Molecular cloning and expression of human trophoblast antigen FDO161G and its identification as 3 β -hydroxy-5-ene steroid dehydrogenase

D. A. Nickson¹, M. W. McBride¹, S. Zeinali¹, C. S. Hawes²,
A. Petropoulos², U. W. Mueller² and R. G. Sutcliffe¹

¹*Institute of Genetics, Glasgow University, Church Street, Glasgow G11 5JS, UK; and*

²*Department of Obstetrics and Gynaecology, Flinders University, Flinders Medical Centre, Bedford Park, South Australia 5042*

Summary. The monoclonal antibody FDO161G reacts with a 43-kDa protein found in human extravillous trophoblast, syncytiotrophoblast, adrenal cortex, interstitial cells of the testis and ovarian follicle cumulus cells. cDNAs for this protein have been isolated from the lambda gt11 library, sequenced, and expressed in COS-7 cells. The protein was identified as 3 β -hydroxy-5-ene steroid dehydrogenase (HSD). The sequence of the HSD protein raises questions about its association with cell membrane systems. The lack of reactivity of FDO161G with other tissues suggests that HSD has a limited tissue distribution and that other enzymes may exist in peripheral tissues, which can convert Δ 5 3-hydroxysteroids to Δ 4 3-ketosteroids.

Keywords: trophoblast antigen; cDNA cloning; 3 β -hydroxysteroid dehydrogenase; placenta; man

Introduction

The proteins of the plasma membranes of human trophoblast have attracted investigation for a variety of reasons, including materno–fetal immunology, developmental cell biology, membrane transport and membrane receptors, and the isolation of oncofetal proteins. As a consequence, a large panel has been generated of monoclonal antibodies that react with and define a range of epitopes expressed on plasma membrane proteins. An early compilation of such antibody specificities is in Anderson *et al.* (1987) and more-recent listings are available through the auspices of the WHO Special Programme of Research, Development and Research Training in Human Reproduction, Geneva. One of these monoclonal antibodies, FDO161G, is a mouse monoclonal IgG1 which reacts with a 43-kDa protein in villous syncytiotrophoblast and nonvillous cytotrophoblast from first and third trimester human placentae (Mueller *et al.*, 1987). It also reacts with interstitial cells of the testis, cumulus cells of mature ovarian follicles and cells of the adrenal cortex (U. W. Mueller, C. S. Hawes & W. R. Jones, unpublished).

We report here the molecular cloning and expression of the cDNA for the protein encoding the FDO161G epitope and identify the protein as a key enzyme in hormonal steroidogenesis.

Materials and Methods

Purification of FDO161G protein

The FDO161G monoclonal antibody was coupled to cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia Biosystems, UK) as described by the manufacturers. The monoclonal antibody was purified from murine

ascites fluid by precipitating with an equal volume of 25% (w/v) polyethylene glycol 6000 followed by DEAE-Sephacel ion-exchange chromatography using a linear 0–300mM-NaCl gradient in 20mM Tris buffer, pH 8.0 with 0.02% (w/v) sodium azide.

A term placenta obtained within 2 h of delivery was washed in saline, the membranes and umbilical cord were removed and the tissue was finely minced with scissors. Membrane proteins were solubilized by the addition of an 8mM solution of 3[(cholamidopropyl) dimethylammonio] 1-propanesulphonate (CHAPS; Boehringer Mannheim, Germany) in 20mM Tris-HCl, pH 8.0 buffer with 0.02% (w/v) sodium azide, 5mM EDTA and 1mM phenylmethylsulphonylfluoride (Sigma) in the ratio of 1:10 (w/v). After stirring for 17 h, the insoluble material was removed by centrifugation at 2000 g for 15 min.

The immuno-affinity beads (2 ml + 2 ml nonderivatized Sepharose 4B/41 homogenate) were incubated in batches for 48 h with the solubilized trophoblast preparation. The beads were then recovered by centrifugation at 200 g for 5 min and transferred to columns for washing with fresh homogenization buffer. The immobilized antigen was eluted with 3 volumes 100mM glycine-HCl, pH 2.5 buffer, containing 0.05% (w/v) sodium azide and 8mM CHAPS. The eluate was immediately neutralized with 1.5mM Tris-HCl buffer, pH 8.7 (100 µl/ml eluate). The purity of the isolated protein was assessed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed essentially according to the method described by Laemmli (1970) except that a linear 10–20% polyacrylamide gel gradient was used. SDS-PAGE gels were stained with Coomassie Blue R-250. The isolated protein was quantitated using an LKB laser densitometer. SDS-PAGE gels were dried onto Whatman 3 MM chromatography paper using a Hoeffer gel dryer. The amount of protein on the polyacrylamide gels was determined by reference to ovalbumin (Sigma) as standard.

Affinity gel eluates were electroeluted from SDS-PAGE gels as follows. The appropriate Coomassie-Blue-stained gel slices were cut from the SDS-PAGE gel and equilibrated with a buffer consisting of 100mM Tris-HCl, pH 8.0 with 0.1% (w/v) SDS and 5mM dithiothreitol. The gel slices were loaded into modified glass tubes with dialysis tubing over the lower end. Electroelution was run overnight using 0.1% (w/v) SDS in 50mM ammonium bicarbonate at 50 V.

Amino acid sequencing

The electroeluted protein was concentrated by precipitating with ice-cold methanol (i.e. 100 µl of electroeluted protein with 900 µl of methanol at -20°C). After overnight incubation, the methanol was removed and the pellet resuspended in minimal volume 50mM ammonium bicarbonate solution. The protein was applied to a glass-fibre disc coated with Polybrene, and sequenced in an Applied Biosystems model 470A gas-phase sequenator (Murphy *et al.*, 1987). Using this system, 1–2 pmol of amino acid derivatives can be accurately determined.

To determine the amino acid sequence of the protein, the FDO161G antigen was cleaved with the endoprotease V8 (Boehringer Mannheim) in phosphate buffer. Under these conditions, this enzyme cleaves on the carbonyl side of both aspartic and glutamic acid residues. Peptides arising from this digest were isolated by high-performance liquid chromatography, using the following conditions: column, Deltapack C4 (Waters), 15 µm, 30 nm; 45°C; flow 1 ml/min; solvent A 0.1% trifluoroacetic acid (TFA) in water; solvent B 0.1% TFA in water:acetonitrile 30:70 (v/v); gradient 50% A to 100% B over 17.5 min.

Antiserum production

Polyclonal antiserum to the electroeluted protein was raised in sheep. The immunogen was prepared as follows; electroeluted protein was methanol precipitated and resuspended in 200 µl of 50mM ammonium bicarbonate solution containing 0.1% (w/v) SDS. The procedure of forming an emulsion with a protein containing SDS and Freund's Complete Adjuvant involved forming an emulsion between 0.3 ml of a 3M-KCl solution and 0.5 ml adjuvant using a double syringe method. The electroeluted protein (36 µg) was then blended into the preformed emulsion (V. C. Stevens, personal communication). A sheep was immunized with the resulting emulsion at 3 sites subcutaneously. Three booster injections prepared with Freund's Incomplete Adjuvant were given at weekly intervals, beginning 3 weeks after the initial immunization.

Sheep antiserum sampled at week 8 was affinity purified by passage over the electroeluted protein immobilized on Sepharose. The electroeluted protein (~1 mg) was coupled to 2 ml CNBr-activated Sepharose 4B as described for the monoclonal antibody. Antiserum diluted 1 in 5 with 20mM Tris buffer, pH 8.0 containing 150mM-NaCl and 0.02% (w/v) sodium azide was incubated with the beads overnight. After thorough washing with buffer in a column, bound antibody was eluted with glycine buffer and subsequently neutralized as described above. The efficiency of the antibody purification was examined by diaminobenzidine staining intensity on placental tissue sections (Mueller *et al.*, 1987).

Molecular cloning

A *lambda gt11* library containing 1.5×10^6 independent clones was constructed from term placental mRNA by oligo(dT)-primed reverse transcription (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989). The library was immunoscreened (Young & Davies, 1985) with affinity purified polyclonal antibodies to the 161G protein at a dilution of 1/50 (0.01 mg/ml). The second antibody was rabbit anti-sheep IgG (heavy and light chains) conjugated with alkaline phosphatase. It was used at a dilution of 1/5000 (0.14 µg/ml). Colour was developed with 5-bromo-4-chloro-3

indolyl phosphate and nitroso-blue tetrazolium (Promega Corporation: protoblot-immunoscreening system). Lytic plaques were screened with cDNA probes (Maniatis *et al.*, 1982). Probes were labelled by random priming (Feinberg & Vogelstein, 1983) with a ^{32}P dATP (3000 Ci/mmol).

Hybridization was carried out overnight at 65°C in $\times 5$ SSC $5\times$ Denhardt's solution, 0.2% SDS and 100 µg/ml salmon sperm DNA, followed by a single wash at room temperature in $1\times$ SSC, 0.1% SDS for 15 min and 2 washes at 65°C in $1\times$ SSC 0.1% SDS each for 15 min.

Polymerase chain reaction (PCR). Selected plaques were picked as BBL-agarose plugs (Maniatis *et al.*, 1982), which were twice freeze-thawed in 50 µl H_2O to release phage DNA. The supernatant was used as a source of template for PCR reactions, which were carried out in 50 µl final volume in Taq buffer (50mM-KCl, 100mM Tris-HCl pH 8.4/15mM-MgCl₂/0.1% gelatin), containing 8 µl of a dNTP solution (1.25mM dGTP/1.25mM dCTP/1.25mM dATP/1.25mM dTTP) and 1 µM of PCR primers (Clontech Cat: 6451-1 and 6461-1) that prime from the lambda arms on either side of the EcoRI cloning site. 1 unit of Taq polymerase was used per reaction. Annealing was carried out for 0.5 min at 50°C and extension at 72°C for 2 min.

Dideoxy DNA sequencing. Lambda gt11 inserts were cloned into pUC18/19 and sequenced with a Sequenase kit, USB version 2. Subclones of inserts were sequenced using primers both to pUC and to sections of the cDNA inserts.

Transfection of COS-7 cells and panning. Full length cDNA clones were subcloned into the EcoRI polylinker site of pcDNA1 (Invitrogen), a vector based on pCDM8 of Seed & Aruffo (1987). Constructs were amplified in *Escherichia coli* MC1061/P3 cells and purified plasmid was transfected into COS-7 cells by DEAE dextran (Lopata *et al.*, 1984; Ausubel *et al.*, 1987). For panning (Seed & Aruffo, 1987), bacteriological culture dishes (Falcon) were prepared using an affinity-purified, goat antimouse Ig (Sera-Labs). The transfected COS cells were incubated with 1/10–1/50 dilutions of the first antibody FDO161G in PBS/EDTA 1mM/0.02% NaN₃ at 4°C for 30 min. Unbound antibody was separated by centrifugation through 2% Ficoll 400. The cells were resuspended in PBS/EDTA/5% FCS and placed in the panning dishes. Cells with first antibody bound to their surface were complexed with second antibody and retained on the panning dishes.

Immunoperoxidase staining of freeze-dried paraffin embedded sections

For freeze-dried tissue sections, first trimester human placentas were processed for freeze drying (Stein *et al.*, 1985). Frozen tissue blocks were freeze dried over phosphorus pentoxide at -45°C and 2.66 Pa for 45 h. Tissue was then vacuum-embedded in paraffin wax at 60°C for 2 h. Sections 5 µm thick were cut. After dewaxing in xylene for 10 min followed by 5 min in acetone, the sections were kept wet with Tris-buffered saline before incubation with the respective monoclonal antibody. For thick skin, frozen sections (5 µm) were cut. The tissue was obtained within 6 h post-mortem, with consent. Tissues were frozen in liquid nitrogen, and used either immediately or stored at -70°C for no longer than 2 months.

A monoclonal antibody (FDO114G) produced in our laboratory, which reacts with Type IV collagen, acted as a positive control. All sections were processed for immunoperoxidase staining as described by Mueller *et al.* (1987). Sections were counterstained with Mayer's haemalum and mounted with DePex (Gurr).

Staining cytospun COS cells. After cells had been cytospun onto microscope slides and fixed with acetone for 15 s, they were allowed to rehydrate in TBS (Tris-buffered saline, pH 7.4) for 5 min. After removing excess TBS, 100 µl of diluted antibody 1/10 in TBS was added to each slide, which was then incubated for 15–30 min at room temperature, in a humid chamber. The slides were washed twice in TBS. After removing excess TBS, 100 µl 1/50 in TBS of FITC-conjugated antimouse Ig (DAKO) was added to each slide and incubated for 20–40 min. After washing the slides in TBS, cells were mounted with 30 µl of wet mountant (glycerol/TBS (v/v)) combining propidium iodide.

Results

The lambda gt11 cDNA library was screened with affinity-purified, sheep anti-161G. Positive plaques were picked, replicated and rescreened twice. Consistently positive plaques were observed at a frequency of 10^{-5} plaques. One clone, 1/6, was 1096 bp long; it was sequenced on both strands and contained an open reading frame of 742 bp and a 354-bp untranslated region which did not terminate with a poly(A) site or poly(A) tail (Fig. 1). Clone 1/6 was used as a probe to isolate further clones from the original library by screening lytic plaques. Confirmed positive plaques were found at a frequency of 3×10^{-4} and 48 of these were assessed for insert size by PCR. Clone B3 was 1562 bp long. Restriction mapping showed that it included the sequence of clone 1/6 (Fig. 1). Clone B3 contained a complete open reading frame beginning with an AUG codon in a good

consensus sequence for translational initiation (Kozac, 1983). This was preceded by 33 bp of 5' untranslated sequence containing a stop codon and an in-frame ATG at position 28. The latter ATG was in a weaker consensus sequence for translational initiation (Kozac, 1983). The deduced amino acid sequence of the open reading frame from nucleotide 34 (Table 1) is in agreement with our *N*-terminal amino acid sequence which shows no evidence of an additional *N*-terminal dipeptide.

Fig. 1. Structure of the cDNA clones B3 and 1/6. The dark bars represent DNA sequence obtained off both strands of the respective cDNAs. The translational stop codon is shown at position 1153. The 33 base pairs upstream of the initiation codon are shown, including the inframe stop codon. Sizes are in base pairs; B = BamHI, Bx = BstXI, H = HincII, H3 = HindIII, P = PstI, S = SacI.

Table 1. Protein sequence of the human 161G/3 β -hydroxy-5-ene steroid dehydrogenase polypeptide deduced from cDNA clones 1/6 and B3. The sections underlined were also subjected to protein sequencing on purified 161G protein. The deduced and observed amino acid sequences were identical except at position 6, as shown. This discrepancy was subsequently found to be due to an ambiguity in the amino acid data

	MTGWSCLVTG H	AGGFLGQRII	RLLVKEKELK	EIRVLDKAFG	PELREEFSKI
51	QNKT ⁶ KLTVLE	GDILDEPFLK	RACQDVSVII	HTACIIDVFG	VTHRESIMNV
101	NVKG ⁶ TQLLLE	ACVQASVPVF	IYTSSIEVAG	PNSYKEIIQN	GHEEEPLENT
151	WPAPYPHSKK	LAEKAVLAAN	GWNLKNGGTL	YTCALRPMYI	YGEGRFLSA
201	SINEALNNNG	ILSSVGKFST	VNPVYVGNA	WAHILALRAL	QDPKKAPSIR
251	GQFYIISDDT	PHQSYDNLNY	TLSEKFLRL	DSRWSFPLSL	MYWIGFLLEI
301	VSFLLRPIYT	YRPPFNRIHV	TLSNSVFTFS	YKKAQRDLAY	KPLYSWEEAK
351	QKTVEWVGS	VDRHKETLKS	KTQ*		

Nine independent cDNA clones were isolated from the library by probing with clone 1/6. They represented two sequence variants at codon 338 of the open reading frame. Six clones contained the sequence CGA GAT GTG for codons 336–338; and 4 had the sequence CGA GAT TTG. The first sequence contains a *Bgl*II restriction site (AGATCT); both sequences encode the amino acid sequence *arg*, *asp* and *leu*. All the clones were sequenced for 80 bp around this site and no other sequence variation was found. There is now evidence that this variation in codon 338 is a common polymorphism (Russell *et al.*, 1991).

Clone B3 was transferred to the EcoRI site of the mammalian expression vector pcDNA1 and the construct was transfected into COS-7 cells. Control transfections were carried out using either no DNA, vector DNA alone, or CD2 (Seed & Aruffo, 1987). After 2 days the cells were extracted, cytospun onto slides, fixed and stained with FDO161G. About 5% of cells were positive for FDO161G (Fig. 2). The staining was strong; background staining was negligible. Panning with

immobilized antibody and immunofluorescence on cells in solution gave no indication that the FDO161G was on the outside of the COS cell. Sections of human skin, liver and placenta were tested with the FDO161G antibody; only placental trophoblast stained positively (Fig. 3).

Fig. 2. Immunofluorescence staining of COS-7 cells transfected with the expression vector containing B3. First antibody was FDO161G.

Discussion

We have shown that clone B3 contains an open reading frame encoding a polypeptide that can be expressed in COS-7 cells and detected with FDO161G. In-situ hybridization located the gene corresponding to clone 1/6 to chromosome 1p13.1 (Morrison *et al.*, 1991), within the region to which 3 β -hydroxysteroid dehydrogenase-5-ene-isomerase (HSD) had been localized (Berube *et al.*, 1989). We subsequently found that clones B3 and 1/6 had a nucleotide sequence identical to the open reading frame and 3' untranslated sequence of HSD (The *et al.*, 1989), with the exception of the polymorphic site in codon 338 (Russell *et al.*, 1991).

The pattern of histological staining shown by FDO161G (Mueller *et al.*, 1987; and U. W. Mueller, unpublished) agrees closely with that reported for the localization of HSD activity, namely in adrenal, ovarian follicle cumulus cells, interstitial cells of the testis and placenta (Zoller & Weisz, 1978, 1979; Ishii-Ohba *et al.*, 1986a, b; Ishimura *et al.*, 1988; Thomas *et al.*, 1989). The agreement between these immunochemical and enzymatic studies is in marked contrast to the claims that HSD activity is found in other tissues such as human skin and liver (Bongiovanni, 1984; Parks *et al.*, 1971; Rosenfield *et al.*, 1980; Pang *et al.*, 1983, 1985). No staining with FDO161G was observed in these tissues. Since the specificity of this monoclonal antibody has been demonstrated in transfected COS cells (Fig. 2), we conclude that there is little or no expression of the HSD exons described in this paper in tissues other than adrenals, gonads and placenta. We cannot rule out the

Fig. 3. Strong reactivity of FDO161G with the outer syncytiotrophoblast cell layer of first trimester placenta, detected by the immunoperoxidase technique. Freeze-dried, paraffin-embedded section; $\times 65$.

possibility that alternative enzyme activities exist in these tissues. Three different forms of HSD have been described in rat with wider tissue distributions (Zhao *et al.*, 1990, 1991). In human genomic blots probed with clone B3 we observed several clear bands of hybridization, suggesting the existence of other HSD-related sequences (D. Gaffney, A. J. Russell & R. G. Sutcliffe, unpublished). Other nonsteroidogenic enzymes have activities similar to HSD. A well-characterized example is the ability of human liver alcohol dehydrogenase containing gamma subunits (ADH gamma) to oxidize 3 β -hydroxy-5 β -steroids to 3-keto derivatives with NAD as cofactor (McEvily *et al.*, 1988).

The protein sequence of HSD raises several points of interest (Table 1). HSD uses NAD as cofactor and the sequence most similar to an NAD-binding β - α - β motif (Wierenga *et al.*, 1986) lies at the amino terminus, from Ser⁵ to Asp³⁶. The central portion of this HSD sequence is GXGGXXG (residues 10–16), which resembles the consensus: GXGXXG (Wierenga *et al.*, 1986). Variations in this GXGXXG region have been observed for yeast alcohol dehydrogenase (GXGXXA, see Wierenga *et al.*, 1986), though the sequence for human ADH α , β and γ is GXGGXG (Ikuta *et al.*, 1986). The significance of the extra residue in the HSD consensus is not clear. The isomerase activity of HSD requires NAD or NADH as an allosteric effector. We are making site-directed mutations in the GXGGXXG sequence to find out which glycines are required and whether mutations affect the dehydrogenase and isomerase activities equally. The enzyme HSD has been regarded as an integral membrane protein of the endoplasmic reticulum and is commonly purified from microsomal extracts of cells (see Ishii-Ohba *et al.*, 1986a, b; Ishimura *et al.*, 1988; Thomas *et al.*, 1989). However, the sequence of the enzyme shows little sign of typical hydrophobic α -helical transmembrane sequences. Two hydrophobic regions between residues 76–92 and 284–305 have mean hydrophobicities (mean ΔG /residue; von Heijne 1981, 1985) of -3.0 and -4.9 , respectively. Whilst the latter may be sufficiently long and hydrophobic to act as a transmembrane region, there is no hydrophobic leader-like sequence close to the amino-terminal

end. More probably the protein is either hydrophobic enough to be adsorbed to the cytoplasmic face of the endoplasmic reticulum, or some form of glycolipid modification anchors it there. Such a cytoplasmic mode of attachment may explain the ease with which enriched preparations of HSD are obtained from placental trophoblastic microvilli. A similar explanation may relate to the observation that HSD can be co-purified with mitochondria (see Ishimura *et al.*, 1988; Thomas *et al.*, 1989), even though immuno-electron microscopy has failed to detect HSD in bovine adrenal mitochondria (Ishimura *et al.*, 1988). However, this issue will depend on whether the weakly basic amino-terminal section of HSD (Table 1) can act as a leader into mitochondria.

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