Successive Expression and Activation of NFAT Family Members during Thymocyte Differentiation*

Received for publication, September 20, 1999, and in revised form, January 20, 2000

Satoko Adachi, Yoshiharu Amasaki, Shoihiro Miyatake, Naoko Arai, and Makoto Iwata

From the Integrative Projects, Mitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo 194-8511, Japan, Department of Molecular and Developmental Biology, Institute of Medical Sciences, The University of Tokyo, CREST, Minato-ku, Tokyo 108-0071, Japan, and Department of Immunology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California, 94304-1104

DIFFERENTIATION OF IMMATURE CD4+CD8+ THYMOCYTES TO MATURE CD4+ OR CD8+ T CELLS IS INDUCED BY POSITIVE SELECTION AND APPEARS TO INVOLVE CALCINEURIN-DEPENDENT ACTIVATION OF NFAT, A FAMILY OF TRANSCRIPTION FACTORS. NFATx IS PREDOMINANTLY EXPRESSED IN CD4+CD8+ THYMOCYTES, WHEREAS NFATp AND NFATc ARE EXPRESSED AT MUCH LOWER LEVELS IN THE THYMUS THAN IN MATURE T CELLS. HOWEVER, HOW OR WHEN EACH NFAT MEMBER IS INVOLVED IN THE DIFFERENTIATION PATHWAY IS UNCLEAR. USING AN IN VITRO MODEL SYSTEM WHERE ISOLATED CD4+CD8+ THYMOCYTES CAN SURVIVE AND DIFFERENTIATE INTO SEMI-MATURE CD4-LINEAGE T CELLS, WE SUGGEST THAT LOW CALCINEURIN ACTIVITY SUSTAINED FOR APPROXIMATELY 20 HOURS IS REQUIRED FOR CELL SURVIVAL AND DIFFERENTIATION. ACCORDINGLY, THE DNA BINDING ACTIVITY OF NFAT SLOWLY INCREASED DURING THE STIMULATION OF 20 HOURS TO INDUCE THE DIFFERENTIATION. NFATX SIGNIFICANTLY CONTRIBUTED TO THE EARLY RISE, BUT THE LATER INCREASE WAS MOSTLY DUE TO NFATAC ACTIVATION. MEANWHILE, THE EXPRESSION OF NFATX mRNA DECREASED AND THAT OF NFATAC mRNA INCREASED. THE DNA-BINDING ACTIVITY OF NFATP WAS DETECTABLE BUT LOW THROUGHOUT THE STIMULATION. NFATP BECAME DOMINANTLY ACTIVE AFTER THE SEMI-MATURE T CELLS DIFFERENTIATED INTO MATURE AND ACTIVATED CD4 T CELLS. THESE FINDINGS SUGGEST THAT NFATX AND NFATAC SUCCESSIVELY PLAY ROLES IN T CELL DEVELOPMENT.

Clonal selection of immature T cells occurs at the CD4+CD8+ stage in the thymus. Useful clones are rescued from apoptosis and differentiate into mature CD4+CD8- or CD4-CD8+ single-positive (SP) cells by positive selection (1). Positive selection is considered to be based on weak interactions between T cell receptors (TCR) and major histocompatibility complex-encoded molecules and to require proper levels of signals through TCR/CD3 and accessory molecules such as LFA-1, CD4, and CD8 (2–4). Signaling molecules including ZAP-70, Vav, p21ras, c-Raf, classical Ca2+-dependent protein kinase C, and calcineurin (CN) are suggested to be involved in this event (5–11). CN activation is dependent on Ca2+ mobilization. The active CN dephosphorylates the transcription factor NFAT that resides in the cytoplasm and accelerates its translocation to the nucleus. Among the four members of the NFAT family, NFATp (NFAT1 or NFATc2), NFATc (NFAT2 or NFATc1), and NFATx (NFAT4 or NFATc3) are expressed in lymphoid organs, whereas NFAT3 (NFATc4) is expressed in nonlymphoid organs (12–15). NFATp is constitutively expressed in mature T cells. NFATc is expressed in thymus and peripheral lymphoid organs at low levels, and its transcripts are markedly increased in mature T cells upon activation through the TCR complex (13). NFATx is predominantly expressed in CD4+CD8+ thymocytes (16). In NFATx-deficient mice, the number of SP thymocytes is about half that of normal mice, and their CD4+CD8+ thymocytes are more sensitive to glucocorticoid-induced apoptosis than normal CD4+CD8+ thymocytes (16). NFATc-deficient mice have cardiac valvular defects and are embryonic-lethal. Thus, by using RAG-deficient blastocyst complementation, it was shown that young chimeric mice lacking NFATc have reduced numbers of thymocytes (17, 18). On the other hand, NFATp deficiency does not cause any significant alteration in thymocyte differentiation (19, 20). Thus, positive selection may involve NFATx and NFATc activation and induce succession of NFAT family members. It is not known, however, when or how the activation and succession of NFAT family members occurs during positive selection. To resolve this issue, it is necessary to perform quantitative and kinetic analysis of positive selection in the absence of other cell types such as thymic epithelial cells.

We have established an in vitro model system for positive selection in which isolated CD4+CD8+ thymocytes survive and differentiate into semi-mature T cells committed to the CD4- or CD8-T cell lineage by using defined combinations of the calcium ionophore ionomycin (IM) and the protein kinase C activator PMA (21–23). The combinations of drugs were originally chosen to mimic the anti-apoptotic effect of proper cross-linking of TCR/CD3 and CD4, CD8, or LFA-1 on thymocytes (10, 24, 25). Combinations of IM and PMA are known to mimic antigenic stimulation in mature T cells. However, the concentration ranges required for thymocyte survival and differentiation are narrower and lower than those that induce proliferation of mature T cells (26), indicating that the stimulation intensities for differentiation have to be relatively low and within a narrow range.

The duration as well as intensity of stimulation are crucial for differentiation (21–23). In the present study, we found that FK506 affected thymocyte differentiation even when it was added to the culture at the late stage of the stimulation. Thus, sustained activities of CN and NFAT are likely to be required

* This work was supported in part by grants from the Ministry of Education, Sports, Science, and Culture of Japan, the Ministry of Public Welfare of Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Integrative Projects, Mitsubishi Kasei Institute of Life Sciences, 11 Minamimioya, Machida-shi, Tokyo 194-8511, Japan. Tel.: 81-427-24-6397; Fax: 81-427-24-6316; E-mail: iwata@libra.ls.m-kagaku.co.jp.
2 The abbreviations used are: SP, single-positive; [Ca2+]i, intracellular calcium level; CN, calcineurin; EMSA, electrophoretic mobility shift assay; IM, ionomycin; TCR, T cell receptor; PMA, phorbol 12-myristate 13-acetate; Ab, antibody; mAb, monoclonal Ab; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; IL, interleukin.
for the differentiation. From the semi-mature T cells that are committed to the CD4-T cell lineage in vitro, mature and activated CD4 SP T cells are induced by secondary-pulse stimulation with a combination of IM and PMA followed by incubation with IL-2 (22). By using the in vitro system, we analyzed kinetic changes in the expression and activation of NFAT family members during the differentiation of isolated CD4+CD8+ thymocytes into mature CD4 T cells. Although it has been difficult to induce and detect the NFAT DNA binding activity in murine CD4+CD8+ thymocytes, we could detect the activities and observe the succession of expression and DNA binding activities of NFAT family members in these cells. These changes in NFAT members were induced by a limited number of defined stimuli without thymic microenvironments or other cell types, suggesting that the changes may be intrinsically programmed along with T cell development.

**Experimental Procedures**

**Mice**—BOG8 TCRαβ transgenic mice were generated as described previously (21). The TCRαβ is specific for an ovalbumin-derived peptide (P271-285) and is restricted to I-Ak. The mice of RAG-2(-/-) background were bred with BALB/c (Becton Dickinson, Lincoln Park, NJ), and were analyzed for co-receptor expression. The gate for viable cells was set by incubating with 7-AAD (Becton Dickinson, Lincoln Park, NJ) and FACScan Research Software (Becton Dickinson, Lincoln Park, NJ). Viable cells were enumerated by trypan blue dye exclusion. The recovery (%) of viable cells at the end of culture and that at the start of culture, were enumerated by trypan blue dye exclusion. The recovery (%) of viable cell number at the end of culture and that at the start of culture, was estimated by BCA protein assay kit (Pierce). To analyze AP-1 DNA binding activity, nuclear extracts were prepared by the method described by Schreiber et al. (30).

**Anti-NFAT Abs**—A StuUball 426-bp fragment of mouse NFATx cDNA (corresponding to amino acid residues 331-462 of the mouse NFATx protein (31)) was subcloned into pGEX-SX-3 vector (Amersham Pharmacia Biotech), and truncated mouse NFATx protein fused to glutathione S-transferase was bacterially expressed. The resulting recombinant protein was purified and used for immunizing rabbits to raise an anti-mouse NFATx anti-serum (termed DN97). The anti-murine NFATx mAb and the anti-murine NFATp antiserum were purchased from Affinity Bioreagents (Golden, CO) and Upstate Biotechnology (Ann Arbor, MI), respectively.

**Plasmid Construction and Expression of NFAT Proteins**—pME-mNFATx1 and pME-mNFATc are expression plasmids containing mouse NFATx and NFATc full-length cDNA under the control of the Srα promoter in the pME18S mammalian expression vector (31, 32). pME-mNFATC1 expression plasmid was generated by subcloning an EcoRI/HindIII fragment from pHPEF-mNFATC1 (33) (a generous gift from Dr. A. Rao) encoding full-length mouse NFATC1 cDNA into the pME18S vector cleaved by EcoRI/SpeI. Transfection of plasmids into COS-7 cells by the DEAE-dextran method and the subsequent preparation of cytosolic extracts were carried out as described previously (29, 34).

**EMSA—NFAT-DNA binding reactions were performed by incubating 5 x 10^5 of nuclear extracts with 0.05 pmol of a radiolabeled double-stranded oligonucleotide probe for 20 min at room temperature in 15 μl of the binding buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM MgCl2, and 0.1 mM EDTA). The double-stranded oligonucleotide probe for 20 min at room temperature was pelleted by high speed centrifugation at 220,000 g for 15 min. The nuclear proteins in the pellet were dissolved in 20 μl of buffer C by vortexing for 30 min at 4 °C and stored at −80 °C until use. All buffers were supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin. Protein DNA binding activity was estimated by BCA protein assay kit (Pierce). To analyze AP-1 DNA binding activity, nuclear extracts were prepared by the method described by Schreiber et al. (30).

**Flow Cytometric Analysis and Sorting**—For two-color flow cytometric analysis of CD4 and CD8 expression, cells were stained with labeled Abs; phycoerythrin-conjugated anti-CD4 mAb (RM4–5) and fluorescein isothiocyanate-labeled mAb to CD8 (53–6.7) (Pharmingen). Viable cells were gated by forward and side scattering with a FACScan flow cytometer and FACScan Research Software (Becton Dickinson, Lincoln Park, NJ), and were analyzed for co-receptor expression. The gate for viable cells was determined by using propidium iodide exclusion and Pattern-A-Gate software (Becton Dickinson). For sorting of CD69+ and CD69 subsets, thymocytes from BALB/c mice were stained with fluorescein isothiocyanate-labeled mAb to CD69 (H1.2F3) (Pharmingen). Viable cells were gated by forward by side scattering and were sorted into CD69+ and CD69− subpopulations with FACStar plus and Consort 30 software programs (Becton Dickinson).

**Plasmid Construction and Expression of NFAT Proteins**—To analyze NFAT DNA binding activity, nuclear extracts were prepared from thymocytes as described previously (29), with modifications. Briefly, 1 to 2 x 10^6 cells were washed with cold phosphate-buffered saline and resuspended in buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM MgCl2, and 0.1 mM EDTA). The suspension was mixed with an equal volume of buffer B (buffer A plus 0.1% Nonidet P-40), and the resulting nuclei were pelleted by brief, low speed centrifugation. The nuclear pellet was washed with buffer A, resuspended in 200 μl of buffer C (50 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, and 10% glycerol) containing 0.3 mM ammonium sulfate for 10 min at 4 °C and vortexed vigorously for 30 min at 4 °C. The nuclear debris was pelleted by high speed centrifugation at 220,000 x g for 45 min. The nuclear proteins in the supernatant were precipitated by the addition of an equal volume of 3.0 M ammonium sulfate (pH 7.9). The suspension was incubated on ice for 2 to 3 h and centrifuged at 110,000 x g for 15 min. The nuclear proteins in the pellet were dissolved in 20 μl of buffer C by vortexing for 30 min at 4 °C and stored at −80 °C until use. All buffers were supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin.
of cells that had been stimulated for 2 h with IM/PMA (Fig. 3A).

The addition of FK506, but not rapamycin, at the beginning of the culture inhibited differentiation of the cells and induced apoptosis (Fig. 2 and data not shown). FK506 is an immunosuppressant that inhibits CN activation through binding to FKBP12, whereas rapamycin is another immunosuppressant that binds to FKBP12 without affecting CN activity (36).

The results suggest that the incubation period as well as the drug concentrations were crucial for the induction of CD4⁺CD8⁻ thymocyte differentiation.

The addition of FK506, but not rapamycin, at the beginning of the culture inhibited differentiation of the cells and induced apoptosis (Fig. 2 and data not shown). FK506 is an immunosuppressant that inhibits CN activation through binding to FKBP12, whereas rapamycin is another immunosuppressant that binds to FKBP12 without affecting CN activity (36). FK506 was moderately effective when it was added 15 h after the start of culture with IM/PMA (Fig. 2, D and G) but did not affect differentiation or survival when it was added after the stimulation period (data not shown). These findings suggest that sustained CN activity is required for differentiation.

**Kinetic Changes in the DNA Binding Activities of NFAT Family Members in T Cells during Differentiation of CD4⁺CD8⁻ Thymocytes to CD4 SP T Cells in Vitro**—Since it is known that the activation of CN in T cells is precisely followed by the activation of its major target NFAT, we analyzed NFAT activation during thymocyte differentiation induced by the transient stimulation with IM and PMA in vitro. DNA binding activity of NFAT was detected by EMSA in the nuclear extract of cells that had been stimulated for 2 h with IM/PMA (Fig. 3A). The NFAT DNA binding activity was increased thereafter and
reached a maximum within 15 h of stimulation. Multiple bands were detected during the stimulation, but most of them became undetectable in the presence of an excess amount of cold DNA probe with an NFAT-binding site (Fig. 3B). Thus, there may be multiple forms of NFAT-DNA complexes. After removing IM/PMA from the culture, the NFAT-DNA complexes quickly disappeared (Fig. 3A).

To analyze the contribution of each NFAT family member, the super shift assay was performed with specific Abs. The Abs to NFATc and NFATp were commercially available, but the Ab to mouse NFATx was produced as described under "Experimental Procedures." Its specificity was confirmed by using COS-7 cells expressing mouse NFAT members (Fig. 4). The DNA binding activity of NFATx was detected in the nuclear extract of cells stimulated for 2 h with IM/PMA, but it decreased after 15 h of stimulation (Fig. 5, lanes 2 and 6). These supershift bands were specific for NFATx-DNA complexes, since neither control Abs with the nuclear extract nor the anti-mouse NFATx anti-serum without the nuclear extract induced a similar band (data not shown). However, the intensity of the main band of NFAT-DNA complexes without Abs was rather enhanced in the presence of the anti-mouse NFATx anti-serum in some experiments (Fig. 5, lanes 1 and 2). It was partly due to a nonspecific effect of the anti-serum, since the anti-serum alone induced a nonspecific band at almost the same position (data not shown). The addition of the anti-NFATp antiserum supershifted the NFATp-DNA complex (Fig. 5, lanes 4 and 8). The intensities of the supershift bands indicated that the DNA binding activity of NFATp was induced after 2 h of stimulation with IM/PMA and was almost unchanged after 15 h of stimulation. The anti-NFATc mAb also supershifted NFATc-DNA complex (Fig. 5, lanes 3 and 7), although the retardation of the mobility was less than in the case of the anti-NFATx and anti-NFATp antisera. This may be partly because the anti-NFATc mAb recognizes a single epitope on NFATc, and the predicted molecular size of NFATc is smaller than that of NFATx or NFATp (29). The intensities of the supershift bands indicated that the DNA binding activity of NFATc was induced after 2 h of stimulation with IM/PMA and was dramatically increased over 15 h of stimulation.

Co-operation of NFAT and AP-1 (Fos/Jun) or AP-1-related factors is thought to be required for the maximal NFAT tran-
Succession of NFAT Members during Thymocyte Differentiation

Kinetic Changes in the Expression of NFAT Family Genes in T Cells during the Differentiation of CD4\(^+\)CD8\(^-\) Thymocytes to CD4\(^+\)SP T Cells in Vitro—To examine whether the expression of NFAT genes alters along with the differentiation of thymocytes in vitro, we analyzed the mRNA levels of NFAT members during culturing. mRNA was obtained from aliquots of cells harvested before and after 2 or 15 h of stimulation with IM/PMA.

Kinetic Changes in the Expression of NFAT Family Genes in T Cells during the Differentiation of CD4\(^+\)CD8\(^+\) Thymocytes to CD4SP T Cells in Vitro—To examine whether the expression of NFAT genes alters along with the differentiation of thymocytes in vitro, we analyzed the mRNA levels of NFAT members during culturing. mRNA was obtained from aliquots of cells harvested before and after 2 or 15 h of stimulation with IM/PMA.
Succession of NFAT Members during Thymocyte Differentiation

Fig. 6. NFATp is dominantly responsible for the NFAT DNA binding activity in the mature and activated CD4 SP T cells differentiated from CD4~CD8~ thymocytes in vitro. The CD4-lineage committed cells were induced from CD4~CD8~ thymocytes as described in Fig. 3 and were restimulated for 17 h with 0.2 μg/ml IM and 3.0 ng/ml PMA. After removing the drugs, these cells were further cultured for 48 h with 50 units/ml of mouse rIL-2. DNA binding activity of NFAT was assessed by EMSA (lane 1) as described in Fig. 3. The NFAT-DNA complexes were characterized by super shift assay (lanes 2 to 4) with Abs to NFAT family members as described in Fig. 5. A similar result was obtained by another independent experiment.

PMA and from those harvested after maturation. The expression levels of NFATx, NFATc, and NFATp genes were assessed by semi-quantitative RT-PCR. As shown in Fig. 7, during the first stimulation, the expression of NFATx mRNA decreased quickly, whereas that of NFATc mRNA significantly increased. The expression of NFATp mRNA increased after 15 h of stimulation. After maturation of the cells, the mRNA expression of NFATc dramatically decreased corresponding to the removal of drugs. The mRNA levels of NFATp remained high at the final stage. Thus, succession of NFAT members indeed occurred during the differentiation and maturation of CD4~CD8~ thymocytes in vitro.

Physiological Changes in the Expression of NFAT Family Genes in Normal Thymocytes During Positive Selection—It is known that CD69 is transiently expressed in the cells undergoing positive selection or the cells that had been selected recently in the thymus (43, 44). Accordingly, during the differentiation of CD4~CD8~ thymocytes in vitro, CD69 expression gradually increased and reached the maximum level after 15 h of stimulation and gradually decreased after removing IM/PMA from the culture medium (Fig. 8A). To test if the succession of NFAT members observed in vitro is in accord with physiological changes in the expression of NFAT family genes, we analyzed expression levels of these genes in CD69~ and CD69~ subsets of normal thymocytes. In general, the majority of CD69~ cells are CD4~CD8~ thymocytes at the pre-selection stage (roughly about 90%); the rest of CD69~ cells are mainly at the post-selection stage (44). As shown in Fig. 8, C and D, the expression level of NFATx mRNA in CD69~ cells was lower than that in CD69~ cells. In contrast, the expression levels of NFATc and NFATp mRNA in CD69~ cells was higher than those in CD69~ cells. The results indicate that the changes in mRNA expression of NFAT members during normal thymic differentiation are paralleled by the changes in the in vitro culture system.

DISCUSSION

Several research groups including ours have previously shown that the immunosuppressant FK506 or cyclosporine A inhibits positive selection in fetal thymus organ cultures or reaggregated cultures (9–11). The major requirements for thymocyte-positive selection are to rescue CD4~CD8~ thymocytes from apoptosis and to induce differentiation of the cells to the CD4 or CD8 SP stage. FK506 inhibits the IM/PMA-induced differentiation of CD4~CD8~ thymocytes in suspension cultures (Fig. 2) and annuls the anti-apoptotic effect of IM/PMA (10, 25), suggesting that both the inhibition of apoptosis and the induction of differentiation are dependent on FK506-sensitive reactions. Rapamycin, another immunosuppressant that binds to FKBP12 without affecting CN activity, fails to inhibit differentiation (data not shown) or to annul the anti-apoptotic effect (10). The CD4~CD8~ lymphoma RLm6 becomes CD4 SP by incubation with the same combination of IM/PMA, and differentiation or transition of the cells is also inhibited by FK506 (38). Furthermore, RLm6 transfected with an expression vector encoding an active form of CN became CD4 SP upon stimulation with PMA alone (38). These findings collectively suggest that CN plays a pivotal role in Ca2+ signaling for positive selection.

IM induces a sustained increase in intracellular Ca2+ level ([Ca2+]i), thereby inducing CN activation. Prolonged elevation of [Ca2+]i, levels and CN activation in T cells are dependent on the capacitative calcium entry, a process that couples the release of calcium from intracellular stores with the influx of extracellular Ca2+ through specialized calcium channels (45, 46). Upon stimulation through the TCR/CD3 complex, immature thymocytes show a much lower increase in [Ca2+]i, compared with mature T cells (47, 48). However, co-stimulation through some of the accessory molecules has been shown to enhance and/or prolong the TCR/CD3-mediated increase in [Ca2+]i, in immature thymocytes (10, 49, 50) and to potentiate the calcineurin-dependent anti-apoptotic effect (10). Although IM directly releases Ca2+ from the intracellular stores and brings extracellular Ca2+ into the cells (51, 52), the dose and duration of IM treatment are crucial for the survival and differentiation of CD4~CD8~ thymocytes (Figs. 1 and 2) (21, 22), indicating that IM effectively mimics the calcium signal induced by TCR- and accessory molecule-mediated stimulation. Indeed, in accord with the IM/PMA-induced thymocyte differentiation, it has been reported that stimulation of isolated CD4~CD8~ thymocytes by cross-linking of TCR and some of the accessory molecules overnight, followed by culturing without stimulation, results in the differentiation and commitment of the cells to the CD4-T cell lineage (53).

To maintain the NFAT activated and localized in the nucleus, a sustained increase in [Ca2+]i, is required (54, 55). The activation and nuclear localization of NFAT precisely follows the activation of CN in T cells and can even last for many hours depending on increased levels of [Ca2+]i (56–58). It has been suggested that positive selection involves sustained interactions with the thymic microenvironment and that CN-mediated signaling is required at least until CD4~CD8~ cells become CD69~ (11). The active CN dephosphorylates NFATs that reside in the cytoplasm and induces their translocation to the nucleus. NFATx is preferentially expressed in CD4~CD8~ thymocytes, and mice lacking NFATx have impaired development of CD4 and CD8 SP thymocytes and peripheral T cells partly due to increased apoptosis of CD4~CD8~ thymocytes (16). Thus, NFATx appears to play an important role in the successful generation of T cells. However, it has been difficult to induce
Succession of NFAT Members during Thymocyte Differentiation

Fig. 7. mRNA expression levels of NFAT members changed during the differentiation of CD4⁺CD8⁻ thymocytes into mature CD4 SP T cells in vitro. Differentiation of CD4⁺CD8⁻ thymocytes to mature and activated CD4 SP T cells was induced as described in Fig. 6. An aliquot of the cells was recovered from the culture at the indicated time, and mRNA expression in the cells was assessed by semi-quantitative RT-PCR. A, the reverse transcribed cDNA from thymocytes stimulated with IM/PMA for 0, 2, and 15 h and that from mature and activated CD4 SP T cells were serially diluted, and each diluted sample was subjected to PCR amplification for NFATx, NFATc for 32 cycles, NFATp for 39 cycles, and β-actin for 25 cycles. The PCR products were resolved by electrophoresis on a 1.2% agarose gel containing ethidium bromide. B, the bands corresponded to the exponential amplification phase were analyzed, and the intensities of NFAT cDNA-derived bands were normalized with those of β-actin cDNA-derived bands. Relative expression levels of mRNA of each NFAT member were calculated. A representative result of three experiments is shown.

and detect NFAT DNA binding activity itself in nuclear extracts of murine CD4⁺CD8⁻ thymocytes (39, 40). By using human fetal thymocytes, NFATx DNA binding activity could be induced and detected in CD4⁺CD8⁻ thymocytes for the first time by stimulating the cells with A23187/PMA (29). The nuclear extraction method and/or the low ionic strength conditions for EMSA rather than the drug concentrations might be crucial in detecting the activity. In the present study, by employing a much weaker stimulation with IM/PMA and the same nuclear extraction method, we could also induce and detect the DNA binding activity of NFATx in the nucleus of murine CD4⁺CD8⁻ thymocytes (Fig. 5). Transient stimulation of isolated CD4⁺CD8⁻ thymocytes with this combination of drugs results in differentiation of the cells to semi-mature CD4 SP and CD4⁺CD8⁻ T cells that are committed to the CD4-T cell lineage (21, 22). DNA binding activities of NFATp and NFATc were also detected in the nucleus of stimulated murine CD4⁺CD8⁻ thymocytes (Fig. 5). In human fetal CD4⁺CD8⁻ thymocytes stimulated with 0.25 μM A23187 and 5 ng/ml PMA for 5 h, NFATx was almost the sole contributor to the NFAT DNA binding activity, although the DNA binding activities of NFATc and NFATp might be slightly induced (29). This small discrepancy may be partly due to the differences in stimulation conditions, cell preparation methods, or species. Especially, it is noteworthy that a marked increase in the DNA binding activity of NFATc depends on sustained stimulation (15 h) of the cells without a significant loss of viability (Figs. 3 and 5), whereas in the previous studies, the cells were stimulated for relatively short periods.

Both the mRNA expression and DNA binding activity of NFATc dramatically increased and remained high until the end of the first stimulation in our system (Figs. 5 and 7). On the other hand, the expression levels of NFATx mRNA decreased during the first stimulation period (Fig. 7), and the DNA binding activity of NFATx protein decreased after the early rise (Fig. 5). Since calcineurin activity appears to be required almost throughout the stimulation period to induce differentiation of the cells to the CD4 T-cell lineage (Fig. 2), both the early rise of NFATx activity and the late increase in NFATc activity may contribute to the differentiation of CD4⁺CD8⁻ thymocytes to the semi-mature stage. Indeed, in the normal thymus, the expression levels of NFATc mRNA in CD69⁺ cells were higher than those in CD69⁻ cells, whereas the expression levels of NFATx mRNA in CD69⁻ cells were lower than those in CD69⁺ cells (Fig. 8). CD69 is considered to be transiently expressed in the cells undergoing positive selection or the cells that had been selected recently in the thymus (43, 44). In chimeric mice lacking NFATc, reconstitution of thymus is quantitatively impaired (17, 18). Thus, the deficiency of NFATx and that of NFATc differentially affect T cell development, suggesting that NFATx and NFATc have distinct roles during T cell development. Since NFATx binds to the distal NFAT-binding site in the IL-2 promoter and a NFAT-binding site in the IL-4 promoter with weaker affinity than NFATc and NFATp (14, 59), the DNA binding specificity of NFATx may be somehow different from that of the other members. NFATx may mainly act through unspecified DNA sequences different from the known NFAT-binding sites (29). However, it is also possible that the two members can replace each other to some extent since the deficiency of either NFATx or NFATc alone does not absolutely prohibit developmental programs (16–18). There is no evidence that NFATp deficiency affects thymic development (19, 20). On the other hand, NFATc deficiency and NFATp/NFATx deficiency differentially affect the production of Th1/Th2 cytokines by peripheral T cells (17, 18, 60). The DNA binding activity of NFATp was detected in our system but was kept at low levels during the first stimulation period. However, after the secondary pulse stimulation with a combination of IM/PMA followed
by IL-2 treatment, NFATp became the major NFAT member (Fig. 6 and 7). The resultant cells were phenotypically and functionally mature and activated T cells as described previously (22). Thus, the expression and activation of NFATx, NFATc, and NFATp were sequentially changed during the T cell development and maturation in vivo. Since the cells were only exposed to a limited number of stimuli and not microenvironments in the thymus or peripheral lymphoid organs, the findings suggest that the changes in NFAT members may be intrinsically programmed and may reflect alterations in patterns of gene expression along with T cell development.

In the thymus it is likely that interactions between thymocytes and stromal cells induce not only an increase in [Ca\(^{2+}\)]\(_i\), but also other signals including those that activate protein kinase C and those that suppress the protein kinase C signaling in thymocytes through TCR and accessory molecules (61, 62). The net signals may determine the fate of each thymocyte. The combination of IM and PMA may bypass the complexity of TCR- and accessory molecule-proximal signals in CD4\(^+\)CD8\(^+\) thymocytes in the same way that combinations of higher concentrations of IM and PMA bypass the early consequence of TCR cross-linking in mature T cells but mimic its late consequence including gene expression (37). It is known that TCR cross-linking activates both p21\(^{ras}\) and protein kinase C and that protein kinase C activation with PMA directly induces p21\(^{ras}\) and Raf activation, followed by activation of the mitogen-activated protein kinase cascade (37). Recently, Kaye and co-workers (63) indicated that slow accumulation of active extracellular signal-regulated kinase is critical for IM/PA-induced differentiation.

By using a reporter gene with the promoter region of IL-2 gene, it has been shown that PMA enhances the transcriptional activity of NFAT depending on p21\(^{ras}\) activation and that the enhanced activity is likely to involve co-operation between NFAT and AP-1 (37). Since treatment of CD4\(^+\)CD8\(^+\) thymocytes with PMA or IM alone fails to induce positive selection (21), the expression of genes essential for positive selection may also depend on the co-operation of NFAT with other transcription factors such as AP-1 and NF-xB. The transcriptional regulation of positive selection may involve LKLF, Notch, IRF-1, and Egr (64), but is as of yet largely unknown. The in vitro differentiation system used in the present study may also provide a useful tool for finding the responsible genes.

Acknowledgments—We thank Dr. K.-I. Arai for kind support, Dr. A. Rao for pGLP3-mNFATIC, S. Kamijo and co-workers for their help in producing transgenic mice, Y. Shirota-Someya for her technical assistance, and A. Nakamura, M. Shiomi, and Y. Kishi for secretarial assistance.

REFERENCES

1. Egerton, M., Scollay, R., and Shortman, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2579–2582
2. Rasmussen, C., and Powlak, B. J. (1989) J. Immunol. 143, 1467–1471
3. Zuniga-Pflucker, J. C., MacCartney, S. A., Weston, M., Long, D. L., Singer, A., and Kruisbeek, A. M. (1989) J. Exp. Med. 169, 2085–2096
4. Fine, J. S., and Kruisbeek, A. M. (1991) J. Immunol. 147, 2825–2836
5. Nakash, T., Motomura, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chen, A. C., and Loh, D. Y. (1995) Nature 376, 435–438
6. Fischer, K.-D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A., and Guidos, C. (1995) Nature 374, 474–477
7. Swan, K. A., Alberola-Ila, J., Gross, J. A., Appleby, M. W., Forbush, K. A., Thomas, J. F., and Perlmutter, R. M. (1986) EMBO J. 14, 276–283
8. Alberola-Ila, J., Forbush, K. A., Seeger, R., Krebs, E. G., and Perlmutter, R. M. (1995) Nature 373, 620–623
9. Wang, C.-R., Hashimoto, K., Kubo, S., Yokochi, T., Kubo, M., Suzuki, M., Suzuki, K., Tada, T., and Nakayama, T. (1995) J. Exp. Med. 181, 927–941
10. Zhao, Y., and Iwata, M. (1995) Int. Immunol. 7, 1387–1396
11. Wilkinson, R. W., Anderson, G., Owen, J. J. T., and Jenkinson, E. J. (1995) J. Immunol. 155, 5284–5291
12. McCaffrey, P. G., Lu, C., Kerpola, T. K., Jain, S., Badalain, T. M., Ho, A. H., Burgeon, E. B., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A., and Hogan, P. G. (1993) Science 262, 759–764
13. Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmernann, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994) Nature 369, 497–502
14. Hoey, T., Sun, Y.-L., Williamson, K., and Xu, X. (1995) Immunity 2, 461–472
15. Maeda, E. S., Naito, Y., Tomokushti, H., Campbell, D., Saito, F., Manourm, C., Arii, K.-I., and Ari, Y. (1995) Mol. Cell. Biol. 15, 2697–2706
16. Okuka, M., Ho, I.-C., de la Brousse, F. C., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1998) Immunity 29, 2085–2096
17. Ranger, A. M., Hodge, M. P., Gravallese, E. M., Okuka, M., Davidson, L., Alt, F. W., de la Brousse, F. C., Hoey, T., Grusby, M., and Glimcher, L. H. (1998) Immunity 8, 125–134
18. Yoshida, H., Nishida, H., Takimoto, H., Marenge, L. E. M., Wakham, A. C., Bouchard, D., Kung, Y.-Y., Ohleki, T., Shahinian, A., Bachmann, M., Ohasagi, P. S., Penninger, J. M., Crabtree, G. R., and Mak, T. W. (1998) Nature 398, 115–124
19. Hodge, M. R., Ranger, A. M., Charles de la Brousse, F., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1996) Immunity 4, 397–405
20. Xanthoudakis, S., Vila, J. P., Show, K. T. Y., Lu, C., Wallace, J. D., Bozza, P. T., Curran, T., and Glimcher, L. H. (1996) EMBO J. 15, 889–895
21. Ohoka, Y., Kuwata, T., Tozawa, Y., Zhao, Y., Mukai, M., Motegi, Y., Suzuki, R., Yokoyama, M., and Iwata, M. (1996) Int. Immunol. 8, 297–306
22. Ohoka, Y., Kuwata, T., and Iwata, M. (1997) J. Immunol. 158, 5707–5716
23. Ohoka, Y., Hanooka, S., and Sato, K. (1991) J. Immunol. 146, 643–648
24. Zhao, Y., Tozawa, Y., Iseki, R., Mukai, M., and Iwata, M. (1995) J. Immunol. 154, 6546–6554
25. Iwata, M., Ohoka, Y., Kuwata, T., and Asada, A. (1996) Stem Cells 14, 632–641

FIG. 8. Thymocyte positive selection in vivo is also accompanied by a decrease in the expression of NFATX mRNA and increases in the expression of NFATc and NFATp mRNA. A, CD4\(^+\)CD8\(^+\) thymocytes were stimulated with IM/PMA for 20 h and further cultured for 24 h in the absence of drugs (44 h in total). An aliquot of the cells was recovered at the indicated time, and the surface expression of CD69 was assessed. B, thymocytes from normal BALB/c mice were stained with an anti-CD69 mAb and sorted to obtain CD69(+) and CD69(-) and CD69(-) aliquots of the cells were recovered at the indicated time, and the surface expression of CD69 was assessed. B, thymocytes from normal BALB/c mice were stained with an anti-CD69 mAb and sorted to obtain CD69(+) and CD69(-) subsets. C and D, expression levels of each NFAT member mRNA in CD69(+) and CD69(-) subsets were assessed by semi-quantitative RT-PCR. Relative expression levels of NFAT mRNA were calculated as described in Fig. 7. A representative result of three experiments is shown.
Successive Expression and Activation of NFAT Family Members during Thymocyte Differentiation
Satoko Adachi, Yoshiharu Amasaki, Shoichiro Miyatake, Naoko Arai and Makoto Iwata

J. Biol. Chem. 2000, 275:14708-14716.
doi: 10.1074/jbc.275.19.14708

Access the most updated version of this article at http://www.jbc.org/content/275/19/14708

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 23 of which can be accessed free at http://www.jbc.org/content/275/19/14708.full.html#ref-list-1