Journal of Immunological Methods 354 (2010) 45–52

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

A receptor-independent, cell-based JAK activation assay for screening for JAK3-specific inhibitors

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ARTICLE INFO

Article history:
Received 8 November 2009
Received in revised form 22 January 2010
Accepted 26 January 2010
Available online 4 February 2010

Keywords:
JAK
STAT
TEL fusion protein
JAK3 inhibitor

ABSTRACT

New immunosuppressive compounds with less systemic toxicity that could replace calcineurin inhibitors are urgently needed. For identification of specific inhibitors of JAK3, a potential new drug target, from large chemical libraries we developed a cell-based screening system. TEL–JAK fusion proteins composed of an oligomerization domain of TEL and kinase and/or pseudokinase domains of JAKs provided constitutive activation of JAKs without receiving a signal from the cytokine receptors. These fusion proteins also induced STAT5b phosphorylation in the absence of cytokine receptors. Both the kinase and pseudokinase domains of JAKs were required for full activation of the JAKs, and four copies of STAT5 response elements provided the greatest luciferase activity. The sensitivity and specificity of the system was evaluated using specific JAK3, JAK2, or MEK inhibitors. Thus, we generated a receptor-independent, cell-based selective screening system for specific JAK3 inhibitors, which is easily convertible to a high-throughput screening platform.

1. Introduction

Transplantation is the final treatment option for patients with organ failure, and the long-term survival of patients depends heavily on the effective use of immunosuppressants (Meier-Kriesche et al., 2006). The use of cyclosporine A and tacrolimus has tremendously increased the 5-year survival rate of organ-transplanted patients (Sam and Leehey, 2000). As calcineurin inhibitors prevent the induction of an immune tolerance mechanism by directly inhibiting the NFAT pathway (Heissmeyer et al., 2004), the inevitable continuous use of these drugs can lead to long-term immunosuppression and, in turn, recurrent infections. Additionally, these drugs can cause severe systemic side effects including nephrotoxicity, hypertension, and neurotoxicity (Liptak and Ivanyi, 2006). Therefore, new immunosuppressants with selectively targeting specific molecules with fewer side effects are required.

Janus kinase 3 (JAK3) is an intracellular signaling component for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, which share a common γ (γ_c) chain surface receptor. γ_c chain cytokines modulate the development, activation, proliferation, and survival of T, B, NK, and NKT cells (Rochman et al., 2009). As JAK3 expression is restricted to the immune system, JAK3 is a good molecular target for the development of novel

Abbreviations: JAK, Janus kinase; STAT, Signal transducer and activator of transcription; IL, Interleukin; γ_c chain, Common γ chain; TEL, Translocated Ets Leukemia/ETV6; GM-CSF, Granulocyte macrophage colony-stimulating factor; MEK, Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

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doi:10.1016/j.jim.2010.01.010

immunosuppressants (O'Shea et al., 2004; Borie et al., 2004). Recently, JAK3-specific inhibitors such as CP-690,550 and PNU156804 have been shown to prolong graft survival in rodent models of heart transplantation and in cynomolgus monkeys receiving kidney transplants (Changelian et al., 2003; Borie et al., 2005; Stepkowski et al., 2002). Although JAK3-specific inhibitors might be good immunosuppressive reagents, there is no easy way to identify novel effective compounds in a large chemical library.

In this study, we designed a cell-based, high-throughput screening system to identify JAK3-specific inhibitors. To develop a cytokine-independent system, we used a receptor-independent activation strategy naturally found in some leukemias (Lacronique et al., 2000, 1997). Fusion of the Ets-family transcription factor TEL to JAK2 in an acute T cell lymphoblastic leukemia cell line has been shown to allow the cytokine-independent proliferation of hematopoietic cells (Lacronique et al., 1997). TEL–JAK fusion proteins bind each other through the TEL oligomerization domain, after which the closely localized JAKs are auto-phosphorylated and activated. The activated JAKs, in turn, phosphorylate STATs, and the phosphorylated STATs dimerize and translocate into the nucleus, where they function as transcription factors (Lacronique et al., 2000). Unlike most STATs that are recruited to cytokine receptors for activation (Leonard and O'Shea, 1998), STAT5a and STAT5b, can directly bind to JAK molecules in the absence of cytokine receptors (Fujitani et al., 1997) and be activated by them. By using constructs expressing the TEL–JAK fusion protein, STAT5b, and a reporter gene regulated by dimerized phospho-STAT5, our system provides 1) receptor-independent JAK activation; 2) receptor-independent, JAK-dependent STAT activation; and 3) a sensitive activated-STAT reporter assay system for cell-based high-throughput screening for potential JAK3 inhibitors (Fig. 1).

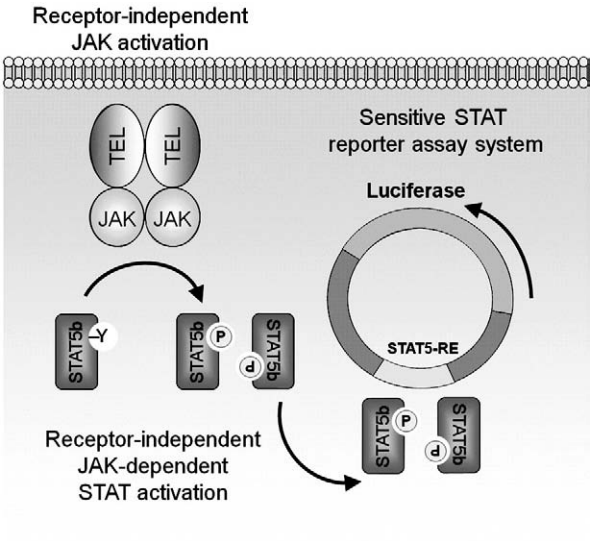

Fig. 1. The receptor-independent, cell-based JAK3-specific inhibitor screening system. The JAK-STAT signaling pathway was constitutively activated through the use of a TEL–JAK fusion protein; receptor-independent, JAK-dependent STAT activation; and a sensitive activated-STAT reporter assay.

2. Materials and methods

2.1. TEL–JAK fusion protein and STAT5b expression system

Total RNA was isolated from human peripheral blood mononuclear cells using TRIZOL (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI) and random hexamers. One microliter of the first-strand cDNA reaction mixture was used in the subsequent PCR reactions. The primer sets used are: TEL, 5′-CTA GGC TAG CCC TGA TCT CTC TCG CTG TGA-3′/5′-AAT TGA ATT CAC AGT CTG CTA TTC TCC CAA TG-3′; JAK1 (JH1), 5′-GTA CGG TAC CCC AAC TGA AGT GGA CCC CAC-3′/5′-GGC CGC GGC CGC TGC TTC TTA TTT TAA AAG TGC TTC A -3′; JAK1 (JH2-1), 5′-GTA CGG TAC CCT CAA GAA GGA TCT GGT GCA-3′/5′-GGC CGC GGC CGC TGC TTC TTA TTT TAA AAG TGC TTC A-3′; JAK2 (JH1), 5′-AAT TGA ATT CGG TGC CCT AGG GTT TTC TGG T-3′/5′-GGC CGC GGC CGC TTT GGT CTC AGA ATG AAG GTC A-3′; JAK2 (JH2-1), 5′-AAT TGA ATT CGT GGA TGT ACC AAC CTC ACC A-3′/5′-GGC CGC GGC CGC TTT GGT CTC AGA ATG AAG GTC A-3′; JAK3 (JH1), 5′-AAT TGA ATT CAT TCG TGA CCT CAA TAG CCT CA-3′/5′-GGC CGC GGC CGC AAG GTC ACA CAG CCA GTC AA-3′; JAK3 (JH2-1), 5′-AAT TGA ATT CAA CCT GAT CGT GGT CCA GAG-3′/5′-GGC CGC GGC CGC AAG GTC ACA CAG CCA GTC AA-3′; STAT5b, 5′-GCC GAG CGA GAT TGT AAA CC-3′/5′-CCA CCA TGC ACA GAA ACA CT-3′. Amplified human TEL, JAK1(JH1), JAK1(JH2-1), JAK2(JH1), JAK2(JH2-1), JAK3(JH1), JAK3(JH2-1), and STAT5b DNA were cloned into the pGEM-T easy vector (Promega) and then subcloned into the pcDNA3.1(+) expression vector using the restriction enzyme sites, generating the TEL–JAK1(JH1), TEL–JAK1(JH2-1), TEL–JAK2(JH1), TEL–JAK2(JH2-1), TEL–JAK3(JH1), TEL–JAK3(JH2-1), and STAT5b expression plasmids.

2.2. Reporter system containing STAT5b responsive elements

The STAT5 responsive element (RE) was synthesized as a fifteen-nucleotide, double-stranded DNA molecule (5′-AATTTCCTGGAAATT-3′, BIONIX, Seoul, Korea) and blunt-end ligated into the SmaI-digested pTA-Luciferase vector (pTA-luc, Clontech, Mountain View, CA). The sequence and number (1, 2, or 4) of the STAT5 REs were verified by DNA sequencing using an ABI automated sequencer (Perkin Elmer, Boston, MA).

2.3. Transfection and JAK activity assay

The CV-1 (CCL-70) and TF-1 (CRL-2003) cell lines were used in this study. These cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD) at 37 °C and 5% CO_2. For the TF-1 cell line, 2 ng/ml recombinant human GM-CSF was added to the culture. Transfection was performed using the Lipofectamine 2000 transfection reagent (Invitrogen), following the manufacturer's recommended protocol. Briefly, 2×10^5 cells/well (in a 6-well plate) or 6×10^3 cells/well (in a 96-well plate) were transfected with various combinations of the TEL–JAK1(JH1), TEL–JAK1(JH2-1), TEL–JAK2(JH1), TEL–JAK2(JH2-1), TEL–JAK3(JH1), TEL–JAK3(JH2-1), STAT5b, pTA-luc, STAT5 RE(1)/pTA-luc, STAT5 RE(2)/pTA-luc, and STAT5