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Pim-1 Kinase and p100 Cooperate to Enhance c-Myb Activity

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Summary

The *pim-1* oncogene is regulated by hematopoietic cytokine receptors, encodes a serine/threonine protein kinase, and cooperates with c-*myc* in lymphoid cell transformation. Using a yeast two-hybrid screen, we found that Pim-1 protein binds to p100, a transcriptional coactivator that interacts with the c-Myb transcription factor. Pim-1 phosphorylated p100 in vitro, formed a stable complex with p100 in animal cells, and functioned downstream of Ras to stimulate c-Myb transcriptional activity in a p100-dependent manner. Thus, Pim-1 and p100 appear to be components of a novel signal transduction pathway affecting c-Myb activity, linking all three to the cytokine-regulated control of hematopoietic cell growth, differentiation, and apoptosis.

Introduction

The *pim-1* protooncogene was first identified as a common site of proviral integration in murine retrovirus-induced T cell lymphomas, and elegant studies using transgenic mice have documented its ability to cooperate with c-*myc* in lymphoid cell transformation (reviewed in Jonkers and Berns, 1996). Expression of the *pim-1* mRNA and protein can be induced by a number of cytokines that signal through specific receptors and membrane-associated protein tyrosine kinases such as Jak2 (Dautry et al., 1988; Lilly et al., 1992; Joneja and Wojchowski, 1997; Sakai and Kraft, 1997). The *pim-1* gene maps to chromosomes 6p21 and 17 in human and mouse, respectively, and it is expressed in a wide variety of cell types and tumors (Stewart and Rice, 1995; Liang et al., 1996), although highest expression is found in hematopoietic tissues and testes (Amson et al., 1989; Meeker et al., 1990; Wingett et al., 1992). The gene encodes a serine/threonine protein kinase whose overexpression can enhance the survival of hematopoietic cells (Moroy et al., 1993; Joneja and Wojchowski, 1997). However, despite the fact that recognition sites for Pim-1 phosphorylation have been identified in artificial substrates (Saris et al., 1991; Friedmann et al., 1992), no intracellular targets for Pim-1 phosphorylation have been reported. Thus, the functional roles of Pim-1 in signal transduction, lymphopoiesis, and tumorigenesis remain obscure.

The protooncogene c-*myb* encodes a DNA-binding transcriptional activator, which is linked to the regulation of cell proliferation, differentiation, and apoptosis, and which is expressed in a variety of proliferating cell types including immature hematopoietic cells as well as testes and some types of differentiating epithelia (reviewed in Ness, 1996). Expression of c-*myb* is required for fetal hematopoiesis (Mucenski et al., 1991), and c-*myb* mRNA levels are elevated in several types of human tumors, suggesting that the c-Myb protein plays a role in both normal hematopoiesis as well as tumorigenesis (reviewed in Ness, 1996). Two different avian leukemia viruses, avian myeloblastosis virus (AMV) and E26, express truncated and mutated forms of c-Myb, which transform and regulate the differentiation of hematopoietic cells in tissue culture and induce leukemias in animals (Graf, 1998).

In normal cells, c-Myb regulates the differentiation and proliferation of immature hematopoietic and lymphoid precursors, and it is presumed to be regulated by upstream signaling pathways (Graf, 1998). Indeed, several distinct regulatory mechanisms have been shown to affect c-Myb protein activity (reviewed in Ness, 1996; Hunter, 1998). For example, the N-terminal DNA binding domain can interact with the C-terminal EVES motif (Dash et al., 1996), a component of the negative regulatory domain (Dubendorff et al., 1992; Dini and Lipsick, 1993), suggesting that c-Myb is regulated by intramolecular interactions. The c-Myb protein is phosphorylated at multiple sites (reviewed in Ness, 1996), and oncogene-encoded protein kinases can cooperate with c-Myb to activate alternate sets of target genes (Kowenz-Leutz et al., 1997). Also, c-Myb interacts with several cellular proteins including p300/CBP (Oelgeschläger et al., 1996), p160 (Tavner et al., 1998), c-Maf (Hedge et al., 1998), and the peptidyl-prolyl isomerase Cyp-40 (Leverson and Ness, 1998). Cyp-40 can disable the DNA binding activity of c-Myb, but the AMV v-Myb protein escapes inhibition due to several acquired mutations that disrupt the Cyp-40 binding site (Leverson and Ness, 1998). Finally, the DNA binding domains of both c-Myb and v-Myb can interact with the transcriptional coactivator p100 (Dash et al., 1996), a protein first identified by

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its ability to bind the Epstein-Barr virus protein EBNA2 (Tong et al., 1995). However, despite this progress, there is still no link between c-Myb transcriptional activity in the nucleus and upstream signaling pathways that might regulate the protein in hematopoietic cells.

Here we describe a novel signal transduction pathway linking the protein kinase Pim-1, the transcriptional coactivator p100, and the transcription factor c-Myb. Previous studies have implicated both Pim-1 and c-Myb as regulators of similar cellular events, including proliferation, differentiation, and apoptosis. Our results suggest that p100 functions in a signaling cascade that is likely to regulate the fate of hematopoietic cells and to play a key role in the development of lymphoid and perhaps other types of mammalian tumors.

Results

Pim-1 Interacts with p100

A number of studies have linked the Pim-1 protein kinase to growth control and leukemogenesis, although little is known about the mechanism of its activity. We employed a yeast two-hybrid screen to identify cellular proteins that could interact with Pim-1 and potentially serve as substrates or regulators of its kinase activity. Because the kinase activity of the wild-type LexA-Pim-1 protein allowed it to activate transcription of the reporter genes on its own, a kinase-inactive (PimMt) version was used to screen a library of cDNA clones prepared from mouse embryo mRNA (Hollenberg et al., 1995). The library cDNAs were expressed from a yeast vector as fusions to the heterologous transcriptional activation domain from VP16. Approximately 1×10^7 yeast transformants coexpressing cDNA clones and the LexA-PimMt bait construct were tested in the two-hybrid assay, and 36 clones were recovered that were able to activate two separate reporter genes in a strictly Pim-1-dependent fashion.

One cDNA clone, designated S3, encoded a protein that appeared to interact quite strongly with LexA-PimMt. To test whether S3 would also interact with wild-type Pim-1, a modified two-hybrid assay designed to give lower background levels (Durfee et al., 1993) was used. The Pim-1 bait protein fused to the GAL4 DNA-binding domain did not activate the reporter genes on its own (Figure 1 and data not shown), and the VP16-S3 fusion protein interacted equally well with both the kinase-inactive and wild-type versions of Pim-1, but not with any control proteins tested, such as Lamin (Figure 1 and data not shown), suggesting that the interaction between S3 and Pim-1 was specific. Nucleotide sequence analysis indicated that S3 encoded a fragment of p100, a transcriptional coactivator that has been shown to interact with the v-Myb transforming protein and its cellular counterpart, c-Myb (Dash et al., 1996). At the amino acid level, the murine p100 fragment was completely conserved with the corresponding 108–amino acid region of human p100, which spanned residues 449–554 in the published human protein sequence (Tong et al., 1995).

Pim-1 Phosphorylates p100 In Vitro

In vitro kinase assays were devised to determine whether Pim-1 could specifically phosphorylate the p100 protein.

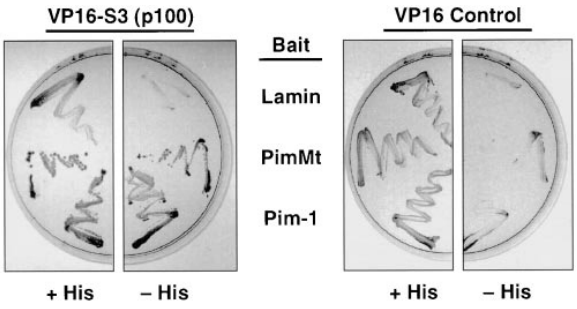

Figure 1. Pim-1 and p100 Interact in a Yeast Two-Hybrid Assay

Yeast strains expressing the VP16 activation domain alone (VP16 control) or VP16 fused to the S3 fragment of p100 (VP16-S3) were mated with strains expressing GAL4 DNA-binding domain fused to the control protein Lamin or to either a kinase-inactive (PimMt) or wild-type (Pim-1) version of mouse Pim-1. Each diploid strain was streaked on nonselective medium containing histidine (+His) or selective medium lacking histidine (−His), as indicated. The ability of two proteins to interact was judged by their capacity to induce growth on selective (−His) medium.

Wild-type or kinase-inactive mutant forms of Pim-1 were expressed in bacteria as GST fusion proteins and then purified using glutathione agarose beads. The recombinant GST-Pim-1 phosphorylated both itself and artificial substrates such as histone H1 in vitro (Figures 2B and 2C). Furthermore, Pim-1 phosphorylated a bacterially expressed GST-S3 fusion protein, but not a control, nonrecombinant GST protein (Figure 2B), suggesting that the S3 region of p100 serves as both a binding site and an in vitro phosphorylation target site for Pim-1. Besides the S3 fragment, Pim-1 was also able to phosphorylate two larger fragments of p100 spanning amino acid residues 1–340 and 340–595, respectively (Figures 2A and 2C), as well as bacterially expressed, full-length human p100 (data not shown). A third p100 fragment derived from residues 654–886 at the C terminus was not phosphorylated by Pim-1. Although the Pim-1 phosphorylation sites in p100 have not yet been mapped, these results suggest that Pim-1 is capable of phosphorylating at least two distinct sites in the p100 protein. Phosphoamino acid analysis indicated that phosphorylation by Pim-1 was directed primarily to serine residues with trace phosphorylation on threonine (data not shown), consistent with the description of Pim-1 as a serine/threonine protein kinase. As expected, the mutant form of Pim-1 had no protein kinase activity (Figure 2B).

Pim-1 and p100 Form a Complex in Animal Cells

A coimmunoprecipitation experiment was used to test whether p100 and Pim-1 interact in living cells. In brief, plasmids expressing wild-type Pim-1 alone or in combination with a FLAG-epitope-tagged version of human p100 were transfected into chick embryo fibroblasts (CEF). After 2 days, the cells were radiolabeled with ^{35}S-methionine and cysteine, cell extracts were prepared, and the resulting protein complexes were immunoprecipitated using specific antisera and then analyzed by SDS gel electrophoresis and autoradiography (Figure 3). In CEF cells expressing only Pim-1, the ectopically expressed kinase was efficiently immunoprecipitated

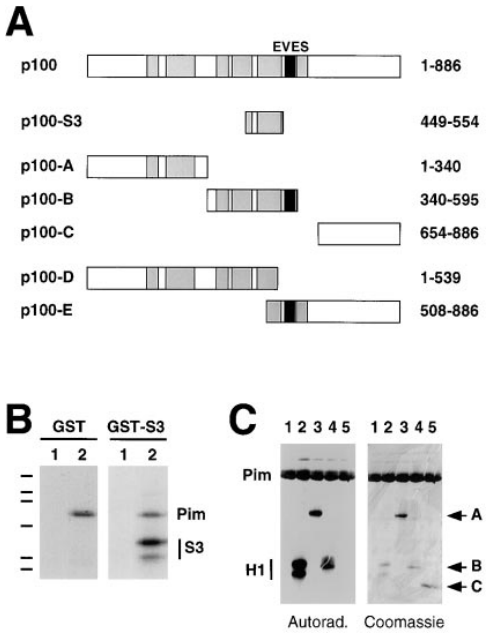

Figure 2. Pim-1 Phosphorylates p100 In Vitro

(A) Structural domains in human p100. The diagrams represent full-length (top, 1–886) or various deletion mutants of human p100 protein. The EVES domain previously shown to interact with c-Myb is shaded black. The other shaded regions are domains of highest similarity between the human and *C. elegans* p100 proteins. The p100-S3 fragment of p100 was identified in the two-hybrid screen described in Figure 1. The other fragments were constructed as described in Experimental Procedures. The amino acid residues contained in each fragment are indicated at right, using the human p100 numbering system.

(B) Pim-1 phosphorylates the S3 fragment of p100. GST-only control protein (GST) or GST fused to the S3 fragment of p100 (GST-S3) was expressed in *E. coli*, purified to near homogeneity, and then incubated in a kinase reaction mixture containing [γ-^{32}P]ATP with bacterially expressed forms of mutant (lanes 1) or wild-type (lanes 2) Pim-1 protein, as described in Experimental Procedures. The samples were analyzed by SDS gel electrophoresis, and the resulting autoradiogram is shown. The wild-type Pim-1 kinase has autophosphorylation activity, and its migration is indicated at right (Pim), as is the migration of the recombinant S3 fragment of p100. The bars at left indicate the migration of molecular weight markers of size (from top) 201, 117, 78, 49, 33, and 25 kilodaltons, respectively.

(C) Pim-1 phosphorylates p100 in vitro. Murine Pim-1 protein was expressed in *E. coli* and purified to near homogeneity as described in Experimental Procedures. The Pim-1 was added to in vitro kinase reactions containing [γ-^{32}P]ATP either by itself (lane 1), with the control substrate histone H1 (lane 2), or with the recombinant, bacterially expressed pA, pB, and pC fragments of human p100 described above (lanes 3–5, respectively). After the samples were analyzed by SDS gel electrophoresis, the gel was stained to visualize the proteins (Coomassie, right panel) and then subjected to autoradiography to detect phosphorylated proteins (autorad, left panel). The positions of Pim-1, which has autophosphorylation activity, as well as histone H1 and the three p100 fragments A, B, and C, are indicated.

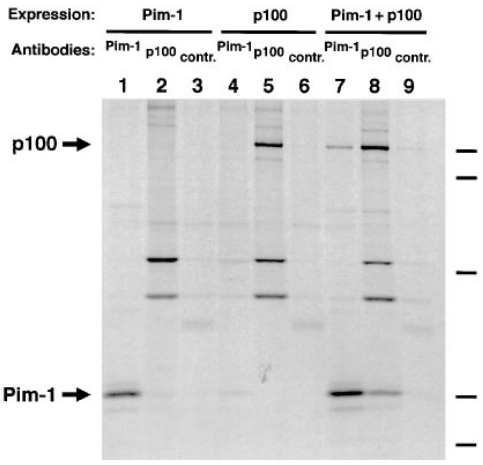

Figure 3. Coimmunoprecipitation of Pim-1 and p100

Chick embryo fibroblasts were transfected with plasmids expressing Pim-1 (lanes 1–3), a FLAG epitope-tagged version of p100 (lanes 4–6), or both (lanes 7–9). After 2 days, the cells were radiolabeled with ^{35}S-methionine for 2 hr, then extracts were prepared and protein complexes were immunoprecipitated using antibodies specific for Pim-1 (lanes 1, 4, and 7) or p100 (anti-FLAG; lanes 2, 5, and 8), or with control, nonimmune antibodies (lanes 3, 6, and 9). The proteins were analyzed by SDS gel electrophoresis followed by autoradiography. The positions of the p100 and Pim-1 proteins are indicated. The bars at right indicate the migration of molecular weight markers of size (from top) 104, 81, 48, 35, and 28 kilodaltons, respectively.

using anti-Pim-1 antibodies (Figure 3, lane 1), but not with anti-p100 (anti-FLAG epitope) or control, nonimmune antibodies (lanes 2–3). Similarly, in cells expressing only p100, the ectopically expressed protein was specifically immunoprecipitated only by anti-p100 antibodies (lane 5). However, in cells coexpressing both proteins, Pim-1 antibodies were able to immunoprecipitate Pim-1 along with a significant fraction of the p100 protein (lane 7), and p100 antibodies immunoprecipitated both p100 and Pim-1 (lane 8). In parallel assays, mutant and wild-type Pim-1 proteins bound p100 with similar affinities (data not shown). Control antibodies immunoprecipitated only background level amounts of both proteins (lanes 3, 6, and 9). Similar results were obtained using antibodies directed against the p100 protein and plasmids expressing wild-type p100 (data not shown), ruling out any role of the FLAG epitope in the observed interactions. Purified, bacterially expressed versions of Pim-1 and p100 were also able to interact in vitro (data not shown), suggesting that Pim-1 bound directly to p100 without the need for any additional cellular proteins. In addition, the Pim-1 protein that was recovered in a complex with p100 retained histone kinase activity (data not shown), indicating that association with p100 did not inhibit the protein kinase activity of Pim-1. These results confirm that the full-length p100 and Pim-1 can interact directly with each other, and suggest that the two proteins form a stable complex in living cells.

Activation of the *mim-1* Promoter by Ras and Pim-1

The c-Myb transcription factor controls hematopoietic cell growth and differentiation, and has regulated DNA binding and transcriptional regulatory activities, but has not been linked to signal transduction pathways originating in the cytoplasm. We used transient transfection assays to investigate whether Pim-1 and p100 might cooperate to influence the activity of c-Myb and the expression of Myb-responsive promoters. We started with the avian HD-3 erythroblast cells as a model system, since these cells express high levels of c-Myb protein capable of activating reporter constructs as well as

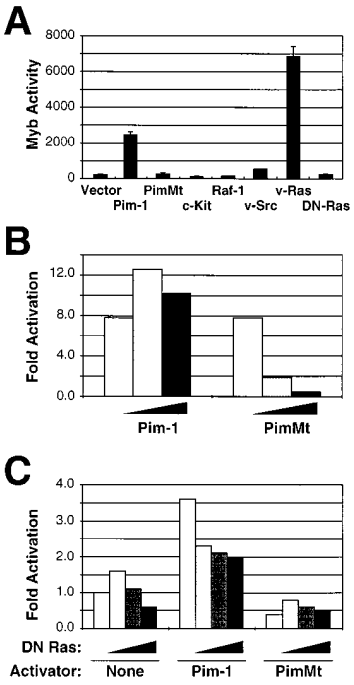

Figure 4. Pim-1 and Ras Activate c-Myb

(A) Stimulation of Myb-responsive promoters by Pim-1 and Ras. HD-3 erythroblasts were transfected with the Myb-responsive reporter plasmid derived from the chicken *mim-1* gene along with control, nonexpressing vector plasmid or plasmids expressing wild-type Pim-1 or a kinase-inactive mutant of Pim-1 (PimMt), c-Kit, wild-type c-Raf (Raf-1), v-Src, activated Ras (EJ-Ras), or a dominant-negative allele of Ras (DN-Ras). The bars indicate activity of the promoter in arbitrary units. The data shown are representative of several independent experiments.

(B) Pim-1 activates downstream of Ras. HD-3 erythroblasts were transfected with a *mim-1* gene reporter plasmid alone or with plasmids expressing activated Ras. The transfections also included additional nonrecombinant expression vector (open bars) or either 50 ng (shaded bars) or 100 ng (black bars) of plasmids expressing either wild-type (Pim-1, left panel) or kinase-inactive mutant (PimMt, right panel) forms of Pim-1. The results are plotted as fold activation by Ras, relative to the no-Ras samples. Note that overexpression of mutant Pim-1 blocks the activation by Ras.

(C) Effect of dominant-negative Ras. HD-3 erythroblasts were transfected with a *mim-1* gene reporter plasmid without an activator (none, left panel) or with plasmids expressing wild-type (Pim-1, middle panel) or mutant (PimMt, right panel) forms of Pim-1, as indicated at the bottom. In addition, the samples received 0 (open bars), 50, 100, or 150 ng (black bars) per well of plasmid expressing a dominant-negative allele of Ras (DN-Ras). The results are plotted as fold activation, relative to the no-activator, no-DN-Ras sample. Note that Pim-1 activates 2- to 4-fold, no matter how much DN-Ras is expressed (compare left and middle panels).

endogenous Myb-responsive genes (Ness et al., 1993). In brief, HD-3 cells were transfected with a reporter plasmid derived from the chicken *mim-1* gene, along with plasmids expressing Pim-1 or other proteins known to be involved in signaling pathways. As shown in Figure 4A, *mim-1* promoter activity was stimulated approximately 10-fold by ectopic expression of Pim-1, but not by a nonrecombinant control plasmid (vector) or a plasmid expressing a mutant, kinase-inactive version of Pim-1 (PimMt).

To determine whether the effect of Pim-1 was specific, we tested the effects of several other oncogenes that express protein kinases, but found that c-Kit, c-Raf (and v-Mil, an activated form of c-Raf; data not shown), and v-Src had little or no effect on *mim-1* promoter activity (Figure 4A). Since c-Raf is known to activate the mitogen-activated protein (MAP) kinase p42mapk (Howe et al., 1992), these results suggested that Pim-1 stimulated expression of the *mim-1* promoter via a MAP kinase-independent mechanism. However, as shown in Figure 4A, the *mim-1* promoter was dramatically stimulated by cotransfection of a plasmid expressing an activated form of Ras (v-Ras), but not by a plasmid expressing a dominant-negative mutant allele of Ras (DN-Ras). Interestingly, Ras had no effect on *mim-1* promoter activity when the transfection assays were performed in nonhematopoietic cell lines, such as COS7 cells (data not shown), which do not express c-Myb, suggesting that Ras expression increased the activity of c-Myb.

Since immature hematopoietic cells are known to express Pim-1 (Amson et al., 1989; Nagata and Todokoro, 1995; Liang et al., 1996), additional cotransfection assays were performed to test whether Ras and Pim-1 were signaling via a common pathway. In brief, HD-3 cells were transfected with the *mim-1* promoter reporter plasmid as described above, along with plasmids expressing either wild-type or mutant versions of Pim-1 or Ras. In the experiment shown in Figure 4B, expression of activated v-Ras led to an approximately 8-fold stimulation of *mim-1* promoter activity, which was increased to 12-fold when Ras and Pim-1 expression plasmids were transfected together. In contrast, overexpression of the mutant allele of Pim-1 almost completely blocked the ability of v-Ras to activate the *mim-1* promoter (Figure 4B, right panel). In the experiment shown in Figure 4C, ectopic Pim-1 expression led to a nearly 4-fold increase in *mim-1* promoter activity (compare open bars) in HD-3 cells. When plasmids expressing dominant-negative Ras were included, the activity of the promoter dropped somewhat. However, even when the maximum amount of plasmid expressing dominant-negative Ras was present, ectopic Pim-1 expression still led to a 4-fold increase in promoter activity (compare black bars). Taken together, these results suggest that, at least in HD-3 erythroblasts, Ras expression stimulates the activity of Pim-1, which then acts downstream to stimulate the *mim-1* promoter.

Several results suggested that Pim-1 specifically increased the activity of c-Myb and activated the *mim-1* promoter through the Myb binding sites. As diagrammed in Figure 5, the *mim-1* promoter contains three binding sites for Myb proteins, labeled A, B, and C. The A site has the highest affinity for v-Myb and c-Myb proteins and was shown previously to be most important for activation of the promoter by Myb (Ness et al., 1989, 1993). In the experiment shown in Figure 5, expression of Pim-1 (black bars) resulted in a 3- to 4-fold activation of the wild-type *mim-1* promoter. However, despite the fact that it still contained two lower affinity Myb binding sites, a mutant promoter with the A site inactivated (Ness et al., 1989) had less basal activity and was only minimally affected by ectopic expression of Pim-1. Deletion of the B and C sites, or mutation of the adjacent binding site for NF-M, the avian homolog of C/EBPβ that is required for expression of the endogenous *mim-1* gene in myeloid cells (Ness, 1996), had no affect on the ability

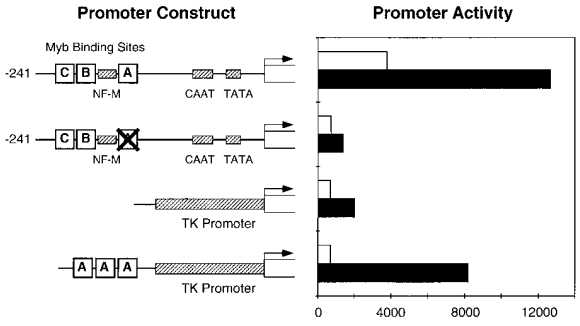

Figure 5. Activation by Pim-1 Requires Myb Binding Sites

The structure of the *mim-1* promoter and reporter gene constructs is shown at left. The high-affinity Myb-binding A site is indicated, as are the lower affinity B and C sites, the major NF-M (C/EBPβ) binding site, and the canonical CAAT and TATA boxes, all of which have been described (Ness et al., 1989, 1993). The arrow and open box indicate the direction of transcription and the luciferase reporter gene, respectively. In the second construct, the mutated A site is indicated by an X. The bottom two constructs represent the minimal HSV thymidine kinase promoter, either without or supplemented with three synthetic Myb-binding A site oligonucleotides, as indicated. Each construct was tested in HD-3 erythroblast cells, as described in Figure 4, either alone (open bars) or along with a plasmid expressing wild-type Pim-1 (closed bars). Results are plotted horizontally, in arbitrary units.

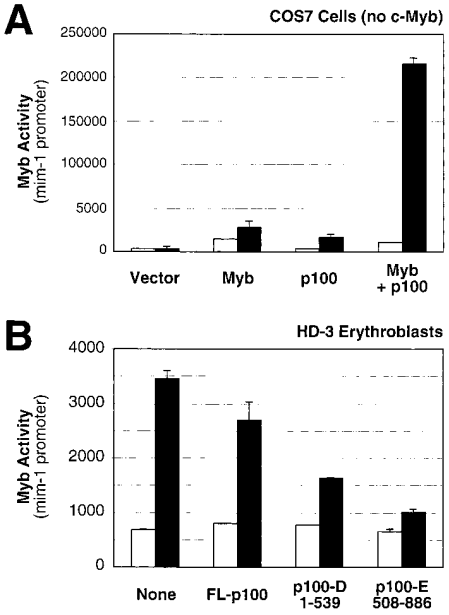

Figure 6. Upstream Activation of c-Myb Requires p100

(A) Pim-1 and p100 cooperate to activate c-Myb. COS7 cells were transfected with the Myb-responsive *mim-1* reporter plasmid plus empty expression vector or plasmids expressing c-Myb, p100, or both, as indicated. The samples also received either additional empty vector (open bars) or plasmid expressing wild-type Pim-1 (closed bars). Transfection results are shown as the average of two independent trials, and transfection efficiencies were monitored by analyzing the expression of the c-Myb and p100 proteins using a Western blotting assay (data not shown).

(B) Mutant p100 blocks c-Myb activation. HD-3 erythroid cells were transfected with a reporter plasmid derived from the *mim-1* promoter, along with nonrecombinant expression vector (none) or plasmids expressing full-length p100 (FL-p100) or the p100-D or p100-E deletion mutants described in Figure 2. In addition, each sample received control vector (open bars) or plasmid expressing wild-type Pim-1 (closed bars). The data shown here are representative of results from numerous independent experiments.

of Pim-1 to activate the *mim-1* promoter (data not shown). Western blot controls showed that Pim-1 expression did not alter c-Myb protein levels (data not shown). Also, the ability of Pim-1 to stimulate the *mim-1* promoter was completely blocked by coexpression of a dominant-negative repressor version of c-Myb (Badiani et al., 1994), suggesting that Pim-1 stimulation was dependent on c-Myb transcriptional activity (data not shown).

Although ectopic expression of Pim-1 did not activate control promoters, such as the RSV LTR (data not shown), it did cause a modest increase in the activity of the minimal promoter from the HSV thymidine kinase (*TK*) gene (Figure 5). However, this effect was significantly enhanced when the promoter was supplemented with several synthetic Myb-binding oligonucleotides (Figure 5). Thus, although overexpression of the Pim-1 kinase appears to activate some promoters in a manner that is not Myb-dependent, indicating that it can influence multiple signaling pathways in hematopoietic cells, greatest activation of the *mim-1* promoter was dependent on the presence of high-affinity Myb binding sites. Taken together, these results suggest that Pim-1 increased the transcriptional activity of c-Myb.

Ras and Pim-1 Activate through p100

The interactions between Pim-1 and p100 suggested that they could constitute components of a novel signal transduction pathway influencing c-Myb activity. We used transfection assays to determine whether Myb, Pim-1, and p100 would cooperate to activate a Myb-responsive promoter in cells that usually do not express c-Myb. As shown in Figure 6A, the *mim-1* promoter reporter construct had very little activity when transfected into monkey COS7 epithelial cells but was activated several-fold by ectopic expression of c-Myb. Expression of Pim-1 (closed bars) had little or no effect on its own but led to increased promoter activity in the presence of c-Myb. Similarly, expression of only p100 had no effect on the promoter, or on the activity of c-Myb, although the combination of p100 plus Pim-1 led to a modest stimulation. However, simultaneous expression of c-Myb, Pim-1, and p100 led to a marked increase in the activity of the Myb-responsive promoter. In parallel assays, expression of p100 did not affect the levels of c-Myb protein made by the transfected cells, and mutants of p100 lacking the Myb-binding EVES domain (Dash et al., 1996) failed to cooperate with Pim-1 in c-Myb activation (data not shown). These results suggest that Pim-1 and p100 cooperate to stimulate the transcriptional activity of c-Myb.

The data described above suggested that p100 was acting as an important mediator between c-Myb and upstream regulators such as Pim-1 and Ras. We hypothesized that mutant p100 proteins should have a dominant-negative activity, capable of blocking the signal transduction pathways that activate c-Myb. To test this idea, HD-3 erythroblast cells were transfected with a Myb-responsive reporter plasmid either with or without Pim-1 expression vectors, as described above. However, the samples also received either nonrecombinant

expression vector DNA or recombinant plasmids expressing either wild-type or various deletion mutant forms of p100. As shown in Figure 6B, ectopic expression of Pim-1 led to a 5- to 10-fold increase in *mim-1* promoter activity. Although overexpression of full-length p100 (FL-p100) had little effect, two different deletion mutants of p100 were able to almost completely block the activation by Pim-1. The most effective inhibitory allele of p100 was the mutant p100-E, comprising amino acid residues 508–886 of p100. This mutant lacked several of the most highly conserved domains in the protein (see Figure 2) but included the Myb-binding EVES domain (Dash et al., 1996) and a portion of the S3 region that binds Pim-1 (Figures 1 and 2). None of the p100 expression vectors had any effect on the basal level of *mim-1* promoter activity observed in the absence of Pim-1 (open bars), or on the activity of control promoters such as the thymidine kinase promoter or RSV LTR, which were tested in parallel (data not shown), ruling out the possibilities that they interfered with general transcription processes or blocked *mim-1* promoter expression through some indirect mechanism. Thus, dominant-negative alleles of p100 block the activity of Pim-1 and prevent the activation of c-Myb. We conclude that p100 is a necessary component of a signaling pathway allowing the protein kinase Pim-1 to regulate the activity of the c-Myb transcription factor.

Discussion

The Pim-1 serine/threonine protein kinase cooperates with activated c-*myc* to induce lymphoid tumors, and Pim-1 expression is regulated by cytokines that control the growth and differentiation of immature hematopoietic cells. Pim-1 is expressed in and regulates the fate of many of the same cell types that express c-Myb, making it an ideal candidate for a component of a signal transduction cascade regulating c-Myb activity. We now describe a physical interaction between Pim-1 and p100, a transcriptional coactivator previously shown to bind c-Myb and influence its activity, and show that Pim-1 and p100 cooperate to regulate the transcriptional activity of c-Myb. These results define a previously undescribed signal transduction cascade involving Pim-1, p100, and c-Myb, and lead to a hypothesis for the role of all three proteins in hematopoietic cell transformation.

A Signal Transduction Pathway from Pim-1 to c-Myb

While c-Myb can be phosphorylated at several sites and can interact with a number of cellular proteins, its activity has not previously been linked to signaling from any specific plasma membrane receptor or protein kinase pathway. Our results show that Pim-1 can stimulate the transcriptional activity of Myb proteins and that this effect requires p100, a coactivator which binds c-Myb. Interestingly, the results using dominant-negative signaling molecules suggested that Pim-1 activity was affected by Ras and that the ability of Ras to stimulate c-Myb activity depended on functional Pim-1. Although we can not definitively rule out that Ras and Pim-1 affect separate pathways leading to c-Myb, our results suggest that the activity of Pim-1 is regulated by Ras, probably through a pathway that does not involve c-Raf or p42mapk.

The mechanism of c-Myb regulation by Pim-1 has not yet been addressed. One possibility is that p100 plays an adaptor role, mediating a direct interaction between c-Myb and Pim-1. This could result in the direct phosphorylation of c-Myb by the Pim-1 protein kinase. Phosphorylation has been shown to affect c-Myb activity (Lüscher et al., 1990; Aziz et al., 1995; Oelgeschläger et al., 1995), and Pim-1 can phosphorylate bacterially expressed fragments of c-Myb in vitro (A. B. D. and S. A. N., unpublished data). However, specific Pim-1 phosphorylation sites have not been localized in c-Myb, nor has Pim-1 been shown to phosphorylate full-length, wild-type c-Myb, so this mechanism remains speculative. Another possibility is that Pim-1 phosphorylates and regulates the activity of p100, perhaps altering its affinity for or ability to interact with c-Myb. Although we have not yet been able to demonstrate that Pim-1 specifically phosphorylates p100 in vivo, if such phosphorylation plays a regulatory role, it could be substoichiometric in nature and therefore quite difficult to detect. Alternatively, the biological substrate for Pim-1 could be an as yet unidentified protein component in the Pim-1/p100/Myb complex. We showed previously that overexpression of p100 in fibroblasts, which do not express Pim-1, could inhibit the transcriptional activity of c-Myb in transfection assays (Dash et al., 1996). In light of our current results, the most likely role for p100 might be that of a regulated coactivator, able to stimulate c-Myb activity, but only in the presence of Pim-1. Although we have shown that p100 and Pim-1 can interact in transfected cells, we have not yet shown that such complexes occur when the proteins are expressed at their normal levels. However, we have found that some cell lines appear to express mutant, perhaps dominant-negative alleles of p100, which could complicate such assays (F. C. O. and S. A. N., unpublished data). Additional experiments with cytokine-dependent primary cells will likely be required to resolve this issue fully.

We showed recently that the human cyclophilin protein Cyp-40 could bind to and inhibit the activity of c-Myb in vitro but that the activated v-Myb protein contained point mutations which allowed it to escape this negative regulatory mechanism (Leverson and Ness, 1998). Since p100 bound c-Myb and v-Myb with similar affinities (Dash et al., 1996) and Pim-1 stimulates the activity of v-Myb as well as c-Myb (J. D. L. and S. A. N., unpublished data), it is likely that Cyp40-mediated repression operates through a mechanism which is independent of p100 binding. Experiments with purified components will likely be required to resolve this issue.

Implications for Pim-1 Function in Leukemias

The ability of *pim-1* to cooperate with c-*myc* in lymphoid tumor induction has been well-documented (Jonkers and Berns, 1996), although the molecular mechanism of this cooperation has remained obscure. Our results raise the possibility that one function of Pim-1 activation in tumors is to stimulate c-Myb activity, and suggest

that cooperation between activated forms of the c-Myc and c-Myb transcription factors may be significant.

There are several reasons to suspect that Myb and Myc proteins could cooperate to regulate the growth and differentiation of hematopoietic cells. For example, both oncogenes were originally discovered as the transforming components of avian leukemia-inducing retroviruses (Bishop, 1991), and the expression of both genes is tightly and coordinately controlled during hematopoietic cell differentiation (Kohlhuber et al., 1993; Lachman and Skoultchi, 1984). In transformed myeloid cells, Myc and Myb proteins have complementary functions, since v-Myc stimulates cytokine-dependent proliferation without affecting differentiation, while v-Myb regulates the differentiation of cells that remain cytokine-dependent for growth (Ness et al., 1987).

However, the suggestion that lymphoid tumors result from cooperation between Myc and Myb raises an important question. In transgenic mice expressing an activated c-*myc* gene, why do retroviral insertions result in activation of *pim-1* rather than c-*myb*? There are at least two possible answers to this question. First, the effects of Pim-1 may be more complex than merely stimulating c-Myb activity. For example, Pim-1 could stimulate the activity of additional transcription factors besides c-Myb, so that activation of c-Myb alone is insufficient to duplicate the effects of Pim-1 overexpression. Alternatively, if c-Myb is the primary functional target, Pim-1 activation may mimic the effects of multiple activating mutations in c-Myb, which are unlikely to occur during simple retrovirus insertions into the c-*myb* gene. Indeed, both forms of v-Myb have sustained numerous activating mutations, including truncation at both ends as well as fusion to other proteins (reviewed in Ness, 1996). Interestingly, transgenic mice expressing v-Myb alleles develop lymphoid tumors with a long latency (Badiani et al., 1996), mimicking the original results observed with single Pim-1 or c-Myc transgenes. In addition, recent results suggest that activated c-*myc* genes can accelerate this v-Myb-induced lymphoid tumor formation (K. Weston, personal communication). Thus, both v-Myb and Pim-1 can cooperate with Myc, suggesting that they activate similar pathways in lymphoid cells. However, additional assays in transgenic mouse systems will likely be required to resolve the issue of whether Myb proteins and Pim-1 lie in the same biochemical pathway.

This work describes biological assays for Pim-1 kinase activity and function, and links the activity of Pim-1 to Ras activation, independent of p42mapk. Our preliminary results suggest that the ability of Ras to activate Pim-1 is cell line dependent, suggesting that other cellular components may also be involved (J. D. L. and S. A. N., unpublished data). The availability of assays for Pim-1 activity should allow us to delineate the pathways leading from cell surface receptors to the activation of Pim-1, p100, and c-Myb.

The Role of p100 in Growth Control and Oncogenesis

Several types of evidence suggest that p100 plays a role in the regulation of cell proliferation, or in cellular transformation. The p100 protein also interacts with the products of two different oncogenes, the c-Myb and v-Myb transcription factors, as well as the protein kinase Pim-1, all of which transform or regulate the growth and differentiation of immature hematopoietic cells. Although it is expressed ubiquitously (J. D. L., F. C. O., and S. A. N., unpublished data), there is some evidence that the subcellular localization of p100 may be regulated (Tong et al., 1995). However, its ability to interact with both viral (EBNA2 and v-Myb) and cellular (Pim-1 and c-Myb) transforming proteins suggests that it plays an important regulatory role. It will be interesting to test whether p100 and Pim-1 also affect the activity of other Myb family member transcription factors, such as A-Myb or B-Myb. Further studies should lead to a better understanding of the role of p100 and the mechanism through which it signals to the cellular proteins that control growth, differentiation, and the cell cycle.

Experimental Procedures

Yeast Two-Hybrid Assays

The murine *pim-1* cDNA (Cuypers et al., 1984) was amplified in a polymerase chain reaction (PCR) using PfuI polymerase (Stratagene) and primers 5′-CCGGAATTCATGCTCCTGTCCAA-3′ and 5′-CGT TCTGCAGCTGCTACTTGCT-3′. The products were digested with EcoRI and PstI and inserted into the vector pBTM116 (Hollenberg et al., 1995). A fragment of Pim-1 containing the K67M mutation was generated using primers 5′-GCTTAATGGCCACCGGCAA-3′ and 5′-GCCGGTGGCCATTATGCATGTGGAGAA-3′ followed by subcloning with EaeI. The expression library consisted of randomly primed, size-selected cDNA fragments prepared from 9.5- and 10.5-day-old mouse embryo RNA, inserted into the pVP16 vector (Hollenberg et al., 1995). Plasmids were introduced into yeast strain L40, and candidate interacting proteins were double-selected for growth on histidine-deficient plates (plus 25 mM 3-aminotriazole) and β-galactosidase production.

Interactions were confirmed using a second two-hybrid system (Durfee et al., 1993), for which the wild-type and mutant Pim-1 proteins were fused in-frame with the yeast GAL4 DNA-binding domain. For this purpose, the *pim-1* sequences were amplified with primers 5′-CCATGCCATGGTCCTGTCCAA-3′ and 5′-CGTTCTGCAGCTGCT ACTTGCT-3′, digested with NcoI and PstI, and inserted into the pAS2 vector. GAL4 fusion proteins were expressed in the yeast strain Y190, which carries *HIS3* and *lacZ* reporter genes, but lacks endogenous GAL4.

Nucleotide sequences obtained using an Applied Biosystems automated sequencing apparatus were analyzed using software from the Genetics Computer Group of the University of Wisconsin (Devereux et al., 1984).

Plasmids and Expression Vectors

The CMV-Myb plasmid has been described, as have plasmids expressing c-Raf (Bruder et al., 1992), v-Src (DeLorbe et al., 1980), c-kit (Hayman et al., 1993), EJ-Ras and DN-Ras (Kowenz-Leutz et al., 1997), and FLAG-p100 (Tong et al., 1995). The p100 expression plasmids were prepared by transferring the wild-type full-length or FLAG epitope-tagged versions of the human p100 cDNA (Tong et al., 1995) to the expression vector pCDNA3 (Invitrogen). The CMV-Pim-1 plasmids were prepared using primers 5′-TTAGAATTCCATAT GCTCCTGTCCAAGATC and 5′-ACACTCGAGGGCTGCTACTTGCT GGAT, the high-fidelity polymerase PfuI (Stratagene), and the wild-type or kinase-inactive (K67M) mutant template plasmids (Hoover et al., 1991). For addition of a C-terminal FLAG epitope tag, the latter primer was replaced with 5′-CAACTCGAGCTACTTGTCATCG TCGTCCTTGTAGTCGGATCCCGGTGACAGACT. The resulting PCR products were digested with appropriate restriction enzymes, cloned into the vector pCDNA3, and checked by nucleotide sequencing before use. The p100-C deletion mutant was prepared by subcloning the NheI-XbaI fragment directly, and the other p100 deletion mutants were prepared by PCR as described above, using

the following primer sets: p100-A (5′), ACAGAATTCAAGCGGGG CATCATCAAGA; p100-A (3′), CTAGAATTCAGCTTCACAACAATGGC; p100-B (5′), GAAGAATTCACCATGGGCGATTACAAGACGAT; p100-B (3′), TAGCGGCCGCCATCGATGTGCAGCCAG; p100-D (5′), ACA GAATTCAAGCGGGGCATCATCAAGA; p100-D (3′), TAAGAATTCGC CTCTGGGGCATTCAA; p100-E (5′), AGGAATTCTCCATGGACGTC TTCAGTGGTTCTC; and p100-E (5′), AGTCTAGACCCTCCTTAGC GGCTG.

Transfections, Coimmunoprecipitations, and Kinase Assays

Rabbit antibodies were produced using a bacterially expressed fragment of the human p100 cDNA, encoding the C-terminal 232 amino acids (fragment p100-C, described above), as antigen. Antibodies specific for the Myb DNA-binding domain have been described (Dash et al., 1996). Antibodies specific for Pim-1 were prepared using bacterially expressed protein as antigen (E.-M. R. et al., unpublished data). Commercially available anti-FLAG M2 mAb beads were used as directed by the manufacturer (Kodak).

Primary chicken embryo fibroblast (CEF) cells were plated in 6-well dishes (Corning) and transfected using Lipofectamine or Lipofectamine Plus (GIBCO BRL) according to the manufacturer's instructions. After 48 hr, the cells were washed twice with PBS and once with methionine-free and cysteine-free medium (GIBCO BRL) supplemented with 10% dialyzed serum. Cells were labeled for 2 hr with 0.5 mCi ^{35}S-Trans (ICN) in 1.0 mL Met/Cys-free medium at 37°C, harvested in cold PBS, collected by centrifugation, and resuspended in 25.0 mM Tris (7.4), 5 mM KCL, and 1 mM $MgCl_2$ supplemented with 1.0 μg/ml each of the protease inhibitors chymostatin, leupeptin, antipain, and pepstatin A plus 1.0 mM each of PMSF and benzamidine, before using aliquots for immunoprecipitation assays (Klempnauer et al., 1984). Although the Pim-1/p100 complexes could be detected from both cytoplasmic and nuclear extracts, the latter often gave less background.

Kinase assays (Hoover et al., 1991) were performed with GST fusion proteins expressed in *E. coli* and purified using glutathione agarose beads (Leverson and Ness, 1998). Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Purified histone H1 (Sigma) was included as an artifical control substrate.

Transfections and Reporter Gene Assays

Tissue culture cells were grown as described previously (Dash et al., 1996) and transfected in 24-well dishes using Lipofectamine (GIBCO BRL) according to the manufacturer's instructions, using a total of 0.6 μg plasmid DNA and 2 μl of Lipofectamine in 0.2 ml of Optim-MEM serum-free medium (GIBCO/BRL) per well. For most assays, 0.25 μg of the *mim-1* gene reporter plasmid (Ness et al., 1989, 1993) and 50 ng of each expression vector were used per well. For titration experiments, expression vectors varied from 50 ng to 150 ng per well. Transfection results were representative of at least three independent experiments and were reported as the average of duplicate or triplicate assays. Where indicated, error bars represent the average distance from the mean, for duplicate assays. Transfection efficiencies were monitored in representative samples by following the expression of cotransfected reporter constructs or by direct Western blot analysis of expressed proteins.

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