P-selectin, an adhesion receptor for leukocytes, is constitutively expressed by megakaryocytes and endothelial cells. Synthesis of P-selectin is also increased by some inflammatory mediators. We characterized a previously identified κB site (−218GGGGGTACCCCC−209) in the promoter of the human P-selectin gene. The κB site was unique in that it bound constitutive nuclear protein complexes containing p50 or p52, but not inducible nuclear protein complexes containing p65. Furthermore, the element bound recombinant p50 or p52 homodimers, but not p65 homodimers. Methylation interference analysis indicated that p50 or p52 homodimers contacted the guanines at positions −218 to −214 on the coding strand and at −210 to −207 on the noncoding strand. Changes in the three central residues at −213 to −211 altered binding specificity for members of the NF-κB/Rel family. Mutations that eliminated binding to NF-κB/Rel proteins reduced by −40% the expression of a reporter gene driven by the P-selectin promoter in transfected bovine aortic endothelial cells. Overexpression of p52 enhanced P-selectin promoter activity, and co-overexpression of Bcl-3 further induced promoter activity in a κB site-dependent manner. In contrast, overexpression of p50 repressed reporter activity; this repression was prevented by co-overexpression of Bcl-3. Similar phenomena were observed with reporter gene constructs driven by two tandem P-selectin κB sequences linked to the SV40 minimal promoter. These data suggest that Bcl-3 differentially regulates the effects of p50 and p52 homodimers bound to the κB site of the P-selectin promoter. This site may be a prototype for κB elements in other genes that bind specifically to p50 and/or p52 homodimers.

Regulation of the Human P-selectin Promoter by Bcl-3 and Specific Homodimeric Members of the NF-κB/Rel Family

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K B Element in the P-selectin Gene

some cells, where they may prevent binding of inducible complexes containing members of the first group of proteins such as p65 (30). Bcl-3, a protein structurally related to IxBp, associates bound p50 homodimers from DNA, allowing heterodimers containing p65 to bind and transactivate (31). In contrast, Bcl-3 forms a ternary complex with p52 homodimers on DNA, resulting in transactivation (32, 33). There is disagreement as to whether Bcl-3 can form ternary complexes with p50 homodimers on DNA that transactivate (32, 33). One difficulty in interpreting the roles of p50 and p52 homodimers in gene expression has been the inability to identify K B elements that bind only to these proteins.

In this paper we characterize the properties of the K B element in the human P-selectin promoter. We find that this K B site has the unique property of binding only to p50 and p52 homodimers. Interactions of Bcl-3, p50 homodimers, and the K B element augment transcription. In contrast, interactions of p50 homodimers with the K B element augment repression; however, repression is prevented by co-expression of Bcl-3. These data suggest that differential interactions of Bcl-3 with p50 and p52 homodimers regulate the constitutive and inducible expression of the P-selectin gene, and perhaps other genes with K B sites specific for these homodimers.

EXPERIMENTAL PROCEDURES

Cells, Proteins, Antibodies, and Expression Plasmids—CHRF-288 human megakaryocytic cells were maintained in Fisher’s medium supplemented with 20% horse serum. BAEC, HUVEC, HL-60 cells, HEL cells, and Jurkat cells were cultured as described previously (11). Recombinant p50 and p49 (a variant form of p52) were obtained from Promega. Purified, bacterially expressed p50 (amino acids 1–503) and p65 were gifts from Dr. Craig Rosen (34). Antibodies against p50, p52, and c-Rel were obtained from Santa Cruz. The expression plasmid encoding Bcl-3, driven by the Rc/CMV promoter, was a gift from Dr. Craig Rosen (34). Antibodies against p50, p52, and c-Rel were obtained from Santa Cruz. The expression plasmid encoding Bcl-3, driven by the Rc/CMV promoter, was a gift from Dr. Craig Rosen (34). Antibodies against p50, p52, and c-Rel were obtained from Santa Cruz. The expression plasmid encoding Bcl-3, driven by the Rc/CMV promoter, was a gift from Dr. Craig Rosen (34). Antibodies against p50, p52, and c-Rel were obtained from Santa Cruz. The expression plasmid encoding Bcl-3, driven by the Rc/CMV promoter, was a gift from Dr. Craig Rosen (34). Antibodies against p50, p52, and c-Rel were obtained from Santa Cruz. The expression plasmid encoding Bcl-3, driven by the Rc/CMV promoter, was a gift from Dr. Craig Rosen (34). Antibodies against p50, p52, and c-Rel were obtained from Santa Cruz.

To determine whether Bcl-3 can form ternary complexes with p50 homodimers, we synthesized a K B element that is functionally related to the element in the H-2Kb gene that binds constitutive p50 and p52 homodimers as well as inducible p50/p52 heterodimers (42–44) (Fig. 1A). As shown in Fig. 2, a 50- to 200-fold excess of an unlabeled oligonucleotide encompassing the H-2Kb promoter sequence prevented Seq B from forming complexes with p50 homodimers, with nuclear extracts. We next synthesized a shorter oligonucleotide, termed Seq B, that contained only the sequence from –222 to –200 immediately downstream of the p50/p65 boundary element (Fig. 1A). Seq B, when labeled, formed only complex I, and complex formation was specifically inhibited by addition of unlabeled Seq 1, Seq B, or the H-2Kb probe (Fig. 2). These results indicate that Seq B contains a K B element that is functionally related to the element in the H-2Kb promoter.

RESULTS

The Sequence from –232 to –192 in the 5′-Flanking Region of the P-selectin Gene Forms Five DNA-Nuclear Protein Complexes—We previously showed that deletion of the sequence from –249 to –197 in the 5′-flanking region of the human P-selectin gene decreased expression of a reporter gene in transfected BAEC by –40% (11). This region contains putative recognition elements for ETS proteins, certain zinc finger proteins induced by phorbol esters, and members of the NF-κB/Rel family of proteins. To determine whether nuclear proteins bound to this region, we synthesized a 41-base pair double-stranded oligonucleotide encompassing the sequence –232 to –192, which contained all the putative regulatory elements (Fig. 1A). The labeled oligonucleotide, termed Seq 1, formed five complexes with nuclear extracts from the megakaryocytic cell line CHRF-288 (Fig. 1B) and from all other cell types tested (BAEC, HEL cells, Hy.EA926 hybrid endothelial cells, and Jurkat cells, data not shown). Formation of all complexes was prevented by addition of a 100-fold excess of unlabeled Seq I probe, but not by an unlabeled probe containing an unrelated GATA element (11) (Fig. 1B). Complex II was resolved into separate complexes, termed IIA and IIB, only after electrophoresis for longer periods. Complexes IIA and IIB were not consistently formed by all nuclear extracts from a given cell type.

The K B Element in the P-selectin Promoter Binds Homodimers—Expression of p50 or p52 but Not Homodimers or Heterodimers Containing p65—The putative K B element in Seq I is very similar to a K B element in the murine H-2Kb gene that binds constitutive p50 and p52 homodimers as well as inducible p50/p52 heterodimers (42–44) (Fig. 1A). As shown in Fig. 2, a 50- to 200-fold excess of an unlabeled oligonucleotide encompassing the H-2Kb promoter sequence prevented Seq B from forming complex II, but not the other complexes, with nuclear extracts. We next synthesized a shorter oligonucleotide, termed Seq B, that contained only the sequence from –222 to –200 immediately downstream of the p50/p65 boundary element (Fig. 1A). Seq B, when labeled, formed only complex I, and complex formation was specifically inhibited by addition of unlabeled Seq 1, Seq B, or the H-2Kb probe (Fig. 2). These results indicate that Seq B contains a K B element that is functionally related to the element in the H-2Kb promoter.

To determine whether Seq B bound constitutive p50 or p52 homodimers as well as inducible NF-κB dimers containing p65, we first performed gel shift assays with nuclear extracts from BAEC treated with or without phorbol myristate acetate, which induces degradation of IκBα, and release of p50/p65 complexes into the nucleus (Fig. 3A). The labeled H-2Kb probe formed two complexes, the faster migrating complex, found in both unstimulated and stimulated cells, corresponded to constitutively expressed p50 or p52 homodimers. The slower moving complex represented p50/p65 heterodimers that were more abundant in extracts from stimulated cells (43). The labeled Seq B also formed the faster moving complex, but not the slower moving complex. Furthermore, unlabeled Seq B pre-
vented the labeled H2-Kb probe from forming the faster moving complex, but not the slower moving complex (Fig. 3B). These results suggest that the P-selectin κB site, unlike the H2-Kb κB element, interacts with p50 or p52 homodimers but not inducible dimers containing p65. In other experiments, Seq B failed to form slower moving complexes with nuclear extracts from HUVEC treated with LPS for 2, 4, or 24 h, or from BAEC treated with TNF-α for 2 or 4 h (data not shown). Because dimers containing c-Rel also migrate to the nuclei of stimulated HUVEC (45), this result suggests that Seq B does not bind inducible dimers containing c-Rel.

To confirm the differential specificities of the Seq B and H2-Kb probes, we performed gel shift assays with purified, recombinant p50, p52, and p65; each of these proteins forms homodimers when expressed in bacteria (34). Seq B formed complexes with p50 and p52 homodimers, but not with p65 homodimers, whereas the H2-Kb probe formed complexes with all three proteins (Fig. 4A). To determine whether complexes IIa and IIb represented interactions of Seq I with p52 and p50 homodimers, we preincubated nuclear extracts or purified p50 or p52 with specific antibodies prior to gel shift analysis. To resolve complex IIa from complex IIb, electrophoresis was performed for a longer period such that the faster migrating complexes III and IV exited the gel. Antibodies to p50 supershifted complex IIb to a slower migrating position, and antibodies to p65 supershifted complex IIa (Fig. 4B). In contrast, antibodies to c-Rel had no effect on either complex. None of the antibodies affected formation of complex I. Collectively, these data indicate that the P-selectin κB element interacts with constitutively expressed nuclear complexes consisting of p50 or p52 homodimers, but not inducible nuclear complexes containing p65.

Characterization of the Nucleotides in the P-selectin κB Sequence That Contact p50 and p52 Homodimers—We used methylation interference analysis to characterize the sites on the P-selectin κB element that bind p50 and p52 homodimers. As shown in Fig. 5, methylation of five guanines at −218 to
-214 in the coding strand and four guanines at -210 to -207 in the non-coding strand suppressed binding to p52 and p50. Methylation of the guanine at -212 on the coding strand also prevented binding to both proteins, although the effect was more pronounced for p52.

The three core nucleotides at -213 to -211 in the P-selectin \( \kappa B \) element differ from those in the \( \kappa B \)-h element, suggesting that they are important for recognition specificity. We used gel shift assays to test the effects of some changes in the core sequence of the \( \kappa B \) element of Seq I. Substitution of the G at -212 with C or A preserved recognition specificity for p50 and p52 homodimers, whereas substitution to T also conferred recognition specificity for p50 and p52 homodimers. Therefore, we asked whether p50, p52, and Bcl-3 regulate \( \kappa B \)-dependent expression (14). The previously studied genes have \( \kappa B \) elements that bind both p50 and p52 homodimers as well as heterodimers containing p65. In contrast, the \( \kappa B \) site in the P-selectin promoter binds only p50 and p52 homodimers. Therefore, we asked whether p50, p52, and Bcl-3 regulate \( \kappa B \)-dependent expression of the P-selectin gene. BAEC were co-transfected with plasmids encoding various combinations of these proteins with a luciferase reporter gene driven by the P-selectin promoter containing a wild-type or mutated \( \kappa B \) element. Co-expression of p52 augmented luciferase expression driven by the wild-type promoter, but not the promoter with the mutated \( \kappa B \) element (Fig. 7A). Co-expression of increasing amounts of Bcl-3 with a constant amount of p52 further increased luciferase expression by the wild-type, but not the

![Fig. 4. The P-selectin \( \kappa B \) element binds p50 and p52 homodimers but not p65 homodimers](image)

![Fig. 5. Methylation interference analysis of the nucleotides in the P-selectin \( \kappa B \) element that contact p50 and p52 homodimers](image)
mutant, promoter. The expression observed without co-transfection of p52 or Bcl-3 may reflect the basal functions of the endogenously expressed proteins (42, 44, 46). Similar stimulatory effects were observed with a reporter gene driven by an SV40 minimal promoter linked to two copies of wild-type Seq B, but not Seq B containing a mutated kB element (Fig. 7B). The mutated Seq B oligonucleotide also failed to interact with p50 or p52 in gel shift assays (data not shown).

In sharp contrast, co-expression of p50 repressed luciferase expression driven by the wild-type P-selectin promoter (Fig. 8A). However, co-expression of increasing amounts of Bcl-3 prevented the inhibitory effects of p50 on reporter gene expression. Similar effects were observed with the reporter gene containing two copies of wild-type Seq B linked to the SV40 minimal promoter (Fig. 8B). The p50 construct in these experiments encompassed residues 1–503, whereas the p50 protein generated by proteolysis in intact cells may span only the first 400 amino acids (33). It has been suggested that only homodimers containing the larger form of p50 repress kB-dependent gene expression (33). However, we found that a p50 construct encoding residues 1–401 had similar inhibitory effects on P-selectin reporter gene expression (data not shown). These data indicate that the function of the kB element in the P-selectin promoter is differentially regulated by interactions of Bcl-3 with p52 and p50 homodimers.

Cells Expressing P-selectin Also Transcribe mRNAs for Bcl-3 and the Precursors of p50 and p52—Endothelial cells and megakaryocytes constitutively synthesize P-selectin in vivo (47). Northern blot analysis of RNA from HUVEC and the megakaryocytic HEL and CHRF-288 cell lines, which also constitutively express P-selectin (41), identified transcripts for NF-kB1 (p105), NF-kB2 (p100), and Bcl-3 (Fig. 9). The presence of these transcripts is consistent with a role of Bcl-3, p50, and p52 in regulating the expression of P-selectin in megakaryocytes and endothelial cells.

**DISCUSSION**

We defined a unique kB site in the P-selectin promoter that recognized homodimers containing p50 and p52, but not homodimers or heterodimers containing p65. Interactions of Bcl-3 with p50 and p52 homodimers differentially regulated the activity of the kB site. The gene for P-selectin may be a prototype for other genes whose expression may be regulated by kB sites that do not bind inducible heterodimeric NF-κB complexes.

Inducible NF-κB complexes containing p65 activate gene transcription in response to a variety of inflammatory signals (14). These complexes are normally sequestered in the cytoplasm by IκBα. Upon cellular stimulation, IκBα is phosphoryl-
Similar results were obtained in another experiment. The data in experiment. Similar results were obtained in two other experiments.

...stitutively expressed in the nucleus, at least in some cells (14). In contrast, p50 and p52 homodimers are constitutively expressed in the nucleus, at least in some cells (14). Most reports suggest that p50 homodimers do not transactivate and degraded, releasing the p65-containing heterodimers to the nucleus. In contrast, p50 and p52 homodimers are constitutively expressed in the nucleus, at least in some cells (14). Most reports suggest that p50 homodimers do not transactivate gene expression (14). Instead, it has been proposed that p50 homodimers serve as repressors of gene activation by competing with p50/p65 heterodimers for binding to \( \kappa B \) elements (30, 31). For example, p50 homodimers in nuclear extracts of unstimulated T cells bind the \( \kappa B \) element in the IL-2 gene (30). Upon antigenic stimulation, fewer p50 homodimers bind to the element, whereas p50/p65 heterodimers that have moved to the nucleus then bind. Activation of the IL-2 gene is correlated with the change in binding profiles. Notably, inhibitors of protein synthesis prevent the loss of binding of p50 homodimers as well as activation of the IL-2 gene, suggesting that a newly synthesized protein sequesters p50 homodimers in the nucleus. This protein might correspond to Bcl-3, which is inducibly expressed in some cells (48) and dissociates p50 homodimers from bound DNA (31). In contrast, ternary complexes of Bcl-3 and p52 homodimers may activate expression of some genes (32, 33). For example, the \( \kappa B \) site in the H-2K\( ^{b} \) promoter is required for constitutive gene expression, and expression is correlated with binding of nuclear p52 homodimers to the \( \kappa B \) element (42, 44).

A difficulty in interpreting previous studies of p50 and p52 homodimers is that the \( \kappa B \) elements of genes encoding proteins such as IL-2 and H-2K\( ^{b} \) also bind p65-containing heterodimers. Furthermore, variable levels of such heterodimers have been found in cells in the absence of overt stimulation (14). Thus, it has not been clear whether p50 and p52 homodimers regulate gene expression directly, or function indirectly by affecting binding of p65-containing heterodimers to \( \kappa B \) elements. Because the \( \kappa B \) site in the P-selectin gene did not bind p65, the role of the interactions of Bcl-3 with p50 and p52 homodimers could be more clearly assessed. Mutations of the \( \kappa B \) element that abolished binding to p50 and p52 homodimers reduced gene expression directed by the P-selectin promoter in transfected BAEC. Co-expression of p52 and Bcl-3 augmented expression in a concentration-dependent manner. In contrast, co-expression of p50 repressed expression, but this repression was prevented by co-expression of Bcl-3. These data suggest that p50 and p52 homodimers compete for the P-selectin \( \kappa B \) site. In this model, binding of Bcl-3 to DNA-bound p52 homodimers activates gene expression, whereas binding of Bcl-3 to DNA-bound p50 homodimers results in their dissociation from DNA, allowing p52 homodimers to bind. The model predicts that the constitutive expression of P-selectin is partially regulated by the relative amounts of p50, p52, and Bcl-3 in megakaryocytes and endothelial cells; basal levels of mRNA encoding all three proteins were detected in the cultured endothelial cells and megakaryocytic cell lines that we examined. The function of the \( \kappa B \) site may also be regulated by inflammatory stimuli. LPS increases transcripts for the precursors of p50 and p52 in cultured HUVEC, although the relative amounts of these transcripts were not quantified (22, 49). Mitogen stimulation increases transcripts for Bcl-3 in peripheral blood mononuclear cells (48). Phosphorylation of Bcl-3 may also affect its activity (33, 46).

Regulation of P-selectin expression probably requires cooperative interactions of proteins binding to the \( \kappa B \) site with proteins binding to other elements in the promoter/enhancer. Mutation of the \( \kappa B \) element reduced but did not eliminate constitutive expression in BAEC. A GATA element downstream of the \( \kappa B \) site was previously demonstrated to be required for optimal P-selectin expression, and several other putative regulatory elements in the promoter have been identified (11). An oligonucleotide encoding Seq I, which spanned the area immediately surrounding the \( \kappa B \) site, formed several other complexes with nuclear proteins. These and other proteins may positively or negatively regulate binding of p50 or
p52 homodimers to the κB site, and may affect the ability of Bcl-3 to activate gene expression.

Methylation interference analysis indicated that p50 and p52 homodimers contacted four adjacent guanines on each half-site of the P-selectin κB element, consistent with a preference for these homodimers to bind symmetrical half-sites (42, 50). The κB elements in the P-selectin and H-2κB genes are similar, except that the three residues separating the two half-sites differ. These three residues contribute to recognition specificity, since the H-2κB element also binds p50/p65, and certain substitutions of these residues in the P-selectin element conferred binding to p50/65 as well as to p50 and p52 homodimers. The sequences of the P-selectin κB element and the altered versions that retained specificity for p50 and p52 homodimers were not identified by random amplification of sequences by polymerase chain reaction (50). We hypothesize that other genes have κB elements that bind only p50 and/or p52 homodimers. One candidate is the gene encoding Bcl-3, which has an intrinsic autoregulatory function. Bcl-3 could recognize the κB site, and may affect the ability of Bcl-3 to activate gene expression.

Expression of the gene for P-selectin is clearly regulated in response to inflammatory stimuli. TNF-α and/or form activating complexes with p52 homodimers bound to the κB element and/or form activating complexes with p52 homodimers bound to the κB element.

Expression of the gene for P-selectin is clearly regulated differently than that of the genes encoding the endothelial adhesion receptors E-selectin, VCAM-1, and ICAM-1. Transcriptional induction of the latter genes by LPS, IL-1, and adhesion receptors E-selectin, VCAM-1, and ICAM-1 is positively autoregulated by its protein product. Bcl-3 could be positively autoregulated by its protein product.
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