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Cutting Edge: TCR-Induced NAB2 Enhances T Cell Function by Coactivating IL-2 Transcription

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TCR engagement leads to the up-regulation of genetic programs that can both activate and inhibit T cell function. The early growth receptor (Egr) proteins Egr-2 and Egr-3 have recently been identified as TCR-induced negative regulators of T cell function. NAB2 (NGFI-A-binding protein 2) is both a coactivator and a corepressor of Egr-mediated transcription and has been implicated in regulating Schwann cell myelination. In this report we demonstrate that NAB2 is induced by TCR engagement and that its expression is enhanced by the presence of costimulation. The overexpression of NAB2 enhanced IL-2 production while small interfering RNA to NAB2 markedly inhibited IL-2 expression. Mechanistically, we demonstrate that NAB2 enhances IL-2 transcription by acting as a coactivator for Egr-1. Indeed, chromatin immunoprecipitation analysis reveals that NAB2 is recruited to the Egr-1 binding site of the IL-2 promoter. Taken together, our findings identify NAB2 as a novel coactivator of T cell function. The Journal of Immunology, 2006, 177: 8301–8305.

The NGFI-A-binding proteins NAB1 and NAB2 were originally described as corepressors of early growth response (Egr)1 gene-mediated transcription (1, 2). Although neither NAB1 nor NAB2 possess DNA binding domains, they inhibit transcription by binding to Egr-1, Egr-2, and Egr-3. For example, the binding of the NCD1 domain of NAB2 to the R1 domain of Egr-1 has been shown to inhibit nerve growth factor-induced expression of TGF-β, matrix metalloproteinase-3, and p21 (3). Interestingly, NAB2 has also been shown to act as a coactivator of Egr-1-induced transcription of the luteinizing hormone (4). Recently, with the generation of NAB1- and NAB2-null mice, their physiologic roles in promoting neuron myelination have been elucidated (5). In addition, dysregulation of NAB2 expression has also been linked to tumor development (6). Although high levels of NAB2 expression have been described for the thymus, a role for NAB2 in peripheral T cells has yet to be elucidated (2).

In T cells, Egr-1, Egr-2, and Egr-3 are markedly up-regulated upon TCR engagement (7, 8). Egr-1 plays an important role in T cell activation and promotes the up-regulation of IL-2, TNF, CD154, and IL-2R (9–11). In contrast, our group and others have demonstrated that Egr-2 and Egr-3 act as inhibitors of T cell function (8, 12–14). Indeed, both Egr-2 and Egr-3 promote the expression of TCR-induced FasL (12, 13). In addition, overexpression of Egr-2 and Egr-3 inhibits IL-2 production and proliferation independently of enhancing cell death (8, 14). Furthermore, T cells from Egr-null mice are resistant to peptide-induced anergy in vivo (8). In an effort to better understand the mechanisms by which Egr-2 and Egr-3 inhibit T cell function, we mined a microarray data set of TCR-induced genes and found that NAB2 was up-regulated upon TCR engagement. In light of the role of NAB2 as a corepressor, we hypothesized that NAB2 might facilitate Egr-2- and Egr-3-mediated inhibition of T cell activation. In fact, contrary to our initial hypothesis, in this report we demonstrate that NAB2 is a novel coactivator of T cell function by promoting Egr-1-mediated IL-2 production.

Materials and Methods

Mice

B10.A/AtTac−1(Tg)TCRCyt5CC7-I[KO]/Rag2 were purchased from Taconic Farms (model 004094-MM). All animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD).

Cells, Abs, and reagents

A.E7, a CD4+ Th type 1 clone specific for pigeon cytochrome c (81-104), was grown and maintained as described (15). Jurkat T cells (clone E6-1) were maintained in RPMI 1640 (BioSource International) supplemented with FBS (Invitrogen Life Technologies). A.E7 stimulations were conducted with 1 μg/ml anti-CD3 (clone 2C11; BD Pharmingen). Some cultures were supplemented with ascites fluid containing the 37.51 mAb to CD28 (a gift from J. Allison, Memorial Sloan-Kettering Cancer Center, New York, NY) at a final dilution of 1/1000. Anti-Krox20 (Egr-2 specific; clone PRB-236P) was purchased from Covance. Anti-EGR1 (clone sc-110) was purchased from Santa Cruz Biotechnology. Anti-actin was purchased from Sigma-Aldrich. Anti-NAB2 was a gift

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3 Abbreviations used in this paper: Egr, early growth response; ChIP, chromatin immunoprecipitation; CSA, cyclosporin A; DNNAB2, dominant negative NAB2; siRNA, small interfering RNA.
from Dr. J. Johnson (Institute for Immunology, Munich, Germany) and has been described previously (16).

Microarray analysis

Microarray analysis was conducted on A.E7 T cells as previously described (8, 17).

Real-time RT-PCR

Real-time PCR was conducted as previously described (8) using primers and probes specific for Egr-2 (forward, 5'-GTGCCCCACTGCTATCCAGAAG-3'; reverse, 5'-AGGATCTTCCGGAGTAAG-3'; and probe, 5'-TTGTAGTGTCCGCGCATTCCTTGCA-3'), Egr-1 (forward, 5'-GATGCTCTCCGCTGAGATCCTC-3'; reverse, 5'-GTGAGTGA-3'; and probe, 5'-GCCCTTCGGCTCC-3'), and NAB2 (forward, 5'-CAGAGATGCTGCGGAATGCTG-3'; reverse, 5'-GCTACCAAGGATCGATC-3'; and probe, 5'-TGGGAGATGTTGGAGAGATC-3').

Plasmids and vectors

Lentiviral vectors containing Egr-2 and Egr-3 (Lenti-Egr-2 and Lenti-Egr-3) were generated as previously described (8). NAB2 and dominant negative NAB2 (DN-NAB2) expression vectors were generated as previously described (2) and inserted into the EF.CMV.GFP lentivirus vector. The IL-2-luciferase construct was a gift from Dr. J. Ragheb (National Institutes of Health, Bethesda, MD). Negative control small interfering RNA (siRNA) and NAB2 siRNA (sequence 5'-GGAGAGUUUCUGGAGACUGT-3') were purchased from Ambion.

Transfection

Jurkat T cells were transfected as previously described (8). For siRNA experiments, human PBMCs were isolated from whole blood by Ficoll-Paque (GE Healthcare) and T cells were isolated as recommended by the Pan T cell isolation kit from Miltenyi Biotec. Enriched T cells were transfected according to the Nucleofector human T cell kit (Amaxa Biosystems) protocol.

Chromatin immunoprecipitation (ChIP) assay

Jurkat T cells were stimulated with 50 ng/ml PMA for 2 h. A ChIP assay was then performed according to manufacturers protocol (Upstate Biotechnology). For immunoprecipitation, 1 μg of anti-NAB2, anti-Egr-1, or anti-Glut1 was used. SYBR Green PCR (Bio-Rad) was conducted using primers specific to luciferase (forward, 5'-CATTTCATCCGCTTGAAAGAT-3'; reverse, 5'-GCTACCAAGGATCGATC-3'). For endogenous IL-2 promoter ChIP assays, 5C.C7 CD4+ T cells were stimulated with 1 μg/ml plate-bound anti-CD3 and anti-CD28 for 8 h. A ChIP assay and PCR were conducted as previously described. The primers used for IL-2 promoter PCR were 5'- ATCTCCTCCTTGGCGTTTGC-3' (forward) and 5'-CCCCTACACTGACGTAAGT-3' (reverse).

Results and Discussion

NAB2 is up-regulated during TCR stimulation

Previously, using a novel statistical algorithm our laboratory had identified the transcription factors Egr-2 and Egr-3 as mediating TCR-induced inhibition of T cell function (8, 17). For this analysis, A.E7 T cell clones (which are Th1 lineage cytokine and transcription factors specific) were mock stimulated or stimulated with anti-CD3 or anti-CD3 plus cyclosporin A (CSA) for 2, 4 and 6 h and RNA was pooled and analyzed by Affymetrix mouse GeneChips U74A, U74B, and U74C. Using this approach, we identified NAB2 as being only minimally expressed in resting T cells and induced upon TCR engagement (Fig. 1A). This up-regulation is partially blocked by CSA, suggesting that NFAT contributes to NAB2 regulation. Additional analysis of our screen identified NAB1 (Fig. 1B) (another corepressor of Egr-mediated transcription) as being constitutively expressed in T cells. In our previous report we demonstrated that Egr-2 and Egr-3 were up-regulated following TCR engagement in a CSA-sensitive manner, whereas Egr-1 was found to be elevated upon TCR engagement in a CSA-insensitive fashion (8). Egr-4, which is the only family member that cannot interact with the NAB proteins, was found to be constitutively expressed in T cells (8). NAB2 had previously been described as both a corepressor and coactivator of Egr-mediated transcription, and thus we wanted to understand its potential function in T cells. First, we sought to confirm the inducible expression of NAB2. A.E7 T cells were stimulated with anti-CD3 alone or with anti-CD3 and anti-CD28, and NAB2 expression was determined by real-time PCR. NAB2 RNA was rapidly induced by CD3 stimulation and reached maximal expression at 3 h (Fig. 1C). The addition of CD28 costimulation had no affect on NAB2 RNA expression as compared with anti-CD3 alone. For comparison, Egr-1 expression was induced by anti-CD3 alone but did not appear to be regulated by TCR engagement (Fig. 1D). Like NAB2, costimulation did not affect Egr-1 levels. In contrast as we have previously reported, Egr-2 levels are markedly up-regulated and remain persistently induced by anti-CD3 alone (8), and this induction is abrogated by the presence of costimulation (Fig. 1E). Next, we wanted to determine the effect of TCR engagement and costimulation on NAB2 protein expression. Interestingly, at the protein level NAB2 expression did not precisely correlate
with the induction of RNA. In T cells stimulated with anti-CD3 plus anti-CD28 there is an increase in NAB2 expression at 8 h that increases and remains elevated up to 24 h (Fig. 1F). However, in multiple experiments NAB2 protein levels were consistently lower in T cells stimulated with anti-CD3 alone. Thus, although costimulation does not effect NAB2 RNA expression, it results in enhanced expression of the NAB2 protein. For comparison, we also determined the kinetics of IL-2 transcription. As can be seen in Fig. 1G, the transcription of IL-2 peaks around 16 h, simultaneously with high levels of the NAB2 protein.

**NAB2 enhances T cell activation**

We initially focused on NAB2 in an effort to define cofactors for Egr-2- and Egr-3-mediated inhibition of T cell function. Interestingly, NAB2 expression was associated with activating conditions and the NAB2 protein was associated with peak IL-2 transcription. As such, we wanted to determine the function of NAB2 with regard to enhancing or inhibiting T cell function. First, we developed lentiviral vectors that express GFP plus Egr-2, Egr-3, NAB2, and a form of NAB2 that lacks the NCD2 domain responsible for interaction with transcriptional machinery (DNNAB2). Jurkat T cells were transfected with these constructs as well as a reporter construct consisting of 2.2 kb of the IL-2 promoter driving luciferase expression. Overexpression of NAB2 led to an increase in IL-2 promoter-driven luciferase activity when compared with the empty vector control (Fig. 2A). In contrast, overexpression of Egr-2 and Egr-3 inhibited IL-2 promoter-driven reporter activity. Furthermore, overexpression of DNNAB2 inhibited IL-2 promoter-driven luciferase activity to the same degree as Egr-2 and Egr-3. The fact that the dominant negative form of NAB2 inhibits T cell function suggests that endogenous NAB2 is playing an important role in the induction of the IL-2 promoter. In addition to enhancing IL-2 promoter activity, we also examined the effect of NAB2 overexpression on IL-2 production. As predicted by our transfection data, overexpression of NAB2 enhanced the secretion of IL-2 in response to PHA stimulation (Fig. 2B).

To complement the overexpression data we next addressed the effect of knocking down NAB2 on T cell activation. Human CD4+ T cells were isolated from PBMC and transduced with either nonspecific control siRNA or siRNA specific for NAB2. The cells were rested for 48 h and then stimulated with anti-CD3 plus anti-CD28. Western blot analysis revealed that NAB2 expression levels were markedly reduced in the T cells transfected with the NAB2-specific siRNA primers as compared with the controls (Fig. 2C). Functionally, knocking down NAB2 led to a decrease in proliferation as well as a marked decrease in IL-2 production (Fig. 2, D and E). Thus, consistent with our expression data showing that NAB2 is maximally expressed under conditions of activation, our experiments overexpressing NAB2, overexpressing DNNAB2, and knocking down NAB2 all indicate that NAB2 is an important activator of IL-2 production in T cells.

**NAB2 enhances Egr-1 mediated IL-2 production**

Although NAB2 was originally described as a corepressor of Egr-mediated transcription, it can also act as a coactivator (4). Because our functional data demonstrate that NAB2 enhances T cell function but that Egr-2 and Egr-3 are inhibitors of T cell activation, we hypothesized that NAB2 might be collaborating with Egr-1 in T cells. Egr-1 is induced by TCR engagement and has been shown to promote the transcription of IL-2, the IL-2R, CD-154, and TNF (9–11). We observed that the kinetics of TCR-induced Egr-1 expression precedes and overlaps the up-regulation of NAB2. Because Egr-1 has previously been shown to enhance IL-2 transcription by binding to an Egr-1 binding site in the IL-2 promoter (7), we wanted to determine whether NAB2 is recruited to the IL-2 promoter upon activation in an Egr-1 dependent fashion. Recall, NAB2 does not contain a DNA binding domain and thus must interact with one of the Egr proteins to be recruited to a promoter (18). Jurkat T cells were transfected with luciferase reporter constructs containing the wild-type IL-2 promoter or a mutant promoter in which the Egr-1 binding site (−300 to −290) was destroyed. The cells were stimulated and ChIP analysis was performed using anti-NAB2, anti-Egr-1, or a control Ab (anti-Glut-1). The binding of NAB2 or Egr-1 to the native or mutant IL-2 promoter was assessed by using PCR primers specific for luciferase. In the absence of stimulation, neither Egr-1 nor NAB2 bound to the IL-2 promoter (Fig. 3A). Upon activation, both Egr-1 and NAB2 were recruited to the IL-2 promoter. The specificity of this finding is demonstrated by the fact that the Glut-1 Ab failed to precipitate the IL-2 promoter even under activating conditions. Importantly, the recruitment of Egr-1

![FIGURE 2](http://www.jimmunol.org/DownloadedFrom/8303.png)
and NAB2 were both abrogated by mutation of the Egr-1 binding site. These observations support the hypothesis that NAB2 binds to the IL-2 promoter by interacting with Egr-1.

Next, we wanted to determine whether we could inhibit the ability of NAB2 to enhance IL-2 promoter-mediated transcription by mutating the Egr-1 binding site. Jurkat T cells were transfected with either an empty vector control construct or a NAB2 overexpression construct and the wild-type or mutant IL-2 promoter-reporter constructs, stimulated with PHA, and then assayed for luciferase activity. As we have previously shown (Fig. 2A), the overexpression of NAB2 enhanced reporter activity in the cells transfected with the wild-type IL-2 reporter construct (Fig. 3B). As expected, the overall reporter activity of the Egr-1 mutant IL-2 reporter construct was lower than that of the wild-type construct because the mutation eliminates the ability of Egr-1 to contribute to IL-2 promoter-driven transcription. As predicted by our ChIP experiments, eliminating the ability of the IL-2 promoter to recruit Egr-1 also prevents NAB2 from enhancing IL-2 promoter-driven transcription. That is, the overexpression of NAB2 failed to enhance the IL-2 promoter-driven reporter activity when the Egr-1 site of the promoter was mutated. Thus, the ability of NAB2 to bind to the IL-2 promoter and enhance IL-2 transcription is dependent upon its ability to coactivate Egr-1-mediated transcription. Finally, we wanted to confirm that NAB2 could bind to the endogenous IL-2 promoter. ChIP analysis was performed on primary SC.C7 transgenic T cells. As seen in Fig. 3C, upon activation both Egr-1 and NAB2 are recruited to the endogenous IL-2 promoter.

In summary, we have identified NAB2 as a novel TCR-induced transcriptional coactivator of IL-2 production. Interestingly NAB2 was identified by high dimensional analysis as being up-regulated in a Ca\(^{2+}\)-dependent fashion in B cells (19). Inasmuch as NAB2 was originally described as a corepressor, the authors hypothesized that NAB2 was part of an inhibitory pathway contributing to B cell tolerance. Indeed, before our analysis our bias was that NAB2 would be an inhibitor of T cell function. However, our data clearly demonstrate that NAB2 expression is maximal under conditions leading to T cell activation and that functionally NAB2 enhances T cell function by enhancing IL-2 production. The sustained up-regulation of NAB2 in the presence of costimulation suggests that NAB2-induced T cell activation plays a role in the ability of CD28 to enhance T cell function. Along these lines, a divergence between NAB2 RNA levels and protein expression in the presence of costimulation suggests a posttranscriptional mechanism of regulation. Interestingly, analysis of the 3’ untranslated region of NAB2 demonstrates a sequence that matches a highly conserved microRNA binding site, providing a potential explanation for our observation (J. D. Powell, unpublished observations). For example, expression of the angiotensin type 1 receptor is inhibited by the microRNA mir-155. TFG-β signaling leads to the down-regulation of mir-155 and the consequent up-regulation of the angiotensin type 1 receptor (20).

Our findings further expand the role of Egr family members in regulating T cell function. In the presence of costimulation, Egr-1 and NAB2 promote T cell activation. In contrast, in the absence of costimulation Egr-2 and Egr-3 inhibit T cell function. Based on EMSA, it has previously been reported that the Egr binding site of the IL-2 promoter binds Egr-1 and not Egr-2 and Egr-3 (21). However, by using ChIP analysis we are currently exploring the possibility that Egr-2 and Egr-3 mediate their inhibition in part by binding to the IL-2 promoter. In contrast, in addition to IL-2, Egr-1 has been implicated in the up-regulation of TNF, CD154, and IL-2R\(\beta\) (9–11). It remains to be determined whether NAB2 also acts as a coactivator for these genes. Along these lines, it has been proposed that NAB2 acts as a coactivator at promoters that contain isolated Egr sites and as a corepressor for promoters that contain multiple tandem Egr sites (4). Inasmuch as IL-2 has a single Egr-1 site in the proximal promoter, our data fits this paradigm.
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Disclosures

The authors have no financial conflict of interest.

References


