Available online at www.sciencedirect.com
ScienceDirect
Gene 413 (2008) 58–66
GENE
www.elsevier.com/locate/gene

Transcription of the *LAT* gene is regulated by multiple binding sites for Sp1 and Sp3

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Received 19 November 2007; received in revised form 29 January 2008; accepted 31 January 2008
Available online 9 February 2008
Received by A.J. van Wijnen

Abstract

The *LAT* gene encodes an adaptor molecule that links receptor engagement to critical downstream signaling events. Previously, we identified the proximal promoter for the human *LAT* gene and found that it contains binding sites for members of the Ets and Runx transcription factor families. In the present study, we show that the promoter also contains 5 GC-rich elements that contribute to promoter activity and that are capable of binding the transcription factors Sp1 and Sp3. Overexpression of either Sp1 or full-length Sp3 was shown to augment *LAT* promoter activity, while siRNA-mediated knockdown of each transcription factor was demonstrated to have an inhibitory effect. We also discovered a cell-type specific DNase hypersensitive site that maps to the Sp1/Sp3 and adjacent Ets and Runx binding sites. Collectively, these results provide compelling data that implicates Sp1 and Sp3 in the transcriptional regulation of the human *LAT* gene.

Keywords: Gene regulation; Promoter; T cells; Mast cells

1. Introduction

The *LAT* gene encodes a 36–38 kDa protein that facilitates intracellular signal transduction processes following activation of cell surface receptors (Horejsi et al., 2004; Samelson, 2002). Receptor engagement results in the inducible phosphorylation of LAT, which allows it to bind (either directly or indirectly) various signaling molecules, including members of the Grb2 family (Grb2, Gads, Grap), Vav, SLP-76, Sos, and PLCγ. The activation of these and other signaling proteins through their interactions with LAT results in additional biochemical events, such as an influx of calcium and activation of the Ras signaling pathway, and eventually leads to changes in cell function.

LAT plays an important role in cell types where it is expressed, which are limited to T cells, mast cells, Natural Killer (NK) cells, and platelets (Facchetti et al., 1999; Weber et al., 1998; Zhang et al., 1998). It has been shown that LAT is required for the development of T cells in the thymus and for the activation of mature T cells to elicit an immune response (Finco et al., 1998; Zhang et al., 1999). LAT is also required for mast cell survival and their activation following engagement of the high affinity receptor for immunoglobulin E, for NK cell-mediated cytotoxicity, and for collagen, collagen-related peptide, and convulxin-mediated activation of platelets (Jevremovic et al., 1999; Judd et al., 2002; Klem et al., 2002; Pasquet et al., 1999; Saitoh et al., 2000; Yamasaki et al., 2007).

Previously, we found that the 5′ proximal promoter for the *LAT* gene extends approximately 800 bp upstream of the translation start site and contains binding sites for Ets and Runx transcription factors (Finco et al., 2006). In the present study, we demonstrate that the promoter region also contains 5 GC-rich DNA elements that contribute to *LAT* promoter activity. We show that these sites are capable of binding Sp1 and Sp3 and,

Abbreviations: LAT, linker of activation in T cells; EMSA, electrophoretic mobility shift assay; KLF, Kruppel-like factor; NK, Natural Killer; NE, nuclear extract; comp., competitor.

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doi:10.1016/j.gene.2008.01.020

consistent with this observation, that overexpression of Sp1 or full-length Sp3 stimulates *LAT* promoter activity. Moreover, siRNA-mediated inhibition of Sp1 or Sp3 leads to a significant decrease in promoter function. Finally, we discovered a DNase I hypersensitive site that maps to the promoter region encompassing the Sp1/Sp3 binding sites and that is restricted to cells that express the *LAT* gene.

2. Materials and methods

2.1. Cell lines and plasmids

The Jurkat T cell line (clone E6-1), the HeLa S3 cell line, and the Schneider's Drosophila line 2 (SL2) were obtained from American Type Culture Collection. HMC-1 mast cells were provided by Dr. Joseph H. Butterfield (Mayo Clinic, Rochester Minnesota). Jurkat cells were grown in RPMI-1640, HeLa cells in DMEM, SL2 cells in Schneider's Drosophila Medium, and HMC-1 cells in RPMI-1640 or IMDM. All media was supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

The pPAC-Sp1 expression vector was obtained from Robert Tjian (University of California, Berkley), while pPAC-Sp3 (pPAC-Sp3L), which encodes the full-length isoform of Sp3 (Sp3L), and pPAC Sp3Δ1.+2.AUG (pPAC-Sp3S), which encodes a short isoform of Sp3 (Sp3S), were provided by Dr. Guntram Suske (Philipps-Universität Marburg, Germany). Construction of the pGL3-basic plasmid (Promega Corp.) containing the −1000 to −1 region upstream of the *LAT* translation start site has been described previously (Finco et al., 2006). The internal control pRL-TK *Renilla* luciferase vector was purchased from Promega Corp.

Site-directed mutagenesis of the putative Sp1/Sp3 binding sites was conducted using the QuikChange® II Site-Directed Mutagenesis kit (Stratagene). The base pair changes were as follows (changed bases in bold and underlined): The Sp A site was changed from CGGGTGGG to CG**AC**TGGG, the Sp B site was changed from GGGGTGGG to GG**AC**TGGG, the Sp C site was changed from GGGGCGGG to G**ACT**CGGG, the Sp D site was changed from CGGGCGGG to CG**AC**CGGG, and the Sp E site was changed from CGGGCGGG to CG**AC**CGGG. Reporter constructs containing mutations in multiple Sp1/Sp3 sites introduced the same base pair changes in each site as listed above and were constructed using templates that already contained mutations in other Sp1/Sp3 sites. All plasmids created for these studies were sequenced to ensure that unwanted mutations had not been incorporated during their construction.

2.2. Electrophoretic mobility shift assay (EMSA)

Jurkat and SL2 nuclear extracts were prepared as previously described (Finco et al., 2006; Suske, 2000). Protein concentrations were determined using the Bio-Rad DC protein assay. The ^{32}P labeled probes used in the EMSA were prepared by filling in annealed oligonucleotides with the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dCTP and other dNTPs followed by purification using NucTrap probe purification columns (Stratagene). The sequences of the filled in probes used in EMSAs are CCACTGGCGGGTGGGTGGGAAGGGGGC for the Sp A/B probe, GTGGGAAGGGGGCGGGTG-CAGCCGGCT for the Sp C, and CCGCAGGCGGGCGG-GAGGGCGGGCACGGAG for the Sp D/E probe. For probes with mutations in one or more Sp sites, the sequences of the mutated Sp sites were identical to those in the mutated promoter constructs described above.

For EMSAs, 5 μg of Jurkat nuclear extract or 20 μg of SL2 extract was used in DNA binding reactions containing 10 mM Tris, pH 8.0, 60 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 2 μg poly(dI/dC). For competition experiments a 100-fold molar excess of unlabeled competitor DNA was also added, while for supershift experiments 1 μl of antibody was used. The Sp1 (sc-59X), Sp2 (sc-643X), and Sp3 (sc-644X) antibodies used in supershift experiments were purchased from Santa Cruz Biotechnology, Inc. Samples were incubated on ice for 15 min, 20 K cpm of labeled probe was then added, and samples again incubated for an additional 30 min prior to loading onto a 4.5% nondenaturing polyacrylamide gel. Samples were electrophoresed at 4 °C and 20 mA using a 0.5× TBE running buffer. After the separation of complexes, the gel was dried and exposed overnight to Kodak Biomax film at −80 °C using an intensifying screen.

2.3. Transient transfection

Jurkat cells were transiently transfected by electroporation at 950 μF and 240 V using 1×10^6 cells, 2.5 μg of pGL3 firefly luciferase reporter plasmid, and 0.25 μg of the internal control phRG-TK *Renilla* luciferase plasmid. HMC-1 cells were similarly transfected except that electroporation was performed at 750 μF and 375 V. Following transfection, cells were resuspended in their respective media, incubated 20–24 h, then harvested and assayed for firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System (Promega Corporation). The ratio of firefly to *Renilla* luciferase activity was calculated for each sample and the value obtained using the promoterless pGL3-basic vector was set at one-fold activation. The value for the wild-type *LAT* promoter, which equaled 10–15-fold activation, was set at 100%.

SL2 cells were transfected using the calcium phosphate procedure and 1.5×10^6 cells, 2.5 μg of firefly luciferase reporter plasmid and either empty pPAC vector, pPAC-Sp1, pPAC-Sp3, or pPAC-Sp3Δ1.+2.AUG. Empty pPAC vector and pBluescript II KS+plasmid DNA were also added as necessary to keep the amount of pPAC and total DNA the same for each sample. Twenty-four hours following transfection, cells were washed and then incubated an additional 24 h before harvesting. Luciferase activity for each sample was then obtained and normalized based on the amount of protein as quantitated using the Bio-Rad DC Protein Assay. The activity of the wild-type promoter was set at either 1-fold or 100%.

Duplicate or triplicate samples were included for all transfections and the average and standard error of the mean (s.e.m.) for each reporter plasmid or condition were calculated from three or more independent transfection experiments using at least two preparations of each test plasmid.