Recombination activation gene-2-deficient blastocyst complementation analysis reveals an essential role for nuclear factor I-A transcription factor in T-cell activation

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Received 5 January 2011, accepted 5 April 2011

Abstract

Nuclear factor I (NFI)-A is a member of the NFI family of transcription factors implicated in regulation of granulocyte differentiation. However, its role in the lymphoid lineage is not known. NFI-A deficiency results in perinatal lethality, thus precluding analysis of the role of NFI-A in lymphocyte development and function. Using recombination activation gene-2-deficient (RAG-2<sup>−/−</sup>) blastocysts and embryonic stem cells with homozygous NFI-A gene deletion, we show an essential role for NFI-A in T-cell activation. NFI-A<sup>−/−</sup>/RAG-2<sup>−/−</sup> chimeric mice had normal distributions of CD4<sup>+</sup>CD8<sup>−</sup> double negative, CD4<sup>−</sup>CD8<sup>+</sup> double positive, CD4<sup>−</sup>CD8<sup>−</sup> and CD4<sup>−</sup>CD8<sup>+</sup>-single positive cells in the thymus and spleen and lymph nodes. However, NFI-A<sup>−/−</sup>/RAG-2<sup>−/−</sup> mice had severely reduced thymus size and hypocellularity. The decrease in thymocytes and peripheral T cells in NFI-A<sup>−/−</sup>/RAG-2<sup>−/−</sup> chimeric mice is attributed to proliferative defects associated with decreased blast transformation, CD69 expression and DNA synthesis in response to T antigen receptor stimulation. Interestingly, NFI-A-null T cells showed increased levels of c-myc transcription that is inhibited in response to antigen receptor-mediated activation. These studies demonstrate for the first time a requirement for the NFI-A transcription factor in antigen receptor-induced T-cell activation events.

Keywords: activation, lymphocyte, thymocyte development, transcription factor

Introduction

Nuclear factor I (NFI)-A is a member of the NFI family of transcription factors that bind to a conserved TTGGC<sub>N2</sub>GCCAA DNA motif through a putative α-helical segment (1). NFI-A proteins mediate initiation of adenoviral replication in vitro and transcription of many cellular and viral genes (1–6). The NFI gene family is highly conserved from chickens to humans and is composed of Nfi-a, Nfi-b, Nfi-c and Nfi-x (7, 8). The proteins encoded by these genes, sometimes referred to as CCAAT-binding transcription factors or CTF, contain a highly conserved 220 amino acid N-terminal region that mediates DNA binding and dimerization (8, 9). Different NFI proteins exhibit variation within their COOH terminal domains that results in distinct transcriptional modulatory functions (8, 9). Gene diversity, alternative splicing and unrestricted dimerization add multiple levels of diversity to regulation of gene expression by NFI family of proteins. The occurrence of multiple NFI genes in vertebrates, their differential expression during mouse development and the expression of NFI-dependent genes in multiple organs including brain, liver, muscle and other terminally differentiated...
NFI-A transcription factor in T cell activation

NFI-A, a transcription factor in T cell activation (7). Toward this goal, we generated chimeric mice by injecting cell proliferation in general and lymphoid lineage in particular to delineate the role of NFI-A in the CNS and perinatal lethality (3). Expression of NFI-A in spleen indicated. Targeted deletion of NFI-A protein in mice resulted in lack of corpus callosum associated with ventricular dilation indicated. The role of NFI-A in the lymphoid lineage has not been evaluated.

Methods

Generation of chimeric mice

RAG-2−/− mice used in these studies were backcrossed 10 generations onto the C57Bl/6 background. The targeting construct used in the generation of targeted ES cells is described elsewhere (3). The NFI-A−/− heterozygous ES cells were subjected to high G418 selection to obtain the NFI-A−/− ES cell clones as described by us previously (17). The NFI-A−/− ES cell or wild-type NFI-A+/− ES cells were injected into 3.5-day post coitus blastocysts obtained from RAG-2−/− mice to generate the NFI-A−/− → RAG-2−/− chimeric mice. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Southern blot analysis

Genomic DNA samples, prepared from the ES cells or indicated tissues from the RAG-2, NFI-A+/− → RAG-2−/− or NFI-A−/− → RAG-2−/− chimeric mice, were digested with HindIII, fractionated on a 0.8% non-denaturing agarose gel, transferred to nylon membranes and probed with an NFI-A-specific probe as described previously (3). The relative levels of the 7.0-kb wild-type and 5.0-kb mutant NFI-A alleles were quantified by phoshorimaging (Molecular Dynamics).

Expression analysis of NFI-A, NFI-B, NFI-C and NFI-X

Real-time quantitative PCR was performed using primers specific for each NFI gene with β2 microglobulin used as normalization. The following primers were used:

mβ2microglobulin—B2MM AGACTGATACATACGCTG- 
CAG 119 bp product and B2MM C GCAGGGTCAAAAT- 
GAATCTTCAAG; mα total mNFI-AE23 TGCGGATCTTGTACA- 
TCGACG 128 bp product and mNFI-AE4C2 ACCTGATGT- 
GACAAACGCGCAC 133 bp product and mNFI-BE23 CTCCTATTACAT- 
TGGTACAG 147 bp product and mNFI-C2E4C CACACCT- 
GAGCGTACAAGCT and mNFI-D2E4C GTGGGTCTCAGAC 148 bp product and mNFI-DE4C CCAGCTTGTACATTCCAGAC.

Total RNA was isolated using TRIzol Reagent (GIBCO/ 
BRL) following the manufacturer’s instructions. cDNA was generated from 2 to 5 μg of RNA using Superscript (GIBCO/BRL) following the manufacturer’s random-prime protocol. Nfi-a, Nfi-b, Nfi-c and Nfi-x transcript levels were normalized to β2 microglobulin levels by real-time quantitative PCR using a Bio-Rad real-time thermocycler with SYBR-green detection and the deltadelta Ct method as recommended by the manufacturer.

Reagents

Hamster anti-mouse CD3 antibody (2C11.2) was purchased from BD Pharmingen. Phorbol myristate acetate (PMA) and calcium ionophore (ionomycin) were obtained from Calbiochem (La Jolla, CA, USA). Propidium iodide was purchased from Sigma Chemical Co (St Louis, MO, USA). Fluorochrome-labeled anti-CD4 (PE), anti-CD8 (FITC), anti-CD3 (FITC), anti-TCRαβ (PE), anti-TCRγδ (FITC), anti-IgM (FITC), anti-B220 (PE) and anti-CD69 (FITC) were purchased from BD Pharmingen.

Culture conditions and assay for DNA synthesis

Thymocytes and splenic T cells were prepared as described previously, using commercially available T-cell enrichment columns (R&D system) (17). Thymocytes (4.0 × 10⁷) or splenic T cells were stimulated with plate immobilized anti-CD3 antibody (15 μg ml⁻¹) or PMA (100 ng ml⁻¹) ± ionomycin (0.5 mg ml⁻¹) in 96-well tissue culture plates, in 200 μl volumes. DNA synthesis was measured by incorporation of [³H]thymidine, for 18 h following 40 h of stimulation. Results are presented as the geometric mean of counts per minute ± standard error from triplicate cultures.

Flow cytometry

Single cell suspensions (0.5 to 1 × 10⁶) of thymus, spleen, bone marrow or lymph node cells from 8-week-old mice (n = 5) were stained with fluorochrome-labeled anti-CD4 (PE), anti-CD8 (FITC), anti-CD3 (FITC), anti-TCRαβ (PE), anti-TCRγδ (FITC), anti-IgM (FITC) and anti-B220 (PE) or anti-CD69 (FITC) as described previously (18). All samples were analyzed on an EPICS ELITE ESP flow cytometer (Hialeah, FL, USA). At least 20,000 events gated for live lymphocytes based on forward and side scatter were collected for each sample.

Results

Generation of NFI-A−/− → RAG-2−/− chimeric mice

The NFI-A wild-type genomic locus, the targeting vector used to mutate the NFI-A locus and the disrupted NFI-A locus are schematically shown in Fig. 1(a). The targeting vector replaces part of exon 2 resulting in disruption of the NFI-A locus (3). HindIII digestion of the wild type and the targeted loci resulted in 7- and 5-kb fragments, respectively, identified with the probe shown in Fig. 1(a). Southern blot analysis of DNA from NFI-A+/− wild type, NFI-A−/− heterozygous and NFI-A−/− homozygous ES cells generated by high G418 selection revealed the expected 7- and 5-kb-targeted fragments (Fig. 1b). The generation of null NFI-A mutation.
expression of NFI-B, NFI-C and NFI-X transcripts were observed in NFI-A\(^{+/+}\) and NFI-A\(^{+/−}\) ES cells indicating the absence of compensatory modulation of other NFI family members. The NFI-A\(^{+/−}\) and NFI-A\(^{+/+}\) ES cells were used to reconstitute RAG-2\(^{−/−}\) blastocysts, to generate NFI-A\(^{+/−}\)→RAG-2\(^{−/−}\) and NFI-A\(^{+/+}\)→RAG-2\(^{−/−}\) chimeric mice, respectively. Southern blot analysis of genomic DNA prepared from kidney, lung, heart and intestine from NFI-A\(^{+/−}\)→RAG-2\(^{−/−}\) chimeric mice revealed the expected 7-kb wild-type band (Fig. 2a). Figure 2(b) shows contribution of injected mutant ES cell to 40–62% chimerism in the kidney, lung, heart and intestine as determined by the relative levels of the targeted and wild-type alleles detected by Southern blot analysis as described by us previously (17).

**Developmental analysis of NFI-A\(^{+/−}\) lymphocytes in NFI-A\(^{+/−}\)→RAG-2\(^{−/−}\) chimeric mice**

Analysis of NFI-A in various tissues has revealed NFI-A binding activity in spleen and T-cell lines, suggesting a role for NFI-A in T-lymphocyte development and/or function (ref. 19 and data not shown). In order to directly test the role of NFI-A in lymphocyte development, thymocytes from RAG-2\(^{−/−}\), NFI-A\(^{+/−}\)→RAG-2\(^{−/−}\) and NFI-A\(^{+/−}\)→RAG-2\(^{−/−}\) chimeric mice were stained with fluorochrome-labeled anti-CD4, anti-CD8, anti-TCR\(β\) or CD3 antibodies. As previously reported, the RAG-2\(^{−/−}\) mice exhibited developmental arrest in CD4\(^+/\)CD8\(^−\) double negative stage resulting in the absence of CD4\(^+/\)CD8\(^+\) double positive (DP) or CD4\(^+/\)CD8\(^−\) single positive (SP) or CD4\(^−\)CD8\(^+\) SP T cells (Fig. 3) (17). In contrast to

was confirmed by reverse transcription-PCR analysis of the cDNA prepared using RNA isolated from the NFI-A\(^{+/+}\) and NFI-A\(^{+/−}\) ES cells, using primers specific for the NFI-a, NFI-c and NFI-x microglobulin transcripts. The data are represented as moles of NFI gene transcripts per mole of β2 microglobulin.
RAG-2−/− mice, NFI-A−/−→RAG-2−/− chimeric mice revealed a normal distribution of CD4+CD8− SP, CD4+CD8+ SP and CD4+/CD8− SP T cells comparable to NFI-A+/+→RAG-2−/− chimeric mice (Fig. 3). The ratio of CD4+CD8− SP and CD4+CD8+ SP cells were comparable in both the NFI-A+/+→RAG-2−/− and the NFI-A−/−→RAG-2−/− chimeric mice. Consistent with the normal development in the thymus, flow cytometric analysis of splenocytes and lymph node cells revealed normal profiles and distribution of CD4 and CD8 SP cells (Fig. 3 and data not shown). Further, staining for the surface IgM and the B220 molecules showed comparable development of mature B cells in the spleen and bone marrow in NFI-A+/+→RAG-2−/− and NFI-A−/−→RAG-2−/− chimeric mice (Fig. 3 and data not shown).

T-cell antigen receptor-induced proliferative defect in NFI-A−/−→RAG-2−/− chimeric mice

In contrast to the observed normal distribution of CD4 SP, CD8 SP cells and CD4+/CD8− DP cells, the thymi and spleen from NFI-A−/−→RAG-2−/− chimeric mice were consistently smaller in size by visual appearance and ~60% loss in weight compared with the equally reconstituted NFI-A+/+→RAG-2−/− chimeric mice. Consistent with the decreased size and weight, the total thymocytes in NFI-A−/−→RAG-2−/− chimeric mice were significantly reduced in numbers by >80% (Fig. 4a). This is further reflected by the corresponding decrease in the absolute numbers of CD4+CD8− and CD4−CD8+ SP and CD4+CD8− SP cells and CD4+CD8+ SP cells in the thymus. To directly test if the decreased in cell number is due to limited expansion of T cells, the proliferative potential of NFI-A−/−→RAG-2−/− and NFI-A+/+→RAG-2−/− thymocytes was determined in response to stimulation with T-cell antigen receptor using plate immobilized anti-CD3 antibody or PMA and ionomycin. Activation through the TCR resulted in a 60–80% decrease in the proliferation of NFI-A−/− T cells compared with NFI-A+/+ T cells. Interestingly, NFI-A−/− T cells showed no decrease, in response to stimulation with PMA and ionomycin, when compared with NF-1A+/+ T cells (Fig. 4b). The decreased proliferation of the NFI-A−/− T cells is not due to differences in the levels of CD3 molecules in NFI-A+/+ and NFI-A−/− T cells as evidenced by comparable levels of surface expression of CD3 [NFI-A+/+ mean fluorescence intensity (MFI) = 149 ± 12 versus NFI-A−/− MFI = 155 ± 8, n = 3)]. Further the antigen receptor-induced defect in DNA synthesis is not restricted to thymocytes as activation of purified splenic T cells from NFI-A−/− mice also resulted in decreased DNA synthesis (Fig. 5c). This appears to be due to an effect on the early stage of T-cell activation as the NFI-A−/− T cells exhibited a significant decrease in anti-CD3-induced blast transformation (~88% compared with NFI-A+/+ cells) as detected by forward scatter profiles (Fig. 5a). Further, consistent with the TCR-induced activation defect in NFI-A-deficient T cells, decreased expression of activation antigen CD69 was observed in NFI-A−/− T cells compared with NFI-A+/+ T cells, in response to anti-CD3 stimulation (Fig. 5b). Consistent with the decreased activation, supernatants obtained from anti-CD3-stimulated NFI-A−/− T cells exhibited ~80% reduction in IL-2 levels compared with NFA-1+/+ T cells (data not shown). The decreased proliferation observed in NFI-A−/− T cells is not due to either endogenous or CD3-induced modulation of CD3 signaling molecules such as GRB-2, LAT, LCK, ZAP-70 or PKB as both NFI-A+/+ and NFI-A−/− T cells showed comparable expression of these gene transcripts (Fig. 6a). In contrast to
Fig. 5. Defective activation of NFI-A−/− T cells in response to antigen receptor stimulation. Purified splenic T cells (4.0 × 10⁶ ml⁻¹) from NFI-A+/+ → RAG-2−/− and NFI-A−/− → RAG-2−/− chimeric mice were stimulated through the T-cell antigen receptor using plate immobilized anti-CD3 antibody. The cells were harvested at 36 h after stimulation and analyzed by FACS. Panel (a) shows the forward (FSC) and side scatter (SSC) profiles of the activated cells. The numbers in each panel shows the percentage of cells undergoing blast transformation. Panel (b) shows the expression of the activation marker CD69, in anti-CD3-activated NFI-A+/+ and NFI-A−/− T cells. The numbers indicate the mean fluorescence intensity (MFI) of CD69+ cells in the total activated T cells. Panel (c) shows the DNA synthesis in NFI-A+/+ or NFI-A−/− T cells stimulated with the anti-CD3 as measured by [³H]thymidine incorporation. The cells were cultured 48 h and then pulsed with 1 μCi [³H]thymidine for 18 h. The error bars indicate the mean and standard deviation of triplicate cultures. The results are representative of two independent experiments.

Fig. 6. NFI-A-null T cells show increased c-Myc transcription that is inhibited in response to antigen receptor-mediated activation. Purified T cells from NFI-A+/+ → Rag-2 or NFI-A+/− → Rag-2−/− mice were stimulated with media or immobilized anti-CD3 (10 μg ml⁻¹). Total RNA was isolated 24 h following stimulation and the expression of GRB-2, LAT, LCK, ZAP70, CD3ζ, PKB and HPRT transcripts were analyzed by semiquantitative real-time PCR as described previously (18). HPRT was used as an internal control. Panel b shows increased c-Myc transcription that is inhibited in response to antigen receptor-mediated activation in NFI-A−/−. This is in contrast to induction of c-Myc in NFI-A+/+ T cells. Results are representative of three independent experiments.

Discussion

NFI-A has been implicated in the expression of many cellular and viral genes. Recently, its role in human granulopoiesis has been established through interplay between C/EBPα and mir 223 (16). The role of NFI-A in lymphocyte development or proliferation has not been previously analyzed. The RAG-2−/− blastic complementation model described in this report describes a role for NFI-A in T-cell proliferation and activation
NFI-A\textsuperscript{−/−} and NFI-A\textsuperscript{+/−} T cells. Understanding the molecular mechanisms and role of NFI-A in regulation of c-Myc will provide insights into the role of NFI-A in antigen receptor-induced T-cell activation and functional differentiation.

**Funding**

This work was supported in part by American Cancer Society to N.M and National Institutes of Health (HD34908 to R.M.G.).

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**References**


