The Protein Kinase C βII Exon Confers mRNA Instability in the Presence of High Glucose Concentrations

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Previous studies showed that short term exposure of cells to high glucose destabilized protein kinase C (PKC) βII mRNA, whereas PKCβ mRNA levels remained unaltered. Because PKCβ mRNAs share common sequences other than the PKCβII exon encoding a different carboxyl terminus, we examined PKCβII mRNA for a cis-acting region that could confer glucose-induced destabilization. A β-globin/growth hormone reporter construct containing the PKCβII exon was transfected into human aorta and rat vascular smooth muscle cells (A10) to follow glucose-induced destabilization. Glucose (25 mM) exposure destabilized PKCβII chimeric mRNA but not control mRNA. Deletion analysis and electrophoretic mobility shift assays followed by UV cross-linking experiments demonstrated that a region introduced by inclusion of the βII exon was required to confer destabilization. Although a cis-acting element mapped to 38 nucleotides within the βII exon was necessary to bestow destabilization, it was not sufficient by itself to confer complete mRNA destabilization. Yet, in intact cells antisense oligonucleotides complementary to this region blocked glucose-induced destabilization. These results suggest that this region must function in context with other sequence elements created by exon inclusion involved in affecting mRNA stability. In summary, inclusion of an exon that encodes PKCβII mRNA introduces a cis-acting region that confers destabilization to the mRNA in response to glucose.

Protein kinase C (PKC), a serine/threonine kinase, comprises a family of 12 isozymes that have been implicated in signaling pathways affecting cellular processes such as cell proliferation and differentiation, apoptosis, tumor promotion, transcriptional activation, and hormone production (1). The PKC isozymes exhibit differential cellular distribution and substrate specificity. The conventional PKC isozymes, which are Ca²⁺−dependent and activated by phospholipid and diacylglycerol, include PKCα, PKCβI, PKCβII, and PKCγ. PKCβI and PKCβII are encoded by the same gene, and are translated from alternatively spliced products of PKCβ pre-mRNA. The inclusion of the PKCβII exon in the 3′-region through alternative splicing results in the PKCβII mRNA. This pattern of splicing generates a stop codon at the βII-βI boundary such that the βI exon, with its coding sequence and 3′-UTR, now becomes the 3′-UTR of PKCβI mRNA (Fig. 1). As a result, the PKCβI and PKCβII mRNAs differ only by the sequence of the included PKCβII exon, and the proteins differ only by their carboxy-terminal 50–52 amino acids, respectively (1).

We have previously shown that acute hyperglycemia down-regulates PKCβII, but not PKCβI, at the mRNA and protein levels in vascular smooth muscle cells (2). To understand the mechanism by which elevated glucose down-regulates PKCβII expression in vascular smooth muscle cells, earlier studies were carried out to determine at what level suppression of PKCβII expression occurred. These studies clearly demonstrated that high glucose (10–30 mM), at levels that commonly occur in hyperglycemia associated with diabetes mellitus, exerted some regulation at the level of transcription, but that the most dominant level of regulation occurred post-transcriptionally through increased destabilization of PKCβII mRNA via a cytoplasmic nuclease activity (2). PKCβI and other isozyme-specific PKC mRNAs were not destabilized. The effect of elevated glucose (25 mM) was independent of osmotic controls, because mannitol did not down-regulate PKCβII mRNA expression (2, 3). We have also shown that PKCβI and PKCβII enzymes have opposite signaling roles in cell division, where PKCβII signaling suppresses vascular smooth muscle cell proliferation by attenuating G1/S transition and high glucose treatment, which down-regulates PKCβII mRNA and protein, and stimulates vascular smooth muscle cell proliferation (4, 5). High glucose also suppressed insulin effects on glucose uptake, another PKCβII-dependent process (3).

The regulation of mRNA stability has emerged as an important mechanism for controlling gene expression. Depending on the system, half-lives of mRNA range from a few minutes to days. The decay rates of many eukaryotic mRNAs are regulated by developmental or environmental stimuli. Most of the mechanisms that control mRNA stability share
common features, and determinants of mRNA stability have been shown to reside in the 5' -cap, 5'-UTR, coding region, poly(A)-tail, and the 3'-UTR (or the AU-rich elements) such that each may play some role in regulating mRNA decay rates (6–11).

Although it is now clear that the decreased stability of PKCβII mRNA correlates with cell exposure to high concentrations of glucose, the molecular mechanism contributing to the regulation of PKCβII expression by altering its mRNA stability remained to be defined. Here, using deletion and competition analyses, we identify a ~38-nucleotide sequence within a region of secondary structure in the alternatively spliced exon that binds factors in vitro in a manner dependent on the sequence and the availability of factors present only in the cytosol of glucose-treated cell extracts. The degree to which protein-RNA complex was formed was dependent on the presence of the 38-nr region. These data suggest that insertion of the βII exon specifies glucose responsive destabilization of PKCβII mRNA. Because PKCβII and PKCβI mRNAs differ only by the inclusion of the PKCβII exon through alternative splicing, we propose that a cis-acting region introduced by inclusion of the PKCβII exon defines glucose-mediated mRNA destabilization. To our knowledge this is the first characterization of an element that is introduced by alternative splicing that allows for metabolite regulation of stability of a specific mRNA.

### EXPERIMENTAL PROCEDURES

#### Cell Culture—
The vascular smooth muscle cell line (A10, ATCC CRL 1476), derived from rat aorta, was grown in Dulbecco's modified Eagle's medium (with 5.5 mM glucose) containing 10% fetal bovine serum, 100 units penicillin G, and 100 μg of streptomycin sulfate/ml at 37 °C in a humidified 5% CO2, 95% air atmosphere in either 6-well or 100-mm plates. Cells were grown to ~90% confluency and medium was changed every 4 days. Cell synchronization was achieved by serum deprivation (0.5% fetal bovine serum) for 48 h as demonstrated previously (12).

Primary cultured human aortic smooth muscle cells (Clonetics, San Diego, CA) were grown in smooth muscle growth medium (Clonetics) containing 5.5 mM glucose, 5% fetal bovine serum, 10 ng/ml human recombinant epidermal growth factor, 390 ng/ml dexamethasone, 50 μg/ml gentamicin, and 500 μg/ml amphotericin-B at 37 °C in a humidified 5% CO2, 95% air atmosphere. Cells were grown to ~90% confluency and medium was changed every 5 days.

#### Materials—
The isopropyl 1-thio-β-D-galactopyranoside (IPTG). Oligo probe labeling kit (Prime-a-Gene) was purchased from Promega. Ribosome T7 RNA probe synthesis kit and Ampliscribe T7 transcription kit were purchased from Epicentre Technologies. RNase A, RNase T1, heparin, and RNase inhibitors were purchased from Sigma.

#### Lipofectamine Transfections—A10 cells were grown and synchronized by serum deprivation for 48 h in either 6-well or 100-mm plates. The pβG or pβG-PKCβII expression vectors were transfected in serum-free medium with the Lipofectin-DNA complex. Lipofectin reagent was purchased from Invitrogen. Following 4 h of incubation, the Lipofectin-DNA complex was washed off with 1× Dulbecco's phosphate-buffered solution and replaced with fresh medium containing 2% serum. For stably transfect A10 cell selection, 0.7 mg/ml G-418 was added to the media. It was changed every 4 days, and 10–14 days later the colonies were pooled and maintained in the appropriate Eagle’s medium (10% fetal bovine serum) with 0.2 mg/ml G-418.

#### mRNA Half-life Determination—pβG-PKCβII stable transfectants and pβG stable transfect A10 cells were plated into 100-mm dishes. 50 μg/ml DRB (5,6-dichloro-1β-D-ribofuranosylbenzimidazole) dissolved in 95% ethanol was added to the plates 30 min prior to the transcription sample and the 0-h RNA sample was isolated. 25 mM glucose was added to the glucose-treated plates. RNA samples were isolated from normal (5.5 mM glucose) and glucose-treated (25 mM glucose) pβG-PKCβII and pβG dishes at 2, 4, 6, 10, and 18 h. In a separate control, an equivalent amount of 95% ethanol was added.

#### Isolation of RNA and Northern Blot Analysis—
Total cellular RNA was isolated from 100-mm plates using Tri-Reagent (Molecular Research Center, Inc.). RNA samples (10 μg) were prepared in formamide, formaldehyde, and 1× MOPS, and fractionated on 1.2% agarose-formaldehyde gels. Ethidium bromide was added in the loading buffer for visualization and quantitation of 18 S and 28 S RNA. After fractionation, the integrity and loading of RNA was assessed under UV light (12–14). The size-fractionated RNA was then capillary transferred to Hybond membranes (Amersham Biosciences), and cross-linked to membranes by baking at 80 °C in a vacuum oven for 2 h. Membranes were hybridized overnight at 42 °C with 2 × 10^6 cpm of the β-globin probe (labeled with [α-32P]dCTP by nick translation as described (16)) per ml of hybridization buffer. Membranes were washed with high stringency conditions; label was detected and quantitated using a Amersham Biosciences PhosphorImaging system.

#### Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—
Total RNA was isolated from control or glucose-treated A10 cells or aortic smooth muscle cells and 2 μg was used to synthesize first strand cDNA using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. A 368-bp cDNA fragment served as the starting point for PCR and contained the 3'-most 216-bp exon specific for PKCβII (5'-GATATATGCACCCTGCGCTTCTG-GGCTTGAGGGG-3') and downstream antisense primer was (5'-AACCACCGACTTGCCCAAGGG-3'). Sense and antisense primers for β-actin (number 5402-3) were obtained from Clontech. To detect PKCβI and βII, the upstream sense primer corresponded to the C4 kinase domain common to both PKCβI and PKCβII (5'-CGTATATCGGCCCCTTGTG-3') and the downstream antisense primer was specific for PKCβII (5'-CGATCTAGTGGCAAGATCTGTTG-3') (16). PCR was performed using platinum Taq DNA polymerase (from Invitrogen) on 10% of the reverse transcriptase reaction product. Following amplification in a Biometra Triblock Thermocycler (β-globin: 94 °C, 1 min; 58 °C, 1 min; and 72 °C, 3 min for 30 cycles; PKCβII and βII: 95 °C, 30 s; 68 °C, 2 min for 35 cycles), 20% of the amplified products were resolved in a 1.2% agarose gel or 5% of products were resolved on 6% PAGE gels and detected by silver staining. The PCR reaction was optimized for linear range amplification to allow for quantification of products.

#### cDNA Probe Preparation—The βSV-βG vector (obtained from Dr. Norman C. Curthoys, Colorado State University) was digested with HindIII and BglII to obtain a 507-bp fragment, which was isolated from agarose gel using the QIAquick gel extraction kit (Qiagen). The β-globin probe was labeled with [α-32P]dCTP using Prime-a-Gene (Promega) to a specific activity of 109 disintegrations/min.

#### Chimeric M. Minigene—
The parent vector pβG contains the strong viral promoter derived from the long terminal repeat of the Rous sarcoma virus followed by the transcriptional start site, the 5'-nontranslated region, the entire coding sequence, and two introns from the rabbit β-globin gene, a multicloning site containing four unique restriction sites, and the 3'-nontranslated region of the bovine growth hormone (Bovine β-Globin: 94 °C, 3 min; 58 °C, 1 min; and 72 °C, 3 min for 30 cycles; PKCβII and βII: 95 °C, 30 s; 68 °C, 2 min for 35 cycles), 20% of the amplified products were resolved in a 1.2% agarose gel or 5% of products were resolved on 6% PAGE gels and detected by silver staining. The PCR reaction was optimized for linear range amplification to allow for quantification of products.

#### PKCβII Probe Preparation—The PKCβII probe [obtained from Dr. D. Norman C. Curthoys, Colorado State University] was digested with HindIII and BglII to obtain a 507-bp fragment, which was isolated from agarose gel using the QIAquick gel extraction kit (Qiagen). The β-globin probe was labeled with [α-32P]dCTP using Prime-a-Gene (Promega) to a specific activity of 109 disintegrations/min.
extracted from the gel (Fig. 1A). Each gel extraction kit, digested with SpeI and XhoI, and purified. The PKCβII cDNA was ligated into the SpeI and XhoI sites of the multicloning region of pGEM vector. The construct, pβG-PKCβII, was verified by restriction mapping and deoxyribonucleotide sequencing.

Using PCR primers for C4 (last common domain) as sense primer with the SpeI site synthesized upstream (5′-CTGATATAGTT- TGGCGCGCTGAAAGGGAACCG-3′) and for β exon antisense primer with the XhoI site synthesized downstream (5′-CCGAGGCTACACATACTACTTCTAGACCT-3′), PKCβII exon without the β exon (286 bp product) was amplified. This insert contained 70 bp corresponding to the C4 exon and the entire PKCβII exon. The product was gel purified and ligated into the SpeI and XhoI sites of the multicloning region of pGEM vector. The construct, pβG-PKCβII, was verified by restriction mapping.

The region of instability comprising 38 nucleotides was synthesized with the SpeI and XhoI restriction enzyme sites to facilitate cloning into the pGEM vector: 5′-AATCCTACGTAGTATTTAAAAACCCCAAGTCT- AAGAGCTCTAGTGAATTTTTAAAACCCGAAGTC-3′. The construct, pβG-PKCβII, was verified by restriction mapping.

As a control, the analogous carboxy-terminal region of PKCδ (C4-VS6) was digested using BstXI and XhoI (450 bp), purified, and ligated into the multicloning region of the β vector. The construct, pβG-PKCs, was verified by restriction mapping.

In Vitro Transcription Vectors—The 404-bp PKCβII cDNA product corresponding to the β exon and flanking regions described above was obtained by PCR amplification using sense primer to the upstream PKCβ common C4 domain (5′-CTGATATAGTTTGCGCGCTGAAAGGGAACCG-3′) and antisense primer to β exon (5′-GGATCCATGTACGGTTCCTGGAAGG-3′) such that the exon-included PKCβII mRNA was amplified. This PKCβII cDNA piece was cloned into the pcR-Blunt vector (Invitrogen) such that transcripts could be generated from the upstream T7 RNA polymerase promoter.

In Vitro Transcript Preparation—The RNA probes were generated by consecutive restriction digestion of the pcR-Blunt-PKCβII vector. Riboprobe A (RPA) was the full-length PKCβII insert described above, linearized at the SpeI site and flanking regions. Riboprobe B (Rpb) was the PKCβII insert linearized at 175 bp with Rsal within the PKCβII exon such that the PKCβ-specific exon was eliminated; riboprobe C (Rpb) was linearized at 137 bp with HpaI, which cut within the PKCβII-specific exon; riboprobe D (Rpd) was linearized at 102 bp with SspI, which cut within the PKCβII-specific exon. After digestion, the riboprobes were purified and their sizes and linearity were confirmed following size fractionation on agarose gels. One μg of each linearized plasmid DNA was further used for in vitro transcription with the Ampliscribe kit (Epitcience) for competitor unlabeled probes or with Riboscribe kit (Epitcience) for transcribing labeled RNA probes using T7 RNA polymerase at 37 °C for 2 h in the presence of nucleotides, RNAse inhibitor, and ribonuclease inhibitor according to the manufacturer’s instructions. The non-specific unlabeled probes were from analogous fragments of PKCδ (described above), PKCβI (15), and β-actin, and were prepared in a similar manner. For labeled RNA probes, 5 μl of [α-32P]CTP (3000 Ci/mmol) was used in the reaction and 1 μl of RNAse-free DNase I was then added and incubated for 15 min at 37 °C to remove the template DNA. The transcripts were precipitated using 5 μl ammonium acetate and incubated for 15 min on ice followed by a 70% ethanol wash. The pellet was resuspended in RNAse-free water. The integrity of RNA probes was confirmed and probes were purified by 6% native polyacrylamide gel electrophoresis.

Cytosplasmic Extract (S100) Preparation—In brief, A10 cells incubated in either high (25 μM) or low (5 μM) glucose (high glucose) for 4 h were washed with 1× phosphate-buffered saline, gently scraped, and centrifuged at 1850 × g for 10 min. The packed cells were re-suspended in hypotonic buffer (10 mM HEPES, pH 7.9, 4.5 μM MgCl2, 10 μM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μM dithiothreitol, 10 μM leupeptin, 10 μM antipain), allowed to incubate on ice, and homogenized in a Dounce homogenizer with 60 strokes using type B pestle. The nuclei were pelleted by centrifuging at 3300 × g for 15 min, and the cytoplasmic extract was mixed with 0.11 volume of cytoplasmic buffer (0.3 M HEPES, pH 7.9 at 4 °C, 1.4 μM KCl, 0.03 μM MgCl2). After centrifugation for 1 h in a Beckman type 50 rotor at 40,000 rpm, the supernatant (S100) was collected, and centrifuged at 185,000 g for 1 h (100,000 × g) at 4 °C. An aliquot was used to determine the protein concentrations using the Bradford protein assay (18).

RNA Electrophoretic Mobility Shift Assay—S100 extracts from A10 cells exposed to low (5.5 μM) glucose and high (25 μM) glucose concentrations containing 3 μg of protein were incubated with 3 μg of yeast tRNA and 10 units of RNase inhibitor in a final volume of 10 μl of RNA shift buffer (12 mM HEPES, pH 7.9, 10 mM KCl, 10% glycerol, 5 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl2) for 10 min at room temperature. 100–300-Fold excess specific cold competitors or 100-fold excess non-specific cold competitors quantified spectrophotometrically using a Pharmacia Gene Quant were added to the binding reactions and incubated for 5 min at room temperature. Labeled RNA probes Rpa, RpB, Rpb, RpC, or RpD (described above) to an activity of ~1 × 106 cpm were added and incubated for 20 min at room temperature. RNase T1 was added to the binding reaction to digest the unbound RNA. Because the cytoplasmic extracts may contain proteins that bind to negatively charged polyacrylamide, like nucleic acids, heparin was added to the reaction at a concentration that could not affect nonspecific binding (19, 20, 210). RNase T1 was added and further incubated for 15 min at room temperature, followed by the addition of 5 μg/ml heparin to reduce nonspecific binding, for 10 min on ice. Samples were separated on a 10% polyacrylamide gel in 0.5× TBE buffer. Gels were dried and exposed to Amersham Biosciences PhosphorImaging screen.

UV Cross-linking of RNA-Protein Complexes—RNA-protein binding reactions were carried out as described above for the RNA electrophoretic mobility shift assay. To demonstrate specificity, the 38-nucleotide sequence (50 nt) described above, or 50 nt of a 20-nucleotide antisense 2′-O-(2′-methoxyethyl) (MOE) oligonucleotide (AS 25847) targeting a portion of the 38-nucleotide region, 5′-CTGATATAGTTGCGCGCTGAAAGGGAACCG-3′, were added to binding reactions. As a control, another 20-nucleotide antisense MOE oligonucleotide (AS 25649), 5′-GAAGTTGGAGGTTGCCTGCTGGTCT-3′, upstream of this region or a scrambled 20-mer MOE oligonucleotide was used. Following heparin addition, the samples were transferred to a 96-well plate, and irradiated for 10 min in Stratalinker (Stratagene) on ice. Laemmli’s buffer was added to the sample, which was then boiled for 5 min and separated on a 10% SDS-polyacrylamide gel. Gels were dried and exposed to Amersham Biosciences PhosphorImaging screens. To control for non-specific protein/RNA interactions, 1 μg of micrococcous nuclea (Sigma) was added to control and glucose-treated cell extracts with 70 μM EDTA in addition to the labeled probe. There was no difference in the UV cross-linked bands observed in the presence of micrococcous nuclea to either control or high glucose-treated cytosolic extracts.

RESULTS

Effects of the PKCβII Exon Insertion on the Stability of β-globin mRNAs—Previous experiments had suggested that insertion of the 3′-PKCβII exon via alternative splicing introduced a glucose-responsive instability region that was not present in the PKCβI mRNA (2). To test this, a corresponding region consisting of 70 nucleotides in the last domain common to both PKCβI and PKCβII (C4), the PKCβII-specific exon (V5) (216 nucleotides), and 119 nucleotides of the flanking β exon were cloned into an expression vector (pβG) to form a vector expressing β-globin-PKCβII mRNA (see Fig. 2, a and b). The resulting chimeric vector, pβG-PKCI, was under the regulation of a viral promoter and contained a polyadenylation sequence, eliminating regulatory effects on transcription and nuclear decay. The pβG-PKCI vector was transiently transduced into A10 cells, a rat clonal vascular smooth muscle cell line, and also into human aorta smooth muscle cells. Aorta smooth muscle cells respond to glucose-induced destabilization of PKCβI mRNA in an analogous manner to A10 cells and provide a primary cell model for corroborating glucose effects in human cells (2). As demonstrated by RT-PCR analysis (Fig. 2c), acute exposure (2 h) to high glucose (25 mM) resulted in a decrease (>80%) in the chimeric β-globin-PKCβII mRNA, when compared with the same cells exposed to control levels of low glucose (5.5 mM). In A10 cells transfectected with the parent vector, pβG, β-globin mRNA levels remained unaltered after exposure to high glucose concentrations. Levels of β-actin mRNA remained unchanged in low glucose and high glucose-treated pβG-PKCI and pβG-transfected cells.

To further rule out the possibility of non-specific effects caused by inserting the fragment of PKCβI cDNA into the...
PKCPβII cDNA: A schematic representation of the cDNA showing conserved (C1–4) and variable (V1–5) regions as deduced from sequence analysis. b. PKCPβII cDNA insert, comprised of the entire 216 bp of the C-terminal exon for PKCPβII, 70 bp of the common domain, C4, and 118 bp of the βI exon, subcloned into the parent vector pβG at the SpeI and XhoI sites. The pβG (obtained from N. P. Curthoys, Colorado University) is a chimeric gene containing a strong viral promoter derived from the long terminal repeat of the Rous sarcoma virus followed by the transcriptional start site, the 5’-untranslated region, the entire coding sequence, and two introns from the rabbit β-globin genomic DNA; a multicloning site containing four unique restriction sites into which 404 bp from COOH-terminal PKCPβII I exon, cytoplasmic extracts from A10 cells were examined. The PKCPβII I exon functions as an extended 3’-UTR (Fig. 1). c. Total RNA was extracted from human aorta smooth muscle cells (AoSMC) transiently transfected with pβG-PKCPβII chimeric or a pβG empty vector stability reporter system and exposed to either normal (5.5 mM) or high (25 mM) glucose for 2 h. 2 µg of RNA was used in the RT-PCR analysis using primers for β-globin A, 100-bp DNA marker; B, normal glucose; C, high glucose. The experiment was repeated five times and similar results were obtained.

| Cell type | A10 | AoSMC | A10 |
|-----------|-----|-------|-----|
| 5.6 mM glucose | 120 | 120 | 120 |
| 25 mM glucose | 120 | 120 | 120 |

The Effect of Glucose on the Half-life of β-Globin-PKCPβII mRNA—Half-life determinations of β-globin-PKCPβII mRNA were carried out in the presence of the transcriptional inhibitor, DRB. DRB was used rather than actinomycin D, because actinomycin D, commonly used to inhibit RNA polymerase II, has also been reported to inhibit translation (21), and in some instances to also inhibit mRNA degradation (22, 23). Stable A10 cell transfectants of pβG-PKCPβII or pβG were pre-treated with DRB, and then incubated with low (5.5 mM) or high (25 mM) glucose for various times up to 6 h. Northern blot analysis of total RNA shown that only 20% of the β-globin-PKCPβII mRNA remained after exposure to high (25 mM) glucose concentrations, compared with β-globin-PKCPβII mRNA in the presence of control (5.5 mM) glucose concentrations within 2 h (Fig. 4). This is in agreement with the results presented in Fig. 2. High glucose concentrations reduced the half-life of the β-globin-PKCPβII mRNA to 45 min, effectively decreasing the amount of the mRNA.

The Effect of Glucose-induced Destabilization by the βI Exon in the Absence of the βI Exon—Inclusion of the PKCPβII-specific exon in many cell types results in mature PKCPβII mRNA in which PKCPβII exon functions as an extended 3’-UTR (Fig. 1). Exon inclusion produces new C4-βII and βI-βI junctions as shown in Fig. 2c. Hence, to examine the influence of glucose on mRNA stability that may be mediated by insertion of the βI exon, a new construct without the 3’-βI exon was examined. The β-globin-PKCPβII-I vector was transiently transfected into A10 cells, and cells were exposed to high or low glucose (control). As shown (Fig. 5), high glucose also destabilized the β-globin-PKCPβII-IββI mRNA. The extent of destabilization was less than that observed for the β-globin-PKCPβII chimeric mRNA shown in Fig. 2c. Approximately 50% of the β-globin-PKCPβII-IββI mRNA was degraded compared with >80% degradation of the β-globin-PKCPβII chimeric mRNA in cells treated with high glucose. These results suggested that inclusion of the βI exon was necessary to confer full glucose-sensitive mRNA degradation, but the C4-βII exon junction alone was not sufficient to produce the same level of glucose-induced destabilization as observed for the complete 3’ PKCPβII mRNA sequence, including both the C4-βII and βI-βI exon junctions as shown in Fig. 2c.

Cytoplasmic Extracts from A10 Cells Treated with High Glucose Concentrations Specifically Retard a Labeled PKCPβII Exon Transcript—Initial studies demonstrated that a nuclease activity present in the cytosolic extracts from glucose-treated cells mediated PKCPβII mRNA destabilization (2). To further investigate regions important for destabilization within the PKCPβII exon, cytoplasmic extracts from A10 cells were exam-
Glucose-regulated Instability Element in PKCβII mRNA

MOE Antisense Oligonucleotides Block Glucose-induced Destabilization of PKCβII mRNA—Because of the limitations of the β-globin system to map glucose-responsive elements within the PKCβII mRNA, a third approach was taken to further demonstrate that this 38-nt region was associated with destabilization. Antisense (AS) MOE oligonucleotides (20-mers) were designed to “walk” complementary to this putative 38-nt PKCβII mRNA instability region. MOE modifications are resistant to exo- and endonuclease degradation and do not support cleavage of hybridized mRNA by RNase H. Furthermore, these oligonucleotides bind with high affin-
ity to the complementary mRNA sequences (24). AS oligonucleotides 25646 and 25648 spanned consecutive regions while AS 25647 overlapped these sequences (Fig. 8a). AS 25645 and AS 25649 corresponding to upstream (not shown) and downstream sequences, served as controls because they are outside the 38-nt region. To evaluate specific binding and targeting of the antisense, as a separate control, a scrambled sequence (AS 25581) was used. A10 cells were transfected with the AS oligonucleotides (50 nM) and then exposed to 25 or 5.5 mM glucose. As shown in Fig. 8b, AS 25647 blocked high glucose-induced destabilization. AS 25646 and AS 25648 blocked destabilization to a lesser extent. The downstream AS 25649 did not block glucose effects. Scrambled control sequences did not block destabilization (data not shown). This approach further mapped the relevant element to 20 nucleotides within this 38-nt region and suggested that the sequence was important for protein interaction.

**UV Cross-linking Detected Association of Proteins**—To provide insight into the basis for the mobility shift and nature of the components that bind to the PKCβII mRNAs in response to high glucose exposure, UV cross-linking experiments were performed using transcripts RpA and RpB, which demonstrated specific binding by mobility shift assays. Binding assays were carried out in parallel with the RNA shift assays, but were further subjected to UV light to generate covalent bonds between the 32P-labeled RNA transcript and associated proteins, digested with RNase A, and separated by SDS-polyacrylamide gel electrophoresis. Under these conditions a single band was observed at 10–14 kDa for the control extracts (Fig. 9), and extracts from high glucose-treated cells showed a 5-fold increase in intensity over that observed for extracts from control cells exposed to low glucose. Longer exposure times did not elucidate any other bands. An excess 38-nucleotide sequence, corresponding to the HpaI-BglII region identified in mobility shift assays, competed for protein binding with RpB (Fig. 9). Unlabeled probes RpA and RpB competed for protein binding, respectively, with labeled RpA and RpB, but unlabeled RpC, RpD, and PKCβ probes did not compete for protein binding following UV cross-linking (data not shown). To control for possible nonspecific protein interactions, micrococcal nuclease (1 μg) was added to cell extracts in the presence of excess EGTA. The affinity of this exogenous protein binding to labeled

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electrophoretic mobility assay showed that a 38-nt region near the 38-nucleotide/HpaII and H9252 cells contain a small molecular weight protein (10,000

results were taken to demonstrate that cell extracts from A10 reactions abolished complex formation (data not shown). These unaffected (data not shown). Addition of proteinase K to the

instability in the PKCII exon monitored by RNA of RNA was used in the RT-PCR analysis using primers for the C4 domain and lV5 domain (indicated by arrows in a), such that PKCβII and PKCβIII products were detected simultaneously. The experiment was repeated at least four times to ensure reproducibility.

RpB in control and high glucose-treated cell extracts remained unaffected (data not shown). Addition of proteinase K to the reactions abolished complex formation (data not shown). These results were taken to demonstrate that cell extracts from A10 cells contain a small molecular weight protein (10,000–14,000) that specifically binds to a PKCβIII mRNA region containing the 38-nucleotide HpaI-BglII sequence, and that the efficiency of binding, as measured by UV cross-linking, increases in response to high glucose.

Antisense Corresponding to a Portion of the HpaI-BglII Region Blocked Protein Interaction and Glucose-induced Destabilization—To demonstrate whether the protein binding to the mRNA was involved in its destabilization, antisense MOE oligonucleotide (Fig. 8) complementary to a portion of the HpaI-BglII region and shown to block destabilization, was used as a competitor in the UV cross-linking experiments. The 20-mer, AS 25647, was shown to compete for protein binding with RpB (Fig. 9). Another antisense oligonucleotide upstream of this region, AS 25649, did not block the protein interaction with RpB or glucose-induced destabilization. Hence, the association of a low molecular weight protein with a specific sequence in the PKCβII carboxyl terminus exon was required for glucose-induced destabilization of the mRNA.

DISCUSSION

The previously reported observation that only PKCβII and not PKCβI was subject to glucose-induced mRNA destabilization by a cytoplasmic nuclease activity suggested that the sequence within the PKCβII exon was responsible. The results of this investigation indicate that when this region is inserted into a β-globin reporter gene, glucose-dependent instability is introduced and the mRNA half-life is markedly reduced.

Deletion analysis of the PKCβII exon monitored by RNA electrophoretic mobility assay showed that a 38-nt region near the middle of the exon was required for this interaction. Furthermore, the addition of an RNase H-resistant antisense MOE oligonucleotide that targeted 20 nt within the 38-nt region abolished the interaction. However, when inserted into the β-globin reporter gene, the 38-nt region by itself was not sufficient to confer glucose-dependent destabilization, indicating that other sequences within the PKCβII exon are necessary. In fact, the destabilization was maximal when a portion of the PKCβ exon was present in addition to the PKCβII exon. This difference highlights the importance of the context in which the βII exon is placed and may reflect differences in factors such as translation rate, secondary structure, and intracellular localization, which may also influence mRNA stability (25).

Computer modeling of the RNA sequence encoded by the C4-βII specific exon indicates that the 38-nt region would likely form a stem-loop structure. The complementary antisense that spanned an AU-rich element and the sequence at the base of a putative stem-loop structure blocked glucose-induced destabilization of PKCβII mRNA as well as protein interaction. This possible secondary structure may, in part, explain some structural features of the context necessary for the cis-acting element to function.

The antisense oligonucleotide that blocked destabilization also targeted an AU motif. One group of cis elements that may mediate mRNA instability are the AU-rich elements (AREs or AUREs) (see Ref. 25 for review). These elements have been associated with AU-rich element-binding proteins. Analysis of the PKCβII complete mRNA indicates 46% AU content. However, the RpB probe containing the PKCβII exon contains 58% AU and the 38-nt fragment contains 66% AU. Furthermore, the 38-nt fragment contains an extended pentamer sequence AAUUAUA that has been identified as a putative AU-rich element-binding protein target. It has also been identified as conferring mRNA instability in the epidermal growth factor receptor transcript (26). Other U-rich sequences identified by Levine et al. (27) as possible AU-rich element-binding protein targets are also present in the PKCβII exon. It is interesting to note that the c-fos mRNA contains two domains: an AUUUA pentamer region and a U-rich region (28). Both regions appear...
to be necessary for maximal RNA destabilization. In the case of PKCβII, however, these elements occur in the coding region and not the 3‘-UTR. They are also introduced in a regulated fashion via exon inclusion.

Our results support the proposal that the PKCβII exon, introduced by hormone-regulated alternative splicing of a common pre-mRNA (16), not only specifies a different 52-amino acid carboxyl-terminal end in the protein, but also confers glucose responsive instability to the PKCβII mRNA in vascular smooth muscle cells. High glucose concentrations increased the levels of a low molecular weight protein that binds to this region and may target the mRNA molecule for degradation. To our knowledge, this is the first report that defines an mRNA instability element present within an exon that responds to regulation by an external stimulus, acute high glucose concentrations in a mammalian cell. The scheme shown in Fig. 10 illustrates how hormone-regulated alternative splicing inserts a region into the mRNA that under conditions of high glucose concentrations, results in the destabilization of the mRNA encoding an important regulatory protein.

Taken together with previous reports by this laboratory showing that the alternative splicing of PKCβ mRNA is regulated by insulin (5, 16, 29), this study highlights the possible integration of metabolic regulation mediated between nutrient (glucose) and endocrine (insulin) controls on vascular smooth muscle cell gene expression. Significantly, it is known that hyperglycemia can further increase smooth muscle cell proliferation that may contribute to the development of atherosclerotic lesions in diabetic subjects (30). In view of the results presented here, hyperglycemic episodes could result in the rapid destabilization of PKCβII mRNA. As a consequence, destabilization would remove PKCβII signaling that has been shown previously to repress smooth muscle cell proliferation (31–33), and may therefore help explain the contribution of acute hyperglycemic incidences to the increased risk of vascular disease in diabetes.

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The Protein Kinase C βII Exon Confers mRNA Instability in the Presence of High Glucose Concentrations
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