Glucose Catabolism in Cancer Cells

THE TYPE II HEXOKINASE PROMOTER CONTAINS FUNCTIONALLY ACTIVE RESPONSE ELEMENTS FOR THE TUMOR SUPPRESSOR p53

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The p53 tumor suppressor is found to be mutated and abundant in a wide variety of tumors. Within tumors showing rapid growth, the Type II isoform of hexokinase is also highly expressed to facilitate high rates of glucose catabolism, which in turn promote their rapid proliferation. We previously reported isolation of the proximal promoter of the Type II hexokinase gene from the highly glycolytic hepatoma AS-30D (Mathupala, S. P., Rempel, A., and Pedersen, P. L. (1995) J. Biol. Chem. 270, 16918–16925). Here, we show that a p53 protein, exhibiting two point mutations in its cDNA, is abundantly expressed in the AS-30D hepatoma. Co-expression studies showed that p53 overexpression significantly and reproducibly activated the Type II hexokinase promoter. Two functional p53 motifs were identified within this promoter by footprint and gel retardation analyses. Presence of functional p53 response elements on the Type II hexokinase promoter and the positive regulatory effect on the promoter by the mutant p53 indicates that in rapidly growing liver tumors, and perhaps in many other tumors as well, this highly abundant p53 protein plays a role in maintaining a high glycolytic rate. This is the first report of a possible link between loss of cell cycle control in rapidly growing cancer cells and their high glycolytic phenotype.

Mutations within the p53 gene represent one of the most common genetic aberrations in tumorigenesis (1–3). Whereas the wild type p53 (wt p53) negatively regulates cell growth and division, the mutant forms lack the ability to suppress or control cell cycle progression. It is now generally accepted that the tumor suppressor function of wt p53 is a result of its ability to act as a cell cycle checkpoint protein, thus halting the cell cycle in the G1 phase if and when DNA damage occurs to a normal cell. Considerable evidence has accumulated for regulation of transcription as one of the primary mechanisms of wt p53 action, where the p53 protein binds to a specific motif on gene promoters and thus transactivates the genes to bring about the suppression of cellular transformation (4, 5). Two repeats of a 10-bp motif PuPuPuC(A/T)(T/A)GPyPyPy have been described as the common DNA binding site of wt p53 (6, 7). An improved high affinity motif, PuPuCATGPyCPy, where the G and A at positions 2 and 5, respectively, are critical determinants in p53-DNA binding has been reported also (7, 8). Mutant p53 (mut p53) proteins are reported to show a dominant-negative effect by forming oligomeric complexes with the wt p53 prior to DNA binding, which brings about a change in conformation and subsequently a loss of affinity of wt p53 for DNA. However, recent reports (5, 9–14) also suggest that, in addition to this inactivating effect by mut p53, at least in some cases, the mutant forms can even promote the growth of the parental tumor cell and therefore exhibit an oncogenic gain-of-function of their own (15, 16). This endogenous dominant-oncogenic function is seen more clearly when mut p53 is transleted into p53-deficient cells (15, 17, 18). This contrasts with earlier studies which suggested that mut p53 is unable to bind DNA and transactivate gene transcription (4, 6).

Analogous to the p53 protein, which plays a pivotal role in regulating cell cycle progression at the gene level, hexokinase plays a major role in metabolic regulation, notably in highly glycolytic tumor cells (19). Overexpression of hexokinase, in particular the Type II isoform, induces the capacity of tumor cells, at least in part, to catabolise glucose at high rates (20–22), one of the most common biochemical signatures of such malignant tissues. This enhanced metabolism not only increases the production of biosynthetic precursors essential for cell growth, but maintains a high rate of ATP production under low oxygen (hypoxic conditions). Therefore, it is interesting to inquire whether there is a relationship between loss of cell cycle control, increased abundance of mutated p53, and Type II hexokinase gene transcription. As described below, p53 motifs are located within the tumor Type II hexokinase promoter, and mutated p53 does interact with the promoter in cancer cells to activate transcription of this key metabolic enzyme.

EXPERIMENTAL PROCEDURES

Materials

[α-32P]dATP (3000 Ci/mmol) and [α-35S]dATP (1000 Ci/mmol) were from NEN Life Science Products. The isolation, sequence, and structure of the Type II hexokinase gene promoter has been reported previously (22). PGL-2 vector series (pGL2-Basic, pGL2-Control) and PSV-β-galactosidase control vector were from Promega. Chemiluminescence measurements and mammalian cell transfections were carried out as described previously (22). Restriction enzymes, DNA-modifying enzymes, and tissue culture media were from Life Technologies, Inc., or from Sigma.

Methods

Tumor Cells—AS-30D hepatoma cells, a model tumor cell line exhibiting a high glycolytic rate (23), were propagated in the peritoneal cavity of female Sprague-Dawley rats (100–150 g) exactly as described previously (24). For nuclear extract preparation, the cells were purified in RPMI 1640 media, followed by a phosphate-buffered saline wash.

RNA Isolation—Total RNA was isolated from AS-30D hepatoma cells

22776 This paper is available on line at http://www.jbc.org
using RNaseA B according to the manufacturer’s instructions (Tel-Test, Inc., Friendswood, TX).

Isolation of Hepatocytes—Hepatocytes were isolated from non-hepato-ринized female Sprague-Dawley rats (100–150 g) by the collagenase perfusion method (25) with minor modifications as follows. Post-perfu- sion, 1 mL of hepatocytes was resuspended in an equal volume of Hepato- cell Wash Medium (4 °C) (Life Technologies, Inc.). Viable hepatocytes were separated by sedimentation and washed once in phosphate-buff- ered saline prior to the nuclear extract preparation.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from hepatoma or hepatocyte nuclei according to the method of Dignam et al. (26) and stored at –80 °C until use.

Reverse Transcriptase-PCR Cloning of p53—The p53 coding region cDNA of AS-30D hepatoma cells was cloned by reverse transcriptase-PCR, using oligonucleotides complementary to a reported normal rat liver p53 cDNA sequence (27). The forward and reverse oligonucleotides were GC GAATTCC ATG GAG GAT TCA CAG TCG GAT and CC TCTAGA TCA GTC GTC AGG CCC CAC, respectively. The primers contained an EcoRI site and an XbaI site for cloning purposes at their 5’ termini, respectively. Reverse transcriptase-PCR was carried out using the Superscript Preamplification System (Life Technologies, Inc.) for first strand cDNA synthesis. PCR amplification was performed in the presence of both Taq DNA polymerase (Perkin-Elmer) and Pfu DNA polymerase (Taq Extender, Stratagene) to minimize reading er- rors. The PCR product was cloned into the EcoRI-XbaI site of the pUC18 plasmid vector. p53 cDNA was sequenced in both orientations by using the Sanger dyeoxy chain termination method (28).

Plasmid Construction, Transfection, and Reporter Gene Analysis—For co-transfection studies, the p53 cDNA was subcloned into the EcoRI-SalI sites of the pCI-Neo mammalian expression vector (Promega Biotech Inc., Madison, WI) using the EcoRI position on the original cloning primer and SalI position of the pUC18 (20) multiple cloning site. Orientation and sequence integrity were verified by DNA sequenc- ing in both orientations. The Type II hexokinase promoter-luciferase reporter gene construct, transfection conditions, and reporter gene assay method have been described previously (22). For co-expression studies, 2.5 μg of the p53 expression plasmid was used with 5 μg of the Type II hexokinase promoter-luciferase gene construct, per 25 × 106 AS-30D hepatoma cells.

Western Analysis—For Western blotting, samples of nuclear extract from hepatocytes or hepatoma were separated on a 7.5% SDS-polyacry- lamide gel and then transferred onto polyvinylidene difluoride (Bio-Rad) membranes. The membranes were probed with an anti-p53 monoclonal antibody specific for the C-terminal region of human p53 (Catalog No. OP03, Oncogene Science) and detected by enhanced chemiluminescence (ECL System, Amersham).

Gel Mobility Shift Assays—Protein-DNA complexes were allowed to form in 4 mM HEPES (pH 7.9), 12% (v/v) glycerol, 85 mM NaCl, 0.3 mM MgCl₂, 0.04 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM dithiothreitol. In a volume of 15 μL, the nuclear extract (8–12 μg of protein) was preincubated for 10 min with 0.85 μg of sonicated salmon sperm DNA (20 mM HEPES, pH 7.4, 5 mM dithiothreitol, 1 mM MgCl₂, 60 mM KCl) to which 2 ng (approximately 500,000 cpm) of the 32P-labeled probe was added and then added (–4 ng, approximately 120,000 cpm). Incubation was continued for 30 min at 25 °C. Samples were then electrophoresed on a 4% non-denaturing polyacrylamide gel in a low ionic strength buffer (7 mM Tris-HCl, 3.5 mM sodium acetate, 1 mM EDTA, pH 7.9) at 12.5 V/cm for 3–4 h at 4 °C. For competition experiments, a competitor unlabeled double-stranded probe was incubated in the binding mixture before the labeled probe was added.

DNase I Footprint Analysis—DNase I footprint assays were adapted from methods by Kingston (30) and Lane and co-workers (31). DNA binding reactions were performed in a 35-μL volume of binding buffer (20 mM HEPES, pH 7.4, 5 mM dithiothreitol, 1 mM MgCl₂, 60 mM KCl) with 2 ng (approximately 500,000 cpm) of DNA template end-labeled by Klenow fill-in reactions (30), 1000 ng of sonicated salmon sperm DNA as competitor DNA, and variable amounts of protein. Reactions were incu- bated for 20 min on ice followed by a 60-s digestion at 25 °C with 0.3 to 5 μL of a freshly diluted DNase I solution (0.05 μg/μL). The digestion reactions were terminated by the addition of 100 μL of buffer (1% SDS, 20 mM EDTA, 200 mM NaCl, 250 μg/mL yeast RNA). The sample was extracted with phenol-chloroform and precipitated with 2 volumes of ethanol. The DNA pellets were dried and resuspended in sequence loading buffer (99% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), incubated 5 min at 68 °C, and loaded on a 6% polyacrylamide, 8.3 M urea sequencing gel. The gels were dried and autoradiographed for 1 to 6 h prior to film development.

RESULTS AND DISCUSSION

The Tumor Type II Hexokinase Promoter Contains Strong Consensus Motifs for p53—To identify novel cis elements within the Type II hexokinase promoter isolated from AS-30D hepatoma (22), the 4.3-kilobase pair promoter sequence was analyzed against a transcription factor data base (32). The results indicated the presence of a pair of p53 response elements (Fig. 1A) at −4250 and −4195, in close proximity to a region presently identified as containing numerous other cis elements, including response elements for glucose and insulin (22). Upon close examination, it was found that each p53 element was located at the center of a perfect 54-bp direct repeat sequence (−4276 to −4223 and −4222 to −4169, TAC- CATGG...TTTTAAA) (Fig. 1B). The p53 consensus sites located within these repeats, AGGCATGCTTC, were also closely similar to a reported high affinity p53 consensus motif (PuG-PuCATGPyCPy) identified by combinatorial library screening.

FIG. 1. A, position of the p53 elements within the tumor Type II hexokinase Promoter. The two p53 motifs (p53) described are located within the distal 4-kilobase pair region of the Type II hexokinase promoter at positions −4250 and −4195. The positions (22) of the (IRE), insulin response element (IRE), cAMP response elements (cAMP), and the TATA and CAAT boxes are indicated. A nucleotide sequence of the promoter region that contains the two p53 motifs. The two p53 elements are outlined (hatched boxes). The arrow above position −4273 indicates the labeling position for DNase I footprint analysis. The nucleotide region used as the probe for footprinting is highlighted. The arrows below the sequence indicate the two 54-bp direct repeat sequences. C, SDS-PAGE profile for the nuclear proteins in the 40–55-kDa range. Lanes 1–3, 8, 16, and 24 μg of hepatocyte nuclear extract; lanes 4–6, 8, 16, and 24 μg of hepatoma nuclear extract. D, Western blot analysis of the hepatocyte and AS-30D hepatoma nuclear extracts. A duplicate blot of the SDS-PAGE profile shown in C was probed using a p53 monoclonal antibody (see “Methods”). The positions of the molecular mass markers (66 and 45 kDa) are indicated.
and by in vitro transcriptional assay (8) methods, where the G at the second position and the A at the fifth position were reported to be critical for enhanced affinity toward the p53 oligomer.

The p53 Protein Is Highly Abundant in AS-30D Hepatoma Cells—The Western analyses of the nuclear extracts from hepatocytes and AS-30D hepatoma cells (Fig. 1, C and D) clearly show that the p53 protein is highly abundant in the model hepatoma cell line. On a comparative basis, when similar amounts of total nuclear protein were assayed, a signal for p53 could not be detected for the hepatocyte extract. This result implicates possible mutations within the p53 protein of the hepatoma, as mutations often generate a protein with a longer half-life (33). The half-life of wt p53 is known to be between 6 and 20 min, whereas the most common p53 mutants maintain a half-life of 4–12 h (33). This results in accumulation of mutated p53 within tumor cells and is clearly detectable by immunochromatographic techniques. The p53 signal observed for the AS-30D hepatoma extract examined here indicates a similar accumulation of the protein within these cells, suggesting the presence of a mutant p53.

p53 of the Highly Glycolytic AS-30D Hepatoma Does Contain Point Mutations—To determine whether the p53 protein of AS-30D hepatoma cells exhibit one or more mutations, the transcribed p53 message was subjected to reverse transcriptase-PCR cloning. Analysis of the primary sequence of the cloned p53 from AS-30D hepatoma cells, when compared with the normal rat liver wt p53 sequence (27) (Fig. 2), shows two point mutations, at position 103 (Gly to Ser), and 256 (Glu to Gly). The first mutation at position 103 is located in proximity to the second conserved region of p53, whereas the mutation at position 256 is located on the C-terminal end of the fourth conserved region. When the primary sequence is aligned with the human wt p53 (Fig. 2), the mutations observed for the AS-30D hepatoma are located at positions 105 (Gly) and 258 (Glu) of the human wt p53 sequence, at the periphery of the core DNA binding domain (amino acids 102 to 292) (33). However, both mutations, when compared with the most common human p53 point mutations identified so far, lie outside the positions where the mutations result in inactivation of DNA binding ability. Whether the two mutations observed impart a greater stability to p53 in hepatoma cells and whether the mutations confer stronger DNA binding ability remains to be elucidated. It should be noted that both mutations lie outside the transactivating domain of p53 (amino acids 20–42) and the oligomerization domain (amino acids 300–391). Furthermore, the nuclear localization signal motif (33) also remains unaffected.

A DNA Segment within the Tumor Type II Hexokinase Promoter Containing the p53 Response Element Binds Sequence-Specific Factors in Vitro—A gel mobility shift assay was performed using a double-stranded nucleotide fragment corre-
sponding to the putative p53 response element region (−4259 to −4173) of the tumor Type II hexokinase promoter. Two major protein-DNA complexes were formed between the p53 element-containing DNA probe and the AS-30D hepatoma nuclear extract. These complexes were competitively disrupted by addition of unlabeled p53 probe DNA, but were not disrupted by the addition of a nonspecific competitor DNA amplified from the same Type II hexokinase promoter (Fig. 3). These results indicate that the p53 response element region used here is sufficient to specifically bind AS-30D hepatoma nuclear factors in vitro.

The p53 Sequence Motifs Identified on the Distal Tumor Type II Hexokinase Gene Activation by p53
**II Hexokinase Promoter Are Protected from DNase I by the AS-30D Hepatoma Nuclear Extract—**A nuclear extract was prepared from AS-30D hepatoma cells as described under “Methods.” In the presence of this extract both p53 motifs identified on the hexokinase promoter are seen to be protected (Fig. 4), indicating that the tumor Type II hexokinase promoter engages in specific interactions with the mutated p53 nuclear protein. The footprint data provide us the first direct evidence that the p53 motifs are in fact functional within the promoter. In addition, the motif AGGCATGTTC not only retains a perfect consensus sequence for p53 binding (PuPuPuC(A/T)(A/G)PyPyPy), but also contains a Gly at position 2 and an Ala at position 5 which are reported to be critical for high affinity p53 binding activity and transactivation (7, 8).

**Co-transfected Mutant p53 Activates Tumor Type II Hexokinase Gene Expression in Vivo—**To test for the effects of over-expressed mut p53 on Type II hexokinase gene transcription, the mut p53 cDNA isolated from the AS-30D hepatoma cells was placed under control of a cytomegalovirus promoter-driven mammalian expression vector pCI-Neo (see “Methods”). The expression plasmid was co-transfected with the Type II hexokinase promoter-luciferase construct (22) into AS-30D hepatoma cells (Fig. 5A). Control co-transfection experiments contained the pCI-Neo vector that lacked the Type II hexokinase cDNA insert. Eight independent experiments were performed to test for regulation of the tumor Type II hexokinase gene promoter by the overexpressed mutant p53. Fold activations as high as 1.91 (calculated over that observed for the pCI-Neo control and normalized against a β-galactosidase expression vector) were obtained. The average fold activation was 1.51 in the eight experiments with a S.D. of ±0.277 (Fig. 5B). The results obtained clearly show the transactivating effect of the Type II hexokinase promoter by overexpressed mut p53. It should be noted that the above activation was observed over the basal mutant p53 levels already present in the AS-30D hepatoma cells. Therefore, it is quite likely that in the absence of the endogenous mutant p53, that a much higher fold activation would be obtained upon co-expression of the mutant p53.

**Conclusions and Implications—**The above experiments clearly demonstrate for the first time the ability of a mutant p53 protein to transactivate a gene that is critical for maintaining the high glycolytic capacity, and therefore the survival, of a rapidly growing tumor. This novel finding is consistent with recent reports describing transactivating effects of mutant p53 on various genes (9–14). This is in contrast to previous reports that mutant p53 only functions in tumor cells to debilitate the ability of wt p53 to transactivate genes involved in cell cycle regulation (4, 5). The findings that the mut p53, abundantly present in most tumor cells, can in fact function or induce a gain-of-function in activating certain genes is physiologically reasonable, as the mut p53 protein is present in quantities that are significantly higher than that necessary for its dominant-negative effect on wt p53. This is the first report of a possible link between loss of cell cycle control and one of the most common biochemical signatures of cancer cells, i.e. their propensity to catabolize glucose at high rates.

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