Modulation of the Human Protein Kinase Cα Gene Promoter by Activator Protein-2†

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ABSTRACT: Protein kinase Cα (PKCα) is a phospholipid-dependent protein−serine/threonine kinase that plays a major role in intracellular signaling pathways associated with transformation and tumor progression. Glioblastoma multiforme (GBM) and GBM cell lines exhibit increased levels of PKCα compared to normal brain tissue that relates to their proliferative and invasive potential. To investigate the transcriptional regulation of PKCα, the 5′-flanking sequence of the human PKCα gene was cloned and its promoter activity assessed in U-87 GBM cells. This sequence contained a TATA-less promoter region and a single transcription start site within an initiator sequence. Basal promoter activity was restricted to a region spanning −227 to +77 relative to the transcription start site. DNase I footprinting revealed multiple activator protein-2 (AP-2) binding sites and one Sp1 binding site within this region, and point mutations of two AP-2 elements resulted in a loss of DNA binding and transcriptional activation. Overexpression of Sp1 in either U-87 or insect cells increased transcription from the −227/+77 promoter region, whereas overexpression of AP-2 increased transcription only in insect cells. Cis activation of the promoter in U-87 cells was increased by phorbol esters but not by cyclic AMP or phosphatidylinositol 3-kinase inhibitors. These results provide evidence that cis activation of the basal promoter of the human PKCα gene occurs through an AP-2-dependent, phorbol ester-responsive pathway, which suggests an autoregulatory manner of transcription in GBM.

PKC[1] is a family of phospholipid-dependent protein−serine/threonine kinases that play a major role in intracellular signaling pathways associated with transformation and tumor progression (*1*−*4*). PKC consists of 12 closely related isoforms that are subdivided into four groups based upon cofactor requirements and structural variations (*5*). The conventional isoforms (α, *β*1, *β*2, *γ*) are catalytically dependent upon calcium, diacylglycerol, and phospholipid, the novel isoforms (*δ*, *ϵ*, *η*, *θ*) are calcium-independent but require diacylglycerol and phospholipid for activation, the atypical isoforms (*ζ*, *ι*, *λ*) are not dependent on any conventional PKC cofactor, and the more distantly related PKC*μ*/PKD is activated by diacylglycerol and phospholipid. The individual PKC isoforms differ in their substrate specificity, cellular and subcellular distribution, and tissue-specific expression, and it is likely that differential activation of PKC isoforms by second messengers such as diacylglycerol, arachidonic acid, and phosphoinositides plays a unique role in their regulation and function (*1*).

Among the conventional isoforms, PKCα is expressed in virtually all tissues and is particularly abundant in poorly differentiated and invasive tumors such as GBM, which is refractory to current modalities of therapy (*6*−*8*). PKCα is highly expressed in GBM cell lines (*8*−*11*), and inhibition of PKCα by either an antisense cDNA or antisense oligonucleotide effectively suppresses proliferation and tumor growth (*11*, *12*). PKCα also negatively regulates one or more proapopotic survival and proliferative pathways in GBM cells such as those associated with p53 and insulin-like growth factor binding protein-3 (*13*, *14*).

In this report, we describe the isolation and characterization of the 5′-flanking sequence of the human PKCα gene. Our data demonstrate that basal transcription is dependent predominantly upon transcription factor AP-2 in a phorbol ester-responsive manner and suggest a mechanism by which PKCα may be upregulated in GBM.

EXPERIMENTAL PROCEDURES

Genomic Library Screening. Approximately 1×10^6 clones from a human placental genomic library constructed in the EMBL-3 vector (Clontech) were screened with a 27-mer oligonucleotide spanning −3 to +24 relative to the translation start codon of the human PKCα cDNA (*15*). Plaques were immobilized on a nylon membrane (Qiagen),

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[1] Abbreviations: AP-2, activator protein-2; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; GBM, glioblastoma multiforme; Inr, initiator element; TK, thymidine kinase; 5′-UTR, 5′-untranslated region; PCR, polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate.

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prehybridized for 2 h at 50 °C, and hybridized overnight at 50 °C to the ^{32}P-labeled 27-mer probe in a solution containing 30% formamide, 5 × SSPE, 1% SDS, 5 × Denhardt's solution, 0.1% gelatin, and 50 μg/mL denatured salmon sperm DNA. Blots were washed stepwise to a final stringency of 0.1 × SSC and 0.1% SDS at 48 °C. The sequence reported in this study can be obtained from GenBank under accession no. AF395829.

Primer Extension Analysis. A ^{32}P-labeled oligonucleotide (60 ng) complementary to sequence −9 to −26 relative to the translation start codon was annealed for 20 min at 58 °C with 10 μg of total RNA prepared from U-87 cells using Trizol (Life Technologies). RNA was transcribed with AMV reverse transcriptase at 42 °C for 30 min (Primer Extension System, Promega), and the extended product was separated by electrophoresis in a 6% polyacrylamide/urea gel, dried, and analyzed by autoradiography at −70 °C for 1 day.

RNase Protection Assay. A riboprobe spanning the sequence −162 to −549 relative to the translation start codon was prepared by PCR and subcloned into pZeroBlunt (Invitrogen). A 387 bp riboprobe was generated with T7 RNA polymerase and hybridized overnight at 42 °C to 15 μg of total RNA prepared from U-87 cells in a buffer containing 80% deionized formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), and 1 mM EDTA. The hybridization mixture was digested with RNase A and RNase T1 (RPAII, Ambion), and the protected fragments were precipitated and separated by electrophoresis in a 6% polyacrylamide/urea gel, dried, and analyzed by autoradiography at −70 °C for 1 day.

Promoter Constructs and Mutations. All promoter constructs were synthesized by PCR amplification using *Pfu* polymerase and cloned into pZeroBlunt (Invitrogen). All constructs, except as noted, were amplified with the reverse primer, 5′-GGAGAGTCGGGCTGGTGCTG-3′, and the following forward primers: 5′-AGGCAGGTGCAGGCTCTTGAAGC-3′ (−1571/+77), 5′-CTGAGGGGTTAGGAGGTGAGC-3′ (−606/+77), 5′-AGTGTTCCCAGCACCGCAA-3′ (−227/+77), and 5′-CTCGTCCTCGCGGCCTC-3′ (−106/+77). Construct (−606/−26) was generated with the forward primer, 5′-CTGAGGGGTTAGGAGGTGAGC-3′, and the reverse primer, 5′-CTTCCTACTCGCGGCGGCAGA-3′. Construct (−1571/+227) was generated with the forward primer, 5′-AGGCAGGTGCAGGCTCTTGAAGC-3′, and the reverse primer, 5′-CAACCACCTCTTGCCTCC-3′. The PCR-amplified products were cloned into pZeroBlunt, and their orientation was checked by restriction enzyme digestion and sequencing. pZeroBlunt constructs were digested with *Kpn*I/*Xho*I and subcloned into pGL3-basic containing the firefly luciferase gene (Promega). Site-specific mutations were introduced into the (−227/+77) promoter construct using the GeneEditor in vitro site-directed mutagenesis system (Promega). The following oligonucleotides were used to generate mutations: 5′-GGGGCATGCGCGGCTAGCGGTGCCGCTCC-3′ to mutate the −72/−54 Sp1 site from GGTGGGCGGTGCCGCTCC to GGCTAGCGGTGCCGCTCC; 5′-CTCGCCGCACCCTTGCGCGCGCCCGG-3′ to mutate the −141/−129 AP-2 site from CACCCCCGCGCGCG to CACCCTTGCGCGCG; 5′-CGCTCAGCGCCGTTGCCGCCGCACC-3′ to mutate the −168/−154 AP-2 site from GCGCCGCCGCCGCCG to GCGCCGTTGCCGCCG; 5′-CGCACTCGGTTTGGTCCGCTCAGC-3′ to mutate the −176/−185 Ets-1 site from TCGGTCCGGT to TCGGTTTGGT; 5′-AGTGTTCCCAGCATTGCAAGGCACTCGC-3′ to mutate the −204/−217 AP-2 site from GCACCGCAAGGCAC to GCATTGCAAGGCAC. All mutations were verified by sequencing.

The pTriEx-AP-2 and pTriEx-Sp1 plasmids were constructed by ligating either the AP-2 or Sp1 cDNA lacking start and stop codons in-frame into the *Eco*RV and *Pvu*II sites of the expression vector pTriEx-1 (Novagen). pPac0 and pPacSp1 insect cell expression vectors (*16*) were provided by Dr. Robert Tjian, University of California at Berkeley, and pPacAP-2α was provided by Dr. Trevor Williams, University of California at Berkeley.

Reporter Gene Assays. U-87 cells were seeded at a density of 2 × 10^5 cells in a six-well plate and grown in DMEM medium supplemented with 10% fetal bovine serum, 5 mM glutamine, and 50 μg/mL gentamicin at 37 °C. One day after seeding, cells were transfected using Lipofectin (Life Technologies) and the indicated amounts of pGL3-basic containing the various PKCα promoter sequences and 500 ng of pRL-TK containing the *Renilla* luciferase gene under the control of the HSV TK promoter (Promega) to determine transfection efficiency. Lipofection was carried out for 7−14 h, and cells were then transferred to complete medium for 48 h. Sf9 cells were transfected in a similar manner except that cells were seeded at a density of 1 × 10^6 and grown at 25 °C in a six-well plate in Ex-Cell 400 (JRH Biosciences) medium supplemented with 5% fetal bovine serum. U-87 cell lysates were prepared with passive lysis buffer (Promega), centrifuged at 14000*g* for 2 min at 4 °C, and 5 μL of supernatant was used to determine luciferase activity using the dual luciferase assay system (Promega). Sf9 cell lysates were prepared with passive lysis buffer (Promega) and sonication before centrifugation. Luminescence was determined in a Lumat LB 9501 Berthhold luminometer.

Drug treatment with 2-*O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma), phorbol 12,13-dibutyrate (PDBu) (Sigma), wortmannin (Sigma), forskolin (Sigma), lithium chloride (LiCl) (Sigma), Ro318220 (Roche), LY294002 (Exelixis), and R_p-cAMP and S_p-cAMP (Biolog) was carried out 24 h after transfection for the time indicated.

DNase I Footprinting. The −227/+77 fragment of the PKCα promoter was cloned into pZeroBlunt and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Footprinting was carried out using the core footprinting system (Promega). Briefly, sense and antisense probes were generated by digestion with *Eco*RV and *Spe*I, respectively. The ^{32}P-labeled probe (10000−50000 cpm) was incubated on ice with 2 footprinting units of purified AP-2 or Sp1 (Promega) for 30 min in buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, and 1 mM DTT. An equal volume of buffer containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was added, the solution was incubated at room temperature for 1 min, and the reaction was stopped by the addition of a buffer containing 20 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/mL yeast tRNA. Samples were extracted with phenol/chloroform, precipitated with ethanol, and analyzed by electrophoresis in 6% polyacrylamide/urea gels. G and G + A sequencing reactions of the probes were carried out by the Maxam−Gilbert method (*17*).

Gel Shift Assay. Gel shift assays were performed with the following double-stranded oligonucleotides: human PKCα promoter sequences −73/−53, 5′-CGGTGGGCGGTGC-CGCTCCGG-3′, −146/−125, 5′-GCCGCACCCCCGCGCG-CGCCCG-3′, and −222/−201, 5′-TCCCAGCACCGCCCG-GCACTCG-3′; AP-2 consensus sequence (Promega) 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′. Nuclear extracts (10 μg of protein) were incubated for 30 min on ice with 0.2 ng of ^{32}P-labeled oligonucleotide (20000 cpm) in a volume of 20 μL containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 50 μg/mL poly(dI-dC). For competition, unlabeled oligonucleotide was incubated with nuclear extract on ice for 15 min prior to the addition of the labeled probe. For supershift analysis, 1 μL of AP-2 or Sp1 antibody (Santa Cruz Biotechnology) was incubated with nuclear extract on ice for 30 min, followed by the addition of the labeled probe and further incubation. The DNA−protein complexes were analyzed on a 4% polyacrylamide gel in 0.5 × TBE buffer at 350 V at room temperature. The gels were dried and exposed to Kodak X-Omat films at −70 °C.

Immunoblotting. Cell extracts were separated by SDS−PAGE and transferred onto nitrocellulose membranes (Schleicher and Schuell). Membranes were blocked for 1 h with 5% nonfat dry milk in TBST and then incubated for 1 h with either an AP-2α rabbit polyclonal antibody or a mouse Sp1 monoclonal antibody (Santa Cruz Biotechnology) diluted to 1 μg/mL in TBST. Following washing in TBST, membranes were incubated for 1 h with either goat anti-rabbit IgG or goat anti-mouse IgG, and antigen was detected by chemiluminescence with the ECL system (Amersham).

RESULTS

Cloning the PKCα 5′-Flanking Region. A human genomic library was screened with a 27-mer oligonucleotide to the 5′-UTR, and three clones were identified by restriction mapping with *Bam*HI that had an average insert size of approximately 12 kb. Southern hybridization identified a 6 kb genomic fragment (Figure 1A) that contained exon 1 and 174 bp of open reading frame, followed by a 251 bp intron sequence with a splice donor site. A 1857 bp fragment immediately upstream to the translation start site was sequenced, and promoter regulatory elements were identified (Figure 1B). The 5′-flanking region to the translation start site contained 201 bp of sequence identical to the human PKCα 5′-UTR reported previously (*15*) with two exceptions, the absence of a G at −4 and the presence of a G at −23 relative to the ATG translation start codon. The human PKCα sequence showed some regions that were homologous to the murine PKCα promoter (*18*), viz., the translation start site and the first exon. However, the murine and human genes differed in their transcription start sites, wherein the human PKCα gene contained a single start site at −228 bp upstream to the translation start site, and the murine promoter contained two initiation sites at −919 and −925 bp relative to the translation start site. Only the +618/+722 sequence in the mouse PKCα promoter showed homology to any part of the human PKCα basal promoter region (−86/+18), which contained one Sp1 and one AP-2 element.

The PKCα 5′-flanking region was analyzed using the computer database TRANSFAC (*19*) to identify putative cis-acting regulatory elements (Figure 1B). This region lacked TATA and CAAT boxes but contained an Inr surrounding the transcription start site. The 5′-flanking region was GC-rich and contained multiple consensus elements for Sp1, AP-1, AP-2, and Ets-1.

PKCα Transcription Start Site. The transcription start site was mapped initially by primer extension analysis (Figure 2A). An 18-mer oligonucleotide complementary to the sequence −9 to −26 relative to the translation start codon was used for reverse transcription of RNA prepared from U-87 cells. A single 228 nt extended product indicated the transcription start site to be 236 nt upstream to the translation ATG codon (Figure 2A). The transcription start site was confirmed by RNase protection analysis with a riboprobe spanning −162 to −549 relative to the translation start codon (Figure 2B). The protected fragment was 74 bp, which was consistent with the results of primer extension analysis. The start site was flanked by an Inr consensus sequence that is common in TATA-less promoters (*20*).

Characterization of the PKCα Promoter Region. Deletion constructs of the 5′-flanking region were cloned into a luciferase reporter plasmid and tested in U-87 cells (Figure 3). The −227/+77 and −606/+77 constructs exhibited the strongest promoter activity, whereas the −1571/+77 sequence had 60% of the activity of the −227/+77 construct, suggesting possible negative regulatory elements between −1571 and −606. Deletion of the 3′ end in −606/−26 resulted in virtually complete loss of activity, in agreement with the location of the transcription start site. Further 5′ deletion from −227 to −106 completely abolished transcriptional activity, indicating that the basal promoter region was contained within the −227 to −106 sequence. Human breast carcinoma MCF-7 cells and human GBM A172 cells also exhibited a pattern of transcriptional activation that was similar to U-87 cells (results not shown). These results indicate that the first 227 bp upstream to the translation start site contain the transcriptional elements necessary for basal promoter activity.

To further characterize the transcriptional elements in the −227/+77 promoter region, DNase I footprinting was performed in both the sense and antisense directions (Figure 4A). Recombinant human AP-2 bound to regions at +21/+1, −37/−68, −72/−107, −129/−141, −154/−173, and −198/−220. Three of the six AP-2 binding sites were located in the −227/−106 sequence that was found to be necessary for basal transcriptional activity. Footprinting with purified Sp1 revealed one protected region at −54/−72 (Figure 4B).

To corroborate the AP-2 binding sites between −227 and −106, the AP-2 elements were mutated, and footprinting was performed (Figure 5). AP-2 binding was lost by point mutations at −136/−137, −161/−162, and −213−214 in the −129/−141, −154/−173, and −198/−220 AP-2 elements, respectively (lanes 3, 4, and 6). The Ets-1 site at −179/−180 (lane 5) was also mutated but was not affected by AP-2.

The transcriptional activity of the mutated constructs was evaluated further by reporter gene activity (Figure 6). Mutation of the −129/−141 or −198/−220 AP-2 sites resulted in a dramatic reduction in luciferase activity, whereas mutation of the −154/−173 AP-2 site and the Ets-1 consensus site at −176/−185 did not significantly affect

FIGURE 1: Sequence of the 5′-flanking region of the human PKCα gene. (A) Genomic organization of the 5′-flanking region. (B) Sequence of the 5′-flanking region. The region from −1620 to +425 is shown, and the transcription start site (+1) is underlined in bold. The ATG translation start codon is in bold, and intron 1 is in lowercase letters. Sequence identifications of putative regulatory elements were identified using the TRANSFAC database (*19*) and are underlined with the associated factor indicated above. The arrows indicate either the sense (→) or antisense (←) direction.

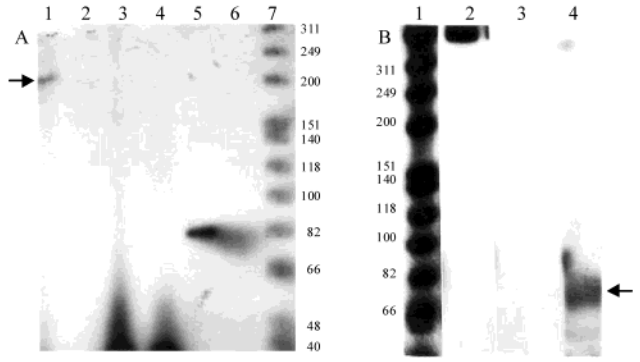

FIGURE 2: Identification of the transcription initiation site. (A) Primer extension analysis: (lane 1) 236 nt extension product from U-87 cell mRNA (arrow); (lanes 3 and 4) no RNA; (lane 5) 87 nt extension product of the actin mRNA control; (lane 7) *Hin*fI-digested ϕX174 DNA markers; (lanes 2 and 6) empty. (B) RNase protection assay: (lane 1) *Hin*fI-digested ϕX174 DNA markers; (lane 2) unhybridized 387 nt riboprobe; (lane 3) hybridization with yeast *Torula* RNA; (lane 4) 75 nt protected fragment from U-87 cell mRNA (arrow).

activity, and mutation of the Sp1 site at −54/−72 resulted in a modest reduction in activity.

Since the AP-2 and Sp1 binding sites seen in DNase I footprinting assays were determined with purified transcription factors, gel shift assays were performed with U-87 nuclear extracts to determine association of AP-2 and Sp1 with the PKCα promoter in GBM cells. A probe corresponding to the Sp1 response element at −73/−53 bound to U-87 nuclear extracts (Figure 7A, lane 2) and was unaffected by a consensus AP-2 oligonucleotide (Figure 7A, lanes 3 and 4). Probes corresponding to AP-2 binding sites at −146/−125 and −220/−201 both bound U87 nuclear extracts (Figure 7A, lanes 6 and 10) and were specifically competed by the consensus AP-2 oligonucleotide (Figure 7A, lanes 7, 8, 11, and 12). A probe for the consensus AP-2 site also bound U87 extracts and was competed by the cold AP-2 probe (Figure 7A, lanes 13−16). Supershift assays with an AP-2 antibody were unsuccessful; however, the −73/−53 probe formed a complex with Sp1-enriched nuclear extracts from Sf9 cells that was shifted by the Sp1 antibody, and the −222/−201 probe, as well as the AP-2 consensus probe, bound AP-2-enriched nuclear extract from Sf9 cells that reacted with the AP-2 antibody (Figure 7B).

Regulation of PKCα Promoter Activity by AP-2 and Sp1. To determine the effect of AP-2 and Sp1 expression on promoter activity, U-87 cells were cotransfected with the −227/+77 reporter construct and plasmids encoding either AP-2 or Sp1 (Figure 8A). Expression of AP-2 in U-87 cells had little effect on promoter activity, a result most likely due to the presence of a high level of endogenous AP-2 (Figure 8D); however, expression of Sp1 markedly increased transcription. When the concentration of Sp1 plasmid was held constant and the concentration of AP-2 plasmid was

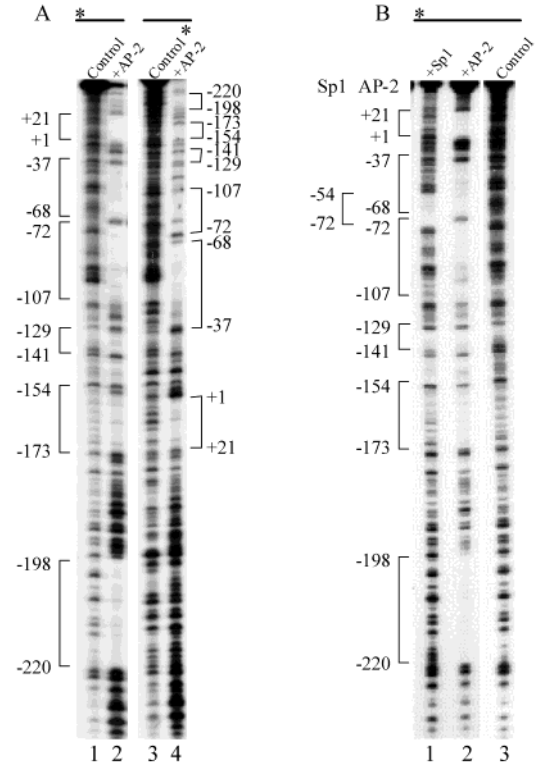

FIGURE 4: AP-2 and Sp1 binding to the PKCα promoter. (A) DNase I footprint analyses of the ^{32}P-end-labeled 304 bp fragment spanning −227/+77. Footprinting was carried out in the sense (left) and antisense (right) directions. The probes were incubated with buffer (lanes 1 and 3) or with 2 footprint units of purified human AP-2 (lanes 2 and 4). After digestion, DNA sequences were resolved in a 6% polyacrylamide/urea gel. Brackets designate the AP-2 protected sequence as determined by Maxam−Gilbert G and G + A sequencing reactions (not shown). (B) DNase I footprint analysis using the sense probe described in (A). The probe was incubated either with buffer (lane 3) or with 2 footprint units of purified human Sp1 (lane 1) or AP-2 (lane 2). Brackets designate the AP-2 and Sp1 protected sequences.

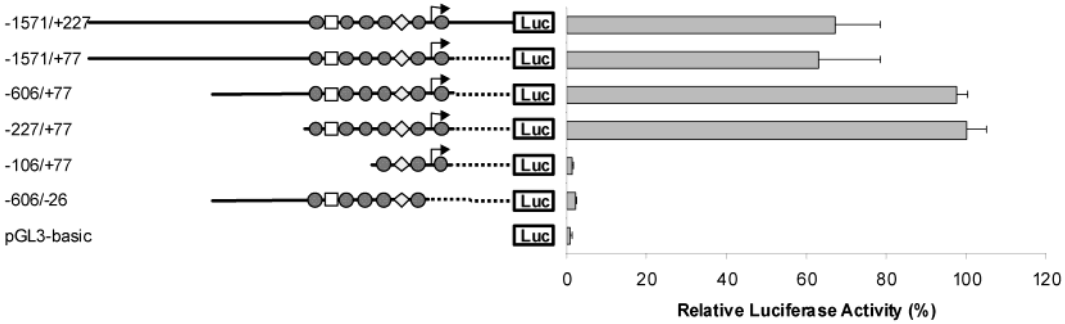

FIGURE 3: Luciferase reporter gene activity under the control of the human PKCα promoter. The schematic diagram on the left represents each of the constructs designated by the region they span relative to the transcription start site (bent arrow). AP-2 (shaded circle), Ets-1 (open square), and Sp1 (shaded diamond) elements are shown. U-87 cells were cotransfected with 2 μg of each reporter gene construct and 0.5 μg of pRL-TK to correct for transfection efficiency. Luciferase activity is normalized for transfection efficiency and expressed as a percentage of the activity obtained with the −227/+77 promoter construct (100%). Transfections were performed in duplicate, and the results are the mean ± SD of three to four experiments.

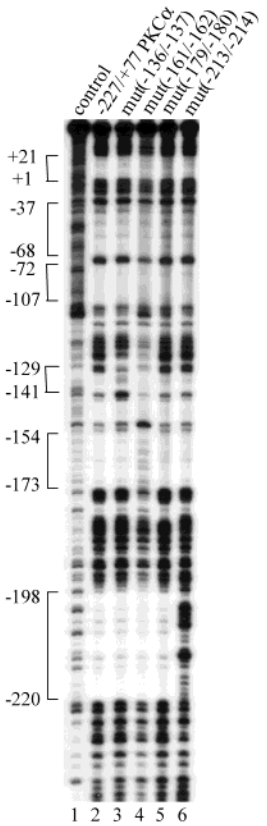

FIGURE 5: Effect of AP-2 consensus element mutations on the binding of AP-2. DNase I footprint analyses of constructs containing point mutations at each of the three AP-2 sites at −136/−137, −179/−180, and −213/−214 as well as a point mutation in a putative Ets-1 site at −179/−180. The DNA fragment was end-labeled with ^{32}P in the sense strand. Lanes: (1) control probe incubated with buffer; (2) control probe incubated with 2 footprint units of purified human AP-2; (3−6) constructs with mutations at −136/−137, −161/−162, −179/−180, and −213/−214, respectively, incubated with 2 footprint units of purified human AP-2. After digestion, DNA sequences were resolved in a 6% polyacrylamide/urea gel.

increased, reporter gene activity was inhibited, suggesting that AP-2 can function as a negative regulatory factor in the presence of high levels of Sp1. Promoter activity was also assessed in Sf9 insect cells (Figure 8B), which lack endogenous Sp1 and contain low levels of AP-2 (Figure 8E). Luciferase activity in Sf9 cells was highly dependent on AP-2 and Sp1 (Figure 8B), although basal transcription was less than 0.1% of that in U-87 cells. AP-2 did not affect Sp1 activity when both transcription factors were expressed. To determine whether mutation of the AP-2 binding sites abrogates transactivation of the PKCα promoter by AP-2, Sf9 cells were cotransfected with PKCα promoter constructs with mutations at these sites and AP-2 expression plasmids (Figure 8C). The mutated constructs, Mut(−136/−137) and Mut(−213/−214), both showed slight increases in transcription with increasing amounts of AP-2; however, their transactivation was significantly less than that of the wild-type construct, −227/+77 PKCα.

Effects of Signal Transduction Inhibitors on Promoter Activity. To investigate the effect of second messenger signaling pathways on promoter activity, the effects of several modulators were examined (Table 1). Short-term (2 h) treatment with TPA did not affect reporter gene activity from the −227/+77 promoter construct in U-87 cells, but long-term (24 h) treatment with either TPA or PDBu greatly increased transcription. Ro 318220, a selective inhibitor of conventional PKC isoforms, surprisingly did not affect transcriptional activity, suggesting that PKC does not directly regulate AP-2 activity. Neither R_p-cAMP, a PKA inhibitor, S_p-cAMP, a PKA agonist, nor forskolin, an activator of adenylyl cyclase, significantly affected activity. LY294002 and wortmannin, inhibitors of PdIn-3-kinase, and LiCl, an inhibitor of GSK3β, did not significantly affect reporter gene activity.

Since 24 h PDBu treatment increased transcription from the PKCα promoter, gel shift and western blot analyses were performed to determine if DNA binding and protein expression were affected (Figure 9). Neither 2 nor 24 h PDBu treatment affected Sp1 binding to the −73/−53 promoter region (Figure 9A); however, 24 h PDBu treatment decreased AP-2 binding to the −146/−125 and −220/−201 promoter regions, as well as to the AP-2 consensus sequence. PKCα levels in U-87 cells treated for 24 h with PDBu were markedly reduced, whereas the levels of AP-2 and Sp1 were unchanged (Figure 9B).

The effect of PDBu on luciferase activity was evaluated further using reporter constructs with mutations in the −129/−141 and −198/−220 AP-2 elements (Figure 10). Although luciferase activity was markedly reduced by either AP-2 element mutation, PDBu treatment stimulated reporter gene activity under the control of either promoter mutation, although to a lesser extent than with the control promoter construct.

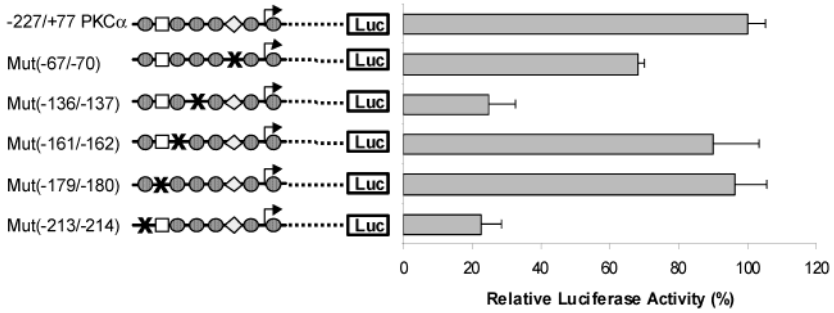

FIGURE 6: Effect of AP-2 element mutations on promoter activity. AP-2, Ets-1, and Sp1 elements within the −227/+77 promoter region were mutated and tested for reporter gene activity as described in Figure 3. AP-2 (shaded circle), Ets-1 (open square), and Sp1 (shaded diamond) elements are shown, and mutations are indicated by an **X**. U-87 cells were cotransfected with 2 μg of the indicated construct and 0.5 μg of pRL-TK. Promoter activity is normalized and expressed as a percentage of activity of the −227/+77 control promoter construct (100%). Transfections were performed in duplicate, and the results are the mean ± SD of three experiments.

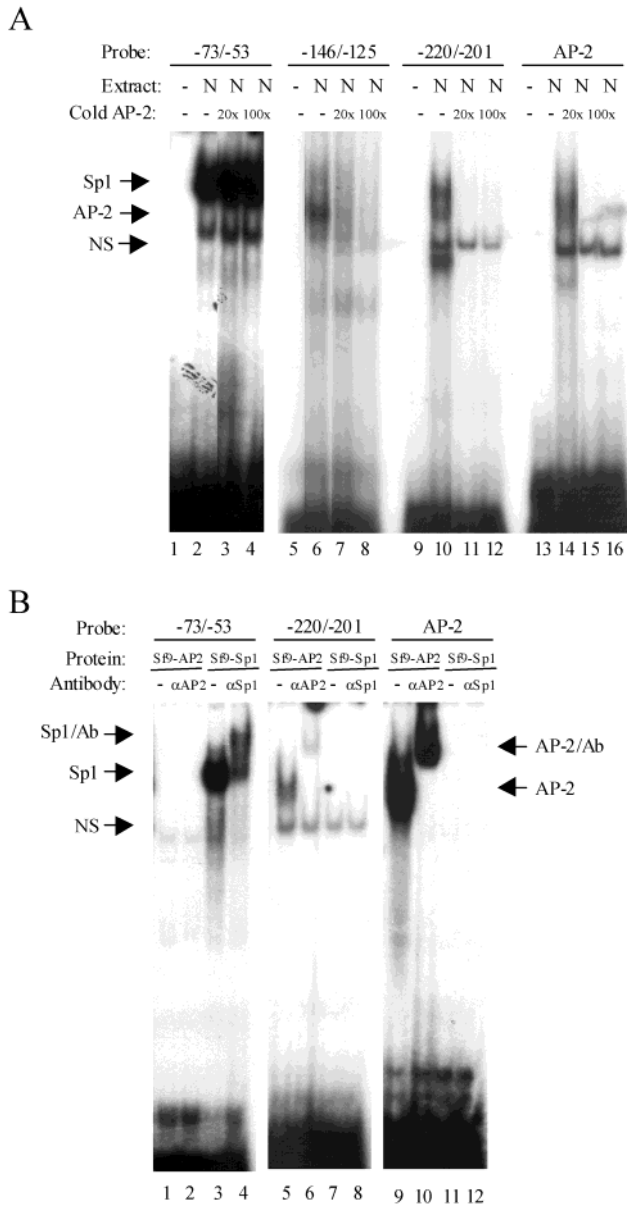

FIGURE 7: DNA-binding activity of AP-2 and Sp1 to the PKCα promoter in U-87 and Sf9 cells. (A) DNA binding was measured by gel shift assay using 5′-end-labeled DNA probes spanning −73/−53 (lanes 1−4), −146/−125 (lanes 5−8), and −220/−201 (lanes 9−12), as well as an AP-2 consensus oligonucleotide (lanes 13−16). Assays were carried out with probe alone (−) (lanes 1, 5, 9, and 13) or with U-87 nuclear extracts (lanes 2−4, 6−8, 10−12, and 13−16). Competition for binding was carried out with a 20-fold or 100-fold molar excess of unlabeled AP-2 consensus oligonucleotide (lanes 3, 4, 7, 8, 11, 12, 15, and 16). Sp1 and AP-2 denote specific complexes, and NS denotes nonspecific binding. (B) Gel shift assays were carried out with extracts from Sf9 cells expressing either AP-2 (lanes 1, 2, 5, 6, 9, and 10) or Sp1 (lanes 3, 4, 7, 8, 11, and 12). Antibodies to either AP-2 (lanes 2, 6, 10) or Sp1 (lanes 4, 8, 12) were added to the DNA−protein complexes. Sp1 and AP-2 denote specific complexes, NS denotes nonspecific binding, and Sp1/Ab and AP-2/Ab denote supershifted complexes.

DISCUSSION

The present study describes for the first time the 5′-flanking sequence of the human PKCα gene. The PKCα promoter region lacked both TATA and CAAT boxes, and transcription was initiated from a single start site within an Inr element, which is believed to be functionally equivalent to a TATA box for directing accurate transcription initiation from a single site (*20*). The promoter regions of the murine PKCα (*18*) and the human PKC*β*, PKC*γ*, and PKC*η* genes (*21*−*23*) also lack classical TATA and CAAT boxes, but transcription of these genes, unlike the human PKCα gene, occurs from multiple transcription start sites. Although 1571 bp of sequence upstream of the PKCα transcription start site were active in U-87 cells for reporter gene activation, basal transcription was controlled mainly by the region −227 to −106, which contained two functional AP-2 elements at −126/−141 and −198/−220. The human PKC*β* promoter also contains several potential Sp1 and AP-2 regulatory elements. AP-2 is known to regulate gene expression in response to diverse signaling pathways mediated by retinoids (*24*), cAMP, and phorbol diesters (*25*, *26*). Although the PKCα promoter was unresponsive to cAMP-elevating agents and to short-term treatment with TPA in U-87 cells, long-term treatment with TPA significantly stimulated promoter activity in a manner similar to that reported for the PKC*β* and PKC*γ* promoters (*22*). Since AP-2 is highly expressed in cells originating from the neural crest (*27*) from whence GBM arises, it is not surprising that U-87 cells contained substantial levels of AP-2 as well as high transactivation activity, suggesting the importance of AP-2 in driving PKCα transcription.

Several genes as well as viral promoters are regulated by AP-2 including the human metallothionein-IIA (*25*), human growth hormone (*28*), human T-cell leukemia virus type I (*29*), human proenkephalin (*26*), acetylcholinesterase (*30*), Na^+/H^+ exchanger (*31*), mouse mammary tumor virus long-terminal repeat (*32*), human insulin-like growth factor binding protein-5 (*33*), and MXI1 (*34*). In the context of these promoters, AP-2 was found to act as both a transcriptional activator and a repressor. Our data also confirm this biphasic activity (Figure 8), which may reflect the complement of coactivators and corepressors in a particular cell type (*35*, *36*) as well as the intracellular level of AP-2, which when highly expressed results in transcriptional self-interference (*37*).

An Sp1 response element overlapping with an AP-2 element was identified at −54/−72; however, mutation of this site resulted in only a small reduction in promoter activity in U-87 cells, suggesting that it was not significantly involved in basal transactivation. Overexpression of Sp1 in U-87 and Sf9 cells, however, dramatically increased transcription, indicating a degree of cooperativity with endogenous transcription factors or coactivators. Cooperativity between AP-2 and Sp1 has been described in the regulation of several genes including human protein C inhibitor (*38*), lens-specific major intrinsic protein (*39*), matrix metalloproteinase-2 (*40*), human lysosomal acid lipase (*41*), and acid sphingomyelinase (*42*). In the latter two instances, cooperativity was increased by long-term phorbol diester treatment (*41*, *42*). However, despite the stimulatory effects of TPA on PKCα promoter activity in U-87 cells, no synergy was observed between AP-2 and Sp1 in either U-87 or insect cells (Figure 8). Phorbol esters have been found to induce AP-2 activity without affecting AP-2 mRNA and protein expression (*25*), suggesting that their effect on the activity of the PKCα promoter may be mediated in part through posttranslational modification of AP-2. This was evident in the two promoter constructs containing a mutated AP-2 element, where PDBu treatment increased reporter gene activity from either of the

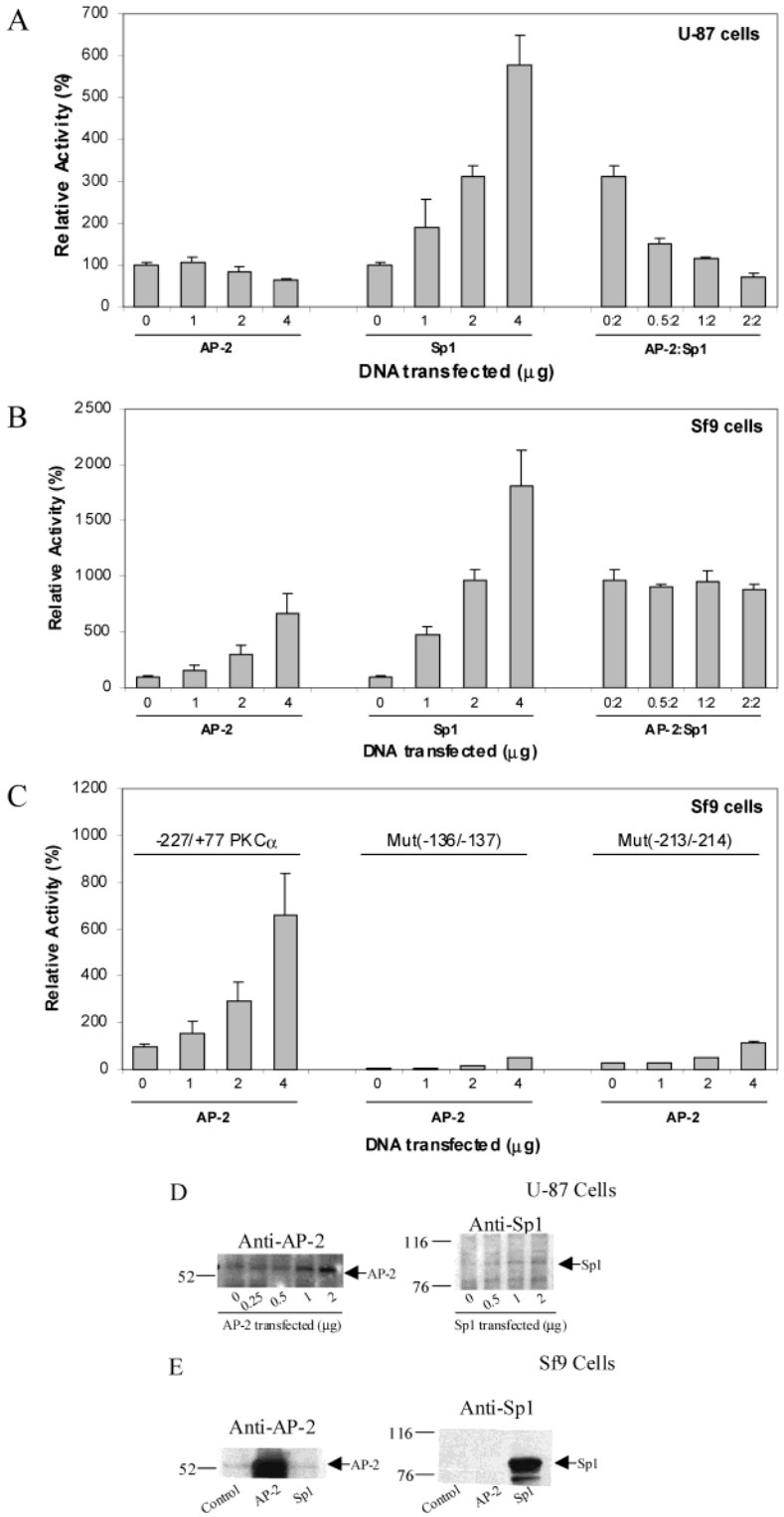

FIGURE 8: Effect of AP-2 and Sp1 expression on PKCα promoter activity in U-87 and Sf9 cells. (A) The effect of AP-2 and Sp1 on promoter activity was determined by transfecting U-87 cells with 2 μg of the −227/+77 promoter construct and increasing amounts of plasmid encoding AP-2 or Sp1. When Sp1 and AP-2 were coexpressed, the amount of Sp1 plasmid was held constant at 2 μg. (B) The effect of AP-2 and Sp1 on promoter activity was determined by transfecting Sf9 cells with 2 μg of the −227/+77 promoter construct and increasing amounts of plasmid encoding AP-2 and Sp1. When Sp1 and AP-2 were coexpressed, the amount of Sp1 plasmid was held constant at 2 μg. (C) The effect of mutating the AP-2 response elements was determined by cotransfecting Sf9 cells with either the wild type (−227/+77) or mutated [Mut(−136/−137) and Mut(−213/−214)] promoter constructs and increasing amounts of AP-2 expression vector. In (A), (B), and (C), promoter activity was normalized to *Renilla* luciferase activity and is expressed as a percentage of the activity of the −227/+77 construct that was cotransfected with an equivalent amount of empty vector. Each value is the mean ± SD of duplicate determinations from three experiments. (D) U-87 cells were transfected with varying amounts of plasmid encoding AP-2 or Sp1, and cell extracts were separated on 10% polyacrylamide gels by SDS−PAGE, transferred onto nitrocellulose, and probed with either a rabbit polyclonal AP-2α antibody or a mouse monoclonal Sp1 antibody. (E) Sf9 cells were infected for 48 h with either the empty (control) or the vector encoding AP-2 or Sp1. Western blotting was performed as in (D).

Table 1: Effects of Signal Transduction Modulators on PKCα Promoter Activity

compound	activity[a] (% of control)
control	100 ± 4
TPA, 2 h, 100 nM	108 ± 1
TPA, 24 h, 100 nM	404 ± 60
PDBu, 2 h, 100 nM	107 ± 9
PDBu, 24 h, 100 nM	439 ± 58
Ro 318220, 24 h, 1 μM	105 ± 19
R_p-cAMP, 24 h, 50 μM	104 ± 17
S_p-cAMP, 24 h, 50 μM	88 ± 3
forskolin, 18 h, 10 μM	95 ± 7
LY 294002, 24 h, 25 μM	107 ± 1
wortmannin, 24 h, 10 μM	80 ± 8
LiCl, 8 h, 10 mM	99 ± 18

[a] Each value is the mean ± SD of at least three experiments.

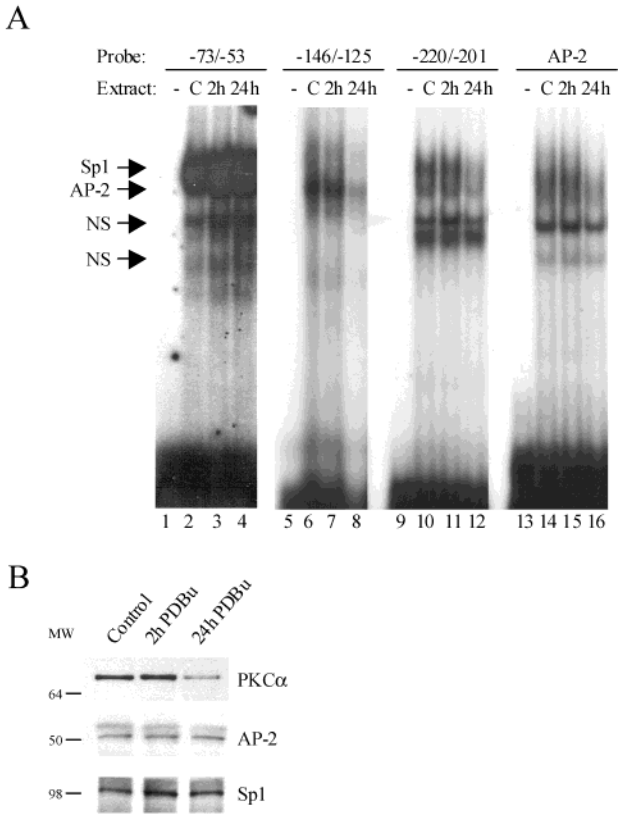

FIGURE 9: Effect of PDBu treatment on AP-2 and Sp1 binding to the PKCα promoter and on AP-2, Sp1, and PKCα levels in U-87 cells. (A) Gel shift assays were carried out with probes spanning −73/−53 (lanes 1−4), −146/−125 (lanes 5−8), and −220/−201 (lanes 9−12), as well as an AP-2 consensus oligonucleotide (lanes 13−16). Assays were carried either in the absence (−) (lanes 1, 5, 9, and 13) or in the presence of nuclear extract from untreated (C) (lanes 2, 6, 10, and 14) or 2 h (2h) (lanes 3, 7, 11, and 15) or 24 h (24h) (4, 8, 12, and 16) PDBu-treated U-87 cells. Sp1 and AP-2 denote specific complexes, and NS denotes nonspecific binding. (B) Western analysis of nuclear extracts from either untreated (control) or 2 h (2h) and 24 h (24h) PDBu-treated U-87 cells for expression of PKCα, AP-2, or Sp1.

constructs despite their reduced activity (Figure 10). This suggests that the phorbol ester effect may be regulated primarily through these AP-2 elements and that the two sites may function independently.

PDBu treatment of U-87 cells for 24 h reduced PKCα expression, which likely reflects increased proteolysis as noted previously (*43*). This treatment also resulted in a reduction in AP-2 binding to the PKCα promoter region and increased transcription from the PKCα promoter. These results suggest a feedforward mechanism in which transcription from the PKCα promoter is increased in response to the low levels of PKCα.

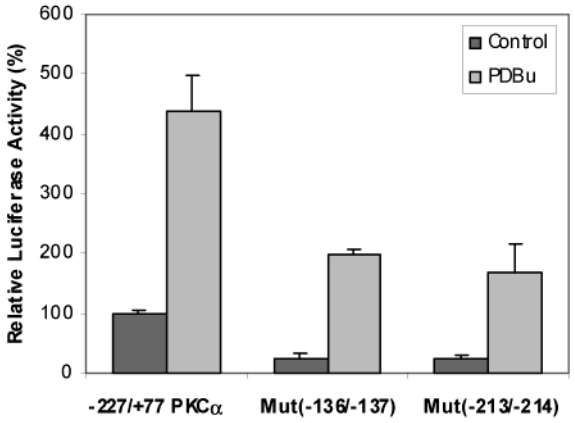

FIGURE 10: Effect of PDBu treatment on the activity of the PKCα promoter containing AP-2 element mutations. U-87 cells were cotransfected with 2 μg of the indicated construct and 0.5 μg of pRL-TK. Cells were treated for 24 h with either DMSO (control) or 100 nM PDBu and harvested 24 h later. Promoter activity is normalized and expressed as a percentage of the −227/+77 control promoter construct (100%). Each value is the mean ± SD of duplicate samples from three experiments.

In summary, the basal promoter of the human PKCα gene is dependent on AP-2, which functions in the phorbol ester-sensitive signaling pathway that suggests an autoregulatory mode of transcription.

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