Bromocriptine Transcriptionally Activates the Multidrug Resistance Gene (pgp2/mdr1b) by a Novel Pathway*

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The P-glycoprotein (Pgp) reversing agent, reserpine, induces MDR1 mRNA and PGP protein in human colon carcinoma cells (Schuetz, E. G., Beck, W. T., and Schuetz, J. D. (1996) Mol. Pharmacol. 49, 311–318) and in H35 rat hepatoma cells. Reserpine’s interference with cellular dopamine utilization suggested that dopamine and dopaminergic pathways might be important physiological regulators of PGP expression. Initial studies demonstrated that the H35 cells express the D2 dopamine receptor. Pgp protein and pgp2/mdr1b mRNA was increased (maximum of 10- and 8-fold, respectively) by the potent D2 dopamine receptor agonists bromocriptine, R-(–)-propylnorapomorphine hydrochloride, and quinpirole, and Pgp protein induction was blocked by D2 receptor antagonists spiperone and clozapine. D2 receptor agonist induction of pgp2/mdr1b mRNA was paralleled by transcriptional activation of the pgp2/mdr1b promoter but blocked by pretreatment with the D2 dopamine receptor antagonists, spiperone, eticlopride, and clozapine. Cotransfection of a D2 dopamine receptor expression vector enhanced bromocriptine’s transcriptional activation of the pgp2/mdr1b promoter. The G-protein, Gαq, is required for bromocriptine transcriptional activation because the G-protein inhibitor, pertussis toxin, suppressed bromocriptine’s activation of pgp2/mdr1b transcription and co-transfection of a dominant negative Gαq abrogated bromocriptine activation of pgp2/mdr1b. Gα proteins can transduce signals by activation of mitogen-activated protein kinases (MAPKs), and because Raf-1 is a known activator of MDR1, we tested for Raf-1 involvement. Co-transfection of a dominant negative Raf-1 failed to block bromocriptine induction of pgp2/mdr1b, and bromocriptine treatment caused no phosphorylation of the MAP kinase kinase substrates p42 and p44, demonstrating that the MAP kinase pathway was not involved. These are the first studies demonstrating transcriptional activation of an MDR gene by dopamine receptor agonists and that this activation occurs by a signal transduction pathway requiring the D2 dopamine receptor coupled to a functional G-protein.

The multidrug resistance (MDR) gene family encodes a small family of plasma membrane ATP-dependent efflux transporters, referred to as P-glycoproteins (PGPs) (1). The MDR genes are part of a small gene family that is composed of three members in rodents and two in humans (2–7) for which cDNAs have been isolated and characterized. Full-length cDNAs for mouse mdr1 (3, 8), mouse mdr3 (3), and human MDR1 (4) but not mouse mdr2 (6) or human MDR2 (MDR3 (9)) can confer the multidrug-resistant phenotype when transfected and overexpressed in drug-sensitive cells. High levels of expression of the multidrug resistance gene (MDR1) commonly occur in human cancers derived from normal tissues that express PGP, such as carcinomas of the liver, colon, kidney, and pancreas and may contribute to the drug resistance of these cancers. The PGPs are involved in the transport of a variety of substances such as peptides (10), endogenous steroids (11), and xenobiotics (12) and may, under certain physiological conditions, function as a chloride ion channel (13). Since we and others (14–16) have shown that PGP expression and transcription can be regulated by substances it transports (e.g., steroids), it seemed possible that agents that interfered with the pump, but had no known cytotoxic effect (e.g., reversing agents), might provide insight into endogenous physiological pathways regulating PGP gene expression.

Although a number of the transcription factors that regulate the multidrug resistance genes have been identified (17, 18) very little is known about the molecular signals activating PGP expression in response to putative substrates, ligands, or modulators. In one example, Fojo et al. (19) demonstrated that PGP reversing agents, such as verapamil and cyclosporin A, increase MDR1 mRNA expression in a human colon carcinoma cell line. We and others (20) have found that a variety of agents, including the MDR1 reversing agent reserpine, increase MDR1 gene expression in these same cells. In similar studies we have also found that reserpine induces the amount of pgp2/mdr1b mRNA in the H35 rat hepatoma cell line by transcriptional activation of the pgp2/mdr1b gene; however, the mechanism by which the pgp2/mdr1b gene is activated by reserpine is unknown.

Because reserpine up-regulates the synthesis of dopamine

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The abbreviations used are: MDR, multidrug resistance; MDR1, human multidrug resistance gene or mRNA encoding the drug transporting PGP protein [P-glycoprotein]; pgp1/mdr1a and pgp2/mdr1b refer to the two drug transporting rat MDR genes or mRNA encoding Pgp; NPA, R-(–)-propylnorapomorphine; RT-PCR, reverse transcriptase-polymerase chain reaction; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; BSV, Rous sarcoma virus; bp, base pair(s); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

2 K. Furuya, J. Thottassery, and J. Schuetz, unpublished observations.
P-glycoprotein Regulation in H35 Cells by Bromocriptine

(21–23), inhibits the dopamine transporter (24, 25), and increases dopamine receptor RNA (18, 26), we hypothesized that dopamine or dopaminergics might serve as endogenous physiological regulators of MDR gene expression. Using H35 hepatoma (27) we have defined the initial components of the D2 dopamine receptor signal transduction cascade leading to transcriptional activation of pgp2/mdr1b. We used specific D2 and D1 dopamine receptor agonists and antagonists, as well as D1 and D2 receptor expression vectors, to define the role of the classical D2 dopamine receptor in pgp2/mdr1b gene activation. In total, these studies reveal a novel D2 dopamine receptor-mediated transcriptional activation pathway for the pgp2/mdr1b gene in the H35 rat hepatoma cells that is coupled to G-proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—Reuber H35 rat hepatoma cells (American Type Culture Collection, Rockville, MD) were maintained in a minimal essential medium containing 10% fetal calf serum supplemented with penicillin, streptomycin, and glutamine at 37 °C in 5% CO2. All drugs used at a final concentration of 10 μM, except where stated otherwise. Pertussis toxin was used at a final concentration of 100 ng/ml of media. Drug-containing medium was changed every 24 h with freshly supplemented medium. (R)-(-)-Propylparomorphine hydrochloride (NPA), quinpirole, SCH23390, spiperone, SKF38393, clozapine, bromocriptine, eticlopride, and pertussis toxin were obtained from Research Biochemicals (Natick, MA). All other drugs and chemicals were obtained from Sigma.

Northern Blot Analysis—Total RNA was isolated from cells pooled from one 100-mm tissue culture dish using the phenol-chloroform method (28). Northern blot analysis was performed as described previously (29) on 20 μg of total RNA. The integrity of the RNA and evenness of loading after transfer to a positively charged membrane (Magna NT, MSI Separations, Westminster, MA) was confirmed by comparison of the 28S and 18S ribosomal bands which were apparent with ethidium bromide staining. Blots were probed with a specific pgp2/mdr1b oligonucleotide (14) labeled with [γ-32P]ATP using the 5'-DNA terminus labeling kit (Life Technologies, Inc.). These same blots were re-probed with a cDNA probe for cyclophilin (kindly provided by Dr. J. Sutcliffe (30)).

RT-PCR—pgp2/mdr1b—First strand cDNA was prepared by reverse transcription of 8 μg of total RNA using 200 ng of random primers (Pharmacia Biotech Inc.) and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The amount of first strand cDNA used in polymerase chain reaction (PCR) amplification was increased stepwise from 12.5 to 200.0 ng. PCR reactions were performed in 100-μl final volumes using rat pgp2/mdr1b gene-specific primers (Center for Biotechnology, St. Jude Children’s Research Hospital, University of California, San Francisco, CA). The pgp2/mdr1b sensor primer corresponded to bp 3533–3562, pgp2/mdr1b anti-sense primer corresponded to bp 3835–3864 of the cDNA sequence (31, 32). Aliquots of the PCR reaction were then separated on a 1.0% NuSieve, 0.5% agarose gel, demonstrating a 332-bp amplification product. The gel was transferred to nylon membrane and probed with an internal pgp2/mdr1b oligonucleotide. Amplification of a 202-bp fragment of the glyceraldehyde-3-phosphate (GAPDH) cDNA (using published oligonucleotide sequences (33)) was chosen as an internal control for normalization because its level in cells in tissue culture has been shown to be independent of culture confluence and xenobiotic treatment (33, 34). Quantitative comparisons were made over the linear range of amplification for each treatment group after each blot was probed with a GAPDH or pgp2/mdr1b oligonucleotide specific to internal sequences of the amplimer and densitometric measurement of band intensity.

RT-PCR: Dopamine Receptors—To demonstrate the presence of dopamine receptor mRNA in H35 cells, first strand cDNA reverse-transcribed from H35 rat hepatoma cell total RNA was used in the PCR assay (35). Oligonucleotide primer pairs used to amplify the D1 dopamine receptor and D2 dopamine receptor short form (D2S) and long form (D2L) were synthesized (Center for Biotechnology, St. Jude Children’s Research Hospital) using the sequences published by Rao et al. (35). D1, D2S, and D2L dopamine receptor expression vectors (36) were used as specific, positive controls (kindly provided by Dr. S. Senogles, University of Tennessee, Memphis).

Immunoblot Analysis—Crude membranes were extracted from H35 rat hepatoma cells using a modified method of Lee et al. (37). Cells were scraped from the dishes in phosphate-buffered saline and were pelleted at 10,000 × g for 4 °C. The pellet was resuspended in membrane storage buffer (MSB; 100 mM potassium phosphate (pH 7.4), 1.0 mM EDTA, 20% glycerol, 1 mM diethiothreitol, 20 μM butyrylhydroxotoluene, and 2 mM phenylmethylsulfonyl fluoride) and lysed for 30 s at 30% power with an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany). The crude membranes were isolated by centrifugation at 10,000 × g for 5 min at 4 °C. This crude membrane pellet was resuspended in a small volume of MSB. Protein determinations were done using the method of Lowry et al. (38). Thirty-five μg of crude membrane proteins were resuspended in standard Laemmli sample preparation buffer (39) and were immediately subjected to polyacrylamide gel and resolved overnight. Proteins were transferred to Protran® nitrocellulose filters (Schleicher & Schuell) as described previously (37, 40). Filters were incubated sequentially with primary polyclonal rabbit anti-mdr(1-3) IgG (Onco-gene Science, Uniondale, NY) and peroxidase-conjugated anti-rabbit IgG and developed using the Amer sham enhanced chemiluminescence detection system. The relative amount of Pgp was determined by densitometric analysis.

Vectors—A 519-bp fragment containing the promoter of the pgp2/mdr1b (~369 to +150 bp) gene was amplified by PCR and fused to either a luciferase or chloramphenicol reporter as described previously (14). Activatedraf-1 and dominant negativeraf expression vectors were provided by Dr. John Cleveland (St. Jude Children’s Research Hospital, Memphis, TN). Gα16 and Gαi2 were provided by Dr. Brian Kobilka (University of California, San Francisco, CA). Wild-typeGαq and dominant negative GαqS48C (41) were from Dr. Melvin Simon (California Institute of Technology, Pasadena, CA).

Transient Transfection—H35 rat hepatoma cells were subcultured by trypsinization and plated at 3–4 × 105 cells per 60-mm tissue culture dishes. When the cells had reached approximately 25–35% confluence, they were transfected for 18 h with 10 μg of plasmid DNA by the calcium-phosphate co-precipitation method (42). The H35 cells were then washed once with medium, and fresh medium with drug was added. After a 24-h treatment cells were harvested for either chloramphenicol acetyltransferase or luciferase assays.

β-Galactosidase Assay—H35 cells were co-transfected with 10 μg of a RSV promoter-driven β-galactosidase expression plasmid to normalize for transfection efficiency (43). The β-galactosidase assay was performed using standard methods (43).

Chloramphenicol Acetyltransferase (CAT) Assay—After washing once with phosphate-buffered saline, the cells were briefly incubated in a CAT harvest buffer (150 mM NaCl, 40 mM Tris (pH 7.4), 5 mM EDTA). The cells were scraped from the 60-mm tissue culture dishes, and cell-associated activity was assayed as described previously (14, 44) with the exception that the H35 cell protein extracts (60 μg) were heat-inactivated for 15 min at 65 °C to destroy endogenous acetylase activity. CAT activity relative to the untreated control dishes was determined after subtraction of background activity obtained from mock transfected control dishes.

Luciferase Assay—H35 cells were washed twice in phosphate-buffered saline, incubated for 15 min in Reporter Lysis buffer (Promega, Madison, WI), and scraped from the culture dishes. Lyase protein concentrations were determined using the method of Lowry et al. (38). Luciferase activity in 20 μg of cell protein extract was measured according to the manufacturer’s instructions (Luciferase Assay Kit, Promega, Madison, WI) using an Optocomp I Luminometer (MGM Instruments, Hamden, CT) with a counting window of 10 s.

MTT Assay—The assay was performed essentially as described (46). H35 cells were subcultured by trypsinization and plated at various densities in 96-well microtiter plates. Fresh medium was added before drug treatment. After a 24-h treatment medium was aspirated, and cells were washed with phosphate-buffered saline, and the MTT reagent was added to a final concentration of 2 mg/ml. Following a 3-h incubation period at 37 °C the plates were spun at 500 × g for 5 min, the MTT reagent aspirated, dimethyl sulfoxide was added, and the plates were read using a Thermomax microplate reader at the test wavelength of 590 nm and the reference 650 nm. The assay was read within the linear range with an r2 = 0.94 when comparing cell number versus the absorbance ratio.

Phosphorylation—H35 rat hepatoma cells were plated into 2 ml of complete medium at a density of 2.5 × 105 cells/well in six-well plates (Corning-Costar, Cambridge, MA). After 2 days of incubation at 37 °C in a humidified atmosphere, the medium was removed from the adhered cells, and 2 ml of serum-free medium was added to each well. After 2 days of starvation, the quiescent cells were stimulated by direct addition of bromocriptine (10 μM) or fetal bovine serum (10%). Cells were incubated at 37 °C in 5% CO2 for the duration outlined by the time
course assay; stimulation was terminated by removal of the medium. The cells were then washed with 2 ml of ice-cold phosphate-buffered saline prior to lysis for Western blot analysis.

Cells were lysed with Laemmli sample preparation buffer and were briefly sonicated. The cell lysates were heated at 95 °C for 5 min, cooled on ice, and then centrifuged at 14,000 × g for 5 min prior to gel electrophoresis. Cell lysate proteins were loaded onto a 10% SDS-polyacrylamide minigel with resolution at 200 V for approximately 45 min followed by electrotransblotting onto polyvinylidene difluoride membrane (0.2-micron pore size, Bio-Rad) at 100 V for 1.5 h at 4 °C. Immunohostaining was performed at room temperature. The membrane was blocked for 1 h in 5% non-fat dry milk (Bio-Rad) and incubated overnight with the phospho-specific MAPK antibody. Rabbit polyclonal phospho-specific MAPK antibody (New England Biolabs, Beverly, MA) was raised against a synthetic phosphotyrosine peptide (3rd to 5th lanes) that spanned the region where alternate splicing creates either the D2L (long) or the D2S (short) isoforms of the D2 dopamine receptor expressed in the liver. We used PCR primers (35) that spanned the region where alternate splicing creates either a long (D2L) or short form (D2S) of the D2 dopamine receptor to generate a cDNA from H35 cells. The D2L and D2S (28 amino acids shorter than D2L) dopamine receptor isoforms can readily be distinguished on agarose gels (Fig. 1). The specificity of the D2 dopamine receptor oligonucleotides for the D2 receptor was demonstrated by testing them against templates of cloned authentic D2L, D2S, or D1 dopamine receptors (Fig. 1). Since the D2L and D2S share common sequences we found that amplification readily occurred using the D2L and D2S dopamine receptor templates as anticipated, whereas no amplification was observed using the unrelated D1 dopamine receptor template. When these same primers were incubated with the H35-derived cDNA, we found amplification of both D2S and D2L dopamine receptor isoforms, with the D2L isoform mRNA amplified to a greater extent. We cannot with certainty state how much of the corresponding proteins are made because of the lack of suitable reagents to detect the D2L and D2S isoforms in these cells.

Next, we treated H35 cells for 24 h with the potent D2 receptor agonist bromocriptine. Bromocriptine treatment resulted in a dose-dependent increase in Pgp expression (2-fold by 0.1 μM drug and up to 10-fold by 100 μM drug) (Fig. 2). Bromocriptine also up-regulated pgp2/mdr1b mRNA (up to 10-fold at 10 μM drug) (Fig. 3A), whereas dopamine was less effective than bromocriptine as an inducer of pgp2/mdr1b mRNA (Fig. 3A). The latter finding can in all likelihood be attributed to the rapid oxidation and cellular metabolism of dopamine in culture (48, 49). We confirmed and extended the Northern blot result by performing RT-PCR with pgp2/mdr1b-specific primers on first strand cDNA prepared from RNA isolated from H35 cells exposed to varying concentrations of bromocriptine (Fig. 3B). pgp2/mdr1b mRNA was dose-dependently increased, to a maximum of 15- and 50-fold above control at 10 and 50 μM bromocriptine, respectively. While bromocriptine has been reported to have some effects on cell viability (50, 51), we found that acute bromocriptine exposure had no effect
on either cell cycle pattern or viability as assessed by the MTT assay. A 24-h treatment of H35 cells with 10 μM bromocriptine produced no significant difference in the tetrazolium dye signal compared with the control cells (bromocriptine produced no significant difference in the tetrazolium dye signal). Total RNA (20 μg) was analyzed by Northern blot, and the 18S and 28S ribosomal RNA bands were visualized under ultraviolet light. Membranes were then sequentially hybridized with a 32P-labeled pgp2/mdr1b oligonucleotide (5'-GAA ATA CTT AGC ACC TCA AAT ACT CCC AGC-3') and a cyclophilin cDNA probe. PCR was performed with serial dilutions of first strand cDNAs prepared by reverse transcription of 8 μg of total RNA extracted from H35 cells cultured in the presence or absence of indicated concentrations of bromocriptine. Amplification was done with pgp2/mdr1b-specific and GAPDH-specific primers, and the PCR products were separated on a 2% agarose gel, transferred to a positively charged nylon membrane, and hybridized. pgp2/mdr1b mRNA expression was normalized to that of GAPDH. Values shown are from a representative experiment.

![Image](image.png)

**FIG. 3. Bromocriptine up-regulates pgp2/mdr1b mRNA expression in H35 hepatoma cells.** A, total RNA was isolated from H35 hepatoma cells cultured in the presence or absence of either 10 μM dopamine or bromocriptine. Total RNA (20 μg) was analyzed by Northern blot, and the 18S and 28S ribosomal RNA bands were visualized under ultraviolet light. Membranes were then sequentially hybridized with a 32P-labeled pgp2/mdr1b oligonucleotide (5'-GAA ATA CTT AGC ACC TCA AAT ACT CCC AGC-3') and a cyclophilin cDNA probe. B, PCR was performed with serial dilutions of first strand cDNAs prepared by reverse transcription of 8 μg of total RNA extracted from H35 cells cultured in the presence or absence of indicated concentrations of bromocriptine. Amplification was done with pgp2/mdr1b-specific and GAPDH-specific primers, and the PCR products were separated on a 2% agarose gel, transferred to a positively charged nylon membrane, and hybridized. pgp2/mdr1b mRNA expression was normalized to that of GAPDH. Values shown are from a representative experiment.

![Image](image.png)

**FIG. 4. Effect of D2 dopamine receptor agonists and antagonists on the expression of immunoreactive P-glycoprotein in H35 cells.** H35 cells were cultured for 24 h in the presence of 10 μM of the D2 dopamine receptor agonist, dopamine (D2); the D2 dopamine receptor antagonist, spiperone (Sp); or clozapine (CLZ). Protein from control H35 cells (CT) or cells treated with spiperone or clozapine alone were used as controls.

To further confirm a role for the D2 dopamine receptor in pgp2/mdr1b gene expression, we determined whether endogenous Pgp expression could be altered by a series of known agonists and antagonists specific for the D2 receptor. Treatment of H35 cells with the D2 dopamine receptor agonists, NPA and quinpirole, increased the expression of Pgp (Fig. 4). Agonist induction of Pgp expression was antagonized by pretreatment with the D2 dopamine receptor antagonist spiperone and clozapine, whereas the antagonists themselves had little effect on Pgp expression.

To assess whether bromocriptine transcriptionally activated the pgp2/mdr1b gene, H35 hepatoma cells were transiently transfected with the Pgp2LUC construct containing the pgp2/mdr1b promoter (bp 369 to +150) and treated with bromocriptine (Fig. 5). There was significant induction of Pgp2LUC by bromocriptine (up to 12-fold); maximal transcriptional activation of Pgp2LUC occurred between 10 and 50 μM bromocriptine (Fig. 5A) with an estimated EC50 of approximately 0.5 μM. Moreover, the transcriptional activation of the pgp2/mdr1b promoter was specific because neither the vector control (pGL2-Basic) nor RSV-LUC (Fig. 5A) was transcriptionally activated by bromocriptine. Similar bromocriptine-mediated activation of the identical pgp2/mdr1b promoter when it was fused to a CAT reporter (Pgp2CAT) (14) (Fig. 5B) ruled out the possibility that transcriptional activation of the pgp2/
A 3.5-fold increase in luciferase activity was seen in the expression vectors for the D2 dopamine receptor transcriptional activation of the D2 dopamine receptor. We reasoned that we could enhance bromocriptine activity by adding bromocriptine to the D2 dopamine receptor. Inhibition of the D2 dopamine receptor antagonists of lower affinity (clozapine, eticlopride, and spiperone) for 1 h. 24 h post-treatment, cells were lysed and harvested for luciferase assay. Values shown represent the average of 3 or 4 separate experiments. Vertical bars represent standard deviations. B, transfected cells were treated with 1 μM bromocriptine, 10 μM SCH23390, or bromocriptine and SCH23390 with SCH23390 added 1 h before bromocriptine for 24 h, and luciferase activities were determined.

To determine the ligand specificity of the transcriptional activation of the pgp2/mdr1b gene, we transiently transfected H35 cells with only Pgp2LUC and treated the transfectants with ligands for the following receptors: dopamine, adrenergic, serotonin, and Sigma receptor agonists (Table I). Addition of the D1 receptor agonist, SKF38393 at doses from 0.1 to 50 μM, or addition of agonists for other receptors (adrenergic, serotonin, and Sigma) did not transcriptionally activate the pgp2/mdr1b promoter thus demonstrating that only D2 dopamine receptor ligands transcriptionally activate the pgp2/mdr1b promoter.

We next evaluated whether pharmacological antagonists of the D2 dopamine receptor could block the transcriptional activation of the pgp2/mdr1b promoter. H35 cells were transiently transfected with Pgp2LUC and treated with the transfectants with ligands for the following receptors: dopamine, adrenergic, serotonin, and Sigma receptor agonists (Table I). Addition of the D1 receptor agonist, SKF38393 at doses from 0.1 to 50 μM, or addition of agonists for other receptors (adrenergic, serotonin, and Sigma) did not transcriptionally activate the pgp2/mdr1b promoter thus demonstrating that only D2 dopamine receptor ligands transcriptionally activate the pgp2/mdr1b promoter.

The D2 dopamine receptor upon binding its ligand activates a transmembrane signaling pathway coupled to G protein before converging on other cellular effector molecules (36, 54). To examine the coupling of D2 dopamine receptor activation to a G protein and its role in bromocriptine activation of the pgp2/mdr1b promoter, we transiently transfected H35 cells with Pgp2LUC. Prior to bromocriptine treatment, we applied pertussis toxin to interfere with the coupling between the endogenous D2 dopamine receptor and the heteromeric G-pro-
teins (36). Cells were then treated with varying concentrations of bromocriptine (Fig. 8A). Pertussis toxin treatment did not alter basal pgp2/mdr1b or thymidine kinase promoter activity.2 In contrast, pertussis toxin suppressed bromocriptine induction of the pgp2/mdr1b promoter at all doses of bromocriptine. These studies indicate that a majority of the bromocriptine-elicited activation of the pgp2/mdr1b promoter requires coupling with Gi/Go.

To define the Gi protein involved in bromocriptine signal transduction, we co-transfected increasing amounts of dominant negative Gi2 S48C along with the pgp2/mdr1b promoter (Fig. 8B). At low amounts of dominant negative Gi2, bromocriptine induction of pgp2/mdr1b transcription was unaffected. As the concentration of co-transfected Gi2 increased, bromocriptine failed to transcriptionally activate the pgp2/mdr1b promoter. The effect of Gi2 was specific because Gi2 had no effect on the thymidine kinase promoter.5 To control for the possibility that Gi2 might produce nonspecific effects we also co-transfected a G-protein not known to couple with D2 receptors, Go (36, 54). Co-transfection of wild-type Go, had no effect on bromocriptine transcriptional activation of pgp2/mdr1b (Fig. 8C). Combined with the pertussis toxin findings, these data show that pgp2/mdr1b transcriptional activation by bromocriptine requires functional Gi2.

Some Gi/Go-coupled receptors, such as the thrombin receptor, are known to stimulate the MAP kinase pathway in a pertussis toxin-sensitive manner (55, 56). Because the Raf-1 MAP kinase pathway has been proposed as a control point in the regulation of MDR1 transcription (57, 58) and, furthermore, because the induction of pgp2/mdr1b by D2 dopamine receptor agonists is specifically abrogated by a Go2 dominant negative, we reasoned that the Raf-1 MAP kinase pathway might be involved in the downstream signal transduction cascade for bromocriptine activation of pgp2/mdr1b. To assess a potential role of Raf-1 MAP kinase, a plasmid expressing a dominant negative Raf (59) was co-transfected in varying amounts into H35 cells to determine if bromocriptine's activation of pgp2/mdr1b could be blocked (Fig. 9A). Consistent with the previous findings reported for the human MDR1 promoter (58) the dominant negative Raf suppressed the pgp2/mdr1b promoter with maximal suppression being over 80%. However, the dominant negative Raf had no effect on pgp2/mdr1b tran-
Bromocriptine does not perturb the MAP kinase pathway. H35 cells were cultured in the presence of 10 μM bromocriptine and harvested at the indicated times. Cell lysates were analyzed on immunoblots with a phospho-specific MAPK antibody. To demonstrate that the MAP kinase pathway was functional, H35 cells were grown in the absence of serum for 48 h and then supplemented with 10% fetal bovine serum (FBS)-containing medium.

**FIG. 10.** Bromocriptine does not perturb the MAP kinase pathway. H35 cells were cultured in the presence of 10 μM bromocriptine and harvested at the indicated times. Cell lysates were analyzed on immunoblots with a phospho-specific MAPK antibody. To demonstrate that the MAP kinase pathway was functional, H35 cells were grown in the absence of serum for 48 h and then supplemented with 10% fetal bovine serum (FBS)-containing medium.

**DISCUSSION**

We and others (20, 60) have previously shown that the PGP reversing agent reserpine can increase MDR1/Pgp expression in vitro in rat and human cells and can activate transcription of the pgp2/mdr1b promoter.² Because reserpine, a dopamine reuptake inhibitor, can affect expression of the dopamine receptor (18, 26) and since dopamine receptors are expressed in the liver (35, 61) and the H35 hepatoma cells (Fig. 1), we hypothesized that reserpine might induce pgp2/mdr1b by altering the amount of an endogenous substrate (dopamine) that serves as a natural intracellular controller of pgp2/mdr1b gene expression in H35 cells. In the present study, we have shown that a D2 dopamine receptor ligand, bromocriptine, can increase Pgp and pgp2/mdr1b mRNA expression in H35 rat hepatoma cells and that this correlates with increased transcriptional activity of the pgp2/mdr1b promoter. The specific involvement of the D2 dopamine receptor in bromocriptine transcriptional activation of the pgp2/mdr1b promoter was strongly indicated because (a) transcriptional activation was specific for D2 dopamine receptor agonists, (b) agonist activation of pgp2/mdr1b transcription could be blocked by D2 dopamine receptor antagonists, and (c) pgp2/mdr1b promoter activation by bromocriptine was enhanced only by the D2 dopamine receptor.

The signal transmitted by the D2 dopamine receptor, in the H35 cells, required a functional Gi as demonstrated by (a) the dramatic suppression of bromocriptine activation of the pgp2/mdr16 promoter by pertussis toxin, and (b) the specific abrogation of bromocriptine transcriptional activation by the dominant negative Gαi2. These findings support the idea that a D2 dopamine receptor initiated transmembrane signal transduction pathway being mediated by the Gαi2. While D2 receptor activation would lead heterotrimeric G-proteins to dissociate and activate downstream signaling pathways, either via Gαi GTP or G-protein βγ subunits, the cis-elements mediating transcriptional activation of pgp2/mdr1b are unknown. While Gαi can lead to AP-1 activation, and the pgp2/mdr1b promoter contains an AP-1 site (14), Gαi activation of AP-1 requires the MAPK pathway that our findings show is not involved in bromocriptine activation of pgp2/mdr1b. It is also possible that bromocriptine mediates its effect through transcription factors that are themselves directly regulated by dopaminergic compounds. Clearly our future studies with deletion constructs of the pgp2/mdr1b promoter will delineate the important cis-elements and additional intracellular signals required for pgp2/mdr1b transcriptional activation by bromocriptine.

Since the type and amount of dopamine receptor varies from tissue to tissue (35, 61, 62), the specific dopamine receptor isoform expressed may be an important factor controlling Pgp expression. In normal rat liver, Giros et al. (61) detected the long form of the D2 dopamine receptor by Northern blot analysis. Rao et al. (35) similarly found in rats that the long form of
the D2 dopamine receptor was detectable in normal liver by RT-PCR assay. Similarly, we found that both the long and short forms of the D2 dopamine receptor were detectable, but we have no explanation for why the alternatively spliced form would be detected in H35 cells and not in normal liver. Nevertheless, both the short and long forms of the D2 dopamine receptor couple via the guanine nucleotide-binding protein, G/Gα. Although the D2 dopamine receptor isoforms appear to utilize the same G-protein, it is clear that in different tissues the second messenger pathways significantly differ. For instance, in the pituitary, the D2 dopamine receptor couples via a G-protein to produce a decrease in cAMP by inhibition of adenylyl cyclase (36, 63). In contrast, in isolated lactotrophs, D2 dopamine receptor activation results in activation of K+ channels or Ca2+ currents (63, 64). Other studies have suggested that activation of the D2 dopamine receptor leads to induction of phosphoinositide hydrolysis (65) or potentiation of technical assistance of Amber Troutman is gratefully acknowledged.

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REFERENCES

1. Juranka, P. F., Zastawny, R. L., and Ling, V. (1989) FASEB J. 3, 2583–2592
2. Gros, P., Creop, J., and Houssam, D. (1986) Cell 47, 371–380
3. Devault, A., and Gros, P. (1990) Mol. Cell. Biol. 10, 1652–1663
4. Ueda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. (1991) Proc. Natl. Acad. Sci. U. S. A. 84, 3004–3008
5. Chen, C., Chiu, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1988) Cell 52, 351–369
6. Gros, P., Raymond, M., Bell, J., and Houssam, D. (1988) Mol. Biol. Cell. 2, 2790–2797
7. van der Blik, A. M., Kooman, P. M., Schneider, C., and Borst, P. (1988) Gene (Amst.) 71, 401–411
8. Guild, B. C., Mulligan, R. C., Gros, P., and Houssam, D. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1595–1599
9. Schinkel, A. H., Roelfs, M. E. M., and Borst, P. (1991) Cancer Res. 51, 2628–2633
10. Sharma, R. C., Inoue, S., Roitelman, J., Schimke, R. T., and Simoni, R. D. (1992) J. Biol. Chem. 267, 5731–5734
11. Shulkin, A. H., Roelfs, M. E. M., and Borst, P. (1993) J. Biol. Chem. 268, 23120–23127
12. Endicott, J. A., and Ling, V. (1989) Ann. Rev. Biochem. 58, 157–171
13. Valverde, M. A., Diaz, M., and Selveda, F. V. (1992) Nature 355, 830–833
14. Schuetz, J. D., Silverman, J. A., Thottassery, J. V., Furuya, K. N., and Schuetz, E. G. (1995) Cell 81, 1341–1348
15. Altmann, S., Stein, W. D., Goldenberg, S., Kane, S. E., Pastan, I., and Gottesman, M. M. (1995) J. Biol. Chem. 270, 27137–27142
16. Kuo, M. T., Julian, J., Hussain, F., Song, R., and Carson, D. D. (1995) J. Cell. Physiol. 164, 132–141
17. Tissari, R. A., and Lillgals, M. S. (1993) J. Neurochem. 61, 231–238
18. Butlerait, P., and Friedman, E. (1993) J. Neurochem. 60, 366–371
19. Herzog, C. E., Tsuchiya, M., Bates, S. E., and Fujino, A. T. (1993) J. Biol. Chem. 268, 2946–2952
20. Schuetz, E. G., Beck, W. T., and Schuetz, J. D. (1996) Mol. Pharmacol. 49, 311–318
21. Biguet, N. F., Buda, M., Lamouroux, A., Samolyk, D., and Mallet, J. (1996) EMBO J. 5, 287–291
22. Pasciuti, G. M., Morgan, D. G., Johnson, S. A., Millar, S. L., and Finch, C. E. (1990) J. Neurochem. 55, 1793–1796
23. Muller, R. A., Tohen, H., and Axelrod, J. (1969) Mol. Pharmacol. 5, 463–469
24. Reith, M. E. A., Jacobsen, A. E., Rose, K. C., Bennek, M., and Zimanyi, I. (1991) J. Pharmacol. Exp. Ther. 259, 1188–1196
25. Diliberto, P. A., Jaffe, R. A., and Cubeddu, L. X. (1989) J. Pharmacol. Exp. Ther. 248, 644–653
26. Jaber, M., Fournier, M. C., and Bouch, B. (1992) Mol. Brain Res. 15, 189–194
27. Reuber, M. D. (1961) J. Natl. Cancer Inst. 26, 891–897
28. Xie, W., and Rothblum, L. I. (1991) BioTechniques 11, 325–327
29. Furuya, K. N., Gebhardt, R., Schuetz, E. G., and Schuetz, J. D. (1994) Biochim. Biophys. Acta 1219, 636–644
30. Danielson, P. E., Fors-Petter, S., Brown, M. A., Calavetta, L., Douglass, J., Milner, R. J., and Sutcliffe, J. G. (1988) DNA (N. Y.) 7, 261–267
31. Silverman, J. A., Rannie, H., Gant, T. W., and Thorgeisson, S. S. (1991) Gene (Amst.) 100, 229–236
32. Deuchar, K. L., Duthie, M., and Ling, V. (1992) Biochem. Biophys. Acta 1130, 157–165
33. Jhalani, S. B., Kuehl, R. M., Jr., and Roundtree, W. S. (1992) Ann. Rev. Biochem. 61, 231–238
34. Rao, D., McKelvy, J., Kebabian, J., and MacKenzie, R. (1990) J. Biol. Chem. 265, 25027–25029
35. Kahan, C., Seuwen, K., Meloche, S., and Pouyssegur, J. (1992) J. Biol. Chem. 267, 7354–7358
36. Kahan, C., Seuwen, K., Meloche, S., and Pouyssegur, J. (1992) J. Biol. Chem. 267, 13569–13575
37. Li, Y. X., Gaitas, F., Rabag, A., Rabagh-Thomas, J., and Chap, H. H. (1995) EMBO J. 14, 2519–2526
38. Kim, S. H., Lee, S. H., Kwak, H. N., Kang, C. D., and Chung, B. S. (1996) Cancer Lett. 99, 199–205
39. Cornwell, M. M., and Smith, D. E. (1993) J. Biol. Chem. 268, 15347–15350
40. Okawa, H., Varma, R., Gillis, B., Bruder, J. T., Rapp, U. R., Davis, L. S., and Geppert, M. T. D. (1993) EMBO J. 12, 4357–4373
41. Bhat, U. G., Winter, M. A., Pearce, H. L., and Beck, W. T. (1995) Mol. Pharmacol. 48, 682–689
42. Giros, B., Sokoloff, P., Martres, M. P., Riou, J. F., Emorine, L., and Schwartz, J. C. (1989) Nature 343, 923–926
43. Montmayeur, J. P., Bao, A., Amlaiky, P., Marie, L., and Basset, J. (1991) FEBS Lett. 278, 239–243
44. Cirelli, O., Buzaw, J., and Grandy, D. K. (1993) Annu. Rev. Pharmacol. Toxicol. 33, 261–307
45. Brown, N. A., and Seabrook, G. R. (1995) Br. J. Pharmacol. 113, 459–466
46. Vallar, L., Fuca, C., Magni, M., Albert, P., Buzaw, J., and Meldolesi, J. (1990) J. Biol. Chem. 265, 10329–10332
47. Pompelli, D., Platt, C., Giros, B., Sokoloff, P., Martres, M. P., and Schwartz, J. C. (1991) Nature 353, 164–167

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