RUNX1-RUNX1 Homodimerization Modulates RUNX1 Activity and Function*

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RUNX1 (AML1, CBFa2, PEBP2αB) is a transcription factor essential for the establishment of the hematopoietic stem cell. It is generally thought that RUNX1 exists as a monomer that regulates hematopoietic differentiation by interacting with tissue-specific factors and its DNA consensus through its N terminus. RUNX1 is frequently altered in human leukemia by gene fusions or point mutations. In general, these alterations do not affect the N terminus of the protein, and it is unclear how they consistently lead to hematopoietic transformation and leukemia. Here we report that RUNX1 homodimerizes through a mechanism involving C terminus-C terminus interaction. This RUNX1-RUNX1 interaction regulates the activity of the protein in reporter gene assays and modulates its ability to induce hematopoietic differentiation of hematopoietic cell lines. The promoters of genes regulated by RUNX1 often contain multiple RUNX1 binding sites. This arrangement suggests that RUNX1 could homodimerize to bring and hold together distant chromatin sites and factors and that if the dimerization region is removed by gene fusions or is altered by point mutations, as observed in leukemia, the ability of RUNX1 to regulate differentiation could be impaired.

RUNX1, also known as AML1, CBFa2, and PEBP2αB, is the DNA binding subunit of a highly conserved transcription factor that regulates differentiation. Studies of targeted mutagenesis have shown that RUNX1 and its co-factor CBFB are essential for the establishment of definitive hematopoiesis in the murine developing embryo (1–6). In vitro studies showed that RUNX1 regulates the expression of several factors modulating the expression of receptors, cytokines, and enzymes that regulate the hematopoietic programs (7–12). More recently, several investigators suggested that RUNX1 also has role in the proliferation and cell renewal of the hematopoietic progenitor cells (13, 14). RUNX1 contains several domains with specific functional activity. At the N terminus there is the evolutionarily conserved DNA binding domain (runt domain (RD))3 (15, 16) that interacts with several hematopoietic factors and the methyltransferase SUV39H1 (17–19). Approximately in the center of the protein there is the transactivation domain, which directly interacts with co-activators (CBP/p300, MOZ) and co-repressors (Sin3A) modulating the gene-transcription activity of RUNX1 (20, 21). This region also contains a nuclear matrix targeting signal and an inhibitory domain (22, 23). At the C terminus, the protein terminates with a pentapeptide perfectly conserved throughout evolution that interacts with the repressor TLE (24–26).

RUNX1 is frequently targeted in human leukemia by three separate mechanisms. RUNX1 is point-mutated in familial platelet disorder and myelodysplastic syndrome leading to amino acid substitution or premature termination of translation. These mutations generally impair either the ability of the protein to bind efficiently to DNA or the transactivating activity of RUNX1, leading therefore to a loss of RUNX1 function (27–31). Alternatively RUNX1 is profoundly altered by chromosomal rearrangements leading to fusion proteins that generally display opposite or deregulated activity compared with the wild type RUNX1 (32–39). More recently it was shown that the RUNX1 locus is also the target of a genomic amplification that results in transcription overexpression and presumably in overall gain of function in lymphoblastic leukemia (40–43). Of these three mechanisms, perhaps the one best studied at the molecular level is the generation of fusion proteins after chromosomal translocations. A common feature of the fusion partners of RUNX1 is their ability to associate with chromatin remodeling factors and to form dimers through their own self-interaction domains that are invariably retained in the fusion proteins with RUNX1. Thus, it is generally thought that through this self-interaction domain the fusion proteins acquire the ability to recruit to RUNX1-regulated promoters inappropriate transcription complexes that deregulate hematopoietic gene programs (44–45). Given the crucial role of correct gene expression during hematopoiesis, it is likely that the minimal disturbance of gene expression could indeed have a dominant effect in the final ratio between progenitor cells and differentiated cells and in the abundance and balance of the hematopoietic mature cells. This model is based in part on the presumption that RUNX1 is unable to interact with itself or with the fusion proteins. However, here we show that RUNX1 is capable of self-interaction through the C terminus in a region previously described as an inhibitory domain (22). When

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2 The abbreviations used are: RD, runt domain; HA, hemagglutinin; IP, immunoprecipitation; NLS, nuclear localization signal; aa, amino acids; ID, inhibitory domain; GST, glutathione S-transferase; M-CSF, macrophage colony-stimulating factor; G-CSF, granulocyte-CSF; CSFR, CSF receptor.

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expressed separately, this domain represses in trans the transcription regulatory activity of the normal RUNX1 and disrupts the hematopoietic differentiation of the 32Dc3 cells, suggesting that self-interaction regulates the transcription activity of RUNX1, and loss of autoregulation could affect the ability of RUNX1 to execute correctly the differentiation programs. These results introduce a new level of complexity to the generally accepted model of leukemic transformation by RUNX1 fusion proteins.

**EXPERIMENTAL PROCEDURES**

**Construction of Deletion Mutants**—All the deletion mutants were generated by standard or PCR-mediated cloning techniques and inserted in pCMV-FLAG (Sigma) or in a modification of pCMV-FLAG in which the HA epitope replaced the FLAG epitope (pCMV-HA) or in modified pCMV/myc/nuc (Invitrogen) with a FLAG or HA epitope inserted. When necessary, a nuclear localization signal (NLS) was added in-frame before the stop codon. All the clones were DNA-sequenced to confirm that no sequence error had been introduced.

Specifically, for construction of FLAG-RUNX1-ID (F-ID), FLAG-RUNX1-IDs (F-IDs), HA-RUNX1-ID (H-ID), and HA-RUNX1-IDs (H-IDs), DNA fragments corresponding to amino acids 346–411 (ID) and amino acids 372–411 (IDs) were amplified by PCR and subcloned in modified pCMV/myc/nuc downstream of FLAG or HA epitope. For construction of HA-RUNX1-RD (H-RD), FLAG-RUNX1-RD (F-RD), FLAG-RUNX1-ΔRD (ΔRD), and HA-RUNX1-ΔRD (ΔRD), DNA fragments corresponding to amino acids 346–411, amino acids 1–177 and amino acids 1–177 and amino acid 185–453 were amplified by PCR and subcloned in pCMV-FLAG or pCMV-HA.

FLAG-RUNX1-ΔRDΔID (ΔRDΔID) was generated by overlapping PCR. Briefly, two sets of primers (AML1–1387 and AML1–371R and primers AML1–371F and AML1–1917SalI) were used to generate overlapping fragments excluding the region between amino acids 371 and 411. These two fragments were annealed and amplified with primers AML1–1387 and AML1–1917SalI all were maintained as described (47). The 32Dc3 cells were maintained in RPMI containing 10% newborn bovine serum. DNA-transfection of adherent cells was performed by calcium phosphate precipitation (Invitrogen) or with the Escort IV transfection kit (Sigma) according to the manufacturer’s instructions. The cells were harvested 48 h after transfection and treated as described (46). Proteins separation on denaturing gels and their transfer to a polyvinylidene difluoride membrane were carried out as described (46). The protein bands were identified with commercially available monoclonal rat antibody to the HA epitope (Roche Applied Science) and monoclonal mouse antibody M2 (Sigma) or goat antibody ECL (Bethyl) to the FLAG epitope.

Co-IP assays were performed by FLAG beads (Sigma). The 32Dc3 cells were maintained in RPMI containing 10% newborn bovine serum and interleukin-3 supplement. To generate stable expression of integrated genes, the 32Dc3 and the SW13 cell lines were infected or transfected, selected with G418, and maintained as described (47). The 32Dc3 cells were differentiated as described (48).

**GST Fusion Pulldown Assays**—The expression and purification of GST fusion proteins and their interaction with RUNX1 were carried out as described (49).

**Reporter Gene and Mammalian Two-hybrid Assays**—We used NIH-3T3 cells for reporter gene studies with the RUNX1-responsive M-CSFR promoter linked to the luciferase reporter gene (pM-CSFR-luc) containing the −416 to +71 region of the human M-CSFR promoter (50). For normalization of transfection efficiency, we used pRL-TK plasmid (Promega) that expresses Renilla luciferase. Each assay was repeated at least three times. For mammalian two-hybrid assays, FLAG-RUNX1 and FLAG-RUNX1-ΔRD were fused in-frame to the activation domain of VP16. HA–RUNX1-ΔRD was cloned in-frame to the DNA binding domain of GAL4. The plasmid pG5Luc (Promega) with five GAL4 binding sites upstream of the luciferase coding sequence was used as reporter plasmid. Plasmid pRL-TK was an internal control. The assays were carried out by transient transfection in HeLa cells and the luciferase readings were recorded with a Promega Dual-Luciferase Assay System by luminometer (Turner Design TD20/20).
**RUNX1 Function Is Regulated by RUNX1-RUNX1 Homodimerization**

*Immunofluorescence Analysis*—HeLa cells were cultured on glass slides in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% newborn bovine serum. The initial concentration of the cells was 1 × 10⁶ cells/ml. After 24 h the transfected cells were fixed in 100% methanol at −20 °C and processed for indirect immunofluorescence. As primary antibodies, we used rat monoclonal anti-HA (Roche Applied Science) and mouse monoclonal anti-FLAG (Sigma). Alexa Fluor 594 goat anti-rat IgG (H+L) conjugate and Alexa Fluor 488 goat anti-mouse IgG (H+L) highly cross-adsorbed (Molecular Probes) were used as secondary antibodies. Background binding of the antibodies was reduced by preincubation with bovine serum albumin (5% in phosphate-buffered saline, Sigma). Cells were incubated with primary antibodies for 1 h at room temperature, washed in phosphate-buffered saline, and then incubated with the secondary antibodies for 45 min. The specific binding and cellular distribution of the antigens were assessed by fluorescence microscopy (Zeiss). To visualize the nucleus, we used 4′,6-diamidino-2-phenylindole (Sigma).

**RESULTS**

RUNX1 Homodimerizes in Vitro and in Vivo—The promoters of several genes regulated by RUNX1 often contain multiple DNA consensus motifs for RUNX1 binding. These recognition sequences can be found next to each other or separated by a few dozen to a few hundred base pairs, and almost always they are located next to the recognition site of other factors (C/EBPs, GATA1, PU.1, etc.) that cooperate with RUNX1 in gene regulation. The organization of these promoters suggest that RUNX1 could interact with itself to either stabilize its own DNA interaction in adjacent sites or to facilitate/stabilize long range interactions by bringing together the proteins located next to distant RUNX1 sites. To determine whether RUNX1 is capable of self-interaction, we used co-IP assays of 293T cells transiently co-transfected with HA- and FLAG-tagged RUNX1. The results shown in Fig. 1A indicate that RUNX1 interacts with itself (lane 3, top panel). To confirm these results and determine whether the interaction is direct, we performed a GST pulldown assay. We used bacterially expressed and purified GST-RUNX1 and in vitro translated RUNX1. The results shown in Fig. 1B indicate that in vitro translated RUNX1 interacts with GST-RUNX1 immobilized on a solid support.

The DNA binding domain of RUNX1, RD, located at the N terminus of the protein, is very highly charged and tends to interact non-specifically with other proteins. The occurrence of nonspecific interaction could be especially evident at high level of protein expression. Therefore, to avoid potential false positive results, we generated a stable RUNX1-expressing SW13 cell line, SW13-FLAG-RUNX1 (S-F-R), by transfection and selection. To ensure that the level of stably expressed RUNX1 was low, we selected those clones in which the expression of RUNX1 was detected after immunoprecipitation but not by Western blotting (not shown). We used these low expressing cell lines to verify RUNX1-RUNX1 interaction after transient transfection. Untransfected SW13 cells were used as the control. The cells were lysed 48 h after transfection, and the proteins were co-immunoprecipitated with anti-FLAG antibody, separated by electrophoresis, transferred to a membrane, and analyzed with anti-HA antibody. The results show that HA-RUNX1 co-precipitates with FLAG-RUNX1 (Fig. 1C, lanes 5 and 6, top panel), indicating that RUNX1 interacts with itself. Anti-FLAG antibody did not precipitate HA-RUNX1 in SW13 cells (Fig. 1C, lanes 2 and 3, top panel). Lanes 4–6, bottom panel, confirm the presence of immunoprecipitated FLAG-RUNX1 in S-F-R cell lysates. Lanes 8, 9, 11, and 12, top panel, show the expression of HA-RUNX1 transiently transfected in normal SW13 and S-F-R cells.

**FIGURE 1.** RUNX1 interacts with itself. A, 293T cells were transiently transfected with HA-RUNX1 alone (lane 2) or together with FLAG-RUNX1 (lane 3). Two days after transfection, the proteins were immunoprecipitated (IP) with anti-FLAG antibody. The proteins from the immuno complex, and the cell lysates (CL) were separated by electrophoresis, transferred to a membrane, and analyzed by Western blotting (WB) with anti-HA (top panel) and anti-FLAG (bottom panel) antibodies. FLAG-RUNX1 efficiently interacts with HA-RUNX1 (lane 3, top panel). Anti-FLAG antibody did not precipitate HA-RUNX1 itself (lane 2, top panel). Lanes 5 and 6 show the expression of transiently transfected proteins in the cell lysates. Lanes 1 and 4 represent mock-transfected cells. B, recombinant GST-RUNX1 was generated in E. coli cells and tested for its capacity to interact with RUNX1 itself. Purified GST-RUNX1 conjugated to glutathione-Sepharose beads was incubated with in vitro translated (IVT) RUNX1. After extensively washing the beads were subjected to SDS-PAGE, and the separated proteins were analyzed by autoradiography. Lane 1 shows the in vitro translated ³⁵S-labeled RUNX1 input. ³⁵S-Labeled RUNX1 interacts with purified GST-RUNX1 (lane 2) but not with GST (lane 3). C, stably transfected RUNX1-expressing SW13 cells, SW13-FLAG-RUNX1 (S-F-R), and naive SW13 cells as control were transfected with HA-RUNX1. Two days after transfection, the proteins in the cell lysates were analyzed as described in A. Lanes 5 and 6, top panel, indicate the self-interaction of RUNX1. Anti-FLAG antibody did not precipitate HA-RUNX1 in SW13 cells (lanes 2 and 3, top panel), Lanes 4–6, bottom panel, confirm the presence of immunoprecipitated FLAG-RUNX1 in S-F-R cell line. The expression of transiently transfected HA-RUNX1 is shown in the right top panel. The lower right panel shows that the level of stably transfected RUNX1 is too low to be detected by Western blotting. F, FLAG; H, HA; R, RUNX1. SW13 cell line is indicated as S.
RUNX1 Function Is Regulated by RUNX1-RUNX1 Homodimerization

**The RUNX1-RUNX1 Interaction Involves the C Termi

nus of the Protein**—To map the interaction domain, we tested the interaction of RUNX1 separately with either the N terminus, consisting mostly of the runt-domain (RUNX1-RD), or the C terminus, which does not contain runt domain (RUNX1-ΔRD). The diagram of the plasmids used in this study is shown in Fig. 2. We used co-IP assays of SW13-FLAG-RUNX1 (S-F-R) cells and normal SW13 cells for transient transfection with the N terminus (RUNX1-RD) or the C terminus (RUNX1-ΔRD). The results shown in Fig. 3A indicate that full-length RUNX1 is unable to co-precipitate with RUNX1-RD (lane 4, top panel). However, the C terminus of the protein, RUNX1-ΔRD, co-precipitates with RUNX1 (Fig. 3B, lane 4, top panel). Anti-FLAG antibody did not precipitate the RUNX1-ΔRD in SW13 cells (Fig. 3B, lane 2, top panel). The expression of RUNX1 in S-F-R cells is shown in lanes 3 and 4 of Fig. 3, A and B, lower panels. The expression of HA-tagged transiently transfected RUNX1-RD or RUNX1-ΔRD in SW13 cells and S-F-R cells is confirmed in the top panels (lanes 6 and 8).

The **C Terminus of RUNX1 Interacts with Itself**—To confirm these results, we generated the SW13 cell line SW13-HA-RUNX1-ΔRD (S-H-ΔRD) that expresses the stably integrated C terminus (RUNX1-ΔRD) and used these cells in co-IP assays with the C terminus itself or with the runt domain (RUNX1-RD). Full-length RUNX1, RUNX1-ΔRD, or RUNX1-RD was transiently transfected in S-H-ΔRD cells or in normal SW13 cells. After co-IP, the proteins were separated and analyzed by Western blotting. The results of the assay, shown in Fig. 4, indicate that RUNX1 and the C terminus (lanes 6 and 7, top panel) but not the runt domain (lane 8, top panel) are able to interact with the stably expressed C terminus (HA-RUNX1-ΔRD). This interaction does not occur in control cells (lanes 1–4, top panel) or in untransfected cells (lane 5, top panel). After the detection of the proteins with HA antibody, the blot was stripped and hybridized to anti-FLAG antibody (lanes 1–8, bottom panel). The right side of the figure shows the expression of the proteins in cell lysates by Western blotting. These results indicate that C terminus of RUNX1 interacts with itself but not with the N terminus runt domain.

**The Inhibitory Domain Is the Major Interaction Region**—Somatic or inherited point mutations introducing frameshifts or stop codons that inhibit translation of the C terminus are often detected in RUNX1 patients (28–30). Through its interaction with other factors, the C terminus of RUNX1 carries out several functions that are necessary for RUNX1 signaling. Between amino acids (aa) 291 and 371 there is a region that interacts with co-activators and modulates gene transcription. This region is generally indicated as an activation domain and also contains a nuclear matrix targeting signal (aa 324–353). By deletion mutation analysis it was previously reported that a short amino acid region between aa 372 and 411 functions as a transcription inhibitory domain (ID). Based on these functional boundaries we generated several mutants, shown in Fig. 2, that were used to further map the interaction region within the C terminus of RUNX1. We first generated two fragments containing the inhibitory domain (ID) spanning aa 346–411 and including part of nuclear matrix targeting signal (named ID).
**RUNX1 Function Is Regulated by RUNX1-RUNX1 Homodimerization**

**FIGURE 4.** The C terminus of RUNX1 interacts with itself in stably transfected cell line. Stably transfected RUNX1-ΔRD-expressing SW13 cells, SW13-HA-RUNX1-ΔRD (S-H-ΔRD), and naive SW13 cells as control were transfected with FLAG-tagged RUNX1, RUNX1-ΔRD, and RUNX1-ΔRD and analyzed as described in Fig. 1. FLAG-tagged RUNX1 and RUNX1-ΔRD are able to interact with stably expressed HA-RUNX1-ΔRD (lanes 6 and 7, top panel), but FLAG-RUNX1-ΔRD is unable to co-precipitate HA-RUNX1-ΔRD (lane 8, top panel). Anti-FLAG antibody did not precipitate HA-RUNX1-ΔRD in S-H-ΔRD cell line (lane 5, top panel). The right panels show the expression of stably transfected RUNX1-ΔRD and transiently transfected FLAG-tagged RUNX1, RUNX1-ΔRD, and RUNX1-ΔRD (bottom) in the cell lysates. F, FLAG; H, HA; R, RUNX1; IP, immunoprecipitate; CL, cell lysates; WB, Western blot. SW13 cell line is indicated as S.

aa 372–411 (named IDs). Both fragments were HA- or FLAG-tagged at the N terminus and included a NLS at the C terminus to provide appropriate localization. In addition, we generated two internal deletion mutants in the C terminus lacking aa 372–411 (RUNX1-ΔRDΔID) or aa 351–411 (RUNX1-ΔRDΔ351–411). These two mutants were FLAG-tagged at the N terminus and included a NLS at the C terminus.

First, we tested the ID region using co-IP assays in 293T cells. As shown in Fig. 5A, differently tagged ID regions efficiently co-precipitate together (lane 3, top panel). The importance of aa 351–411 for interaction is confirmed with the larger C terminus deletion mutant that does not have this region (RUNX1-ΔRDΔ351–411) because this mutant does not significantly interact with the ID (lane 5, top panel). The smaller C terminus deletion mutant lacking aa 372–411 (RUNX1-ΔRDΔID) interacts weakly with the ID (lane 4, top panel), indicating that the major interaction surface is contained between aa 372 and 411. The assays were repeated with the smaller IDs fragment spanning aa 372–411. The results (Fig. 5B) indicate that this short region consisting only of the inhibitory domain co-precipitates with itself (lane 5, top panel) but not with the C terminus with the larger (RUNX1-ΔRDΔ351–411) or the smaller (RUNX1-ΔRDΔID) deletion (lanes 6 and 7, top panel). Lanes 8–12 in Fig. 5A and lanes 9–15 in Fig. 5B show the expression of the transfected proteins in cell lysates. Lanes 1 and 7 in Fig. 5A and lanes 8 and 16 in Fig. 5B are mock-transfected controls. To determine whether the interaction region extends at either side of the ID, we generated two clones that contain only the proximal or distal region of the C terminus flanking the ID (RUNX1-185–351 and RUNX1-411–453), see the schematic in Fig. 2). Each of these clones included a NLS and was used in co-IP assays. The results (Fig. 5C) show that the ID efficiently co-precipitates with the C terminus (RUNX1-ΔRD, lane 6, top panel). In contrast, the proximal and distal fragments aa 185–351 and aa 411–453 interact weakly with the C terminus (lanes 7 and lane 8, top panel). Lanes 13–16 show the expression of the proteins. Taken together these results indicate that the C terminus of RUNX1 interacts with itself and that the major region of interaction spans the inhibitory domain between aa 372 and 411. They also suggest that the interaction expands weakly both at the left and right sides of the inhibitory domain.

**Full-length RUNX1 Lacking the ID Region Does Not Interact with Itself**—To exclude artifacts due to high protein expression, we generated an SW13 cell line expressing stably transfected FLAG-tagged ID (S-F-ID) and tested the cells with full-length RUNX1. The results (Fig. 6A) show that RUNX1 co-precipitates with the small ID fragment (lane 4, top panel). Anti-FLAG antibody did not precipitate HA-RUNX1 in SW13 cells (lane 2, top panel). Lane 3 and 4, lower panels, show the expression of FLAG-ID in the cell line. Lanes 6 and 8, top panels, show the expression of transiently transfected HA-RUNX1. To exclude additional interaction sites in the intact RUNX1, we generated a RUNX1 deletion mutant missing only aa 351–411 (RUNX1-Δ351–411). This clone was first tested in SW13 cells expressing the C terminus (S-H-ΔRD). The results (Fig. 6B) show that only the intact full-length RUNX1 (lane 5, top panel) but not the RUNX1 internal deletion mutant (lane 6, top panel) interacts with the C terminus (HA-RUNX1-ΔRD). Anti-FLAG antibody did not interact with the C terminus (lane 4, top panel). Lanes 10–12, top panel, show the expression of the C terminus (HA-RUNX1-ΔRD) in S-H-ΔRD cells. The bottom panels show the expression of transiently transfected proteins in SW13 and in S-H-ΔRD cells. These assays were repeated with the SW13 cells expressing stably transfected HA-RUNX1 (S-H-R) to compare the interaction of RUNX1 with itself or with RUNX1-Δ351–411 or with the ID. The results (Fig. 6C) show that RUNX1 and the ID region efficiently interact with full-length RUNX1 (lanes 6 and 8, top panel) but not with the internal deletion mutant RUNX1-Δ351–411 (lane 7, top panel). Anti-FLAG antibody did not interact with HA-RUNX1 (lane 5, top panel). Lanes 13–16, top panel, show the expression of HA-RUNX1 in S-H-R cell line. The bottom panels show the expression of transiently transfected proteins in SW13 cells and S-H-R cell line.

**Full-length RUNX1 Can Support the Nuclear Localization of the Nuclear Import-defective Inhibitory Domain**—Immunofluorescence microscopy analysis is a powerful tool to visualize the location of a protein within the cell. It is well known that RUNX1 is a nuclear factor and that the nuclear localization signal was identified within the RD. It was previously reported that the C terminus itself does not localize to the nucleus. Therefore, if our results are correct and if indeed the two proteins interact in vivo, then we should be able to observe their co-localization to the nucleus where the C terminus would be recruited by RUNX1. To determine whether this occurs, we transiently transfected HeLa cells with either RUNX1 or the ID region or the two proteins together. The results (Fig. 7) confirm that RUNX1 (left panels) is nuclear, but the ID region (center panels) is distributed in both nucleus and cytoplasm. However, when both RUNX1 and RUNX1-ID were co-expressed in HeLa
cells, the ID region was visualized only in the nucleus, confirming the interaction in vivo (right panels).

The Mammalian Two-hybrid System Confirms C terminus–C Terminus Interaction in Vivo—We used the mammalian two-hybrid assay to confirm that RUNX1 and the C terminus interact in vivo. The C terminus of RUNX1 was fused in-frame to the DNA binding domain of Gal4 and used as bait for the assay. The full-length RUNX1 or the C terminus (RUNX1-ΔRD) were separately fused to the transactivation domain of the herpes virus protein VP16 and were used as prey. The plasmids were transiently co-transfected in HeLa cells with constant amount of a plasmid expressing Renilla luciferase as a control for transfection efficiency. As seen in Fig. 8 there was a significant increase in luciferase activity only when the C terminus was present in both prey and bait plasmids as shown in panel 4, reporting the results for GAL4-ΔRD and VP16-ΔRD co-transfection, and in panel 6, reporting GAL4-ΔRD and VP16RUNX1 co-transfection. Various combinations of plasmids in which the C terminus was expressed in only one of them or in none of them did not induce significant activation (bars 1, 2, 3, and 5).

The Inhibitory Domain of RUNX1 Impedes the Transactivation Activity of RUNX1—We thought that the RUNX1-RUNX1 interaction could have a role in the regulation of RUNX1 transactivation potential. Therefore, we performed reporter gene assay with the M-CSF receptor promoter linked to the luciferase gene. This promoter contains a RUNX1 consensus and is expressed in only one of them or in none of them did not induce significant activation (bars 1, 2, 3, and 5). The Inhibitory Domain of RUNX1 Inhibits the Transactivation Activity of RUNX1—It is well known that RUNX1 co-regulates myeloid differentiation in cooperation with other factors. To evaluate the functional importance of the ID region in myeloid differentiation, we introduced the ID in the retroviral vector and infected 32Dcl3 cells, the ID region was visualized only in the nucleus, confirming the interaction in vivo (right panels).

The Inhibitory Domain of RUNX1 Impairs G-CSF-induced Differentiation of 32Dcl3 Cells—It is well known that RUNX1 co-regulates myeloid differentiation in cooperation with other factors. To evaluate the functional importance of the ID region in myeloid differentiation, we introduced the ID in the retroviral vector and infected 32Dcl3 cells, the ID region was visualized only in the nucleus, confirming the interaction in vivo (right panels).

FIGURE 5. The ID is the major interaction region in the C terminus of RUNX1. (A) 293T cells were transiently transfected, respectively, with HA-RUNX1-IDs alone (lanes 2 and 8) or in combination with FLAG-RUNX1-IDs (lanes 3 and 9), FLAG-RUNX1-ΔRDΔID (lanes 4 and 10), or FLAG-RUNX1-ΔRDΔ351–411 (lanes 5 and 11). Two days after transfection the proteins were analyzed as described in Fig. 1. FLAG-RUNX1-IDs efficiently interacts with HA-RUNX1-IDs (lane 3, top panel), whereas FLAG-RUNX1-ΔRDΔID interacts weakly with HA-RUNX1-IDs (lane 4, top panel). FLAG-RUNX1-ΔRDΔ351–411 does not interact with HA-RUNX1-IDs (lane 5, top panel). The right panels show the expression of transiently transfected proteins in the cell lysates. Lanes 1 and 7 represent mock-transfected cells; IP, immunoprecipitate; CL, cell lysate; WB, Western blot. (B) SW13 cells were transiently transfected with HA-RUNX1-IDs alone (lanes 1 and 9); FLAG-RUNX1-IDs alone (lanes 2 and 10), or with HA-RUNX1-IDs (lanes 5 and 13); FLAG-RUNX1-ΔRDΔID alone (lanes 3 and 11) or with HA-RUNX1-IDs (lanes 6 and 14); FLAG-RUNX1-ΔRDΔ351–411 alone (lanes 4 and 12) or with HA-RUNX1-IDs (lanes 7 and 15). Two days after transfection the proteins were analyzed as described in Fig. 1. HA-RUNX1-IDs interacts with FLAG-RUNX1-IDs (lane 5, top panel) but does not interact with FLAG-RUNX1-ΔRDΔID or FLAG-RUNX1-ΔRDΔ351–411 (lanes 6 and 7, top panel). The right panels show the expression of transiently transfected proteins in the cell lysates. Lanes 8 and 16 represent mock-transfected cells. (C) stably transfected SW13 cells expressing RUNX1-ΔRunt, SW13-HA-RUNX1-ΔRunt (S-H-ΔRD), and naive SW13 cells as control were transfected with FLAG-tagged RUNX1-IDs (lanes 7, top panel) or FLAG-RUNX1-ΔRDΔ351–411 (lanes 6 and 8, top panel). The right panels show the expression of stably transfected proteins in the cell lysates. Lanes 8, 16 represent mock-transfected cells. F, FLAG; H, HA; R, RUNX1; IP, immunoprecipitate; CL, cell lysate; WB, Western blot. SW13 cell line is indicated as S. (bars 1–4).
ral vector MSCV (murine stem cell virus) and infected the hematopoietic murine cell line 32Dcl3. These cells grow in an undifferentiated state in interleukin-3 and differentiate into granulocytes in response to G-CSF. After neomycin selection, the infected cells were carefully washed to remove interleukin-3 and were cultured with 10 ng/ml G-CSF. In general, 32Dcl3 cells will undergo complete differentiation in about 8–10 days. As controls, we used the same cell line stably transfected with the empty vector. As a further control, we also examined 32Dcl3 cells that were infected with MSCV expressing the RUNX1 region included between aa 185 to 381 (left region). As shown in Fig. 5C, this region interacts very weakly with the C terminus. After 6 days of culture with G-CSF, the control cells, the RUNX1-185–385 cells, and the ID cells were analyzed after Wright-Giemsa staining (Fig. 10A). At 40×/H11003 magnification it was possible to recognize the difference between the normal polymorphism of control and RUNX1-185–385 cells compared with the complete monomorphism of the ID cells (data not shown). At 100×/H11003 magnification, we found that whereas control

FIGURE 6. The isolated inhibitory region interacts with RUNX1. A, stably transfected SW13 cells expressing RUNX1-ID, SW13-FLAG-RUNX1-ID (S-F-ID), and naive SW13 cells as control were transfected with HA-RUNX1 and analyzed as described in Fig. 1. Lane 4 in the top panel confirms the interaction of RUNX1 and RUNX1-ID. Anti-FLAG antibody did not precipitate HA-RUNX1 in SW13 cells (lane 2, top panel). Lanes 3 and 4, bottom panel, confirm the presence of immunoprecipitated FLAG-RUNX1-ID in S-F-ID cells. The expression of transiently transfected SW13 cells expressing RUNX1-ΔRnt, SW13-FLAG-RUNX1-ΔRnt (S-F-Δ), and naive SW13 cells as control were transfected with FLAG-tagged RUNX1 or RUNX1-Δ351–411 and analyzed as described in Fig. 1. FLAG-RUNX1 interacts with stably expressed HA-RUNX1-ΔRnt (lane 5, top panel), but FLAG-RUNX1-Δ351–411 is unable to co-precipitate HA-RUNX1-ΔRnt (lane 6, top panel). Anti-FLAG antibody did not precipitate HA-RUNX1-ΔRnt in S-F-ΔRD cells (lane 4, top panel). The right panels show the expression of stably transfected SW13 cells expressing FLAG-tagged RUNX1 and RUNX1-Δ351–411 (bottom). C, stably transfected SW13 cells expressing RUNX1, SW13-HA-RUNX1 (S-H-R), and naive SW13 cells as control were transfected with FLAG-tagged RUNX1, RUNX1-Δ351–411, or

RUNX1-ID and analyzed as described in Fig. 1. FLAG-RUNX1 and FLAG-RUNX1-ID efficiently interact with stably expressed HA-RUNX1 (lanes 6 and 8, top panel), but FLAG-tagged RUNX1-Δ351–411 interacts very weakly with HA-RUNX1 (lane 7, top panel). Anti-FLAG antibody did not precipitate HA-RUNX1 (lane 5, top panel). The right panels show the expression of stably transfected SW13 cells expressing FLAG-tagged RUNX1, RUNX1-Δ351–411, and RUNX1-ID (bottom). F; FLAG; H; HA; R; RUNX1; IP, immunoprecipitate; CL, cell lysates; WB, Western blot. SW13 cell line is indicated as S.
and RUNX1-185–385 cells had the appearance of normal granulocytes and monocytes without significant atypical aspects, in which the maturation stages were represented until the myelocyte and monocyte stages, the ID cells showed a severe impairment in differentiation. Virtually all the cells were blocked at promyelocytes with frequent atypical features as agranular cytoplasm, N/C asynchrony, or increase in N/C ratio. In addition, in the ID culture it was possible to describe some very immature cells with a notable increase in the N/C ratio, chromatin irregularities, evident nucleoli, and immature azurophil cytoplasm devoid of granules. These cells were not evident in the control and RUNX1-185–385 cells. The block in differentiation was confirmed by fluorescence-activated cell sorter analysis of the myeloid lineage-specific GR1 marker. The results showed that although starting at day 4 of G-CSF treatment, this marker appeared in normal 32Dcl3 cells but was not present in the 32Dcl3 cells expressing the ID region (Fig. 10).

**FIGURE 8.** The mammalian two-hybrid assay confirms C terminus-C terminus interaction in vivo. HeLa cells were transfected with pG5Luc and GAL4-ARD or VP16-RUNX1 or VP16-ΔRD as indicated. Normalized reporter gene activity was recorded with a Promega Dual-Luciferase assay system by luminometer. There was a significant increase in luciferase activity in bar 4 (GAL4-ARD and VP16-ΔRD co-transfection) and in bar 6 (GAL4-ARD and VP16-RUNX1 co-transfection).

**FIGURE 9.** The inhibitory domain (ID) represses the transactivation activity of RUNX1. NIH-3T3 cells were transfected with 3 µg of pM-CSFR-luc alone (bars 1 and 5) or with RUNX1 (bars 6–9) in the absence (bar 6) or presence (bars 7–9) of increasing amounts of the isolated inhibitory region ID. As expected, RUNX1 activates the promoter (bar 6); the addition of the inhibitory region reduces promoter activation in a dose-dependent manner. The results represent three independent experiments.

**FIGURE 10.** The ID deregulates the differentiation of 32Dcl3 cells. A, naïve 32Dcl3 cells or 32Dcl3 cells stably expressing the inhibitory region ID or the distal region RUNX1-(185–351), taken as additional control, were induced to differentiate by culture with G-CSF. After 6 days, the cells were analyzed after Wright-Giemsa staining. Expression of the inhibitory domain blocked the differentiation program. B, naïve 32Dcl3 cells or 32Dcl3 cells stably expressing the inhibitory region ID were induced to differentiate by culture with G-CSF. After 2, 4, and 6 days, the cells were analyzed by fluorescence-activated cell sorter. The expression of the GR1 myeloid differentiation marker was inhibited in the cells expressing the ID. Gr1-PE, phycoerythrin-labeled Gr1 marker.

**DISCUSSION**

Among the genes that are associated with human leukemia, RUNX1 is perhaps one of the most studied because of its role in normal hematopoiesis and the many ways in which it is altered either in expression or in sequence in both myeloid and lymphoid malignancies. The combined efforts of many investigators have shown that RUNX1 is a DNA-binding protein that interacts with tissue-specific transcription factors and with members of the chromatin remodeling complexes. Therefore, in general it is thought that RUNX1 specifically binds to its DNA consensus and regulates gene transcription by interacting with adjacent transcription factors. RUNX1 regulates transcription factors with which it recruits chromatin modifiers (51). Genes such as ETO, myelodysplastic syndrome 1/EVI1, and TEL, which fuse in-frame to RUNX1 after chromosomal translocations and interact with their own sets of transcription regulators, contain a homodimerization domain that is maintained in the fusion...
proteins, and therefore, it has long been proposed that through this dimerization the RUNX1 fusion proteins assemble inappropriate transcription complexes that deregulate hematopoietic programs regulated by RUNX1 and ultimately lead to the development of leukemia (52, 53).

A frequent feature of RUNX1-regulated promoters is the presence of multiple consensus motifs for RUNX1 binding, which could be either closely spaced or separated by hundreds of base pairs (54). Although the existence of multiple consensus motifs is observed also in promoters regulated by other members of the RUNX1 family (55), it has not received much attention, and its significance, if any, has not been considered. Here we present data which strongly indicate that RUNX1 interacts with itself and that this interaction is probably a regulatory mechanism through which RUNX1 could control its own activity. On this background, the existence of multiple DNA binding sites in promoters regulated by RUNX1 members acquires new importance and should perhaps be reconsidered because there are ways in which these sites could play a significant role in controlling RUNX1-dependent gene regulation. For example, it was shown that in the regulation of the bone-related osteocalcin gene, RUNX2, is required for the extensive chromatin remodeling that accompanies gene activation, and it was proposed that activation of the promoter requires bending of chromatin around a nucleosome flanked by RUNX2 sites. Importantly, next to these sites there are those of other factors that are essential promoter regulators. According to the proposed model, these factors would be brought together by a chromatin turn on itself of more than 180 degrees (55). If this is true, then chromatin bending would clearly be facilitated and stabilized by the two RUNX2 proteins that interact with each other through their C termini facilitating the assembly of the transcriptional complex. RUNX1 also interacts with several proteins involved in chromatin remodeling, signal transduction, and cell cycle progression with which it forms discrete multiprotein complexes that can be visualized as punctate sites within the chromatin (51, 56). It was, therefore, proposed that RUNX factors could function as molecular scaffolds to organize regulatory complexes for combinatorial transcription control required for lineage commitment and tissue-specific gene expression (56). In its function as molecular scaffold, again the ability of RUNX1 to interact with itself would be a stabilizing property in the assembling of high order complexes.

The ability of RUNX1 to interact with itself assumes significance in normal gene regulation or chromatin remodeling; however, given how RUNX1 is mutated in leukemia, this property acquires more importance. Aside from the t(12;21), all chromosomal translocations cloned so far involving RUNX1 generate fusion proteins that lack the C terminus of RUNX1 (51). There is no doubt that the fusion proteins play a dominant role in the development or progress of leukemia. Indeed, it is generally accepted that the differentiation blocks associated with the fusion proteins originate from erroneous transcription complexes, which inappropriately contain interacting factors and chromatin modifiers recruited by the RUNX1 fusion partner. The effect of these inappropriate complexes on the normal transcription regulation directed by RUNX1 is further enhanced by the multimerization of these fusion proteins, which often normally assemble in tetrameric complexes or in complexes of higher order (57). Whereas this mechanism is quite likely to be a major pathway leading to RUNX1 deregulation, it is not excluded that the normal interaction of two RUNX1 molecules located at distant sites could be inhibited by the presence of the fusion proteins, and we propose that the inability of the fusion proteins lacking the C terminus to bring together factors located at distant sites could also impair the combinatorial factors assembly regulated by RUNX1. Finally, several missense and nonsense point mutations associated with myelodysplastic syndrome and familial platelet disorder occur in the runt domain or distal to the runt domain. The majority of runt-domain missense mutations generate RUNX1 proteins unable to bind to the DNA but still expressing an intact C terminus, which could interact with the C terminus of normal RUNX1 but fail to bring together distant chromatin sites. On the other hand, nonsense mutations in the runt domain or distal to the runt domain lead to a protein that although still capable of occupying a DNA site, lacks the C terminus and is, therefore, unable to form a chromatin complex. In conclusion, whereas this report introduces a new complexity in understanding the mechanisms regulating RUNX1 functions and their deregulation, it also could help to better understand the normal regulatory control of this protein and the pathways that are disrupted by RUNX1 mutations in human leukemia.

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