The bradykinin type 2 receptor (BK2) is a developmentally regulated G protein-coupled receptor that mediates diverse actions such as vascular reactivity, salt and water excretion, inflammatory responses, and cell growth. However, little is known regarding regulation of the BK2 gene. We report here that the rat BK2 receptor is transcriptionally regulated by the tumor suppressor protein p53. The 5′-flanking region of the rat BK2 gene contains two p53-like binding sites: a sequence at −70 base pairs (P1 site) that is conserved in the murine and human BK2 genes; and a sequence at −707 (P2) that is not. The P1 and P2 motifs bind specifically to p53, as assessed by gel mobility shift assays. Transient transfection into HeLa cells of a CAT reporter construct driven by 1.2-kilobases of the BK2 gene 5′-flanking region demonstrated that the BK2 promoter is dose dependently activated by co-expression of wild-type p53. Co-expression of a dominant negative mutant p53 suppresses the activation of BK2 by wild-type p53. Promoter truncation localized the p53-responsive element to the region between −38 and −94 base pairs encompassing the p53-binding P1 sequence. Furthermore, p53-mediated activation of the BK2 promoter is augmented by the transcriptional co-activators, CBP/p300. Interestingly, removal of the P2 site by truncation or site-directed deletion amplifies p53-mediated activation of the BK2 promoter. These results demonstrate that the rat BK2 promoter is a target for p53-mediated activation and suggest a new physiological role for p53 in the regulation of G protein-coupled receptor gene expression.

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The Bradykinin Type 2 Receptor Is a Target for p53-mediated Transcriptional Activation*

The type 2 bradykinin receptor, BK2, is a developmentally regulated G protein-coupled receptor that mediates diverse actions, such as vascular reactivity, salt and water excretion, inflammatory responses, and cell growth. However, little is known regarding regulation of the BK2 gene. We report here that the rat BK2 receptor is transcriptionally regulated by the tumor suppressor protein p53. The 5′-flanking region of the rat BK2 gene contains two p53-like binding sites: a sequence at −70 base pairs (P1 site) that is conserved in the murine and human BK2 genes; and a sequence at −707 (P2) that is not. The P1 and P2 motifs bind specifically to p53, as assessed by gel mobility shift assays. Transient transfection into HeLa cells of a CAT reporter construct driven by 1.2-kilobases of the BK2 gene 5′-flanking region demonstrated that the BK2 promoter is dose dependently activated by co-expression of wild-type p53. Co-expression of a dominant negative mutant p53 suppresses the activation of BK2 by wild-type p53. Promoter truncation localized the p53-responsive element to the region between −38 and −94 base pairs encompassing the p53-binding P1 sequence. Furthermore, p53-mediated activation of the BK2 promoter is augmented by the transcriptional co-activators, CBP/p300. Interestingly, removal of the P2 site by truncation or site-directed deletion amplifies p53-mediated activation of the BK2 promoter. These results demonstrate that the rat BK2 promoter is a target for p53-mediated activation and suggest a new physiological role for p53 in the regulation of G protein-coupled receptor gene expression.

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was ligated into the reporter plasmid pCAT3Basic (Promega) from the chloramphenicol acetyltransferase (CAT) open reading frame. For the generation of promoter constructs with serial deletions in the 5′-upstream region, the original construct (∼1184 + 55/CAT) was digested with XhoI (∼1087), NcoI (∼827), BglII (∼635), and EcoRI (∼563). The −384, −200, −94, and −38/CAT promoter constructs were generated by polymerase chain reaction using the −1184 + 55/CAT construct as a DNA template. Partial (15/20 bp) or complete (20/20 bp) deletion of the p53-binding site at −707 to −688 was performed by the QuickChange™ Site-directed Mutagenesis System (Stratagene, La Jolla, CA) following the manufacturer's recommendations. All constructs were sequenced to verify the sequence and orientation by manual DNA sequencing using T7 Sequenase (Amersham Pharmacia Biotech) or by automated DNA sequencing (Applied Biosystems, Model 373A).

**Tissue Culture and Transfections**—HeLa cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc.) at 37 °C in a humidified incubator with 5% CO2. Cells were plated in duplicates in six-well plates at 1.8 × 104 cells/well in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum 1 day prior to transfection. Cells were transfected with 1.2 μg of DNA per well of pCAT vectors, driven by various BK2 promoter fragments. A control β-galactosidase vector pSVZ (Promega, 0.5 μg of DNA/well) was co-transfected with BK2-pCAT plasmids to correct for transfection efficiency. CMV-driven expression vectors for wild-type or mutant p53 (33) were co-transfected using DNA/well. Transfections were performed using LipofectAMINE PLUS Reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. Four hours after transfection, fresh medium was replaced and cell extracts were prepared 24 h later using a reporter lysis reagent (Promega). After normalization for β-galactosidase activity in the lysate, aliquots of cell lysates were analyzed for CAT activity by the addition to the aliquot of 20 μl of 4 μM acetyl-CoA, 70 μl of 1 M Tris-Cl (pH 7.8), 5 μl of 25 μCi/ml [14C]chloramphenicol (Amersham), and H2O to a final volume of 150 μl. The mixture was incubated at 37 °C for 1 h. The reaction was extracted with 1 ml of ethyl acetate and the ethyl acetate evaporated in a Speedvac evaporator. Samples were suspended in 25 μl of ethyl acetate and spotted 2 cm above the edge of a plastic-backed TLC sheet. Thin layer chromatography was run in 19:1 chloroform/methanol, and the sheet was air-dried and placed on film for autoradiography. The optical density of the radiolabeled spots was measured by densitometry and CAT activity was calculated as percent acetylated product.

**Oligonucleotides**—The oligonucleotide sequences used were as follows (double-stranded): consensus p53, 5′-TAGGCATGTCTAGGCATG-3′ (positions 688) (2). Underlined bases denote sequence variation from the consensus p53 element. A, schematic showing the promoter-reporter BK2 (pBK2-CAT) construct and the relative positions of the p53-binding sites, P1 and P2. B, sequence comparisons of the P1 and P2 sites with the consensus p53 binding sequence (14). Underlined bases denote sequence variation from the consensus p53 element. C, species conservation of P1 site.

p53 recognition sequence (14) is underlined (see also Fig. 1B). Whereas the P1 sequence is conserved in the human (41, 42) and mouse3 BK2 genes, the P2 site is not (Fig. 1C).

**Specific Binding of p53 to the P1 Element Identified in the BK2 Promoter**—We next examined whether p53 binds to these putative p53-binding sites (P1 and P2) using EMSA. As a source of p53, we used nuclear extract from SVT-2 embryonic fibroblasts as well as recombinant p53. Western blots using the p53 antibodies, PAb421 (Oncogene Science), and FL-393 (Santa Cruz), showed that SVT-2 cells contain p53 (data not shown). EMSA assays utilizing a 32P-labeled BK2-P1 oligonucleotide and SVT-2 nuclear extracts revealed two major DNA-protein complexes (Fig. 2A, lane 2). The addition of the p53-activating antibody, PAb421 (lane 1, 2 μl) resulted in enhanced DNA binding activity (Fig. 2A, lanes 3 and 4), demonstrating the presence of p53 in the complexes. To test the specificity of the DNA-protein interactions, EMSA competition experiments were performed (Fig. 2B). Incubation of the consensus p53 oligoduplex probe with SVT-2 nuclear extracts produced two major DNA-protein complexes (Fig. 2A, lane 1). The addition of 100-fold molar excess of unlabeled consensus p53 oligoduplex eliminated both band shifts (Fig. 2B, lane 2). An unlabeled oligonucleotide corresponding to the p53-binding site in the p21 gene prevented the formation of the higher mobility complex when added at 100-fold molar excess (Fig. 2B, lane 3) and both low and high mobility complexes when added at 200- and 300-fold excess (Fig. 2B, lanes 4 and 5). An 100–200-fold molar excess of unlabeled BK2-P1 oligoduplex also competed efficiently with binding to the consensus p53 probe (Fig. 2B, lanes 6 and 7). In contrast, a P1 probe containing point mutations in the p53 BK2-P1 sequence did not produce any specific band shifts (Fig. 2B, lanes 8–10, see figure legend). Finally, the band shift produced by binding of nuclear proteins to the P1 probe was greatly diminished upon addition of 100-fold molar excess of unlabeled p53 consensus sequence (Fig. 2B, lanes 11, 12).

To further document the specific binding of p53 to the p53-like motif in the P1 site, we examined the binding of the P1 duplex to purified p53 (Fig. 2C). Binding of p53 to the P1

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oligoduplex (Fig. 2C, lane 1) is competed, albeit not completely, by 100-fold excess of unlabeled oligoduplex corresponding to the p53-binding site in the p21 promoter (lane 2) or the p53 consensus (lane 3). However, when added at 200-fold excess, the p53 consensus sequence and the p53 motif in the p21 gene compete efficiently with the BK2-P1 for binding to purified p53 (Fig. 2D). Binding of the p53 consensus oligoduplex to purified p53 (Fig. 2C, lane 4) is abolished or attenuated by 100-fold excess of unlabeled p53 consensus sequence (lane 5), p53 motif in the p21 gene (lane 6) or BK2-P1 (lane 7). These cross-experiment results demonstrate that p53 binds specifically to the P1 sequence in the BK2 promoter. In addition, the results suggest that the binding affinity of the p53 site in BK2-P1 to p53 may be higher than that of the consensus p53 sequence or the p21 gene p53 site. We speculate that the difference in mobility of the DNA-protein complexes in SVT-2 nuclear extracts (two major bands) as compared with recombinant p53 (one major band) may be related to the presence of p53-interacting proteins in the former but not the latter.

p53-dependent Transactivation of the BK2 Promoter—In HeLa cells, p53 is bound to the human papillomavirus E6 protein, which targets p53 for degradation (43) and renders these cells functionally deficient in p53. Other studies have shown that these cells can be used to study p53-mediated transcriptional responses (33). HeLa cells were transiently transfected with CAT vectors containing segments of the BK2 promoter with and without a CMV promoter-driven p53 expression plasmid. As shown in Fig. 3A, cells co-transfected with pCMV-p53(wt) and seven different BK2 promoter-CAT constructs, pBK2(−1184), (−827), (−635), (−563), (−384), (−200), and (−94), showed a clear induction of CAT activity only in the presence of wild-type p53. In contrast, no increase in CAT expression was observed in cells co-transfected with pCMV-p53(wt) and pBK2(−38) (Fig. 3A). Wild-type p53 stimulated pBK2(−1184) or pBK2(−94)-driven CAT expression in a dose-dependent manner (Fig. 3B). These results suggest that the p53 response element in the BK2 promoter is located in the region between −38 to −94 bp, which is consistent with the location of the P1 p53-binding site.

To test the specificity of the stimulatory effect of wild-type p53 on BK2 promoter-driven CAT transcription, HeLa cells were co-transfected with a CMV promoter-driven expression plasmid, pCMV-p53E258K, which encodes a dominant negative mutant of p53, with a Glu → Lys change at amino acid 258 (33). Co-transfection with this p53 mutant which lacks the ability to bind DNA failed to induce BK2 promoter-driven CAT expression (data not shown and Fig. 5B), and in experiments in which both pCMV-p53(wt) and pCMV-p53E258K were co-transfected, the mutant p53 repressed the activation of the BK2 promoter by wild-type p53 in a dose-dependent manner (Fig. 3C and D).

Effect of CBP/p300 on p53-mediated Activation of the BK2 Promoter—CBP/p300 are transcriptional co-activators that bind and activate a number of transcription factors including the p53 protein (18–21, 44, 45). Accordingly, we examined the effect of CBP/p300 on p53-mediated transcriptional responses of the BK2 promoter. As shown in Fig. 4, A and B, co-transfection of viral promoter driven expression plasmids for CBP/p300 (0, 10, and 50 ng of DNA) into HeLa cells augmented the p53-mediated activation of BK2 promoter in a dose-dependent manner. The transactivation by CBP/p300 most likely occurs via the p53-like motif at the P1 site (located at −70 to −94), rather than via the NF-κB site which overlaps with the upstream BK2-P2 motif, for two reasons. First, CBP/p300 activates CAT transcription from the pBK(−94) construct, which lacks the NF-κB site (Fig. 4C). Second, deletion of the BK2-P2 site (see below) destroys the NF-κB site, yet this mutant construct (with an intact P1 site) remains responsive to CBP/p300 (data not shown).

The P2 p53 Site Modulates p53-mediated Activation of BK2 Promoter—In addition to the proximal P1 p53-like binding site, the rat BK2 5′-flanking region contains a second p53-like motif at positions −707 to −688 (Fig. 1B). Unlike P1, P2 is not conserved in the human BK2 gene. This DNA sequence binds specifically to p53 present in SVT-2 cells’ nuclear extracts as indicated by the enhanced DNA binding activity following
treatment with the PAb421 antibody (Fig. 5A). Specificity of the complex was demonstrated by competition by unlabeled p21 and BK2-P2 (Fig. 5A) but not by irrelevant oligoduplex (Jun/AP-1) (data not shown). To determine the functional relevance of the P2 site, we compared the effect of wild-type p53 on the activity of BK2 promoter constructs either containing (pBK-1184) or lacking (pBK-635) the P2 site. As shown in Fig. 5B, p53-mediated transactivation is approximately 2-fold higher in pBK2-635 than pBK2-1184. To validate this finding, we utilized a deletion mutagenesis approach in which we removed either 3/4 (ΔP215bp) or 4/4 (ΔP220bp) pentamers of the P2 site. As shown in Fig. 5, C and D, co-transfection of pBK2(Δ1184) construct with a fixed amount, 50 ng, of pCMV-p53(wt), and increasing amounts (0, 50, 100, and 500 ng) of pCMV-p53(mutant) showing that the dominant negative mutant p53 suppresses the transcriptional activation of the BK2 promoter by wild-type p53.

**DISCUSSION**

The bradykinin type 2 receptor, BK2, is a heptahelical G protein-coupled receptor. Upon ligand activation, BK2 engages numerous second messenger systems such as phospholipase C and D, phosphoinositol 3-kinase, protein kinase C, and tyrosine kinases. Among the nuclear factors activated by BK are AP-1 (9) and NF-κB (46) which are believed to mediate the effects of BK on growth, differentiation, and gene expression of inflammatory cytokines and growth factors. Activation of BK2 on endothelial cells mediates nitric oxide release and vasorelaxation, consistent with the hypertensive phenotype of BK2-deficient mice (47–49).

In the present study, transient transfections into HeLa cells of promoter-reporter constructs containing serial deletions of the BK2 5′-flanking region localized the p53 activation effect to a 56-bp region extending from −38 to −94 of the promoter. This region contains a p53-like binding motif (P1 site) at −70 to −50. This finding together with the sequence-specific binding of p53 to the P1 site, the inhibition of p53-mediated activation by co-expression of a mutant p53, and the species conservation of this site strongly implicate this DNA element in p53-mediated activation of the BK2 promoter.

Cyclic AMP response element-binding protein and p300 (CBP/p300) are structurally related activators of numerous transcription factors including p53. CBP/p300 possess intrinsic histone acetyltransferase activity (18–21). Acetylation of lysine residues in the N-terminal domains of histones facilitates gene activation, perhaps by reducing histone tail affinity for DNA, leading to a more favorable transcription factor binding to nucleosomal DNA. CBP/p300 acetylate components of the basal transcription machinery, such as TFIIIEβ and TFIIIF in vitro (50–52). Furthermore, p300 acetylates p53 on its C terminus thereby enhancing its DNA binding activity in vitro (21). In our model, co-expression of CBP/p300 with p53 augmented the transactivation function of p53. Based on these results, we propose that the interaction of p53 with the P1 site acts as a
Mediated Transactivation of the BK2 Promoter

We also identified a second p53-like binding site (P2) at position -707 of the rat BK2 gene. This site differs from P1 in many respects. First, unlike P1, P2 is not conserved in the mouse or human BK2 genes. Second, removal of P2 by truncation or deletion amplified p53-mediated transactivation. It is not clear how the P2 site down-modulates the transcriptional activity of p53 that is exerted at the level of the proximal promoter. One possibility may involve the displacement of an activator protein from binding to the same regulatory element, as recently reported in the c-fos proto-oncogene (40). Indeed, the P2 sequence overlaps with a putative binding site recently described in the rat a-fetoprotein gene (40). In addition, p53 is highly expressed in certain tissues during development (3). Our preliminary studies have shown a remarkable overlap in the expression of p53 and BK2 in the developing kidney (54). Thus, it is conceivable that p53 may have a role in the developmental regulation of the BK2 gene, as has recently been proposed for the a-fetoprotein gene (40). In addition, p53 is induced and activated by cellular injury and chronic inflammation (55) and this may represent a new mechanism to activate BK2 gene expression in response to inflammation (5, 6).

The biological significance of p53-mediated regulation of BK2 gene expression remains unclear. We speculate that endogenous p53 may be important in the regulation of BK2 expression under at least two conditions. Both p53 and BK2 are highly expressed in certain tissues during development (3). Our preliminary studies have shown a remarkable overlap in the cellular expression of p53 and BK2 in the developing kidney (54). Thus, it is conceivable that p53 may have a role in the developmental regulation of the BK2 gene, as has recently been proposed for the a-fetoprotein gene (40). In addition, p53 is recruiting vehicle for CBP/p300 to the vicinity of the proximal promoter thereby activating the basal transcription machinery.

We also transfect HeLa cells with pBK2-(P2Δ94) or pBK2-(P2Δ1184) containing (5) or lacking (Δ365) the P2 site. Insertion of P2 amplifies the p53-mediated activation. pBK2-(P2Δ365) and pBK2-(Δ1184) (n = 3 assays in duplicates). A DNA-binding mutant p53 fails to activate the BK2 promoter. C, representative CAT assay showing the effect of deletion of the BK2-P2 site on p53-mediated activation of the BK2 promoter. HeLa cells were transfected with 1.2 µg each of the promoter-reporter plasmids. BK2 promoter activity is increased in a dose-dependent manner by wild-type p53. Removal of 3/4 or 4/4 pentamers of the P2 site amplifies the p53-mediated activation. D, quantitative analysis of the CAT assay in C; white bar, pBK2-(Δ1184); gray bar, pBK2-(P2Δ365); black bar, pBK2-(P2Δ94); similar results were obtained in three independent experiments.

\[ \text{pCMV-p53} \quad \text{pRSV-CBP} \quad \text{pRSV-p300} \]

\[ \text{+} \quad \text{+} \quad \text{+} \]

\[ \text{pCMV-p53} \quad \text{pRSV-CBP} \quad \text{pRSV-p300} \]

\[ \text{+} \quad \text{+} \quad \text{+} \]

\[ \text{pCMV-p53} \quad \text{pRSV-CBP} \quad \text{pRSV-p300} \]

\[ \text{+} \quad \text{+} \quad \text{+} \]

\[ \text{FIG. 5. The upstream BK2-P2 sequence modulates p53-mediated transactivation. A, EMSA gels. }^{32} \text{P-Labeled oligoduplexes corresponding to the BK2-P2 sequence (5'-ACTCTTGCTGGTCTTCCCT-3') were incubated with SVT-2 nuclear extracts ( } \sim 10 \mu \text{g protein/sample) in the presence or absence of the p53-activating antibody, PA421 (1 µl). The position of the band shift is indicated by an asterisk (*). Addition of PA421 enhanced the DNA binding activity, while addition of unlabeled p53 sequence from the p21 gene attenuated the binding. B, HeLa cells were co-transfected with pBK2 reporter constructs either containing (Δ1184) or lacking (Δ365) the P2 site with expression plasmids for wild-type (wt) or mutant (mut/p53). CAT activity is 2-fold higher in pBK2 (Δ365) than pBK2 (Δ1184) (n = 3 assays in duplicates). A DNA-binding mutant p53 fails to activate the BK2 promoter. C, representative CAT assay showing the effect of deletion of the BK2-P2 site on p53-mediated activation of the BK2 promoter. HeLa cells were transfected with 1.2 µg each of the promoter-reporter plasmids. BK2 promoter activity is increased in a dose-dependent manner by wild-type p53. Removal of 3/4 or 4/4 pentamers of the P2 site amplifies the p53-mediated activation. D, quantitative analysis of the CAT assay in C; white bar, pBK2-(Δ1184); gray bar, pBK2-(P2Δ365); black bar, pBK2-(P2Δ94); similar results were obtained in three independent experiments.} \]

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