Nuclear Factors that Mediate Intrathymic Signals Are Developmentally Regulated

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Summary

Thymocytes mature through several stages of development, defined by cell surface markers such as CD3, CD4, and CD8, in response to environmental cues. Signal transduction resulting from lymphocyte–stromal cell interactions is likely to activate inducible transcription factors which in turn govern stage-specific gene expression. In this report we show that inducible transcription factors such as AP-1 and NF-AT are constitutively nuclear, in response to intrathymic signals, in freshly isolated thymocytes at all stages of maturation. In $CD4^+CD8^+$ double positive (DP), but not in the more immature $CD4^-CD8^-$ double negative (DN) thymocytes, constant stimulus from the thymic environment is required to maintain nuclear AP-1. Thus, disruption of the thymus and incubation of thymocytes at 37°C downregulates DNA binding by nuclear factors AP-1 and NF-AT. Similar treatment of thymocytes has previously been shown to downregulate CD3 ζ chain phosphorylation and increase T cell receptor CD3 expression on DP thymocytes, which is a feature of repertoire selection. Since mature T cells maintain inducible nuclear factors in an inactive form until an encounter with antigen, we propose that downregulation of nuclear DNA binding proteins may reflect another feature of this stage of T cell maturation.

T cell differentiation in the thymus proceeds via a series of discrete intermediates that have been characterized largely on the basis of expression of cell surface molecules such as CD4, CD8, and the TCR–CD3 complex (1–3). During T cell development the most immature thymocytes lack all of these markers and are referred to as double negative ([DN][1] $CD4^-CD8^-$) cells. T cell development is blocked at the DN stage in mice deficient in either recombination activation genes, RAG 1 or RAG 2, the TCR β chain (for a review see reference 4), or in mice carrying the SCID mutation (5). Mice deficient in the lymphocyte-specific protein tyrosine kinase $p56^{lck}$, or expressing a dominant negative mutant of $p56^{lck}$, also attenuate development at the DN stage, indicating a role for this enzyme at this stage of T cell development. DN thymocytes mature to cells that express both CD4 and CD8 molecules, called double positive (DP) cells. Recent evidence suggests that transgenic expression of a functionally rearranged TCR β chain gene in RAG-deficient mice (for a review see reference 4), or SCID mice (5) allows maturation of DN thymocytes to the DP stage. Cross-linking the TCR ϵ chain on the surface of DN thymocytes in fetal thymic organ cultures (FTOCs) (6), and in young adult RAG 1 or 2 deficient mice (7, 8), also mediates progression to the DP stage of development. However, expression of the α chain is required for subsequent maturation steps (4). DP cells expressing the α/β TCR are subject to positive selection, during which cells bearing TCRs that recognize self-MHC molecules are selected for further differentiation, as well as to negative selection, the process by which self-reactive T cells are eliminated (9). Selected thymocytes finally mature to single positive (SP) cells expressing high levels of TCR, and either CD4 or CD8.

Transitions between the different stages of developing thymocytes are likely to be modulated by signals provided by the thymic environment, since intact thymus is crucial for the developmental process (10). Lymphocyte–stromal cell interactions are known to phosphorylate the TCR ζ chain (11) and activate the protein tyrosine kinase $p56^{lck}$ (12). Cellular differentiation, resulting in expression of stage-specific genes, must be governed by the activation of inducible nuclear DNA binding factors. Several groups have studied DNA binding proteins in fetal (13, 14) and adult thymocytes (15–17). We have studied the expression of nuclear factors associated with mature T cell activation, during T cell maturation using RAG $2^{-/-}$ mice as a source of thymocytes at the DN stage, and TCR β^+ RAG $2^{-/-}$ or BALB/c mice as a source of thymocytes at the DP stage of development. Here we show

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; EMSA, electrophoretic mobility shift assay; RAG, recombination activation gene; SP, single positive.

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that: (*a*) nuclear proteins that bind AP-1 and NF-AT DNA binding motifs are constitutively nuclear in response to intrathymic signals; (*b*), lymphocyte–stromal cell interactions are essential to maintain the activated nuclear phenotype in DP but not in DN thymocytes; and (*c*), DN but not DP thymocytes reinduce AP-1 in response to stimulation in vitro. These results indicate that activation of inducible nuclear factors is exquisitely modulated during thymocytes maturation.

Materials and Methods

Mice. BALB/c mice were bred and maintained at the Redstone facility of the Dana Farber Cancer Institute. RAG $2^{-/-}$ and TCR β^+ RAG $2^{-/-}$ mice were maintained in a pathogen-free environment at the animal facility at Children's Hospital in Boston.

In Vivo mAb Treatment of RAG $2^{-/-}$ Mice. RAG $2^{-/-}$ mice were injected intraperitoneally with 300 μg of purified anti-CD3 ϵ mAb, 145-2C11 (18), or anti-TCR β chain mAb H57 (19). Thymuses were removed 6 d after injection for analysis.

Preparation and Treatment of Thymocytes. Thymuses from BALB/c, RAG $2^{-/-}$, and TCR β^+ RAG $2^{-/-}$ were isolated, thymocytes were released into single cell suspension, filtered through a nylon mesh, and red blood cells were removed by lysis with Tris/NH_4Cl solution at room temperature for 5 min (20). Thymocytes in single cell suspension were either maintained on ice for the duration of the experiment, generally 3 h, or incubated at 37°C in a 5% CO_2 atmosphere with or without 10 ng/ml PMA and 20–200 nM ionomycin, at 4 × 10^6 cells/ml. SP thymocytes from BALB/c mice were purified, as described before (20), by incubation with anti-CD8 mAb (21), for 30 min on ice followed by rabbit complement (Cederlane Laboratories Ltd., Hornby, Ontario, Canada)-mediated lysis at 37°C for 45 min, for two rounds.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Analysis. Nuclear extracts were prepared by hypotonic lysis and extraction of nuclei with high salt buffer as described before (22). Protein concentrations were determined using the Bradford assay (Pierce, Rockford, IL) and equivalent amounts of extracts used for in vitro binding assays. Binding assays were carried out in a final volume of 10 μl containing 6 mM Hepes, pH 7.9, 60 mM NaCl, and 6% glycerol for 15 min using indicated probes. Anti-Fos-1 antibody was a gift from J. Jain (Dana Farber Cancer Institute) (23), anti-Fos-2 and anti-Jun antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclic AMP response element binding protein (CREB) antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). NF-AT probe was derived from the murine IL-2 promoter (16). AP-1 probe used was generated by annealing synthetic oligonucleotides containing the AP-1 site from the metallothioenine gene promoter (24). SRF-SP-1 probe was derived from the IL-2Rα promoter region (25). The probes were labeled with polynucleotide kinase and γ-[^{32}P]ATP. Binding reactions were analyzed by electrophoresis through 4% polyacrylamide gels in 0.5X Tris-borate buffer.

Results

Nuclear Factors AP-1 and NF-AT Are Constitutively Nuclear, and in DNA Binding Form in Thymocytes In Vivo. Total BALB/c thymocytes and purified mature SP (CD4$^+$ CD8$^-$) thymocytes were cultured at 37°C in the presence or absence of PMA and ionomycin. Nuclear extracts prepared from such cells were analyzed by electrophoretic mobility shift assays (EMSA) using probes that bind NF-κB and AP-1 nuclear factors. 3-h treatment with PMA and ionomycin at 37°C resulted in induction of AP-1 in the SP thymocytes (Fig. 1, lane *9*), but not in the total thymocytes (Fig. 1, lane *5*). NF-κB binding was observed in both total thymocytes (Fig. 1, compare lanes *2* and *3*), as well as in CD4$^+$ CD8$^-$ SP thymocytes (Fig. 1, compare lanes *6* and *7*). Binding assays were normalized to the amount of nuclear protein used in the assays, and in most experiments, a probe containing binding sites for serum response factor (SRF) and the transcription factor SP-1, derived from the human IL-2Rα gene promoter, was used as an additional normalization criterion. Proteins binding to this probe showed minimal variation between extracts made from thymocytes incubated under various conditions. These observations are consistent with those of others (14–16), and indicate that total thymocytes do not induce AP-1 in response to PMA and ionomycin. One interpretation of these studies is that the majority of thymocytes lack expression of the proteins that bind the AP-1 motif.

We found, however, that nuclear extracts prepared from freshly isolated thymocytes contained nuclear proteins that bound to the NF-AT and AP-1 DNA elements in EMSA. Both factors were also detected in nuclear extracts from thymocytes kept on ice for 3–6 h (Fig. 2 *A*, lanes *1* and *4*), but were significantly diminished in extracts prepared from thymocytes incubated at 37°C for the same duration (Fig. 2 *A*, lanes *2* and *5*). Furthermore, PMA and ionomycin treatment of thymocytes did not induce the NF-AT or AP-1 binding factors found in freshly isolated thymocytes (Fig. 2 *A*, lanes *3* and *6*). It is important to note that with the AP-1 probe two

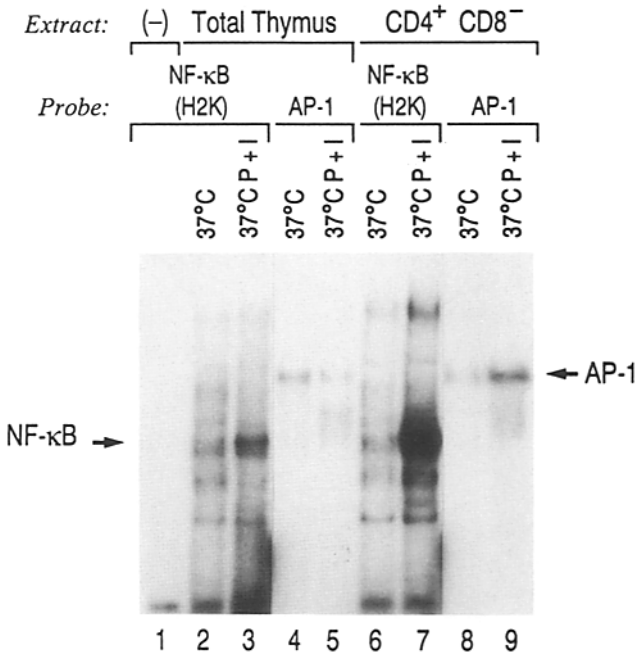


Figure 1. PMA and ionomycin treatment does not induce AP-1 in adult thymocytes. Extracts were prepared from total thymocytes (lanes *2–5*) or from purified CD4$^+$ CD8$^-$ thymocytes (lanes *6–9*) from BALB/c mice. EMSA was carried out using a probe for NF-κB (lanes *1–3* and *6* and *7*) or AP-1 (lanes *4* and *5* and *8* and *9*). Nuclear extracts were derived from thymocytes incubated at 37°C (lanes *2*, *4*, *6*, and *8*) or with PMA (10 ng/ml) and ionomycin (200 nM) (lanes *3*, *5*, *7*, and *9*).

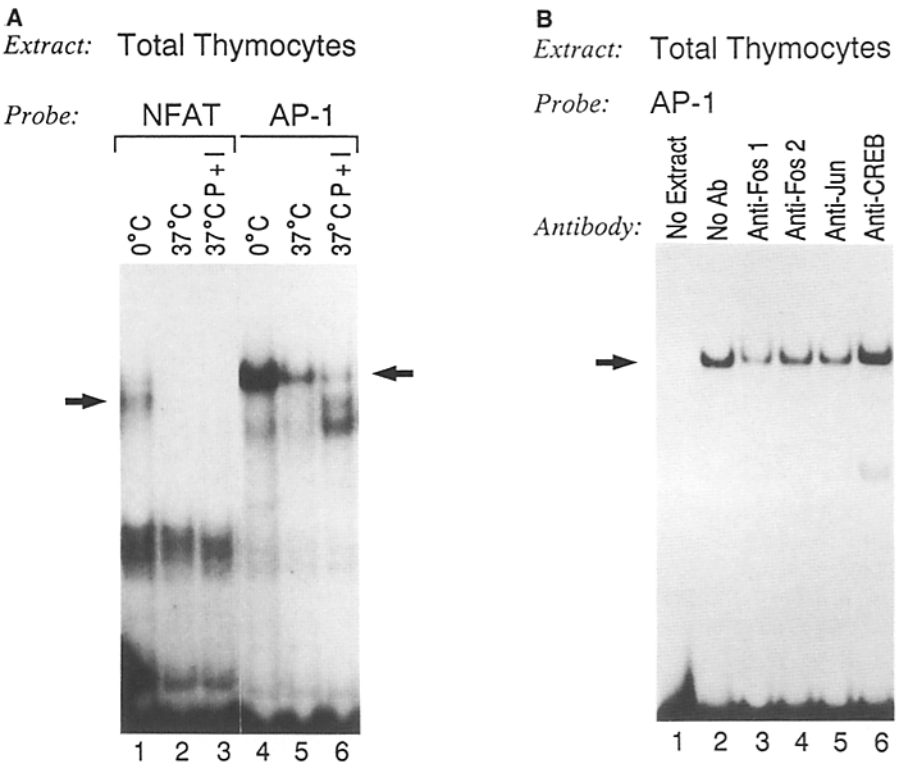


Figure 2. Nuclear factors binding to AP-1 and NF-AT motifs are constitutively nuclear in freshly isolated thymocytes. (*A*) EMSA was carried out using the NF-AT probe (lanes *1–3*) and AP-1 probe (lanes *4–6*). Extracts were from single cell suspension of thymocytes kept on ice for 3 h (lanes *1* and *4*); incubated at 37°C for 3 h (lanes *2* and *5*); activated with PMA and ionomycin at 37°C for 3 h (lanes *3* and *6*). (*Arrow, left*) Position of the NF-AT complex; (*arrow, right*) the AP-1 complex. (*B*) EMSA was carried out using AP-1 probe and nuclear extracts from thymocytes kept on ice. The binding reaction contained no extract, (lane *1*), and no antibody, (lane *2*); anti-Fos (lanes *3* and *4*); anti-Jun (lane *5*); and anti-CREB (lane *6*) antibodies.

new, faster migrating nucleoprotein complexes were seen in extracts from PMA- and ionomycin-induced thymocytes (Fig. 2, lane *6*). These bands result from specific binding to the AP-1 probe and are distinct from the band induced by the intrathymic interactions. Chen and Rothenberg (16) have previously shown that the AP-1/CRE site from the −161 to −143 region of the IL-2 5′ flanking region bound proteins from the Fos, Jun, and CREB families in thymocyte extracts stimulated by PMA and ionomycin. To determine which proteins are induced by intrathymic signals, we included antibodies directed against Fos and Jun or the CREB family of proteins in the binding reactions. As seen in Fig. 2 *B*, two anti-Fos and one anti-Jun antibody diminished binding to the AP-1 probe whereas anti-CREB antibody had no effect. Anti-CREB antibody generated a "supershifted" band in EMSA with thymocyte extract and a CRE probe (data not shown). Residual binding to the AP-1 probe in the presence of the anti-Fos and anti-Jun antibodies may result from proteins of the Fos and Jun family not recognized by the antisera or from the presence of other DNA binding proteins that may bind to the site in the absence of Fos and Jun proteins. We conclude that inducible transcription factors that bind to AP-1 and NF-AT probes are constitutively nuclear in immature thymocytes, presumably in response to lymphocyte–stromal cell interactions. Making single cell suspensions disrupts these signals resulting in the observed loss of DNA binding activity. Unlike mature SP thymocytes and T cells, where PMA and ionomycin treatment induces NF-AT and AP-1 binding factors, we noted that stimulation of total thymocytes did not reinduce these nuclear factors. Panned DP thymocytes, lacking SP thymocytes and contaminating stromal cells also show the same DNA binding profile as total thymocytes (data not shown). These results suggest that intracellular activation pathways are operative in vivo, and are not mimicked by PMA and ionomycin or anti-CD3 ϵ mAb (data not shown).

Inactivation of AP-1 DNA Binding Activity Is Developmentally Regulated: Analysis of DN Thymocytes from RAG 2$^{-/-}$ Mice. In mice deficient for either RAG 1 or 2 gene, the thymus remains small and populated with DN cells. Introducing a rearranged TCR β chain gene into this background results in a normal-sized thymus consisting mainly of DP cells (for a review see reference 4). These DP thymocytes express the β TCRs at the cell surface instead of α/β TCRs expressed on the surface of normal DP thymocytes. β^+ RAG 2$^{-/-}$ mice provide a population of cells arrested at an early DP stage of T cell differentiation. To investigate the expression and induction of AP-1 during DN to DP stage of differentiation of thymocytes, we examined nuclear extracts prepared from RAG 2$^{-/-}$ and TCR β^+ RAG 2$^{-/-}$ thymocytes by EMSA.

Nuclear extracts from DN thymocytes from RAG 2$^{-/-}$ mice contain AP-1 binding proteins (Fig. 3 *A*, lane *1*). This binding was not significantly reduced in cells incubated at 37°C (Fig. 3 *A*, lane *2*). PMA and ionomycin treatment did not increase the level of AP-1 DNA binding activity significantly (Fig. 3 *A*, lane *3*). The SRF-SP-1 probe derived from the human IL-2Rα gene promoter showed the same

level of binding in extracts made from thymocytes incubated under various conditions (Fig. 3 *A*, lanes *4–6*). We conclude that inducible nuclear factors binding to AP-1 are constitutively nuclear in the most immature thymocytes. However, the mechanisms that inactivate DNA binding upon incubation of thymocytes in single cell suspension at 37°C, are not present in these cells.

DP Thymocytes Require the Thymic Environment to Maintain AP-1 in DNA Binding Form in the Nucleus. In contrast to what was observed in RAG $2^{-/-}$ DN thymocytes, AP-1 binding activity present in DP thymocytes obtained from TCR β^+ RAG $2^{-/-}$ mice, kept at 0°C (Fig. 3 *B*, lane *1*) was substantially decreased upon 37°C incubation (Fig. 3 *B*, lane *2*). This was similar to that observed with total adult thymocyte extracts as shown in Fig. 2, lanes *4* and *5*. Furthermore, in several independently generated extracts from TCR β^+ RAG $2^{-/-}$ thymocytes, we observed little or no induction of the AP-1 binding activity in response to PMA and ionomycin stimulation (Fig. 3 *B*, lane *3*). The doublet of faster migrating nucleoprotein complexes was seen in extracts from PMA and ionomycin-treated DP thymocytes from TCR β^+ RAG $2^{-/-}$ mice as well. As before, the IL-2Rα–derived SRF-SP-1 probe served as an additional normalizing control (Fig. 3 *B*, lanes *4–6*). We conclude, therefore, that the property of thymocytes to inactivate DNA binding to the AP-1 probe, upon 37°C incubation, is developmentally regulated, coinciding with transition of the thymocytes to the DP stage. Inactivation of DNA binding by NF-AT also appears to be similarly regulated (data not shown). During this transition, the cells also lose the ability to reinduce AP-1 or NF-AT by in vitro stimulation.

DP Thymocytes Generated by Treatment of RAG $2^{-/-}$ Mice with Anti-CD3 ϵ Antibody Exhibit Characteristics of an Intermediate Between DN and DP Thymocytes. Anti-CD3 ϵ mAb treatment of DN thymocytes from RAG 1– or 2-deficient mice induces differentiation of the DN cells to the DP stage (6–8). We injected anti-CD3 ϵ mAb into young adult RAG $2^{-/-}$ mice as described previously (8). Briefly, 300 μg of mAb was injected on day 1 into RAG $2^{-/-}$ mice and 6 d later, 100–300 × 10^6 largely DP cells were isolated (Fig. 4 *A*). Thymocytes from RAG $2^{-/-}$ mice did not express CD4 or CD8 (Fig. 4 *A*, *top*). The majority of thymocytes from β^+ RAG $2^{-/-}$ mice expressed CD4 and CD8, but there were no mature cells that expressed only CD4 or CD8 (Fig. 4 *A*, *middle*). The majority of thymocytes from RAG $2^{-/-}$ mice injected with anti-CD3 ϵ mAb were DP whereas a few were DN (Fig. 4 *A*, *bottom*) (7, 8).

The nuclear properties of thymocytes generated by anti-CD3 ϵ mAb treatment were assessed by EMSA analysis of nuclear extracts from thymocytes isolated from anti-CD3 mAb–treated mice. Thymocytes incubated on ice showed AP-1 DNA binding activity (Fig. 4 *B*, lane *1*). Incubation of thymocytes at 37°C inactivated DNA binding by the AP-1 probe but not to the same extent as in the DP thymocytes from either normal mice or the RAG $2^{-/-}$ mice expressing the TCR β chain (Fig. 4 *B*, lane *2*). PMA and ionomycin treatment of these cells reinduced the same AP-1 binding complex and the two new faster migrating bands (Fig. 4 *B*, lane *3*). PMA and ionomycin treatment of DN thymocytes induced the slowest migrating band seen in freshly isolated thymocytes (Fig. 3 *A*, lane *3*), whereas stimulation of DP thymocytes from TCR β–expressing RAG $2^{-/-}$ mice and

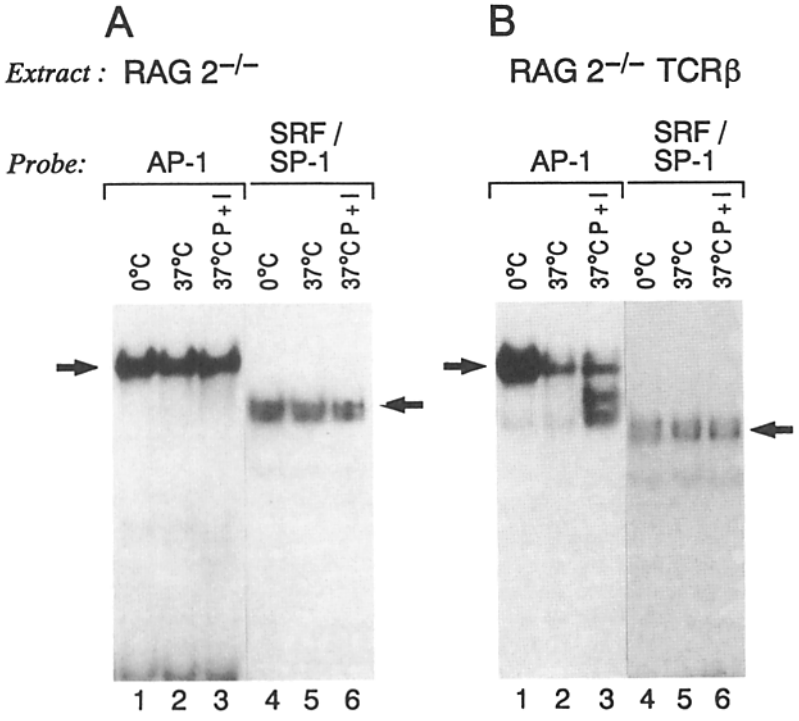
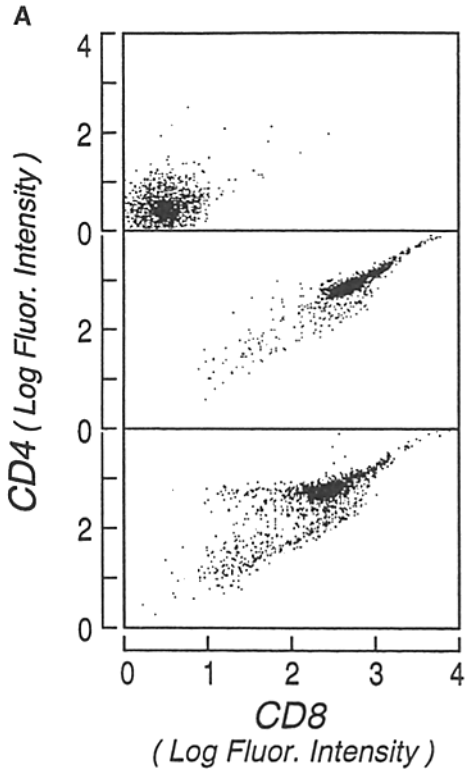


Figure 3. DP, but not DN thymocytes require the thymic microenvironment to maintain AP-1 binding proteins in the nucleus. (*A*) Nuclear extracts were prepared from single cell suspension of thymocytes from RAG $2^{-/-}$ mice. Thymocytes were kept on ice for 3 h (lanes *1* and *4*); at 37°C for 3 h (lanes *2* and *5*); at 37°C for 3 h in the presence of PMA (10 ng/ml) and ionomycin (200 nM), (lanes *3* and *6*). (*B*) Nuclear extracts prepared from TCR β^+ RAG $2^{-/-}$ mice. Thymocytes were kept on ice for 3 h (lanes *1* and *4*); at 37°C for 3 h (lanes *2* and *5*); at 37°C for 3 h in the presence of PMA (10 ng/ml) and ionomycin (200 nM), (lanes *3* and *6*). EMSA was carried out with specific probes as indicated above each set of lanes.



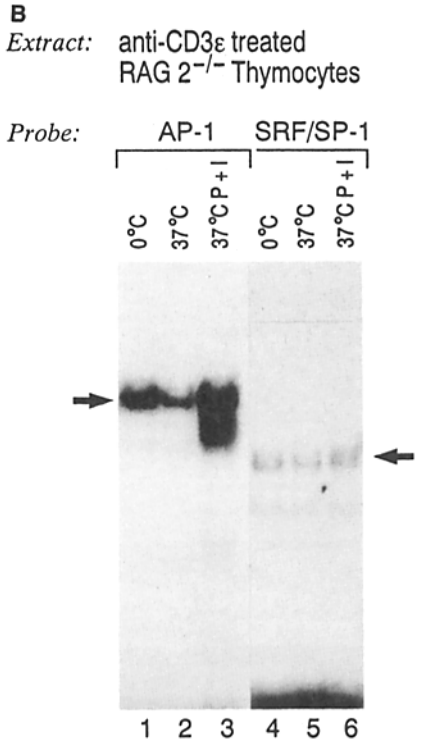


Figure 4. Anti-CD3 ϵ mAb treatment of RAG $2^{-/-}$ mice leads to the generation of immature DP thymocytes. (*A*) Thymocytes from RAG $2^{-/-}$ mice (*top*), β^+ RAG $2^{-/-}$ mice (*middle*), and RAG $2^{-/-}$ mice injected with anti-CD3 ϵ mAb (*bottom*), were stained with FITC-labeled anti-CD8 and PE-labeled anti-CD4 mAbs. (*B*) Nuclear extracts were prepared from thymocytes generated by in vivo injection of anti-CD3 ϵ mAb in RAG $2^{-/-}$ mice, thymocytes were maintained on ice (lanes *1* and *4*); incubated at 37°C (lanes *2* and *5*); or incubated at 37°C with PMA (10 ng/ml) and ionomycin (200 nM) (lanes *3* and *6*). EMSA was carried out with the specific probes indicated above each set of lanes.

normal mice did not induce the slowest migrating band but induced two faster moving bands (Fig. 3 *B*, lane *3*). Thus, the properties of anti-CD3 ϵ mAb–induced DP cells are intermediate between DN and DP thymocytes with regards to inactivation of DNA binding upon incubation at 37°C, and similar to the DN cells in the ability to reinduce AP-1 in vitro. Since freshly isolated thymocytes from RAG $2^{-/-}$ mice that have been or have not been treated with anti-CD3 ϵ mAb show DNA binding to the AP-1 probe, it is unlikely that the induction of AP-1 DNA binding activity is simply the result of cellular activation by CD3 ϵ chain cross-linking. We, therefore, propose that these nuclear characteristics identify an "early DP" stage, that may be an intermediate in normal T cell development.

Discussion

AP-1 and NF-AT DNA binding activities were detected in nuclear extracts from freshly isolated thymocytes. These data suggest that interactions between thymocytes and stromal cells result in signals that activate nuclear factors AP-1 and NF-AT. These data are consistent with previously reported observations that intrathymic signals result in TCR ζ chain phosphorylation, which is a phenotype of an activated T cell, and also activate the protein tyrosine kinase $p56^{lck}$. The cell surface molecules that mediate the intrathymic signals are largely unknown, however, a role for CD4 as been postulated in the modulation of TCR ζ chain phosphorylation and $p56^{lck}$ activation (11, 12).

Incubation of thymocyte single cell suspensions at 37°C resulted in the loss of DNA binding activity by AP-1 and NF-AT. This suggests that thymocyte–stromal cell interactions are required to maintain the nuclear factors in DNA binding form. Inactivation of DNA binding takes place in the absence of protein synthesis (data not shown) but requires energy because cells incubated on ice retain DNA binding. The downregulation of nuclear factors is developmentally regulated, being most prominent in β/β and α/β TCR expressing DP thymocytes, but not in immature DN thymocytes. These data correlate with the reported observation that incubation of DP thymocytes in single cell suspension results in dephosphorylation of the TCR ζ chain and upregulation of cell surface expression of the TCR–CD3 complex.

The nuclear characteristics of DP cells induced by anti-CD3 ϵ mAb treatment of RAG $2^{-/-}$ mice share features of both DN and DP thymocytes. Intrathymically induced AP-1 was significantly downregulated upon incubation of the cells at 37°C, although not to the same extent as in DP thymocytes from normal or RAG $2^{-/-}$ TCR β transgenic mice. However, AP-1 DNA binding activity was strongly induced upon PMA and ionomycin treatment, as has been seen only

in DN thymocytes from RAG $2^{-/-}$ or SCID mice. Phenotypic analysis of thymocytes from anti-CD3 ϵ mAb–injected mice showed a majority of DP cells and only a small number of DN cells. These data can be interpreted to indicate that the majority of cells in the DP population generated by anti-CD3 ϵ mAb treatment are at an intermediate stage during DN to DP transition. If this is the case, it is possible that the difference between the DP cells generated by cross-linking CD3 ϵ and by transgenic expression of the TCR β chain, reflect additional signals transmitted via the β TCR–gp33 complex (26) and associated surface molecules. Alternatively, it cannot be ruled out that all the cells did not receive the signals upon cross-linking the CD3 ϵ chain equally and the data reflect a mixed population of cells at various stages of differentiation.

The analysis of inducible factors presented in this study, and previously published data regarding phosphorylation of the CD3 ζ chain or activation of $p56^{lck}$, could be a result of signals received by thymocytes that maintain these cells through their differentiation programs, or a result of death signals received by unselected thymocytes. We favor the first interpretation because these factors are constitutively nuclear in the earliest DN stage of development, at which stage it is unlikely that the cells have been signaled to die. Mature T cells maintain inducible nuclear factors in an inhibited often cytosolic form until activated by an encounter with antigen. Upon antigen-mediated stimulation, inducible nuclear factors are activated to translocate to the nucleus and bind DNA (27). We speculate that these proteins are activated in immature thymocytes in response to developmental signals during the DN and DP stages and are downregulated before transition to the mature SP stage.

As mentioned before, several groups have studied the induction of inducible nuclear factors in thymocytes at different stages of development (14–16). In all of these papers, the question addressed was that of nuclear factors induced upon stimulation of purified thymocytes, at various stages of maturation, and correlated with the capacity of thymocytes to produce IL-2. The results suggested that whereas immature DN and mature SP thymocytes produced IL-2 and activated AP-1 and NF-AT, DP thymocytes did not respond similarly. The interpretation was that the lack of NF-AT and AP-1 in DP thymocytes reflected their inability to make IL-2. The implication that thymocytes at the DP stage are incapable of expressing and activating NF-AT and AP-1 is misleading. This conclusion has been changed by our observation that DP thymocytes do express NF-AT and AP-1, presumably in response to intrathymic signals. The fact that DP thymocytes do not make significant amounts of IL-2 in vivo (28), in spite of activated NF-AT and AP-1 complexes, suggests that these factors may be necessary, but are not sufficient for transcription of the IL-2 gene.

In conclusion, the ability of thymocytes at different stages of development to modulate DNA binding nuclear factors is exquisitely regulated. Immature thymocytes at DN and DP stages of development activate AP-1 and NF-AT in response to intrathymic signals. Disruption of the intrathymic signals leads to inactivation of DNA binding by AP-1 and NF-AT in DP thymocytes but not in DN thymocytes. Furthermore, immature DN thymocytes activate AP-1 upon stimulation in vitro but DP thymocytes do not, even though the proteins involved in these interactions are expressed in these cells. Incubation of thymocyte single cell suspension at 37°C has been previously shown to decrease tyrosine phosphorylation of TCR ζ chain and increase cell surface expression of the TCR–CD3 complex. Because SP thymocytes express high levels of TCR–CD3, the increased CD3 expression on DP thymocytes upon 37°C incubation has been postulated to mimic part of the maturation response. Since mature T cells maintain nuclear factors in an inhibited form, to be activated upon encountering antigen, we speculate that the mechanisms resulting in the inactivation of transcription factors may also be a part of the maturation step that ultimately produces SP cells. As a consequence, SP cells that emerge would not contain DNA binding AP-1 or NF-AT in the nucleus, until restimulated by antigen during an immune response.

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