A Chimeric Serine/Threonine Kinase Receptor System Reveals the Potential of Multiple Type II Receptors to Cooperate with Transforming Growth Factor- β Type I Receptor

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Submitted August 9, 1996; Accepted December 10, 1996
Monitoring Editor: Carl-Henrik Heldin

Receptor-type serine/threonine kinases (RSKs) have been organized into two distinct classes known as types I and II on the basis of sequence similarity. However, experiments have shown ligand specificities in the two classes and as a result type I and type II receptors can often bind to a common ligand. The transforming growth factor- β (TGF- β) specific receptors represent such a case, where both type I and II receptors (T β RI and T β RII) are observed. Of additional interest is the observation that heteromeric associations of type I and II receptors can also enable signaling. To further elucidate the function of various RSKs, the extracellular domains of both α and β chains from human granulocyte-macrophage colony-stimulating factor receptors were linked to transmembrane cytoplasmic domains of RSKs. Chimeric receptors of human granulocyte-macrophage receptor (hGMR) α with T β RI and hGMR β with T β RII were expressed in murine pre-B cell-derived Ba/F3 cells. These chimeras formed heteromeric complexes, transmitted TGF- β signals, and were down-modulated in response to human granulocyte-macrophage colony-stimulating factor. However, experiments utilizing these chimeric receptors in different combinations revealed that only heteromeric associations of transmembrane cytoplasmic domains mediated signaling and down-modulation. Chimeric receptors with transmembrane cytoplasmic domains of activin receptor type II and bone morphogenetic protein receptor type II also provided signals in conjunction with chimeric T β RI. As a result, these type II receptors may share a common potential to signal via T β RI. hGMR-RSK chimeric receptors may be useful tools for the identification and characterization of the divergent signals mediated by individual RSKs.

INTRODUCTION

The transforming growth factor- β (TGF- β) ligand family consists of more than 20 proteins, including TGF- β , activin, bone morphogenetic protein (BMP), muellerian-inhibiting substance, and glial cell line-derived

neurotrophic factor (Lin *et al.*, 1993). This family is implicated in the regulation of various cellular processes including cell growth, differentiation, and development (Massague, 1990; Roberts *et al.*, 1990; Kingsley, 1994). TGF- β has biological activities including inhibition and promotion of cell growth and differentiation, control of cell migration, adhesion and invasion of tumor cells, as well as regulation of extracellular matrix formation (Roberts *et al.*, 1990; Sporn and Roberts, 1992), and TGF- β is also a potent immu-

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noregulator (Roberts *et al.*, 1990; Stavnezer, 1995). Mice deficient in TGF- β 1 have uncontrolled inflammatory responses which lead to an early death by waste syndrome (Shull *et al.*, 1992; Kulkarni *et al.*, 1993). Class switching of the immunoglobulin (Ig) heavy chain in B cells is also regulated by TGF- β and leads to an increase in the population of IgA-positive B cells (Stavnezer, 1995).

TGF- β interacts mainly with distinct cell surface proteins known as receptor type I, type II, and type III (Massague, 1987, 1992). Only type I (T β RI) and type II (T β RII) receptors account for signaling and both of these have been cloned (Lin *et al.*, 1992; Franzen *et al.*, 1993; He *et al.*, 1993; Lawler *et al.*, 1994; Suzuki *et al.*, 1994; Tomoda *et al.*, 1994). Both receptor types contain an intracellular protein serine/threonine kinase domain, and searches for other members of the family have identified receptors for both types for activin (Mathews and Vale, 1991; Attisano *et al.*, 1992; Donaldson *et al.*, 1992; Ebner *et al.*, 1993; He *et al.*, 1993; Matsuzaki *et al.*, 1993; ten Dijke *et al.*, 1993, 1994a; Carcamo *et al.*, 1994) and BMP (Koenig *et al.*, 1994; ten Dijke *et al.*, 1994b; Liu *et al.*, 1995; Nohno *et al.*, 1995; Rosenzweig *et al.*, 1995) within the past few years. Since the TGF- β ligand family has various biological effects, it is important to clarify how these type I and type II receptor-type serine/threonine kinases (RSKs) coordinate to exert such a wide range of biological responses.

RSK signal transduction is best characterized in the TGF- β receptor system (Wrana *et al.*, 1992, 1994; Attisano *et al.*, 1993; Franzen *et al.*, 1993; Basing *et al.*, 1994). This is because mutant mink lung epithelial cells lacking functional receptor subunits (Boyd *et al.*, 1989; Laiho *et al.*, 1990) enabled numerous studies of TGF- β receptor function. Cloned T β RI and T β RII proved to be bona fide components of functional TGF- β receptors using these cells (Wrana *et al.*, 1992, 1994; Attisano *et al.*, 1993; Franzen *et al.*, 1993; Basing *et al.*, 1994). However, major difficulties in studying recombinant T β RI and T β RII signal transduction lie in the presence of endogenous TGF- β receptors. Most culture cells express TGF- β receptors, although the relative levels of different types of receptors vary with the cell type (Massague, 1990). In TGF- β -responsive cells, the simple overexpression of exogenous T β RI or T β RII cannot be applied to assess function since the effect of endogenous receptors would not make for a valid interpretation of the results.

Although mutant cell lines with defective receptor subunits would facilitate analysis of other ligand-specific receptors such as activin and BMP, such mutants are not so readily available for a given cell line. One approach to overcome this problem is to generate chimeric receptors. For this purpose, we selected the extracellular domains of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) receptors

(Gearing *et al.*, 1989; Hayashida *et al.*, 1990) and linked them to transmembrane cytoplasmic domains of RSKs. There are several advantages for using the hGM-CSF receptor. First, hGM-CSF receptor is composed of an α -chain and β -chain (hGMR α and hGMR β) which undergo heteromeric association in response to hGM-CSF. Since the granulocyte-macrophage colony-stimulating factor (GM-CSF) is species specific (Arai *et al.*, 1990), the hGM-CSF receptor chimera expressed on murine cells can specifically be activated by hGM-CSF. Finally, the expression of chimeric receptors on the cell surface can be monitored by flow cytometry using monoclonal antibodies.

We used murine pro-B cell-derived Ba/F3 as host cells to study the functions of our chimeric receptors. This is a useful system since TGF- β affects both growth inhibition and transcriptional activation. Using this system, we were able to show that chimeric hGMR-RSK receptors transmit TGF- β signals in response to hGM-CSF. Heteromeric but not homomeric association of the transmembrane cytoplasmic T β RI and T β RII domains accounted for signaling. Furthermore, flow cytometric analysis revealed that expression of chimeric receptors on the cell surface was down-modulated following incubation with hGM-CSF. Although association of activin receptor type II (ActRII) or binding protein receptor type II (BMPRII) with T β RI is not demonstrated, ActRII and BMPRII chimeric receptors can mediate TGF- β signals along with the T β RI chimeric receptor. Thus, transmembrane cytoplasmic domains of these type II receptors probably share a common function. We propose that hGMR-RSK chimera may be a unique system to examine the function of individual RSKs.

MATERIALS AND METHODS

Cells and Reagents

A murine interleukin (mIL) 3-dependent pro-B cell line, Ba/F3, was maintained in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 50 U/ml penicillin, 50 μ g/ml streptomycin, and 0.25 ng/ml of recombinant mIL-3 produced in silkworm (*Bombyx mori*). COS7 cells were maintained in DMEM supplemented with 10% FCS, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Recombinant hGM-CSF produced in *Escherichia coli* was provided by Schering-Plough Co. (Bloomfield, NJ). Recombinant TGF- β 1 was purchased from King Brewery Co. (Maebashi, Japan). Mouse monoclonal antibody against hGMR α (GMA-1) and rat monoclonal antibody against hGMR β (5A5) were prepared as described elsewhere (Watanabe *et al.*, 1992).

Plasmid Construction

First, we constructed four chimeric receptor cDNAs (α I, α II, β I, and β II; Figure 1) as follows. The extracellular domains of hGM-CSFR α and β subunits were obtained from pCEV4-hGMR α and pME18S-hGMR β , respectively (Watanabe *et al.*, 1993). A 0.3-kb *SacI*-*EcoRI* fragment of pCEV4-hGMR α was replaced with a double-strand DNA cassette fragment composed of 5'-CCTGGAGTGAAGCCAT-TGAATTTGGTTCTGACGACGGGG-3' and 5'-AATTCCCCGT-CGTCAGAACCAATTCAATGGCTTCACTCCAGGAGCT-3' to

generate pSR α - α N. pSR α - α N contains the entire extracellular domain of hGMR α (amino acids 1–320) and an *Eco*RI site at the 3' end. A 1.8-kb *Bss*HII-*Xba*I fragment of pME18S-hGMR β was replaced with a double-strand DNA cassette composed of 5'-CGCGCTCCTGGGACACCGAGTCCGAATT-3' and 5'-CTAGAATTCGACTCGGTGTCCAGGAG-3' to generate pSR α - β N. pSR α - β N contains the entire extracellular domain of hGMR β (amino acids 1–438) and an *Eco*RI site at the 3' end. pSR α - α N and pSR α - β N were used to provide the extracellular domains of the chimeric receptors.

An A5–3 clone (Tomoda *et al.*, 1994), which is essentially a truncated T β RII cDNA that contains the entire transmembrane cytoplasmic domains (amino acids 125–503 of T β RII), was ligated into the *Eco*RI sites of pSR α - α N and pSR α - β N to generate pSR α - α I and pSR α - β I, respectively.

Polymerase chain reaction (PCR) was performed using a T β RII cDNA (provided by N. Ueno; Suzuki *et al.*, 1994) as the template and the following as the primers: 5'-CGAATTCTGTGGTCAT-TATCCA-3' and 5'-GGGGAAGATCTTGACACAGCCAC-3'.

The resulting 368-bp fragment was subcloned into a pT7–7 vector (Novagen, Madison, WI) and was cut at a unique *Mun*I site in the cDNA and a *Kpn*I site in the vector. A truncated T β RII (amino acids 185–592) was generated by inserting a 3-kb *Mun*I-*Kpn*I fragment of the original T β RII cDNA into this site. The *Eco*RI fragment including the truncated T β RII cDNA was further subcloned into *Eco*RI sites of pSR α - α N and pSR α - β N to generate pSR α - β II and pSR α - β III, respectively.

Next, we constructed two chimeric receptors harboring transmembrane cytoplasmic domains of ActRII and BMPRII (see Figure 7A) as follows.

PCR was performed using ActRII cDNA (Yamada, unpublished results) as the template and the following as the primers: 5'-GAATTCCTGCTCTATTCCTTGG-3' and 5'-CCTGTACACCCAAAATGCCA-3'.

The resulting 75-bp fragment was subcloned into the pT7–7 vector and cut at the *Bsr*G1 site. A truncated ActRII (amino acids 140–513) was generated by inserting a 1.3-kb *Bsr*G1 fragment of ActRII cDNA into this site. The *Eco*RI fragment including the truncated ActRII cDNA was further subcloned into pSR α - β N to generate pSR α - β ARII.

PCR was performed using T-ALK cDNA (BMPRII cDNA; provided by M. Kawabata; Kawabata *et al.*, 1995) as the template and the following as the primers: 5'-GAATTCATAATCATGTCTTGGCATCAG-3' and 5'-TCTAGATCAAGAGAGGGTTC-3'.

The resulting 159-bp fragment was subcloned into the pT7–7 vector and cut at the *Xba*I site. A truncated T-ALK (amino acids 151–1038) was generated by inserting a 2.7-kb *Xba*I fragment of T-ALK cDNA into this site. The *Eco*RI fragment encoding the truncated T-ALK cDNA was subcloned into pSR α - α N to generate pSR α - α T.

Chimeric receptor constructs harboring the extracellular domain of human granulocyte-macrophage receptor β -chain (hGMR β) were further subcloned into pKU2-hyg, a version of the SR α expression plasmid carrying a hygromycin-resistant gene. Sequences of all of the DNA portions derived from cassette mutagenesis or PCR and all of the junctions of the constructs were confirmed.

Stable Transfection

Transfection of Ba/F3 cells was performed using electroporation as described previously (Watanabe *et al.*, 1993). To obtain Ba/F3 cells expressing α I and β II (M series), 2×10^6 cells in 200 μ l of phosphate-buffered saline with 2 μ g of pKU2-hyg- β II and 8 μ g of pSR α - α I were subjected to electroporation at 250 kV, 960 μ F (Gene Pulsar; Bio-Rad, Richmond, CA). Cells were then plated onto 96-well plates, and after 24 h, selection was started by changing the medium to one containing 1.2 mg/ml hygromycin. Hygromycin-resistant clones appeared after 7–10 days. This same procedure was then repeated for all of the following pairs of plasmids: pKU2-hyg- β I and pSR α - α I, pKU2-hyg- β II and pSR α - α II, pKU2-hyg- β II and pSR α - α I, pKU2-

hyg- β ARII and pSR α - α I, and pKU2-hyg- β I and pSR α - α T, to obtain clones which will be referred to as the J, K, L, P, and B series, respectively.

Flow Cytometry Analysis

All of the hygromycin-resistant clones were subjected to flow cytometric analysis as described elsewhere (Watanabe *et al.*, 1992). Briefly, cells were washed twice with staining solution using phosphate-buffered saline containing 5% FCS and 0.02% sodium azide. Then about 10^5 cells were incubated at 4°C for 1 h in 50 μ l of staining solution containing 150 ng of GMA-1 (an anti-GMR α -antibody) or 150 ng of 5A5 (an anti-GMR β -antibody). After again washing twice with staining solution, samples were stained for 30 min at 4°C with 250 ng of fluorescein isothiocyanate-conjugated goat anti-mouse IgG fragment (for GMA-1) or rabbit anti-rat IgG fragment (for 5A5) in 50 μ l of staining solution. Cells were finally washed twice and suspended in 100 μ l of staining solution. Flow cytometric analysis was performed with a Becton Dickinson FACScan (Mountain View, CA) using FACScan software, LYSIS II.

[3 H]Thymidine Incorporation Assay

DNA synthesis was measured by the extent of [3 H]thymidine incorporation. Aliquots (100 μ l) containing 2×10^4 cells suspended in RPMI 1640 with 5% FCS and mIL-3 with or without hGM-CSF were incubated for 16 h. [3 H]thymidine (70 Ci/mmol) at 1 μ Ci was added to each well and the preparation was further incubated for 3 h. The cells were harvested using a cell harvester and the 3 H radioactivity incorporated was determined using a liquid scintillation spectrophotometer. Radioactivity data were always averaged over three wells.

Luciferase Assay

Transient transfection of luciferase reporter plasmids into Ba/F3 clones was achieved by electroporation. Briefly, 5×10^6 cells were washed twice with OPTI-MEM (Life Technologies, Inc., Gaithersburg, MD), resuspended in 0.2 ml of OPTI-MEM, then 5 μ g of pFL-1 or pFL-3 were added, and an electronic pulse was given at 200 kV, 960 μ F (Gene Pulsar; Bio-Rad). Cells were kept in RPMI 1640 with 5% FCS and 0.25 ng/ml mIL-3 for 6 h, divided into six plates, and then leaving two plates unstimulated, the remaining four were stimulated with either TGF- β (5 ng/ml) or hGM-CSF (5 ng/ml). After incubation for 16 h, cells were harvested for the luciferase assay, the activity of which was measured using the luciferase assay substrate (Promega, Madison, WI) and a luminometer (model LB9501; Berthold, Germany). Protein concentration was measured using bicinchoninic acid reagents (Pierce, Rockford, IL), and the luciferase activity was calculated as relative light unit-background per μ g of protein. Fold induction was calculated by dividing the luciferase activity in the TGF- β - or hGM-CSF-treated groups with the luciferase activity in the untreated groups.

Immunoprecipitation Assay

COS7 cells (1×10^6 cells) were transiently transfected with pSR α - α I and pSR α - β II using the DEAE-dextran method (Watanabe *et al.*, 1993). Transiently transfected COS7 cells and M3, L4, J8, and K8 cells (1×10^7) were homogenized in 100 μ l of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.2% Nonidet P-40, and 10 μ M phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, and 2 μ g/ml leupeptin using a pentype homogenizer for 10 s. The homogenate was centrifuged at 15,000 rpm for 5 min, and GMA-1 or 5A5 antibodies were added to the supernatant at the final concentration of 20 μ g/ml. Samples were kept on ice for 2 h, protein G-Sepharose was added, and the samples were shaken gently for another 1 h. Immune complexes were first collected by centrifugation and washed three times with lysis buffer. Then these complexes were boiled in loading buffer and subjected to SDS-PAGE followed

by immunoblotting using the 5A5 antibody. Detection was performed using horseradish peroxidase-linked protein A and the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom) according to the protocol supplied by the manufacturer.

RESULTS

Ba/F3 Clones Expressing Chimeric Receptors Transmit Growth Inhibitory Signal in Response to hGM-CSF

To develop a system for examining the function of T β R1 and T β R2 in TGF- β -responsive cells, we constructed chimeric receptors α I, α II, β I, and β II (Figure 1) as described in MATERIALS AND METHODS. Of

Figure 1. Schematic representation of the hGMR-T β R chimeric receptors expressed in Ba/F3 cells. α I is composed of an extracellular domain of hGMR α and a transmembrane cytoplasmic domain of T β R1. β II is composed of an extracellular domain of hGMR β and a transmembrane cytoplasmic domain of T β R2. α II is composed of an extracellular domain of hGMR α and a transmembrane cytoplasmic domain of T β R2. β I is composed of an extracellular domain of hGMR β and a transmembrane cytoplasmic domain of T β R1. M, L, J, and K series of Ba/F3 stable clones were transfected with expression vectors for α I and β II, α II and β I, α I and β I, and α II and β II, respectively.

the hygromycin-resistant clones selected after transfection with pSR α - α I and pKU2-hyg- β II (M series), several independent clones were subsequently analyzed for the expression of α I and β II as well as in the variation in [3 H]thymidine incorporation with mIL-3 and hGM-CSF. Figure 2 shows the results for representative clones. M1 expressed neither α I nor β II, and [3 H]thymidine incorporation was not affected by hGM-CSF. M3 and M8 expressed both α I and β II, at different levels, and hGM-CSF significantly reduced [3 H]thymidine incorporation at all concentrations of mIL-3. The inhibition of [3 H]thymidine incorporation was most significant at lower concentrations of mIL-3. M11 expressed β II without α I and [3 H]thymidine incorporation was not affected by hGM-CSF. Thus, Ba/F3 cells expressing both α I and β II has reduced

Figure 2. Expression of both α I and β II in M series clones correlates with hGM-CSF-dependent growth inhibition. Upper panels of M1, M3, M8, and M11 show the flow cytometry analysis using monoclonal anti-hGMR α or anti-hGMR β antibodies (dark area) and control antibodies (white area) as indicated. Lower panels show [3 H]thymidine incorporation of these clones with increasing doses of mIL-3 with hGM-CSF (5 ng/ml) or without hGM-CSF as indicated.

levels of [^3H]thymidine incorporation in the presence of hGM-CSF. Thus, clones expressing both αI and βII were retained for subsequent studies.

We then asked whether Ba/F3 clones expressing chimeric receptors with other combinations of transmembrane cytoplasmic domains would respond to hGM-CSF. By transfecting Ba/F3 cells with pSR α - αI and pKU2-hyg- βI (J series), pSR α - αII and pKU2-hyg- βII (K series), or pSR α - αII and pKU2-hyg- βI (L series), hygromycin-resistant stable clones could be selected and then subjected to flow cytometric analysis. Clones positive for staining with both GMA-1 and 5A5 at this stage were also selected for additional studies. In a similar manner to the M series in Figure 2, there were noticeably different levels of expression in the corresponding chimeric receptors in each of the cell series. These independent clones were subjected to a [^3H]thymidine incorporation experiment in a test medium containing 0.02 ng/ml mIL-3 and varying concentrations of hGM-CSF. M3, M7, and M8, which express αI and βII at different levels, reduced the level of [^3H]thymidine incorporation by hGM-CSF in a dose-dependent manner (Figure 3A). The maximum inhibition was 85–95% and the half maximum inhibition occurred at about 0.3 ng/ml hGM-CSF. The levels of [^3H]thymidine incorporation of J3, J4, J8, and J10, which express αI and βI (Figure 3B), and K3, K8, K9,

and K11, which express αII and βII (Figure 3C), were not affected by hGM-CSF. On the other hand, [^3H]thymidine incorporation of L4 and L8, which express αII and βI , was dose dependently suppressed by hGM-CSF (Figure 3D). Both clones were maximally suppressed by 90% and the half maximum inhibition occurred at about 0.3 ng/ml hGM-CSF (Figure 3D). All of the clones tested responded to TGF- β and growth was suppressed by up to 90%. These results indicate that expression of chimeric receptors with a combination of heterologous domains of T βRI and T βRII correlates with ligand-dependent inhibition of [^3H]thymidine incorporation.

Ba/F3 Cells Expressing Chimeric Receptors Activate Transcription in Response to hGM-CSF

To examine whether chimeric receptors would transmit transcriptional signals, we introduced a luciferase reporter gene carrying a TGF- β -responsive element from a germ-line *Ig α* constant region gene, *Ig α -TGF- β -RE*. *Ig α -TGF- β -RE* was identified as a tandem repeat in the regulatory element of the *Ig α* gene, which mediates the transcriptional enhancement by TGF- β (Figure 4A). As multiple but not single copies of *Ig α -TGF- β -RE* confer TGF- β inducibility to a heterologous promoter (Lin and Stavnezer, 1992), we used reporters

Figure 3. Effect of hGM-CSF on [^3H]thymidine incorporation of Ba/F3 clones expressing chimeric receptors. Ba/F3 cells expressing αI and βII (A; M3, M7, and M8), αI and βI (B; J3, J4, J8, and J10), αII and βII (C; K3, K8, K9, and K11), and αII and βI (D; L4 and L8) were stimulated by increasing doses of hGM-CSF and subjected to a [^3H]thymidine incorporation assay as described in MATERIALS AND METHODS. The level of [^3H]thymidine incorporation is expressed as a percentage of incorporation relative to control cells that are not stimulated.

Figure 4. Transcriptional activation of reporter genes carrying $I\alpha$ -TGF- β -RE in Ba/F3 stable clones expressing chimeric receptors. (A) Diagram of pFL-1 and pFL-3 reporter which carry one and three repeats of $I\alpha$ TGF- β -RE (shaded boxes) on a c-fos promoter from -71 to +109 and a luciferase gene. The sequence of $I\alpha$ TGF- β -RE (-127 to -105 of germ-line α promoter) is shown at the bottom. The shaded boxes represent the tandem repeat. (B) pFL-1 or pFL-3 were transiently transfected into Ba/F3, M3, J4, K8, or L8, as indicated, and luciferase activity was determined after stimulation with TGF- β (5 ng/ml) or hGM-CSF (5 ng/ml). All of the data are presented as fold induction of the unstimulated groups.

consisting of a minimal c-fos promoter carrying one (pFL-1) or three (pFL-3) copies of $I\alpha$ -TGF- β -RE (Figure 4A). pFL-1 or pFL-3 was transiently transfected to Ba/F3, M3, J4, K8, and L8, which were the representative clones from each series, and luciferase activity was measured after stimulation with TGF- β (5 ng/ml) or hGM-CSF (5 ng/ml; Figure 4B). It was immediately clear that pFL-1 transfected into Ba/F3 or M3 was not activated with either TGF- β or hGM-CSF (Figure 4B, columns 1 and 2). In contrast, pFL-3 transfected into Ba/F3 was activated (about 20-fold) by TGF- β but not by hGM-CSF (Figure 4B, column 3), indicating that Ba/F3 cells are responsive to TGF- β . These responses of pFL-1 and pFL-3 in Ba/F3 cells were comparable to those in other B lymphoma cell lines (Lin *et al.*, 1992). Importantly, pFL-3 transfected into M3 was significantly activated (about 22-fold) not only by TGF- β but also by hGM-CSF (Figure 4B, column 4), which suggests that transcription activity is being mediated. However, pFL-3 transfected into J4 or K8 was only

activated by TGF- β (Figure 4B, columns 5 and 6). Furthermore, pFL-3 transfected into L8 was significantly activated (about 18-fold) by TGF- β or hGM-CSF (Figure 4B, column 6). These results indicate that expression of chimeric receptors with the combination of heterologous transmembrane cytoplasmic domains of T β RI and T β RII correlates with ligand-dependent transcriptional activation.

hGM-CSF Induces Association of Chimeric Receptors

To examine whether hGM-CSF would induce oligomerization of α I and β II, a coimmunoprecipitation experiment was carried out. COS7 cells transiently transfected with pSR α - α I and pSR α - β II or M3 cells were treated both with and without hGM-CSF (5 ng/ml) for 10 min, and cell lysates were prepared. Preparations were immunoprecipitated with antibodies 5A5 or GMA-1, and the immune complexes were subsequently analyzed by immunoblotting using 5A5. As shown in Figure 5A, β II was immunoprecipitated with 5A5 in cells both treated and untreated with hGM-CSF. In contrast, GMA-1 mediated β II coimmunoprecipitation only with hGM-CSF-treated cells. These results indicate that α I and β II form a heteromeric complex in response to hGM-CSF. With J4, L8, and K8 clones (Figure 5B), GMA-1 coimmunoprecipitation occurred only in a hGM-CSF-dependent manner. These results indicate that chimeric receptors form a complex in the presence of hGM-CSF. The duplicated bands of β I observed in J4 and L8 are likely to be due to the differences in glycosylation, since the extracellular domain of hGMR β was seen to be glycosylated (Muto *et al.* 1996).

hGM-CSF Reduces the Level of Expression of the Chimeric Receptors on the Cell Surface

To study whether treatment with hGM-CSF would affect expression of α I and β II on the cell surface, M3 was first incubated with and without hGM-CSF (5 ng/ml) for 3 h and followed by flow cytometric analysis. α I and β II expression for M3 treated with hGM-CSF was significantly lower than that for untreated cells (Figure 6A, upper and middle panels). Cell surface α I and β II remained low after a 16-h incubation in medium containing hGM-CSF. When M3 was returned to medium without hGM-CSF and further cultured for 6 h, the expression of α I and β II recovered to the initial levels. In contrast, TGF- β had no significant effect (Figure 6A, lower panels).

We then studied the variation of J4, K8, and L8 chimeric receptor expression with hGM-CSF treatment (Figure 6B). Expression of α I and β I (for J4) and α II and β II (for K8) were very similar in both hGM-CSF-treated and untreated experiments (Figure 6B, middle and upper panels). In contrast, the expression

A **B**

Figure 5. hGM-CSF induces association of the chimeric receptors. (A) COS7 cells transiently transfected with α I and β II or M3 cells were treated with or without hGM-CSF. Cell lysates were subjected to immunoprecipitation against GMA-1 or 5A5 antibody and subsequently analyzed by immunoblotting using 5A5 antibody as described in MATERIALS AND METHODS. (B) J4, L8, and K8 clones were treated with or without hGM-CSF (5 ng/ml), and cell lysates were subjected to immunoprecipitation using GMA-1 and analyzed by immunoblotting using 5A5 as described in MATERIALS AND METHODS.

of α II and β I on hGM-CSF-treated L8 was significantly lower than that for untreated cells (Figure 6B, bottom panels). These results indicate that association of heteromeric transmembrane cytoplasmic domains of T β RI and T β RII is required for the reduction of chimeric receptors on the cell surface.

Chimeric Receptors with Transmembrane Cytoplasmic Domains of Other Type II RSKs Can Cooperate with Chimeric T β RI

To gain further insight into the function of other type II RSKs, a chimeric receptor using ActRII and hGMR β (β ARII) and another using BMPRII and hGMR α (α T) were generated as described in MATERIALS AND METHODS. pSR α - α I and pKU2-hyg- β ARII (P series) or pSR α - α T and pKU2-hyg- β I (B series) were transfected into Ba/F3 cells to obtain stable clones (Figure 7A). Representative P13, expressing β ARII and α I, and B520, expressing α T and β I, were investigated for their ability to respond to hGM-CSF. As shown in Figure 7B, [3 H]thymidine incorporation was dose dependently suppressed by hGM-CSF in P13 and B520 by 30% and by 70%, respectively. Maximum growth inhibition of P13 and B520 was lower than that of M3 or L8, and, furthermore, several other P and B series clones (expressing both receptor subunits) also exhibited lower growth inhibitory responses. Thus, the partial response is likely to be due to inefficient signaling by these chimeric receptors rather than clonal differences.

The pFL-3 reporter plasmid was also transfected into P13 and B520, and luciferase activity was determined after stimulation with TGF- β or hGM-CSF. As shown in Figure 7C, pFL-3 was activated less efficiently by hGM-CSF than TGF- β , indicating that the signal for transcription is also carried by these cells.

Chimeric receptors' expression on the cell surface of P13 and B520 was determined by flow cytometric analysis. β ARII and α I expressed by P13 (Figure 7D, upper panels) as well as α T and β I expressed by B520 (Figure 7D, lower panels) decreased in response to hGM-CSF. Taken together, these results demonstrate that chimeric receptors with transmembrane cytoplasmic domains of ActRII or BMPRII can mediate signal transduction in conjunction with chimeric T β RI.

DISCUSSION

Chimeric Molecules of hGM-CSF and TGF- β Receptors Transmit Signals in Ba/F3 Cells

We constructed various chimeric molecules by coupling extracellular domains of hGM-CSF receptors with transmembrane cytoplasmic domains of RSKs and examining their signaling potentials in Ba/F3 cells. All of the Ba/F3 clones expressing α I and β II or α II and β I responded to hGM-CSF and the growth was suppressed. In contrast, none of the cells expressing α I and β I or α II and β II responded to hGM-CSF. These results indicate that combination of the transmembrane cytoplasmic domains of T β RI and T β RII is required and sufficient for ligand-dependent growth inhibition.

As TGF- β mediates growth inhibition and transcription activation signals, we then examined whether the

Figure 6. Flow cytometric analysis of the expression levels of chimeric receptors on the cell surface. (A) Control M3 cells (upper panels) or M3 cells treated with hGM-CSF (middle panels) or TGF- β (lower panels) for 3 h were subjected to flow cytometric analysis to determine the expression level of α I and β II expressed on the cell surface. Shaded and open areas in the left panels represent cells stained by GMA-1 and anti-mouse IgG, as a control, respectively. Shaded and open areas in the right panels represent cells stained by 5A5 and anti-rat IgG, as a control, respectively. (B) Effect of hGM-CSF on chimeric receptors expressed on J4 (α I and β I), K8 (α II and β II), L8 (α II and β I). Untreated cells (shaded areas indicated by the arrow) or cells treated with hGM-CSF (open areas indicated by the arrow) were stained as in A. The control staining is also shown as indicated.

chimeric receptors activate transcription. In preliminary experiments, we used a luciferase plasmid driven by a PAI-1 promoter and three 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements, but these were inefficient indicators for the TGF- β response in Ba/F3 cells. Therefore, we used a reporter gene with TGF- β -responsive elements from a germ-line *Ig α* constant region gene, which has been characterized in a B cell lymphoma cell line (Shockett *et al.*, 1991). Our results showed that pFL-3, which has three copies of this element, provides an efficient indicator for the TGF- β response in Ba/F3 cells. Using this reporter gene, we have proved that the α I and β II or α II and β I combinations are capable of transmitting TGF- β transcriptional signals, whereas α I and β I or α II and β II combinations cannot. We interpreted this to mean that chimeric receptors with transmembrane cytoplasmic domains of T β RI and T β RII can induce TGF- β responses, which mediate to growth suppression and transcriptional activation in Ba/F3 cells. These results

are consistent with previous studies done using different T β RI and T β RII chimeric receptors, which dem-

Figure 7 (on facing page). Characterization of Ba/F3 stable clones P13 (expressing β ARII and α I) and B520 (expressing α T and β I). (A) Schematic representation of chimeric receptors expressed in P13 and B520. β ARII is composed of an extracellular domain of hGMR β and a transmembrane cytoplasmic domain of ActRII. α T is composed of an extracellular domain of hGMR α and a transmembrane cytoplasmic domain of BMPRII. (B) [3 H]Thymidine incorporation of P13 and B520. P13 and B520 were stimulated with increasing doses of hGM-CSF and were subjected to a [3 H]thymidine incorporation assay. The level of [3 H]thymidine incorporation is expressed as a percentage of incorporation relative to control cells that are not stimulated. (C) Activation of pFL-3 reporter in P13 and B520. pFL-3 was transiently transfected in M3, P13, and B520, and luciferase activity was monitored after stimulation with TGF- β (5 ng/ml) or hGM-CSF (5 ng/ml) as indicated. (D) Reduced surface expression of chimeric receptors on P13 and B520. A13 and B520 were incubated with or without hGM-CSF (5 ng/ml), and flow cytometric analysis was performed to determine the expression level of chimeric receptors on the cell surface.

Figure 7 (cont).

onstrated that heteromeric but not homomeric association of the T β RI and T β RII generate signals (Okadome *et al.*, 1994; Vivien *et al.*, 1995).

Association of Chimeric Receptors: Comparison between the TGF- β and hGM-CSF Receptor Systems

Chimeric receptors α I and β II (M series), α I and β I (J series), α II and β II (K series), and α II and β I (L series) all associated in the presence of hGM-CSF. Thus, heteromeric oligomerization of the extracellular domain of hGMR α and hGMR β is sufficient for creating ligand-dependent chimeric receptors. This hGM-CSF-induced complex formation (for α I and β II or α II and β I) is reminiscent of the TGF- β -induced complex formation of T β RI and T β RII. There also appear to be some similarities in the mode of ligand-receptor subunit interaction between the TGF- β and hGM-CSF systems. In the case of the TGF- β receptor, TGF- β binds to T β RII and thereby recruits T β RI, which does not bind to TGF- β by itself (Wrana *et al.*, 1994). In the case of the hGM-CSF receptor, hGMR α binds to hGM-CSF albeit at a low affinity (Gearing *et al.*, 1989) and along with hGMR β (which do not bind to hGM-CSF by itself) forms a high-affinity receptor (Hayashida *et al.*, 1990). In addition, it has been suggested that high-affinity receptor assembly also involves the recruitment and stabilization of hGMR β (Eder *et al.*, 1994).

T β RII forms a homodimer in the absence of TGF- β , which does not signal by itself (Yamashita *et al.*, 1993; Chen and Derynck, 1994; Henis *et al.*, 1994). However, the addition of T β RI to the T β RII homodimer results in a ligand-inducible receptor complex (Weis-Garcia *et al.*, 1996), probably consisting of a heterotetramer of T β RI and T β RII (Yamashita *et al.*, 1993). On the other hand, hGMR β also forms a homodimer in the absence of hGM-CSF (Muto *et al.*, 1996). Although the exact stoichiometry or activation mode of the chimeric receptors remains to be studied, oligomerization of the two distinct receptor systems suggest that β II or β I might form a homodimer in the absence of hGM-CSF, to which α I or α II is recruited after binding to hGM-CSF.

Dynamic Modulation of Chimeric Receptors Expressed on the Cell Surface

Flow cytometric analysis revealed that treatment with hGM-CSF reduced the level of α I and β II expressed on the cell surface. This apparent down-modulation occurred at a relatively rapid time course (3 h) and was not accompanied by a significant decrease in cellular α I and β II levels determined by immunoblotting. In addition, TGF- β did not affect the level of α I and β II, suggesting that this down-modulation is not due to post-receptor events, such as inhibition of transcription and translation or secondary effects due to growth inhibition. Since internalization of TGF- β receptors

has been demonstrated using 125 I-labeled TGF- β cross-linking experiment (O'Grady *et al.*, 1991), we assume, although not proven, that this down-modulation of the chimeric receptors may be a consequence of ligand-induced internalization. This phenomenon was observed in L8, which expresses α II and β I, but not in J4, which expresses α I and β I, or K8, which expresses α II and β II. This indicates that heteromeric association transmembrane cytoplasmic domains are required for down-modulation. We do not know at present whether the down-modulation observed for chimeric receptors holds true for wild-type TGF- β receptors with regard to its mechanism and magnitude, and additional studies including mutational analysis are needed to clarify its mechanism.

Potentiality for Other Type II RSKs to Cooperate with T β RI

Using hGMR-RSK chimeric receptors with transmembrane cytoplasmic domains of ActRII and BMPRII, we have shown that they are also capable of mediating growth inhibitory and transcription activation signals in cooperation with the transmembrane cytoplasmic domain of T β RI. Even though the cytoplasmic domains of ActRII and BMPRII share only about 40% sequence identity with T β RII, they did initiate a similar set of responses in the context of the chimeric receptor system. Accumulating data indicate that the type I receptor is the primary determinant of signals generated by RSKs. T β RII has been shown to associate and phosphorylate T β RI, revealing a hierarchy of receptor subunits in signal transduction (Wrana *et al.*, 1994). Also, constitutively active T β RI, which has a signaling ability without T β RII, has been engineered. This means that T β RI is probably the downstream of receptor complex (Wieser *et al.*, 1995). Our results are consistent with these notions, since β ARII and α I or α T and β I generated signals characteristic for T β RI. The growth inhibitory and transcriptional signal provided by activin was seen to be mediated by ActRII and ActRIB, the kinase domain of which shares 90% amino acid identity with T β RI (Carcamo *et al.*, 1994). Considering the homology between T β RI and ActRIB, it is not so surprising that transmembrane cytoplasmic domains of ActRII generated signals with transmembrane cytoplasmic domains of T β RI in chimeric receptors. In contrast, transmembrane cytoplasmic domains of BMPRII generating signals with transmembrane cytoplasmic domains of T β RI is an enigma. BMPRII was originally identified in a yeast two-hybrid system using the cytoplasmic domain of T β RI (Kawabata *et al.*, 1995; Liu *et al.*, 1995). Thus, both cytoplasmic domains have the potential to interact with each other. However, studies using radiolabeled BMP-2, 4, or OP-1/BMP-7 did not detect interaction between BMPRII and T β RI at the cellular level (Liu *et al.*, 1995; Rosen-

zweig *et al.*, 1995). Therefore, it would seem that BMP-2, 4, or OP-1/BMP-7 does not oligomerize BMPRII and T β RI to generate signals. In fact, we do not currently know whether BMPRII transmembrane cytoplasmic domains can combine with T β RI in any signaling event. However, if there is a ligand that oligomerizes BMPRII with T β RI, it is conceivable that it could generate the signals specified by T β RI.

ActRI has recently been shown to interact with both ActRII and BMPRII, suggesting a possible cross-talk between activin and BMP signals at the receptor level (Liu *et al.*, 1995; Rosenzweig *et al.*, 1995). Evidently, the possibility of cross-talk between other type I and type II RSKs at a wider range needs to be investigated by examining other members of the TGF- β family as potential ligands, as they become available in a recombinant form. Whether or not such interaction has a physiological role remains to be determined.

We have developed a hGMR-RSK chimeric receptor system which allows us to study the ligand-dependent signaling of RSK. This system may provide a useful tool for investigating the individual roles of RSKs.

ACKNOWLEDGMENTS

We are grateful to Dr. Suzuki and Dr. Ueno for T β RII cDNA, Dr. Kawabata and Dr. Miyazono for BMPRII cDNA, and Dr. Stavnezer for pFL reporter plasmids. We also thank Dr. Swindells for critically reading the manuscript.

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