The Megakaryocyte/Platelet-Specific Enhancer of the α2β1 Integrin Gene: Two Tandem AP1 Sites and the Mitogen-Activated Protein Kinase Signaling Cascade

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Distinct members of the integrin family of cell adhesion receptors are expressed at different times during hematopoietic differentiation. The expression of some integrin receptors, such as the αβ1 integrin on erythroid cells, is required for differentiation; the expression of other receptors, such as αβ1 and αIIbβ3 on megakaryocytes and platelets, is required for normal function. The αβ1 integrin mediates platelet adhesion to collagen in vessel walls and is essential for the formation of a normal platelet plug. Lack of αβ1 integrin expression by platelets, either due to congenital abnormalities or the development of myelodysplastic/myeloproliferative disorders, is associated with bleeding. The regulation of integrin gene expression during hematopoietic differentiation, specifically megakaryocytic differentiation, has been a major focus of our laboratory.

Expression of the αβ1 integrin is exquisitely regulated by distinctly different transcriptional regulatory elements in epithelial cells, fibroblasts, megakaryocytes, and platelets. To evaluate the molecular mechanisms by which differentiation-dependent expression of the αβ1 integrin is regulated during megakaryocytic differentiation, we have used several models of megakaryocytic differentiation including K562 cells, a pluripotent hematopoietic cell line, and DAMI cells, a megakaryocytic cell line. K562 cells cultured in the presence of 40 mmol/L phorbol dibutyrate (PDB) acquired megakaryocytic features, including the expression of the platelet/megakaryocyte integrins αβ1 and αβ1. The increased αβ1 integrin expression was a result of increased steady-state levels of αβ1 mRNA due to transcriptional activation of the αβ1 integrin gene.

AP1 activation suggested a role for the mitogen-activated protein kinase (MAPK) signaling pathway in regulating αβ1 integrin gene expression. Inhibition of the MAP kinase cascade with PD98059, a specific inhibitor of MAPK kinase 1, prevented the expression of the αβ1 integrin subunit in cells induced to become megakaryocytic. We provide a model of megakaryocytic differentiation in which expression of the αβ1 integrin gene requires signaling via the MAP kinase pathway to activate two tandem AP1 binding sites in the αβ1 integrin enhancer.

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Materials and Methods

Cell cultures and transfection assays. The K562 cell line obtained from the American Type Culture Collection (Rockville, MD) and the DAMI cell line obtained from Dr Robert I. Handin (Harvard Medical School, Boston, MA) were propagated in RPMI 1640 medium. Megakaryocytic differentiation was induced to become megakaryocytic. We provided a model of megakaryocytic differentiation in which expression of the αβ1 integrin gene requires signaling via the MAP kinase pathway to activate two tandem AP1 binding sites in the αβ1 integrin enhancer.

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Gel mobility shift analysis. Nuclear extracts were obtained from uninduced and phorbol-induced K562 cells by isolating nuclei, as previously described. The cells were washed with phosphate-buffered saline (PBS) and lysed in 0.5% Triton X-100 in 10 mL of STEK buffer (30% sucrose, 40 mM Tris, pH 7.5, 400 mM NaCl, and 12 mM MgCl₂). After pelleting, the nuclei were washed in TMN buffer (100 mM KCl, 7.5, 10 mM NaCl, and 10 mM MgCl₂). The proteins were extracted from the nuclei using a buffer containing 20 mM KOH/LEPAES, 20% glycerol, 0.35 mM L/L K/LCl, 0.1 mM L/LEPTA, 0.5 mM EDTA, 10 µg/mL aprotinin, and 10 µg/mL leupeptin. After incubation with gentle rotation for 1 hour at 4°C, the sample was centrifuged at 100,000gRfor 2 hours. The supernatant was collected and the concentration of nuclear proteins in the extracts was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). The gel-purified double-stranded 75-bp DNA fragment extending from bp −1503 to −1578 or the double-stranded oligonucleotides were end-labeled with 32P-dCTP using Klenow DNA polymerase. Oligonucleotides containing a single AP1 consensus element (5’-CGCGTATGACTCGCGCGAGC-3’), a consensus element with a 2-bp mutation of the AP1 site (AP1m; 5’-CGCTTGA TGACTTGGCCGGAA-3’), a single Sp1 consensus element (5’-TTT GCATCGCGCCGGCCGAGC-3’), or an early growth response (Egr) consensus element (5’-GATCCAGCGCGCCGAGC-3’), were obtained from Santa Cruz Biotech, Inc (Santa Cruz, CA). Additionally, 1 to 2 µg of nuclear protein extract was added to 20,000 counts per minute of labeled DNA in 15 µL of binding buffer (10% glycerol, 25 mM NaCl, pH 7.5, 100 mM L/KCl, 5 mM MgCl₂, 50 mM L/EDTA, 1 mM L/dithiothreitol, and 2 mM phenylmethylsulfon fluoride) and incubated on ice for 15 minutes. Some incubations contained an excess of unlabeled competitor DNA fragment, or oligonucleotide, as indicated. To indicate inhibition experiments, the indicated amount of anti-c-fos or anti-c-jun antibody (Santa Cruz Biotech, Inc) was preincubated with the nuclear extract for 18 hours at 4°C before the addition of 32P-labeled probe. The reactions were then analyzed by polyacrylamide gel electrophoresis using 6% acrylamide/Bis (19:1) in Tris, pH 8.8.

α2-CAT fusion constructs. The pα2 CAT 2592-CAT and pα2 1426-CAT constructs were generated by restriction enzyme digestion of the original pα2 5000-CAT construct at the restriction enzyme sites 3’ and 5’ Sac I (−1426), respectively, as described earlier. The 3’ Stu I/Sac I fragment was then inserted into the blunt-ended Sal I site within the multiple cloning site of the SV40 CAT plasmid (Promega, Madison, WI). Two SV40CAT constructs containing the −1426 to −2592 region in opposite orientations were selected by restriction enzyme and sequence analyses. A series of deletion mutants of the 1166-bp (bp −1426 to −2592) were generated by restriction enzyme digestion of the −1426/−2592 SV40 CAT plasmid at the restriction sites XmnI (−1842), NsiI (−1655), Mnl I (−1578) and MvaI (−1503). The −1426/−1655 SV40 CAT plasmid was used as a template for polymerase chain reaction (PCR) to generate the site-specific mutations that disrupt the AP2, GATA, or both binding sites in the construct. The −1503/−1578 SV40 CAT construct was used as a template for PCR-directed mutagenesis to disrupt the constructs containing site-specific mutations that disrupt the two AP1 binding sites, including 5’ AP1m (−1503/−1578 SV40 CAT), 3’ AP1m (−1503/−1578 SV40 CAT), and Dbl AP1m (−1503/−1578 SV40 CAT). The following primers were used for the GATA and AP2 mutant constructs: GATAm (5’-GTTATT TCC CCC CAC CCC CAG ATT TAA AAC AC-3’), AP2 (5’-GTT ATT TCC CCC TTT CCC CAG AGA TAA AAC AC-3’), or AP2/GATAm (5’-GTT ATT TCC CCC CAG ATT TAA AAC AC-3’). The following primers were used for the AP1 mutant constructs: 5’ AP1m (−1503/−1578 SV40 CAT), 5’ GAA ATG TAT GGG AGT GTT TGG-3’, 3’ AP1m (−1503/−1578 SV40 CAT) (5’-CCT TC ATG CTT AGT GAA ATG-3’), and Dbl AP1m (−1503/−1578 SV40 CAT) (5’-CCA AAC ACT CCC ATG CAT TTC AGT CGG ATA GAA-3’). A common 3’ primer from the M13 vector sequence was used in all reactions. All constructs were completely sequenced by the dideoxy-nucleotide chain termination method of Sanger et al to ensure that the site-specific mutations were incorporated with no error. Mutations in the full-length CAT construct pα2 2592-CAT were produced by site-directed mutagenesis.

Flow cytometric analysis. Flow cytometric analysis was performed on uninduced K562 cells, K562 cells induced for 6 days with PDB, or K562 cells incubated with PD98059 for 15 minutes before induction with PDB. Single cells (1 × 10⁶) were incubated with the monoclonal anti-α2 antibody (PIE6) at a concentration of 2 µg/mL for 45 minutes at 4°C. Cells were washed three times and incubated with 2.5 µg/mL of a secondary goat antimouse coupled to fluorescein (Tago, Inc, Burlingame, CA) for 45 minutes at 4°C, washed twice, and resuspended in PBS. Fluorescein-labeled cells were analyzed using a FACScan instrument (Becton Dickinson, Mountain View, CA).

RESULTS

A 229-bp region of the distal 5’ flank functions as an α2 integrin subunit enhancer. Three distinct regions of the 5’ flank of the α2 integrin gene were required for high-level gene expression in cells with megakaryocytic features (Fig 1). To verify that the region between bp −1426 and −2592 served enhancer function in cells with megakaryocytic features, the 1,166-bp fragment was placed in the irrelevant promoter construct SV40 CAT in both 5’ to 3’ and 3’ to 5’ orientations (Fig 2A). Both the 5’ to 3’ and 3’ to 5’ −1426/−2592 SV40 CAT constructs, independent of orientation, directed high-level, reporter gene activity in K562 cells induced with phorbol dibutyrate to undergo megakaryocytic differentiation (Fig 2B and data not shown). The −1426/−2592 SV40 CAT constructs directed only low-level CAT activity in uninduced K562 cells. The enhancer activity of the 1,166-bp region in the irrelevant SV40 CAT construct responded to induction in a manner similar to the activity of the original intact promoter/ enhancer construct containing the entire 5’ flank of the α2 integrin gene, as shown in Fig 2A and B. The orientation-independent activity of the region between bp −1426 and −2592 in an irrelevant promoter construct established its role as an enhancer.

To determine which elements within the 1,166-bp region were required for enhancer activity in cells undergoing megakaryocytic differentiation, a series of deletion mutants of the −1426/−2592 SV40 CAT construct were constructed as shown in Fig 2A. This series of deletion mutants localized strong enhancer activity to the region between bp −1426 and −1655 relative to the activity of SV40 CAT in uninduced K562 cells (Fig 2B). The construct −1426/−1655 SV40 CAT enhanced activity comparable to that of the longer enhancer constructs −1426/−1842 SV40 CAT and −1426/−2592 SV40 CAT (Fig 2A and B). These findings suggested that the enhancer was located in the 229-bp region between bp −1426 and −1655.

AP2 and GATA elements do not mediate enhancer function. Sequence analysis of this 229-bp region showed a single GATA box between bp −1628 to −1684 adjacent to an AP2 site between bp −1637 to −1644 and two tandem, but inversely oriented AP1 binding sites at bp −1543 and −1557 (Fig 1). Based on data from other laboratories suggesting that members of the GATA family are involved in erythromegakaryocytic differentiation, we initially focused on the role of the GATA and
AP2 sites in megakaryocytic enhancer activity. To evaluate the role of these two potential binding sites in enhancer activity, mutations in the AP2 site, the GA TA site, or both sites that are known to eliminate protein binding were constructed by PCR-directed mutagenesis of the $2^{1426}$/$2^{1655}$ SV40pCA T construct (Fig 3A). Activity of the mutant constructs in uninduced and induced K562 cells was compared with the activity of the intact $2^{1426}$/$2^{1655}$ SV40pCA T construct in uninduced K562 cells (Fig 3B). The activity of the intact $2^{1426}$/$2^{1655}$ SV40pCAT construct in uninduced K562 cells was assigned a value of 1.0. Results are represented as the mean and standard deviation of at least three separate experiments. The $2^{1426}$/$2^{1655}$ SV40pCAT construct directed at least 10-fold greater enhancer activity in K562 cells induced by hematopoietic cytokines than in uninduced K562 cells. In contrast to the loss of enhancer activity we expected upon mutation of the AP2 or GATA sites, mutation of either the AP2 site, the GATA site, or both sites increased enhancer activity twofold to threefold in both uninduced and induced K562 cells. Clearly, the AP2 and GATA sites do not mediate enhancer activity for the α2 integrin gene and may serve to diminish enhancer activity in hematopoietic cells.

Two tandem, inversely oriented AP1 sites demonstrate full enhancer activity. We then turned our attention to the potential role of the two tandem AP1 sites with dyad symmetry located between bp $-1543$ and $-1557$. To focus only on the two tandem AP1 sites, a shorter double-stranded DNA fragment extending from bp $-1503$ to $-1578$ that included both AP1 binding sites but eliminated 77 bp of the 5' region including the GATA and AP2 sites and 77 bp of 3' elements including the GATA and AP2 sites contained all of the enhancer activity of the longer construct $2^{1426}$/$2^{1655}$ SV40pCAT in induced K562 cells. To determine the role of one or both AP1 sites, mutation of either the 5' AP1 site, the 3' AP1 site, or both sites was prepared in the shorter construct $2^{1503}$/$2^{1578}$ SV40pCAT, as shown in Fig 4A. The ability of the mutant constructs to direct reporter gene activity in the irrelevant promoter construct SV40pCAT was compared with the activity of the nonmutated construct $2^{1503}$/$2^{1578}$ SV40pCAT and the longer $2^{1426}$/$2^{1655}$ SV40pCAT construct in both induced and uninduced K562 cells. In contrast to the high level of reporter gene activity generated by the intact $2^{1503}$/$2^{1578}$ SV40pCAT construct, the activity of the constructs with mutations of either the 5' AP1 site, the 3' AP1 site, or both sites was markedly reduced to levels only slightly above background in both uninduced and induced K562 cells (Fig 4B). These results indicate that the two adjacent but inversely oriented AP1 binding sites play an important role in enhancer activity of the α2 integrin gene in K562 cells induced to become megakaryocytic. The tandem AP1 binding sites appear responsible for both enhancer gene activity in K562 cells induced to differentiate...
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A schematic diagram of the $\alpha_2$ promoter-CAT construct and the megakaryocytic enhancer in the irrelevant promoter construct SV40pCAT. (A) The construct $\alpha_2$-2592-CAT contains the entire 5' flanking region from bp -2592 to -109 of the $\alpha_2$ integrin gene upstream to the CAT structural gene. The construct -1426/-2592 SV40pCAT consists of the megakaryocyte enhancer region extending from bp -1426 to -2592 in the irrelevant promoter construct SV40pCAT. Constructs -1842/-2592 SV40pCAT, -1426/-1842 SV40pCAT, -1426/-1655 SV40pCAT, and -1426/-1542 SV40pCAT were derived from the construct -1426/-2592 SV40pCAT. (B) The 1,166-bp region of the distal 5' flank serves as a megakaryocytic enhancer. The constructs $\alpha_2$-2592-CAT, -1426/-2592 SV40pCAT, -1842/-2592 SV40pCAT, -1426/-1842 SV40pCAT, -1426/-1655 SV40pCAT, and -1655/-1842 SV40pCAT were transfected in parallel with the SV40pCAT plasmid that contains the strong viral promoter SV40 without an enhancer into either uninduced or induced K562 cells. Cotransfection with RSV-luciferase was used to control for transfection efficiency. After 48 hours of incubation, cell extracts were assayed. After normalization for transfection efficiency, CAT activity of the constructs was determined using thin-layer chromatography and differential extraction. The mean and standard deviation of CAT activity of the mutated constructs in uninduced or induced K562 cells from three separate experiments was determined relative to the activity of SV40pCAT in uninduced K562 cells, which was assigned a value of 1.0.

To further demonstrate that the two AP1 sites within the distal 5' flanking region are required for expression of the $\alpha_2$ integrin gene in cells with megakaryocytic features, either one or both of the AP1 binding sites were mutated in the context of the original $\alpha_2$ integrin gene promoter/enhancer construct, $\alpha_2$-2592-CAT. Identical mutations of either the 5' AP1 site, the 3' AP1 site, or both AP1 sites, as outlined above, were introduced into the original $\alpha_2$-2592-CAT construct by site-directed mutagenesis. Mutation of either the 5' AP1 site, the 3' AP1 site, or both AP1 sites completely eliminated reporter gene activity mediated by the full-length construct containing the entire 5' flank of the $\alpha_2$ integrin gene in both induced and uninduced K562 cells (Fig 5), just as it did for the simpler reporter construct (Fig 4B).

The two AP1 sites serve enhancer function in DAMI cells. The enhancer activity of the two AP1 sites in DAMI cells, another model of megakaryocytic differentiation, was established. Uninduced DAMI cells express low levels of the $\alpha_2\beta_1$ integrin. In DAMI cells, $\alpha_2\beta_1$ integrin expression is markedly increased by the addition of PDB. Reporter gene activity of the $\alpha_2$-2592-CAT construct with mutations of the 5' AP1 site, the 3' AP1 site, and both AP1 sites was compared with the activity of the original $\alpha_2$-2592-CAT construct in uninduced and induced DAMI cells. The $\alpha_2$-2592-CAT construct exhibited low levels of reporter gene activity in uninduced DAMI cells and high levels of reporter activity in induced DAMI cells. Mutations of either the 5' AP1 site, the 3' AP1 site, or both AP1 sites completely eliminated reporter gene activity of the $\alpha_2$-2592-CAT in induced K562 cells, which was assigned the value of 1.0.
CAT construct in uninduced and induced DAMI cells as well (Fig 5). These findings provide additional evidence that the tandem AP1 sites located in the distal 5' flank of the α2 integrin gene are required for α2 integrin gene activity in hematopoietic cells either poised to become megakaryocytic or induced with phorbol dibutyrate to acquire megakaryocytic features.

DNA-protein complex formation with the tandem AP1 binding sites. To identify transcription factors that specifically interact with the α2 integrin enhancer, binding of nuclear proteins from uninduced and induced K562 cells to the 75-bp double-stranded DNA fragment (bp −1503 to −1578) containing the two tandem AP1 sites was evaluated by gel mobility shift assays. Nuclear proteins from both uninduced K562 cells and K562 cells induced to differentiate for 4 days with phorbol dibutyrate bound the 75-bp enhancer element (Fig 6A). Nuclear extracts from both uninduced K562 cells and K562 cells induced with phorbol dibutyrate formed a complex of intermediate migration (B). Nuclear extracts from uninduced K562 cells also formed a faster migrating complex (C) that was not as prominent with extracts from induced cells. Furthermore, nuclear extracts from K562 cells induced to develop megakaryocytic features bound the 75-bp DNA fragment to form an additional, slower migrating complex (A) that was not as abundant with extracts from uninduced cells. Formation of all three DNA-protein complexes could be specifically inhibited by the unlabeled 75-bp DNA fragment but not by an irrelevant 119-bp double-stranded DNA fragment. The unlabeled 75-bp fragment containing a mutation of the 5' AP1 site, the 3' AP1 site, or both AP1 sites (Dbl AP1m) failed to inhibit complex formation (Fig 6B, data not shown). More rapidly migrating, nonspecific complexes, as judged by the inability of the unlabeled DNA fragment to compete, were present in some experiments.

The involvement of two tandem AP1 consensus elements in the enhancer suggests a role for the AP1 family of transcription factors in α2 integrin gene regulation. A double-stranded
oligonucleotide containing only a single consensus AP1 site specifically bound nuclear extracts from both uninduced and induced K562 cells (Fig 7A, B, and C). The complex formed between the consensus AP1 oligonucleotide and nuclear extracts from induced K562 cells was more abundant than the complex formed with nuclear extracts from uninduced K562 cells. Complex formation between the consensus AP1 site and nuclear extracts from both uninduced and induced K562 cells was partially inhibited by 10-fold and almost completely inhibited by 20-fold addition of the identical unlabeled oligonucleotide and completely inhibited by 10-fold or 20-fold addition of the 75-bp enhancer fragment but was not inhibited by the AP1 consensus oligonucleotide with a 2-bp mutation (AP1m; Fig 7A and C). In addition, an oligonucleotide containing the consensus binding site for the Egr family of transcription factors failed to inhibit complex formation (Fig 7B).

Antibodies against c-fos/c-jun heterodimers prevent DNA-protein complex formation. The AP1 consensus element of the \( \alpha_2 \) integrin enhancer is separated by one nucleotide (TGANTCA).\(^{36}\) c-fos/c-jun heterodimers prefer such DNA targets. In contrast, ATF/CREB-containing dimers prefer AP1 consensus elements separated by two nucleotides (TGANNTCA).\(^{37}\) Antibodies against c-fos or c-jun family members inhibited complex formation between K562 nuclear extracts and the oligonucleotide containing the AP1 consensus site, but not oligonucleotides containing the SP1 or NFκB consensus sites in a concentration-dependent manner under our experiment conditions (Fig 7A and data not shown).

Under similar experimental conditions, antibodies against c-fos also inhibited formation of complexes B and C between the 75-bp enhancer fragment and uninduced K562 cell extracts, as shown in Fig 8. Anti–c-fos antibodies inhibited complexes A and B formed between the 75-bp enhancer and nuclear extracts from induced K562 cells. An antibody against c-jun family members diminished formation of complex B formed by the 75-bp enhancer and uninduced nuclear extracts and formation of complexes A and B between the 75-bp fragment and extracts from induced K562 cells. These findings suggest that members of the c-fos and c-jun family bind to the 75-bp enhancer fragment of the \( \alpha_2 \) integrin gene.

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**Fig 6.** Gel mobility shift analysis of the megakaryocyte enhancer. (A and B) Gel mobility shift analysis was performed with \(^{32}\)P end-labeled double-stranded DNA fragment containing the 75-bp enhancer region (bp – 1503 – 1578) with two tandem AP1 binding sites and 1 μg of nuclear protein from either uninduced K562 cells (K562) or K562 cells induced for 4 days (4d Ind) with phorbol. Three major DNA-protein complexes are indicated as A, B, and C. DNA-protein complexes were formed in the absence of competitive inhibitor (ø) (A and B), in the presence of a non-specific (NS) inhibitor consisting of 119-bp double-stranded DNA fragment from the coding region of the \( \alpha_2 \) cDNA (A), or in the presence of 75-bp DNA fragment containing mutations to both AP1 binding sites (Dbl AP1m) (B). The presence of an unlabeled specific inhibitor (75 bp) inhibited DNA-protein complex formation (A and B). Gel mobility shift analysis was performed as described in Materials and Methods.
Fig 7. A consensus AP1 site forms DNA-protein complexes. (A, B, and C) Gel mobility shift experiments were performed with \(^{32}\)P end-labeled double-stranded oligonucleotide containing a single AP1 consensus binding site and 1 \(\mu\)g of nuclear extract from uninduced K562 cells (K562) or phorbol ester-induced K562 cells (4d Ind) (A, B, and C). A DNA-protein complex formed in the absence of competitive inhibitor (\(\Theta\)) (A, B, and C), in the presence of an identical oligonucleotide with a 2-bp mutation in the consensus binding site (AP1m) (A), in the presence of the unlabeled specific 75-bp enhancer region (bp 1503–1578) with two tandem AP1 binding sites (C), or in the presence of an irrelevant double-stranded oligonucleotide containing the consensus binding site for transcription factors of the early growth response family (Egr) (B). The unlabeled AP1 oligonucleotide inhibited DNA-protein complex formation (A, B, and C). Monoclonal or polyclonal antibodies against either c-fos or c-jun (at the indicated concentration) were preincubated with nuclear extracts for 18 hours at 4°C before the addition of \(^{32}\)P end-labeled probe, as described in Materials and Methods. Antisera against both c-fos and anti–c-jun inhibited DNA-protein complex formation in a concentration-dependent manner. The molar excess of unlabeled competitor or the concentration of antibody is indicated. Gel mobility shift analyses were performed as described in Materials and Methods.
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A role for the MAPK cascade in α2 integrin gene regulation.

The signaling cascades that lead to AP1 activation include numerous mitogen activated protein kinase intermediates and transcriptional induction of c-fos and c-jun proteins. Recent observations showed that inhibition of MAPK kinase 1 (MAPKK1) prevented some features of phorbol-induced megakaryocytic differentiation, including expression of the αIIb and β3 integrin genes. The role of MAPKK1 in regulating α2 integrin gene expression was therefore evaluated. As shown previously, uninduced K562 cells fail to express detectable levels of the α2β1 integrin (Fig 9). After 6 days of PDB induction, expression of the α2β1 integrin is greatly increased. The addition of PD98059, the MAPKK1 specific inhibitor, to K562 cells 15 minutes before the addition of PDB completely eliminated α2β1 integrin subunit expression, as shown in Fig 9.

The role of the MAPK cascade in activating the α2 promoter/enhancer was assessed. As shown in Figs 2B and 10, the reporter gene activity of the original intact α2 integrin promoter/enhancer construct pα2-2592-CAT was high in induced K562 cells and at background levels in uninduced K562. Reporter gene activity of the pα2-2592-CAT in K562 cells treated with both PDB and PD98059 was markedly reduced compared with the reporter activity in PDB induced K562 cells (Fig 10). Inhibition of the MAPK kinase cascade diminished the function of the α2 integrin promoter/enhancer. Based on these findings, we propose a model by which activation of the MAPK cascade leads to α2 integrin gene expression in cells induced to become megakaryocytic (Fig 11).

DISCUSSION

In earlier studies we demonstrated that expression of the α2 integrin gene in cells with megakaryocytic features, both induced K562 cells and DAMI cells, is regulated by three distinct regions of the 5’ flank of the α2 integrin gene containing both positive and negative elements. The three regions include a core promoter between bp −30 and −92, a silencer between bp −92 and −351, and megakaryocytic enhancers between bp −1426 and −2592. Analysis of these regions showed that inhibition of MAPK cascades led to activation of the megakaryocytic enhancer of the α2 integrin gene (Fig 11). Extracellular stimuli such as phorbol dibutyrate under our experimental conditions or growth factors during thrombopoiesis directly or indirectly activate the MAPK signaling cascade.

The MAP kinase cascade involves at least three distinct signaling pathways, including the extracellular regulated kinases (ERK), jun kinase, and p38 MAPK. The best delineated pathway initiated via growth factor receptors or phorbol esters initiates a cascade from ras, raf, MAPKK1 (MEK1), or MAPKK2 (MEK2) to ERK1 or ERK2. The activated ERK proteins then translocate to the nucleus to phosphorylate and activate nuclear transcription factors, such as ternary complex factors (TCF). ERK activation directly increases AP1 activity via c-jun activation and increased c-fos synthesis, leading to an increase in c-fos/c-jun heterodimerization and DNA binding.

A role for the MAP kinase pathway in megakaryocytic differentiation was originally suggested by the ability of phorbol esters to initiate megakaryocytic differentiation and induce the expression of megakaryocyte-specific genes. A direct role for the MAP kinase pathway in megakaryocytic differentiation has been demonstrated by a number of investigators. Megakaryocytic differentiation of K562 cells induced with phorbol dibutyrate, including the morphologic aspects of cell adhesion and cell spreading and the expression of the megakaryocytic genes encoding the αIIb and β3 integrin subunits, was either partially or completely inhibited by PD98059, the MAPKK1 specific inhibitor. In addition, constitutive activation of the pathway via expression of the constitutively active mutants of MAPKK1 or MAPKK2 induced the expression of the platelet/megakaryocytic-specific genes αIIb and β3. The regulatory elements required for expression of megakaryocyte-specific proteins αIIb, platelet factor 4 (PF4), glycoprotein Ib, and c-mpl have been mapped. Adjacent GATA and c-mpl binding sites identified in the promoters of the αIIb, PF4, glycoprotein Ib, and c-mpl are essential for transcriptional activation of these genes.

The importance of these transcriptional activation factors in the role of the adjacent GATA and AP2 sites in...
enhancer activity of the α2 integrin gene. Surprisingly, the GATA and AP2 sites did not serve enhancer function and served to slightly repress the activity of the distal enhancer of the α2 integrin gene. Although the architecture of the α2 promoter/enhancer that consists of a promoter, a silencer, and a distal enhancer resembles other megakaryocytic genes, the enhancer is distinctly different and consists of two adjacent but inversely oriented AP1 binding sites. Mutation of either or both AP1 sites markedly reduced enhancer activity in the irrelevant promoter construct SV40pCAT, as well as in the intact α2 promoter/enhancer construct extending through the distal 5’ flank of the α2 integrin gene. Consensus AP1 binding sites have not been demonstrated to play an important role in the enhancer activity of other megakaryocytic genes. However, the inverse orientation of the two AP1 sites resembles the dyad symmetry element (DSE) required for c-fos induction after 12-0-tetradecanoyl phorbol-13-acetate (TPA)-induced megakaryocytic differentiation of K562 cells. AP1, a sequence-specific transcription factor composed of c-fos and c-jun family members, was initially identified as the factor that mediates TPA induction. Activation and expression of AP1 is induced by multiple stimuli, including growth factors, cytokines, UV irradiation, and the different members of the MAPK cascade.

The NF-E2 binding site consists of either one or tandem, extended AP1 binding sites. A possible role for NF-E2 in α2 integrin gene regulation was considered. The NF-E2 binding sites in the hypersensitivity site 2 (HS2) enhancer of the β globin gene resemble the two tandem AP1 binding sites in the
α₂ integrin enhancer. However, the additional -G- in the GCTGAGTC sequence that is required for NF-E2 binding is absent from both of the tandem AP1 binding sites in the α₂ integrin enhancer. In addition, although nuclear proteins from induced K562 cells bind to an oligonucleotide containing the consensus NF-E2 binding site and the unlabeled oligonucleotide containing the NF-E2 consensus binding site successfully inhibits the binding of nuclear proteins to NF-E2, the unlabeled NF-E2 oligonucleotide fails to compete for binding to the megakaryocyte enhancer region (data not shown). In addition, expression of the hematopoietic restricted component p45 failed to induce megakaryocytic differentiation or expression of the α₂β₁ integrin (data not shown).

Based on the studies presented here, the MAPK pathway is required for α₂ integrin gene expression in cells induced to differentiate along the megakaryocytic lineage. Two tandem and inversely oriented AP1 sites in the α₂ integrin enhancer bind to members of the AP1 family of transcription factors. We propose a model by which either PDB, under experimental conditions, or growth factors such as thrombopoietin (TPO), under physiologic conditions, activate the MAPK cascade and ultimately lead to α₂ integrin gene expression in cells induced to be megakaryocytic. The mechanisms by which PDB activates c-fos/c-jun family members is well delineated. TPO has been shown to directly activate the MAPK cascade after binding to c-Mpl on megakaryocytic progenitors. This model provides a physiologically relevant series of events that lead to expression of the α₂β₁ integrin.

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The Megakaryocyte/Platelet-Specific Enhancer of the \( \alpha_2 \beta_1 \) Integrin Gene: Two Tandem AP1 Sites and the Mitogen-Activated Protein Kinase Signaling Cascade

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