Biochimie 91 (2009) 996–1002

Contents lists available at ScienceDirect

Biochimie

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

Research paper

A novel non-phosphorylated potential antitumoral peptide inhibits STAT3 biological activity☆

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ARTICLE INFO

Article history:
Received 6 May 2009
Accepted 12 May 2009
Available online 24 May 2009

Keywords:
STAT3
Peptide inhibitor
Immunofluorescence
Cellular effects

ABSTRACT

STAT3 (Signal Transducer and Activator of Transcription 3) is an SH2 (Src Homology 2)-domain containing transcription factor that plays a key role in cancer, by regulating as a dimer the expression of genes implicated in the main processes of the tumorigenesis. Therefore, STAT3 and more particularly its dimeric form has emerged as promising targets for cancer therapy. STAT3 dimerization occurs through reciprocal interaction between the SH2 domain of one monomer and the phosphorylated tyrosine residue of a second one. One strategy to design small inhibitors of STAT3 dimerization, consists in using phosphotyrosine-based peptidomimetics targeted to the SH2 domain. We have tested whether a high affinity phosphotyrosyl peptide ligand **P1** (AYRNRY*RRQYRY) ($K_d = 0.34$ μM), issued from combinatorial chemistry, could inhibit STAT3 dimerization and biological activity. This ligand was found to disrupt dimerization with an IC_{50} of 9 μM. Further biological evaluation using a STAT3-dependent cell line demonstrated that its Antennapedia-vectorized form **P1a** interacted with STAT3 within the cell, resulting in a significant effect on cell proliferation and expression of cell cycle and apoptosis regulators controlled by STAT3. More importantly, these studies identified unexpectedly a non-phosphorylated vectorized peptide **P2a**, as another potent inhibitor of STAT3 biological activity and thus give further insight for the development of novel inhibitors.

1. Introduction

Signal Transducer and Activator of Transcription 3 (STAT3) is a latent cytoplasmic transcription factor that regulates cellular processes such as proliferation, differentiation and survival, in response to numerous cytokines and growth factors [1–3]. STAT3 is composed of an amino-terminal domain, a coiled-coil domain, a DNA-binding domain, a linker, a Src Homology 2 (SH2) domain, and a transcriptional activation domain. Upon stimulation, STAT3 is rapidly phosphorylated on Tyr^{705}, by activated tyrosine kinase receptors [1] or by receptor-associated tyrosine kinases such as Janus kinases (JAKs) or Src kinases [4]. Phosphorylated STAT3 form dimers, through reciprocal SH2-pTyr705 interactions [3,5]. The dimeric form of STAT3 translocates to the nucleus, binds to specific DNA sequences, and regulates transcription of target genes. Persistent activation of STAT3 has been associated to oncogenic transformation. So STAT3 has been shown to be constitutively activated in a wide variety of cancers including breast cancer [6], head and neck cancers [7] and prostate cancer [8] as well as multiple myeloma [9] and melanoma [10]. The mechanism by which STAT3 contributes to oncogenesis lies in its ability to up-regulate the expression of genes encoding regulators of cell cycle (Cyclin D_1 [11,12] and c-Myc [13]), cell survival (Bcl-x_L [9,11,14] and Mcl-1 [10]) and angiogenesis (VEGF) [15]. In malignant cells harbouring abnormal STAT3 activity, inhibition of STAT3 signaling is sufficient to repress the induction of these genes, resulting in growth arrest and apoptosis. Therefore, targeting STAT3 appears as a promising strategy for cancer therapy [16–19].

Abbreviations: Ahx, 6-amino hexanoic acid; Antp, Antennapedia sequence; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIPEA, diisopropylethylamine; GPK, GMP-protein dependant kinase; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STAT3, signal transducer and activator of transcription 3; Y* or pTyr, phosphotyrosine residue

☆ This work was supported by La Ligue Nationale Contre le Cancer (Comité National et Comité 93, Equipe Labellisée 2006), Jennifer Dourlat benefited a grant by La Ligue Nationale Contre le Cancer.

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doi:10.1016/j.biochi.2009.05.006

Several approaches have been developed for interfering with STAT3 signaling, including inhibition of STAT3 phosphorylation, dimerization or DNA-binding [20–26] (see the references in recent reviews [27,28]). Since the dimerization step represents a key event in STAT3 pathway, a powerful strategy consists of preventing pTyr-SH2 domain interaction with the use of phosphopeptides, peptidomimetics, and more recently small chemical molecules. The peptides or peptidomimetics are generally derived from protein sequences that bind to STAT3-SH2 domain [22,23,29], and the small molecules from screening [30–32] or computer-aided rational design [33,34]. Such recently discovered small molecules have given very promising results on cancer cells and on *in vivo* animal cancer models [33].

The first phosphotyrosyl peptide PY*LKTK, derived from the dimerization interface of STAT3, was shown capable of inhibiting STAT3 dimerization and of suppressing its biological activity when conjugated to a cell-penetrating sequence [22]. In the same way, another peptide Y*LPQTV, issued from the STAT3-SH2 domain docking sites in gp130, was identified as a more potent *in vitro* STAT3 dimerization inhibitor, but was not further evaluated against tumor cells [23].

More interestingly, a non-endogenous phosphopeptide AYRNRY*RRQYRY, identified through combinatorial chemistry, showed a high and selective affinity toward STAT3 [35]. Here we report the use of the latter sequence as a tool to evaluate the effect of such phosphopeptide on STAT3 dimerization and biological activity. So the ability of this peptide to disrupt STAT3 dimerization was examined *in vitro* and its effect on STAT3 cellular localization and biological activity was then investigated on a STAT3-dependent cell line. These experiments allowed us to validate the phosphopeptide as a STAT3 dimerization inhibitor in cells and more importantly to discover an Antennapedia-conjugated non-phosphorylated peptide, RNRYRRQYRY-Ahx-Antp, as another potent inhibitor of STAT3 biological activity.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA, USA). STAT3, phospho-Tyr^{705} STAT3, phospho-Erk1/2 and Cyclin D_1 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), Bcl-x_L antibody was purchased from BD Biosciences (Heidelberg, Germany). The peroxidase-conjugated anti-mouse and anti-rabbit secondary antibody, nitrocellulose membranes, Western-blot chemiluminescence reagent were purchased from Amersham Biosciences (Buckinghamshire, England). Texas Red dye-conjugated AffiniPure Donkey anti-rabbit secondary antibody was obtained from Jackson Immunoresearch (West Grove, PA, USA). Il-6 stimulated HepG2 nuclear extracts and TransAM™ STAT3 Assay kit were from Active Motif (Rixensart, Belgium).

2.2. Cell lines and culture

v-Src transformed and non-transformed NIH 3T3 fibroblasts were grown in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, 4.5 g/L glucose, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2.

2.3. Peptide synthesis

Synthesis of peptides was carried out by solid phase on a Wang resin on an A433 synthesizer (Applied Biosystems) following the standard Fmoc chemistry protocol using HBTU/HOBt/DIPEA as coupling agents. Wang resin and suitably protected amino-acids were purchased from Novabiochem. Phosphotyrosine derivative, with phosphate diprotected by methyldiphenylsilyl(ethyl) moiety [36], was from Bachem.

For the synthesis of fluorescein labeled peptides, peptidyl resin was coupled with 6-*O*-(carboxymethyl)fluorescein ethyl ester, which was synthesized as described [37]. Briefly, Fmoc-deprotected resin-bound peptide was treated overnight in *N,N*-dimethylformamide (DMF) with 5 equivalents of 6-*O*-(Carboxymethyl)fluorescein ethyl ester and 5 equivalents of the coupling agents BOP/HOBt in the presence of 15 equivalents of DIPEA. The peptidyl resin was then washed with DMF and dichloromethane and dried *in vacuo*.

Peptide was cleaved from the polymer support and freed from side-chain protections in trifluoroacetic acid/water/triisopropylsilane (95:2.5:2.5 in volumes) for 4 h and then precipitated in cold diethyl ether after evaporation of trifluoroacetic acid. Crude peptide was collected and purified by semi-preparative reversed-phase HPLC on a Vydac C_{18} column (10 × 250 mm) with acetonitrile/water gradient containing 0.1% trifluoroacetic acid. The peptide fractions were collected, lyophilised and analysed by reverse-phase HPLC on a Vydac C18 column (4.6 × 250 mm). The molecular weight of peptide was verified by ion electrospray mass spectrometry.

2.4. Evaluation of STAT3 dimerization inhibition by ELISA

The DNA-binding activity of STAT3 was evaluated by an ELISA using the TransAm™ STAT3 Assay kit according to manufacturer's instructions. Briefly, IL-6 stimulated HepG2 nuclear extracts were preincubated for 30 min with peptides and incubated in 96-well plates coated with immobilized oligonucleotides containing a consensus (5′-TTCCCGGAA-3′) binding site for STAT3. STAT3-dimer binding to the oligonucleotide was detected by incubation with primary antibody specific to the DNA-bound STAT3 dimer, and the complexes were visualized then by an anti-IgG horseradish peroxidase conjugate, and quantified at 450 nm with a reference wavelength at 630 nm. DB_{50}, corresponding to the dose which reduces DNA-binding by 50%, was determined from a sigmoidal dose–response using GraphPad Prism (GraphPad Software, San Diego, CA).

2.5. Immunofluorescence and confocal microscopy on NIH 3T3/v-Src fibroblasts

NIH 3T3/v-Src cells were seeded in 10% FBS at a density of 2×10^5 cells in 35 mm coverglass dishes (IWAKI) and allowed to adhere for 24 h. Cells were then starved in medium containing 0.5% FBS for 24 h and they were incubated with control DMSO or peptides (50 μM for 15 min or 20 μM for 2 h).

After indicated times, cells were washed with PBS and fixed with 3% formaldehyde for 15 min. Cells were then permeabilized with 0.5% Nonidet P-40 for 5 min and non-specific interactions were blocked with 1.5% Donkey serum in PBS for 45 min. STAT3-primary antibody diluted in blocking solution was then incubated at 4 °C overnight. Following this treatment, cells were washed and incubated with Texas Red-labeled secondary antibody for 2 h at room temperature. Nuclei were detected using DAPI mounting solution and glass coverslips were added. Confocal laser scanning microscopy was then performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Göttingen, Germany).

2.6. Cell growth inhibition assay

v-Src transformed or non-transformed NIH 3T3 fibroblasts were seeded at a density of 10^5 cells per well into 6-well dishes and