Role of NFAT Proteins in \textit{IL13} Gene Transcription in Mast Cells*\textdagger

Silvia Monticelli\textdagger\textdagger, Deborah C. Solymar\textdagger\textdagger,\textdagger\textdagger and Anjana Rao\textdagger\textdagger\textdagger

From the \textdagger Department of Pathology, Harvard Medical School, and CBR Institute for Biomedical Research, Boston, Massachusetts 02115, the \textdagger Division of Biotechnologies Applied to Medical Sciences, University of Milan, 20133 Milan, Italy, and the \textdagger Graduate Program in Immunology, Division of Medical Sciences, Harvard Medical School, Boston, Massachusetts 02115

Th2 and mast cells are participants in the asthmatic response to allergens, and both cell types produce the cytokines interleukin (IL)-4 and IL-13. IL-13 in particular is both necessary and sufficient for experimental models of asthma. The transcription factor NFAT plays a central role in cytokine transcriptional regulation in both cell types. Here, we analyze the molecular basis of \textit{IL13} gene transcription in Th2 and mast cells. We show that NFAT1 is the major NFAT protein involved in regulating \textit{IL13} transcription in mast cells. Although NFAT2 is correctly expressed and regulated in mast cells, it does not contribute to \textit{IL13} gene transcription as shown by analysis of cells lacking NFAT2 and cells expressing a constitutively active version of NFAT2. The difference between NFAT1 and NFAT2 appears to be due to a preferential synergistic interaction of NFAT1 with GATA proteins in the \textit{IL13} promoter. We suggest that mast cells lack a co-activator protein that stabilizes the binding of NFAT2 to the \textit{IL13} promoter by interacting either with NFAT2 itself or with a DNA-bound complex of NFAT2 and GATA proteins.

Th2 cells and mast cells are both central players in the pathophysiology of asthma (1). Th2 cells are characterized by the ability to produce a specific panel of cytokines, IL-4,\textsuperscript{1} IL-5, and IL-13. These cytokines are also produced by mast cells (2, 3), even though mast cells and Th2 cells derive from two different compartments of the immune system (myeloid and lymphoid respectively). Expression of Th2-type cytokines is strongly associated with asthma; this is demonstrated by the fact that human asthma has been linked to a region of chromosome 5q containing the \textit{IL4}, \textit{IL5}, and \textit{IL13} genes (4). Although IL-4 and IL-5 have been implicated in asthma, IL-13 is now thought to be especially critical. In animal models of allergic asthma, blockade of IL-13 markedly inhibited allergen-induced airway hyperresponsiveness, mucus production, and eosinophilia. Furthermore, IL-13 delivery to the airways was able to cause all of these effects. IL-13 is thus both necessary and sufficient for experimental models of asthma (5, 6).

In Th2 cells, production of the cytokines IL-4, IL-5, and IL-13 is controlled by cooperation between two families of transcription factors, the GATA and NFAT families (3, 7–9). The calcium-regulated transcription factor NFAT consists of four family members, three of which (NFAT1 (p, c2), NFAT2 (c, c1), NFAT4 (x, c3)) are expressed in both cell types (3, 9–11). Targeted disruption of the genes encoding individual NFAT family members suggests that there are cell type- and gene-specific differences in their ability to regulate gene transcription in activated cells (11, 12). The GATA family of transcription factors is also essential for IL-4, IL-5, and IL-13 by Th2 cells, as shown by increased and decreased expression of these cytokines in transgenic mice overexpressing wild-type GATA3 (7) and a dominant-negative version of GATA3 (8) respectively. In addition to playing a role in Th2 cytokine expression, GATA3 is important for T cell lineage development and for embryonic brain development (13, 14). However, GATA3 is not expressed by mast cells, leading to the possibility that other GATA family members regulate IL-4, IL-5, and IL-13 cytokine expression by mast cells. GATA1 expression is restricted to hematopoietic lineage cells and plays an important role in erythroid lineage development (2, 15–21). Disruption of murine GATA2 results in embryonic lethality characterized by a major loss of blood cells and reduction in hematopoietic precursors. In the mast cell lineage, GATA2 appears to be essential for mast cell differentiation, since GATA2\textdagger\textdagger--/-- yolk sac progenitors generate only macrophages, while GATA1 appears to regulate the mature phenotype of tissue mast cells (22–24).

Despite the fact that targeted gene disruption of individual NFAT members leads to disparate phenotypes, specific comparison of constitutively active versions of NFAT1 and NFAT2 in Th2 cells suggests that these two family members have equivalent roles in expression of \textit{IL4} and possibly other cytokine genes (25, 26). Thus the intrinsic ability of NFAT transcription factors to activate gene expression could be similar for certain target genes, and in these cases the different phenotypes of the gene-disrupted mice could be due to differences in the expression pattern of the different NFAT proteins. However, other target genes could show real differences in their ability to be activated by different NFAT members, for instance because co-activator proteins discriminated between the individual members. The existence of combinatorial synergy between NFAT and GATA proteins would increase this possibility, because co-activators that bridged these proteins would need to interact effectively with both protein partners in cells.

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\textdagger Recipient of a Lady Tata Memorial Trust fellowship.

\textdagger\textdagger Student in the Graduate Program in Immunology, Division of Medical Sciences, Harvard Medical School and a Predoctoral Fellow of the Ryan Foundation.

\textdagger\textdagger\textdagger To whom correspondence should be addressed: Harvard Medical School & CBR Inst. for Biomedical Research, Warren-Alpert Bldg., Rm. 152, 280 Longwood Ave., Boston, MA 02115. Tel.: 617-278-3260; Fax: 617-278-3260; E-mail: aro@chb.med.harvard.edu.

\textdagger The abbreviations used are: IL, interleukin; HA, hemagglutinin; ES, embryonal stem; BMMC, bone marrow-derived mast cells; ESMC, ES cell-derived mast cells; PMA, phorbol 12-myristate 13-acetate; CaA, cytoplasmic antibody; mAb, monoclonal antibody; GFP, green fluorescent protein; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; CA, constitutively active.
In this study, we analyze the molecular basis of IL13 gene transcription in mast cells. We show that NFAT1 plays a predominant role in this process, whereas NFAT2 makes only a minor contribution, despite the fact that it is expressed and properly regulated in mast cells. In chromatin immunoprecipitation assays performed in mast cells, NFAT1 but not NFAT2, binds the IL13 promoter, and only NFAT1 effectively synergizes with GATA factors to activate transcription from the IL13 promoter. However, both NFAT1 and NFAT2 can physically interact with GATA proteins in co-immunoprecipitation assays. We discuss the possibility that cell type-specific co-activators might stabilize specific NFAT-GATA combinations bound to DNA.

EXPERIMENTAL PROCEDURES

Plasmids—Expression plasmids encoding HA-tagged NFAT1 and NFAT2, and retroviral expression plasmids encoding constitutively active (CA)-NFAT1 and CA-NFAT2, have been described (26–28). GATA expression vectors were obtained from S. Orkin (GATA1 and GATA2) and K. Murphy (GATA3). The luciferase plasmid driven by the human IL13 promoter (29) was a gift from D. B. Lewis.

Bone marrow-derived Mast Cells (BMMC) and ES Cell-derived Mast Cells (ESMC) — BMMC were generated from bone marrow cells isolated from femurs and tibias of 8-week-old C57Bl/6 mice as described (3). Cells were maintained 4–12 weeks in medium containing 50% WEHI-3 (ATCC) conditioned supernatant as a source of IL-3. For generation of ESMC, wild-type and homozygous NFAT2−/− ES cells were differentiated to embryoid bodies as described (30, 31). Briefly, ES cells were maintained in Dulbecco’s modified Eagle’s medium on fibroblast-feeder cells in the presence of 1000 units/ml leuke- mia inhibitory factor (LIF) (Invitrogen). Prior to differentiation, ES cells were passaged two times on gelatinized plates, and medium was changed to Iscove’s modified Dulbecco’s medium. ES cells were then trypsinized and resuspended at a concentration of 7.5–12.5 × 10^5 cells/ml in Iscove’s modified Dulbecco’s medium differentiation medium (containing 15% fetal calf serum, 2 mM l-glutamine (Invitrogen), 300 μg/ml transferrin (Roche Applied Science), 4 × 10^{-4} M methyl-β-thio-galactoside, 50 μg/ml ascorbic acid (Sigma), 5% protein-free hybridoma medium (PFFM-II, Invitrogen)) in Petri-grade dishes for about 11 days. Embryoid bodies were then disrupted in trypsin, and single cell suspensions were cultured in mast cell media (RPMI with 50% WEHI-3 conditioned supernatant, and c-Kit ligand (20 μg/ml)). For both BMMC and ESMC, differentiation was assessed by metachromatic staining of cytoplasmic basophilic granules with toluidine blue and by surface staining of membrane FcERI and c-Kit (3).

For stimulation, BMMC and ESMC were treated with 20 nM PMA and 2 μM ionomycin (Calbiochem). Alternatively, BMMC and ESMC were sensitized with 3 μg/ml anti-dinitrophenol rat IgE (Zymed Laboratories Inc.) after which FcERI-bound IgE were cross-linked with mouse anti-rat IgG F(ab),/H11032/2 fragments (Jackson ImmunoResearch Laboratories) at 25 μg/ml as described (3). Where indicated, 2 μM cyclosporin A (CsA) was added 20 min prior to stimulation.

T Cell Differentiation and Stimulation—T cell differentiation was performed as described (32). Briefly, for Th2-polarizing conditions, IL-4, anti-IL-12, and anti-IFN-γ Ab were added. For stimulation, cells were treated with 20 nM PMA and 2 μM ionomycin for the indicated times; where indicated, 2 μM CsA was added 20 min prior to stimulation. The D10 Th2 clone (D10.G4.1, (33)) was used for some experiments.

Retroviral Transduction—The Phoenix ecotropic packaging cell line (kindly provided by G. P. Nolan) was transiently transfected with expression vectors for NFAT and GATA proteins. The total DNA amount was adjusted to 10–20 μg in each transfection. Cells were transfected at room temperature by electroporation in serum-free medium with pulses of 270 V, 960 microfarads (Jurkat) and 450 V, 500 microfarads (CFTL15). Twenty-four hours after transfection, cells were stimulated with 2 μM ionomycin and 20 μM PMA. Eight hours after stimulation, cells were harvested, and cell extracts were used for luciferase and Renilla activity using an automated luminometer (Berthold) and a dual-luciferase reporter assay system (Promega) following the manufacturer’s instructions. Luciferase data were normalized for renilla readings (without TK) and in some experiments for the total protein content in the lysate.

RESULTS

NFAT1 Participates in IL13 Gene Transcription in Mast Cells while NFAT2 Does Not—We examined the features of IL13 gene transcription by wild-type, NFAT1−/− and NFAT2−/− mast cells. Mast cells lacking NFAT1 were obtained by culturing bone marrow cells from NFAT1-deficient mice in the presence of IL-3 as described previously (3). This method could not be used to obtain NFAT1-deficient BMMC because NFAT2−/− mice display very early embryonic lethality (39, 40). Instead we obtained ESMC by culturing wild-type and NFAT2−/− ES cells with IL-3 and c-Kit ligand. We first showed that BMMC and ESMC expressed similar levels of IL13 and NFAT2 and that their levels of NFAT2 were elevated compared with Th2 cells (Fig. 1A). We also confirmed that BMMC and ESMC show very similar levels and kinetics of cytokine gene transcription in response to PMA/ionomycin stimulation or IgE cross-linking of the FcERI receptor (data not shown). As demonstrated previously (3), NFAT1−/− BMMC are strongly impaired in their ability to transcribe the IL13 gene: indeed, the low levels of IL13 transcripts produced by NFAT1−/− BMMC were comparable with those produced by wild-type BMMC in the presence of CsA (Fig. 1B). In contrast, NFAT2−/− ESMC produced completely normal levels of IL13 transcripts with kinetics identical to those of wild-type ESMC (Figs. 1, C–E).

This result suggested that there was a fundamental difference in the ability of NFAT1 and NFAT2 to regulate IL13 gene expression in mast cells. Loss of NFAT1 was associated with a striking drop in IL13 mRNA induction, while loss of NFAT2 linked with 1% formaldehyde. Chromatin was isolated, sheared by sonication, and immunoprecipitated with antibodies against NFAT1 (6G.1 and 7B21; Ref. 35), NFAT2 (7A6, Affinity Bioreagents), or acetylated histone 4 (AcH4, Upstate Biotechnology). Cross-links were reversed by heating, and the presence of selected DNA sequences in the immunoprecipitated DNA was assessed by PCR followed by agarose gel electrophoresis and ethidium bromide staining. The primers used for the IL13 promoter were as follows: forward, 5′-CTTTCTTATAGCCGCC- CAC; reverse, 5′-CAGAGGCCTCATGAGCCCAAGAG (227 bp product).

Immunocytochemistry—Endogenous NFAT2 was detected with monoclonal antibody 7A6 (Affinity Bioreagents), followed by Cy3-conjugated anti-IgG Ab (Jackson ImmunoResearch Laboratories).

Immunoprecipitation and Western Blotting—Antibodies against HAT and GATA proteins and GATA proteins were purchased from Santa Cruz Biotechnology. To analyze endogenous proteins, whole cell extracts were prepared by boiling pellets directly in Laemmli sample buffer. For co-immunoprecipitation experiments, HNK293 cells were transfected with the appropriate expression vectors by calcium-phosphate precipitation and reassembled in Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, and protease inhibitors). Immunoprecipitations were performed overnight at 4 °C with 1–5 μg of antibody, and the conjugate was collected with protein A or protein G (Sigma) 50% slurry.

ELISA Analysis of Cytokine Production—IL-13 levels in culture supernatants were measured in a sandwich ELISA using two different monoclonal antibodies (one of them biotinylated) that recognize different epitopes on the IL-13 protein (R&D Systems).

Reporter Assays—Cell transfections and reporter assays were performed as described (36, 37). Typically, 10^5 CFTL15 (38) or Jurkat cells were transiently transfected with 100 ng of TK-renilla plasmid (Promega), 1 μg of IL13 promoter-luciferase reporter plasmid, and 5–10 μg of expression vectors for NFAT and GATA proteins. The total DNA amount was adjusted to 10–20 μg in each transfection. Cells were transfected at room temperature by electroporation in serum-free medium with pulses of 270 V, 960 microfarads (Jurkat) and 450 V, 500 microfarads (CFTL15). Twenty-four hours after transfection, cells were stimulated with 2 μM ionomycin and 20 μM PMA. Eight hours after stimulation, cells were harvested, and cell extracts were used for luciferase and Renilla activity using an automated luminometer (Berthold) and a dual-luciferase reporter assay system (Promega) following the manufacturer’s instructions. Luciferase data were normalized for renilla readings (without TK) and in some experiments for the total protein content in the lysate.
had no discernible effect (Fig. 1). This is in contrast to Th2 cells where both NFAT1 and NFAT2 contribute to expression of several cytokine genes (26, 41).

To evaluate independently whether NFAT1 and NFAT2 differed in their ability to support IL13 gene transcription, we introduced CA versions of NFAT1 and NFAT2 into bone marrow-derived mast cells. CA-NFAT1 and CA-NFAT2 bear multiple Ser/H11022Ala substitutions in phosphorylated serine residues of the regulatory domain (26, 28). We showed previously that these proteins are equivalent in their ability to support transcription of the endogenous IL4 gene in Th2 cells (26). However, as shown in Fig. 2, only CA-NFAT1 was capable of supporting IL13 expression in mast cells.

Freshly isolated bone marrow cells were infected, in two independent experiments, with internal ribosomal entry site (IRES)-GFP retroviruses encoding CA-NFAT1 and CA-NFAT2. The cells were then cultured with IL-3 and SCF to promote mast cell differentiation and sorted a week later for GFP-positive cells expressing the CA-NFAT proteins. After 1 month of culture under differentiating conditions, the cells showed the morphological features of mast cells (data not shown). Immunoblotting with an antibody to the HA epitope tag showed that expression levels of the CA-NFAT proteins paralleled the levels of GFP expression by FACS (Fig. 2, A and B). Experiment 1 shows high CA-NFAT1 but low CA-NFAT2 expression, while Experiment 2 shows low CA-NFAT1 and moderate CA-NFAT2 expression (Fig. 2, A and top panel of Fig. 2B). However, in no case were the constitutively active proteins overexpressed relative to endogenous NFAT; immunoblotting with specific anti-NFAT1 and anti-NFAT2 antibodies (anti-67.1 and 7A6, respectively) showed no increase in band intensity at the expected positions of the CA-NFAT proteins (asterisks), indicating that the CA-NFATs constituted only a very minor fraction of total NFAT (Fig. 2B, bottom panels).

To evaluate cytokine production driven by the CA-NFATs, the infected populations were stimulated in the presence of the
calcineurin inhibitor CsA. This strategy permits selective assessment of the transcriptional function of the CA-NFATs without interference from endogenous NFATs. Cells were left unstimulated or were stimulated with ionomycin and CsA for 24 h, and culture supernatants were tested for IL-13 levels by ELISA (Fig. 2C). Under these conditions, cells expressing the high level of CA-NFAT1 produced IL-13 even under resting conditions (Fig. 2C, Experiment 1); stimulation with ionomycin and CsA resulted in increased IL-13 production, presumably because IL-13 expression is potentiated by parallel activation of other (calcineurin-independent) calcium signaling pathways. Note that for these experiments, we stimulated cells with ionomycin alone rather than with PMA/ionomycin; although this results in an overall lower level of IL-13 expression, it eliminates a substantial background of PMA-stimulated, NFAT-independent IL-13 production (see Fig. 1B) that obscures the effect of the CA-NFATs. In Experiment 2, a low level of CA-NFAT1 was able to up-regulate IL-13 production in ionomycin/CsA-stimulated mast cells, while a higher level of CA-NFAT2 was not (Fig. 2C, Experiment 2). Consistent with the very low expression of introduced CA-NFAT1 relative to endogenous NFAT1 (Fig. 2B), the amount of IL-13 produced by CA-NFAT1 expressing cells is quite low in both experiments. Nevertheless, the level of IL-13 expression correlated very well with the level of CA-NFAT1 expression (compare Fig. 2B, top panel with Fig. 2C). Taken together, these experiments reinforce the hypothesis that NFAT1 can activate IL-13 production in mast cells but NFAT2 cannot.

NFAT2 Is Correctly Regulated in Mast Cells—One potential reason for the apparent inability of NFAT2 to activate IL13 expression in mast cells could be that mast cells do not properly regulate NFAT2. To test this possibility, we examined the behavior of NFAT2 in wild-type mast cells by Western blotting and immunocytochemistry (Fig. 3). Stimulated mast cells showed normal dephosphorylation and nuclear translocation of NFAT2 (Fig. 3, A and B), as well as normal induction of the short inducible isoform of NFAT2 as previously observed in Th2 cells (Fig. 3C (42, 43)). Each of these processes was blocked by the calcineurin inhibitor CsA (Fig. 3 and data not shown) as expected, since calcineurin is required for NFAT dephosphorylation, nuclear translocation, and transcriptional activity (11), and since NFAT autoactivates the distal (inducible) promoter of NFAT2 (42, 43). Together these data suggest that the inability of NFAT2 to up-regulate IL-13 expression results from a failure of DNA binding, transcriptional activity, or both.

NFAT1, but Not NFAT2, Binds the IL13 Promoter in Mast Cells—The results presented above could reflect selective binding of NFAT1 to IL13 regulatory regions; alternatively, NFAT1

**Fig. 2. CA-NFAT1, but not CA-NFAT2, induces IL-13 expression in BMMC.** Bone marrow cells were isolated from the femur and tibia of a BALB/c mouse. After 1 day in culture, cells were retrovirally transduced with GFP-RV, HA-tagged CA-NFAT1-RV or HA-tagged CA-NFAT2-RV. Two independent experiments are shown, in which infection efficiency with the three different retroviruses ranged between 7.5 and 39%. After 1–2 weeks in culture, GFP+ cells were sorted and kept in culture for a subsequent 2 weeks in 50% WEHI-3 conditioned medium to allow mast cell differentiation. Note that in Experiment 1, expression of CA-NFAT1 is much higher than that of CA-NFAT2. In Experiment 2, CA-NFAT1 expression is lower than that of NFAT2 but expression levels are more closely approximated. A, GFP expression of the sorted populations after 4 weeks in culture. B, Western blot of 4-week-infected BMMC, using anti-HA, anti-NFAT1, or anti-NFAT2 antibodies. C, 4-week-infected BMMC were either left unstimulated or were induced with ionomycin for 24 h after 30-min pretreatment with CsA. IL-13 protein levels in culture supernatants were assessed by ELISA.
and NFAT2 might bind the IL13 promoter equivalently but differ in their ability to activate transcription at this site. To differentiate between these possibilities, we evaluated binding of endogenous NFAT1 and NFAT2 to the IL13 promoter in T cells and mast cells by ChIP assays (Fig. 4). As expected, both NFAT1 and NFAT2 bound the IL13 promoter in T cells (Fig. 4A), but surprisingly, only NFAT1 bound substantially to the IL13 promoter in mast cells (Fig. 4B and C). As shown in Fig. 4A, this is not due to lower expression of NFAT2, since NFAT2 levels are actually slightly elevated in mast cells compared with Th2 cells.

We compared the ability of NFAT1 and NFAT2 to transactivate the IL13 promoter in mast cells. The CPTL15 mast cell line (38) was used for these experiments because of the poor transfectability of primary BMMC and ESMC. Expression of NFAT1 or GATA1 alone in CPTL15 cells did not result in induction of luciferase activity driven by the IL13 promoter, while combined expression of NFAT1 and GATA1 led to strong synergistic activation (Fig. 6, A and B). Strong synergy was also

**Fig. 3.** NFAT2 is regulated normally in mast cells. A, all NFAT2 isoforms undergo normal dephosphorylation in stimulated mast cells. Wild-type and NFAT2−/− ESMC were either left unstimulated or stimulated for the indicated amounts of time with PMA and ionomycin. Whole cell extracts were analyzed by immunoblotting with an anti-NFAT2 antibody (mAb 7A6), or acetylated histone 4 (AcH4, Upstate Biotechnology), and the presence of NFAT2 (7A6), or acetylated histone 4 (AcH4, Upstate Biotechnology), and the presence of NFAT2 to the IL13 promoter in T cells but not mast cells suggested that its binding might be stabilized in T cells by a nuclear partner protein not expressed in mast cells. Members of the GATA family of transcription factors were obvious candidates, since (as shown in Fig. 5A) T cells and mast cells express nonoverlapping sets of GATA proteins, and since NFAT and GATA proteins synergize to activate the IL13 promoter in mast cells (see below and Fig. 6A). Western blotting of cell lysates showed that T cells express GATA1 and GATA2 but not GATA3 (Fig. 5, B–D). HEK293 cells were transfected with expression vectors for GATA proteins and HA-tagged NFAT proteins in different combinations. Immunoprecipitation was performed with either anti-HA or anti-GATA antibodies, and immune complexes were analyzed for the presence of interacting proteins. Although the extent of co-immunoprecipitation varied slightly in different experiments, there was no systematic difference between the ability of NFAT1 and NFAT2 to interact with any of the three GATA proteins (Fig. 5, B–D); in particular, NFAT2 interacted effectively with both GATA1 and GATA2, the GATA proteins expressed in mast cells (Fig. 5, B and C).

**Fig. 4.** NFAT2 binds the IL13 promoter (prom) in Th2 cells but not in mast cells. A, ChIP assay monitoring binding of NFAT1 and NFAT2 to the IL13 promoter in primary Th2 cells. Cells were left unstimulated (−) or stimulated for 3 h with PMA/ionomycin (+). Chromatin was immunoprecipitated with antibodies against NFAT1 (67.1), NFAT2 (7A6), or acetylated histone 4 (Ach4, Upstate Biotechnology), and the presence of IL13 promoter sequences was assessed by PCR followed by ethidium bromide staining. Both NFAT1 and NFAT2 bind to the IL13 promoter in Th2 cells. B, ChIP assay showing that only NFAT1 binds the IL13 promoter in ES-derived mast cells. C, ChIP assay showing that only NFAT1 binds the IL13 promoter in BMMC.

**Impaired Cooperation of NFAT2 with GATA Proteins in Mast Cells**—Based on the conservation of DNA contact residues, all four NFAT proteins are expected to bind identical DNA elements (44). Thus our finding that NFAT2 bound to the IL13 promoter in T cells but not mast cells suggested that its binding might be stabilized in T cells by a nuclear partner protein not expressed in mast cells. Members of the GATA family of transcription factors were obvious candidates, since (as shown in Fig. 5A) T cells and mast cells express nonoverlapping sets of GATA proteins, and since NFAT and GATA proteins synergize to activate the IL13 promoter in mast cells (see below and Fig. 6A). Western blotting of cell lysates showed that T cells express GATA1 and GATA2 but not GATA3 (Fig. 5, B–D). HEK293 cells were transfected with expression vectors for GATA proteins and HA-tagged NFAT proteins in different combinations. Immunoprecipitation was performed with either anti-HA or anti-GATA antibodies, and immune complexes were analyzed for the presence of interacting proteins. Although the extent of co-immunoprecipitation varied slightly in different experiments, there was no systematic difference between the ability of NFAT1 and NFAT2 to interact with any of the three GATA proteins (Fig. 5, B–D); in particular, NFAT2 interacted effectively with both GATA1 and GATA2, the GATA proteins expressed in mast cells (Fig. 5, B and C).
**Fig. 5. NFAT1 and NFAT2 interact with equivalently with GATA family proteins.**

A. Western blot (WB) for GATA proteins expressed in Th2 cells and mast cells. 10^6 D10 Th2 cells and ESMC were lysed in Laemmli sample buffer and analyzed by immunoblotting for expression of the GATA proteins GATA1, GATA2, and GATA3. Note that a nonspecific band of ~50 kDa (ns) also stains with the GATA1 antibody. Th2 cells express only GATA3, while mast cells express only GATA1 and GATA2. B, GATA1 co-immunoprecipitates with both NFAT1 and NFAT2. HEK293 cells were co-transfected with expression vectors for GATA1 and either HA-tagged NFAT1 or NFAT2. Top panel, whole cell lysates were immunoprecipitated (IP) with a monoclonal anti-HA antibody, and co-immunoprecipitation of GATA1 was assessed by Western blotting. Middle panel, equivalent representation of HA-NFAT1 and HA-NFAT2 in the immunoprecipitates (IP) was confirmed by immunoblotting with polyclonal anti-HA antibodies. Lower panel, expression of GATA1 in the transfected cells was assessed by immunoblotting cell lysates with anti-GATA1.

C. GATA2 coimmunoprecipitates with both NFAT1 and NFAT2. HEK293 cells were co-transfected with expression vectors for GATA2 and either HA-tagged NFAT1 or NFAT2. Top panel, whole cell lysates were immunoprecipitated (IP) with a monoclonal anti-HA antibody, and co-immunoprecipitation of GATA2 was assessed by Western blotting. Middle panel, equivalent representation of HA-NFAT1 and HA-NFAT2 in the immunoprecipitates (IP) was confirmed by immunoblotting with polyclonal anti-HA antibodies. Lower panel, expression of GATA2 in the transfected cells was assessed by immunoblotting cell lysates with anti-GATA2.

D. GATA3 coimmunoprecipitates with both NFAT1 and NFAT2. HEK293 cells were co-transfected with expression vectors for GATA3 and either HA-tagged NFAT1 or NFAT2. Top panel, whole cell lysates were immunoprecipitated (IP) with an antibody to GATA3, and co-immunoprecipitation of HA-NFAT1 and HA-NFAT2 was assessed by Western blotting with the monoclonal anti-HA antibody. Second panel, equivalent representation of GATA3 in the immunoprecipitates (IP) confirmed by immunoblotting with anti-GATA3. Third panel, whole cell lysates were immunoprecipitated (IP) with a monoclonal anti-HA antibody, and co-immunoprecipitation of GATA3 was assessed by Western blotting. GATA3 (arrow) coimmunoprecipitates with both HA-NFAT1 and HA-NFAT2. A nonspecific band (ns), migrating close to the position of GATA3 in all three lanes, is indicated. Lower panel, expression of GATA3 in the transfections was assessed by immunoblotting cell lysates with anti-GATA3.
observed between NFAT1 and GATA2 (Fig. 6C). Surprisingly, however, NFAT2 was unable to synergize effectively with either GATA1 or GATA2 to activate the IL13 promoter in CFTL15 cells (Fig. 6, B and C). Western blotting of transfected cell lysates with an anti-HA antibody showed that the expressed HA-tagged NFAT1 and NFAT2 proteins were expressed at equivalent levels in the transfected cells (data not shown). As a control, we showed that in Jurkat T cells, the NFAT2-GATA combination was only slightly less effective than the NFAT1-GATA combination (Fig. 6, D and E). In T cells, NFAT1 and NFAT2 are similar in their ability to synergize with GATA proteins at the IL13 promoter.

DISCUSSION

Different members of a transcription factor family often have overlapping functions. This functional redundancy is important in evolution to guard against genetic accidents such as loss or mutation of essential genes. While functional differences have been observed in gene disruption experiments, they are often due to differential expression rather than intrinsically different function. This point has been illustrated for several proteins whose genomic disruption results in embryonic lethality, by showing that introduction of the gene encoding a different member of the same protein family into the chromosomal location occupied by the first has few (if any) deleterious effects (45–49).

We have previously shown that NFAT1 and NFAT2, which are closely related members of a calcium-regulated transcription factor family, have similar effects on regulation of a large number of genes including IL4 in T cells (26). In contrast in this study, we show a marked difference between these two family members on IL13 gene expression in mast cells. We show that both NFAT1 and NFAT2 can drive transcription of the IL13 gene in Th2 cells; but surprisingly, only NFAT1 can drive transcription of the same gene in mast cells. The data constitute an unusual example of differential and cell type-specific gene regulation by different NFAT proteins.

Our evidence for differential regulation is as follows. Loss of NFAT2 in mast cells does not affect IL13 gene expression; similarly retroviral expression of constitutively active NFAT2 in mast cells does not result in IL13 gene expression. In contrast loss of NFAT1 has a major effect; moreover, retroviral expression of CA-NFAT1 increases IL-13 expression, even under conditions where the constitutively active NFAT1 protein is expressed at very low levels in only a minor proportion of cells.

Fig. 6. NFAT1 is a stronger activator of IL13 transcription than NFAT2 in mast cells. 10⁵ Jurkat T cells and CFTL15 mast cells were transiently transfected with an IL13 promoter-luciferase reporter plasmid and the indicated expression vectors encoding NFAT and GATA proteins. Twenty-four hours after transfection, cells were stimulated for 8 h with ionomycin (iono) or PMA/ionomycin as indicated before lysis and reporter assay. Reporter assays were repeated three times for CFTL15 and four times for Jurkat cells. Note that T cells and mast cells require different stimuli for maximal activation of the IL13 promoter. A, CFTL15 cells were transfected with GATA1 and NFAT1 expression vectors, alone or in combination. Note the strong functional synergy between NFAT1 and GATA1. B and C, CFTL15 cells were transfected with the indicated combinations of NFAT and GATA expression vectors. In this mast cell line, NFAT1 synergizes effectively with both GATA1 (B) and GATA2 (C) to activate IL13 promoter expression, whereas NFAT2 is much less effective. D and E, Jurkat cells were transfected with the indicated combinations of NFAT and GATA expression vectors. In T cells, NFAT1 and NFAT2 are similar in their ability to synergize with GATA proteins at the IL13 promoter.
The differences between the two proteins is not due to differential regulation. All isoforms of NFAT2 are expressed in mast cells and dephosphorylated normally upon stimulation; NFAT2 translocates normally to nucleus upon stimulation; and the small isofrom of NFAT2 is up-regulated normally upon stimulation. Nevertheless NFAT2 does not bind to the IL13 promoter in stimulated mast cells, although it does bind in stimulated Th2 cells.

In investigating the mechanism of differential IL13 gene regulation by NFAT1 and NFAT2 in mast cells, we found that NFAT2 was far less effective than NFAT1 in synergizing with the GATA family members present in mast cells to increase IL13 reporter activity. This was despite the fact that overexpression assays in a heterologous cell type (HEK293 cells) showed no obvious difference between NFAT1 and NFAT2 in their ability to interact with GATA proteins. One interpretation is that mast cells lack a co-activator function which stabilizes NFAT2 DNA binding to the IL13 promoter and promotes transcriptional synergy of NFAT with GATA proteins on this promoter.

Like NFAT family members, GATA family members have both redundant and nonredundant activities in vivo and in vitro. The GATA1 germ line mutation in mice results in embryonic lethality due to defective erythroid cell maturation (15); but Takahashi et al. (50) showed that transgenic expression of GATA2 and GATA3 under the transcriptional control of the GATA1 gene could rescue the embryonic lethal phenotype of the GATA1 gene-disrupted mice. Thus the related GATA proteins GATA1 and GATA2 can compensate for several functions of GATA1. However, only GATA1 rescued anemia in adult GATA1-mutant mice, indicating a unique function for GATA1 in this context in vivo. GATA1 and GATA2 also have different functions in mast cells. GATA2 is specifically required for mast cell differentiation, since GATA2/–/– yolk sac progenitors generate only macrophages (22–24). In contrast, mast cell and megakaryocyte lineages can differentiate in the absence of GATA1 (51), suggesting that while GATA2 regulates differentiation, GATA1 is more involved in regulating the mature phenotype of these cells. GATA1, but not GATA2, activates the granule major basic protein promoter in transient transfection assays in Jurkat T cells (52). Finally, GATA1 and GATA2 have been shown to have opposing effects on GATA2 gene expression. GATA2 autoregulates its own expression, but analysis of GATA2 transcriptional regulatory regions showed that GATA1 represses GATA2 transcription through disruption of positive autoregulation and via broad chromatin modifications (53). In this case, GATA1 and GATA2 had opposite functions on the GATA2 promoter, with GATA1 rapidly displacing GATA2 from an autoregulatory region controlled by GATA2, inhibiting cAMP-responsive element-binding protein (CREB)-binding protein recruitment by GATA2, inducing widespread changes in histone acetylation, and ultimately repressing GATA2 gene transcription.

Cooperation of NFAT with GATA family members has been observed in many systems. NFAT:GATA cooperation has been established by synergistic activation of reporter plasmids (35, 54–57), as well as by direct binding of NFAT2 to a DNA-binding “bait” fragment of GATA-4 in a yeast two-hybrid assay (56). There is evidence for preferential interactions involving the endogenous proteins. In co-immunoprecipitation experiments using a skeletal muscle cell line, GATA2 bound only one specific, fully dephosphorylated NFAT2 isoform but no other NFAT members (58). Given our finding of equivalent interactions between GATA2 and either NFAT1 or NFAT2 in HEK293 cells, the selectivity observed in the muscle cell line is most likely attributable to muscle-specific factors. A survey of regulatory regions at which NFAT-GATA cooperation had been reported showed that the spacing between NFAT and GATA binding sites in regulatory regions is quite variable (11), most likely because the zinc fingers of GATA proteins interact with the C-terminal domain of the NFAT Rel homology region (56). This C-terminal domain is attached through a flexible linker to the N-terminal domain of the Rel homology region, which makes base-specific interactions with a core OGA sequence in DNA (59). As a result, the N- and C-terminal domains of NFAT can adopt a large array of possible orientations on DNA (59), potentially permitting the C-terminal domain of the NFAT Rel homology region to interact with GATA proteins bound to a distant DNA element and explaining the observed wide variation in spacing of NFAT and GATA sites.

Our data suggest that mast cells lack a mechanism for stabilizing the binding of NFAT2 to the IL13 promoter, while permitting stable binding of a different NFAT protein, NFAT1, to the same promoter. Since NFAT1 and NFAT2 show complete conservation of DNA contact residues (44), they are likely to occupy identical binding elements in the IL13 promoter. Moreover, given our co-immunoprecipitation data and the degree of sequence conservation in the NFAT and GATA families, interactions between NFAT and GATA proteins are likely to involve similar contact surfaces in every case. Thus the simplest interpretation of our results is that mast cells contain a co-activator protein that preferentially recognizes an NFAT1-GATA combination on DNA, thus stabilizing binding of NFAT1 but not NFAT2 to the IL13 promoter. This would happen if the co-activator recognized regions, such as the transactivation domains, that show significant sequence variability within the NFAT family (11). In this context, the transactivation domain of one isofrom of NFAT2 has been suggested to be important for reporter activity driven by the IL4 promoter in mast cells (60). The mast cell system described here is ideal for identifying co-activator proteins that regulate the transcriptional interactions between NFAT and GATA proteins on the IL13 promoter.

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Silvia Monticelli, Deborah C. Solymar and Anjana Rao

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