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S731 in the transactivation domain modulates STAT5b activity

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Abstract

As mediators of cytokine and growth factor signaling, signal transducers and activators of transcription (STATs) transmit signals from the membrane and cytoplasm to the nucleus. While Y699 phosphorylation is required for STAT5b transcriptional activity, our previous studies show that mutation of two tyrosines in the transactivation domain of STAT5b (Y740/743F) increases Y699 phosphorylation leading to increased transcriptional activity and DNA synthesis in breast cancer cells [A.M. Weaver, C.M. Silva, Modulation of signal transducer and activator of transcription 5b activity in breast cancer cells by mutation of tyrosines within the transactivation domain, Molecular Endocrinology 20 (2006) 2392–2405]. In many instances, phosphorylation of serines in the transactivation domain also modulates STAT5b activity. Here, we demonstrate for the first time that EGF stimulation enhances S731 phosphorylation. Furthermore, we show that the increased activity of the Y740/743F STAT5b mutant requires S731. As STAT5b is implicated in several cancers, understanding how its activity is regulated through tyrosine and serine phosphorylation is vital for the development of potential novel cancer therapeutics.

Keywords: STAT5b; Serine phosphorylation; Tyrosine phosphorylation; Breast cancer

Signal transducers and activators of transcription (STATs) are involved in cytokine and growth factor signaling pathways including those involved in cell differentiation, development, proliferation, and survival [1,2]. While the STAT family consists of seven members (STAT 1, 2, 3, 4, 5a, 5b, and 6), a basic paradigm exists for all STAT activation [3]. STAT activation occurs via phosphorylation of a conserved tyrosine residue located in the carboxy-terminus. This phosphorylation can be mediated by receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), or non-receptor tyrosine kinases, such as c-Src or Janus kinases (JAKs) [4]. Tyrosine phosphorylation subsequently results in phosphotyrosine-SH2 domain mediated dimerization. The STAT dimer then translocates to the nucleus, binds to consensus DNA sequences, and recruits additional transcription machinery to initiate specific gene transcription [5].

STAT5b is activated by a variety of factors, including growth hormone (GH), prolactin (Prl), and epidermal growth factor (EGF), resulting in phosphorylation of Y699 [6–8]. Phosphorylation of this tyrosine is required for dimerization, DNA binding, and transcriptional activity, such that mutation of this tyrosine results in a transcriptionally inactive STAT5b [6,9]. We have previously identified and characterized three additional tyrosine phosphorylation sites (Y725, Y740, Y743) located in the transactivation domain of STAT5b (Fig. 1A) [1,10]. By tryptic digest and site directed mutagenesis, these tyrosines were identified as being phosphorylated upon EGF, but not GH treatment [10]. They are positive (Y725) or negative (Y740 and Y743) regulators of STAT5b activity, such that mutation of Y740 and Y743 results in a basally active STAT5b [1].

In addition to tyrosines, there are several serines located in the transactivation domain of STAT proteins, although their functional implications are both STAT- and cell type-specific [11]. While serine phosphorylation of STAT1 and STAT3 has been shown to be necessary for full transcriptional activity, the regulation and importance of serine

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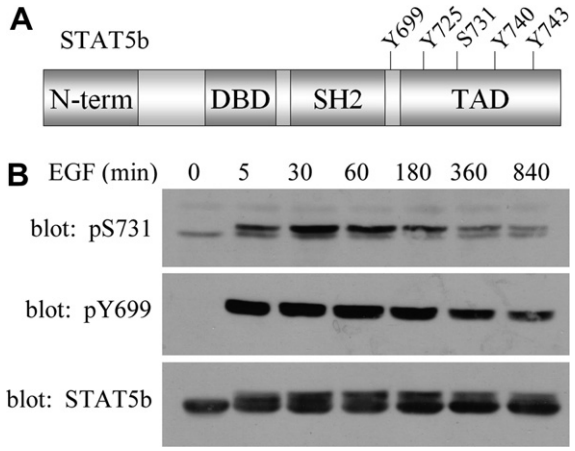

Fig. 1. EGF induced STAT5b phosphorylation. (A) Schematic of STAT5b structure illustrating the conserved domains of the STAT proteins: amino-terminus (N-term), DNA binding domain (DBD), Src homolog domain 2 (SH2), transactivation domain (TAD). The Y and S indicate where tyrosine and serine phosphorylation sites are located. (B) SKBr3 cells stably expressing His-wtSTAT5b were treated with 100 ng/mL EGF for the times indicated. Whole cell lysates were analyzed by immunoblotting with antibodies directed against phospho-S731 STAT5b (top), phospho-Y699 STAT5b (middle) and total STAT5b (bottom).

phosphorylation of STAT5b is poorly understood [11]. In some cell models, STAT5b is constitutively phosphorylated on S731, and this phosphorylation is enhanced upon Prl or GH stimulation [12–14]. Although the kinase that mediates phosphorylation of S731 has yet to be identified, mutation of S731 influences STAT5b transcriptional activity in response to PRL or GH [13–18]. For example, transient transfection of the S731A STAT5b mutant into HepG2 liver cells decreases the transcriptional activity of the GH-induced NTCP-promoter luciferase reporter compared to the corresponding wild type (wt) STAT5b [14]. In contrast, transient transfection of the S731A STAT5b mutant in COS-1 cells increases the transcriptional activity of the β-casein promoter luciferase reporter compared to wtSTAT5b [14]. These data suggest that the effect of serine phosphorylation on STAT5b function may be cell type- and gene-specific. In summary, it is apparent that the role of serine phosphorylation in STAT5b function is more complex than originally appreciated.

STAT5b has developed into a promising target for cancer therapeutics in part due to the ability of STAT5b to regulate the transcription of genes involved in cellular proliferation and survival [2,19]. STAT5b has a fundamental role in the proliferation of breast, head and neck, and prostate cancers [1,20–22]. Additionally, STAT5b is activated by tyrosine kinases that are frequently overexpressed or demonstrate increased activity in these cancers, such as the EGFR, HER2, c-Src, and Brk [1,8,10,23–25]. The inhibition of STAT5b activity either by dominant negative constructs or anti-sense oligonucleotides impairs the transcriptional activity and transforming ability of STAT5b [22,26].

Due to the involvement of STAT5b in cancer, a better understanding of how STAT5b activity is regulated through tyrosine and serine phosphorylation is of the utmost importance. Our previous studies revealed how tyrosines in the transactivation domain either positively or negatively regulate STAT5b activity [1,10]. Since S731 is located between the positive (Y699 and Y725) and negative (Y740 and Y743) regulating tyrosines, we investigated its potential role in modulating STAT5b activity (Fig. 1A).

Materials and methods

Cell lines and transient transfections. The human breast cancer cell line, SKBr3, was obtained from ATCC (Manassas, VA). Cells were passaged twice per week and maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS). SKBr3 cells stably transfected with the His-wtSTAT5b expression vector were generated and maintained in DMEM plus 10% FCS with 400 μg/mL gentamicin. Transient transfections of SKBr3 cells with His-tagged STAT5b constructs were performed using LipofectAMINE Plus (Invitrogen) per manufacturer's directions as previously described [10].

Reagents. Recombinant human epidermal growth factor (rhEGF), molecular weight standards, and all tissue culture reagents were from Invitrogen. The polyclonal STAT5b-specific antibody was developed in our laboratory, as previously described [10]. The monoclonal anti-phospho-STAT5a/5b (Y694/Y699) antibody and anti-phospho-STAT5a/b (S726/S731) were acquired from Upstate Biotechnology. The protease cocktail inhibitor was from Calbiochem, and the acrylamide was obtained from Bio-Rad. Other reagents were of either reagent or molecular biological grade from Sigma.

Cell treatment and analysis of STAT5b. Cells were preincubated overnight in DMEM containing 0.1% bovine serum albumin (BSA). Following preincubation, cells were treated either with media alone (control) or 100 ng/mL rhEGF at 37 °C for the time indicated. After incubation, cells were washed twice in phosphate-buffered saline (PBS). Detergent lysates were prepared as previously described [1]. The His-tagged expressed proteins were isolated using nickel–NTA–agarose magnetic beads (Qiagen) as previously described [1] and analyzed via immunoblotting. Isolated proteins were separated on a 7.5% polyacrylamide gel and analyzed as previously described [1].

Site-directed mutagenesis. The serine point mutation of STAT5b was constructed by designing primers in which the serine would be mutated to an alanine in the nucleotide sequence using the QuikChange site-directed mutagenesis kit (Stratagene).

Luciferase assay. SKBr3 cells were transiently transfected with the STAT5 specific Spi 2.1-containing luciferase reporter plasmid. Forty-eight hours post transfection, lysates were prepared, and luciferase activity was measured as previously described [10]. The luciferase values (arbitrary units), as measured by a Berthold luminometer, were normalized to protein amount.

DNA synthesis assay. SKBr3 cells were transiently transfected using Lipofectamine PLUS (Invitrogen) with His-tagged STAT5b constructs according to manufacturer's recommendations [10]. Twenty-four hours after transfection, cells were serum starved for 18 h, and then incubated with 100 μM of 5-bromodeoxyuridine (BrdU) for an additional 8 h. Cells were fixed and stained with fluorescent antibodies directed against the His-tag and BrdU as previously described [1]. Expression of the His-tagged STAT5b construct and BrdU incorporation into DNA were visualized using a Leica DM RBE Fluorescence microscope (model# RS232C).

Results and discussion

EGF stimulation increases S731 phosphorylation

To investigate basal and EGF-induced S731 phosphorylation of STAT5b, the human breast cancer cell line,