Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 375 (2008) 249–254
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A high-throughput assay for signal transducer and activator of transcription 5b based on fluorescence polarization

Judith Müller, Jochen Schust, Thorsten Berg *

Max Planck Institute of Biochemistry, Department of Molecular Biology, and Munich Center for Integrated Protein Science (CIPSM), Am Klopferspitz 18, 82152 Martinsried, Germany

Received 16 November 2007
Available online 18 January 2008

Abstract

Signal transducer and activator of transcription 5b (STAT5b) is constitutively activated in many human tumors. Activity of STAT5b requires binding of its Src homology 2 (SH2) domain to certain phosphotyrosine-containing sequences. We have developed a high-throughput assay based on fluorescence polarization that allows screening of chemical libraries for compounds that inhibit STAT5b by interfering with the function of its SH2 domain. The assay, which is based on binding between a fluorescein-labeled phosphotyrosine peptide derived from the erythropoietin receptor to the STAT5b SH2 domain, is stable with regard to dimethyl sulfoxide concentration and time and has a Z' value of 0.66 ± 0.11 in a 384-well format.

Keywords: Fluorescence polarization; Assay development; STAT5b; SH2 domain

Signal transducers and activators of transcription (STATs)[1] are latent cytoplasmic transcription factors that transmit signals from cytokine receptors and growth factor receptors to the nucleus [1]. On ligand-induced receptor dimerization, STATs bind to activated cytokine receptors and growth factor receptors via their Src homology 2 (SH2) domains. Receptor-bound STATs subsequently are phosphorylated at a conserved tyrosine residue within the SH2 domain by receptor-associated Janus kinases (JAKs), other cytoplasmic tyrosine kinases (e.g., c-Src), or the intrinsic tyrosine kinase activity of growth factor receptors. Moreover, STATs can be phosphorylated by constitutively activated tyrosine kinases (e.g., v-Src, Bcr-Abl) even in the absence of ligand-induced receptor signaling. Tyrosine-phosphorylated STATs form dimers based on reciprocal phosphotyrosine–SH2 domain interactions that translocate to the nucleus, bind to specific DNA sequences, and thereby regulate transcription of their target genes.

Among the STAT family members, STAT3 and STAT5 are generally considered to be relevant targets for cancer therapy [1–6]. These proteins are constitutively active in a wide range of primary human tumors and tumor-derived cell lines, and inhibition of signaling via these STATs leads to apoptosis of cancer cells. STAT5 is overactive in several kinds of leukemias as well as in breast cancer, uterine cancer, prostate cancer, and squamous cell carcinoma of the head and neck (SCCHN) [6]. Two isoforms of STAT5, dubbed STAT5a and STAT5b, are 93% identical at the amino acid level. STAT5b, but not STAT5a, was reported to potentiate the transforming potential of v-*src* in NIH 3T3 cells [7]. Expression of a dominant negative STAT5b mutant slowed growth of prostate cancer cells in a mouse xenograft model [8]. Similarly, antisense oligonucleotides (ASOs) targeting STAT5b inhibited growth of SCCHN

* Corresponding author. Fax: +49 89 8578 2454.
E-mail address: berg@biochem.mpg.de (T. Berg).

[1] *Abbreviations used:* STAT, signal transducers and activators of transcription; SH2, Src homology 2; JAK, Janus kinase; SCCHN, squamous cell carcinoma of the head and neck; ASO, antisense oligonucleotide; Fmoc, 9-fluorenylmethyloxycarbonyl; DIC, *N,N′*-diisopropylcarbodiimide; HOBt, 1-hydroxy-benzotriazole; DMF, *N,N*-dimethylformamide; NHS, *N*-hydroxysuccinimide; cDNA, complementary DNA; MBP, maltose-binding protein; mP, millipolarization units; DMSO, dimethyl sulfoxide; EPOR, EPO receptor.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.01.017

cells in mice, whereas ASOs against STAT5a had no effect on tumor growth [9,10]. These data demonstrate that despite the high sequence similarity of the STAT5 isoforms, their biological functions may differ in some aspects and also encouraged us to focus on STAT5b.

We recently described an assay based on fluorescence polarization that allows screening of small molecule libraries for inhibitors of the function of the SH2 domain of STAT3 [11]. In an extension of this work, we now describe a homogeneous assay that allows screening of large small-molecule libraries for modulators of the activity of the SH2 domain of STAT5b.

Fluorescence polarization assays have proven to be invaluable for the analysis of binding between two molecules of significantly different molecular weight [12]. The small binding partner is labeled with a fluorophore and excited by linearly polarized light. Thus, only those molecules whose fluorophores have the correct orientation relative to the polarity of the light are excited. If the excited small molecules are unbound, they will continue to rotate rapidly after excitation, resulting in a low overall orientation at the time of fluorescence emission (usually on the order of nanoseconds) that is detected as a low degree of fluorescence polarization. In contrast, binding of the small molecule to its large binding partner will increase its effective molecular weight, resulting in slower tumbling, and thus a more uniform spatial orientation, that is detected as a high degree of fluorescence polarization. An agent that reduces the fraction of fluorophore-labeled small binding partner that is bound to the large binding partner can be recognized by a decrease in the fluorescence polarization. If fluorescence polarization assays are applied to screen small-molecule libraries for bioactive compounds, special attention is needed to filter out test compounds that give a false-positive readout caused by autofluorescence. In case a small organic molecule has excitation/emission properties similar to those of the chosen fluorophore, it is excited by the linearly polarized light itself. Due to its fast tumbling, the polarization of its fluorescence will be low. This artificially reduces the overall fluorescence polarization, accompanied by an increase in overall fluorescence intensity. Therefore, it is crucial to verify that the total fluorescence intensity in the presence of an apparently active test compound is not increased due to compound autofluorescence.

Materials and methods

Peptide synthesis

Peptides were synthesized and purified using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry by the core facility of the Max Planck Institute of Biochemistry and by Peptide Specialty Laboratories (Heidelberg, Germany). Coupling to 5-carboxyfluorescein was performed via *N,N′*-diisopropylcarbodiimide (DIC)/1-hydroxy-benzotriazole (HOBt) activation in *N,N*-dimethylformamide (DMF) [13] or via the *N*-hydroxysuccinimide (NHS) ester. Unless stated otherwise, peptides were synthesized with an N-terminal amino group and a C-terminal carboxyl group. Peptides were analyzed by HPLC and MS.

Cloning and protein expression

Amino acids 136 to 703 of human STAT5b were PCR amplified from placenta complementary DNA (cDNA) and cloned into the *Fse*I/*Asc*I sites of a modified pQE70 vector carrying a C-terminal 6×His tag and an N-terminal maltose-binding protein (MBP) tag. STAT5b was expressed from Rosetta BL21DE3 cells (Novagen) following the published procedure and was purified on Ni–ion exchange resin. After dialysis against a buffer containing 100 mM NaCl, 50 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.1% Nonidet P40, aliquots were snap-frozen in liquid nitrogen and stored at –80 °C before use.

Binding assays

Binding experiments were performed using an Ultra Evolution 384-well plate reader (Tecan). On excitation with linearly polarized light (485 nm), the fluorescence intensities parallel (Int_{parallel}) and perpendicular ($Int_{\text{perpedicular}}$) to the plane of the original excitation were recorded (535 nm). Background fluorescence in wells containing the corresponding buffer only was recorded and subtracted from the values obtained for the samples. Fluorescence polarization is defined as $P = (Int_{\text{parallel}} - Int_{\text{perpedicular}}) / (Int_{\text{parallel}} + Int_{\text{perpedicular}})$. The fluorescence polarization values obtained by this formula were multiplied by the G factor ($G = 0.998$ determined empirically) to correct for imperfections of the optical components of the instrument. Corrected fluorescence polarization values were multiplied by 1000 and expressed in millipolarization units (mP), following the standard procedure for data presentation. Unless otherwise stated in the text and figure legends, the buffer conditions were as follows: 50 mM NaCl, 10 mM Hepes (pH 7.5), 1 mM EDTA, 0.1% Nonidet P40, and 1 mM dithiothreitol. Fluorescent-labeled peptides were dissolved in dimethyl sulfoxide (DMSO) and used at 10 nM. Peptides used for competition binding assays were also dissolved in DMSO. Competition binding assays were performed using STAT5b at a final concentration of 105 nM in the presence of 10% DMSO. All experiments were performed in non-treated black 384-well microtiter plates (Corning). Plates were read 30 min after mixing of all assay components. Binding curves and inhibition curves were fitted using SigmaPlot (SPSS Science Software).

Calculation of Z′

To assess the suitability of the assay for high-throughput screening applications, the Z' value was calculated using the equation $Z' = 1 - (3\ SD_{\text{bound}} + 3\ SD_{\text{free}}) / (mP_{\text{bound}} - mP_{\text{free}})$. The "bound state" was represented by incubating