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IDENTIFICATION OF THE EPITOPE OF A MONOCLONAL ANTIBODY WHICH BINDS TO SEVERAL CYTOCHROMES P450 IN THE CYP1A SUBFAMILY

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Abstract—The monoclonal antibody, 3/4/2, which was raised against purified rat cytochrome P450 isoenzyme 1A1 (CYP1A1) binds to cytochromes P4501A in many species. It was shown by immunoblotting that the antibody binds to CYP1A1 in microsomal fractions prepared from rat, mouse, rabbit, hamster and human. The antibody also binds to cytochrome P450 isoenzyme 1A2 in microsomal fractions prepared from rabbit and human, but not rat or mouse. Using purified isoenzymes in an enzyme-linked immunosorbent assay it was found that the affinity of binding to the two rabbit hydrocarbon-inducible isoenzymes is reduced compared with that for rat CYP1A1. Binding is not affected by denaturation of the antigens. The effects of chemical and enzymatic treatments on rat CYP1A1 showed that the epitope contains a trypsin-sensitive site that includes arginine, but lacks lysine. The epitope does not contain methionine, cysteine, aspartic acid or glutamic acid residues. In addition, digestion of the protein with cyanogen bromide produces a fragment of M_r 20,000 which contains the antibody binding site. By comparing the cross-reactivity of the antibody with the primary structures of CYP1A1 and 1A2 from the rat, mouse, rabbit and human, and by considering the results of the chemical and enzymatic treatments, it was possible to deduce the likely location and structure of the binding site of 3/4/2 on members of the CYP1A subfamily. It is concluded that the epitope for this antibody is Phe-Arg-His-Ser-Ser-Phe, which lies at positions 380–385 in rat CYP1A1. Further, it is predicted from a model of the tertiary structure of eukaryotic cytochrome P450 that a part of this binding site lies within a helix in the native protein.

The cytochrome P450 superfamily of isoenzymes [1] is involved in the metabolism of a diverse range of compounds, including pharmaceutical drugs, toxins and environmental pollutants, as well as endogenous compounds including steroids, prostaglandins and leukotrienes [2]. This exceptionally broad substrate specificity is achieved in part by the large number of isoenzymes of cytochrome P450 and the overlapping substrate specificities of many of the isoenzymes. It is often difficult to establish which isoenzyme(s) contributes to the metabolism of a specific substrate and the use of inhibitory antibodies has proved useful in addressing this problem [3].

Cytochrome P450 expression, content and activity have been studied using antibodies raised against purified isoenzymes [4]. Antibodies raised against synthetic peptides which mimic small regions of the apoprotein have also been shown to be useful [5]. Antisera raised against purified proteins contain a large number of antibody species which bind to a number of different sites on the immunogen. The positions of the major epitopes are largely unknown, although binding sites on some proteins have been determined [6, 7]. Monoclonal antibodies raised against a protein bind to a single site and it has been possible to locate the binding site of many of such antibodies [8]. The interaction between one antibody and its antigen has been determined in detail by X-ray crystallographic analysis of the complex [9]. However, although a number of polyclonal and monoclonal antibodies have been produced against various isoenzymes of cytochrome P450 [10], in only one case has the epitope been determined [11].

The monoclonal antibody, 3/4/2, which was raised against rat cytochrome P450 isoenzyme 1A1 (CYP1A1‡), has been found to bind to a number of members of the 1A sub-family in several species [12]. As the sequences of many isoenzymes of cytochrome P450 have now been determined [1], this raised the possibility that the binding site for this antibody could be located by comparing the primary structures of the various isoenzymes to which the antibody did and did not bind. This approach is similar to that which has been used successfully to determine the location of antibody binding sites on myoglobin, lysozyme and cytochrome *c* [6]. However, for cytochrome P450, relatively few isoenzymes have been both purified and their amino acid sequences determined, and inter-species diversity amongst isoenzymes is generally greater than that of these other proteins. Therefore, in order

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‡ Abbreviations: CYP1A1, cytochrome P450 isoenzyme 1A1; CYP1A2, cytochrome P450 isoenzyme 1A2; PBS, phosphate-buffered saline; MC, 3-methylcholanthrene; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; BSA, bovine serum albumin.

to obtain more information on the structure of the binding site, the effects of modifications of specific amino acid residues and selective proteolysis on antibody binding were also examined. By combining these two approaches it was possible to determine the location of the binding site of the monoclonal antibody 3/4/2 on cytochromes P4501A.

MATERIALS AND METHODS

Materials. All SDS–PAGE reagents were from National Diagnostics (Aylesbury, U.K.), except for pre-stained molecular mass standards which were from the Sigma Chemical Co. (Poole, U.K.). Nitrocellulose filters were obtained from Amersham International plc (Amersham, U.K.). Polystyrene 96-well microtitre plates were from Dynatech Laboratories (Billinghurst, U.K.), and conjugates of immunoglobulins with horseradish peroxidase were from ICN–Flow (High Wycombe, U.K.). Cyanogen bromide-activated Sepharose was obtained from Pharmacia (Milton Keynes, U.K.). Purified rabbit cytochrome P450 isoenzyme 1A2 (CYP1A2) was prepared by K. M. Lewis in this department and was a kind gift. Purified rabbit CYP1A1 was generously supplied by Dr E. F. Johnson (La Jolla, CA, U.S.A.). Microsomal fractions prepared from yeast that had been transfected with either pMA91 containing cDNA encoding human CYP1A1 or with pMA91 lacking this cDNA as described previously [13] were a generous gift from Dr M. Ching (Sheffield, U.K.). All other chemicals were purchased from Sigma or BDH (Dagenham, U.K.) and were of analytical grade or the best equivalent.

Production and purification of the monoclonal antibody. The monoclonal antibody, 3/4/2, was produced from hybridoma cells produced by the fusion of myeloma cells with spleen cells obtained from a mouse immunized with purified rat CYP1A1, as described previously [12]. Actively secreting, exponentially growing hybridoma cells were used to inoculate pristane-treated mice by intraperitoneal injection and the ascites collected as a source of the antibody. The monoclonal antibody was precipitated from the ascites by addition of ammonium sulphate to 50% saturation. The precipitate was collected following centrifugation at 950 *g* for 30 min at 4° and was then washed twice in 50% saturated ammonium sulphate. The washed precipitate was dissolved in a minimal volume of 20 mM triethanolamine–HCl, pH 7.7 and then dialysed four times against 100 volumes of the same buffer. The dialysed material was loaded onto a chromatography column packed with Q-Sepharose Fast Flow ion-exchange resin (22 × 300 mm) equilibrated in the triethanolamine buffer. The column was washed with the triethanolamine buffer to which 35 mM NaCl was added and then the antibody was eluted by the inclusion of 52.5 mM NaCl in the buffer. The antibody was dialysed against phosphate-buffered saline (PBS: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.5) before use. The product was >95% pure as determined by SDS–PAGE and analytical Fast Protein Liquid Chromatography.

Cytochrome P450 purification. The purification of CYP1A1 and CYP1A2 from the hepatic microsomal fraction of rats treated with 3-methylcholanthrene (MC) has been described previously [14]. The isoenzyme preparations were 85–90% pure, as determined by SDS–PAGE and each was free from contamination by the other form, as judged by immunoblotting using a monoclonal antibody which binds to both isoenzymes [14].

Immunological analyses. Enzyme-linked immunosorbent assay (ELISA) was performed by coating the wells of microtitre plates with either purified cytochrome P450 or hepatic microsomal fraction, as described previously [14]. Antibody binding was detected using rabbit anti-mouse IgG or goat anti-rabbit IgG, as appropriate, coupled to horseradish peroxidase. Peroxidase activity was measured using *o*-phenylenediamine and hydrogen peroxide as co-substrates.

Immunoblotting was performed as described previously [14]. Microsomal fractions prepared from liver samples were subjected to SDS–PAGE using 8.5% (w/v) acrylamide gels. Cyanogen bromide-treated proteins were separated on 15% (w/v) acrylamide gels. The proteins or protein fragments were than transferred onto a nitrocellulose filter using a Trans-Blot Cell (Bio-Rad, Hemel Hempstead, U.K.). After transfer, the filter was soaked in a solution of 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS: 20 mM Tris–HCl, 130 mM NaCl, pH 7.4), to reduce non-specific binding, then incubated for 2 hr at room temperature with 1 mg of 3/4/2 diluted in 10 mL of TBS containing 0.1% (w/v) BSA. The filter was then washed five times with TBS containing 0.05% (w/v) BSA and 0.05% (v/v) Tween 20. The binding of 3/4/2 was visualized by incubating the filter for 1 hr at room temperature with rabbit anti-mouse IgG coupled to horseradish peroxidase diluted 1:2000 in TBS containing 0.1% (w/v) BSA. After washing the filter as described above, the peroxidase was detected by the coloured precipitate produced using 4-chloro-1-naphthol and hydrogen peroxide as co-substrates.

Dot-blotting was performed using a Bio-Dot Apparatus (Bio-Rad) by applying samples of 5 μg of microsomal protein in 50 μL of TBS to a nitrocellulose filter. After sample application, the filter was blocked with 3% (w/v) BSA in TBS, then treated with chemical or enzymatic agents as described below. The binding of 3/4/2 was then determined in the same way as described above. The intensity of staining was determined by scanning densitometry using a LKB UltroScan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology, Milton Keynes, U.K.).

Chemical modifications of amino acids and proteolysis. Microsomal protein prepared from rats treated with MC was subjected to reduction by 2-mercaptoethanol and carboxymethylation using iodoacetic acid as described by Crestfield *et al.* [15]. The treated protein was purified from the reactants by gel-filtration chromatography using a Sephadex G-25 column (22 × 300 mm) equilibrated with 0.01 M ammonium bicarbonate. The fractions containing protein were pooled and lyophilized.

Digestion of S-carboxymethylated microsomal

protein by cyanogen bromide was achieved by dissolving 1 mg of protein in 1 mL of 70% (v/v) formic acid and to this was added 4 mg of cyanogen bromide. The reaction mixture was stirred for 18 hr at room temperature. The product was diluted by the addition of 5 mL of deionized water and then lyophilized.

After the application of microsomal protein to nitrocellulose filters for dot-blotting, as described above, the bound protein was subjected to a variety of chemical and enzymatic treatments, as follows:

(i) Free amino groups of the bound proteins were derivatized using maleic anhydride by incubating the filter in 40 mL of 0.2 M sodium phosphate buffer, 8 M urea, pH 9.0 and adding 0.4 g of solid maleic anhydride gradually over 30 min. During this time the pH of the solution was monitored and maintained at pH 9.0 by the addition of 2 M sodium hydroxide. The filter was then washed five times with TBS.

(ii) Arginine groups were temporarily blocked by incubating the nitrocellulose membrane in 0.05 M phenylglyoxal dissolved in 0.1 M *N*-ethylmorpholine acetate, pH 8.0 for 90 min at room temperature. After treatment with trypsin, as described below, phenylglyoxal was removed from the arginine groups by washing the nitrocellulose filter overnight in 0.1 M *N*-ethylmorpholine acetate, pH 8.0.

(iii) Enzymatic hydrolysis was performed using 0.2 mg of enzyme dissolved in 10 mL TBS. Hydrolysis with trypsin (treated with L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone) from bovine pancreas was for 1 hr at room temperature. Treatment with endoproteinase Glu-C (protease from *Staphylococcus aureus* strain V8) was for 20 hr at room temperature, with fresh enzyme added after 4 hr. The respective filters were then washed five times in TBS. In the case of trypsin, 1 mM phenylmethyl sulphonyl fluoride was added to the washing buffer.

After treatment by one, or a combination, of the above methods, the binding of 3/4/2 to the microsomal protein was determined in the same way as described above for filters used in immunoblotting.

Treatment of animals and preparation of hepatic microsomal fractions. Male Wistar rats (200–250 g) were obtained from Harlan Olac Ltd (Bicester, U.K.) and male New Zealand White rabbits (3 kg) were from Froxfield Farms Ltd (Petersfield, U.K.). Male Balb/c mice (6–10 weeks) and Dunkin–Hartley guinea pigs (250–300 g) were from A. Tuck & Son Ltd (Battlebridge, U.K.), and male Golden Syrian hamsters (90–110 g) were supplied by Belgrave Trading Ltd (Lambourn, U.K.). Animal husbandry was as described previously [16]. Groups of animals were left untreated or were administered MC, dissolved in corn oil, at a dose of 80 mg/kg as a single intraperitoneal injection, 48 hr before death. Animals were killed humanely in accordance with approved Home Office procedures appropriate to the species, the livers were rapidly removed and microsomal fractions prepared as described previously [17]. Human liver microsomal samples were prepared similarly from tissue obtained from the human tissue bank at the Royal Postgraduate Medical School [17]. Local Ethics Committee approval and Coroner's permission were obtained for the use of such samples in these studies. The characterization of the human liver microsomal fractions has been reported previously [18].

Computer analyses. The computer programs used were written in BASIC and run on an IBM-AT compatible microcomputer.

Protein sequences. The cytochrome P450 amino acid sequences were obtained by electronic transfer from the European Molecular Biology Library, Heidelberg, Germany.

RESULTS

The binding of 3/4/2 to liver microsomal fractions prepared from various species treated with MC and to human liver microsomes is shown in Fig. 1. The antibody bound to a single band corresponding to CYP1A1 [19] in rats treated with MC, but not in untreated rats. Similar results were obtained in the mouse, the antibody binding to a single protein band in the MC-treated mouse, corresponding to CYP1A1 [19], but not to microsomal fractions prepared from the livers of untreated mice. In the rabbit, the antibody bound to two protein bands, corresponding to CYP1A1 and 1A2 [19], in both untreated and, with greater intensity, MC-treated animals. The antibody also bound to one protein band in the untreated hamster, and after treatment with MC the intensity of this band, which corresponds to CYP1A1 [19], was considerably increased. However, in the guinea pig, the binding of the antibody to a single protein band was more intense in untreated animals compared with those treated with MC. In addition, the antibody bound to a single band in human liver microsomes, previously identified as CYP1A2 [20].

The antibody also recognized human CYP1A1. This was shown by immunoblotting of the microsomal fraction prepared from yeast which had been transfected with a vector containing cDNA encoding human CYP1A1 (Fig. 2). Monoclonal antibody 3/4/2 did not recognize any protein in the microsomal fraction prepared from yeast which had been transfected with the same vector as above, but lacking the cDNA encoding human CYP1A1 (Fig. 2). The M_r of human CYP1A1 and 1A2 were very similar, although CYP1A1 appeared to be slightly heavier than CYP1A2 (Fig. 2), as predicted from their deduced amino acid sequences [21, 22].

The relative affinity of 3/4/2 for purified isoenzymes of cytochrome P450 from the rat and rabbit was determined by ELISA (Fig. 3). Binding was strongest to the immunizing protein, rat CYP1A1, and there was no binding to rat CYP1A2. Binding to rabbit 1A1 and 1A2 was about equal, but substantially lower than to rat 1A1 (Fig. 3a). Denaturation of the isoenzymes prior to and during their application to the microtitre plate using 8 M urea did not effect the subsequent binding of 3/4/2 (Fig. 3b).

These results show that 3/4/2 bound not only to rat CYP1A1, but also to one or both members of the CYP1A sub-family in other species. However, the epitope in the rabbit isoenzymes was clearly not identifical to that in rat CYP1A1. Strong binding to mouse CYP1A1 was observed in immunoblotting, but as a purified preparation of this isoenzyme was

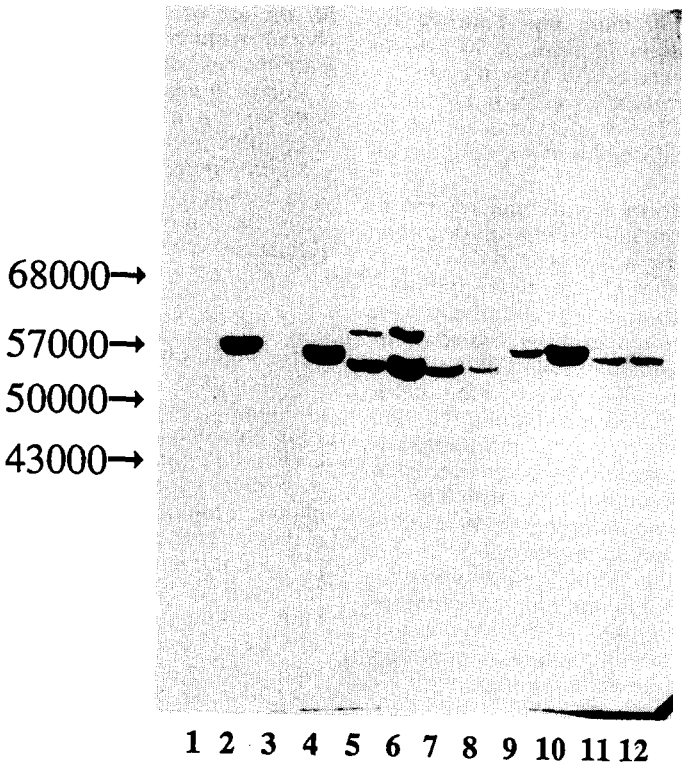

Fig. 1. Immunoblot showing the binding of monoclonal antibody 3/4/2 to cytochromes P450 from various species. Microsomal fractions were prepared from the livers of either untreated animals or following their treatment with MC. To each lane, 20 μg of protein was applied from untreated rat (lane 1), MC-treated rat (lane 2), untreated mouse (lane 3), MC-treated mouse (lane 4), untreated rabbit (lane 5), MC-treated rabbit (lane 6), untreated guinea pig (lane 7), MC-treated guinea pig (lane 8), untreated hamster (lane 9), MC-treated hamster (lane 10), human sample 03009 (lane 11), or human sample 03007 (lane 12). The immunoblot was developed with the monoclonal antibody 3/4/2 as described in the text.

not available relative binding strength could not be determined. Similarly, binding to human CYP1A1 and 1A2 was observed in immunoblotting, but as purified forms of these isoenzymes were not available quantitative binding of the antibody could not be assessed.

The binding of 3/4/2 to rat CYP1A1 was not affected by S-carboxymethylation of cytochrome P450 as shown by immunoblotting and dot-blot experiments (Table 1). Further, hydrolysis of CYP1A1 by cyanogen bromide did not destroy the epitope, although there was a reduction in the M_r of the immuno-positive band to 20,000 (Fig. 4).

The S-carboxymethylated protein was applied to nitrocellulose filteres by dot-blotting and the effect of various chemical reagents and enzymatic hydrolyses on the binding of 3/4/2 was examined (Table 1). Treatment with trypsin destroyed the epitope, thus indicating the presence of lysine and/or arginine residues in the antibody binding site. Derivatization of lysine residues with maleic anhydride did not abolish 3/4/2 binding, nor did it alter the effect of trypsin, suggesting that lysine was not involved in binding. However, the addition of phenylglyoxal, which blocks arginine groups, did prevent antibody binding and also protected the epitope against tryptic digestion. Thus, it was likely that there was an arginine residue in the epitope. Endoproteinase Glu-C did not affect antibody binding, indicating that neither aspartic acid nor glutamic acid formed part of the epitope. The reason for the decrease in 3/4/2 binding after treatment with maleic anhydride and after treatment with phenylglyoxal followed by trypsin is not known, but might involve the modification of amino acids adjacent to the antibody binding site.

A computer algorithm was devised to predict the location of the 3/4/2 binding site on rat CYP1A1. The amino acid sequences of CYP1A1 and CYP1A2 in rat, rabbit and mouse, and CYP1A2 in human were compared on the basis of the reactivity of the monoclonal antibody with them. It was assumed that the epitope comprised a continuous sequence of amino acid residues, as disruption of the antigen's structure by 0.1% SDS (Fig. 1) or 8 M urea (Fig. 3) had no effect on antibody binding. As the size (e) of the epitope was not known analyses were performed setting $e = 4$–7 amino acid residues. First, the sequences of rat, rabbit, mouse and human CYP1A1 and CYP1A2 were aligned using the

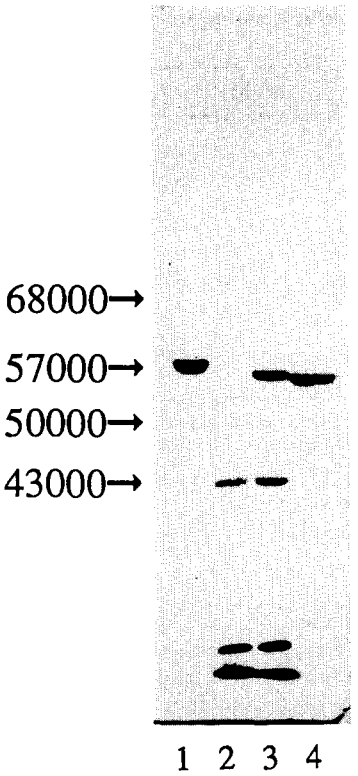

Fig. 2. Immunoblot of human CYP1A1 and CYP1A2 using the monoclonal antibody 3/4/2. To a SDS–polyacrylamide gel (8.5% w/v), the following quantities of microsomal protein were applied: 20 μg from the liver of rats treated with MC (lane 1), 75 μg from yeast transfected with pMA91, but lacking cDNA encoding human CYP1A1 (lane 2), 75 μg from yeast transfected with pMA91 containing cDNA encoding human CYP1A1 (lane 3), and 50 μg from human liver sample 03007 (lane 4). The immunoblot was developed with the monoclonal antibody 3/4/2 as described in the text. Several low M_r bands in the microsomal samples prepared from both yeast preparations can be seen. This was found to be due to binding of the detecting antibody (rabbit anti-mouse horseradish peroxidase), to these proteins. In the absence of 3/4/2 the low M_r bands were still observed, but there was no binding to cytochrome P450.

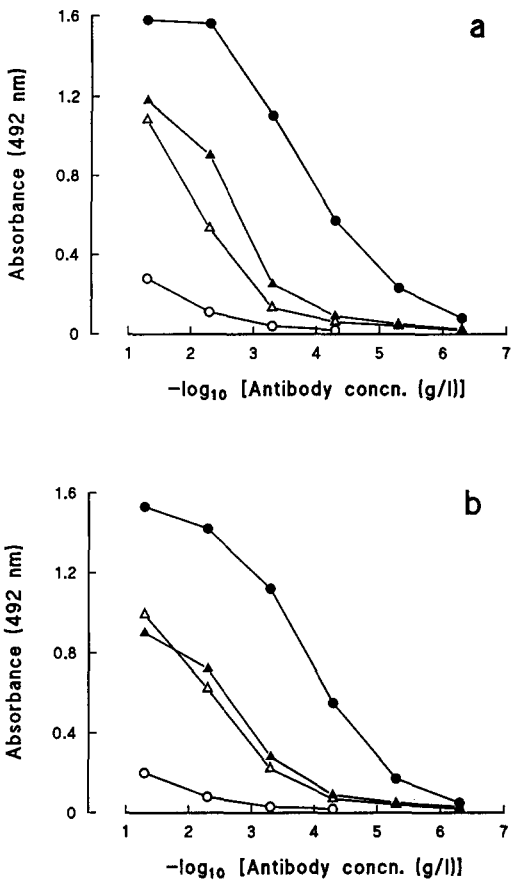

Fig. 3. Relative binding of monoclonal antibody 3/4/2 to purified cytochromes P450. Microtitre plates were coated with rat CYP1A1 (●), rat CYP1A2 (○), rabbit CYP1A1 (▲), or rabbit CYP1A2 (△), in either PBS (a) or PBS containing 8 M urea (b). To each group of wells a series of dilutions of 3/4/2 was added and relative binding was determined as described in the text.

program MULTALIN [23]. The sequence of rat CYP1A1 was then compared with the other sequences. For each value of e, i.e. the number of contiguous amino acids stipulated to form the binding site, comparisons were made between rat CYP1A1 and each of the other isoenzymes throughout the complete length (l) of the aligned proteins at each sequence position (i), from $i = 1$ to $i = l - e + 1$. At each sequence position and for each isoenzyme, the residues from position i to $i + e - 1$ were examined and those that were identical to the corresponding residue in rat CYP1A1 scored 1. Hence, the maximum possible score for any position was e, i.e. where a region was identical to that in rat CYP1A1.

Regions were then sought which fitted a number of criteria relating to the results of immunoblotting and ELISA. Three possible strengths of interaction of the antibody with the various antigens were considered. The first was maximum binding, as found for the immunogen, rat CYP1A1. Such regions had to have a score of e. The second was intermediate binding strength, where the binding was weaker than that of the antibody to the immunogen. These regions were considered to have a score of $e - 1$ for such binding. Finally, where no binding was seen then a significant mismatch score of less than $e - 1$ had to be obtained. From the results of immunoblotting and ELISA the following conditions were defined. (1) There was a significant mismatch between rat CYP1A1 and both rat and mouse CYP1A2. (2) There was intermediate binding strength to the two rabbit isoenzymes. (3) There was definite binding to mouse CYP1A1, human CYP1A1 and human CYP1A2. However, as it was not established in any of these cases whether the binding was maximum or intermediate, it was initially assumed that binding was of intermediate strength.

The computer algorithm was designed to search

Table 1. Effects of treatment of microsomal protein with various reagents on 3/4/2 binding in dot-blotting experiments

Treatment	Antibody binding (A.U. × mm)*	Inference
None	1.136 (0.127)	—
Carboxymethylation	0.966 (0.075)	No cysteine
Trypsin	0.045 (0.031)	Arginine and/or lysine
Maleic anhydride	0.585 (0.094)	No lysine
Maleic anhydride, then trypsin	0.054 (0.030)	No lysine
Phenylglyoxal	0.060 (0.013)	Arginine
Phenylglyoxal, trypsin treatment, then removal of phenylglyoxal	0.741 (0.033)	Arginine
Endoproteinase Glu-C	0.963 (0.066)	No aspartic acid or glutamic acid

Binding of 3/4/2 was detected using anti-mouse-IgG horseradish peroxidase and developed in the presence of horseradish peroxidase substrates as described in the text. The intensity of the dots after development was determined by scanning laser densitometry.

* Values are means ± SD, N = 5.

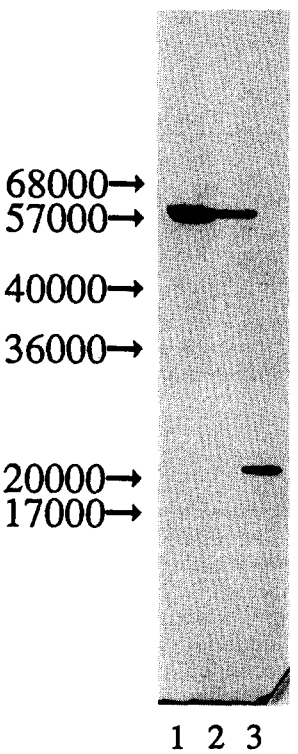

Fig. 4. Immunoblot of rat CYP1A1 following hydrolysis with cyanogen bromide. To each lane, 20 μg of liver microsomal protein from MC-treated rats (lane 1), S-carboxymethylated microsomal protein (lane 2), or cyanogen bromide treated S-carboxylmethylated microsomal protein (lane 3) were applied to a SDS–polyacrylamide gel (15% w/v). After electrotransfer of proteins, the immunoblot was developed with 3/4/2 as described in the text.

through the amino acid sequences and find regions which fulfilled the criteria stated above. Once such regions were identified, any which overlapped with one another were combined to form a larger region. In the simplest version of the algorithm, those regions that fulfilled conditions (1), (2) and (3) were searched for. There were four regions which met these criteria (Table 2). When it was stipulated that binding to mouse CYP1A1 was as strong as to rat CYP1A1, only three regions were selected (Table 2). However, if antibody binding to either human CYP1A1 or 1A2 was considered as maximal then only one region fulfilled all of the criteria of the algorithm (Table 2). Finally, when it was stipulated that binding to mouse CYP1A1, human CYP1A1 and human CYP1A2 was maximal, this identified the same single region as selected above (Table 2).

In general, consideration of values between 4 and 7 for the variable *e* did not affect the predicted epitope size to any great extent (Table 2). This was because overlapping regions were combined to form one region. The majority of the results indicated that the binding site lies within the region 376–385.

The results of specific modifications and hydrolyses of rat CYP1A1 were then considered in the determination of the likely 3/4/2 epitope. Each of the putative antibody binding regions selected by the algorithm was examined for the presence of a trypsin-sensitive site including arginine, but lacking lysine. In addition, there had to be no methionine, cysteine, aspartic acid or glutamic acid residues. Any region not meeting these criteria was rejected. This resulted in the selection of just one region. This was at residues 380–384, or 380–385, depending on the epitope size used in the algorithm. When the epitope size was increased to seven residues, no region that fulfilled all of the criteria of the algorithm was found. When differences in the strength of binding of the antibody were considered, as in the second and third variations of the algorithm, there was no change in the outcome of the epitope identification (Table 2).

DISCUSSION

It has been shown by ELISA and immunoblotting

Table 2. Predictions of the location and structure of the epitope of the monoclonal antibody 3/4/2 on rat CYP1A1

Binding site size (residues)	Basic algorithm	Effect of considering that the epitope on mouse CYP1A1 is identical to that on rat CYP1A1	Effect of considering that the epitope on either human CYP1A1 or human CYP1A2 is identical to that on rat CYP1A1	Effect of considering that the 3/4/2 epitope is identical on CYP1A1 in the rat, mouse and human, and on human CYP1A2
Consideration of immunoblotting/ELISA results only				
4	234–237 SGYP	234–237 SGYP		
	377–385 LETFRHSSF	377–385 LETFRHSSF	377–385 LETFRHSSF	377–385 LETFRHSSF
	501–504 TLKH	501–504 TLKH		
5	376–385 ILETFRHSSF	376–385 ILETFRHSSF	376–385 ILETFRHSSF	376–385 ILETFRHSSF
	500–504 LTLKH	500–504 LTLKH		
6	326–331 ITTAIS			
	376–385 ILETFRHSSF	376–385 ILETFRHSSF	376–385 ILETFRHSSF	376–385 ILETFRHSSF
	499–504 GLTLKH	499–504 GLTLKH		
7	325–332 TITTAISW			
	376–382 ILETFRH	376–382 ILETFRH	376–382 ILETFRH	376–382 ILETFRH
	498–504 YGLTLKH	498–504 YGLTLKH		
Inclusion of chemical/enzymic treatment results				
4	380–384 FRHSS	380–384 FRHSS	380–384 FRHSS	380–384 FRHSS
5	380–385 FRHSSF	380–385 FRHSSF	380–385 FRHSSF	380–385 FRHSSF
6	380–385 FRHSSF	380–385 FRHSSF	380–385 FRHSSF	380–385 FRHSSF
7	None	None	None	None

that monoclonal antibody 3/4/2 is able to bind to microsomal fractions prepared from various species and purified isoenzymes ([12]; present study). Amongst those species studied the amino acid sequences of rat, rabbit, mouse and human CYP1A1 and CYP1A2 have been determined. By comparing the reactivity of 3/4/2 for the various isoenzymes with their primary structure, an attempt was made to determine the structure of the 3/4/2 epitope. Using the information that the antibody bound to rat CYP1A1, mouse CYP1A1, human CYP1A1 and human CYP1A2, but not to CYP1A2 in the rat or mouse, and that the antibody bound with reduced affinity to CYP1A1 and CYP1A2 in the rabbit, four possible antibody binding regions were identified.

It was not possible to determine the relative affinity of the antibody for mouse CYP1A1, human CYP1A1 or human CYP1A2, as purified forms of these isoenzymes were not available to us. If it was assumed that the antibody bound equally well to rat and mouse CYP1A1 then the number of predicted epitopes was reduced by only one, to three regions. However, if it was assumed that antibody binding to either human CYP1A1 or 1A2 was maximal, then just one region was predicted as the 3/4/2 binding site. The expression of CYP1A1 appears to be very low in human tissues. Aryl hydrocarbon hydroxylase activity is induced in the microsomal fraction of placentae of mothers who regularly smoke cigarettes during pregnancy [20]. However, the level of CYP1A1 in these samples is still too low to be detected using 3/4/2 (unpublished observations). Here, binding of 3/4/2 to human CYP1A1 was clearly demonstrated using yeast which had been transfected with cDNA encoding this isoenzyme [13]. However, this does not allow the strength of binding of the antibody to this isoenzyme to be determined. The level of CYP1A2 in human liver microsomal fractions has been determined using 3/4/2, with purified rat CYP1A1 as standard. The levels of CYP1A2 in non-smokers and smokers were 4.7 and 16.3 pmol rat CYP1A1 equivalents/mg protein, respectively [24]. Although Shimada *et al.* [25] found somewhat higher levels of CYP1A2 in human liver samples, in the range 10 to 140 pmol/mg protein, the levels found by Sesardic *et al.* [24] were in agreement with those found by McManus *et al.* [26], which were between 1.1 and 9.1 pmol/mg protein. If 3/4/2 binds to human CYP1A2 less than maximally, as seen with the rabbit isoenzymes, then it is extremely unlikely that human CYP1A2 could be detected by the antibody at these levels (<20 pmol/mg). Thus, it seems likely that 3/4/2 binds to human CYP1A2 as strongly as it does to rat CYP1A1.

The identity of the predicted epitope was confirmed by the stability of the binding site to specific chemical modifications and to enzymatic and chemical hydrolyses of antigen. When this information is used in conjunction with the results of ELISA and immunoblotting in the simplest version of the algorithm, only one region is identified as a possible binding site, and the refinements considered have no effect on this prediction (Table 2). This region, 380–385, fits all of the experimental evidence, i.e. it contains a trypsin-hydrolysable arginine group, but lacks lysine, methionine, cysteine, aspartic acid and glutamic acid. In addition, the position of the methionine residues in the sequence of CYP1A1 means that this region is predicted to occur in an immunoreactive fragment of M_r 19,000 (comprising residues 336–482) following cyanogen bromide

Table 3. Aligned cytochrome P450 sequences in the predicted epitope region

Isoenzyme	Sequence*	Sequence position	Reference
Rat CYP1A1	-Phe-Arg-His-Ser-Ser-Phe-	380–385	[27]
Mouse CYP1A1	- - - - - - -	377–382	[28]
Rabbit CYP1A1	- - - -Thr- - -	380–385	[29]
Human CYP1A1	- - - - - - -	376–381	[21]
Trout CYP1A1	- - - - - - -	381–386	[30]
Rat CYP1A2	-Tyr- -Tyr-Thr- - -	374–379	[31]
Mouse CYP1A2	-Tyr- -Tyr-Thr- - -	374–379	[28]
Rabbit CYP1A2	- - - -Thr- - -	375–380	[32]
Human CYP1A2	- - - - - - -	376–381	[22]
Hamster CYP1A2	-Tyr- -Tyr-Thr- - -	374–379	[33]

* Only those residues differing from the sequence in rat CYP1A1 are shown.

cleavage, in agreement with the observed fragment size of M_r 20,000. The putative epitope in rat CYP1A1 is identical to that found in mouse 1A1, human 1A1 and human 1A2 (Table 3). It is also very similar to the epitope region in the rabbit isoenzymes, which differs by a single amino acid, from Ser to Thr, in both forms (Table 3). The equivalent regions in rat CYP1A2 and mouse CYP1A2 differ by three amino acid residues from rat CYP1A1 (Table 3).

As cytochromes P450 in the CYP1A sub-family from different species are sequenced it will be possible to test this prediction further. For example, in immunoblotting there was clear binding to a protein in the microsomal fraction prepared from MC-treated hamster liver, most likely CYP1A1 [19]. This isoenzyme has yet to be sequenced. However, it is predicted that the 3/4/2 epitope will be strongly conserved in this protein. The antibody does not bind to hamster CYP1A2, consistent with the fact that there are three amino acid differences in the proposed 3/4/2 binding region (Table 3). Also, the sequence of trout CYP1A1 has been determined and the predicted 3/4/2 binding site is identical to that seen in the rat (Table 3). Thus, it is expected that 3/4/2 will bind strongly to trout CYP1A1.

Amongst the species examined here, the epitope for 3/4/2 on CYP1A1 and, to a lesser extent, CYP1A2 is well conserved. The unusual ability of this monoclonal antibody to bind to a number of cytochromes P450 indicates that the epitope lies at a conserved region. Such a region may also have some importance in the structure or the function of the protein. However, examination of the amino acid sequences of these isoenzymes shows that conservation of this region is far from unique. Hence, it is not known if the conservation of this region has any particular significance.

We have produced a model of the three-dimensional structure of cytochrome P450 [34] based on homology of the α-helices of the eukaryotic cytochromes P450 with the structure of cytochrome $P450_{cam}$ (CYP101) which has been determined by X-ray crystallography [35]. Although this model has yet to be refined, it was possible to locate the 3/4/2 binding site at the *C*-terminal end of the K-helix and the adjacent turn region. This region is likely to be on the surface of the protein and accessible for antibody binding, as would be expected for an immunogenic site. Previous workers have shown that it is possible to produce antibodies which bind to helical regions of proteins [36, 37].

Ten binding sites for anti-peptide antibodies have been located on the model of eukaryotic cytochromes P450 [38]. Antibody binding to only three of these regions results in inhibition of enzyme activity [5, 39, 40]. In the model it can be seen that these three regions are adjacent to one another. The predicted binding site for 3/4/2 is not close to this general region and is not pro-inhibitory as expected from its location.

We have successfully raised anti-peptide antibodies to CYP1A1 [14, 38, 40] and to other isoenzymes [5, 14, 38]. These antibodies were all targeted to regions which were likely to be in hydrophilic loops. The predicted epitope for 3/4/2 does not fall within such a region [5, 41]. Hence, it was expected that anti-peptide antibodies directed to this region of CYP1A1 would not bind strongly, if at all, to the protein. Antibodies were successfully raised against a peptide encompassing this region, but they did not bind to CYP1A1 (results not shown). One explanation for the lack of binding of the anti-peptide antibodies is that the preferred structure adopted by the peptide is not the same as the conformation of the region in the intact protein, as suggested previously for other peptides [36, 42]. We also found that, compared with its binding to protein antigens, 3/4/2 bound relatively weakly to short synthetic peptides which contain the epitope (results not shown). These results suggest that the conformation of the antibody binding region of the protein may be quite stable and resistant to denaturation conditions. However, short flexible peptides do not appear to accurately mimic the structure of the epitope on the protein.

In conclusion, the relatively broad binding specificity of the monoclonal antibody, 3/4/2, to cytochromes P4501A which have been sequenced provided sufficient information to determine the location of the antibody binding site. Studies on the stability of the epitope to chemical/enzymatic

treatments provided confirmatory evidence. It should be possible to determine the location of other antibody binding sites in a similar manner provided that the sequences of related proteins are known.

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