 1990; Lopez *et al.*, 1991; Taketazu *et al.*, 1991). Interestingly, the mouse has two distinct β subunits (Gorman *et al.*, 1990; Itoh *et al.*, 1990; Hara and Miyajima, 1992): one (AIC2A, or β_{IL3}) is specific for the mouse IL-3 receptor (mIL-3R), and the other (AIC2B, or β_c) is equivalent to the human β_c and is shared by the three receptors (Devos *et al.*, 1991; Kitamura *et al.*, 1991b; Shanafelt *et al.*, 1991; Takaki *et al.*, 1991; Hara and Miyajima, 1992). These two mouse β subunits have an unusually high degree of sequence homology (91% identical at the amino acid level) (Gorman *et al.*, 1990), and are also homologous to the human β_c (56% identical) (Hayashida *et al.*, 1990). We have previously shown that the β subunits are not only required for high-affinity binding, but are also important for growth signal transduction (Kitamura *et al.*, 1991a; Shanafelt *et al.*, 1991; Hara and Miyajima, 1992; Kitamura and Miyajima, 1992).

The overall structure of GMRα, IL-3Rα and IL-5Rα is similar: they are glycoproteins of ~60–80 kDa having the common motif of the cytokine receptor superfamily in the extracellular domain and they have a small cytoplasmic domain with a short stretch of an amino acid sequence which is conserved among these α subunits (Kitamura *et al.*, 1991b; Miyajima *et al.*, 1992). The mature β subunits are 120–140 kDa and have two repeats of the common motif of the cytokine receptors (Gorman *et al.*, 1990; Hayashida *et al.*, 1990; Itoh *et al.*, 1990). They have a large cytoplasmic domain without any consensus sequence for signaling molecules such as kinases, phosphatases or nucleotide binding proteins. GM-CSF, IL-3 and IL-5 induce rapid protein tyrosine phosphorylation that appears to be important for their function (Morla *et al.*, 1988; Isfort and Ihle, 1990; Kanakura *et al.*, 1990; Murata *et al.*, 1990). It is known that activation of tyrosine kinases leads to activation of *ras* and phosphorylation of *raf*, a Ser/Thr kinase (Satoh *et al.*, 1990; Li *et al.*, 1991). GM-CSF and IL-3 also activate *ras* and induce phosphorylation of *raf* (Carroll *et al.*, 1990;

Kanakura *et al.*, 1991; Satoh *et al.*, 1991). However, activation of *ras* or *raf* alone is not sufficient to abrogate the requirements of GM-CSF and IL-3 (Ihle *et al.*, 1985; Rein *et al.*, 1985), suggesting that activation of multiple signal transduction pathways is needed to stimulate cell growth. Since neither the α or β subunit has an intrinsic tyrosine kinase, there must be additional proteins that mediate a signal from the high-affinity receptors to signaling proteins such as *ras* (Miyajima *et al.*, 1992). In this paper, we describe the identification of the critical cytoplasmic domains of β_c for growth signal transduction and tyrosine phosphorylation by GM-CSF. Our results demonstrate the apparent separation of the β_c-mediated major tyrosine phosphorylation and growth signal transduction.

Results

Construction and expression of mutant human β_c and GMRα cDNAs

A mouse pro-B cell line BaF3 requires mouse IL-3 (mIL-3) for growth, but is not mGM-CSF responsive. BaF3 transfectants expressing high-affinity hGMR ($\alpha\beta_c$) proliferate in response to low concentrations of hGM-CSF, whereas BaF3 transfectants expressing hGMRα alone require >100-fold higher concentrations of hGM-CSF for proliferation (Kitamura *et al.*, 1991a). We have also demonstrated that hGMRα does not form a high-affinity hGM-CSF binding site with either of the mouse β subunits (AIC2A and AIC2B), but is capable of transmitting a growth signal in combination with the mouse common β subunit (AIC2B) in the presence of high concentrations of hGM-CSF. Thus, β_c is not only required for high-affinity hGM-CSF binding, but is also an essential component of growth signal transduction (Kitamura *et al.*, 1991a).

To identify the functional domains of β_c for signal transduction, we constructed a series of cytoplasmic deletion mutants of the human β_c (Figure 1) and coexpressed them in BaF3 cells with the full length hGMRα. These transfectants were designated as BaF3/$\alpha\beta_c$, BaF3/$\alpha\beta_{825}$, BaF3/$\alpha\beta_{763}$, BaF3/$\alpha\beta_{626}$, BaF3/$\alpha\beta_{544}$, BaF3/$\alpha\beta_{517}$, BaF3/$\alpha\beta_{455}$, and BaF3/$\alpha\beta_{int}$. We also established three types of transfectants in an IL-2-dependent mouse T cell line CTLL2; CTLL/$\alpha\beta_{517}$, CTLL/$\alpha\beta_{455}$, and CTLL/$\alpha\beta_{int}$. Moreover, as hGMRα has a cytoplasmic domain of 54 amino acid residues, we made a mutant lacking 50 amino acids from the C-terminus. BaF3 cells coexpressing the hGMRα mutant with the full length β_c (BaF3/$\alpha_{328}\beta$) were used to examine the function of hGMRα.

BaF3 transfectants expressing these mutant receptors were examined for hGM-CSF binding. As summarized in Table I, regardless of the cytoplasmic deletions, all the transfectants exhibited high-affinity hGM-CSF binding sites with almost the same K_d value. Most of the transfectants also exhibited low-affinity sites as well, indicating that the expression level of hGMRα exceeded that of the human β_c in the transfected cells. These results are consistent with the previous result that the cytoplasmic domain of β_c is not required for formation of high-affinity hGMR (Hayashida *et al.*, 1990). The expression level of β_c of the transfectants was also evaluated by using a monoclonal antibody against β_c, CRS1, and we confirmed that the expression level of β_c was not significantly different among the transfectants (data not shown).

Function of the β_c mutants in growth signal transduction

Proliferation potential of the β_c mutants was examined using the BaF3 transfectants (Figure 2). As BaF3 cells express the endogenous mouse common β subunit (AIC2B), transfectants expressing hGMRα can proliferate in the presence of high concentrations of hGM-CSF even if the transfected human β_c is inactive. The $\alpha\beta_{825}$, $\alpha\beta_{763}$, $\alpha\beta_{626}$ and $\alpha\beta_{544}$ transfectants proliferated in the presence of low concentrations of hGM-CSF, and the EC_{50}s are comparable with that of the full length β_c (~ 0.05 ng/ml). These transfectants were able to grow continuously in medium containing 1 ng/ml

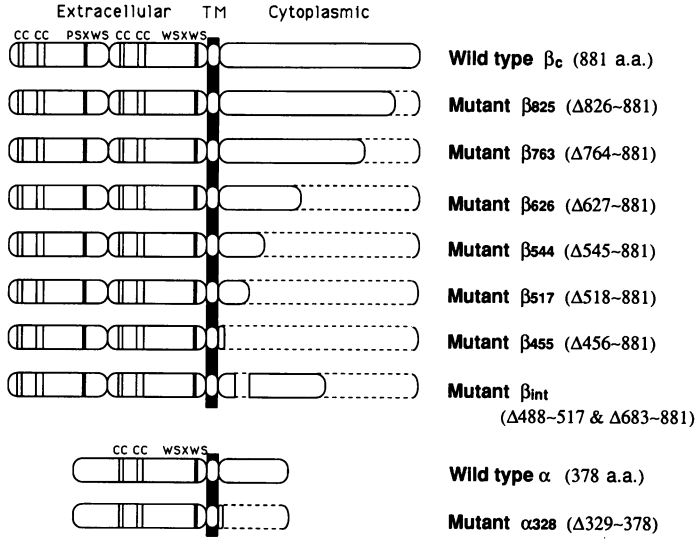

Fig. 1. Structure of mutant β_c and α subunits for hGMR. The extracellular portion of the β_c subunit is divided into two domains, each containing four cysteine residues and the WS motif as described in the Introduction. The deletion mutants of the β_c or α subunits (mutants β_{825}, β_{763}, β_{626}, β_{544}, β_{517}, β_{455}, β_{int} and α_{328}) are shown below the wild-type subunits. The portions deleted in the mutants are shown by the dotted lines, and the numbers of amino acids deleted in each mutant are indicated. The N-terminal signal sequence in all wild-type and mutant subunits is not shown in the figure.

of hGM-CSF. These results indicate that the β_{544} mutant lacking residues downstream of 544 is completely functional with regard to proliferation. In contrast, BaF3/$\alpha\beta_{517}$ showed reduced sensitivity to hGM-CSF; the EC_{50} was ~5 ng/ml, which is 50- to 100-fold higher than the EC_{50} for BaF3/$\alpha\beta_c$ as well as for BaF3/$\alpha\beta_{825}$ to BaF3/$\alpha\beta_{544}$. However, BaF3/$\alpha\beta_{517}$ was able to grow continuously in medium containing 1 ng/ml of hGM-CSF. These results indicate that the cytoplasmic region between 518 and 544 enhances the proliferative response to hGM-CSF, but that this region is not essential for hGM-CSF-mediated growth signal transduction. The same result was obtained with CTLL2 transfectants, CTLL/$\alpha\beta_{517}$, which expressed the truncated β_c and the intact α subunit, but lacked expression of the endogenous mouse β_c (Figures 1 and 2). Thus, hGM-CSF induced growth signals through the β_{517} mutant receptor, but not through the endogenous mouse β_c, indicating that the β_{517} mutant remains capable of transmitting proliferation signals.

A clone (clone #9) of BaF3/$\alpha\beta_{455}$ expressing both high- and low-affinity binding sites for hGM-CSF responded to hGM-CSF only at high concentrations, indicating that β_{455} is not capable of transmitting hGM-CSF-mediated growth signals at physiologic ligand concentrations and that proliferation of this clone is stimulated by the endogenous mouse β_c in combination with hGMRα. Interestingly, another BaF3/$\alpha\beta_{455}$ transfectant (clone #7) expressing only high-affinity binding for hGM-CSF did not respond to hGM-CSF even at high concentrations. Since there was only a high-affinity hGMR in clone #7 (Table I), this clone appeared to express no extra hGMRα that interacted with endogenous mouse β_c. Neither clone #7 nor #9 was capable of being maintained in medium containing 1 ng/ml of hGM-CSF. CTLL2/$\alpha\beta_{455}$ demonstrated high- and low-affinity binding for hGM-CSF (data not shown), but it did not proliferate in response to hGM-CSF. These results indicate that the β_{455} mutant is not capable of inducing proliferation. The β_{455} mutant is 62 amino acids shorter than β_{517}, suggesting that the amino acid sequence between 456 and 517 is important for growth signal transduction in BaF3 and CTLL2 cells. BaF3 and CTLL2 expressing an internal deletion mutant of β_c (β_{int}), which lacks 30 amino acid residues within the 456 to 517 sequence of β_c, proliferated in the presence of low concentrations of hGM-CSF (Figure 2). These results suggest that the transmembrane-proximal portion (32 amino acids) of the cytoplasmic region of β_c is involved in hGM-CSF-induced growth signal transduction.

Table I. Binding profiles of hGM-CSF receptor on BaF3 transfectants[a]

Transfectant	High affinity K_d (pM)	High affinity Binding sites (per cell)	Low affinity K_d (nM)	Low affinity Binding sites (per cell)
Wild type				
$\alpha\beta_c$	93 ± 21	3000–6000	2.8 ± 1.0	26 000–70 000
Mutant				
$\alpha\beta_{825}$	133 ± 33	16 000–39 000	2.7 ± 1.2	30 000–66 000
$\alpha\beta_{763}$	65 ± 40	1000–6000	4.0 ± 2.8	13 000–86 000
$\alpha\beta_{626}$	50 ± 40	2000–16 000	6.4 ± 3.2	21 000–33 000
$\alpha\beta_{544}$	45 ± 32	3000–6000	1.6 ± 0.7	82 000–390 000
$\alpha\beta_{517}$	90 ± 64	4000–14 000	6.0 ± 2.4	140 000–380 000
$\alpha\beta_{455}$[b]	88 ± 50	10 000–34 000	2.8 ± 1.3	40 000–270 000
$\alpha\beta_{int}$	45 ± 15	5000–15 000	7.5 ± 2.5	110 000–620 000
$\alpha_{328}\beta$	77 ± 35	6000–18 000	1.0 ± 0.3	43 000–97 000

[a]Two to four independent clones were analyzed for each mutant.
[b]Clone #7 exhibited only high-affinity receptor (112 pM) with 17 000 binding sites.

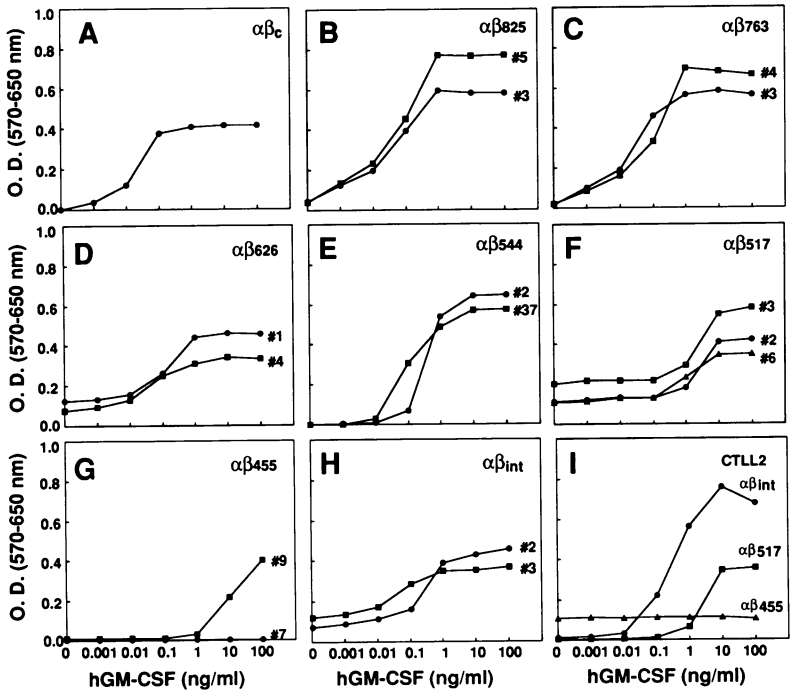

Fig. 2. Growth response of the transfectants expressing the mutant β_c subunits. Transfectants of BaF3 and CTLL2 were incubated for 24 h with 0–100 ng/ml of hGM-CSF, and cell growth was examined by the MTT colorimetric assay. Panels **A**–**H**: BaF3 transfectants expressing the wild type and mutant hGMR as indicated, and # shows the clone number of the transfected BaF3 cells. Panel **I**: CTLL transfectants.

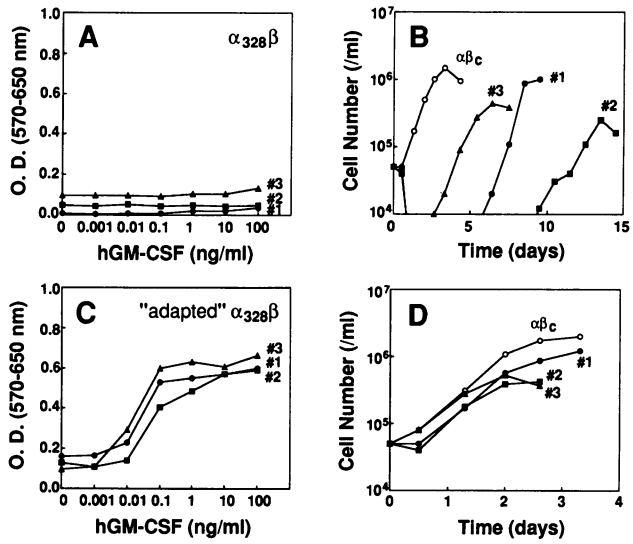

Fig. 3. Proliferation of BaF3/$\alpha_{328}\beta$. Panels **A** and **C**: growth response of the transfectants to hGM-CSF. Three independent clones of BaF3/$\alpha_{328}\beta$ transfectant maintained in medium containing mIL-3 (A) or hGM-CSF (C) were incubated for 24 h with 0−100 ng/ml of hGM-CSF, and cell growth was examined by MTT assay as described in Materials and methods. Panels **B** and **D**: time course of hGM-CSF-dependent proliferation of the transfectants. A BaF3/$\alpha\beta_c$ transfectant and three independent clones of BaF3/$\alpha_{328}\beta$ transfectants growing in medium containing mIL-3 (B) or BaF3/$\alpha\beta_c$ and 'hGM-CSF-adapted' BaF3/$\alpha_{328}\beta$ clones cultured in medium containing mIL-3 for 1 month (D) were washed and inoculated at 5×10^4 cells/ml in medium containing 1 ng/ml of hGM-CSF on day 0. Cell proliferation was monitored by counting viable cell numbers.

Deletion of the cytoplasmic domain of hGMRα

We next examined whether the cytoplasmic domain of hGMRα is necessary for growth signal transduction (Figure 3). BaF3/$\alpha_{328}\beta$ which expresses the cytoplasmic deletion mutant of hGMRα plus the full length β_c exhibits a high-affinity hGMR (Table I). These transfectants were maintained in medium containing mIL-3 and assayed for their response to hGM-CSF. None of the transfectants proliferated in the presence of low and high concentrations of hGM-CSF immediately after transfer to medium containing hGM-CSF (Figure 3A). However, when these cells were cultured in the presence of hGM-CSF instead of mIL-3, a few cells survived and proliferated after a latency of 4−12 days (Figure 3B). These 'hGM-CSF-adapted' cells proliferated in response to hGM-CSF in a dose-dependent manner (Figure 3C), and retained their ability to respond to hGM-CSF after re-exposure to mIL-3 for 1 month (Figure 3D). In contrast, transfectants expressing the full length hGMRα showed no such lag time for proliferation during the transition from medium containing mIL-3 to medium containing hGM-CSF (Figure 3B and D). The same result was obtained with the CTLL2 transfectants (CTLL/$\alpha_{328}\beta$) expressing the mutant hGMRα and normal β_c (data not shown). These results suggest that the cytoplasmic region of hGMRα can modulate hGM-CSF-mediated growth signals, but is not essential for signal transduction in BaF3 and CTLL2 cells.

Tyrosine phosphorylation stimulated by hGM-CSF

Since tyrosine phosphorylation appears to be critical for cytokine signal transduction (Cleveland, 1989; Satoh *et al.*, 1992), we examined the tyrosine phosphorylation pattern induced by hGM-CSF in BaF3 transfectants expressing mutant hGMRs. hGM-CSF induced the rapid appearance of multiple phosphotyrosylproteins in BaF3/$\alpha\beta_c$ which expresses the wild type hGMR. The phosphorylation pattern, with the anti-phosphotyrosine antibody 4G10, was similar to that of parental BaF3 cells stimulated with mIL-3 (Figure 4A, lanes 2 and 4). Both hGM-CSF and mIL-3 induced tyrosine phosphorylation of 95 and 60 kDa proteins. A faint protein band at 75 kDa was also reproducibly observed by stimulation with either mIL-3 or hGM-CSF. In addition, hGM-CSF uniquely induced phosphorylation of a 130 kDa protein, while mIL-3 induced phosphorylation of 128 and 135 kDa proteins.

hGM-CSF induced phosphorylation of proteins of 95, 75 and 60 kDa in BaF3/$\alpha\beta_{825}$ and BaF3/$\alpha\beta_{763}$, similar to the pattern observed in cells expressing the wild-type hGMR (Figure 4A, lanes 6 and 8). However, phosphorylation of a unique 125 or 115 kDa protein was found in BaF3/$\alpha\beta_{825}$ and BaF3/$\alpha\beta_{763}$, respectively, and the 130 kDa phosphorylated protein disappeared in these transfectants, suggesting that these proteins are β_c (see below). hGM-CSF-induced tyrosine phosphorylation of proteins 95, 75 and 60 kDa was significantly reduced in BaF3/$\alpha\beta_{626}$ and BaF3/$\alpha\beta_{544}$ (Figure 4A, lanes 10 and 12). Tyrosine phosphorylation of 75 and 60 kDa proteins was barely detectable in BaF3/$\alpha\beta_{544}$, but tyrosine phosphorylation of the 95 kDa protein was weakly detectable. An alternative monoclonal anti-phosphotyrosine antibody, PY20, clearly detected tyrosine phosphorylation of a 95 kDa protein in BaF3/$\alpha\beta_{544}$ (Figure 4B). However, no significant tyrosine phosphorylation was induced by hGM-CSF in BaF3/$\alpha\beta_{517}$ and BaF3/$\alpha\beta_{455}$ (Figure 4A and B). The 95 and 60 kDa tyrosine phosphorylated proteins were clearly detected in BaF3/$\alpha\beta_{int}$, whereas the 75 kDa protein was not clearly phosphorylated in this transfectant (Figure 4). Thus, phosphorylation of the 95 and 60 kDa proteins appears to be dependent on the cytoplasmic region between 518 and 626 of β_c. However, it is not clear whether this region is also responsible for tyrosine phosphorylation of the 75 kDa protein.

Herbimycin A inhibits the hGM-CSF-mediated growth of BaF3 transfectants

The analysis of tyrosine phosphorylation suggests that major tyrosine phosphorylation induced by hGM-CSF is not required for proliferation. However, it does not prove that

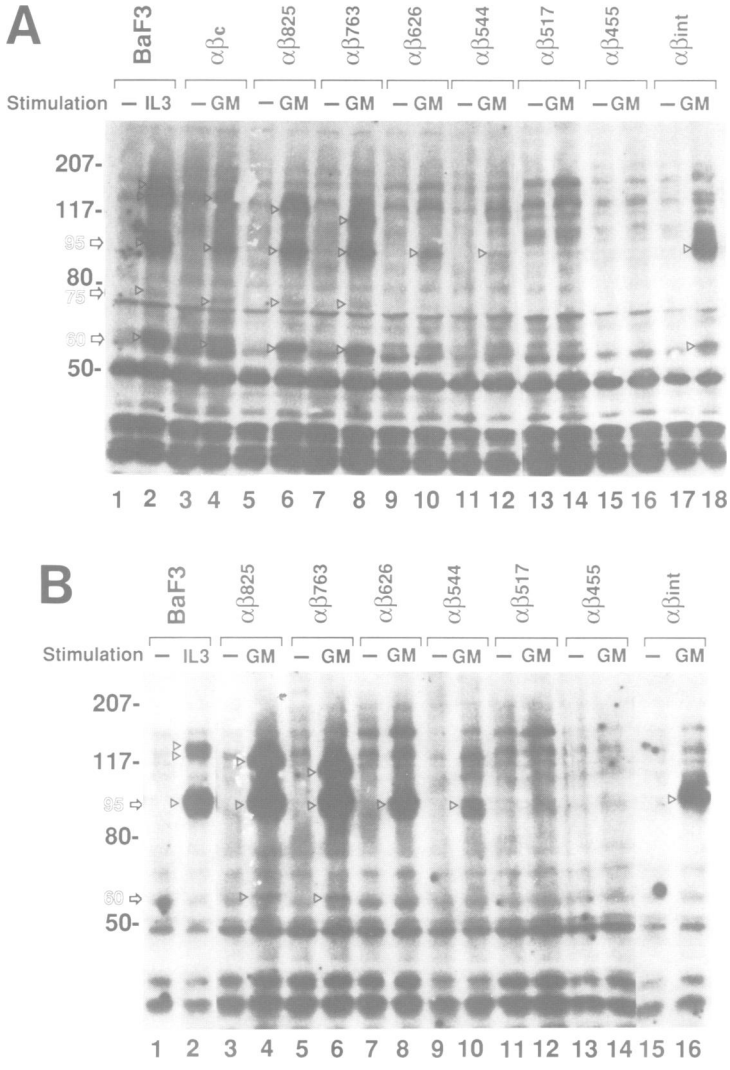

Fig. 4. Tyrosine phosphorylation induced by hGM-CSF stimulation in BaF3 transfectants. Cytokine-depleted cells were either unstimulated (lanes 3, 5, 7, 9, 11, 13, 15 and 17) or stimulated with 10 ng/ml of hGM-CSF for 10 min (lanes 4, 6, 8, 10, 12, 14, 16 and 18). Factor-depleted parental BaF3 cells were also incubated with or without 10 ng/ml of mIL-3 for 10 min (lanes 1 and 2). Cell lysates (1×10^6 cells/lane) were separated by SDS−8% PAGE and immunoblotted with the monoclonal anti-phosphotyrosine antibodies, 4G10 (**A**) and PY20 (**B**). Size standards are shown in kDa on the left side. Proteins phosphorylated by the stimulation are indicated by arrowheads with their respective approximate molecular sizes.

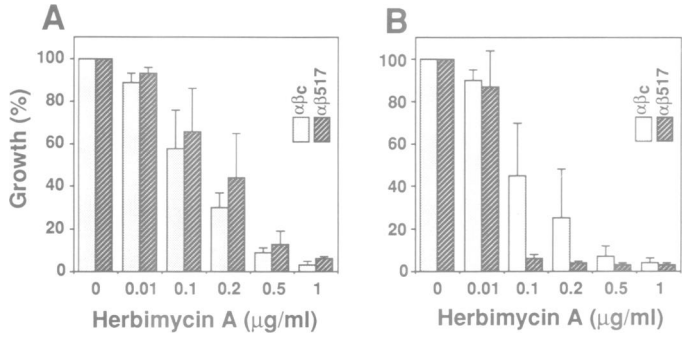

Fig. 5. Effects of herbimycin A on growth of BaF3 transfectants. BaF3/$\alpha\beta_c$ and BaF3/$\alpha\beta_{517}$ were incubated in medium containing 50 ng/ml of mIL-3 (**A**) or hGM-CSF (**B**) for 24 h in the presence of various concentrations of herbimycin A. Cell growth was examined by MTT assay. The data represent the means of three or four separate experiments.

tyrosine phosphorylation is not involved in hGM-CSF-mediated growth signal transduction by the deletion mutant β_{517}. To examine further the contribution of a tyrosine kinase(s) in hGM-CSF-mediated signal transduction, we used herbimycin A, a tyrosine kinase specific inhibitor (Uehara *et al.*, 1989). As previously shown in IL-3-dependent PT18 cells (Satoh *et al.*, 1992), herbimycin A inhibited the growth of BaF3 transfectants in the presence of mIL-3 (Figure 5A). The dose response of BaF3/$\alpha\beta_{517}$ to herbimycin A was almost the same as that of BaF3/$\alpha\beta_c$ when they were

assayed in the presence of mIL-3. In contrast, although herbimycin A inhibited the growth of both transfectants in the presence of hGM-CSF, BaF3/$\alpha\beta_{517}$ was more sensitive to herbimycin A than BaF3/$\alpha\beta_c$ transfectants (Figure 5B). The inhibitory effect of herbimycin A was suppressed by addition of 2-mercaptoethanol, an inhibitor of herbimycin A (data not shown). These results suggest that a tyrosine kinase(s) is involved in the growth signal transduction pathway mediated by hGM-CSF in BaF3/$\alpha\beta_{517}$, although hGM-CSF-mediated major tyrosine phosphorylation was almost undetectable in this transfectant (Figure 4).

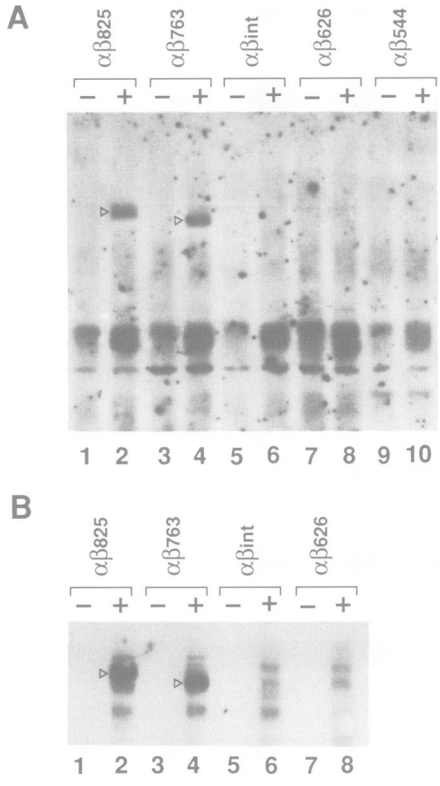

Fig. 6. Analysis of the phosphorylated βc subunit. Factor-depleted cells were stimulated with hGM-CSF and lysed. Samples were incubated with (lanes 2, 4, 6, 8 and 10) or without (lanes 1, 3, 5, 7 and 9) anti-phosphotyrosine antibody, PY20, and the immunoprecipitates were subjected to SDS−PAGE, as described in Materials and methods. Anti-β_c subunit antibody, #159 (**A**), or anti-phosphotyrosine antibody, PY20 (**B**), was used to detect proteins. The 125 and 115 kDa proteins are indicated by arrowheads

Tyrosine phosphorylation of β_c

The 125 and 115 kDa phosphoproteins observed in BaF3/$\alpha\beta_{825}$ and BaF3/$\alpha\beta_{763}$, respectively, may represent the truncated forms of β_c, since the molecular weights of these phosphoproteins were consistent with molecular weights of the β_c mutants estimated from the mutant cDNAs. We examined this possibility by immunoprecipitation. Anti-phosphotyrosine antibody, PY20, was used to immunoprecipitate tyrosine phosphorylated proteins from hGM-CSF-stimulated cells; the immunoprecipitates were analyzed by Western blotting using antibody #159, raised against peptides of β_c and the anti-phosphotyrosine antibody, PY20. Both antibodies detected the 125 and 115 kDa proteins in the anti-phosphotyrosine immunoprecipitates from BaF3/$\alpha\beta_{825}$ and BaF3/$\alpha\beta_{763}$, respectively (Figure 6A and B, lanes 2 and 4), but they failed to detect any specific protein corresponding to the mutant β_c in BaF3/$\alpha\beta_{int}$, BaF3/$\alpha\beta_{626}$ and BaF3/$\alpha\beta_{544}$ (Figure 6A and B). These results indicate that the 125 and 115 kDa tyrosine phosphorylated proteins observed in BaF3/$\alpha\beta_{825}$ and BaF3/$\alpha\beta_{763}$, respectively, were β_c mutants. These results also suggest that the major tyrosine phosphorylation site is present between Ser^{683} and Ser^{763}.

Discussion

We previously demonstrated that both human β_c and mouse β_c (AIC2B) interact with the human GMRα and transmit growth signals in response to hGM-CSF (Kitamura *et al.*, 1991a; Shanafelt *et al.*, 1991). As BaF3 expresses endogenous mouse β_c, transfectants with the complete deletion of the cytoplasmic domain of the human β_c (β_{455}) still respond to hGM-CSF when hGMRα is coexpressed (Figure 2G). However, as human GMRα and mouse β_c are unable to form a high-affinity receptor (Kitamura *et al.*, 1991a), high concentrations of hGM-CSF are needed for proliferation. This result is consistent with the observation that CTLL2/$\alpha\beta_{455}$, which lacked the endogenous mouse β_c expression, did not respond to hGM-CSF even at high concentrations, while they exhibited hGM-CSF binding sites with both high and low affinities. The expression level of GMRα seems to be important for the growth response to hGM-CSF in BaF3/$\alpha\beta_{455}$ cells, i.e. clone #9 of BaF3/$\alpha\beta_{455}$ exhibited both high- and low-affinity hGM-CSF binding and responded to hGM-CSF at only high concentrations, whereas clone #7 showed only high-affinity hGM-CSF binding and did not respond to hGM-CSF

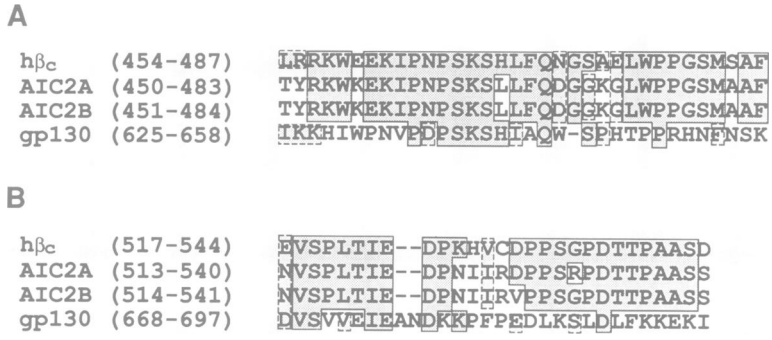

Fig. 7. Cytoplasmic domains of β_c required for growth. Primary sequences of the essential (**A**) and important (**B**) regions of the β_c subunit for transducing the growth signal. The amino acid sequences of the corresponding regions of mouse AIC2A and AIC2B and gp130 (the β subunit of human IL-6 receptor) are also aligned. These two regions are one of the most conserved regions between human β_c and mouse β subunits (78% and 81% identical).

(Figure 2G). These results suggest that hGMRα preferentially interacts with the human β_c, and hGMRα interacts with the endogenous mouse β_c only when the hGMRα level exceeds that of human β_c, i.e. both high- and low-affinity hGM-CSF binding sites are present.

We identified two cytoplasmic domains of β_c that are important for proliferation. A membrane-proximal domain of 32 amino acids was necessary for hGM-CSF-mediated growth signal transduction. The amino acid sequence of this domain is highly conserved among the β subunits, i.e. human β_c, β_{IL3} of the mouse IL-3 receptor (AIC2A) and mouse β_c (AIC2B), as shown in Figure 7A. We also identified a second domain between amino acids 518 and 544 that is important but not essential for growth signal transduction. Deletion of this region reduced sensitivity to hGM-CSF and the extent of stimulation induced by hGM-CSF. However, BaF3/$\alpha\beta_{517}$ and CTLL/$\alpha\beta_{517}$ grew in medium containing hGM-CSF. Therefore, the sequence between 518 and 544 possesses some function for signal transduction, but is not absolutely required in BaF3 and CTLL cells. Most of the amino acid residues in this region are also well conserved among the human β_c, AIC2A and AIC2B (Figure 7B). Recently, Murakami *et al.* (1991) identified two segments in the cytoplasmic region of gp130 (signal transducer of the IL-6 receptor) that are critical for transducing the IL-6-mediated growth signal. These two segments show limited homology to the important domains of β_c which we identified (Figure 7).

The tyrosine phosphorylation pattern of BaF3/$\alpha\beta_c$ cells stimulated with hGM-CSF was similar to that stimulated with mIL-3 (Figure 4), consistent with previous reports (Isfort and Ihle, 1990; Kanakura *et al.*, 1990). Deletion mutants allowed us to identify a cytoplasmic region responsible for major tyrosine phosphorylation. The region between 518 and 626 appears to be responsible for phosphorylation of the 95 and 60 kDa proteins. Since BaF3/$\alpha\beta_{517}$ proliferated in the presence of hGM-CSF without major tyrosine phosphorylation, the most critical β_c region responsible for proliferation and the region responsible for major tyrosine phosphorylation were apparently separated. Since tyrosine phosphorylation is required for IL-3- and GM-CSF-mediated growth signal transduction (Cleveland *et al.*, 1989; Satoh *et al.*, 1992), some proteins must be tyrosine phosphorylated by the GM-CSF stimulation even in BaF3/$\alpha\beta_{517}$. In fact, herbimycin A inhibited hGM-CSF-mediated growth of the BaF3/$\alpha\beta_{517}$ transfectant (Figure 5B), indicating involvement of a tyrosine kinase(s) in hGM-CSF-mediated signal transduction by the β_{517} mutant. These results suggest that minor tyrosine phosphorylated proteins, which were not detected by immunoblotting, are more important for growth signal transduction. The major tyrosine phosphorylated proteins such as 95, 75 and 60 kDa proteins may have functions not directly coupled to proliferation. Interestingly, hGM-CSF-induced proliferation of BaF3/$\alpha\beta_{517}$ was more sensitive to herbimycin A than was hGM-CSF-induced proliferation of BaF3/$\alpha\beta_c$; however, both transfectants showed similar sensitivity to herbimycin A in the presence of mIL-3. These results suggest that multiple tyrosine kinases are activated by the wild type β_c, and that the major tyrosine phosphorylated proteins may also contribute to cell proliferation.

Phosphorylation of membrane receptors is important for the regulation of receptor function and distribution (Ullrich and Schlessinger, 1990). We have demonstrated that β_c is tyrosine phosphorylated by the hGM-CSF stimulation (Figure 6). Eight tyrosine residues at 450, 452, 577, 612, 695, 750, 806 and 856 are present in the cytoplasmic region of β_c. All except Tyr^{450} and Tyr^{856} are conserved between the human and mouse β subunits. Tyr^{750} is most likely the candidate for the tyrosine phosphorylation site, because this residue is surrounded by acidic amino acids (Hunter, 1989) and the truncated β_c lacking Tyr^{750} and Tyr^{695} was not tyrosine phosphorylated (Figure 6). The relation between tyrosine phosphorylation of β_c and tyrosine phosphorylation of the 95, 75 and 60 kDa proteins remains to be established. Since tyrosine kinases are involved in the GM-CSF-mediated growth signal transduction pathway, identification of tyrosine kinases stimulated by hGM-CSF is crucial for further delineation of the mechanisms of signal transduction.

Since the human IL-3, IL-5 and GM-CSF receptors share the same β subunit (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991), similar proliferation signals induced by these cytokines may be mediated by β_c. It is known that although the α subunits of the IL-2 and IL-6 receptors are important to form a high-affinity receptor with the respective β subunit, the IL-2 receptor α subunit is not required for signal transduction in certain types of cell and the cytoplasmic domain of the IL-6 receptor is not required for signal transduction (Hatakeyama *et al.*, 1987; Hibi *et al.*, 1990). However, the presence of a well conserved sequence motif in the cytoplasmic domains of the α subunits of the GM-CSF, IL-3 and IL-5 receptors (Kitamura *et al.*, 1991b) suggests that the cytoplasmic regions of these α subunits may have an important role for signal transduction. Interestingly, cells which express the mutant hGMR lacking the cytoplasmic region of hGMRα required a lag period prior to growth in medium containing hGM-CSF, but they proliferated continuously once they adapted to medium containing hGM-CSF (Figure 3). Induction of tyrosine phosphorylation by the hGM-CSF stimulation was also observed in 'hGM-CSF-adapted' cells, but not in original cells grown in mIL-3 medium (data not shown). As β_c has no known sequence for signaling proteins such as kinases, there must exist alternative signaling molecules capable of interacting with β_c. If this interaction is augmented by the cytoplasmic domain of hGMRα, the mutant hGMR lacking the cytoplasmic domain of GMRα may require high level expression of such signaling proteins. If this is the case, the lag period may represent a process of selecting cells expressing higher levels of such molecules. In addition to proliferation, GM-CSF, IL-3 and IL-5 induce various activities depending on the target cells (Arai *et al.*, 1990), and different biological activities may be mediated by the cytoplasmic domains of different α subunits. Alternatively spliced mRNA for hGMRα encodes a hGMRα isoform that has an identical membrane-proximal cytoplasmic domain but has completely different sequences in the distal cytoplasmic region (Crosier *et al.*, 1991). Therefore, it may be possible that the cytoplasmic domain of hGMRα has an important role in signaling and its activity is regulated by splicing. The functions of the cytoplasmic domains of the α subunits remain to be clarified.

Materials and methods

Cell lines, media and growth factors

A mIL-3-dependent pro-B cell line, BaF3 (Palacios and Steinmetz, 1985) and an IL-2-dependent mouse T-cell line, CTLL2 (Cerottini *et al.*, 1974), were maintained in RPMI 1640 medium supplemented with 10% fetal calf

serum (FCS) and 50 μM 2-mercaptoethanol, in the presence of mIL-3 (100 U/ml) or mouse IL-2 (100 U/ml), respectively. Purified recombinant hGM-CSF produced in *Escherichia coli* was provided by R.Kastelein (DNAX, Palo Alto, CA). mIL-3 expressed in silkworm was purified as described previously (Miyajima *et al.*, 1987)

Antibodies

Rat monoclonal antibody against β_c, CRS1, was prepared using an NIH3T3 transfectant expressing the high-affinity hGMR (Watanabe *et al.*, 1992) and rabbit polyclonal antibody #159 against the β_c peptide sequence (EFEVVYKRLQDSWED) was obtained as described previously (Schreurs *et al.*, 1991). Anti-phosphotyrosine antibodies, PY20 and 4G10 were obtained from ICN Biomedicals Inc. and Upstate Biotechnology Inc., respectively.

Generation of deletion mutants and transfection

The plasmid containing the β subunit cDNA, KH97 was digested with either *Bst*EII (β_{825}), *Sma*I (β_{763}), *Bgl*II (β_{544}) or *Fsp*I (β_{455}) and blunt-ended by the Klenow fragment. The cDNA fragments encoding the N-terminal ends were then isolated by *Xho*I digestion that cleaves the vector sequence adjacent to the cDNA insert. The fragments were inserted between the *Xho*I site and the filled-in *Not*I site of the pME18S vector (K.Maruyama and A.Miyajima, unpublished), a high copy number derivative of pCEV4 (Itoh *et al.*, 1990). As termination codons are present just downstream of the *Not*I site, these cDNA fragments encode truncated β subunits with five additional amino acid residues derived from the vector at their C-terminus. The β_{626} and β_{517} mutants were generated by insertion of a termination codon at the amino acid positions 627 (β_{626}) and 518 (β_{517}), respectively, using site-directed mutagenesis (Kunkel *et al.*, 1987). The β_{int} mutant was generated as follows: *Hpa*I and *Stu*I restriction sites were created by site-directed mutagenesis at amino acid 488 and 517, respectively, on a cDNA truncated at the *Sac*I site (amino acid 682), and the fragment between 488 and 517 was subsequently deleted by ligating the *Hpa*I- and *Stu*I-digested DNA. All these mutated cDNA fragments were inserted in pME18S with a neomycin resistance gene.

A cytoplasmic deletion mutant, α_{328} of the hGMRα was made by creating a termination codon just downstream of the transmembrane domain using the polymerase chain reaction (PCR). The PCR product was then ligated to pCEV4 (Itoh *et al.*, 1990), a prototype vector of pME18S.

A DNA fragment containing either the neomycin resistance or the hygromycin resistance gene was inserted in these constructs. BaF3 cells were transfected by electroporation and transfectants were selected either by G418 (1.5 mg/ml) or hygromycin (1 mg/ml) as previously described (Kitamura *et al.*, 1991a).

Radioligands and binding assays

Recombinant hGM-CSF was iodinated using the Bolton–Hunter reagent (Bolton and Hunter, 1973) as described (Chiba *et al.*, 1990). Binding assays were performed as follows: cells were incubated with iodinated hGM-CSF at 4°C for 1–2 h in the presence or absence of 100-fold excess of unlabeled hGM-CSF. Cell-bound [^{125}I]hGM-CSF was separated from the free ligand by centrifugation through an *n*-butyl phthalate oil layer. The equilibrium binding data were analyzed by the Ligand program (Munson, 1983).

Cell proliferation assays

Cell proliferation was measured by the colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT; Sigma) originally developed by Mosmann (1983). To examine the effect of herbimycin A (Uehara *et al.*, 1989) on transfectants, cells were incubated in medium containing 50 ng/ml of mIL-3 or hGM-CSF with 0–1 μg/ml of herbimycin A. After a 24 h incubation, cell proliferation was measured by the MTT colorimetric assay. For this assay, 2-mercaptoethanol was omitted from the medium.

Immunoblot analysis

Cells were deprived of cytokines for 2.5 h and during the last 30 min cells were incubated with sodium orthovanadate (50 μM). Cells were then stimulated with 10 ng/ml of hGM-CSF or mIL-3 for 10 min and harvested by centrifugation. Cells were lysed in Laemmli's sample buffer (2×10^7 cells/ml) and boiled for 10 min. The proteins were electrophoresed on an SDS–8% polyacrylamide gel and proteins were electrophoretically transferred to a PVDF membrane filter (Millipore) as described previously (Wang *et al.*, 1989). The blotted filter was incubated in TBS (50 mM Tris–HCl pH 7.5 and 150 mM NaCl) containing 3% BSA (Fraction V, Sigma). The filter was incubated with an anti-phosphotyrosine monoclonal antibody, 4G10 or PY20, respectively, at 1 μg/ml in TBS containing 3% BSA and 0.1% sodium azide for 3 h at room temperature, washed four times with TTBS (TBS containing 0.05% of Tween 20) and once with TBS, and incubated with a ^{125}I-labeled F(ab')$_2$ fragment (1 μCi/ml) of goat anti-mouse IgG antibody (Amersham) for 60 min at room temperature. The filter was then washed five times with TTBS, and subjected to autoradiography.

Analysis of the phosphorylated β_c subunit

Cells deprived of cytokines were stimulated with hGM-CSF, harvested and lysed in the modified RIPA buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% NP-40, 0.2% SDS and 0.5% sodium deoxycholate) supplemented with 1 mM PMSF, 0.1% aprotinin, 10% glycerol. Cell lysates were sonicated and centrifuged at 15 000 *g* for 10 min at 4°C. The supernatant was divided into two aliquots. Aliquots were incubated with 40 μl of protein G in the presence or absence of 5 μg of an anti-phosphotyrosine monoclonal antibody, PY20, for 2 h at 4°C. After washing four times with RIPA buffer, immunoprecipitates were dissolved in Laemmli's sample buffer and boiled. The proteins were separated by SDS–PAGE and transferred to a PVDF membrane filter. Either rabbit polyclonal antibody against an oligopeptide of the β subunit or mouse monoclonal antibody, PY20, was used as the first antibody and ^{125}I-labeled F(ab')$_2$ fragments (1 μCi/ml) of donkey anti-rabbit IgG antibody (Amersham) or goat anti-mouse IgG antibody were used as the second antibody, respectively.

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