MOLECULAR AND CELLULAR BIOLOGY, June 1993, p. 3802–3810
0270-7306/93/063802-09$02.00/0

Vol. 13, No. 6

Evidence for Differential Functions of the p50 and p65 Subunits of NF-κB with a Cell Adhesion Model

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Received 13 October 1992/Returned for modification 16 November 1992/Accepted 8 March 1993

The p50 and p65 subunits of NF-κB represent two members of a gene family that shares considerable homology to the *rel* oncogene. Proteins encoded by these genes form homo- and heterodimers which recognize a common DNA sequence motif. Recent data have suggested that homodimers of individual subunits of NF-κB can selectively activate gene expression in vitro. To explore this possibility in a more physiological manner, murine embryonic stem (ES) cells were treated with phosphorothio antisense oligonucleotides to either p50 or p65. Within 5 h after exposure to phosphorothio antisense p65 oligonucleotides, cells exhibited dramatic alterations in adhesion properties. Similar findings were obtained in a stable cell line that expressed a dexamethasone-inducible antisense mRNA to p65. Although antisense oligonucleotides raised against both p50 and p65 elicited a significant reduction in their respective mRNAs, only the cells treated with antisense p50 maintained a normal morphology. However, 6 days following removal of leukemia-inhibiting factor, a growth factor which suppresses embryonic stem cell differentiation, adhesion properties of cells treated with the antisense p50 oligonucleotides were markedly affected. The ability of the individual antisense oligonucleotides to elicit differential effects on cell adhesion, a property dependent upon the stage of differentiation, suggests that the p50 and p65 subunits of NF-κB regulate gene expression either as homodimers or as heterodimers with other *rel* family members. Furthermore, the finding that reduction in p65 expression alone had profound effects on cell adhesion properties indicates that p65 plays an important role in nonstimulated cells and cannot exist solely complexed with the cytosolic inhibitory protein IκB.

The NF-κB transcription factor complex is a pleiotropic activator which participates in the induction of a wide variety of cellular and viral genes (2, 17). The active complex is composed of two subunits designated p50 and p65 (2, 7). The genes encoding p50 (8, 12) and p65 (21, 24) have been cloned, and the N termini of both proteins show considerable homology to the product of the oncogene *rel*.

Although both subunits of NF-κB bind to a common decameric DNA motif as either homo- or heterodimers, it was recently demonstrated that the individual subunits exhibit distinct binding preferences (15). Furthermore, selected DNA motifs that bound only one of these subunits (p50 or p65) were significantly reduced in their ability to recognize NF-κB (i.e., p50/p65) and sometimes could not recognize it at all (15). These findings suggest the existence of rather distinct κB DNA motifs that have differential binding specificities for the Rel proteins, implicating a role for such motifs in selective regulation of gene expression. Consistent with this speculation, transfection studies demonstrate that the p65 subunit can function as a homodimeric transcriptional activator (3, 25). However, the physiological relevance of this observation remains unclear, as it is believed that in resting cells p65 exists in an inactive cytoplasmic complex with the inhibitory molecule IκB (1, 27). The potential role of the individual subunits of NF-κB on regulation of gene expression was examined by using antisense oligonucleotides complementary to p50 and p65. We chose to assess the effects on cell adhesion as a measure of antisense function.

The precise regulatory mechanisms of the molecules involved in cell adhesion (cell adhesion molecules [CAMs]) have not yet been elucidated. Several of these CAMs, such as ICAM-1 (29), vimentin (18), and ELAM-1 (30), have been shown to have NF-κB binding sites. We reasoned that NF-κB may affect cell adhesion by regulating a variety of these adhesion molecules, thus affecting cell growth. By the use of antisense oligonucleotide-mediated inhibition of gene expression, we have investigated the role of the individual subunits of NF-κB in cell adhesion. Our results show that in diverse cell types inhibition of the p65 subunit alone causes significant inhibition of cell adhesion. The inhibition of p50, on the other hand, has no effect on the adhesion of most cell types. However, in the murine ES cell system, the p50 antisense oligonucleotides inhibit cell adhesion in a differentiation-dependent manner. Our results demonstrate that the p65 subunit of NF-κB can function to regulate cell adhesion either as homodimers or as heterodimers with other members of the *rel* family.

MATERIALS AND METHODS

Antisense oligonucleotides. The phosphorothio analogs of the oligonucleotides were synthesized by using an automated synthesizer (model 394; Applied Biosystems, Foster City, Calif.), according to the published protocol (19). The oligomers were purified as described previously (20) with one modification: the oligonucleotides were routinely reextracted with ether (six times) before washing with absolute ethanol. Cells were trypsinized, mixed with the oligonucleotides (30 μM), and plated onto a variety of extracellular

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TABLE 1. Phosphorothio oligonucleotides used in this study

Gene	Species	Sequence (5′ to 3′)[a]	Reference
P65-S	Murine	ACC ATG GAC GAT CTG TTT CCC CTC	6
P65-AS	Murine	GAG GGG AAA CAG ATC GTC CAT GGT	6
P65-S	Human	GCC ATG GAC GAA CTG TTC CCC	7
P65-AS	Human	GGG GAA CAG TTC GTC CAT GGC	7
P50-S	Murine	ACC ATG GCA GAC GAT GAT CCC	4
P50-AS	Murine	GGG ATC ATC GTC TGC CAT GGT	4
P50-S	Human	AGA ATG GCA GAA GAT GAT CCA	8
P50-AS	Human	TGG ATC ATC TTC TGC CAT TCT	8

[a] The oligonucleotide sequences correspond to the 5′ ends of the respective mRNAs and include three or four nucleotides present upstream of the initiation codon.

matrix (ECM)-coated dishes as previously described (11) for 24 h to 2 weeks; in some experiments the oligonucleotides were added every 48 h. In some experiments, cells were also plated first and allowed to attach before addition of the oligonucleotides. The antisense oligonucleotide experiments were repeated three to four times with independently prepared oligonucleotides. Cells were used at different passages.

Cell culture. The embryonic stem (ES) cells (CCE-24; L. Robertson, Columbia University) were routinely grown on 1% gelatin-coated dishes in Dulbecco's modified Eagle's medium containing 15% heat-inactivated fetal bovine serum, 10 ng of human leukemia inhibitory factor (LIF) (UBI, Lake Placid, N.Y.) per ml, and monothioglycerol (Sigma, St. Louis, Mo.) at 4.5×10^{-4} M. Differentiation was initiated by the removal of LIF and subsequent subculturing for 6 to 8 days in medium minus LIF. The cell lines NIH 3T3, Rat-1, PC-12, and S-17 were maintained as described previously (16, 20). Primary human vascular endothelial cells (HUVECs) and primary keratinocytes were from Clonetics Inc., San Diego, Calif. RHEK-1 cells were maintained as described previously (23). An ECM was established from feeder layer fibroblasts by lysing the confluent cultures with 0.5% Triton X-100 containing 3.5×10^{-4} M NH_4OH for 5 min at room temperature, followed by three washes with phosphate-buffered saline.

PCR analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as previously described (20). The p65 primers (i) 5′ GCG GCC AAG CTT AAG ATC TGC CGA GTA AAC 3′ and (ii) 5′ CGC TGC TCT AGA GAA CAC AAT GGC CAC TTG CCG 3′ define an amplicon of 150 bp. The p50 (i) 5′ AAA GGT TAT CGT TCA GTT 3′ and (ii) 5′ TTG TAG ATA GGC AAG GTC 3′ define an amplicon of 250 bp. The GAPDH primers have been described (20). The cytokine receptor primer sequences have been described (26).

Plasmid construction and stable cell lines. A 350-bp fragment of m-p65 cDNA was cloned by RT-PCR from NIH 3T3 cell cDNA, encompassing the ATG initiation codon. The PCR primers included (i) 5′ ACC GCT CGA GCT AGC CCG GGA CCC TGA CCA TGG AC 3′ and (ii) 5′ CCG GAA TTC GCT AGC GCT TCA CAC ACT GGA TCC CCA GG 3′, and the amplified fragment was inserted into the *Nhe*I site of the MAM-Neo-CAT vector (20). Sense and antisense clones were characterized by restriction analysis and confirmed by sequencing. Supercoiled plasmid DNAs were transfected onto PC-12 cells by electroporation as described previously (16).

Nuclear extracts and gel mobility shift assays. Rat PC-12 stable cell lines expressing p65 sense or antisense RNA were used to prepare nuclear extracts (22) in the absence (−) or presence (+) of 10^{-6} M dexamethasone. An oligonucleotide containing the sequence 5′ GTA GGGGACTTTCC GAG CTC GAG ATC CTA TG 3′ was labeled with ^{32}P as described previously (15) and used as a probe. Binding reactions were carried out as described previously (15, 25). Nuclear extracts (≈20 ng) and ^{32}P-labeled probe (0.5 ng; 50,000 cpm) were used in the binding reactions. Complexes were resolved on a 4% nondenaturing polyacrylamide gel; this process was followed by autoradiography.

Other methods. RNA was isolated by RNAzol-B (Cinna Biotecx, Friendswood, Tex.). Northern (RNA) blotting was performed as previously described (20).

RESULTS

Differential effect of inhibition of NF-κB p50 and p65 on cell adhesion. To establish a differential function for the NF-κB subunits, we chose to examine the effects of antisense oligonucleotides complementary to p50 and p65 in a cellular adhesion assay. Modified phosphorothio oligonucleotides to the individual subunits of NF-κB were synthesized (Table 1). We initially chose murine ES cells to test the effects of inhibition of the p50 and p65 subunits of NF-κB. The ES cells were maintained undifferentiated by the presence of LIF; differentiation was initiated by withdrawing LIF from these cultures. Exposure of both the differentiated and undifferentiated ES cells to p65 antisense oligonucleotides caused complete detachment of the cells from the gelatin-coated dishes; the control (sense) p65 oligonucleotide had no effect on the ES cells' adhesion (Fig. 1).

The p50 antisense oligonucleotides exhibited a dramatic effect that was dependent on the differentiational status of the ES cells; in the undifferentiated ES cells, the p50 antisense oligonucleotides had no effect. After removal of LIF for 6 days to allow the ES cells to differentiate, addition of p50 antisense oligonucleotides caused complete detachment of cells, an effect identical to that of p65 antisense oligonucleotides (Fig. 2). The differentiative status of these ES cells was confirmed by monitoring the upregulation of diverse cytokine receptors such as epo-R, c-*kit*, G-CSF-R, and CSF-1R (19a). The antisense oligonucleotide-detached cells were viable by trypan blue exclusion and continued to grow as aggregates in the presence of antisense oligonucleotides (oligonucleotides being replaced every 48 h) for 2 to 3 weeks. Furthermore, the antisense p65-treated ES cells were growth inhibited (40 to 60%) in comparison to p65 sense or p50 oligonucleotides. The growth-inhibitory effects of antisense p65 oligonucleotides were seen with diverse adherent cell lines including K-BALB (fibrosarcoma), B-16 (melanoma), T-47D (breast carcinoma), MCF-7 (breast carcinoma) HT-29 (colon carcinoma), and HOS-MNNG (osteosarcoma)

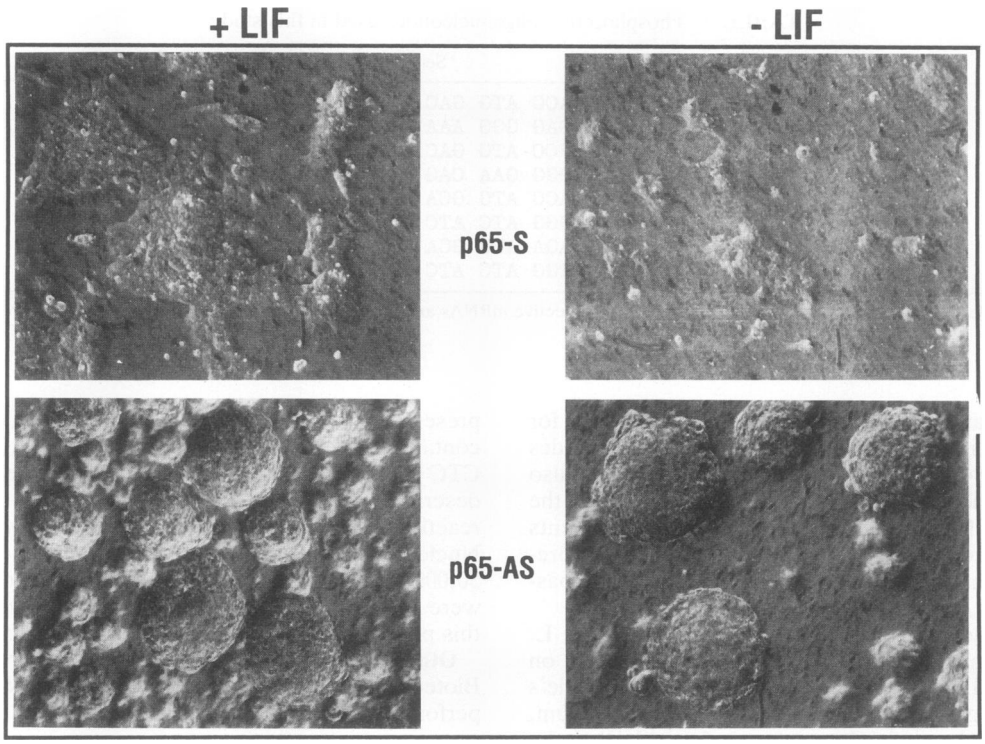

FIG. 1. Antisense oligonucleotides to p65 inhibit ES cell adhesion to a matrix independent of differentiative status. ES cells were cultured differentiated and undifferentiated as described in Materials and Methods. Cells were trypsinized and mixed with phosphorothio oligonucleotides to p65 (30 μM), replated onto gelatin-coated dishes, and photographed after 72 h in culture. Magnification, ×50.

(19a). These results suggested that the effect of these antisense oligonucleotides on ES cell adhesion is highly specific and is not due to a nonspecific toxicity. Several unrelated antisense oligonucleotides had no such effect on ES cell adhesion (19a). The antisense oligonucleotide-treated ES cells grew as an aggregate in suspension, similar to ES cell growth in methyl cellulose cultures (31). Furthermore, the effects of antisense p50 and p65 were transient; when replated in the absence of new oligonucleotides, the antisense oligonucleotide-treated ES cells retained their differentiated or undifferentiated morphology. These results provided us with a strong indication that NF-κB plays an important role in cell-to-substratum adhesion.

Involvement of ECM in p65 antisense-mediated inhibition of ES cell adhesion. Recently we demonstrated that inhibition of a putative adhesion molecule and a tumor suppressor gene, deleted in colorectal cancer (DCC), by antisense oligonucleotides caused detachment of a variety of cells which could be abrogated by plating the cells on diverse ECMs (20). We reasoned that the p65 antisense oligonucleotides' effect on ES cell adhesion could also be overcome by the use of a distinct ECM (Fig. 3 and 4). ES cells plated on fibronectin, laminin, or collagen were completely detached when exposed to p65 antisense oligonucleotides. However, when the ES cells were plated on an ECM generated by lysed feeder-layer cells (Fig. 4), the detachment effect of p65 antisense oligonucleotides was completely abolished. These results suggest that some other adhesion molecule(s) is provided by the total ECM from feeder cells, independent of NF-κB function.

The effect of p65 antisense oligonucleotides on ES cell adhesion is very rapid: within 5 h, the p65 antisense-treated cells showed dramatic alteration in their adhesion property (Fig. 5). These cells were completely detached within 12 to 14 h, whereas neither the p65 sense nor the p50 antisense oligonucleotides had any effect on the adhesion of undifferentiated ES cells for up to 2 weeks.

We next investigated the inhibition of p50 and p65 mRNA expression in these antisense oligonucleotide-treated ES cells by RT-PCR (Fig. 6). The p65 antisense oligonucleotides abolished p65 mRNA expression in both the undifferentiated and differentiated ES cells, while the expression of p50 and that of a housekeeping enzyme, GAPDH, were unaffected in these cells (Fig. 6A). Similarly, the p50 antisense oligonucleotides inhibited p50 mRNA expression regardless of the differentiative status of the ES cells, whereas the p65 and GAPDH mRNA expression were not affected in these cells (Fig. 6B). These results indicate that the differential effects of p65 versus p50 antisense oligonucleotides on ES cell adhesion are due to selective inhibition of the respective mRNA expression. The effect of the p65 phosphorothio oligonucleotides on NF-κB-like binding activity in undifferentiated cells was next investigated with ES and PC-12 cells (Fig. 7). NF-κB-like binding activity was detected in the nuclear extracts of both the cell lines treated with the control p65 sense oligonucleotides, and this activity was sensitive to competition by a 30-fold molar excess of cold oligonucleotide. In contrast, the complex was significantly inhibited in the p65 antisense oligonucleotide-treated cells, suggesting that inhibition of p65 mRNA is associated with inhibition of NF-κB-like activity.

We further investigated whether the inhibition of cell adhesion by p65 antisense oligonucleotides is applicable to other cell types (Table 2). The p65 antisense oligonucleotides caused complete detachment of diverse cell lines and primary cells, effects that were sequence and species specific.

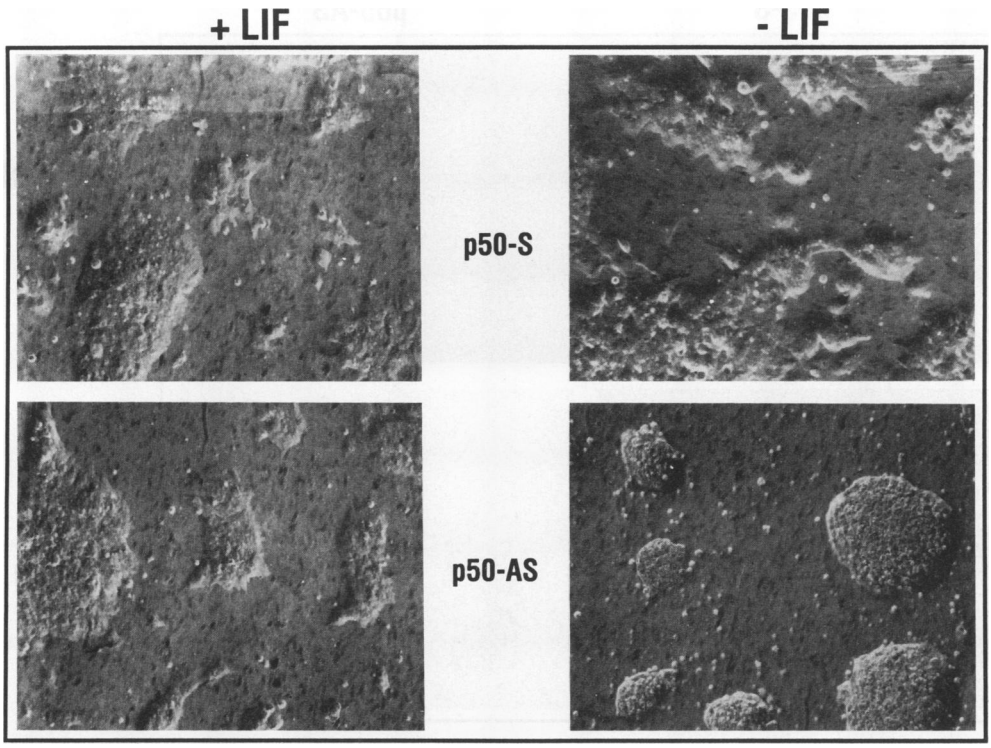

FIG. 2. Differentiation-specific inhibition of ES cell adhesion by p50 antisense oligonucleotides. ES cells were cultured on gelatin-coated dishes and allowed to differentiate for 6 days by withholding LIF. The trypsinized cells were then mixed with the phosphorothio oligonucleotides to p50 (30 μM), plated onto gelatin-coated dishes, and photographed as for Fig. 1. Magnification, ×50.

p50 antisense oligonucleotides had no effect on these cells. These results strongly support a pleiotropic function for NF-κB and a more specific role in cell adhesion for the p65 subunit of NF-κB.

Inducible expression of stable antisense RNA to p65 inhibits cell adhesion. To corroborate the effects of antisense oligonucleotides to p65 on cell adhesion, we generated a stable PC-12 cell line expressing dexamethasone-inducible antisense RNA to p65 utilizing a MAM-Neo-CAT vector (16, 20) and established both control (sense) and antisense transfectants of PC-12 cells. High levels of dexamethasone-inducible antisense RNA to p65 were detected in several independent clones (data not shown). The control sense clones showed no change in cell adhesion properties when treated with dexamethasone (Fig. 8), and the antisense p65 clones showed a normal morphology in the absence of dexamethasone. However, dexamethasone induction of antisense RNA to p65 caused a dramatic effect on cell adhesion to the substratum similar to the effect of antisense oligonucleotides to p65 in these cells. Following the removal of dexamethasone from these antisense clones, the cells reverted to normal morphology.

We next investigated NF-κB binding activity in these transfectants by a gel mobility shift assay (Fig. 9). In the absence of dexamethasone, an NF-κB-like binding activity was observed in both the sense and antisense clones (Fig. 9A) that correlated with no loss of cellular adhesion (Fig. 8). In contrast, induction of p65 antisense RNA by dexamethasone treatment in two independent antisense clones caused a significant reduction in NF-κB-like binding complexes (Fig. 9A) that correlated with a pronounced inhibition of cell adhesion (Fig. 8). On the other hand, dexamethasone treatment of the control sense transfectants did not cause any inhibition of NF-κB-like binding activity (Fig. 9A). The specificity of these NF-κB-like DNA binding complexes was further demonstrated by the ability of a 30-fold molar excess of cold oligonucleotide to inhibit the binding of labeled oligonucleotides in either the sense or antisense extracts (Fig. 9B).

DISCUSSION

The studies reported here were undertaken to examine the function of the individual subunits of the NF-κB transcription factor complex. Although the p50 and p65 subunits form the NF-κB heterodimer, several lines of evidence suggest that these molecules may also function as homodimers or as heterodimers with other proteins. For example, transfection studies demonstrate that p65 is a potent transcriptional activator in the apparent absence of the p50 subunit (3, 25). Similarly, results obtained from in vitro transcription studies suggest that p50 is also a strong transcriptional activator (6, 14). The physiological relevance of both observations remains unexplained. In the case of p65, investigators have failed to observe p65 homodimers in cell extracts (1, 27). Furthermore, it is believed that in unstimulated cells, p65 is present in an inactive cytoplasmic state complexed to the inhibitory protein IκB (1, 27). As for p50, although transactivation has been observed in vitro, transfection studies in mammalian cells have failed to demonstrate activity without the presence of other *rel*-related proteins.

In an attempt to examine the function of the p50 and p65 subunits in a physiological setting, the effect of antisense oligonucleotides complementary to both p50 and p65 was

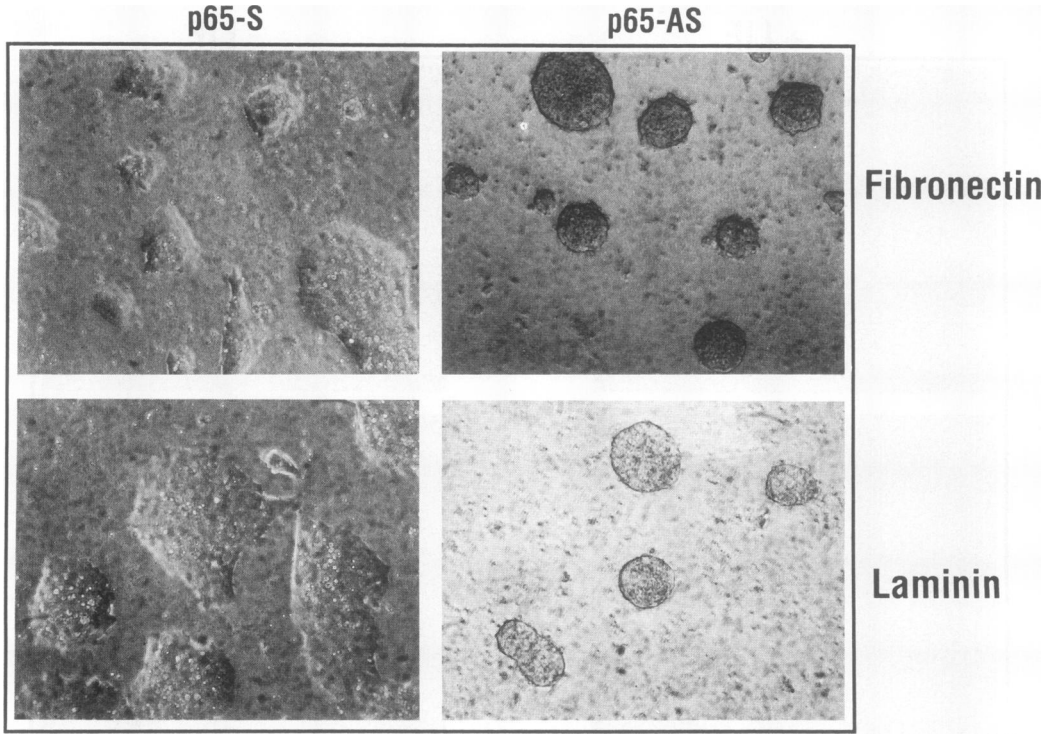

FIG. 3. Effect of ECMs on p65 antisense-mediated inhibition of cell adhesion. Undifferentiated ES cells were cultured in the presence of p65 phosphorothio oligonucleotides (30 μM). The cells were trypsinized and replated onto fibronectin (10 μg/ml) or laminin (10 μg/ml)-coated dishes and photographed after 72 h. Magnification, ×50.

examined. The antisense approach involves introducing oligonucleotides complementary to mRNA into cells (9, 10, 28). The inhibition of gene expression by this approach is highly specific and dependent on the formation of an antiparallel duplex by complementary base pairing between the antisense construct, be it RNA or DNA, and the target mRNA. We used cellular adhesion as an assay to assess antisense function. The model was derived on the basis of the observation that numerous adhesion molecules, including ELAM-1 and ICAM-1, contain NF-κB sites within their 5′ regulatory regions and appear to be regulated by stimuli that induce NF-κB (29, 30).

Exposure of murine ES cells to antisense oligonucleotides to the p65 subunit of NF-κB complex results in dramatic alterations in adhesion properties. This effect occurs within 5 h, independent of differentiative status of the cells, and is reversible. The detached cells are viable, though growth inhibited, and continue to grow as aggregates in suspension in the presence of antisense p65. In addition, the ES cells exposed to p65 antisense oligonucleotides retain their commitment to differentiate when the cells are washed free of oligonucleotides.

In the p65 antisense oligonucleotide-treated ES cells, mRNA expression of p65 is completely inhibited, while expression of p50 or a housekeeping enzyme, GAPDH, was unaffected. The control (sense) p65 oligonucleotide had no effect under identical conditions, suggesting a sequence-specific inhibition of cytoplasmic levels of p65 mRNA. In addition, the NF-κB-like activity was significantly inhibited by antisense p65 oligonucleotides in two different cell lines (ES cells and PC-12 cells).

The inhibition of cell adhesion by p65 antisense oligonucleotides was applicable to diverse cell types tested, supporting a pleiotropic requirement of NF-κB complex for cell adhesion. Our results also demonstrate that individual ECMs such as fibronectin, laminin, or collagen could not overcome the requirement of CAMs regulated by NF-κB function. However, ECMs generated by feeder layer cell lysates had the ability to supplement the requirement of NF-κB function in cell adhesion. This raises the possibility that NF-κB-independent CAMs also are involved in cell adhesion.

The effect of p65 antisense oligonucleotides on cell adhesion is further corroborated by our stable antisense RNA experiments. A dexamethasone-dependent inhibition of cell adhesion to the substratum was seen in stable PC-12 antisense transfectants which expressed high levels of inducible antisense RNA to p65. These results with stable antisense RNA to p65 strongly argue against any nonspecific, sequence-independent inhibition of phosphorothio oligonucleotides on cell adhesion (5). Furthermore, induced expression of p65 antisense RNA caused a significant reduction in NF-κB-like DNA binding activity from nuclear extracts in these stable transfectants. The antisense oligonucleotides as well as antisense RNA to p65 caused the PC-12 cells to grow detached from the substratum as aggregates. This suggests that inhibition of p65 expression interferes with cell-to-substratum, rather than cell-to-cell, adhesion. These stable cell lines should be useful in the eventual identification of specific CAMs regulated by NF-κB.

Inhibition of p50 mRNA by p50 antisense oligonucleotides had no effect on diverse cell types except in the ES cell system, where a dramatic effect was seen. The adhesion of undifferentiated ES cells was not affected by inhibition of p50 expression, but in differentiated ES cells, inhibition of p50 mRNA resulted in pronounced inhibition of adhesion,

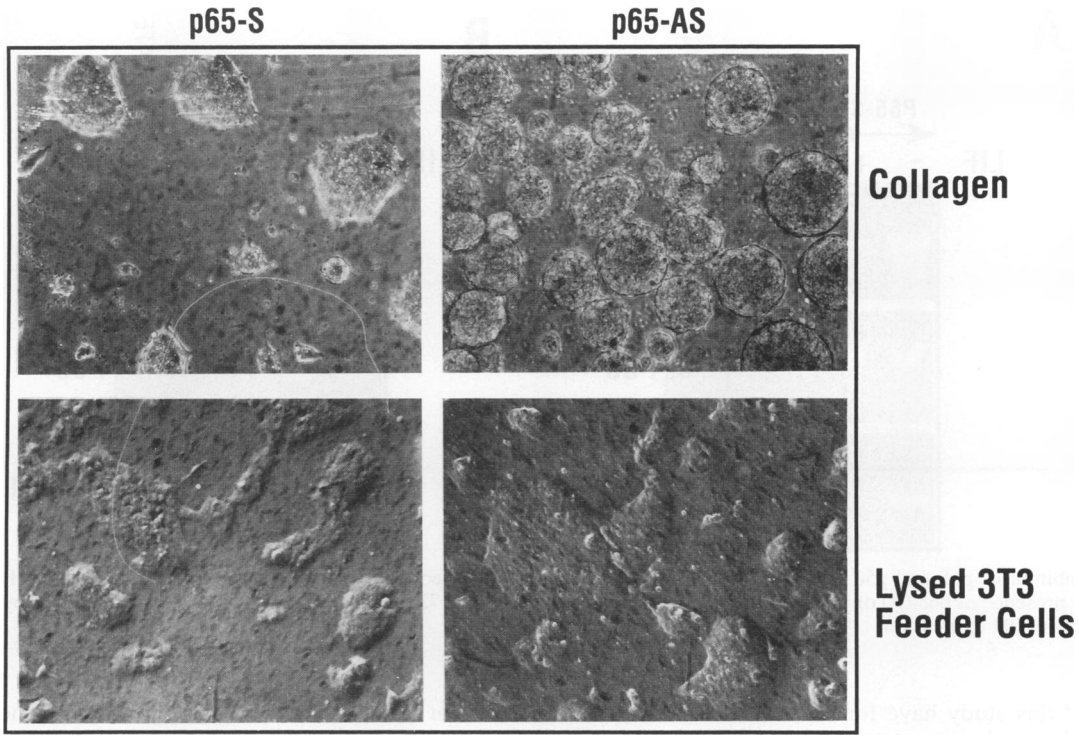

FIG. 4. Abrogation of p65 antisense oligonucleotides' effect on cell adhesion by ECM from feeder layer fibroblasts. Undifferentiated ES cells were cultured in the presence of p65 phosphorothio oligonucleotides (30 μM). The cells were trypsinized and replated onto collagen type IV (5 μg/ml) or onto an ECM generated by lysing the 3T3 feeder cells, and photographed after 72 h. Magnification, ×50.

identical to the effects of p65 antisense. It is not clear whether such a differentiation-specific effect of p50 antisense oligonucleotides is also true in other differentiable cell systems; efforts are under way to address this point. Nonetheless, these results indicate that in undifferentiated ES cells the p65 subunit of NF-κB can either complex with some subunit other than p50 or can function as a homodimer. This is further supported by our observations that inhibition of p65 expression alone in diverse cell types can elicit a dramatic effect on cell adhesion. Under these conditions p50 expression was not affected. Thus in resting cells, p65 can play an essential function in regulating diverse genes involved in cell adhesion and cannot exist solely complexed with cytosolic inhibitory protein IκB (1).

Although the potential target or pathway by which antisense p65 affects adhesion properties was not examined,

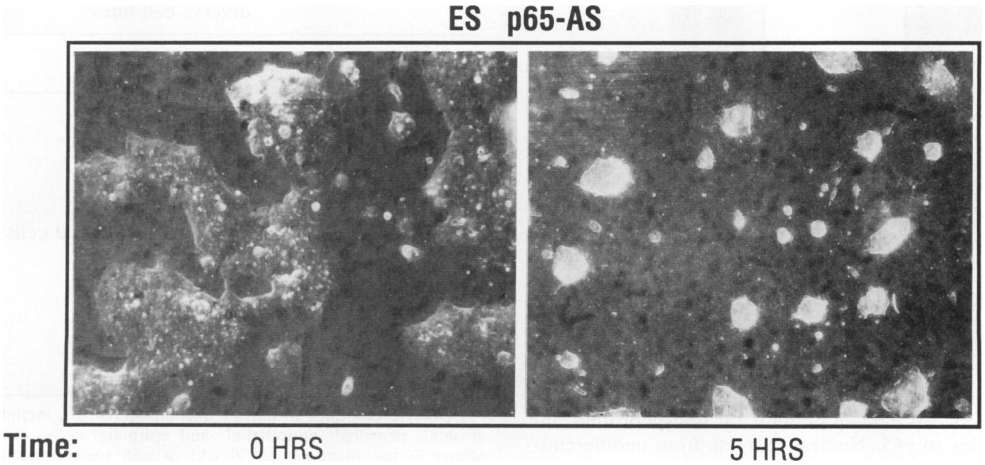

FIG. 5. Rapid inhibition of ES cell adhesion by antisense oligonucleotides to p65. Undifferentiated ES cells were cultured on gelatin-coated dishes for 72 h in the absence of oligonucleotides. Medium was removed and new medium containing 30 μM of antisense or sense (not shown) p65 phosphorothio oligonucleotides was added to the attached cells. An area was marked and photographed immediately (zero time) and after 5 h. Magnification, ×50.

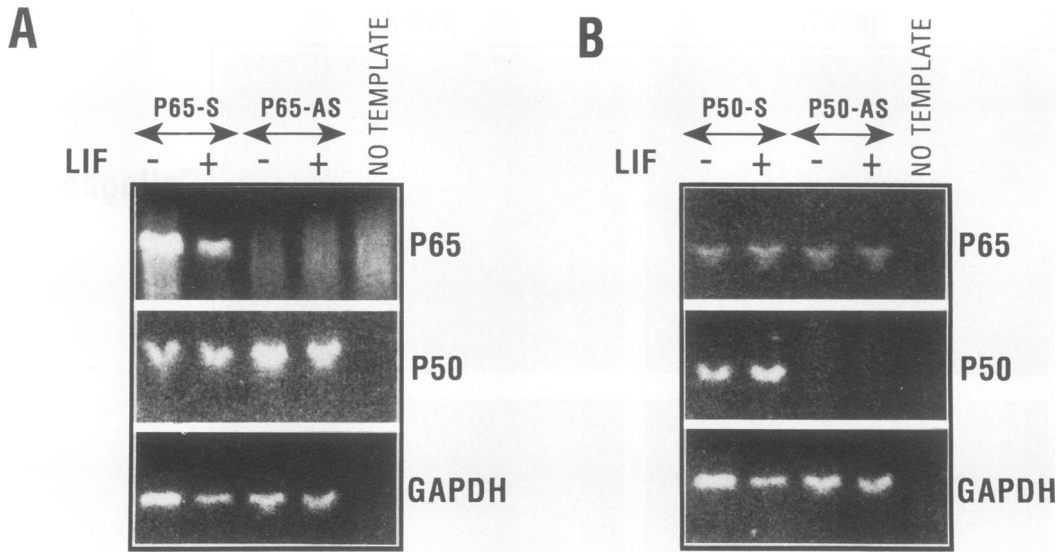

FIG. 6. Inhibition of p65 and p50 mRNA expression by antisense oligonucleotides. Undifferentiated and differentiated ES cells were cultured in the presence of p65 (A) or p50 (B) phosphorothio oligonucleotides for 72 h. Total RNA was isolated and analyzed by RT-PCR for p65, p50, and GAPDH expression.

the results of this study have further implications towards NF-κB function with respect to cellular adhesion. For example, a variety of adhesion molecules such as ICAM-1 (29) and ELAM-1 (30) have been shown to have NF-κB binding sites, suggesting that NF-κB may be involved in the regulation of cell adhesion. However, there is no direct evidence to date that this ubiquitous transcription factor is necessary for any events involved in cell-to-cell or cell-to-substratum interaction. The results reported here demonstrate that at least one property of the p65 subunit involves regulation of adhesion molecules. Whether this is achieved by action of the p65 homodimer or through heterodimeric formation with another protein remains to be established. Furthermore, the observation that antisense p50 oligonucleotides also affect adhesion following differentiation in the ES cell system suggests that the p50 subunit likely regulates expression of a set of adhesion molecules that differ from those which respond to p65. As our studies only utilized the property of cellular adhesion as a model for antisense function, we have no evidence that the CAM molecules themselves, which contain the NF-κB motifs, are responsible for the loss of

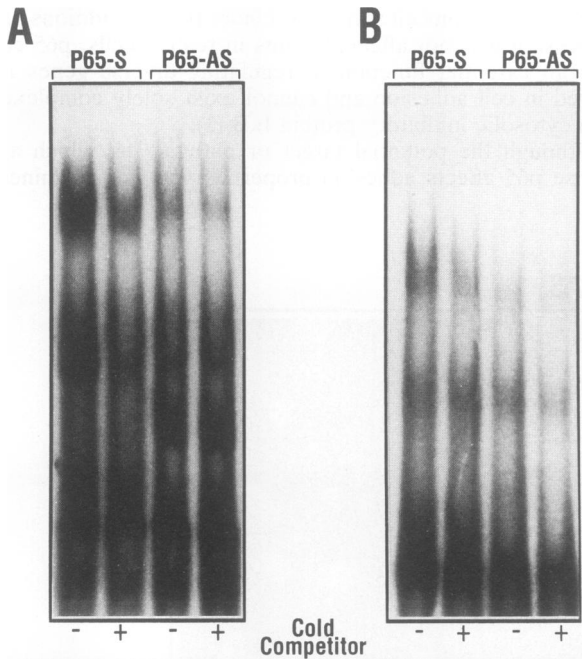

FIG. 7. Inhibition of κB-binding activity by phosphorothio antisense oligonucleotides to p65. Nuclear extracts from undifferentiated ES cells (A) or PC-12 cells (B) treated with the sense or antisense oligonucleotides to p65 for 48 h were prepared. A ^{32}P oligonucleotide corresponding to a consensus κB binding site was used as a probe in a gel mobility shift assay, in the presence or absence of a 30-fold molar excess of the unlabeled κB oligonucleotide.

TABLE 2. Antisense oligonucleotides to p65 inhibit adhesion of diverse cell lines[a]

Cell line	Cell type	Antisense oligonucleotide[b]
Murine		
3T3 Feeder cells (primary)	Fibroblast	m-p65
Rat-1	Fibroblast	m-p65
NIH 3T3	Fibroblast	m-p65
S-17	Bone marrow stromal cells	m-p65
PC-12	Pheochromocytoma	m-p65
Human		
Primary HUVECs	Endothelial	h-p65
RHEK-1	Epithelial	h-p65
Primary keratinocytes	Epithelial	h-p65

[a] Diverse cell lines of murine and human origin, including fibroblasts and stromal, neuronal, endothelial, and epithelial cells, were trypsinized and plated in the presence of 30 μM of p65 phosphorothio oligonucleotides (murine or human) and photographed after 48 to 72 h. Marked effects on cell adhesion were observed with each of the cell lines listed.

[b] The inhibitory effects of antisense oligonucleotides on cell adhesion were species specific. The corresponding sense oligonucleotides had no effect. The p50 sense and antisense oligonucleotides did not inhibit adhesion of these cells.

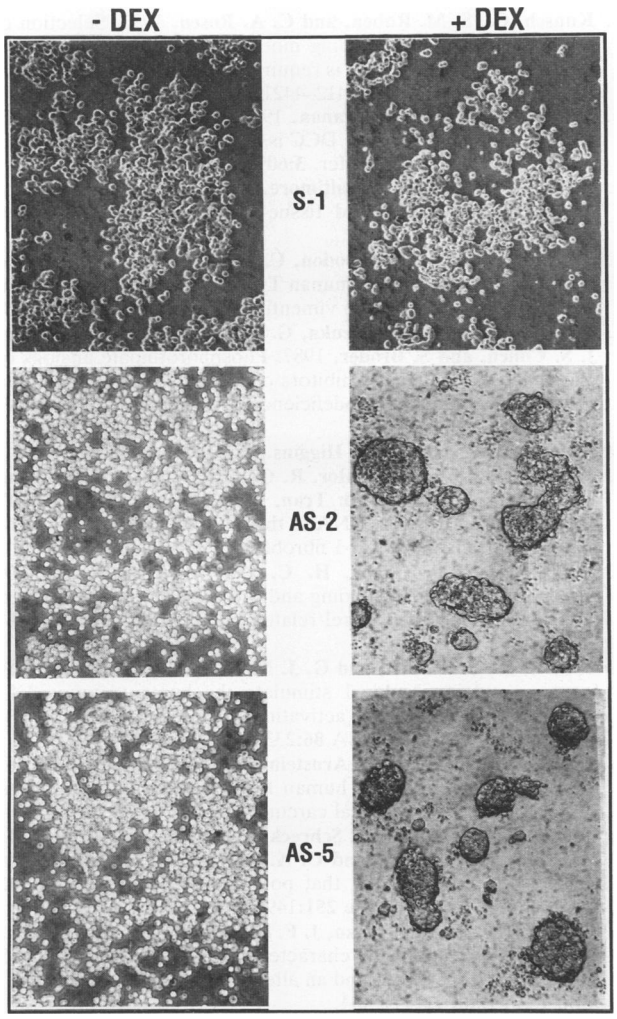

FIG. 8. Inhibition of PC-12 cells' adhesion by inducible antisense RNA to p65. Four sense clones and eight antisense clones were used in this experiment. Representative clones (S-1, AS-2, and AS-5) are shown. Cells were treated with or without dexamethasone (10^{-6} M) for 72 h, replated, and photographed after 24 h. Magnification, ×50.

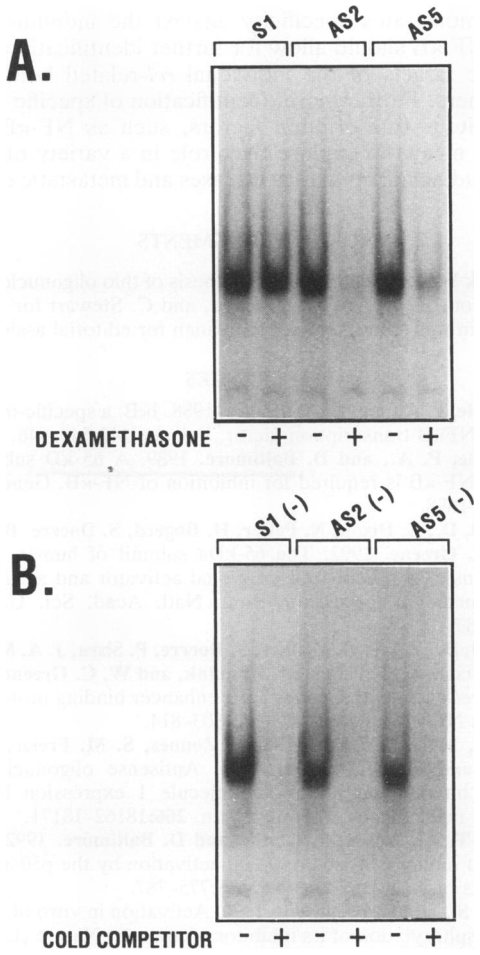

FIG. 9. κB-like binding complexes present in nuclear extract of rat PC-12 transfectants. (A) Inhibition of κB-binding activity by induction of p65 antisense RNA. Nuclear extracts from rat PC-12 cells expressing inducible p65 sense (S-1) or antisense (AS-1 and AS-3) RNA were prepared in the absence (−) or presence (+) of dexamethasone. A ^{32}P oligonucleotide corresponding to a consensus κB-binding site was used as a probe in a gel mobility shift assay. Complexes were resolved on a 4% nondenaturing polyacrylamide gel. (B) Inhibition of κB-binding activity by cold competitor. Complexes observed in the absence of dexamethasone for both the sense (S-1) or antisense (AS-1 and AS-3) cell lines were inhibited by the addition of a 30-fold molar excess of the unlabeled κB oligonucleotide.

adhesion. However, in an independent study we recently identified an integrin (CD 11b) as one of the targets for antisense p65, by using a differentiatable HL-60 cell line (26a).

Our findings have broad implications relating to cellular transformation. Recently, Kitajima et al. (13), utilizing an antisense oligonucleotide similar to the p65 subunit of NF-κB, have demonstrated tumor regression in human T-cell leukemia virus type 1 Tax-transformed tumors in vivo. In addition, these authors have shown growth-inhibitory effects of antisense p65 oligonucleotides on transformed cells in vitro. Our recent evidence suggests that antisense p65 oligonucleotides, but not p50 oligonucleotides, inhibit growth of diverse adherent cells both in vitro and in vivo (10a). The results of these authors, taken in the context of our observations of a block of cellular adhesion by similar antisense p65 oligonucleotides, strongly imply a role for NF-κB-p65-regulatable cell adhesion molecules in transformation.

The product of the v-*rel* oncogene functions as a dominant-negative suppressor of NF-κB (3, 4). Since loss of adherence is a characteristic of transformed cells, it is possible that the v-*rel*-induced transformation might involve inhibition of cell adhesion, an effect similar to that seen with antisense p65. The diminution of NF-κB activity by v-*rel* could be achieved in several ways, the simplest being formation of a v-*rel*-p65 heterodimer that would no longer bind to certain κB motifs recognized by the p65 homodimer. Consistent with this possibility, it was recently shown that within the DNA motifs recognized by each of the individual *rel*-related homodimers, there exists a subset of sequences that selectively recognize only one or two of the individual proteins (15). It was also demonstrated that selected DNA motifs that bound either p50 or p65, but not both, failed to recognize NF-κB. Similar specificities were observed with heterodimers containing the *rel* protein (15).

The observation that the antisense approach taken in this

study demonstrates specificity against the individual subunits of NF-κB should allow for further identification of the respective targets of the individual *rel*-related homo- and heterodimers. Furthermore, identification of specific targets for ubiquitous transcription factors, such as NF-κB, may provide a means to explore their role in a variety of disorders, including inflammatory diseases and metastatic cancer.

ACKNOWLEDGMENTS

We thank W. McComas for the synthesis of thio oligonucleotides; L. Robertson, K. Dorshkind, J. Rhim, and C. Stewart for the cell lines used in this study; and J. Narayanan for editorial assistance.

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