Amplification of the Transketolase Gene in Desensitization-resistant Mutant Y1 Mouse Adrenocortical Tumor Cells*

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As shown previously, mutants of the Y1 mouse adrenocortical tumor cell line that resist agonist-induced desensitization of adenyl cyclase have elevated levels of a 68-kDa protein (designated p68), suggesting a possible relationship between p68 and the regulation of adenyl cyclase activity. In the present study, cDNA cloning and sequencing were used to identify p68 as mouse transketolase. Cells overexpressing p68 exhibited a 17.4-fold increase in transketolase enzymatic activity relative to parental Y1 cells and a 28-fold amplification of the transketolase gene as determined by Southern blot hybridization analysis. Using fluorescent in situ hybridization analysis, the transketolase gene was mapped to mouse chromosome 16B1 and to human chromosome 3p21.2. Transketolase gene amplification was associated with telomeric fusion of the chromosome 16 pair together with the appearance of multiple copies of the transketolase gene throughout a different chromosome. The relationship between overexpression of transketolase and desensitization resistance was evaluated in somatic cell hybrids formed between a desensitization-resistant adrenal cell line and a desensitization-sensitive rat glial cell line. In these hybrids, transketolase overexpression behaved dominantly, whereas desensitization resistance behaved recessively. These results dissociate the desensitization resistance phenotype from overexpression of transketolase and suggest that desensitization resistance may have resulted from disruption of an essential regulatory gene in conjunction with the amplification event. In a variety of cell types, the chronic stimulation of adenyl cyclase by hormones and neurotransmitters often desensitizes the enzyme, rendering it refractory to further stimulation. In our laboratory, this phenomenon has been investigated extensively using Y1 mouse adrenocortical tumor cells and in a family of desensitization-resistant (DR) Y1 mutants (1-5). We have shown that the DR mutation in Y1 cells not only affects desensitization from the endogenous ACTH receptor but also affects desensitization from wild-type mouse $\mu_{2}$-adrenergic and human dopamine D-1 receptors when genes encoding these receptors are transfected into the mutant cell line (2-4). Using ligand binding analyses, we demonstrated that the DR mutation did not affect receptor internalization, a late step in the desensitization pathway, but prevented receptor uncoupling from its guanyl nucleotide-binding regulatory protein (2, 3, 5).

On the basis of these findings, we have suggested that the DR mutation does not reside within the ACTH receptor; rather, it affects an early component of the desensitization pathway that is shared among different receptor signaling systems. A potential insight into regulation of the desensitization pathway came from our observations that the DR phenotype is associated with the overexpression of a 68-kDa protein designated p68 (1-6-8). Among 18 independent subclones of the Y1 adrenal cell line, the level of p68 correlated with the level of ACTH-responsive adenyl cyclase activity and those with high levels of p68 desensitized more slowly and recovered from the desensitized state more quickly than clones with low levels of p68 (1, 6). Inasmuch as p68 has not been identified, we have undertaken the cloning and sequencing of the cDNA encoding this protein. We report that p68 is the mouse transketolase (EC 2.2.1.1; TKT). We show that TKT activity in the DR mutant is 20-fold higher than in parental Y1 cells and that the overexpression of TKT results from amplification of a chromosome segment derived from mouse chromosome 16. Using somatic cell hybridization analyses, we are able to dissociate TKT overexpression from the DR phenotype, suggesting that the DR phenotype likely resulted from a reciprocal gene deletion that accompanied amplification of the TKT gene.

MATERIALS AND METHODS

cDNA Library Screening—Custom cDNA libraries in the bacteriophages $\lambda$gt11 and $\lambda$gt10, respectively (Clontech Laboratories, Inc., Palo Alto, CA) were prepared from poly(A)$^+$ RNA (9) isolated from Y1 mouse adrenocortical tumor cells (10) and from the Y1 derivative, Kin-8 (11). The $\lambda$gt11 library was probed for expression of p68 using a rabbit polyclonal p68 antiserum (8) and $^{125}$I-labeled protein A as described (12). Subsequent screenings of the $\lambda$gt11 and $\lambda$gt10 libraries were performed by DNA hybridization as described by Maniatis et al. (13) using the 600-bp EcoRI fragment from $\lambda$gt11 clone 16 (Fig. 1). The probe was labeled by nick translation in the presence of $[^{32}]$PdCTP using a kit from Life Technologies, Inc. (Canadian Life Technologies, Inc., Burlington, Ontario, Canada).

Rapid Amplification of 5′ cDNA ends (5′-RACE)—The 5′-RACE procedure (14) was used to clone the 5′ end of the p68 transcript. Total RNA was prepared from DR cells overexpressing p68 (1) using guanidine thiocyanate for extraction, followed by centrifugation through CsCl (15). cDNA was synthesized with SuperScript™ reverse transcriptase and C-tailed with terminal deoxynucleotidyltransferase and dcTP using a 5′-RACE System from Life Technologies, Inc. The oligodeoxynucleotide primer used for first strand cDNA synthesis (5′-GGTATGGAAAAAAGCAGCAGCAC-3′) was complementary to the mouse p68 (TKT) sequence from positions 233-256 (Fig. 3). The cDNA was amplified by polymerase chain reaction (PCR) using a kit containing AmpliTaq® DNA polymerase (Perkin Elmer (Canada) Ltd., Rexdale, Ontario, Canada) together with the Anchor Primer provided with the 5′-RACE System and an internal primer (5′-CATGATCTCGGACGGCTGCAGCAGCATGT-3′; Kronem Systems Inc., Mississauga, Ontario, Canada) complementary to the p68 sequence at positions 206-235. PCR was carried out for 36 cycles with a hot start (16); the timing for each cycle consisted of 1-min incubations at 94, 54, and 72°C. At the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U05809.

1 The abbreviations used are: DR, desensitization-resistant; DS, desensitization-sensitive; ACTH, adrenocorticotropic hormone; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; 5′-RACE, rapid amplification of 5′ cDNA ends; TKT, transketolase; DAPI, 4′-6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.
end of the reaction, samples were incubated at 72°C for 10 min to ensure that cDNA synthesis went to completion. An aliquot of the reaction was amplified for an additional 35 cycles under similar conditions, except that the reaction contained the universal amplification primers (17) using Sequenase 2.0 (U. S. Biochemical Corp.) with T7 primers (U. S. Biochemical) or T3 primers (Pharmacia). Reactions carried out in the presence of dGTP or dC dTTP were compared to resolve sequence ambiguities. Data base searches were carried out using the FASTA algorithm (18), and alignments were performed using Geneworks® version 2.21 (IntelliGenetics, Irvine, California). For Northern analysis, total RNA from adrenal cell cultures was prepared as described above, fractionated on a 1.2% agarose gel, blotted onto nylon membranes, and hybridized with a 32P-labeled mouse TKT cDNA from nucleotide 153 to nucleotide 2062 (Fig. 2) or with genomic probes for the mouse immunoglobulin heavy chain gene and the mouse immunoglobulin λ 5 gene.

**Transketolase Activity**—Transketolase activity was assayed in a coupled enzymatic assay as described previously (21). Briefly, cell monolayers were rinsed with phosphate-buffered saline, scraped in buffer containing 50 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1% (w/v) Triton X-100. Cells were homogenized using a Dounce homogenizer with a tight-fitting pestle, and homogenates were centrifuged 100,000 ×g for 90 min at 4°C to obtain cell supernatant fractions. Assays were carried out in quartz cuvettes in a 1-ml reaction volume containing 1 mM MgCl₂, 0.1 mM NADH (Boehringer Mannheim Canada, Laval, Quebec), 2.5 mM d-xylose 5-phosphate (Sigma), glycerol-phosphate dehydrogenase (1 unit) and triosephosphate isomerase (11 units) (Sigma), 100 mM Tris-HCl pH 7.6, and 5-30 μg of cell supernatant. The assay mixture was equilibrated at room temperature for 5 min, and the reaction was initiated by adding ribose 5-phosphate (Sigma) to a final concentration of 2.5 mM to the sample cuvette. Reactions were monitored by measuring the change in absorbance at 340 nm over a period of 20 min; results were converted to units of activity, where 1 unit of activity represents 1 μmol of NADH oxidized × min⁻¹ × mg protein⁻¹. Activity was linear with respect to both enzyme protein and incubation times.

**Gene Mapping**—Gene mapping was performed by SeeDNA Biotech Inc. (North York, Ontario) as described previously (22, 23). Normal mouse chromosomes were prepared from synchronized cultures of spleen lymphocytes (24). Human chromosomes were prepared from syn-
chronized cultures of lymphocytes isolated from cord blood (22). Chromosome spreads from parent and mutant mouse adrenocortical tumor cell lines were prepared from cells arrested in metaphase with 2 μg/ml vinblastin for 18 h (25).

Mouse chromosomes were probed with a partially characterized 20-kb TKT genomic clone isolated from an EMBL3 mouse genomic library. Human chromosomes were probed with mouse TKT cDNA from nucleotide 153 to nucleotide 2062. Probes were labeled with biotinylated dATP, hybridized to the chromosome spreads, and detected with FITC-avidin. Signals were amplified by incubation with biotinylated goat anti-avidin followed by a second round of incubation with FITC-avidin. Chromosome banding patterns were obtained with the chromatin-binding fluorescent dye 4′-6-diamidino-2-phenylindole (DAPI). Chromosomal localization of TKT was made by superimposing photographs of chromosome hybridization signals with photographs of the DAPI banding patterns.

Adenylyl Cyclase Activity—Adenylyl cyclase activity was measured in cell homogenates by measuring the conversion of [2,8-3H]ATP to cAMP in a 5-min reaction at 37 °C as described previously (26). The reaction mixture contained 1 mM disodium ATP (approximately 1.6 × 10⁶ cpm; DuPont Canada), 2 mM MgCl₂, 6 mM theophyllin, 50 μg of albumin, 12.5 μM Tris-HCl, pH 7.7, and approximately 140 μg of enzyme protein in a final volume of 85 μl.

RESULTS
Isolation of p68 cDNA—A λgt11 cDNA library prepared from Y1 cells was screened for p68 cDNA by expression using a polyclonal p68 antiserum (8). An immunoreactive isolate, λgt11-16, was plaque-purified, and found to contain a 900-bp isolate with the TKT probe as chromosome 16.

Identification of p68 as Mouse TKT—The p68 cDNA fragments were sequenced completely from both DNA strands and shown to encode a large open reading frame corresponding to 623 amino acids with a calculated molecular mass of 67,553 Da (Fig. 1). A search of the GenBank™ (27) data bank showed that the p68 sequence was 83% identical with human TKT at the DNA level, 94% identical at the protein level and included conserved amino acids implicated in binding thiamine pyrophosphate (28). The sequencing data thus indicate that p68 is the mouse TKT. Of the 38 amino acids that differed between the mouse and human TKT, the substitutions S30T, S31T, V46E, and A426P in the mouse protein also are seen as polymorphisms in the human gene (28).

The identity of p68 as TKT was further confirmed by demonstrating that extracts from the DR mutant exhibited a 1.74-fold higher level of TKT activity (0.40 ± 0.04 units) compared to extracts from parental Y1 cells (0.023 ± 0.002 units), consistent with the observed amplification of p68 in DR clones.

Chromosomal Localization of TKT—Strong TKT signals were detected on more than 80% of mitotic figures examined and were localized to mouse chromosome 16 and human chromosome 3; background signals were minimal, and positive signals were not detectable on any other chromosomes. A representative example for mouse TKT is shown in Fig. 3. Detailed characterization of 10 mitotic figures further localized the TKT gene to the B1 region of mouse chromosome 16 and to the p21.2 region of human chromosome 3 (Fig. 4).

Basis for Overexpression of TKT in DR Mutant Clones—In order to gain further insight into the relationship between the DR phenotype and overexpression of TKT, we examined TKT gene copy number by Southern blot hybridization using mouse TKT cDNA as a probe. As shown in Fig. 5, Southern blotting genomic DNA from DS and DR cells using TKT cDNA gave similar bands of hybridization that ranged in size from 1 to 12 kb, depending on the restriction endonuclease used for digestion. In each case, hybridization signals for the TKT gene were, on average, 28-fold more intense in the DR mutant than in parental Y1 cells, suggesting that overexpression of TKT resulted from gene amplification. In control experiments, hybridization of enzyme-digested genomic DNA from DS and DR cells with cDNA probes for the ACTH receptor (26) or the regulatory subunit of the type 1 cAMP-dependent protein kinase (29) gave signals of approximately equal intensity (data not shown).
In chromosome spreads prepared from parental Y1 mouse adrenocortical tumor cells, TKT signals also were observed on chromosome 16, and there was no evidence of TKT gene amplification (data not shown). In the DR mutant, however, the TKT gene seemed to be amplified over a large region on a single chromosome (Fig. 6). Since the level of amplification was very high, the morphology of the affected chromosome was completely changed and identification of the affected chromosome was not possible. TKT signals also were evident on the chromosome 16 pair, which showed an abnormal telomeric fusion in the DR mutant (Fig. 6). Additional faint signals seen scattered throughout the chromosome spread are not reproducible and represent background.

As determined by Southern blot hybridization analysis (Fig. 5), other genes associated with the proximal region of mouse chromosome 16, i.e. immunoglobulin λ 1 and mouse immunoglobulin λ 5 (30), were not amplified in the DR mutant clone. Effects on Adenylyl Cyclase Activity—The identification of p68 as TKT raises interesting questions about its contribution to the DR phenotype and the mechanisms responsible for its overexpression in DR cells (6). We considered the possibility that this enzyme, when overexpressed, protects adenylyl cyclase from agonist-induced desensitization. TKT has not been implicated in the regulation of the adenylyl cyclase system previously, and its function in this regard was not readily apparent. We were unable to modify adenylyl cyclase activity in broken DR cells by treatment with TKT antiserum or by adding back purified enzyme to homogenates of parental Y1 cells (data not shown), suggesting that increased expression of TKT per se is not responsible for the DR phenotype.

To further address the relationship between TKT overexpression and desensitization resistance, we evaluated the linkage of these two phenotypes in somatic cell hybrids formed between a DR derivative, Kin-8HGPRT<sup>2</sup>, and the rat glioma cell line, C6TK<sup>2</sup> (25). As we reported previously, Kin-8 cells, like the DR parent, resist ACTH-induced desensitization and produce elevated levels of p68 (approximately 10% of total protein; Ref. 1), whereas in C6 cells, the adenylyl cyclase system is readily desensitized upon continuous exposure to β-adrenergic agonists such as isoproterenol (31) and the levels of p68 are low (approximately 0.1% of total protein; Ref. 8). As determined from Southern blot hybridization analysis using the mouse TKT probe, the TKT genes in two independently isolated Kin-8HGPRT<sup>2</sup> × C6TK<sup>2</sup> hybrid clones, H7 and H8, were amplified to the same extent and gave the same restriction patterns as the Kin-8HGPRT<sup>2</sup> fusion partner (Fig. 7A) and parental DR cells (Fig. 5). Under these same conditions of hybridization stringency, the mouse TKT probe did not give a detectable signal for the TKT gene from the rat glial cell line. As determined by Northern blot hybridization (Fig. 7B), TKT transcripts were markedly abundant in the H7 and H8 hybrids, reaching levels comparable to those seen in Kin-8HGPRT<sup>2</sup> and parental DR cells; these levels of TKT transcript were much higher than those seen in the C6TK<sup>2</sup> fusion partner or in DS cells. These results indicate that the hybrid clones acquired and expressed the amplified TKT gene from the DR parent and that TKT overexpression behaves dominantly in the hybrids.
As shown in Table I, the hybrid clones responded to ACTH, isoproterenol, and NaF with increases in adenylyl cyclase activity. The response to ACTH reflected the contribution of Kin-8HGPRT<sup>2</sup> cells, whereas the response to isoproterenol reflected the contribution of C6TK<sup>2</sup> (Table I). Despite the presence of the amplified TKT gene and overexpression of TKT transcripts, adenylyl cyclase in the hybrid clones was rapidly desensitized upon exposure to ACTH (Fig. 8). Within 1 h of exposure to ACTH, the hybrid clones lost 85% of their hormone-responsive adenylyl cyclase activity. In contrast, the Kin-8HGPRT<sup>2</sup> parent resisted ACTH-induced desensitization and retained 70% of its ACTH-responsive activity after 6 h of continuous exposure to the hormone (Fig. 8). The desensitization induced by ACTH in the hybrid clones was homologous, since the hybrids retained 85–90% of their isoproterenol-stimulated adenylyl cyclase activity after treatment with ACTH (not shown).

**DISCUSSION**

In order to further understand the biochemical and molecular causes of the DR phenotype in mutant Y1 adrenocortical tumor cells, we sought to identify p68 and determine the basis for its overexpression in DR cells. Based on cDNA sequencing results and direct assays of enzymatic activity, we have established that p68 is the mouse TKT. TKT is a thiamine-requiring enzyme that is part of the pentose phosphate metabolic pathway responsible for the synthesis of pentoses and for the generation of NADPH (32). Defects in TKT have been described in a population of alcoholic patients and may contribute to the neuropathological disturbances associated with Wernicke-Korsakoff syndrome (33, 34).

Chromosomal mapping experiments localized human TKT to chromosome 3p21.2 (Fig. 4) and mouse TKT to the B1 region of chromosome 16 (Figs. 3 and 4), a region that appears to be
poorly defined and not yet established as syntenic with human chromosome 3p21 (35). Previous gene mapping studies also had localized the human TKT to chromosome 3p (34, 36); however, the earlier results had placed the TKT gene at 3p14 (36) rather than in the adjacent 3p21.2 region as reported here.

As evidenced from Southern blot and fluorochrome in situ hybridization analyses (Figs. 5 and 6), the overexpression of TKT in DR mutant clones resulted from an approximate 28-fold amplification of the TKT gene. In most examples of gene amplification, the regions involved (referred to as amplicons) are very large and can involve as much as 10,000 kb of DNA (37). Other markers of chromosome 16 proximal to the centromere, such as the immunoglobulin λ genes, are not amplified in the DR mutant (Fig. 5) and thus must be too far away from the TKT gene to have been included in the amplification. Although the basis for amplification of the TKT gene is unknown, it is interesting that TKT shares structural and functional homology with the Rep protein of Streptococcus pneumoniae, a protein required for genetic transformation that functions to promote insertion-duplication mutations in the prokaryotic chromosome (38, 39). As reviewed elsewhere (40, 41), gene amplification may occur through a number of different mechanisms, may involve recombination events (including gene insertions, deletions, and inversions), and is sometimes associated with telomeric fusions (eg. Fig. 6). Amplified genes can exist as self-replicating minichromosomes or as arrays of amplified segments on one or more chromosomes (40), as seen in the case of TKT amplification in the DR mutants (Fig. 6).

To further explore the relationship of TKT gene amplification to desensitization resistance, we examined the linkage of these two phenotypes in somatic cell hybrids between a DR isolate and desensitization-sensitive C6 glioma cells. The hybrids acquired the TKT amplification and overexpressed TKT (Fig. 7) but failed to resist ACTH-induced desensitization of adenylyl cyclase (Fig. 8). These results clearly dissociate TKT gene amplification from the DR phenotype and indicate that desensitization-resistance behaviors repressively in the hybrid. On the basis of these results, we suggest that desensitization resistance may have resulted from a recombination event that disrupted or mutated a gene required for the desensitization process rather than from amplification of TKT itself or from coamplification of a closely linked gene.

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