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CUTTING EDGE

Cutting Edge: TCR-Induced NAB2 Enhances T Cell Function by Coactivating IL-2 Transcription[1]

Samuel Collins, Lawrence A. Wolfraim,[†] Charles G. Drake,* Maureen R. Horton,[‡] and Jonathan D. Powell[2]**

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.

T he NGFI-A-binding proteins NAB1 and NAB2 were originally described as corepressors of early growth response (Egr)[3] gene-mediated transcription (1, 2). Although neither NAB1 nor NAB2 possesses 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 has 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/AiTac-[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 using 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.

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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′-GTGCCAGCTGCTATCCA GAAG-3′; reverse, 5′-GGCTGTGGTTGAAGCTGGAG-3′; and probe, 5′-TTGTGAGTGCGGGCATCTTGCAA-3′), Egr-1 (forward, 5′-, GATGTCTCCGCTGCAGATCTC-3′; reverse, 5′-TGTCCATGGTGG GTGAGTGA-3′; and probe, 5′-CCCGTTCGGCTCC-3′), and NAB2 (forward, 5′-CAGAGATGGTGCGAATGGTG-3′; reverse, 5′-GCT TCAACAGGGATGCGATC-3′; and probe, 5′-TGGAGAGTGTTGAG AGGATCTTCCGGAGT-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 (DNNAB2) 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, Be-thesda, MD). Negative control small interfering RNA (siRNA) and NAB2 siRNA (sequence 5′-GGAGGAGUUUCUGGAGAUCTT-3′) were pur-chased from Ambion.

Transfection

Jurkat T cells were transfected as previously described (8). For siRNA experi-ments, human PBMCs were isolated from whole blood by Ficoll-Paque (GE Healthcare) and T cells were isolated as recommended by the Pan T cell isola-tion 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 conducted 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 lu-ciferase (forward, 5′-CATTCTATCCGCTGGAAGAT-3′; reverse, 5′-TCATAGCTTCTGCCAACCGA-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 con-ducted as previously described. The primers used for IL-2 promoter PCR were 5′- ATCTCCTCTTGCGTTTGTCC-3′ (forward) and 5′-CCCCATACACTGACTGAATG-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 me-diating TCR-induced inhibition of T cell function (8, 17). For this analysis, A.E7 T cell clones (which are Th1 pigeon cyto-chrome *c* 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. 1*A*). This up-regulation is partially blocked by CSA, suggesting that NF-AT contributes to NAB2 regulation. Additional analysis of our screen identified NAB1 (Fig. 1*B*) (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
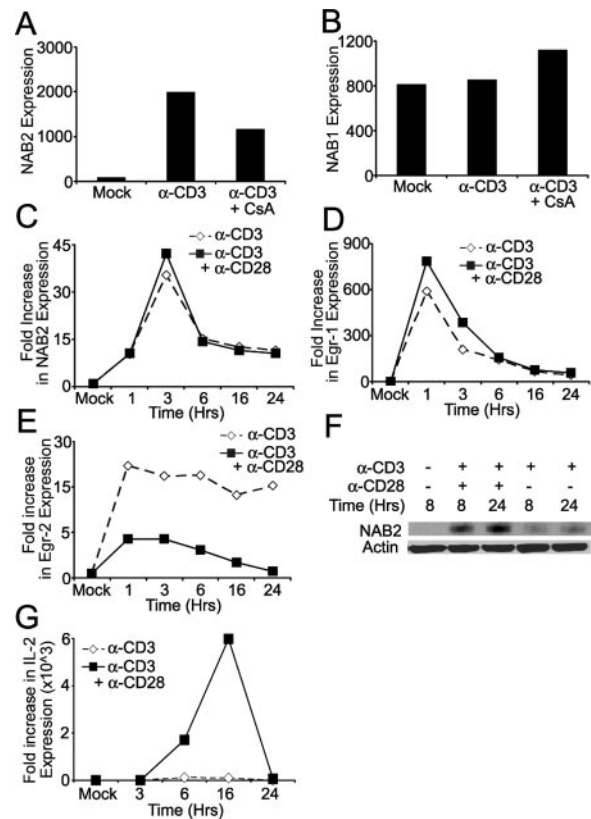

FIGURE 1. *A* and *B*, NAB2 is up-regulated by TCR stimulation. Af-fymetrix gene ChIP analysis was performed on A.E7 T cells mock stimulated or stimulated with anti (α)-CD3 or anti-CD3 plus CSA (CsA). The relative ex-pressions of NAB2 (*A*) and NAB1 (*B*) were determined. *C–E*, Kinetics and stimulation requirements for NAB2. Real-time PCR was performed on A.E7 T cells following stimulation with either anti-CD3 alone or anti-CD3 plus anti-CD28. Expression data for NAB2 (*C*), Egr-1 (*D*), and Egr-2 (*E*) are shown. The *y*-axes represent the fold increase in transcript expression over mock-treated cells. *F* and *G*, Costimulation leads to an increase in NAB2 protein expression. *F*, NAB2 Western blot analysis of lysates from A.E7 T cells stimulated with either anti-CD3 or anti-CD3 plus anti-CD28. Actin levels are included as load-ing controls. *G*, Real-time PCR was performed on A.E7 T cells following stim-ulation with either anti-CD3 alone or anti-CD3 plus anti-CD28. Expression data are for IL-2 and are presented as the fold increase over mock-treated cells. All experiments were performed at least three times.

cells (8). NAB2 had previously been described as both a core-pressor 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 stimula-tion and reached maximal expression at 3 h (Fig. 1*C*). The ad-dition 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 but with consis-tently faster kinetics (Fig. 1*D*). Like NAB2, costimulation did not affect Egr-1 levels. In contrast as we have previously re-ported, Egr-2 levels are markedly up-regulated and remain per-sistently induced by anti-CD3 alone (8), and this induction is abrogated by the presence of costimulation (Fig. 1*E*).

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
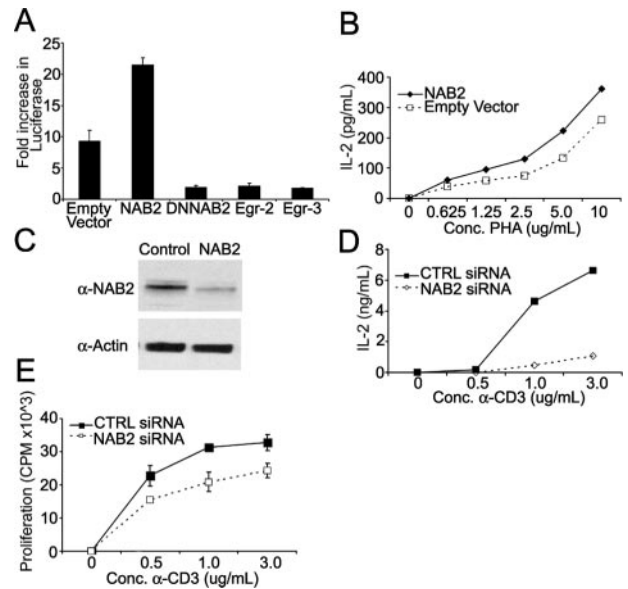

FIGURE 2. NAB2 enhances T cell activation. A, Jurkat T cells were transfected with an IL-2 promoter driving a luciferase reporter construct along with an empty vector or lentivirus vector containing NAB2, DNNAB2, Egr-2, or Egr-3. Equivalent numbers of transfected Jurkat cells were stimulated with PHA for 20 h and luciferase activity was analyzed. B, Jurkat T cells were transfected with either empty vector or lentivirus vector containing NAB2. Overexpressing cells were stimulated for 20 h with PHA, and the IL-2 protein was measured in supernatant fluids by ELISA. C, Human T cells, derived from PBMC were transfected with either control nonspecific siRNA or siRNA specific for NAB2. After 48 h in culture the transfected cells were lysed and analyzed for NAB2 protein expression by Western blot analysis. D, siRNA-transfected human T cells were stimulated with increasing concentrations of anti-CD3 along with anti-CD28 for 48 h, and the supernatant fluid was analyzed for IL-2 production by ELISA. E, siRNA-transfected human T cells were stimulated with increasing concentrations of anti (α)-CD3 along with anti-CD28 for 48 h, pulsed with [^{3}H]thymidine for an additional 16 h, and then proliferation was analyzed by [^{3}H]thymidine incorporation. All experiments were preformed at least three times. Conc, Concentration.

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
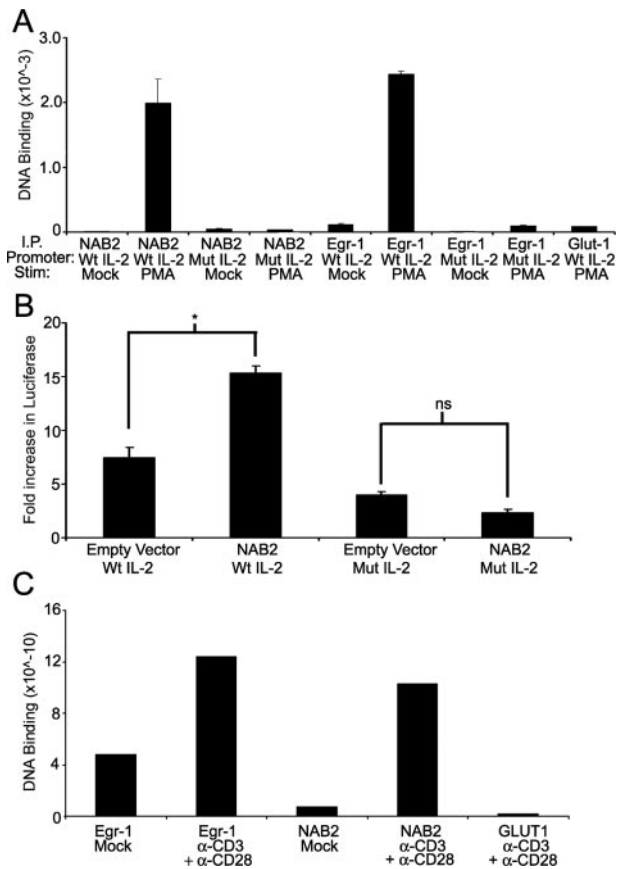
FIGURE 3. NAB2 acts as a coactivator of Egr-1-mediated IL-2 transcription. *A*, Jurkat T cells were transfected with either a wild-type IL-2 promoter (Wt IL-2) driving luciferase or an IL-2 promoter with a mutated Egr-1 binding site (Mut-IL-2). The cells were either mock stimulated or stimulated (Stim.) with PMA for 2 h and subjected to ChIP assay with Abs to either NAB2, Egr-1 or Glut-1 (as a negative control). The data are presented as specific binding of the IL-2 promoter as determined by performing PCR using primers specific for luciferase. I.P., immunoprecipitation. *B*, Jurkat T cells were transfected with a luciferase reporter construct containing either a wild type IL-2 promoter (Wt IL-2) or an IL-2 promoter with a mutated Egr-1 binding site (Mut-IL-2) along with an empty vector or a NAB2 construct, stimulated with PHA for 20 h, and then assayed for luciferase activity. *, $p < 0.05$; ns, not significant. *C*, Primary $CD4^+$ T cells were isolated from spleens of 5C.C7 transgenic mice and were either mock stimulated or stimulated for 8 h with plate-bound anti (α)-CD3 and anti-CD28. A ChIP assay was conducted using Abs to NAB2, Egr-1, or Glut-1 (as negative control). The data are presented as specific binding of the IL-2 promoter as determined by performing PCR using primers specific for the -280 site of the IL-2 promoter. All experiments were performed at least three times.

struct (Fig. 3*B*). 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 reported 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 5C.C7 transgenic T cells. As seen in Fig. 3*C*, 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 micro-RNA binding site, providing a potential explanation for our observation (J. D. Powell, unpublished observations). For example, expression of the angiotensin type I 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 I 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 β (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.

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. 2*A*), the overexpression of NAB2 enhanced reporter activity in the cells transfected with the wild-type IL-2 reporter con-

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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