Cloning and Characterization of a Novel Human Dual Flavin Reductase*

(Received for publication, June 24, 1999, and in revised form, October 15, 1999)

Mark J. I. Paine‡, Andrew P. Garner‡, David Powell§, Jennifer Sibbald¶, Mark Sales¶, Norman Pratt¶, Trudi Smith§, David G. Tew§, and C. Roland Wolf‡

From the §Imperial Cancer Research Fund Molecular Pharmacology Unit, Biomedical Research Centre, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom, ¶SmithKline Beecham Research, King of Prussia, Pennsylvania 19406-0939, and the ¶Cytogenetics Department, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom

Flavoprotein reductases play a key role in electron transfer in many physiological processes. We have isolated a cDNA with strong sequence similarities to cytochrome P-450 reductase and nitric-oxide synthase. The cDNA encodes a protein of 597 amino acid residues with a predicted molecular mass of 67 kDa. Northern blot analysis identified a predicted transcript of 3.0 kilobase pairs as well as a larger transcript at 6.0 kilobase pairs, and the gene was mapped to chromosome 9q34.3 by fluorescence in situ hybridization. The amino acid sequence of the protein contained distinct FMN-, FAD-, and NADPH-binding domains, and in order to establish whether the protein contained these cofactors, the coding sequence was expressed in insect cells and purified. Recombinant protein bound FMN, FAD, and NADPH co-factors and exhibited a UV-visible spectrum with absorbance maxima at 380, 460, and 626 nm. The purified enzyme reduced cytochrome c, with apparent $K_m$ and $k_{cat}$ values of 21 $\mu$M and 1.3 s$^{-1}$, respectively, and metabolized the one-electron acceptors doxorubicin, menadione, and potassium ferricyanide. Immunoblot analysis of fractionated MCF7 cells with antibodies to recombinant NR1 showed that the enzyme is cytoplasmic and highly expressed in a panel of human cancer cell lines, thus indicating that this novel reductase may play a role in the metabolic activation of bioreductive anticancer drugs and other chemicals activated by one-electron reduction.

Flavin-containing enzymes catalyze a broad spectrum of biochemical reactions ranging from oxidase, dehydrogenase, and mono-oxygenase reactions. Most flavoproteins contain either FMN or FAD as prosthetic groups; however, a small number of enzymes contain both cofactors. In mammalian systems, NADPH cytochrome P-450 oxidoreductase (cytochrome P-450 reductase) was the first such enzyme isolated (1), followed by several other dual flavin enzymes including nitric-oxide synthases (NOS)$^{1}$ in higher organisms (3, 4) and CYP102 (5) and sulfite reductase (6) in bacteria. More recently, the cDNA sequence encoding a putative FMN- and FAD-binding enzyme, methionine synthase reductase, has been described (7).

Cytochrome P-450 reductase, the most extensively characterized of these enzymes (8–10), is found in the endoplasmic reticulum of most eukaryote cells and is an integral component of the monoxygenase system transferring electrons from NADPH to cytochromes P-450 via FMN and FAD co-factors. Cytochrome P-450 reductase may also donate electrons to heme oxygenase (11), cytochrome $b_5$ (12), and the fatty acid elongation system (13), and can reduce cytochrome c (14). Both the crystal and NMR structure of the FMN domain of human cytochrome P-450 reductase (15, 16) and the crystal structure of the NH2-terminally truncated form of the rat enzyme (17) have been resolved, providing high resolution structural information on this enzyme class. The amino-terminal region of cytochrome P-450 reductase bears striking amino acid homology with FMN-containing flavodoxins, while the carboxyl-terminal region shows similarities with the FAD-containing ferredoxin-NADP$^+$ reductases, thus leading to the hypothesis that cytochrome P-450 reductase has evolved as a fusion of these two ancestral proteins (18, 19). A carboxyl-terminal cytochrome P-450 reductase-like domain is also a component of the NOS family of enzymes, where it is fused to an amino-terminal heme domain. The NOS reductase domain shuttles electrons from NADPH to the active site iron where the amino acid, l-arginine, is metabolized to nitric oxide (NO) (20).

In addition to its normal physiological functions, cytochrome P-450 reductase plays a role in the reduction of one-electron acceptors such as the therapeutically important anticancer agents mitomycin c (22), adriamycin (23), and the benzotriazine di-N-oxide, tirapazamine (24). Evidence is also emerging that NOS can transfer electrons to these compounds via its reductase domain (25, 26). The expression of these dual flavin reductases will therefore influence the outcome of cancer therapy.

In this study we report the cloning of a novel member of the FNR family containing FMN and FAD as co-factors, which supports the NADPH-dependent metabolism of cytochrome $c$, the quinone anti-neoplastic agent doxorubicin, and menadione. Interestingly, the enzyme, which we have called NR1 (novel reductase 1) appears widely expressed in human cancer cell lines and, therefore, could play a potential role in the activation (or deactivation) of drugs used in cancer therapy.

MATERIALS AND METHODS

Chemicals and Reagents—

The abbreviations used are: NOS, nitric-oxide synthase; PBS, phosphate-buffered saline; FNR, flavodoxin-NADP$^+$ reductase; FISH, fluorescent in situ hybridization; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; EST, expressed sequence tag; TBST, Tris-buffered saline with Tween 20; RB, resuspension buffer.

* This work was supported by SmithKline Beecham and United Kingdom Medical Research Council Grant G9203175. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank$^{TM}$/EBI Data Bank with accession number(s) AF199509.

† To whom correspondence should be addressed. Tel.: 44-1382-632-621; Fax: 44-1382-668278; E-mail: rooney@dundee.ac.uk.

The abbreviations used are: NOS, nitric-oxide synthase; PBS, phosphate-buffered saline; FNR, flavodoxin-NADP$^+$ reductase; FISH, fluorescent in situ hybridization; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; EST, expressed sequence tag; TBST, Tris-buffered saline with Tween 20; RB, resuspension buffer.

This paper is available on line at http://www.jbc.org

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
media were from Life Technologies, Inc. (Paisley, UK), except where stated. All solvents were of HPLC grade (Rathburn Chemicals Ltd., UK).

**Novel Reductase Constructs**—The EST data base was screened for putative FAD- or FMN-containing proteins using the human P-450, PDZ reductase probe sequence (9). Two novel CDAs were identified, one was subsequently reported to be methionine synthase reductase (7) and the other (pNR1-SPORT) was used in these studies. This clone contained a 2506-nucleotide sequence and contained putative FAD- and FMN-binding domains. This CDA in pSPORT (Life Technologies, Inc.) was used as a template for PCR amplification of the sequence for expression in baculovirus as described previously (19). SDS-polyacrylamide gel electrophoresis and immunoblots were carried out using a Mini-PROTEIN II (Bio-Rad) electrophoresis system. Except where indicated, proteins were separated using 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose (Schleicher & Schuell) according to manufacturer's instructions. For immunodetection, the blots were blocked in TBST (20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 0.05% v/v Tween 20) with 5% w/v milk powder (Marvel) overnight at 4 °C with shaking. After washing several times with TBST, blots were incubated with appropriate antibody diluted in TBST, 5% milk powder at room temperature for 1–2 h. The binding of the primary antibodies was detected using a chemiluminescence detection system (Amersham Pharmacia Biotech, ECL). The secondary antibodies used were anti-rabbit IgG and anti-sheep IgG (Scottish Antibody Production Unit, Edinburgh, UK).

**Cell Culture**—The tumor cell lines MCF-7, HepG2, HeLa, and NIH3T3 were cultured in Dulbecco's modified Eagle's medium. PEO1, EJ9, NCI H322, and HT29 cells were cultured in RPMI medium. All cell cultures were supplemented with 10% v/v heat-inactivated fetal calf serum, except for HepG2 cultures, which contained 15% v/v serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in 5% CO2.

To prepare whole cell extracts, approximately 2 × 107 cells were harvested by trypsinization, washed once with PBS, and frozen at −70 °C. Cells were resuspended in 1 ml of 100 mM PBS, 0.25 μM sucrose and sonicated on ice using several pulses with an MSE probe. Following centrifugation at 4 °C, 12,000 × g for 1 h, the supernatant was aspirated and stored at −70 °C. For subcellular fractionation studies, approximately 2 × 108 MCF-7 cells were harvested, washed twice in PBS and homogenized using a 20-ml glass homogenizer in 10 ml of resuspension buffer (RB) consisting of 0.25 μM sucrose, 50 mM HEPES, 1 mM EDTA, 0.2 mM dithiothreitol, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 0.2 mg/ml phenylmethylsulfonyl fluoride. Nuclear material and particulate cell material were pelleted at 10,000 × g for 10 min and resuspended in 5 ml of RB. The resulting nuclear material was centrifuged at 100,000 × g for 1 h, and the resultant pellet was resuspended in 1 ml of RB. The supernatant, containing the cytoplasmic fraction, and the pellet, containing the membrane fractions, were stored at −70 °C.

**Flavin Determination and Spectral Analysis**—FMN and FAD content was determined by HPLC (28) using a Hewlett Packard 1050 HPLC chromatograph and fluorosence detector. Flavins were released from protein-bound material by boiling for 5 min, and denatured protein removed by 20,000 × g centrifugation for 10 min. FMN and FAD were detected by fluorescence (excitation, 450 nm; emission, 520 nm) following isocratic separation (10 mM sodium acetate, pH 6.0, methanol; v/v ratio 78:22) over a 25-cm Spherisorb ODS-2 5-μm column. Authentic FMD and FAD standards purchased from Sigma were used as control. Both were over 98% pure, as judged by HPLC analysis. Absorption spectra were obtained using a Shimadzu 160 UV spectrophotometer.

**Northern Blot**—Human Multiple Tissue Northern blots (CLON-TECH) were hybridized with a 521-base pair cDNA fragment generated by SacI/SmaI restriction endonuclease digestion of pNR1. This probe was radio labeled by incorporation of 32P-dCTP (RadPrime DNA labeling system, Life Technologies, Inc.) and purified using a Chroma Spin TE-30 column (CLONTECH). After a 1-h prehybridization, hybridization was carried out for 1 h using ExpressHyb buffer (CLONTECH) at 68 °C. The membrane was washed twice in 2× SSC, 0.05% sodium dodecyl sulfate for 20 min, twice in 0.1× SSC, 0.05% v/v sodium dodecyl sulfate at 50 °C for 20 min, and exposed to x-ray film at −70 °C with two intensifying screens.

**Chromosomal Location**—The full-length 2.6-kilobase NR1 cDNA segment was digested from pSPORT with EcoRI and HindIII and used as a probe in fluorescence in situ hybridization mapping (FISH). Standard cytogenetic techniques were used to prepare fixed normal lymphocyte slides. The probe was labeled with Spectrum Red dye using a nick translation kit (both Vysis, Downers Grove, IL) using the following kit protocol modifications. Slides were pretreated four times for 2 min in 2× SSC, pH 7.0, and dehydrated through 70%, 85%, and 100% ethanol
Production of Active NR1 in Insect Cells—In order to determine whether the NR1 cDNA codes for a biologically active enzyme, we cloned the cDNA using a baculovirus expression vector. The full-length NR1 coding sequence was subcloned into pFastBac downstream of the very late polyhedron promoter, and the cDNA fused with a 6-histidine-tagged sequence at the amino terminus to facilitate affinity purification by nickel-agarose chromatography. Recombinant baculovirus expression was initiated by the addition of 10 μg of enzyme. The oxidation of NADPH and reduction of doxorubicin and menadione was carried out in 50 mM Tris-HCl (pH 7.5), 1 mM NADPH, and various substrate concentrations at 37 °C. Reactions were monitored at 340 nm using a Shimadzu UV 2000 spectrophotometer. Final doxorubicin concentrations ranged from 20 to 100 μM and NADPH concentrations from 10 to 22.5 μM. Control reactions were carried out in the absence of active enzyme.

**RESULTS**

**Molecular Cloning of a cDNA Encoding a Dual Flavin Reductase**—A DNA fragment was identified, which contained an open reading frame with significant homology to human cytochrome P-450 reductase, following an extensive database search of EST data base libraries. The cDNA insert for the EST was 2452 nucleotides in length (excluding the poly(A) tail) and contained the complete coding sequence for a putative cytochrome P-450 reductase-like enzyme (Fig. 1). Initiation of translation is predicted to be the first in-frame methionine residue based on sequence alignment with human cytochrome P-450 reductase and is preceded by several nucleotides bearing homology to the Kozak sequence consensus (30). There are also in-frame protein stop codons upstream of the predicted start site, which place this ATG codon in good context for the initiation of translation. A 2.5-kb NR1 cDNA was used to screen a human genomic library. Nucleotides corresponding to the Kozak sequence (30) are shown in capital letters; sequences with particular strong sequence conservation in the regions shown in boldface type are in italics. The complete coding sequence shown in Fig. 1 corresponds to a 1791-nucleotide sequence that is in-frame with the deduced amino acid sequence shown in Fig. 2. The coding sequence is 2452 nucleotides in length (excluding the poly(A) tail) and was 2452 nucleotides in length (excluding the poly(A) tail) and contained the complete coding sequence for a putative cytochrome P-450 reductase-like enzyme (Fig. 1). Initiation of translation is predicted to be the first in-frame methionine residue based on sequence alignment with human cytochrome P-450 reductase and is preceded by several nucleotides bearing homology to the Kozak sequence consensus (30). There are also in-frame protein stop codons upstream of the predicted start site, which place this ATG codon in good context for the initiation of translation. A 2.5-kb NR1 cDNA was used to screen a human genomic library. Nucleotides corresponding to the Kozak sequence (30) are shown in capital letters; sequences with particular strong sequence conservation in the regions shown in boldface type are in italics. The complete coding sequence shown in Fig. 1 corresponds to a 1791-nucleotide sequence that is in-frame with the deduced amino acid sequence shown in Fig. 2.
NADPH-binding domain. HPLC analysis of heat-denatured enzyme determined that it released two fluorophores whose retention times exactly matched those of authentic FMN and FAD (Fig. 4B). There were 1.2 and 1.1 mol each, respectively, of FMN and FAD bound per mole of enzyme. NR1 exhibited a UV-visible spectrum similar to cytochrome P-450 reductase (32), possessing absorbance maxima at 380, 460, and 626 nm (Fig. 4C). Like cytochrome P-450 reductase, the addition of NADPH under aerobic conditions caused a decrease in the absorbance at 380 and 460 nm, and an absorbance increase at 580 nm. Furthermore, the UV-visible spectrum of NR1 reduced with NADPH was stable over a 24-h period, which is consistent with the reduction of the flavin co-factors by NADPH and the production of an air-stable semiquinone form (10, 32, 46).

The above data showed that NR1 is a flavoenzyme that binds both FMN and FAD cofactors as predicted from the amino acid sequence. Furthermore, the spectral changes associated with the addition of NADPH indicate that electrons are transferred from NADPH to FAD and FMN, which indicates that NR1 follows the same pattern of electron transfer as in other dual flavin enzymes.

Biological Activity—The cytochrome P-450 reductase family of flavoenzymes are generally capable of reducing the heme-protein cytochrome c, which thus serves as a model substrate for the comparative analysis of enzyme activity and electron transfer. Cytochrome c reducing activity was maximal when it occurred using potassium phosphate concentrations of between 300 and 400 mM and the enzyme had a pH optimum of around 8.0 (data not shown). There was also no detectable enzyme activity using NADH as a reducing cofactor. The conditions for optimal enzyme activity were thus similar to those observed for cytochrome P-450 reductase (9, 10). The kinetic parameters of cytochrome c reduction were compared with human cytochrome P-450 reductase. As shown in Table I, the apparent $K_m$ value of NR1 for cytochrome c was 21 $\mu$M, which was similar to cytochrome P-450 reductase (15 $\mu$M). Reported $K_m$ values of mammalian P-450 reductase for cytochrome c range between 5 and 21 $\mu$M (14, 49, 50). The apparent $k_{cat}$ value was calculated as 1.3 s$^{-1}$, which was approximately 100-fold lower than cytochrome P-450 reductase. NR1 also metabolized a range of one-electron acceptors, including the quinone-containing compounds doxorubicin and menadione (Table II). Although all the activities measured were significantly lower than cytochrome P-450 reductase (in the range 1–4%), they were reasonably similar to activities previously measured (25) for the reductase domain of NOS III (Table II). Taken together, these results indicate that the cloned cDNA encodes an authentic NADPH-dependent reductase enzyme, which is capable of catalyzing the reduction of cytochrome c and one-electron acceptors.

Expression of NR1 in Human Tissue and Cancer Cell Lines—Northern blot analysis of mRNA from human tissue and cancer cell lines indicate two main species of approximately 3 and 6 kilobases in length (Fig. 5). The 3-kilobase mRNA corresponds to the size expected for the full-length NR1 transcript. In human tissues, levels of expression were generally low, with highest levels seen in the placenta (Fig. 5A). In cancer cell lines, highest levels were found in HeLa and colonic adenoma cells followed by myeloid leukemia cells and melanoma cells (Fig. 5B).
Cloning and Characterization of NR1

Comparison of kinetic parameters of cytochrome c reduction by NR1 and cytochrome P-450 reductase

Reactions contained 0.3 M potassium phosphate, pH 7.7, 50 μM NADPH, with varying amounts of cytochrome c. Reactions were preincubated at 37 °C and initiated by the addition of NADPH. Values were determined by Lineweaver-Burke plot analysis and are the mean and standard deviation of three experiments.

| Enzyme       | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $K_{cat}$ |
|--------------|------------|--------------------------|-----------|
| NR1          | 21.3 ± 2.8 | 1.2 ± 0.1                | 1.3       |
| P-450 reductase | 15.2 ± 3.8 | 85.4 ± 13.8              | 109.3     |

Comparison of the reduction of one-electron accepting compounds by NR1, cytochrome P-450 reductase, and NOS III reductase domain

Specific activities were determined by monitoring NADPH oxidation in three independent experiments as detailed under “Materials and Methods.”

| Enzyme       | Ferricyanide | Doxorubicin | Menadione |
|--------------|--------------|-------------|-----------|
| NR1          | 4.5 ± 0.4    | 0.6 ± 0.01  | 0.9 ± 0.02 |
| P-450 reductase | 164.8 ± 2.5  | 15.4 ± 1.3  | 41.9 ± 2.4 |
| NOS III reductase* | ND          | 1.5         | 2.1       |

* NOS III reductase domain (results taken from Ref. 25).

ND, not determined.

Subcellular Localization—From the primary amino acid sequence data, a major difference between NR1 and cytochrome P-450 reductase is the lack of a membrane anchor at the amino-terminal end of NR1. To compare the subcellular distribution of these enzymes, crude subcellular fractionation of MCF-7 cells was carried out by differential centrifugation and NR1 and cytochrome P-450 reductase identified in different fractions by Western blotting (Fig. 7). NR1 was detectable primarily in the 10,000 × g and 100,000 × g supernatant fractions, indicating that the enzyme is associated with the cytoplasmic fraction. There was some signal detectable in the nuclear pellet fraction; thus, possible targeting of NR1 to the nucleus cannot be ruled out. By contrast, cytochrome P-450 reductase was found predominantly in nuclear pellet and microsomal membrane fractions, consistent with its localization to the endoplasmic reticulum. These results indicate that the subcellular localization of NR1 differs from microsomal cytochrome P-450 reductase and is found associated with the cytosolic fraction.

5B). The more prominent high molecular weight band followed a similar profile of expression but appeared to be expressed at higher levels in the tumor cell lines. The nature of the larger mRNA species is unclear. It may represent a partially spliced variant NR1 mRNA, a homologous gene sequence, or possibly a fusion protein between an NR1-related protein and another protein. This is currently under investigation through the characterization of the NR1 gene and its intron/exon organization.

Antibodies were generated against the carboxyl-terminal FAD domain of NR1 in order to identify the native form of the enzyme and to investigate its expression in cancer cell lines. These antibodies were used for Western blot analysis of whole cell extracts from a range of human cancer cell lines derived from different tissues, including ovary (PEO1), breast (MCF7), bladder (EJ9), lung (NCI-H322), colon (HT29), liver (Hep G2), and cervical carcinoma (HeLa). The murine fibroblast NIH3T3 cell line was also analyzed. As shown in Fig. 6A, a ~62-kDa protein of similar size to that predicted from the NR1 primary sequence was detectable at similar levels in all cell lines apart from the murine NIH3T3 cells. The expression profile was significantly different to cytochrome P-450 reductase (Fig. 6B), which showed high levels of expression in HepG2 cells, low levels in MCF7 and HT-29 cell lines, and undetectable levels in the other cell lines. In the murine-derived fibroblast NIH3T3 cell line, NR1 antibodies detected two different sized polypeptides of ~80 and 30 kDa, respectively. Since NR1 genes have so far not been found in mice, it is unclear what relationship these polypeptides have with the human form of the enzyme, but the higher molecular mass protein could indicate the presence of the fusion protein suggested in the Northern blot analysis above. Interestingly, in the HeLa cell line, a high molecular weight protein is also observed in the Western blot consistent with the presence of the 6-kilobase transcript.

**TABLE I**

Comparison of kinetic parameters of cytochrome c reduction by NR1 and cytochrome P-450 reductase

| Enzyme       | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $K_{cat}$ |
|--------------|------------|--------------------------|-----------|
| NR1          | 21.3 ± 2.8 | 1.2 ± 0.1                | 1.3       |
| P-450 reductase | 15.2 ± 3.8 | 85.4 ± 13.8              | 109.3     |

**TABLE II**

Comparison of the reduction of one-electron accepting compounds by NR1, cytochrome P-450 reductase, and NOS III reductase domain

| Enzyme       | Ferricyanide | Doxorubicin | Menadione |
|--------------|--------------|-------------|-----------|
| NR1          | 4.5 ± 0.4    | 0.6 ± 0.01  | 0.9 ± 0.02 |
| P-450 reductase | 164.8 ± 2.5  | 15.4 ± 1.3  | 41.9 ± 2.4 |
| NOS III reductase* | ND          | 1.5         | 2.1       |

* NOS III reductase domain (results taken from Ref. 25).

ND, not determined.

Subcellular Localization—From the primary amino acid sequence data, a major difference between NR1 and cytochrome P-450 reductase is the lack of a membrane anchor at the amino-terminal end of NR1. To compare the subcellular distribution of these enzymes, crude subcellular fractionation of MCF-7 cells was carried out by differential centrifugation and NR1 and cytochrome P-450 reductase identified in different fractions by Western blotting (Fig. 7). NR1 was detectable primarily in the 10,000 × g and 100,000 × g supernatant fractions, indicating that the enzyme is associated with the cytoplasmic fraction. There was some signal detectable in the nuclear pellet fraction; thus, possible targeting of NR1 to the nucleus cannot be ruled out. By contrast, cytochrome P-450 reductase was found predominantly in nuclear pellet and microsomal membrane fractions, consistent with its localization to the endoplasmic reticulum. These results indicate that the subcellular localization of NR1 differs from microsomal cytochrome P-450 reductase and is found associated with the cytosolic fraction.

**FIG. 4.** Purification and characterization of NR1. A, SDS-polyacrylamide gel electrophoresis analysis of the sequential purification of NR1 over nickel-agarose and ADP-Sepharose. From left to right, the lanes contain the 100 × g soluble fraction applied to the column (sup), flow-through (FT), binding buffer wash (BB), 60 mM imidazole wash (60 mM), PBS wash (PBS), 300 mM imidazole eluate (300 mM), ADP-Sepharose column flow-through (ES), 500 mM NaCl wash (NaCl), and 10 mM AMP eluate (10 mM). B, HPLC profile of flavins released by heat-denatured NR1. Peaks 1 and 2 indicate FAD and FMN peaks. The concentration of flavins released was calculated with reference to authentic standards. Twenty nanomoles of FMN and 18 nmol of FAD were released per mg of NR1 (mean of two experiments). C, absorption spectra of purified NR1 (2.6 μM). Trace 1 is the oxidized spectrum; traces 2–5 are spectra reduced with 0.5, 1.5, 2.5, and 3.5 μM NADPH, respectively. The accompanying changes in absorbance units (∆A) at 380 nm (diamond), 460 nm (square), and 580 nm (circle) are shown in the graph in the inset.
DISCUSSION

We have cloned and characterized a novel dual flavin reductase, NR1, which represents a new member of the FNR family of flavoenzymes. NR1 binds FMN, FAD, and NADPH cofactors and shares about 44% similarity with human cytochrome P-450 reductase. Analysis of the prototypical rat cytochrome P-450 reductase crystal structure (17) highlights amino acids of potential functional significance and indicates that there may be close structural similarities. With respect to FMN binding, the isoalloxazine ring of FMN in rat cytochrome P-450 reductase is covered by the phenolic ring of Tyr-140 at the si-side (17). In NR1 the equivalent residues are Thr-61 and Tyr-102, respectively. Thus, the aromatic residue in the Tyr-178 position, which is essential for FMN binding, is conserved while, interestingly, there is a non-aromatic substitution of Tyr-140. Site-directed mutagenesis studies have shown that such a substitution does not necessarily affect FMN binding but may reduce electron transfer efficiency (33).

In cytochrome P-450 reductase, the FAD ring is stacked by the indole ring of Trp-677 while the aromatic residue Tyr-456 lies on the si-side. NR1 contains corresponding aromatic residues in Phe-384 and Trp-596. Strong similarities also exist with the rat cytochrome P-450 reductase peptide fragments 455YYSIAS in Phe-384 and Trp-596. Strong similarities also exist with the rat cytochrome P-450 reductase peptide fragments 455YYSIAS in Phe-384 and Trp-596. Strong similarities also exist with the rat cytochrome P-450 reductase peptide fragments 455YYSIAS in Phe-384 and Trp-596.
The overall conservation in the elements required for co-factor binding and their sequence arrangement indicate that NR1 may be structurally similar to cytochrome P-450 reductase. Like cytochrome P-450 reductase, recombinant NR1 catalyzed the reduction of cytochrome c and various one-electron accepting compounds. Overall, however, the apparent enzymatic activity was significantly lower than human cytochrome P-450 reductase. It is possible that this may be related to the non-atomic substitution of Thr-72 in the Tyr-140 position of the FMN domain, as described above. However, since reduction of potassium ferricyanide by NR1, which occurs via the FAD redox center, was also slower than cytochrome P-450 reductase, it is also possible that amino acid sequence differences in the FAD/NADPH domain may be responsible for different rates of electron transfer. Recent studies have shown that Ser-457, Asp-675, and Cys-630 in rat cytochrome P-450 reductase interact to form a catalytic site for hydride transfer from NADPH to FAD (36). It is notable that, in NR1, Ala-549 corresponds to Cys-630 in the rat enzyme and Cys-629 in the human P-450 reductase sequence shown in Fig. 2. A similar non-conservative amino acid substitution in cytochrome P-450 reductase significantly reduced catalytic activity (36) in this enzyme, and may possibly do so in NR1 as well. A more detailed structural analysis, for example the independent expression of the FAD/NADH domain, will provide more definitive information on the functional relationship between NR1 and cytochrome P-450 reductase.

It has recently been shown that the NOS family of enzymes play an important role in the bioactivation of anti cancer drugs via the reductase domain (25, 26). Rates of reduction of the quinone-containing compounds including the anticancer drugs doxorubicin and menadione were comparable with NOS, with \( k_{\text{cat}} \) values of the NOS III reductase domain measured at 1.45 and 2.07 s \(^{-1} \), respectively (26). Thus, under appropriate physiological circumstances NR1 may also affect the metabolism of one-electron accepting compounds. In this respect, it is interesting that Western blot analysis indicated that NR1 expression was detectable at high levels in a wide range of cancer cells. The enzymatic factors involved in the metabolic activation of bioreductive drugs are complex and not fully understood. In solid tumor tissue, bioreductive enzyme activity is located at different subcellular locations throughout the cell (37). Key bioreductive enzymes are thought to include the cytosolic enzyme DT-diaphorase and the microsomal cytochrome P-450 reductase (37, 47). However, other enzymes with novel activities may well be involved.

The biological role of NR1 is unknown. Clues as to the natural function of genes frequently come from analysis of genetic abnormalities or recurrent chromosomal breakpoints in cancer. We have mapped the gene for NR1 to the telomeric region of the long arm of chromosome 9. There are, however, comparatively few reports of constitutional chromosomal abnormalities, recurrent cancer breakpoints or single gene disorders for this region. Two cases of infants with deletion to 9q34.3 have been recently reported (38, 39), which show that this deletion may be associated with a recognizable pattern of malformation associated with severe developmental delay and respiratory problems (39). Three diseases have also been localized to the region between 9q32 and 9q34. These include limb-girdle muscular dystrophy, characterized by muscle weakness and wasting (40); lethal congenital contracture syndrome, characterized by the fetal akinesia phenotype, with highly focused degeneration of motor neurons in the spinal cord (41); and atypical lateral sclerosis, characterized by slow progressive, distal limb amyotrophy, and severe loss of motor neurons in the brain stem and spinal cord (42).

Further studies investigating tissue-specific expression and interaction with other cellular proteins will help to elucidate the normal function of the gene. Similarities with cytochrome P-450 reductase enzymes indicate that it is likely to transfer electrons from NADPH to the heme- or transition metal-containing center of an appropriate redox partner. Possible redox partners include heme-binding enzymes such as cytochrome P-450 enzymes, or possibly the cobalamin-dependent methionine synthase. Microsomal cytochrome P-450 is unlikely to be the physiological partner, since NR1 lacks an apparent membrane anchor sequence, which is an important requirement for efficient coupling with cytochrome P-450 reductase at the membrane surface (9). Furthermore, we have found that NR1 is unable to reconstitute ethoxyresorufin hydroxylase activity with CYP1A2 in vitro, or CYP 2D6 bufaralol hydroxylase activity when co-expressed in insect Sf9 cells. It seems more likely, therefore, that NR1 may be involved in some other function. One possibility is in methionine synthesis. Methionine synthase is a cobalamin-dependent enzyme that catalyzes the transfer of a methyl group from \( CH_3-H_4 \) folate to homocysteine. During catalysis, accidental build-up of the inactive cob(II)alamin state is prevented by reduction by oxidoreductases (43). Interestingly, the identity of the mammalian proteins that regulate reductive activation of methionine synthase have not been established. However, NADPH-dependent auxiliary redox proteins are known to be involved (44), and the most recent evidence suggests that cytochrome P-450 reductase and cytochrome \( b_5 \) may reactivate methionine synthase (45). Thus, NR1 may represent an alternative pathway for methionine synthase reactivation.

Acknowledgment—We are grateful to Steve Ayivor for assisting with the purification of the FAD domain and the production of NR1 antibodies.

REFERENCES
1. Dignam, J. D., and Strobel, H. W. (1975) Biochem. Biophys. Res. Commun. 63, 845–852.
2. Yasukochi, Y., and Masters, B. S. S. (1976) J. Biol. Chem. 251, 5337–5344.
3. Ilan, R., Ilan, Z., and Cinti, D. C. (1981) J. Biol. Chem. 256, 714–718.
4. Schmidt, H. H. W., Smith, F. M., Nakane, M., and Murad, F. (1992) Biochem. 31, 3243–3249.
5. Narhi, L. O., and Fulee, A. J. (1986) J. Biol. Chem. 261, 7160–7169.
6. Ostreiker, J., Barber, M. J., Ruiger, D. C., Miller, B. E., Siegel, L. M., and Kredich, N. M. (1989) J. Biol. Chem. 264, 15796–15808.
7. Leclerc, D., Wilson, A., Dumax, R., Gajuik, C., Song, D., Watkins, D., Ming, H. Q., Rommens, J. M., Howell, D. S., and Gravel, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3059–3069.
8. Vermillion, J., and Coon, M. J. (1978) J. Biol. Chem. 253, 8812–8819.
9. Shen, L. A., and Kasper, C. B. (1983) in Cytochrome P-450 (Schenkman, J. B., and Greim, H., eds) pp. 35–59, Springer-Verlag, New York.
10. Backes, W. L. (1993) in Cytochrome P-450 (Schenkman, J. B., and Greim, H., eds) pp. 35–59, Springer-Verlag, New York.
11. Schacter, B. A., Nelson, E. B., Marver, H. S., and Masters, B. S. S. (1972) J. Biol. Chem. 247, 3691–3697.
12. Enoch, H. G., and Strittmatter, P. (1981) J. Biol. Chem. 254, 8976–8981.
13. Liu, Z., Tan, K., and Cinti, D. C. (1981) J. Biol. Chem. 256, 10066–10072.
14. Williams, C. H., and Kamin, H. (1962) J. Biol. Chem. 237, 578–595.
15. Zhan, Q., Modi, S., Smith, G., Paine, M. J. L., Wolf, C. R., Tew, D., Lian, L. Y., Roberts, G. C. K., and Driessen, H. P. C. (1999) Protein Sci. 8, 286–306.
16. Barsukov, I., Modi, S., Lian, L. Y., Sze, K. H., Paine, M. J. L., Wolf, C. R., and Roberts, G. C. K. (1997) J. Biomed. NMR 10, 63–75.
17. Wang, M., Roberts, D. C., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J.-J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94 8411–8416.
18. Porter, T. D. (1993) Trends Biochem. Sci. 16, 154–158.
19. Smith, G. C. M. Tew, D. G., and Wolf, C. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8710–8714.
20. Nathan, C. (1992) FASEB J. 6, 3051–3064.
21. Stuehr, D. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 339–359.
22. Keyse, S. R., Fracasso, P. M., Heinbrock, D. L., Rockwell, S., Silgar, S. G., and Sartorelli, A. C. (1984) Cancer Res. 44, 5638–5643.
23. Bachur, N. R., Gordon, S. L., Gee, M. V., and Kon, H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 954–957.
24. W Peyron, M. I., Wolf, C. R., and Workman, P. (1992) Biochem. Pharmacol. 44, 251–259.
25. Vásquez-Vivar, J., Martasek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A.,

\(^2\) M. J. I. Paine, A. P. Garner, and C. R. Wolf, unpublished results.
Cloning and Characterization of NR1

26. Garner A. P., Paine., M. J. I., Rodriguez-Crespo, I., Chinje, E. C., Ortiz de Montellano, P., Stratford, I., Tew, D., and Wolf, C. R. W. (1999) Cancer Res. 59, 1929–1934
27. O’Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W. H. Freeman & Co., New York
28. Klatz P., Schmidt, K., Werner, E. R., and Meyer, B. (1996) Methods Enzymol. 268, 359–365
29. Vermilion, J. L., Ballou, D. P., Massey, V., and Coon, M. J. (1981) J. Biol. Chem. 256, 266–277
30. Kozak, M. (1991) J. Biol. Chem. 266, 19867–19870
31. Church, D. M., Stotler, C. J., Rutter, J. L, Murrell, J. R., Troflatter, J. A., and Buckler, A. J. (1994) Nat. Genet. 6, 98–105
32. Vermilion, J. L., and Coon, M. J. (1978) J. Biol. Chem. 253, 2694–2704
33. Shen, A. L., Porter, T. D., Wilson, T. E., and Kasper, C. B. (1989) J. Biol. Chem. 264, 7584–7588
34. Karplus P. A., Daniels, M. J., and Herriott, J. P. (1991) Science 251, 60–66
35. Scrutton, N. S., Berry, A., and Perham, R. N. (1990) Nature 343, 38–43
36. Shen, A. L, Sem, D. S., and Kasper, C. R. (1999) J. Biol. Chem. 274, 5391–5398
37. Cummings, J., Spanswick, V. J., Tomasz, M., and Smyth, J. F. (1998) Biochem. Pharmacol. 56, 405–414
38. Schimmenti, L. A., Berry, S. A., Tuchman, M., and Hirsch, B. (1994) Am. J. Med. Genet. 51, 140–142
39. Ayyash, H., Mueller, R., Maltby, E., Horsfield, P., Telford, N., and Tyler, R. (1997) J. Med. Genet. 34, 610–612
40. Weiler, T., Greenberg, C. R., Zelinski, T., Nylen, E., Caphlan, M. J., Crumley, Fujikawa, T. M., Morgan, K., and Wrogemann, K. (1998) Am. J. Hum. Genet. 63, 140–147
41. Makela-Rengs, P., Jarvinen, N., Vuspalu, K., Suomalainen, A., Ignatius, J., Sigila, M., Herva, R., Palotie, A., and Peltonen, L. (1998) Am. J. Hum. Genet. 63, 506–516
42. Chance, P. F., Rabin, B. A., Ryan, S. G., Ding, Y., Scavina, M., Crain, B., Griffin, J. W., and Cornblath, D. R. (1998) Am. J. Med. Genet. 51, 140–142
43. Banerjee, R., and Matthews, R. G. (1990) FASEB J. 4, 1450–1459
44. Gulati, S., Chen, Z., Brody, L. C., Rosenblatt, D. S., and Banerjee, R. (1997) J. Biol. Chem. 272, 19171–19175
45. Chen, Z., and Banerjee, R. (1998) J. Biol. Chem. 273, 26248–26255
46. Masters, B. S. S., Bilimoria, M. H., Kamin, H. and Gibson, Q. H. (1965) J. Biol. Chem. 240, 4081–4088
47. Spearman, M. E., Moloney, S. J., and Prough, R. A. (1984) Mol. Pharmacol. 26, 566–573
48. Deleted in proof
49. Philips, A. H., and Langdon, R. G. (1962) J. Biol. Chem. 237, 2652–2660
50. Shen, A., and Kasper, C. B. (1995) J. Biol. Chem. 270, 27475–27480