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SHORT SYNTHETIC PEPTIDES EXPLOITED FOR RELIABLE AND SPECIFIC TARGETING OF ANTIBODIES TO THE C-TERMINI OF CYTOCHROME P450 ENZYMES

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Abstract—An antibody was raised against a synthetic peptide (Ser-Glu-Asn-Tyr-Lys-Asp-Asn) corresponding to residues 290–296 of the cytochrome P450 enzyme, CYP1A2, of both rat and mouse. A cysteine residue attached to the N-terminus of the peptide during synthesis allowed coupling in a specific orientation via the thiol group to the carrier protein, keyhole limpet haemocyanin. Antiserum raised in rabbits bound specifically to CYP1A2 in the rat and mouse. To determine those amino acid residues involved in binding of the antibody, related peptides of various lengths were synthesised and the binding of the antibody was determined by an enzyme-linked immunosorbent assay. These studies show that the minimum epitope is the C-terminal tripeptide sequence, Lys-Asp-Asn. Other than in rat and mouse CYP1A2, this tripeptide is found as an internal sequence in a large number of proteins including bovine fibronectin, chicken gizzard myosin heavy chain, and the P450 enzymes, rabbit CYP3A6 and human CYP3A4, but the antibody did not bind to any of these proteins. However, the antibody did bind to yeast glucose-6-phosphate dehydrogenase in which the tripeptide sequence is the C-terminus. Antibodies raised against a truncated peptide (Tyr-Lys-Asp-Asn), representing the C-terminal half of the peptide, also bound to glucose-6-phosphate dehydrogenase, but failed to bind to CYP1A2; thus although the C-terminal region of the peptide 290–296 is strongly immunogenic, it appears that it is not this population of antibodies that binds to CYP1A2. As antibodies were found to bind strongly to the C-terminus of glucose-6-phosphate dehydrogenase, the C-termini of proteins as targets for anti-peptide antibodies were investigated further by immunising rabbits with four 5-residue peptides which represent the C-termini of the P450 enzymes, CYP1A1, CYP1A2, CYP2E1 and CYP2A6. The peptides were coupled to keyhole limpet haemocyanin through their N-termini via cysteine residues added to the sequences. All four antisera bound specifically to their respective target proteins, as demonstrated by immunoblotting using hepatic microsomal fractions from rat, rabbit and human. It is suggested that this method of antibody production could be of general use for the reliable production of antisera against proteins where their sequence at the C-terminus is known, and such antibodies can be highly specific as they do not bind to internal sequences.

The knowledge that epitopes for antibodies against proteins comprise only a few amino acids led to an appreciation that it was possible to raise protein-reactive antibodies using short synthetic peptides conjugated to carrier protein [1]. The use of this "anti-peptide approach" for the generation of antibodies against protein antigens has become widespread. Antibodies produced by this method have a number of advantages over more conventional methods of raising anti-protein antibodies [2]. These include overcoming the need to purify the protein for use as an immunogen and the ability to direct antibodies to defined regions of the target protein. The specificity of anti-peptide antibodies depends on the primary sequence of the peptide used for immunisation and whether this or similar sequences occur in other proteins. In theory, such binding should be predictable provided that the primary structure of relevant proteins is known. In contrast, for an antiserum produced against a purified protein the potential for cross-reactivity with other proteins is much greater owing to the diversity of antibody populations present which bind to multiple epitopes on protein antigens. In addition, there may be antibodies against impurities in the preparation used to immunise animals, which cannot be removed owing to the lack of suitable immunosorbents. To a large extent the cross-reactivity of all such antibodies is unpredictable. As monoclonal antibodies bind to a single epitope, specificity depends on the occurrence of the epitope, or similar epitopes, on other proteins. This often results in highly specific antibodies. Nevertheless, unexpected cross-reactivity of monoclonal antibodies is frequently observed [e.g. Refs. 3–5].

We have applied the use of anti-peptide antibodies to study P450‡ enzymes. This superfamily of haemoprotein enzymes [6] plays a central role in the disposition of a wide variety of both endogenous and xenobiotic compounds [7]. The enzymes within

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‡ Abbreviations: P450, cytochrome P450; ELISA, enzyme-linked immunosorbent assay; Fmoc, *N*-α-9-fluorenylmethoxycarbonyl; KLH, keyhole limpet haemocyanin; MC, 3-methylcholanthrene.

a family share considerable similarity in their primary structure [6]. Consequently, polyclonal antibodies raised against purified P450 enzymes commonly have a high degree of cross-reactivity with other P450 enzymes within the same sub-family and monoclonal antibodies also frequently bind to more than one enzyme [8]. The use of anti-peptide antibodies provides a means of overcoming this problem as such antibodies show good specificity when targeted towards a small, unique, surface region of the native protein [9, 10].

In our studies, peptides 6 or 7 amino acids long are coupled via a cysteine residue at their N-termini to carrier protein. This approach to antibody generation has been applied to a major polycyclic aromatic hydrocarbon-inducible P450 enzyme in the rat: CYP1A2. Anti-peptide antibodies were raised against a synthetic peptide which correspond to a region of rat CYP1A2, at residues 290–296 [9]. This region was targeted because it is unique to this P450 amongst the known sequences of rat P450 enzymes [9]. It was predicted from a model of the three-dimensional structure of P450 [11] that this region is on the surface of CYP1A2 and accessible to antibodies. This was confirmed by ELISA. The anti-peptide antibodies bound strongly to CYP1A2 in ELISA and were specific for the protein in immunoblotting of microsomal fractions. The antibody also inhibited the activity of CYP1A2 [9].

In the present study, the wider issue of reactivity of the antibody with proteins other than just P450 enzymes and other microsomal proteins was examined. This was done by defining the major antigenic region of the peptide used for immunisation and searching a protein sequence database for other proteins to which the antibody was likely to bind. The results show that cross-reaction of anti-peptide antibodies to protein antigens not only is likely in proteins which contain a sequence identical or very similar to the peptide used for immunisation, but also occurs with protein and peptide antigens which contain even a short C-terminal sequence common to the immunising peptide. As only limited similarity is required, cross-reactivity with the C-termini of proteins is much more likely than binding to residues which occur as internal sequences in the protein. This cross-reactivity appears to be predictable, although at present limited by the number of known protein sequences. The knowledge that antibodies could be produced that bind strongly to C-terminal residues was utilised to produce antibodies against P450 enzymes CYP1A1, CYP1A2, CYP2A6 and CYP2E1.

MATERIALS AND METHODS

Materials. Fmoc amino acids linked to *p*-benzyloxybenzyl alcohol or Kieselguhr/polyamide resin, *N*-α-Fmoc-protected amino acid pentafluorophenylesters and *N*-α-Fmoc-protected amino acid 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine esters were purchased from Novabiochem (Nottingham, U.K.). Nucleosil C_{18} 10 μm high pressure liquid chromatography column was from Jones Chromatography (Hengoed, U.K.) and acetonitrile (grade S) was from Rathburn Chemicals (Walkerburn, U.K.). Sephadex G-15, Sephadex G-25 and cyanogen bromide-activated Sepharose were from Pharmacia (Milton Keynes, U.K.). KLH was from Calbiochem (Nottingham, U.K.). All SDS–PAGE reagents were from National Diagnostics (Aylesbury, U.K.), except for pre-stained molecular weight standards, which were from Sigma (Poole, 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.). Microsomal fractions prepared from human B-lymphoblastoid cell lines transfected with a plasmid vector expressing human CYP2A6, and untransfected cells were purchased from Gentest Corporation (Woburn, MA, U.S.A.). All other chemicals were purchased from Sigma or Merck-BDH (Lutterworth, U.K.) and were of analytical grade or the best equivalent.

Peptide synthesis and conjugation to protein. The rationale for the selection of the peptide Ser-Glu-Asn-Tyr-Lys-Asp-Asn for the production of anti-peptide antibodies has been described previously [9]. This peptide sequence represents residues 290–296 of CYP1A2 [12] and the sequence is unique to this P450 in the rat and mouse amongst the protein sequences currently deposited in the SWISS-PROT database. Here, a series of peptides was synthesised based on this structure. Peptides composed of residues 291–296, 292–296, 293–296, 294–296 and 295–296 plus amino acid residue 296 only, form a series shortened from the N-terminus. In the peptides composed of residues 290–295 and 290–297, the C-terminus was one residue shorter and one residue longer (by addition of glycine), respectively, compared with the immunising peptide. All of these peptides were synthesised with a cysteine residue at the N-terminus through which they were coupled to KLH (see below). Peptide 290–296 was also synthesised without an N-terminal cysteine and was used for coupling to CNBr-activated Sepharose (see below).

Also synthesised were the peptides Cys-Gln-His-Leu-Gln-Ala, Cys-Pro-Arg-Phe-Ser-Lys, Cys-Val-Ile-Pro-Arg-Ser, and Cys-Ser-Phe-Leu-Pro-Arg which represent the C-termini of rat P450 enzymes CYP1A1 [13], CYP1A2 [12], and CYP2E1 [14], and human CYP2A6 [15], respectively, each with cysteine added to the N-terminus for coupling to carrier protein.

All peptides were synthesised manually by a solid-phase method as described previously [16], except that the peptides were purified by gel filtration in 1 M acetic acid using either a Sephadex G-25 column (45 cm × 2.2 cm), or, for peptides smaller than 8 residues, a Sephadex G-15 column (35 cm × 1.5 cm). All products had >90% purity, as determined by high pressure liquid chromatography using a Nucleosil C_{18} column, and a gradient of 0–25% (v/v) acetonitrile in the presence of 0.1% (v/v) trifluoroacetic acid over 10 min at a flow rate of 2 mL/min, with constant monitoring of the eluent at 210 nm. All peptides had the correct composition by amino acid analysis and the correct molecular weight by mass spectrometry.

Cysteine-containing peptides were individually

coupled to KLH using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described by Liu *et al.* [17]. This method allowed the peptides to be coupled in a specific orientation through the thiol group. A "null" conjugate (KLH-Cys) was prepared in the same way by substituting cysteine for cysteinyl peptide.

Antibody production and purification. Male New Zealand White rabbits were immunised with KLH–peptide conjugates by repetitive injections of 0.2 mg of the conjugate in Freund's adjuvant as described previously [9].

Non-cysteinyl peptide 290–296 was coupled to cyanogen bromide-activated Sepharose and used to purify antibodies from rabbit serum by affinity chromatography as described previously [18]. Briefly, Sepharose–peptide 290–296 was mixed with antiserum and then washed sequentially with PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.5), PBS containing 1 M NaCl, and 2% (w/v) formic acid, pH 2.0, containing 0.15 M NaCl. The acid-eluted antibody fraction was neutralised by addition of 0.3 volumes of 3 M Tris–HCl, pH 8.8, then dialysed against PBS. The fraction eluted with PBS containing 1 M NaCl was also dialysed against PBS.

ELISA and immunoblotting. Antibody binding was measured by an ELISA, which was performed as described by Edwards *et al.* [16]. Binding to an antigen is expressed as the effective concentration of antibody that produced 50% of maximum binding (EC_{50}). Immunoblotting was performed as described previously [16].

Preparation of microsomal fractions. Male Wistar rats (200–250 g) were obtained from Harlan Olac Ltd (Bicester, U.K.), male Balb/c mice (6–10 weeks) were from A. Tuck & Son Ltd (Battlebridge, U.K.), and male New Zealand White rabbits (3 kg) were from Froxfield Farms Ltd (Petersfield, U.K.). Rats were either untreated, or treated with 80 mg/kg MC dissolved in corn oil as a single i.p. dose 48 hr prior to killing, or received isoniazid as a 0.1% (w/v) solution in their drinking water for 11 days followed by an i.p. injection of 50 mg/kg in saline 3 hr prior to killing. Mice were treated with the same dose of MC as rats. Rabbits were either untreated or given acetone as a 1% (v/v) solution in their drinking water for 7 days prior to killing. 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 [19]. Human liver samples were obtained from the human tissue bank at the Royal Postgraduate Medical School [19]. Local Ethical Committee permission and Coroner's approval were obtained for their use in these studies. After thawing the samples in ice-cold buffer (0.25 M potassium phosphate, 0.15 M KCl, 1 mM EDTA, pH 7.25) the preparation of the microsomal fraction was as described previously [19, 20]. For these studies a pool of five human liver microsomal fractions was used.

Interrogation of protein database for similar sequences. The identification of proteins contained in release 24 of the SWISS-PROT database [21] which contain specific short peptide sequences either internally or at their C-termini was performed by searching the database in CD-ROM format using a computer program written in BASIC by the present authors and run on an IBM PC compatible microcomputer. Copies of the program are available on request.

Table 1. Comparison of the binding in ELISA of an antibody raised against KLH-peptide 290–296 to related antigens

Antigen	EC_{50} (μg/mL)
Peptide 290–296	0.05 ± 0.01
Peptide 290–297	0.41 ± 0.08**
Peptide 290–295	1.61 ± 0.16**
Peptide 291–296	0.07 ± 0.03
Peptide 292–296	0.05 ± 0.02
Peptide 293–296	0.07 ± 0.03
Peptide 294–296	0.09 ± 0.02
Peptide 295–296	0.14 ± 0.01*
Peptide 296	4.00 ± 0.80**
Null peptide	10.6 ± 2.66*
Purified rat CYP1A1	>20
Purified rat CYP1A2	3.1 ± 0.18**
Hepatic microsomal fraction from MC-treated rats	3.0 ± 0.15**

An acid-eluted fraction of the antiserum was prepared by affinity chromatography as described in the Materials and Methods. Each of the peptide antigens was coupled to KLH and then coated on to microtitre plates. Antibody binding was determined as described in the Materials and Methods. The results are the mean ± SEM of three determinations in each case. Statistical analysis was performed using the Student's *t*-test, each group being compared with the binding to peptide 290–296; * $P < 0.05$, ** $P < 0.01$.

RESULTS

Affinity purification using Sepharose–peptide 290–296 and acid elution yielded 0.64 mg antibody/mL serum. This anti-peptide antibody preparation bound strongly to peptide 290–296 coupled to KLH, but relatively weakly to KLH–Cys (null peptide), indicating that the majority of the antibodies in the purified preparation bound to the peptide and not the carrier protein or linking group (Table 1).

To determine the importance of individual residues in binding, an ELISA was performed using a series of related peptides (Table 1). The purified antibody bound to peptide 290–296 with an EC_{50} of 0.05 μg/mL. In comparison, there was an 8-fold reduction in binding to peptide 290–297 which was extended by 1 amino acid residue at the C-terminus and a 32-fold reduction in binding to peptide 290–295 which was truncated by 1 amino acid residue at the C-terminus. The antibody preparation bound equally well to all of the members of a series of peptides which were reduced in length from the N-terminus compared with the immunising peptide, 291–296, 292–296, 293–296 and 294–296. However, there was a 3-fold reduction in binding to the C-terminal dipeptide Asp-Asn (295–296) and binding to the single C-terminal residue, Asn-296 was reduced 80-

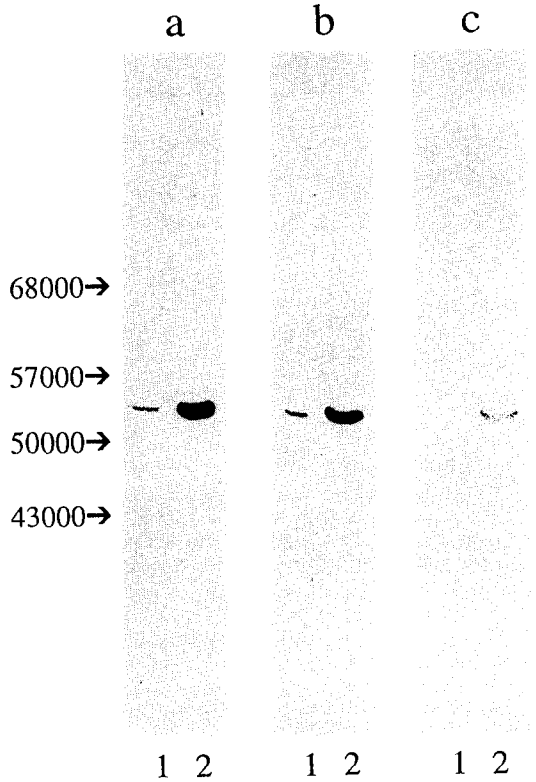

Fig. 1. Binding of crude antiserum and antibody fractions to rat CYP1A2. Immunoblots of 30 μg hepatic microsomal fraction from untreated rat (lane 1) and MC-treated rat (lane 2). The immunoblots were developed with (a) unfractionated antiserum raised against KLH-peptide 290–296, or antibody fractions purified by affinity chromatography and eluted with (b) PBS containing 1 M NaCl or (c) 2% (v/v) formic acid, pH 2.0, containing 0.15 M NaCl.

fold, to a level similar to that for binding to the null conjugate (Table 1).

Although the acid-eluted affinity purified antibody preparation bound to a hepatic microsomal fraction from rats treated with MC and to purified rat CYP1A2, the binding was relatively weak compared with binding to peptide 290–296 (Table 1). There was no detectable binding to purified rat CYP1A1 (Table 1).

The acid-eluted affinity purified antibody fraction only reacted weakly with rat CYP1A2 in immunoblotting (Fig. 1c). This is in contrast to the results of immunoblotting using crude antiserum or an antibody fraction eluted from the affinity column using 1 M NaCl (the yield was 0.15 mg antibody/mL serum), both of which bound strongly to CYP1A2 (Fig. 1). Thus, the antiserum contains at least two populations of antibodies. One was eluted from the affinity column with 1 M NaCl and binds to CYP1A2. The other was eluted under acidic conditions and binds only weakly to CYP1A2 but binds strongly to the C-terminus of the immunising peptide (Table 1).

The specificity of binding of the unfractionated anti-peptide antibody to various protein antigens was investigated by immunoblotting. Replicate nitrocellulose filters were either stained for protein with amido black or developed with the anti-peptide antibody. The amido black stained filter showed that there was abundant electrotransfer of protein in all cases (Fig. 2a). However, the anti-peptide antibody bound only to proteins corresponding to CYP1A2 in the rat and mouse liver microsomal fractions and to yeast glucose-6-phosphate dehydrogenase (Fig. 2b). The antibody did not bind to bovine fibronectin, chicken gizzard myosin heavy chain, rabbit hepatic microsomal fraction, or human hepatic microsomal fraction (Fig. 2b).

An antibody was also raised against the truncated peptide Tyr-Lys-Asp-Asn (residues 293–296 of CYP1A2). This antibody was unable to bind to CYP1A2, but bound strongly to yeast glucose-6-phosphate dehydrogenase in immunoblotting (Fig. 3).

Antibodies raised against the peptides Gln-His-Leu-Gln-Ala, Pro-Arg-Phe-Ser-Lys, Val-Ile-Pro-Arg-Ser, and Ser-Phe-Leu-Pro-Arg, which represent the C-termini of P450 enzymes CYP1A1, CYP1A2, CYP2E1 and CYP2A6, respectively, were also tested by immunoblotting against hepatic microsomal fractions from various animals (Fig. 4). The antibody raised against Gln-His-Leu-Gln-Ala bound to a single band in MC-treated rats, corresponding to CYP1A1, but the antibody did not bind to the hepatic microsomal fraction from untreated rats (Fig. 4a). The antibody raised against Pro-Arg-Phe-Ser-Lys bound weakly to microsomal fraction from untreated rats, and the intensity of this band was greatly increased in rats treated with MC (Fig. 4b). This band corresponds to CYP1A2. The antibody raised against Val-Ile-Pro-Arg-Ser bound to a single band in hepatic microsomal fraction from untreated rats and the intensity of this band was increased following treatment of the rats with isoniazid (Fig. 4c), a known inducer of CYP2E1 [22]. This antibody also bound to a single band in human microsomal fraction (Fig. 4c). The antibody bound to two bands in the rabbit and the intensity of the higher molecular weight protein was increased following treatment of a rabbit with acetone, which is known to induce CYP2E1 [23]. The antibody raised against Ser-Phe-Leu-Pro-Arg bound to recombinant human CYP2A6 and to a single band in the human hepatic microsomal fraction (Fig. 4d).

DISCUSSION

The composition and size of a peptide used to raise antibodies might be expected to influence the specificity of the antibodies for the target protein. Although very short peptide sequences would be expected to occur frequently in proteins, a peptide consisting of seven residues, such as the one studied here, is likely to be unique. Indeed, amongst the sequences listed in the SWISS-PROT database, Ser-Glu-Asn-Tyr-Lys-Asp-Asn occurs only in the enzyme CYP1A2 of rat and mouse. However, it is still possible that the epitope for some of the antibodies present in a polyclonal anti-peptide antiserum does not comprise all of the residues. If this were the case, binding to other proteins is more likely to occur

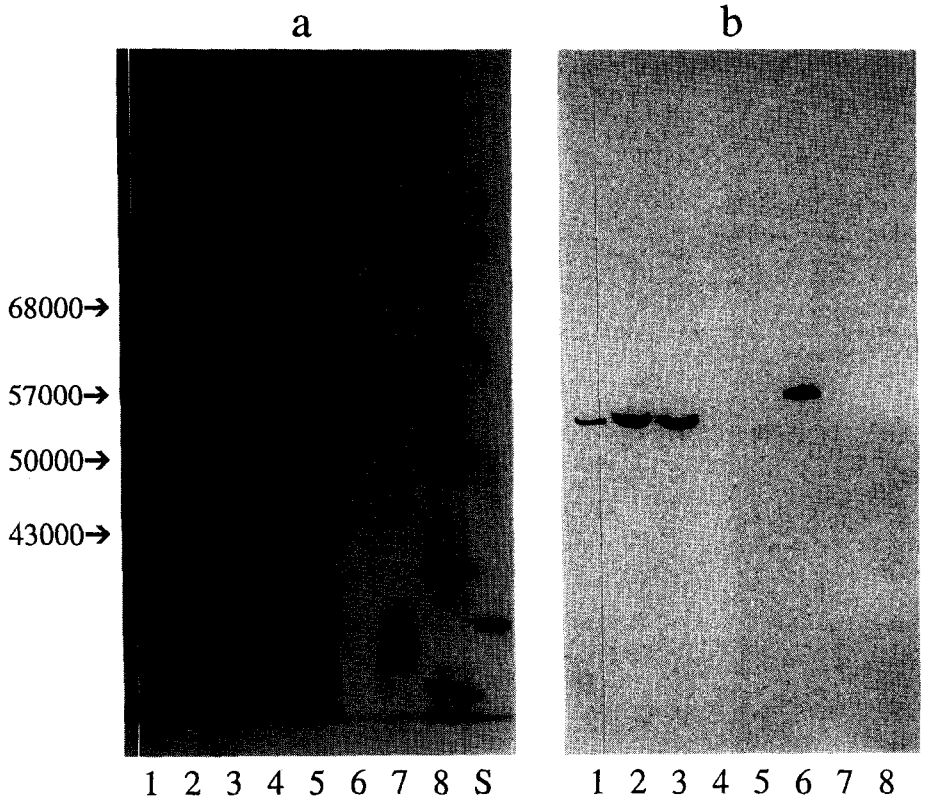

Fig. 2. Binding of antiserum raised against KLH-peptide 290–296 to various antigens. Blots of 30 μg of hepatic microsomal fractions from untreated rat (lane 1), MC-treated rat (lane 2), MC-treated mouse (lane 3), untreated rabbit (lane 4) and human (lane 5), and 2.5 μg of glucose-6-phosphate dehydrogenase (lane 6), 10 μg of fibronectin (lane 7) and 40 μg of myosin (lane 8). Lane S contains pre-stained electrophoresis standards. After electrotransfer, the blots were either (a) stained for protein using amido black, or (b) developed for immunoreactivity using antiserum raised against KLH-peptide 290–296.

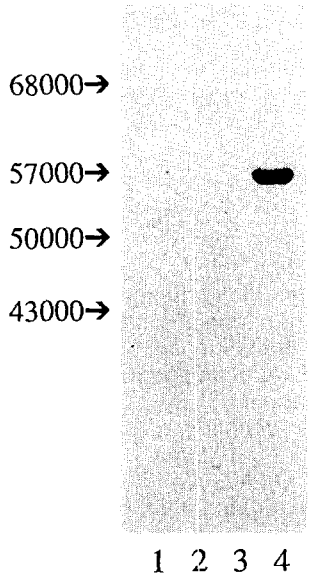

Fig. 3. Binding of antiserum raised against KLH-peptide 293–296 to various antigens. Immunoblot of 30 μg of hepatic microsomal fractions from untreated rat (lane 1), MC-treated rat (lane 2) and MC-treated mouse (lane 3), and 2.5 μg of glucose-6-phosphate dehydrogenase (lane 4), developed with antiserum raised against the truncated peptide 293–296.

and increasing the length of the immunising peptide would not necessarily increase the specificity of the resultant antiserum. In the present study, the binding specificity of an anti-peptide antibody has been characterised and reveals at least two populations of antibody. One population was directed to an internal sequence of the peptide, and the other to the C-terminus, where the free carboxyl group appears to play a role in antibody binding.

Those residues important for antibody binding were determined by ELISA using a purified preparation in which antibodies against the carrier protein and linking group had been removed. An antibody fraction was isolated from the antiserum by affinity chromatography using peptide 290–296 coupled to Sepharose. The fraction eluted under acidic conditions was found to be directed almost entirely against the C-terminal region of the peptide. This was shown in three ways. First, the antibody fraction bound equally well to a series of related peptides progressively shortened from six to three residues in length from the N-terminus. The antibody fraction also recognised the dipeptide Asp-Asn, albeit with reduced affinity. Thus, the minimum epitope was the tripeptide Lys-Asp-Asn. Second, binding to peptide 290–295, which lacks the C-terminal asparagine, was significantly reduced compared with binding to pep-

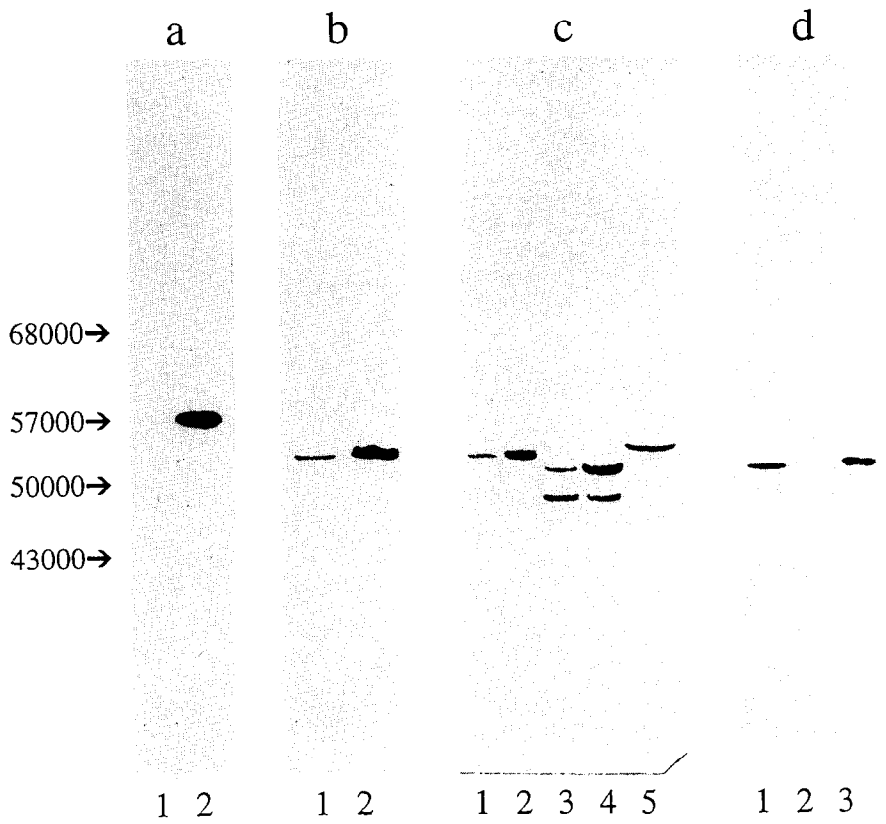

Fig. 4. Binding of antisera raised against short synthetic peptides to their respective target P450 enzymes. (a) Immunoblot of 30 μg of hepatic microsomal fractions from untreated rat (lane 1) and MC-treated rat (lane 2) developed with an antibody raised against Gln-His-Leu-Gln-Ala, which represents the C-terminus of rat CYP1A1. (b) Immunoblot of 30 μg of hepatic microsomal fractions from untreated rat (lane 1) and MC-treated rat (lane 2) developed with an antibody raised against Pro-Arg-Phe-Ser-Lys, which represents the C-terminus of rat CYP1A2. (c) Immunoblot of 30 μg of hepatic microsomal fractions from untreated rat (lane 1), isoniazid-treated rat (lane 2), untreated rabbit (lane 3), acetone-treated rabbit (lane 4) and human (lane 5) developed with an antibody raised against Val-Ile-Pro-Arg-Ser, which represents the C-terminus of rat CYP2E1. (d) Immunoblot of 10 μg of microsomal fractions from lymphoblastoid cells transfected with human CYP2A6 (lane 1), untransfected human lymphoblastoid cells (lane 2), and 30 μg of human hepatic microsomal fraction (lane 3) developed with an antibody raised against Ser-Phe-Leu-Pro-Arg, which represents the C-terminus of CYP2A6.

tide 290–296. This emphasises the importance of the C-terminal asparagine for binding of this antibody fraction. Third, binding of the antibody fraction to peptide 290–297, which was extended by one residue at the C-terminus, was also decreased relative to that to peptide 290–296. Thus, for maximum antibody binding, asparagine must be the C-terminal residue.

The peptide–protein conjugate used for immunisation consisted of the synthetic peptide coupled to KLH in a specific orientation through its N-terminus. Thus, the resultant antibodies were directed against the uncoupled end of the peptide. This has been observed previously for other peptides and their corresponding antibodies [10, 24, 25].

In CYP1A2 Asn-296 forms a peptide bond with Gly-297, as is the case in the peptide 290–297. Therefore, amongst the range of anti-peptide antibodies raised, those which require asparagine as the unblocked C-terminal residue would be inhibited from binding to CYP1A2. Indeed, compared with the binding to peptides the acid-eluted affinity purified antibody fraction bound poorly to CYP1A2 in ELISA. Similarly, this antibody fraction reacted weakly with rat CYP1A2 in immunoblotting. This is in contrast to the results of immunoblotting using crude antiserum or an antibody fraction eluted from the affinity column using 1 M NaCl, both of which bound strongly to CYP1A2. Thus, those antibodies which bind strongly to the C-terminal residues of the peptide cross-react only poorly with the protein antigen. Those antibodies which bind relatively weakly to the immunising peptide, as indicated by their relative ease of elution from Sepharose peptide 290–296, cross-react specifically with the target P450 enzyme.

In the present study, maximum binding of the acid-eluted affinity purified antibody was possible with only the C-terminal tripeptide, and it is remark-

able that binding to even the C-terminal dipeptide was possible. Previous workers have shown that five amino acid residues are generally necessary for antibody binding to continuous epitopes [26]. However, for certain monoclonal antibodies fewer residues may suffice. Tang *et al.* [27] found that a tetrapeptide could completely block the binding of a monoclonal antibody against influenza virus haemagglutinin and Price *et al.* [28] showed that several monoclonal antibodies raised against human epithelial mucin bound to the same epitope, which comprised a 3-amino acid sequence. An extreme case was reported by Khachigian *et al.* [29], who found that a monoclonal antibody which cross-reacted with many proteins did so through the dipeptide lysyl-lysine.

A minimal epitope of Lys-Asp-Asn is not consistent with the specificity of the anti-peptide antibody described here. This sequence occurs commonly amongst proteins (1153 occurrences in 28,154 proteins in SWISS-PROT database) and yet no cross-reactivity with other microsomal proteins was observed. One explanation for this is the location of the sequence within the protein. As discussed above, the antibody species recognising this sequence do so only when it is the C-terminus. A second population of antibodies, and it is these that recognise CYP1A2, bind to an internal peptide sequence. This explains the lack of cross-reactivity with other microsomal proteins. However, it does suggest that those antibodies which bind to the C-terminal epitope should also bind to any protein with Lys-Asp-Asn, or perhaps even just Asp-Asn, at its C-terminus.

In fact, only one of the proteins listed in the SWISS-PROT database has this sequence (-Lys-Asp-Asn) at its C-terminus, yeast glucose-6-phosphate dehydrogenase. However, the tripeptide does occur as an internal sequence in 1152 other proteins in the database, including bovine fibronectin, chicken gizzard myosin heavy chain and the P450 enzymes, rat and mouse CYP1A2 (obviously), rabbit CYP3A6, and human CYP3A4. Although the anti-peptide antibody did bind to yeast glucose-6-phosphate dehydrogenase, it did not bind to bovine fibronectin or chicken gizzard myosin heavy chain. In addition, the antibody did not bind to any protein in microsomal fractions from rabbit or human, thus excluding cross-reactivity with rabbit CYP3A6, and human CYP3A4, which are known to be constitutively expressed at relatively high levels [30, 31]. Thus, the antibody can recognise the tripeptide in protein, but only when it occurs at the C-terminus.

The occurrence of Asp-Asn at the C-terminus of proteins was also determined from the SWISS-PROT database. Although 20 different full-length, non-precursor proteins were identified, the only one of which was readily available, and hence testable, was yeast glucose-6-phosphate dehydrogenase which, as described above, has Lys-Asp-Asn at the C-terminus. Thus, it was not possible to test the binding of the anti-peptide antibody to a protein with just Asp-Asn at the C-terminus.

Antiserum raised against the truncated peptide Tyr-Lys-Asp-Asn (residues 293–296) failed to bind to CYP1A2, indicating that these four residues alone are insufficient to describe the epitope of the protein-reactive antibodies within the antiserum raised against the full-length peptide. However, the antiserum raised against the truncated peptide did bind strongly to yeast glucose-6-phosphate dehydrogenase, presumably at the C-terminus, confirming the immunodominance of the C-terminal residues. The possibility of directing antibodies to the C-terminal residues of P450 enzymes was therefore investigated. It has been predicted from models of the tertiary structure of mammalian P450 that the C-terminus of these enzymes is accessible in the native proteins and is not membrane bound [32, 33]. In addition, the C-terminal residues of these enzymes show considerable variation, so that such antibodies were likely to be specific. These possibilities were tested by synthesising peptides corresponding to the C-termini of rat CYP1A1, CYP1A2, CYP2E1 and human CYP2A6 and raising antibodies against them.

Antibodies were successfully raised against all four peptides and all bound to their target P450 enzymes in immunoblotting. The antibody against Gln-His-Leu-Gln-Ala bound to a single band corresponding to CYP1A1 in hepatic microsomal fraction from MC-treated rats, but not from untreated rats in which this enzyme is absent [34]. There was no cross-reactivity with the other member or the CYP1A subfamily, CYP1A2. Conversely, the antibody against Pro-Arg-Phe-Ser-Lys bound only to CYP1A2, which is present in hepatic microsomal fraction from MC-treated rats but at much lower levels in untreated rats [34], and there was no cross-reactivity with CYP1A1. The antibody against Val-Ile-Pro-Arg-Ser bound to a single band in the hepatic microsomal fraction from untreated rats, corresponding to CYP2E1. The intensity of this band was increased by treating rats with isoniazid, a known inducer of CYP2E1 [22]. The C-terminus of human CYP2E1 is identical to that of the rat enzyme [14]. The antibody bound to a single band in the microsomal fraction from human liver. The antibody also bound to two bands in the rabbit hepatic microsomal fraction. The upper band increased in intensity on treatment of the animals with acetone, and has a similar electrophoretic migration to rat CYP2E1. The intensity of the lower band was not affected by treatment of the animals with acetone. Interestingly, rabbit possesses two enzymes in the CYP2E subfamily, CYP2E1 and CYP2E2, which have identical C-termini [35]. In a previous report it was found that the proteins co-migrate in SDS–PAGE [36]. However, the electrophoretic migration of P450 enzymes is known often to be anomalous [37] so the possibility that the unidentified band represents CYP2E2 cannot be excluded. The antibody against Ser-Phe-Leu-Pro-Arg bound to a single band in microsomal fraction from human lymphoblastoid cells expressing recombinant human CYP2A6 and to a single band with identical molecular weight in human liver microsomal fractions. The predicted C-terminus of CYP2A7 is identical to that of CYP2A6; however, it is unclear as to whether this protein is expressed in human liver [15].

The C-terminus of proteins has been used successfully as a site for antibody production in a number of previous studies [38]. However, the size of the peptides used was relatively large (6–19 residues) and in several cases the orientation of the peptide

coupled to the protein carrier was not considered. A previous attempt to target the C-terminus of rat CYP1A2 using a 17-residue peptide coupled in a heterologous orientation to carrier protein resulted in antibodies which bound to both CYP1A1 and CYP1A2 [39]. Whilst it would seem on first consideration that the longer the peptide, the more specific the antibody, there are several reasons why this will not be so. Firstly, most epitopes comprise about five residues [26], so that a peptide of, say, 15 residues could contain several epitopes. This increases, rather than decreases, the possibility of cross-reactivity. Secondly, in targeting an antibody to a protein antigen, the structure of the target epitope should be known. This should be linear and flexible, to ensure specificity and recognition of both the native and denatured (for immunoblotting) protein. The longer the peptide, the more likely it is that it will adopt some secondary and even tertiary structure in solution, and this may not be the same as that of the peptide region in the native target protein. Such antibodies may fail to recognise the target protein. In the approach adopted here, short peptides, of only five residues, are used and these are coupled in a known orientation, via the N-terminus, to carrier protein. This ensures that the immunodominant epitope, at the C-terminus, is restricted to the specific sequence of interest and is at a known location within the native target protein.

The potential of this approach for targeted antibody production is demonstrated here. Specific antibodies were readily produced in this way against four different forms of P450. Clearly this approach would be applicable to any protein for which the structure of the C-terminus is known, and would involve minimal peptide synthesis. Antibodies could similarly be directed against the N-terminus of proteins. However, for the P450 enzymes this region is occluded by the microsomal membrane [40], which would limit the use of such antibodies. In addition, as the sequence of most proteins is predicted from their cDNA, the extent to which post-translational modification has affected this will not be known. In particular, this has been shown to occur in some P450 enzymes [41].

In conclusion, the binding specificity of an anti-peptide antibody has been characterised in detail. Two populations of antibody species were identified, reflecting different epitopes within the peptide. One of these was directed to an internal sequence and also bound to the target protein antigen. More than four residues appear to be necessary for such binding. Consequently, binding to proteins other than the target is unlikely. The second antibody population was directed to the C-terminus of the peptide and included the free α-carboxyl group. Only three residues are necessary for such binding. Although the small size of the epitope for this population would suggest that binding to proteins other than the target should be more likely, in practice the need for the free terminal residue provides an additional steric constraint so that the specificity of such antibodies is still very high. It has been recognised for some time that the C-terminus of proteins is an attractive target against which to direct anti-peptide antibodies as this site on a potential protein target is often highly accessible for antibody binding [42]. The present study has shown that it is possible to raise highly specific antibodies by targeting a very small sequence comprising the C-terminal residues of a protein. This strategy requires minimal peptide synthesis. The utility of this approach was demonstrated by raising antibodies against four different forms of P450 using peptides 5 residues long. It was also possible to produce specific antibodies against yeast glucose-6-phosphate dehydrogenase using a 4-residue peptide. The minimum length of peptide for reliably producing antibodies was not determined here and may involve consideration of immunogenicity. However, the approach clearly has general applicability in targeting antibodies to proteins of known sequence.

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