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# Spi-1 and Spi-B control the expression of the *Grap2* gene in B cells☆

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**Abstract**

The Ets family members Spi-1 and Spi-B have been implicated in the regulation of genes important for B cell antigen receptor (BCR) signaling. Mice deficient in Spi-B exhibit reduced B cell proliferation in response to BCR cross-linking and impaired T cell-dependent immune responses. This defect is exacerbated in the presence of Spi-1 haplo-insufficiency ($Spi1^{+/-}SpiB^{-/-}$). Tyrosine phosphorylation and calcium mobilization induced by BCR engagement is diminished in $Spi1^{+/-}SpiB^{-/-}$ B lymphocytes, although many key BCR signaling proteins are expressed, suggesting that Spi-1 and Spi-B regulate expression of additional, unidentified signaling molecules. We now demonstrate that expression of the adaptor protein Grap2 is impaired in $Spi1^{+/-}SpiB^{+/-}$ and $Spi1^{+/-}SpiB^{-/-}$ B lymphocytes. Analysis of two alternate murine *Grap2* promoters revealed a functionally important Spi-1 and Spi-B DNA binding element located in the downstream promoter. Ectopic expression of Grap2 in Grap2-deficient B cells reduced the recruitment of BLNK to Igα and the phosphorylation of specific substrates. Regulation of BLNK recruitment was dependent upon the Grap2 proline-rich domain, while modulation of phosphorylation was dependent upon both the proline-rich and SH2 domains. These data indicate that Spi-1 and Spi-B directly regulate the expression of Grap2 and that Grap2 functions to modulate BCR signaling, but that reduced Grap2 expression is unlikely to account for the BCR signaling defects observed in $Spi1^{+/-}SpiB^{-/-}$ B cells.
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*Keywords:* PU.1; GrpL; Gads; Gene regulation; Knockout mice; BLNK; Igα

*Abbreviations:* BCR, B cell antigen receptor; BLNK, B cell linker protein; bp, base pair; Btk, Bruton's tyrosine kinase; cbl, Casitas b-lineage lymphoma; cDNA, complementary DNA; EDTA, ehylenediamine tetra-acetic acid; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; Gads, Grb2-related adaptor downstream of Shc; GFP, green fluorescent protein; Grap, Grb2-like accessory protein; Grap2, GRB2-related adaptor protein 2; Grb2, growth factor receptor-bound protein 2; GrpL, Grb2-like protein of lymphocytes; GST, glutathione-*S*-transferase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IgG, immunoglobulin G; IMDM, Iscove's modified Dulbecco's medium; IP, immunoprecipitation; kb, kilobase; LAT, linker for activated T cells; MOPS, morpholinopropanesulfonic acid; nt, nucleotide; PE, phycoerythrin; PLCγ2, phospholipase C gamma 2; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription–polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SH2, src homology domain 2; SH3, src homology domain 3; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; Syk, spleen tyrosine kinase; TBE, Tris borate EDTA buffer; TBST, Tris buffered saline with Tween; UTR, untranslated region.

☆ GenBank accession nos. AY860455 (*Grap2-1A* promoter) and AY101389 (*Grap2-1B* promoter).

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## 1. Introduction

Spi-1 (also known as PU.1) and Spi-B, two closely related Ets transcription factors, are expressed exclusively in hematopoietic cells. Spi-1 is expressed in many blood cells including B cells, macrophages and neutrophils (Klemsz et al., 1990; Galson et al., 1993; Hromas et al., 1993). In contrast, the expression of Spi-B is restricted to B cells, early T lineage cells and plasmacytoid dendritic cells (Chen et al., 1995b; Su et al., 1996; Schotte et al., 2003). Spi-1 regulates the transcription of many genes expressed in B cells including those encoding the immunoglobulin heavy and light chains, immunoglobulin J chain, major histocompatibility class II genes, Igα, Igβ, Btk, CD20, CD22, CD45 and CD72 (Omori and Wall, 1993; Jabrane-Ferrat and Peterlin, 1994; Andersson et al., 1996; Muller et al., 1996; Bassuk and Leiden, 1997; Himmelmann et al., 1997; Ying et al., 1998; Anderson et al., 2001).

DNA binding site selection has shown that recombinant Spi-1 and Spi-B bind to very similar DNA binding motifs (Ray-Gallet et al., 1995). Indeed, Spi-B can bind to and transactivate numerous Spi-1 target genes (Rao et al., 1999a; Hu et al., 2001; Dahl et al., 2002). Moreover, Spi-1 and Spi-B have been shown to interact with the same co-factors in vitro (Rao et al., 1999b). However, gene targeting experiments in mice have demonstrated that Spi-1 and Spi-B also have unique roles (Scott et al., 1994; McKercher et al., 1996; Su et al., 1997). In particular, Spi-1 is required for the development of B cells, T cells, macrophages and neutrophils (Scott et al., 1994; McKercher et al., 1996), whereas Spi-B is not required for hematopoietic cell development, but is required for mature B cell function (Su et al., 1997).

We have demonstrated that Spi-1 and Spi-B also have overlapping roles in B cell differentiation (Garrett-Sinha et al., 1999; Rao et al., 1999a; Garrett-Sinha et al., 2001; Hu et al., 2001). Mice heterozygous for Spi-1 and null for Spi-B ($Spi1^{+/-}SpiB^{-/-}$) exhibit a 2- to 4-fold reduction in the numbers of immature and mature B cells with normal numbers of pro- and pre-B cells (Garrett-Sinha et al., 1999). The decrease in peripheral B cell numbers is primarily due to higher rates of apoptosis and impaired expression of c-Rel (Hu et al., 2001). B cells isolated from the spleen of $Spi1^{+/-}SpiB^{-/-}$ mice proliferate poorly in response to B cell antigen receptor (BCR) stimulation and exhibit reduced levels of inductive tyrosine phosphorylation and $Ca^{2+}$ mobilization (Garrett-Sinha et al., 1999). Despite the defect in BCR signaling, $Spi1^{+/-}SpiB^{-/-}$ B cells express important membrane-proximal BCR signaling proteins (Ig heavy and light chains, Igα, Igβ, src family kinases, CD45, Syk, BLNK, Btk and PLCγ2) (Garrett-Sinha et al., 1999). Together, these results suggest that mutant B cells fail to express one or more unidentified Spi-1/Spi-B target genes that are critical for BCR-induced tyrosine phosphorylation and $Ca^{2+}$ mobilization.

In this report, we identify a signaling protein Grap2 whose expression is significantly decreased in B cells isolated from mice heterozygous for Spi-1 and null for Spi-B ($Spi1^{+/-}SpiB^{-/-}$). Grap2 (also known as GrpL, Gads, Grf40, GRID or Mona) is an adaptor protein that is expressed in various hematopoietic cells including T cells, B cells, monocytes and megakaryocytes (Bourette et al., 1998; Liu and McGlade, 1998; Qiu et al., 1998; Asada et al., 1999; Law et al., 1999; Ellis et al., 2000; Yankee et al., 2003). In common with the related family members Grb2 and Grap, Grap2 contains a central SH2 domain flanked by two SH3 domains. In addition, Grap2 has a unique proline-rich domain between the SH2 and carboxy-terminal SH3 domains.

The human *Grap2* gene locus has been characterized and shown to harbor two promoter segments that drive expression of alternate 5′ untranslated exons (Guyot et al., 2002). When we cloned and analyzed the homologous murine promoters, we identified a single functional Spi-1/Spi-B binding site in the downstream promoter, which was required for optimal gene transcription. These data suggest that Spi-1 and Spi-B direct the transcription of the mouse *Grap2* gene. To identify the potential function of Grap2 in BCR-mediated signaling cascades, we expressed wild-type or mutant forms of Grap2 in the Grap2-negative mature B cell line A20. Expression of wild-type Grap2 inhibited inductive BLNK phosphorylation and its recruitment to Igα, while PLCγ2 activation was not affected. These data indicate that Spi-1 and Spi-B directly regulate the expression of Grap2 and that Grap2 is involved in regulating BCR signaling cascades, but that reduced expression of Grap2 is unlikely to account for the BCR signaling defects observed in $Spi1^{+/-}SpiB^{-/-}$ B cells.

## 2. Materials and methods

### 2.1. Antibodies

Rabbit anti-GrpL (Grap2) antiserum was raised against the SH2 domain of the murine GrpL protein (Law et al., 1999). For some experiments, we also used rabbit anti-Gads (Grap2) antibody purchased from Upstate Biotechnology (Lake Placid, NY). Mouse anti-phosphotyrosine antibody (Clone 4G10) was also obtained from Upstate Biotechnology. Hamster anti-mouse CD79β (Igβ) antibody (Clone HM79b) and phycoerythrin-labeled rat anti-mouse B220 antibody (clone RA3-6B2) were purchased from BD Biosciences (San Diego, CA). Rabbit anti-PU.1 (Spi-1) antibody (T-21), rabbit anti-PLCγ2 antibody (Q20) and rabbit anti-Grb2 (C-23) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Igα and anti-BLNK antibodies were raised against GST fusions of the C-terminal domain of Igα and the SH2 domain of BLNK. Rabbit anti-Grap antibody was a kind gift from Gensheng Feng (Burnham Institute, La Jolla, California).

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