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Localization of the Domains in Runx Transcription Factors Required for the Repression of CD4 in Thymocytes

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The runt family transcription factors Runx1 and Runx3 are expressed in developing murine thymocytes. We show that enforced expression of full-length Runx1 in CD4⁺CD8⁻ thymocytes results in a profound suppression of immature CD4⁺CD8⁺ double-positive thymocytes and mature CD4 single-positive thymocytes compared with controls. This effect arises from Runx1- or Runx3-mediated repression of CD4 expression, and is independent of positively selecting signals. Runx1 is able to repress CD4 in CD4⁺CD8⁺ double-positive thymocytes, but not in mature splenic T cells. Runx-mediated CD4 repression is independent of association with the corepressors Groucho/TLE or Sin3. Two domains are required for complete Runx-mediated CD4 repression. These are contained within Runx1 aa 212–262 and 263–360. The latter region contains the nuclear matrix targeting sequence, which is highly conserved among runt family transcription factors across species. The presence of the nuclear matrix targeting sequence is required for Runx-mediated CD4 repression, suggesting that Runx transcription factors are stabilized on the CD4 silencer via association with the nuclear matrix.


Immature CD4⁺CD8⁻ double-negative (DN) thymocytes committed to the αβ T cell lineage up-regulate expression of the coreceptors CD4 and CD8 to become double-positive (DP) thymocytes. It is at the DP stage and the intermediate CD4⁺CD8⁻ stage that the association between the CD4 or CD8 coreceptor, TCRβ specificity, and function is established. Cells expressing an TCRβ that recognizes the Ag presentation molecule MHC class I down-regulate the expression of CD4, maturing to a CD8⁻ cytotoxic T cell. Conversely, cells expressing an TCRβ that recognizes the Ag presentation molecule MHC class II fully down-regulate the expression of CD8, maturing to a CD4⁺ T cell. Therefore, the down-regulation of CD4 in DP thymocytes is an integral part of the gene regulatory program leading to the production of CD8⁺ CTLs. The identity of the elements controlling CD4 expression specifically in DP thymocytes is controversial. In the CD4 locus, a combination of distal and proximal enhancers, promoters, and a silencer directs expression of CD4 in CD4⁺ single-positive (SP) and DP thymocytes and silences expression of CD4 in DN and CD8 SP thymocytes (1–5). Several candidates have been proposed for the trans-acting factors responsible for the silencing of CD4 in DP thymocytes that have recently committed to the CD8 T cell lineage. The transcriptional corepressor Hes-1 has been shown to down-regulate the expression of CD4 through binding to the CD4 silencer in collaboration with c-myb (6, 7). However, these studies were done using a T cell clone (DH10) that has already committed to the CD4⁺ Th lineage. ZEB is another transcription factor found to repress CD4 expression, but again only in SP thymocytes, postpositive selection (8). The 434-bp CD4 silencer (3, 4) contains a remarkable five consensus binding sites for runt family transcription factors, making any runt family transcription factors that are expressed in DP thymocytes strong candidates for the factor responsible for down-regulation of CD4 in mature thymocytes. There are three known Runx family members in mice, two of which, Runx1 and Runx3, are expressed at significant levels in the thymus. A transgenic mouse expressing Runx1 under the control of the CD2 promoter shows an increase in the number of CD8 SP thymocytes, even when the thymocytes also express MHC class II-restricted TCR (9). A transgenic mouse expressing the osteoblast-specific runt family member Runx2 ectopically in thymocytes also shows a skewing toward the development of CD8 SP thymocytes (10). These effects have now been shown to result, at least in part, from direct action of Runx proteins on the CD4 silencer. Recent studies have shown that thymocytes with null mutations in Runx1 and Runx3 are not able to silence CD4 appropriately in T cell development (11, 12) and that silencing is dependent upon the presence of runt family sites in the CD4 silencer (13). These studies suggest that Runx1, Runx2, or Runx3 are equally capable of repressing CD4 expression in DP thymocytes by a mechanism that has been evolutionarily conserved since the duplication of the ancestral gene that generated the three mammalian runt family members.

In this study, we focus on Runx1 to define the structural requirements for this repressive activity. Runx1, also known as PEBP2αB, CBFα2, or AML1, is the transcription factor of the runt family (14) that is most highly expressed in hemopoietic stem cells (15) and continues to be highly expressed in thymocytes throughout their development (15, 16). The runt family is characterized by the DNA-binding runt domain, which is highly conserved between runt family molecules from Caenorhabditis elegans, sea urchins, and...
Drosophila, chicken, mice, and humans. Besides the runt domain, Runx1 also contains a C-terminal trans activation domain, a nuclear matrix targeting sequence (reviewed in Refs. 17 and 18), and many sites of interaction with trans activators and corepressors (19–28). Thus, it can serve as the nucleus of a trans-activating complex of proteins, as well as part of a transcriptionally repressive protein complex. It is not known what regulates the switch between the assembly of a transcriptionally active or repressive complex, but it could be influenced by the presence or absence of Runx protein domains in the forms of Runx that predominate in a given tissue. Different forms of Runx1 protein arise from alternative promoter use and alternative splicing. Runx1 is transcribed from two promoters, the distal or proximal. The distal promoter is partly active in T-lineage cells and hemopoietic stem cells (15). Two different N-terminal Runx1 isoforms (derived from distal and proximal promoters) and at least three C-terminal Runx1 isoforms (ΔS, ΔQ, and full-length, derived from alternative splicing) are expressed in the murine thymus. Distal Runx1 is the predominant N-terminal isoform expressed in the thymus; proximal Runx1 is predominant in some myeloid cells. The distal and proximal Runx1 isoforms have functionally different activities in myeloid commitment (15). In addition to promoter use isoforms, there are myriad C-terminal splice isoforms that are expressed in the same tissues that express full-length Runx proteins (15, 29–31). The interaction sites for the two corepressors known to associate with Runx1 map to these C-terminal regions (20, 25).

In this study, we confirm that enforced expression of either full-length Runx1 or Runx3 represses CD4 expression in thymocytes, but not in splenic T cells. We map the domains in these transcription factors that are required for this repression. Our analysis shows that this repressive activity is not dependent on the C-terminal motif that binds the Groucho/TALE corepressor (20, 25, 32, 33). The domain most critical for Runx1-mediated CD4 repression contains the nuclear matrix targeting sequence, which is conserved between Runx1, 2, and 3. This suggests that the Runx-repressive activity is dependent upon subnuclear localization. The conservation of the mechanism of Runx1-mediated CD4 repression implies that this mechanism is used in the regulation of other gene targets potentially involved in the development of hemopoietic stem cells, osteoblasts, or in oncogenesis. Thus, the elucidation of the mechanism of Runx-mediated CD4 repression has broader implications than even the important problem of how CD4 expression is silenced in developing thymocytes.

Materials and Methods

Fetal thymic organ culture (FTOC)

C57BL/6 × DBA F1 hybrids were mated overnight, with resulting fetuses assessed to be 0.5 days postcoitus (d.p.c.). Fetuses were harvested at d.p.c. 14.5–15.5 for host thymic lobes and donor thymocytes and d.p.c. 12.5 for fetal liver donors. Host thymic lobes were washed in DMEM-FTOC medium (DMEM, 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin-streptomycin, 50 μM 2-ME, 1× nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA), 10 mM HEPES, pH 7) plus 1× 3.5 mM deoxyguanosine, 0.3 mM Na2HPO4, 3.9 mM NaHCO3, 0.2% BSA, 1 mM EDTA before incubation with biontinated anti-Ter19, anti-Cr-1, anti-CD19 (BD PharMingen, San Diego, CA), and anti-FcγRIII (Caltag Laboratories, Burlingame, CA). Cells were then incubated with streptavidin microbeads and passed over a MACS column, as directed (Miltenyi Biotec, Auburn, CA). The flow-through fraction was put into culture overnight in six-well plates in DMEM-FTOC plus 50 ng/ml IL-7 and 10% baby hamster kidney cells transduced with mouse kit ligand supernatant as a source of c-kit ligand.

Retroviral constructs

Runx1 clones were inserted into the murine stem cell virus (MSCV) retroviral vector and packaged, as previously described (14). Inserts were generated by PCR with an editing DNA polymerase using 5′ primers designed to generate a BglII restriction site and a consensus Kozak sequence upstream of the initiation methionine. The isolated runt domain construct starts at Runx1 methionine 51 and stops at arginine 178, with the in-frame addition encoding SV40 nuclear localization domain EKKKKKAVDQK (from Stratagene (La Jolla, CA) vector CMV-AD). Internal deletion constructs were made by ligating the PCR-generated flanking regions via an engineered in-frame EcoRI site, which encodes for the amino acids EF in place of the indicated deletion. All amino acid numbering in this study refers to the murine proximal Runx1 isoform. Truncation constructs are named after the last Runx1 amino acid present; internal deletion constructs are named after the first and last amino acids deleted. Cells were infected by centrifuging in six-well plates with 3 ml of retroviral supernatant plus 20 μg/ml lipofectamine (Life Technologies) at 1800 rpm for 1–2 h in a Sorvall RT6000 centrifuge at 30°C or by drawing the supernatant plus lipofectamine through the cells on a cellulose filter by gravity feed. The retroviral supernatant was then replaced by DMEM-FTOC medium. Donor thymocytes used in FTOC were infected once and incubated at 37°C, 7% CO2 for ~3 h before use in FTOC. Donor fetal liver cells were infected with the Runx1 retroviral constructs at 24 h and again at 36 h postinoculation. They were then placed in hanging drop culture with host thymic lobes.

FACS analysis of FTOC

Cells were dissociated from lobes by a 2-h, 37°C treatment with 2 mg/ml collagenase in RPMI 1640 medium plus 20 mM HEPES, pH 7.5. Cells were washed with 5.5 mM KCl, 0.4 mM KH2PO4, 140 mM NaCl, 5.6 mM glucose, 0.3 mM Na2HPO4, 3.9 mM NaHCO3, 0.2% BSA, 1 mM EDTA and incubated with anti-CD4 PE, anti-CD8 CyChrome (BD PharMingen), anti-TCRβ allophecoxyalin (H57-597; BD Pharamingen), anti-TCRγ PE Cy5 (G3L; Accurate Chemical and Scientific, Westbury, NY), and anti-CD3 (500A2), anti-heat-stable Ag (CD24) PE, and anti-CD69 PE (BD Pharamingen). The 7-amoactinomycin D (Molecular Probes, Eugene, OR) was used as a vital dye at 10 μg/ml in the final cell suspension buffer. Cells were run on a FACSCalibur (BD Biosciences, San Diego, CA). FACS data were analyzed using FlowJo (Tree Star, Ashland, OR) software.

The bcl-2 transgenic cell culture

Thymocytes were harvested from the Eq-bcl-2-25 line of bcl-2 transgenic mice (34) (The Jackson Laboratory, Bar Harbor, ME). They were enriched for immature thymocytes by negative selection with anti-CD4 and anti-CD8 Abs directly conjugated to microbeads and passage over a MACS column, as directed (Miltenyi Biotec). Thymocytes were plated in six-well cell culture plates in DMEM-FTOC at a density of 1.6–3.6 × 105 cells/mm2. The thymocytes were infected with retroviral constructs, as described above, and cultured in DMEM-FTOC at 37°C, 7% CO2.

Splenocytes from bcl-2 transgenic mice were enriched for T cells by negative selection with anti-CD19 Ab conjugated to microbeads and passage over a MACS column, as directed (Miltenyi Biotec). Cells were stimulated with plate-bound anti-CD3 and anti-CD28 (each 10 μg/ml) and infected with retroviral constructs 48 h later.
RT-PCR

RT-PCR was performed, as described (15), using the following PCR primers: ACGTCCCGGATGCGACTCCTGCTACC (forward primer exon 4), GTCCCTATCTCTGGTGGTGCCTCAT (reverse primer Δ5 Runx1 3′ untranslated region), and GTACACCCACAGACGTACCGACC (reverse primer Δ6 Runx1 3′ untranslated region). Thymic subsets were prepared, as described (35).

Results

Expression of either distal or proximal full-length Runx1 in fetal thymocytes results in the suppression of CD4/CD8 DP thymocytes and CD4 SP thymocytes, but spares CD8 SP thymocytes

We have previously shown that the distal promoter of distal Runx1 mRNA is highly active in thymocytes and that both full-length and truncated splicing isoforms of the mRNA are produced there (15). Gene dosage is known to be important for the biological activities of Runx1, but it has not been clear which isoforms are most important for the role of Runx1 in the thymus. Therefore, we sought to investigate the functional effect on thymocyte development of the enforced expression of distal or proximal full-length Runx1, because the full-length form includes inhibitory, repressive, and trans-activating domains not present in the alternatively spliced shorter forms that are also naturally expressed in the thymus.

To determine the most direct effects of enforced expression of full-length Runx1 at different, specific stages of thymocyte development, we used a retroviral transduction strategy. Full-length Runx1 cDNAs with sequences encoding the distal or proximal promoter-derived N termini were inserted upstream of the internal ribosome entry site of the retroviral vector MSCV-internal ribosome entry site-green fluorescent protein (GFP), resulting in a bicistronic transcript. The expression levels of Runx1 can thus be tracked by the level of expression of GFP. Fetal thymocytes were harvested at a stage when the majority of thymocytes are CD4+CD8+ TCRβlow (data not shown), and thus not committed to either a CD4+CD8− TCRαβ+ helper or CD4−CD8+ TCRαβ− cytotoxic T cell fate. They were infected with the retroviral vectors encoding full-length distal or proximal Runx1, as well as the control vector encoding GFP alone. Infected fetal thymocytes were incorporated into fetal thymic lobes depleted of endogenous thymocytes and allowed to develop.

Thymocytes infected with the empty control vector expressing only GFP reflect the CD4 and CD8 expression profile seen in GFP-negative uninfected thymocytes (Fig. 1). Compared with these control samples, there is an overall decrease in the number of thymocytes expressing GFP after culture in fetal thymic lobes in those transduced with distal or proximal full-length Runx1. Furthermore, thymocytes...
infected with vectors expressing distal or proximal full-length Runx1 diverge markedly from the GFP-only control in their CD4 expression profiles. Thymocytes expressing either distal or proximal full-length Runx1 show a profound decrease in the percentages of both CD4/CD8 DP and CD4 SP thymocytes compared with GFP-only controls. There is a corresponding increase in the percentage of CD4/CD8 DN and CD8 SP thymocytes in populations of thymocytes expressing either distal or proximal full-length Runx1 compared with GFP-only controls. However, absolute numbers of CD4 SP thymocytes are not greater in populations of cells expressing full-length Runx1 compared with GFP-only controls.

Surprisingly, despite the much higher levels of distal Runx1 than proximal Runx1 naturally expressed in thymocytes (15), there is no difference between the kind or severity of the phenotype between the two isoforms. The phenotype is dose dependent: high levels of GFP and, thus, high levels of distal or proximal Runx1, are not seen in thymocytes expressing high levels of CD4, as compared with thymocytes infected with the control empty vector (data not shown). Only those cells expressing low levels of GFP and, thus, low levels of distal or proximal Runx1, have high levels of CD4 expression (data not shown). In contrast, high levels of GFP and, thus, distal or proximal Runx1, are seen in thymocytes expressing high levels of CD8 (data not shown).

To gain insight into the developmental stage of the thymocytes, we analyzed them for their level of TCRβ expression, which indicates level of maturity. Thymocytes committed to the T cell lineage initially are negative for CD4, CD8, and TCRβ expression, which increases as they mature to the CD4 or CD8 SP thymocyte stage. Murine thymocytes that are negative for TCRβ expression, but positive for CD8 expression, are called immature SPs, because CD8 surface expression may be up-regulated before CD4 and TCRβ surface expression. Also included in the DN TCRβ− and CD8+TCRβ− populations are thymocytes committed to the γδ T cell lineage. Thymocytes transduced with distal or proximal Runx1 have similar TCRβ expression profiles compared with thymocytes transduced with the GFP-only control (Fig. 1) and uninfected thymocytes (data not shown). The only difference seems to be a slight decrease in the average intensity of TCRβ staining within the TCRβ intermediate gate in the thymocytes transduced with distal or proximal Runx1 compared with GFP-only control-transduced thymocytes (Fig. 1). However, the percentages of thymocytes in the TCRβ negative, intermediate, and high gates are similar whether the thymocytes were infected with control GFP only or with full-length distal or proximal Runx1. The increase in the percentage of CD8 SP cells resulting from enforced expression of either distal or proximal Runx1 does not appear to arise from a total block in the development of the thymocytes at the immature SP stage (CD8+TCRβ+), because the percentage of CD8 SP thymocytes is increased over control at all three levels of TCRβ expression (Fig. 1D).

Expression of truncated isoforms of Runx1 conveys a growth advantage to developing thymocytes; expression of full-length Runx1 conveys a growth disadvantage to developing thymocytes. To dissect the mechanism of the effects of Runx1 on thymocyte proliferation and development, we compared full-length Runx1 with naturally occurring truncated isoforms of Runx1 expressed in the thymus (Fig. 2). We have cloned and designated two novel Runx1 isoforms as Δ5 and Δ6 (15), which refers to truncation of the open reading frame before exons 5 and 6, respectively (Fig. 2).
These clones represent use of alternative polyadenylation sites in the introns downstream of exon 4 for Δ5 and downstream of exon 5 for Δ6. They lack DNA-binding and CBFβ-binding capability, nuclear localization sequences, a trans activation domain, a nuclear matrix attachment domain, and a Groucho/TLE repressor binding domain. They are comprised of an N terminus of unknown function plus two-thirds (Δ5) or the entire (Δ6) runt domain. It is unknown whether they have the capacity to act as a naturally occurring inhibitor of full-length Runx1 functions or whether they have no activity.

As noted above, the yields of GFP⁺ cells are much lower for the full-length Runx1 retroviral constructs as compared with the control empty vector in fetal thymocyte donors after 10–15 days of FTOC (Fig. 1). In contrast, both frequencies of GFP⁺ cells and levels of GFP expression per cell are higher for the Δ5 and Δ6 Runx1 constructs than for the control empty vector (data not shown). We investigated whether this difference was actually due to different infection efficiencies or whether it arose due to a growth disadvantage or advantage. Because thymocyte donor cells do not survive long enough in vitro to be used for this assay, fetal liver cells enriched for precursor cells were used as donor cells. The cells were infected, and a sample was analyzed for GFP expression after 48 h in culture, to elicit optimal fluorescence. The remainder of the cells were placed in depleted fetal thymic lobes and cultured for 17 days before FACS analysis. All of the constructs had similar infection efficiencies before FTOC incubation, ~15% of live cells for this experiment. After 17 days of incubation in FTOC, the fraction of cells transduced with the control empty vector is little changed. In contrast, expression of either distal or proximal full-length Runx1 conveys a growth disadvantage to cells in FTOC (Fig. 2D).

This growth disadvantage does not depend on recruitment of the corepressor Groucho/TLE. Runx1 has been shown to act as a repressor through its association with the corepressor Groucho/TLE (20, 32, 33). The corepressor Groucho associates with Runx1 through the last 5 aa (VWRPY) of the C terminus of Runx1. We manufactured a distal Runx1 construct deleted for the last 5 aa (447–451), selectively ablating the Groucho interaction with Runx1 (Dis.d446). The same growth disadvantage is imparted by this Dis.d446 mutant form of distal Runx1, or even by a minimal construct consisting of the DNA binding runt domain plus a nuclear localization sequence (Fig. 2D). This implies that Runx binding to Runx cognate sites in the genome is responsible for the growth disadvantage phenotype.

In contrast, expression of the non-DNA-binding isoforms, proximal or distal Δ5, conveys a growth advantage to cells in FTOC. Surprisingly, even expression of the distal or proximal N-terminal fragments up to the runt domain, especially at higher expression levels, enhances growth in FTOC (Fig. 2D). This suggests that despite the lack of known functional domains present in Δ5 and Δ6 Runx1, they may either interfere with the full-length form’s function or exercise independent functions. The growth advantage conveyed by the isolated N termini suggests that Δ5 and Δ6 derive their growth advantage phenotype in part from the Runx1 N terminus, with or without the runt domain.

**The Runx1 C terminus is necessary for the suppression of CD4/CD8 DP thymocytes and CD4 SP thymocytes by Runx1**

The effect of Runx1 on the appearance of CD4⁺ thymocyte subsets is not simply a byproduct of its growth-inhibitory effect, as shown by the comparison of the activities of different Runx mutant forms. Expression of Δ5 or Δ6 has no appreciable effect on the percentage of DP or CD4 SP thymocytes compared with control. However, the runt DNA binding domain plus nuclear localization sequence also cannot mediate the inhibitory effect on the production of CD4⁺ thymocytes seen with full-length Runx1 (Fig. 1), despite having a growth-suppressive effect similar to that seen with expression of full-length Runx1 (Fig. 2D). This separates the two phenotypes of growth disadvantage and CD4 repression: the former requires only DNA binding and the latter requires the domains downstream of the runt domain. These experiments demonstrate that the Runx1 C terminus is necessary for the suppression of DP thymocytes and CD4 SP thymocytes mediated by expression of full-length Runx1.

An obvious candidate mechanism for the repression of CD4 expression would be Groucho/TLE recruitment via aa 447–451.

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**FIGURE 3.** The Runx1 C terminus downstream of the runt domain and upstream of the Groucho-binding motif is required for Runx1-mediated suppression of DP and CD4 SP thymocytes in FTOC. Fetal thymocytes were infected with control retroviral vector (A), distal (B) or proximal (C) full-length Runx1, distal (D) or proximal (E) Δ5 Runx1, distal (F) or proximal (G) Δ6 Runx1, the runt DNA binding domain with nuclear localization sequence (H), or distal Runx1 lacking the corepressor Groucho binding site (I). Thymocytes were placed in FTOC and analyzed for their GFP, CD4, CD8, and TCRβ expression by FACS at day 10 (A–I). Cells were gated for their viability by forward and side scatter profiles and then for GFP positivity before generating CD4 vs CD8 plots. Percentages are expressed as a percentage of live GFP⁺ thymocytes. Results shown are representative of two independent experiments, with two time points each.
The Dis.d446 Runx1 mutant does not decrease the percentage of CD4 SP thymocytes; however, the percentage of DP thymocytes is still decreased and the percentage of DN and CD8 SP thymocytes is increased, as occurs when full-length Runx1 is expressed (Fig. 3f). This intermediate phenotype implies that some C-terminal functions of full-length Runx1 map to the Groucho interaction domain and others do not. However, the decrease in cell number resulting from expression of Dis.d446 Runx1 (Figs. 2D and 3f) makes it difficult to interpret these results.

**Full-length distal and proximal Runx1 directly down-regulate CD4 expression on DP thymocytes**

Because SP thymocytes are normally generated by MHC-dependent selection events, Runx1 could be perturbing the CD4/CD8 phenotype by affecting the selection process or by direct cell autonomous effects. To examine the effect of Runx1 on CD4+ thymocytes independently of thymic negative and positive selection, we used the Eμ-bcl-2-25 line of bcl-2 transgenic mice, in which the antiapoptotic gene bcl-2 is constitutively expressed in thymocytes (34). We incubate bcl-2 transgenic thymocytes, which are resistant to death by neglect, in suspension culture. This allows us to examine the effect of Runx1 expression with greatly diminished influence from interactions with class I and class II MHC, which normally skew the final readout by the selective proliferation (positive selection) or deletion (negative selection) of certain thymocyte subsets. To be able to monitor the effect of distal and proximal Runx1 on DP thymocytes, these constructs were transduced into the immediate precursors of DP cells.

In suspension culture, bcl-2 transgenic thymocytes differentiate from DN to TCRβ-intermediate and TCRβ-high stages in a few days. The starting population was largely negative for CD4 and CD8 (Fig. 4B) and had negative to low levels of expression of TCRβ (data not shown). Within 16 h of isolation and incubation in a single-cell suspension, the majority of these cells have up-regulated their expression of both CD4 and CD8 (Fig. 4C). At 40 h, TCRβ expression is up-regulated from negative to intermediate levels (Fig. 4E). Subsequently, a few CD4 or CD8 SP thymocytes appear, probably due to the stochastic down-regulation of either CD8 or CD4, as seen in the thymuses of mice with null mutations of class I and class II MHC molecules (36). Thymocytes without the bcl-2 transgene that are cultured under the same conditions die within 24 h (data not shown). The bcl-2 transgenic thymocytes thus allow examination of the molecular events of thymocyte maturation in a more physiologically relevant regulatory context than a transformed thymocyte cell line.

Expression of full-length distal or proximal Runx1 in cells transduced at the DN stage leads to the selective down-regulation of surface CD4 expression in the DP thymocyte population, starting at 16 h when all populations contain many DP thymocytes and intensifying through the time course’s end at 88 h (Fig. 4D). As in the fetal thymic organ cultures, the level of expression of CD8 is unaffected, resulting in the generation of apparent CD8 SPs. GFP+ control and all GFP+Runx1+ viable cell numbers increase from 0 to 66 h (data not shown). This can be explained by the fact that retroviral vectors can stably infect only proliferating cells. Thus, the disappearance of CD4+ thymocytes from populations expressing full-length Runx1 is not due to the death of these cells, but rather results from the selective down-regulation of CD4 in viable cells.

The down-regulation of CD4 in these cultures is not associated with conventional positive selection signals. Thymocytes undergoing positive selection are characterized by an up-regulation of the activation Ag CD69, which is not seen in these cultures (data not shown). In addition, the apparent CD8 SP thymocytes induced by expression of distal or proximal full-length Runx1 do not show the heat-stable Ag down-regulation characteristic of SP thymocytes after they have passed through positive selection and are ready to immigrate to the periphery (data not shown). This indicates that these cells are not mature CD8 SP thymocytes and that CD4 down-regulation by Runx1 is likely to be independent of coreceptor/TCR-MHC interaction.

Expression of the Runx1 mutant incapable of associating with the corepressor Groucho/TLE (Dis.d446) in bcl-2 transgenic thymocytes also results in the down-regulation of surface CD4 expression (Fig. 4). This clarifies the result seen in FTOC, in which the effect on CD4 expression was occluded by a loss of GFP+ cells (Fig. 3f). The C terminus, but not the Groucho interaction domain per se, is necessary for the silencing of CD4 expression by Runx1, confirming results from FTOC.

**Full-length Runx1 expression does not down-regulate surface CD4 expression in mature CD4+CD8- splenic T cells**

To investigate the limit of the responsiveness of the CD4 locus to Runx1-mediated CD4 silencing in development, mature splenic T cells from bcl-2 transgenic mice were infected with control vector, distal or proximal full-length Runx1, distal Runx1 lacking the Groucho binding domain, and distal or proximal Δ5 Runx1 (Fig. 5). In stark contrast to the DP thymocytes in FTOC and in the bcl-2 transgenic suspension culture, CD4 expression was not down-regulated in these cells. Indeed, expression of distal or proximal full-length Runx1 or distal Runx1 lacking the Groucho binding domain in mature CD4+ T cells isolated from the spleen actually slightly up-regulated CD4 expression compared with CD4 expression in uninfected T cells. In these cells, the distal N-terminal isoforms caused stronger up-regulation, consistent with our previous evidence that the distal N terminus enhances DNA binding by Runx1 (15). CD8 expression levels are not affected by the expression of either distal or proximal full-length Runx1. In addition, there is no increase in the small number of CD4+CD8+ splenic T cells, indicating that Runx1 does not trans activate CD4 in CD8+ splenic T cells (data not shown). Distal or proximal Δ5 Runx1 expression in splenic T cells does not alter CD4 expression, suggesting that the C terminus of Runx1 may trans activate, rather than silence, CD4 in CD4+ T cells. This suggests that the mechanism of turning off CD4 expression in DP thymocytes and the mechanism of maintaining the CD4 locus in an “off” configuration in mature CD8+ T cells are distinct. This is consistent with data from mice with a conditional deletion of the CD4 silencer in CD8+ T cells, in which CD4 expression continues to be repressed, whereas deletion of the CD4 silencer in DP thymocytes results in the expression of CD4 throughout thymocyte development and in CD8+ T cells (37, 38).

Expression of either Runx1 or Runx3 results in the down-regulation of CD4 expression in DP thymocytes: essential roles of the nuclear matrix targeting sequence and aa 212–263 for CD4 repression in thymocytes

Enforced expression of Runx1 or Runx2 in the thymus of transgenic mice results in an increase in the production of CD8 SP thymocytes (9, 10). Our results suggest that the increase in CD8 SP thymocytes in these mice results from CD4 silencing in DP thymocytes. Expression of Runx3 has been reported to be required for CD4 silencing (11, 12). This suggests that all three family members have the capacity to silence CD4. In agreement with this hypothesis, enforced expression of Runx3 in bcl-2 Δ2 DP thymocytes results in the down-regulation of CD4 expression (Fig. 6). Because all three Runx family members are capable of silencing CD4, we chose to target conserved regions for deletion, as indicated in Fig. 7. We also deleted a region that is responsible for the
association of Runx1 with the corepressor Sin3A (25), but which is not highly conserved between Runx1, 2, and 3 (Fig. 7). Deletion of the Sin3A association domain (aa 181–210) has no effect on the Runx1-mediated silencing of CD4 in bcl-2+ thymocytes (Fig. 6), indicating that Sin3A interaction with Runx1 is not required to silence CD4. This is in accord with the relative lack of conservation of this domain between the Runx family members. Deletion of the conserved aa 361–451, downstream of the nuclear matrix targeting sequence, also leaves Runx1-mediated CD4 silencing largely intact (Fig. 6). In contrast, deletion of the region (aa 263–360) containing the conserved nuclear matrix targeting sequence (aa 318–358) virtually abrogates Runx1-mediated silencing of CD4. It is unlikely that aa Runx1 263–301 are required for CD4 silencing, because the corresponding amino acids are absent in murine Runx3 (Fig. 7). Deletion of a second region containing conserved amino acid residues that leaves the nuclear matrix targeting sequence intact (aa 212–262; Fig. 7) also decreases Runx1-mediated silencing of CD4, although not to the same extent as deletion of the region containing the nuclear matrix targeting sequence. Deletion of the C terminus while leaving intact the nuclear localization sequences and DNA binding domain causes the Runx protein to lose all activity as a silencer, reducing the effectiveness of CD4 silencing below the level of control. This implies that occupation of the Runx binding sites in the CD4 silencer by the retrovirally expressed protein blocks CD4 silencing by endogenous Runx proteins.

The Runx transcription factor family is highly conserved in vertebrates, and an isoform of Runx3 recently cloned from an elasmobranch (Raja eglanteria, the clearnose skate) includes most, but not all, homologous C-terminal domains of murine Runx3 (Fig. 7).
These results are in good agreement with the evidence derived from transgenic mice expressing Runx1 or Runx2 under the control of T cell lineage-specific promoters and from mice ablated for one or both alleles of Runx1 or Runx3, or Runx target sites in the CD4 gene (9–13). Our results extend in several ways the insights obtained from these steady state analyses of mice with genetic modifications affecting Runx expression. First, not only full-length isoforms of Runx1 mRNA are produced by the intact Runx1 gene, but also the truncated variants investigated in this study. As we show, these appear to act in vivo as competitive inhibitors for the full-length isoforms’ negative effect on thymocyte proliferation or survival. A null mutation in a gene can preclude the ability to express both kinds of forms. We would note that thymocyte population expansion generally, and accumulation of DP thymocytes specifically, may depend in vivo on an adequate level of the truncated isoforms. Thus, a Runx1 null mutation resulting in a lowered thymocyte cell count in vivo does not necessarily imply a requirement for full-length Runx1 and its associated DNA binding, repression, and trans activation domains. The phenotype might also arise from an absence of the truncated Runx1 isoforms. Second, our retroviral transduction system enables us to target specific stages of development for perturbation from a known, normal starting baseline. This is important because gene dosage of Runx family members crucially affects hematopoietic differentiation stages back to the hemangioblast and hemopoietic stem cell (39–42). In mice with a constitutive null mutation or in transgenic mice, the existence of any thymus-populating cells could depend on unknown, compensatory alterations. Finally, this analysis has enabled us to identify specific regions of the Runx1 transcription factor that are most critical for the stage-specific repression of CD4. The results argue against roles for the corepressors Groucho and Sin3A and suggest that the nuclear matrix targeting sequence is involved in this function.

The suspension culture system that we describe using bcl-2+ thymocytes clearly shows that Runx1 or Runx3 can act by specifically down-regulating CD4 expression as soon as CD4 is expressed in DP thymocytes, with ersetz CD8 SP cells accumulating. It remains to be seen whether such disguised DP cells are also the source of the more mature, TCRβ-high CD8 SP cells that can be generated by Runx1-transduced cells in fetal thymic organ culture. Positive selection signaling via MHC class I ligation of the CD8-TCRαβ complex has been postulated to deliver survival signals or to send a signal to down-regulate CD4 expression in normal thymocyte development. However, the short-term suspension culture system shows that Runx1 or Runx3 can act on the CD4 locus independently of positive selection, which does not occur in the single-cell suspension culture. This suggests that Runx repression of CD4 is downstream of signaling from the CD8-TCRαβ complex.

The CD4 locus is responsive to Runx-mediated repression as soon as CD4 is up-regulated in the DP compartment, but loses this responsiveness in CD4+ splenic T cells, in agreement with in vivo data using Runx site mutants of the CD4 silencer (13, 37, 38). Runx family members are the first transcription factors described as capable of down-regulating CD4 specifically in the DP compartment. The fact that cells can respond this way demonstrates that DP cells contain the cofactors needed to carry out the repression. This implies that under normal conditions, the expression of CD4 in DP cells depends on a mechanism that keeps the repressive activity of full-length Runx proteins in check. It is tempting to speculate that the truncated forms of Runx1 could play a role in this antagonism. The DN to DP transition, when CD4 is normally turned on in murine thymocytes, is also the phase of the most rapid...
proliferation, in accord with the growth advantage seen with the expression of the truncated isoforms.

A model in which DP thymocytes receive a signal instructing them to down-regulate CD4 would require the cooperation of a transcriptional repressor active only in those cells. However, in vivo, CD4 expression is not down-regulated until positive selection is well underway. Analyses of the timing of the shut-off of de novo CD4 expression in positive selection (43, 44) imply that the Runx-repressive complex would only be assembled in CD4⁺CD8⁻ DP intermediates. What role does TCR signaling play in mobilizing CD4 repression by Runx factors? Runx3 has been reported to be required for CD4 silencing during thymocyte development into CD8⁺ CTLs (11, 12). Both Runx1 and Runx3 are expressed in the thymus (9, 45). However, neither Runx3 mRNA nor Runx1 mRNA levels differ significantly between CD4 SP and CD8 SP thymocytes. Our finding that enforced expression of either full-length Runx1 or Runx3 can silence CD4 in DP thymocytes supports the hypothesis that signals leading to CD4 silencing in positive selection trigger increased Runx3-repressive activity without an increase in Runx3 mRNA. This increase in

FIGURE 6. Either murine Runx1 or Runx3, but not Runx3 from the skate, can silence CD4 expression in thymocytes. The Runx1 nuclear matrix targeting sequence and Runx1 aa 212–262 are required for complete Runx1-mediated CD4 repression in thymocytes. CD4⁺CD8⁻ bel-2⁺ transgenic thymocytes were infected by murine Runx1, murine Runx3, and R. eglanteria Runx3 retroviral constructs. Schematics of the constructs indicate the runt DNA binding domain (red box) and nuclear matrix targeting sequence (blue box). Thymocytes were infected with retroviral constructs encoding GFP alone (MSCV, 1), distal full-length Runx1 (Dis.full Runx1, 2), murine Runx3 (mRunx3, 3), distal Runx1 truncated at aa 190 (Dis.d190 Runx1, 4), distal Runx1 truncated at aa 211 (Dis.d211 Runx1, 5), isolated runt DNA binding domain with an exogenous nuclear localization sequence (Runt.NLS, 6), distal Runx1 truncated at aa 361 (Dis.d361 Runx1, 7), distal Runx1 with an internal deletion from aa 263–360 (Dis.d263–360 Runx1, 8), distal Runx1 with an internal deletion from aa 212–262 (Dis.d212–262, 9), distal Runx1 with an internal deletion from aa 181–210 (Dis.d181–210, 10), and R. eglanteria (clearnose skate) Runx3 (R. eglanteria Runx3, 11). The GFP-negative (no fill) and GFP-positive (green tint) populations are shown as overlaid histograms with the level of CD4 expression on the x-axis, at 60 h postinfection. Percentages in the overlaid histograms indicate the percentage of CD4 low (range indicated by the horizontal bars) cells in the GFP-positive cell populations (green-tinted histograms). The geometric mean fluorescence intensities of CD4 staining of the GFP-positive cell populations (green-tinted histograms) are indicated below the horizontal bars.
activity could arise from either alternative splicing to include the domains necessary for silencing, or increased translational efficiency. Alternative splicing and regulation by translational efficiency are bypassed by our retroviral constructs; posttranslational modifications and differences in cofactor activity are not. In support of this hypothesis, two groups report that there is normally more full-length Runx3 protein in CD8 SP thymocytes than CD4 SP thymocytes (9, 46), contrasting with the similarity in RNA expression. We have found no difference in the CD4-silencing activity of retrovirally expressed Runx1 and Runx3 in thymocytes treated with mitogen-activated protein kinase inhibitors (data not shown), showing that posttranslational modification of serines in the region C-terminal to the runt domain (47) is not required for Runx-mediated CD4 repression.

We demonstrate that CD4 down-regulation by Runx1 requires the presence of the Runx1 C terminus downstream of aa 210, but does not require the sequences thought to mediate Runx1 interaction with the corepressors Groucho or Sin3A. It is intriguing that deletion of the sequence containing the nuclear matrix targeting sequence abrogates Runx1-mediated silencing of both CD4 in thymocytes and p21Waf1/Cip1 in NIH3T3 cells (25). The Runx nuclear matrix targeting sequence is responsible for the subnuclear localization of this molecule and has been associated with increased trans activation (48–51) or repression (25) of target genes. An attractive possibility is that the nuclear matrix targeting sequence stabilizes Runx binding to the CD4 silencer, and that, in the absence of the nuclear matrix targeting sequence, Runx rapidly diffuses away from the CD4 locus and/or from corepressor molecules associated with sites on the nuclear matrix.

Deletion of aa 212–263 from Runx1 also makes Runx1 less effective as a repressor of CD4 repression, possibly by deleting a site of interaction with an unknown corepressor or by interfering with the recruitment of a histone deacetylase. Murine Runx3 does not include regions corresponding to Runx1 aa 242–297, but represses actively nonetheless, making aa 212–241 more likely to play a critical role. The skate Runx3 isoform that has been cloned to date cannot repress CD4. In addition to regions of Runx1 that are absent in mouse Runx3, the skate isoform of Runx3 lacks an

| FIGURE 7. Alignment of murine Runx1, murine Runx2, murine Runx3, and R. eglanteria Runx3 regions downstream of the runt domain. Amino acids that are identical between the molecules are indicated by an asterisk; those that are similar are indicated by a colon or period. Gaps to facilitate alignment are indicated by dashes. Numbering of amino acids is taken from murine proximal Runx1. The C-terminal portion of the highly conserved runt domain is highlighted in yellow. A putative nuclear localization signal (NLS) is boxed. Amino acids removed from the deletion constructs of distal Runx1 used in Figs. 4 and 6 are highlighted (pink, deletion of aa 181–210; orange, deletion of aa 212–262; underlined, deletion of aa 263–360; and blue, the truncation removing the site of corepressor Groucho/TLE association, aa 447–451). Residues that are conserved between murine Runx1, Runx2, and Runx3, but which are not present in R. eglanteria Runx3 in the region from aa 212–262 (orange) are bolded. The nuclear matrix targeting sequence (NMTS) is highlighted in green. |
additional region corresponding to Runx1 aa 178–241. Runx1 aa residues 178–241 are encoded by Runx1 exon 6. In this region, amino acids potentially required for CD4 silencing by mammalian Runx1, 2, or 3 include Runx1 aa 178–180 (Fig. 7, RHR) and conserved aa from 222–235 (Fig. 7, bolded). Taken together, these results map one of the functions needed to repress CD4 to the part of the protein encoded by Runx1 exon 6. Exon 6 is often spliced out of Runx1 mRNA transcripts from the endogenous gene, and the resulting proteins have been reported to be capable of trans activation (31). Results obtained with deletion mutants of Runx2 and the exon 6-deficient splice isoforms of Runx1 conflict as to whether the amino acids corresponding to Runx1 aa 178–180 are necessary for nuclear localization (52, 53). However, amino acids in the region corresponding to aa 222–235 are conspicuous for their conservation among the mammalian Runx family members and lie within the region corresponding to aa 212–244, which appears to be required for full Runx1-mediated silencing activity. We are therefore investigating the possibility that these conserved amino acids represent an interaction site for a novel corepressor.

We have shown that either Runx1 or Runx3 can down-regulate CD4 expression specifically in DP thymocytes independent of positive selecting signals. Further studies will be necessary to understand the relationship between Runx subnuclear localization and the nature of the control of the switch between Runx-mediated trans activation and repression.

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References


