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G-CSF Receptor-mediated STAT3 activation and granulocyte differentiation in 32D cells

Research Article

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Summary

Granulocyte colony-stimulating factor (G-CSF) receptor (GcR) mediates growth and differentiation signals in the granulocyte/monocyte lineage of hematopoietic cells. To investigate the differentiation signal via GcR, a conditional receptor activation system was constructed. Wild-type and mutant GcRs were controlled by fusion to a molecular switch derived from the hormone binding domain of the estrogen receptor (ER). GcR-associated signaling molecules were analyzed in 32D progenitor cells that possess a potential of granulocyte differentiation. While the wild-type GcR-ER fusion molecule induced a granulocyte differentiation in 32D cells, a substitution of phenylalanine for tyrosine 703 (Y703F) in GcR resulted in a differentiation block. The activation of the JAK1 and JAK2 kinases was indistinguishable between the cells expressing the wild-type fusion and the Y703F mutant, and phosphorylation of the STAT5 transcription factor was comparable, too. On the other hand, tyrosine phosphorylation of STAT3 was significantly decreased following activation of the Y703F mutant compared to the wild-type GcR fusion. The results suggested that tyrosine 703 was responsible, at least in part, for transmitting a differentiation signal via STAT3 in 32D. The fusion system with the estrogen binding domain provides a valuable tool to analyze mutant effector proteins in the natural cellular milieu while bypassing the endogenous counterparts.

I. Introduction

Recent advances in stem cell biology, together with gene transfer technology, have led to the prospect of a new generation of cell therapy. However, many obstacles must be overcome before this vision becomes a reality. One major hurdle is to control transplanted cells in the recipient's body, in particular, to expand the desired cell subsets so that they exhibit therapeutic benefit. We have developed a novel system for selective expansion of genetically modified cells to supplement current gene transfer vectors (Ito et al, 1997; Kume et al, 2002). In this system, the target cells are harnessed with a 'selective amplifier gene (SAG)' which encodes a fusion protein comprising the granulocyte colony-stimulating factor (G-CSF) receptor (GcR) and the hormone binding domain (HBD) of the estrogen receptor (ER). The ER-HBD works as a molecular switch so that the fusion protein generates a GcR-derived growth signal upon binding to estrogen (Mattioni et al, 1994). Besides the prototype SAG encoding a chimera of the full-length GcR and ER-HBD (GcRER), a series of derivative fusion receptors were constructed to attain altered ligand specificity and signal characteristics. The modifications include a deletion of the G-CSF binding site (ΔGcR) (Ito et al, 1997), replacement of the ER with a mutant specific for 4-hydroxytamoxifen (TmR) (Xu et al, 1999), and the substitution of phenylalanine for the most proximal tyrosine residue in the GcR cytoplasmic domain (Y703FGcR) (Matsuda et al, 1999a).

The Y703F mutant is of particular interest because this amino acid substitution apparently led to a differentiation block in myeloid progenitor 32D cells (Matsuda et al, 1999a). To explore the mechanisms of granulocyte differentiation in 32D cells, we examined

JAK-STAT pathways involved in GcR signaling, and identified reduced STAT3 phosphorylation associated with the Y703F mutation.

II. Materials and methods

A. Plasmids and cells

Bicistronic vector plasmids were constructed with the pMX retrovirus backbone and the encephalomyocarditis virus (EMCV)-derived internal ribosome entry site (IRES; nucleotides 259-833 of EMCV-R genome) (Duke et al, 1992; Onishi et al, 1996). pMX/ΔGcRER-IRES-CD8a encodes a fusion protein of ΔGcR and ER-HBD, and murine CD8a as a selectable marker (Fukunaga et al, 1991; Koike et al, 1987; Nakauchi et al, 1985). The Y703F mutation in the GcR part was introduced into this plasmid as previously described (pMX/ΔY703FGcRER-IRES-CD8a) (Matsuda et al, 1999a). The recombinant DNA experiments were carried out following the National Institutes of Health guidelines and approved by the Jichi Medical School Recombinant DNA Research Advisory Board.

The murine myeloid progenitor line 32D and its derivatives were maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia) and 0.5% conditioned medium of C3H10T1/2 cells transfected with a murine IL-3 expression plasmid pBMG-hph-IL-3 (Valtieri et al, 1987; Matsuda et al, 1999a; Xu et al, 1999).

B. Immunoprecipitation and western blotting

32D cells were deprived of serum and IL-3 for 3 hours at a density of 5 x 10^5 cells/ml, and incubated in RPMI medium containing 1 mM Na_3OV_4 for an additional 1 hour at 1 x 10^7 cells/ml. After starvation, cells were stimulated with either 10^{-7} M E_2 (Sigma, St. Louis, MO) or 10^{-9} M recombinant human G-CSF (provided by Chugai Pharmaceuticals, Tokyo, Japan) for given periods, then washed with ice-cold phosphate-buffered saline (PBS) containing 100 μM Na_3OV_4. Subsequently, cells were solubilized in lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin and 2 mM Na_3OV_4) on ice for 30 minutes, and centrifuged for 10 minutes. The soluble proteins were measured by Protein Assay (Bio-Rad, Hercules, CA).

For immunoprecipitation, the cell lysate containing 1 mg of protein was incubated with one of the following antibodies for 8 hours at 4°C: anti-JAK1 (Upstate Biotechnology, Lake Placid, NY), anti-JAK2 (Upstate Biotechnology), anti-STAT3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-STAT5 (C-17; Santa Cruz Biotechnology). The immune complexes were absorbed by protein G-Sepharose beads (Sigma) for 2 hours at 4°C. The beads were washed with the lysis buffer and boiled in sample buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol and 5% 2-mercaptoethanol) for 3 minutes. After centrifugation, the supernatants were subjected to SDS-7.5% polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Yonezawa, Japan). After blocking treatment with 5% bovine serum albumin (Fraction V; Roche Diagnostics, Mannheim, Germany), the membranes were incubated with an anti-phosphotyrosine antibody (4G10; Upstate Biotechnology) for 1 hour at room temperature. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK). In some instances, membranes were stripped by incubation in denaturing buffer (62.5 mM Tris-HCl [pH 6.7], 2% SDS and 100 mM 2-mercaptoethanol) for 30 minutes at 50°C and reprobed with another antibody.

III. Results

A. Construction of conditionally activated G-CSF receptors

Structures of the chimeric receptors used in this study are shown in **Figure 1**. The fusion protein system is based on the fact that ER-HBD functions as an estrogen-specific molecular switch to control heterologous effector proteins, in our case, GcR (Mattioni et al, 1994). GcR belongs to the type I cytokine receptor superfamily, and its cytoplasmic domain comprises functionally distinct subdomains: the membrane-proximal region is sufficient for mitogenic signaling, and the membrane-distal portion is essential for granulocyte maturation (Dong et al, 1993; Fukunaga et al, 1993; Avalos, 1996; Koay and Sartorelli, 1999). All of the four conserved tyrosine residues in the cytoplasmic domain of GcR (at positions 703, 728, 743 and 763 in the murine GcR) are in the membrane-distal region and phosphorylated upon G-CSF stimulation. Among these, the tyrosine at position 703 (Y703) was most prominently phosphorylated and involved in granulocyte differentiation (Yoshikawa et al, 1995). However, previous studies on functional domains of GcR were carried out with ectopically expressed wild-type and mutant molecules in receptor-negative cells. It may be more informative if mutant receptors are analyzed in the natural intracellular environment where the endogenous molecule functions. From this viewpoint, the ER-HBD fusion system provides a valuable experimental tool. Estrogen specifically activates the introduced GcRER (and its derivatives) without influencing the endogenous GcR in the same cell, and the downstream events can be studied independently.

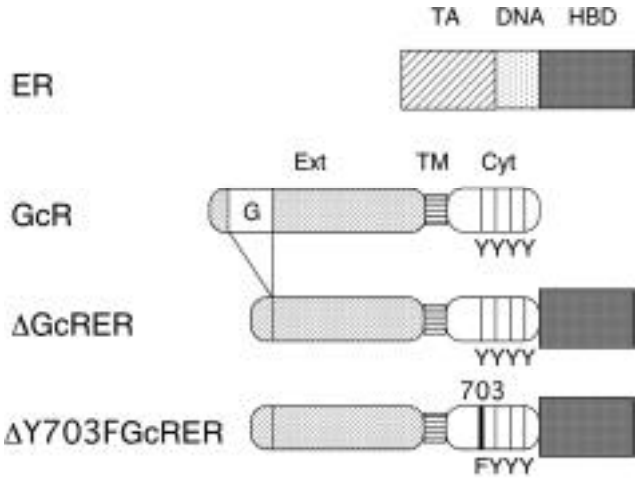

Figure 1. Structures of the chimeric receptors involved in this study. GcRER is a fusion of the full-length murine granulocyte colony-stimulating factor (G-CSF) receptor (GcR) and the hormone binding domain (HBD) of rat estrogen receptor (ER). ΔGcRER is a derivative of GcRER deleted of the G-CSF binding site (amino acids 5-195). ΔY703FGcRER carries a substitution of phenylalanine for a cytoplasmic tyrosine at position 703 (Y703F) in GcR. Ext, extracellular domain; G, G-CSF binding site; TM, transmembrane domain; Cyt, cytoplasmic domain; TA, transactivation domain; DNA, DNA binding domain; YYYY, conserved tyrosine residues in GcR cytoplasmic domain; FYYY, Y703F mutation in GcR.

In our previous report, the biological response to the ΔGcRER- and ΔY703FGcRER-mediated signal was evaluated in murine myeloid progenitor 32D cells (Δ designates a deletion of amino acids 5-195 required for G-CSF binding; Matsuda et al, 1999a). Parental 32D cells are dependent on interleukin-3 (IL-3) for continuous growth, and switching from IL-3 to G-CSF makes the cells differentiate into morphologically mature neutrophils (Valtieri et al, 1987). By retrovirus-mediated gene transfer, stable clones expressing ΔGcRER (32D/ΔGcRER) or ΔY703FGcRER (32D/ΔY703FGcRER) were established and stimulated by estrogen. While estrogen-treated 32D/ΔGcRER cells underwent granulocyte differentiation indistinguishable from that seen in G-CSF-treated cells, 32D/ΔY703FGcRER cells showed a distinct phenotype. Estrogen supported a long-term proliferation of 32D/ΔY703FGcRER with myeloblastic appearance, indicating that the Y703F mutation abrogated the differentiation signal (Matsuda et al, 1999a). This observation prompted us to characterize signaling molecules downstream of GcR in more detail.

Following ligand-induced homodimerization, GcR induces a wide array of intracellular signaling events (Avalos, 1996). Like many other cytokine receptors, GcR has no intrinsic kinase activity; instead, it recruits and activates other cytoplasmic kinases such as Janus kinases (JAKs), signal transducer and activation of transcription (STAT) proteins, Src family kinases and components of the mitogen-activated protein kinase pathway. The activation of JAKs is one of the earliest events in the GcR signaling cascade, followed by the tyrosine phosphorylation of STATs and GcR itself (Nicholson et al, 1994; Dong et al, 1995). Since the signal transduction for granulocyte differentiation has been ascribed to the JAK-STAT pathway, we focused on these molecules in ΔGcRER and ΔY703FGcRER cells.

B. Estrogen-induced phosphorylation of JAK1 and JAK2 via fusion receptors

First, we examined the tyrosine phosphorylation of JAK1 and JAK2. As shown in **Figure 2**, these kinases were not tyrosine-phosphorylated in resting 32D/ΔGcRER and 32D/ΔY703FGcRER cells. Addition of G-CSF rapidly induced phosphorylation of JAK1 and JAK2; this event was induced by dimerization of the endogenous GcR, and maximal activation was observed within 10 minutes (data not shown). Similarly, 10^{-7} M 17β-estradiol (E_2) induced tyrosine phosphorylation of JAK1 and JAK2 in these cells (**Figure 2**). The estrogen-induced activation of JAK1 and JAK2 was mediated by chimeric receptors, at a slower rate than the activation mediated by the endogenous GcR; the maximal phosphorylation was observed 60 minutes after E_2 addition (time course not shown). The difference in kinetics of JAK1/JAK2 phosphorylation may be due to different mechanisms of receptor activation. While G-CSF directly crosslinks GcR at the extracellular domain, the activation of ER-HBD fusion receptors is a ligand-induced derepression that involves other proteins such as HSP90 (Mattioni et al, 1994). Nevertheless, the levels of JAK1/JAK2 phosphorylation were comparable whether the cells were stimulated with G-CSF or estrogen. As shown in **Figure 2**, the levels of estrogen-induced JAK1/JAK2 phosphorylation in 32D/ΔY703FGcRER cells were comparable to those seen in 32D/ΔGcRER cells. Reprobing of the blots with anti-JAK1 and anti-JAK2 antibodies showed that approximately equal amounts of the kinases were loaded on these lanes (not shown). Thus, we concluded that the Y703F mutation had little, if any, effect on the tyrosine phosphorylation of JAK1 and JAK2. Considering that JAK1 and JAK2 are constitutively associated with the membrane-proximal region of GcR which is sufficient to activate them (Nicholson et al, 1994; Dong et al, 1995; Avalos, 1996), it is conceivable that the kinases were not affected by the GcR mutation in the membrane-distal region.

C. Comparable STAT5 phosphorylation following fusion receptor activation

Next, we investigated the activation of STAT proteins in 32D/ΔGcRER and 32D/ΔY703FGcRER cells. It was shown that G-CSF-induced signaling involves STAT1, STAT3 and STAT5 (Tian et al, 1994; de Koning et al, 1996; Tian et al, 1996; Shimozaki et al, 1997; Dong et al, 1998; Chakraborty et al, 1999; Ward et al, 1999). Since the membrane-distal cytoplasmic region of GcR was not required for STAT1 activation (de Koning et al., 1996), we addressed whether the phosphorylation of STAT5 and STAT3 is affected by the Y703F mutation. **Figure 3** shows the time course of STAT5 activation in 32D/ΔGcRER and 32D/ΔY703FGcRER cells (upper panel). STAT5 was not tyrosine-phosphorylated in unstimulated 32D cells, and addition of 10^{-9} M G-CSF induced a rapid phosphorylation of this molecule through crosslinking of the endogenous GcR. On the other hand, 10^{-7} M of E_2 induced a slower and less extensive phosphorylation of STAT5.

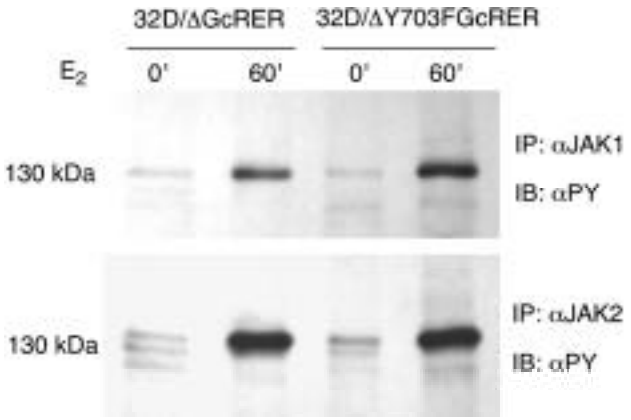

Figure 2. Tyrosine phosphorylation of JAK1 and JAK2. Serum- and cytokine-starved 32D/ΔGcRER and 32D/ΔY703FGcRER cells were harvested before (0') and after 60 minutes (60') of incubation with 10^{-7} M of estradiol (E_2). Lysates from 32D/ΔGcRER and 32D/ΔY703FGcRER cells were immunoprecipitated (IP) with either an anti-JAK1 (αJAK1; upper panel) or an anti-JAK2 (αJAK2; lower panel) antibody. Immunoblotting (IB) was carried out with an anti-phosphotyrosine antibody (αPY).

The estrogen-induced STAT5 activation was comparable in 32D/ΔGcRER and 32D/ΔY703FGcRER cells at 60 minutes after stimulation, and reprobing of the blot with an anti-STAT5 antibody showed that approximately equal amounts of STAT5 were loaded (**Figure 3**, lower panel). The delay in STAT5 phosphorylation may be associated with a slower JAK1/JAK2 activation through estrogen-induced dimerization of the chimeric receptors. The reason for the reduced STAT5 phosphorylation in the E_2-stimulated cells is currently unknown; we speculate that the linking of ER-HBD to the C-terminal of GcR might hinder STAT proteins from freely accessing the membrane-distal region of the receptor. In any case, STAT5 appeared to be phosphorylated to the same extent in 32D/ΔGcRER and 32D/ΔY703FGcRER cells. Others demonstrated that STAT5 was activated even when the membrane-distal region of GcR was deleted or the receptor tyrosine phosphorylation was abrogated (Shimozaki et al, 1997; Tian et al, 1996). Taken together with our observation that JAK1 and JAK2 were activated in both 32D/ΔGcRER and 32D/ΔY703FGcRER cells (**Figure 2**), we concluded that the Y703F mutation did not affect the tyrosine phosphorylation of STAT5.

D. Decrease in STAT3 Activation by Y703F G-CSF Receptor Mutant

Finally, we addressed whether the Y703F mutation in GcR affects tyrosine phosphorylation of STAT3. After cytokine starvation, 32D/ΔGcRER and 32D/ΔY703FGcRER clones were incubated with 10^{-7} M of E_2 for 60 minutes. While estrogen induced a significant tyrosine phosphorylation of STAT3 in 32D/ΔGcRER, only a slight activation of STAT3 was detected in 32D/ΔY703FGcRER clones (**Figure 4**, upper panel, arrow). Reprobing of the membrane with an anti-STAT3 antibody revealed an even loading of STAT3 in these lanes (**Figure 4**, lower panel).

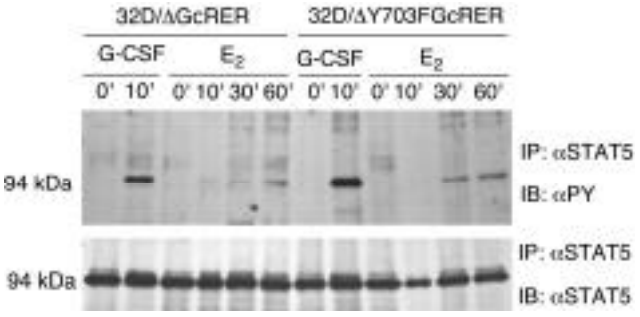

Figure 3. Tyrosine phosphorylation of STAT5. Starved 32D/ΔGcRER and 32D/ΔY703FGcRER cells were harvested before (0') and after 10, 30, and 60 minutes (10', 30', 60') of incubation with 10^{-9} M of G-CSF or 10^{-7} M of estradiol (E_2). Lysates were immunoprecipitated (IP) with an anti-STAT5 antibody (αSTAT5) and immunoblotted (IB) with an anti-phosphotyrosine antibody (αPY; upper panel). The blot was reprobed with the anti-STAT5 antibody to confirm the equal loading of STAT5 (lower panel).

Repeated experiments constantly demonstrated a decreased STAT3 phosphorylation in 32D/ΔY703FGcRER. Consistent with this observation, Tian et al showed that the G-CSF-induced STAT3 activation was greatly abrogated in UT-7epo cell transfectants by deleting a membrane-distal part including Y703 from GcR (Tian et al, 1996). We therefore concluded that Y703 in GcR was involved in STAT3 activation, and that the event is crucial to granulocyte differentiation in 32D cells.

IV. Discussion

The phosphotyrosine residues in GcR create potential docking sites for the recruitment of signaling molecules such as STATs that contain a Src homology 2 (SH2) domain. STAT3 is recruited via the interaction of its SH2 domain with receptor tyrosine residues that are present in a tyrosine-X-X-glutamine (YXXQ) sequence (Stahl et al, 1995). Among four conserved tyrosine residues in the cytoplasmic region of GcR, only Y703 provides a YXXQ motif, accounting for the reduced STAT3 activation by the Y703F mutant. However, there was a residual level of STAT3 activation in ΔY703FGcRER and other GcR mutants devoid of this motif, which suggested the presence of another STAT3 binding site in GcR or some bridging molecule (Avalos, 1996; Chakraborty et al, 1999). We observed a few additional phosphorylated proteins coimmunoprecipitated with STAT3 including a 130 kDa species (**Figure 4**, upper panel, arrowheads). These proteins are yet to be identified; at least they did not react with an antibody against GcR in a subsequent reprobing (data not shown).

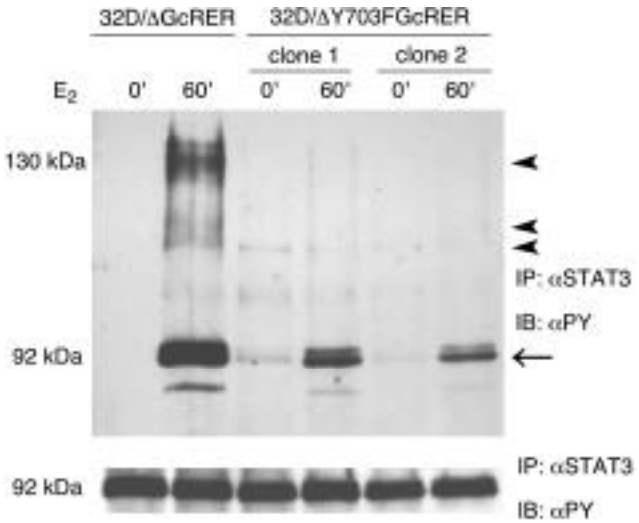

Figure 4. Tyrosine phosphorylation of STAT3. Starved 32D/ΔGcRER and 32D/ΔY703FGcRER (clone 1 and clone 2) cells were harvested before (0') and after 60 minutes (60') of incubation with 10^{-7} M of estradiol (E_2). Lysates were immunoprecipitated (IP) with an anti-STAT3 antibody (αSTAT3) and immunoblotted (IB) with an anti-phosphotyrosine antibody (αPY; upper panel). The blot was reprobed with the anti-STAT3 antibody to confirm the equal loading of STAT3 (lower panel). Besides STAT3 (92 kDa, arrow), several phosphoproteins including a 130 kDa species (arrowheads) were coimmunoprecipitated.

A consensus has been reached that tyrosine phosphorylation of GcR and activation of STAT3 is crucial to granulocyte differentiation, but there remains some controversy over the relative contribution of each tyrosine residue depending on the cells used (Tian et al, 1994, 1996; de Koning et al, 1996; Shimozaki et al, 1997; Chakraborty et al, 1999; Ward et al, 1999). Previous reports employed either GcR-negative cells to examine the function of the receptor and associated molecules, or overexpression of dominant-negative forms of GcR to elucidate the mechanisms for growth and differentiation. By using ER-HBD fusion proteins to bypass endogenous GcR, we herein provided additional data suggesting the major involvement of Y703 in STAT3 activation. It is of particular note that the cells retained the expression of wild-type GcR and downstream signaling molecules, thereby rapidly undergoing granulocyte differentiation in response to G-CSF, indistinguishable from the parent 32D cells (Matsuda et al, 1999a).

Contrary to its promoting function in myeloid cell differentiation, STAT3 was shown to play a central role in the maintenance of the pluripotent phenotype of embryonic stem cells (Matsuda et al, 1999b; Niwa et al, 1998). STAT3 appears to dictate widely divergent instructions such as differentiation and proliferation depending on the cell type. Thus, it is crucial to set up an appropriate venue to study the physiological molecular interaction involving a promiscuous molecule such as STAT3. The HBD fusion system provides a powerful tool to examine the behavior of mutated proteins controlled by specific ligands, in the exact milieu where the wild-type molecules coexist but remain unstimulated.

Acknowledgments

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