SYNTHETIC PEPTIDE ANTIGENS ELICIT MONOCLONAL
AND POLYCLONAL ANTIBODIES TO CYTOCHROME P450 IA2

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Two peptide sequences from cytochrome P450 IA2 were synthesized, coupled to ovalbumin and used as antigens to generate anti-peptide monoclonal and polyclonal antibodies. Antisera to both peptides reacted with rat IA2 but not the structurally similar IA1 form as determined by enzyme-linked immunosorbent assay. However, antisera to both peptides detected both rat IA2 and IA1 on immunoblots. In addition immunoblots of human liver microsomes revealed that both antisera recognized human IA2, but not IA1. Monoclonal antibodies generated against one of the peptides recognized rat IA2 and IA1 but did not detect human IA2. These results demonstrate the utility of anti-peptide antisera as a practical approach for the generation of P450 specific antibodies.   © 1990 Academic Press, Inc.

Cytochrome P-450 enzymes of the mixed function oxidase system metabolize a wide array of endobiotics and xenobiotics, including steroids, prostaglandins, drugs and chemical carcinogens (1,2). The ability to metabolize the large and disparate number of chemicals is a result of the multiplicity of individual P-450 enzymes, many of which possess either unique or overlapping substrate specificities. Elucidation of the role of individual P-450 enzymes in the metabolism of a given substrate is an important goal towards understanding the role of P-450s in metabolism, detoxification and metabolic activation.

Monoclonal and polyclonal antibodies to P-450s are powerful tools that have been employed to characterize P-450 multiplicity via radioimmunoassay, immunopurification and enzyme inhibition (3,4). But, due to a high degree of protein sequence similarity which exists among P-450s that belong to the same gene family (5), many of the antibodies generated to one P-450 crossreact with other related P-450 forms (6-8). Accordingly, these monoclonal and polyclonal antibodies are specific for epitopes of a given P450 family rather than for a single P450. Thus a different approach may be useful to elicit antibodies directed against a single P450 enzyme rather than to a given epitope common to several P450's.

One potential solution is with the use of site specific antibodies, generated by using as the immunizing agent a chemically synthesized peptide sequence which is unique for a given P-450. The advantages of this technique are in restricting the epitope site to the structure of the small peptide and in the relatively large amounts of the antigen that can be obtained by chemical synthesis.   Both of these advantages are generally lacking when the entire molecule is used as the antigen. This report details our successful efforts towards generating monoclonal and polyclonal anti-peptide antibodies which react with the rat P450 IA2 (nomenclature as per ref 9).

MATERIALS AND METHODS

Peptide Synthesis Peptides were synthesized on a BioSearch 9600 automated synthesizer using standard FMOC chemistry. The protected amino acids purchased from BioSearch. Peptide purity was assessed by amino acid composition and by analytical HPLC. The latter was performed on a Spectra-Physics system with Model 8000 pump Model 4270 integrator. The program consisted of a Brownlee C4 cartridge (10 x 25 mm) column and a linear gradient of 0 to 60% acetonitrile, in 0.1% trifluoracetic acid.

Antibody Generation The peptides were coupled to either ovalbumin or bovine serum albumin using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide chemistry (10). Antisera to the ovalbumin-peptides were generated using Freunds Adjuvant and purified by ammonium sulfate precipitation and hydroxyapatite chromatography (11). Monoclonal antibodies were generated as previously described (8).

Microsomes Control and 3-methylcholanthrene-induced rat (MC) microsomes were prepared from either untreated male Sprague-Dawley rats or rats treated for three days with 3-MC (40 mg/kg/d). Human livers were obtained from The University of Miami Kidney Transplant Unit. Liver microsomes were isolated by differential centrifugation (12) and were suspended in buffer containing 0.25 M sucrose and stored at -80°C. Protein content was measured by the Pierce BCA protein assay system (Pierce).

Immunoblots and Enzyme Immunoassay (EIA) Microsomal proteins and immunopurified P-450s (6) were subjected to sodium dodecyl sulfate gel electrophoresis, transferred to nitrocellulose paper for Western blot analysis (13) and developed as previously described (14). The primary antibodies were used at a final titer of 1:50 (anti-D1) or 1:100 (anti-D2). EIA was performed as previously described (15) with the following modifications: the antigens were allowed to bind to the wells in phosphate-buffered saline; the primary antibody was allowed to react overnight and the second alkaline phosphatase conjugated antibody was used at a 1:600 dilution. Control antibodies did not bind in either EIA or Western blot assays.

RESULTS AND DISCUSSION

Two 17-mer peptide sequences present in cytochrome P450 IA2 but not in the structurally similar P450 IA1 form were selected and synthesized. We have termed these peptides D1 and D2. D1 corresponds to an internal amino acid sequence comprised of residues 208-224 with the sequence FPRKSEEMLNLVKSSKD, and D2 corresponds to the carboxyl terminal sequence commencing at residue 497 with the sequence MKPRTCEHVQAWPRFSK.
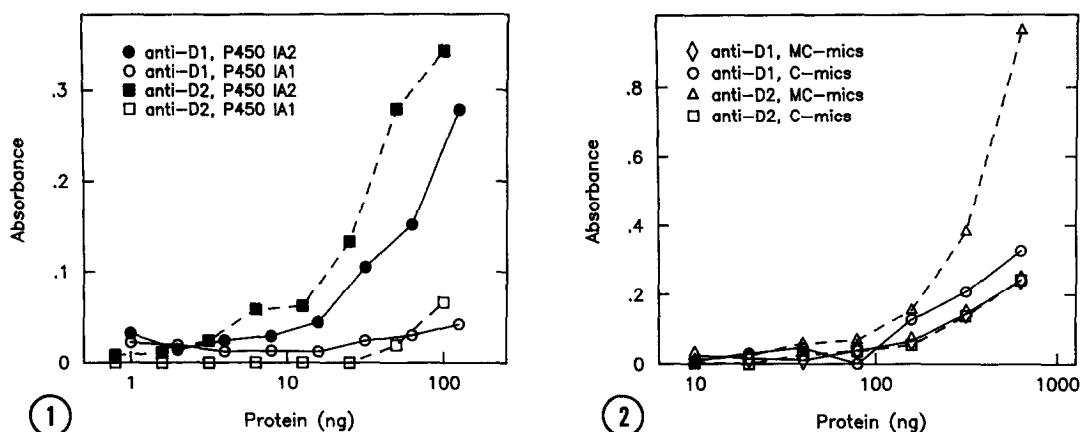
Figure 1: Determination of anti-peptide antisera specificity using purified IA2 and IA1. Antisera generated against either peptide D1 (anti-D1) or peptide D2 (anti-D2) were allowed to react with varying amounts of purified P450 IA2 and P450 IA1. Both antisera were used at a 1:100 dilution.

Figure 2: Determination of anti-peptide antisera specificity using rat liver microsomes obtained from either untreated (C) or 3-methylcholanthrene-induced animals (MC). Conditions are as detailed above.

Antisera were generated using either D1 or D2 coupled to ovalbumin as the immunizing agent. Anti-peptide antisera was initially screened in a dot-blot immunoblot assay, using peptides coupled to bovine serum albumin. All animals which were immunized tested positive and generated antisera having equivalent titers. The results presented in this report detail our findings using one representative antisera to each of the two peptides.

Both anti-D1 and anti-D2 antisera detected P450 IA2, demonstrating 7-fold and 5-fold higher signal to IA2 as compared to IA1, respectively (Fig. 1). Negligible binding to the structurally similar P450 IA1 was observed (Fig. 1). The lower limit of detection of both antisera at a dilution of 1:00 for P-450 IA2 was about 10 ng. These results suggested that these antisera were specific in binding to IA2 relative to IA1. We further examined the affinity of these antisera with respect to P-450s while in the microsomes. Anti-D2 antiserum demonstrated a three fold higher level of binding to MC microsomes as compared to control microsomes (Fig. 2). In contrast, anti-D1 antiserum did not distinguish MC microsomes from control microsomes (Fig. 2). Both antisera detected purified P450 IA2 at a dilution of 1:2000 (using 100 ng of protein), while anti-D1 and anti-D2, at dilutions of 1:8000 and 1:2000 respectively, detected MC microsomes (500 ng) (data not shown).

As microsomes from control or untreated animals are known to contain P450 IA2, it was necessary to ascertain if the EIA data reflected binding of both antisera solely to P450 IA2, or to this P-450 and some other, crossreactive protein. This was especially important with respect to anti-D2 antisera, as this

antisera reacted almost exclusively with purified IA2 but did not distinguish between control and MC microsomes (Figs. 1 & 2). Accordingly, Western blot analysis was performed utilizing electrophoretic mobility as an additional criterion for P-450 identification.

Antisera to both D1 and D2 detected purified P450 IA2 by Western blot analysis (Figs. 3 & 4, lanes 5) but not an equal amount of P450 IA1 (lane 4). The latter, however, was observed when it was overloaded (5-7 fold, data not shown), or in the MC-microsomes (lane 6) in which the level of IA1 greatly exceeds that of IA2 by about 5-10 fold. Both antisera also detected the P450 IA2 band in MC microsomes (lane 6) but not control microsomes (lane 7). However, both antisera reacted with a 54 kD protein in control microsomes (Figs. 3 & 4). Although also present in MC microsomes (lane 6), the level was much lower than that in control microsomes. The detection of this 54 kD protein by antisera directed against two different P450 IA2 peptides suggests that this band is a truncated form of either P450 IA2 or the related IA1, as it is unlikely that an unrelated protein would possess both sequences. Furthermore, the decreased levels of this protein band in samples obtained following induction with 3-methylcholanthrene suggests that this protein is inversely regulated with respect to the positive regulation of P450 IA1 and IA2 following exposure to this chemical agent. The identity of this lower band is currently under investigation to determine if it is related to either P-450 form.

Both antisera also immunoreacted with a protein in human liver microsomes (lanes 1-3, Figs. 3 & 4) whose migration corresponded to that of rat P450 IA2 (lane 5). This result shows that both antisera cross-react with the human orthologs of rat P450 IA2.
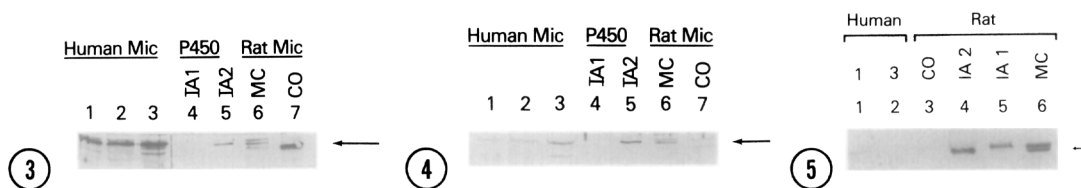

Figure 3: Western Blot analysis of human and rat liver microsomes using anti-D1 antisera. Lanes 1, 2 and 3 contained human liver microsomes (100 µg) from three separate individuals. Lanes 4 & 5 contained immunopurified rat IA1 and IA2 (100 ng each), respectively while lanes 6 and 7 contained 5 µg each of either 3-MC induced rat liver microsomes (MC) or untreated rat liver microsomes (CO). Arrow indicates position of P450 IA2.

Figure 4: Western blot analysis using anti-D2 antisera. Conditions are as detailed above.

Figure 5: Western Blot analysis of human and rat liver microsomes using anti-D2 monoclonal antibodies. Lanes 1, 2 contained human liver microsomes (100 ug, samples 1 & 3 from above). Lane 4 contained control rat liver microsomes (25 µg). Lanes 5 & 6 contained immunopurified rat IA1 and IA2 (100 ng each), respectively while lane 7 contained 5 µg 3-MC induced rat liver microsomes. Arrow indicates position of P450 IA2.

Based on the results obtained above, mice were immunized with OVA-D2 and used for the generation of anti-D2 monoclonal antibodies. One monoclonal, termed d14, was found to give positive binding on a western blot (Fig. 5). The monoclonal antibody bound P450 IA2 and IA1 with near equal intensity. In addition, the monoclonal antibody did not bind to human IA2 (lanes 1 & 2, samples 1 & 3 from Figs. 3 & 4) as did the polyclonal antisera, nor did it bind to the 54 kD protein.

These results demonstrate the utility of the anti-peptide approach towards the generation of anti-P450 antibodies. A recent report described the use of a peptide present in both P450 IA1 and IA2 to elicit antibodies that react with both P450 IA1 and IA2 (16). In contrast, this study utilized peptide sequences unique to P450 IA2. However, while these sequences were unique to IA2, cross-reactivity towards IA1 was observed with both antisera and the monoclonal antibody. The reason for this cross-reactivity is unclear at present and is under further investigation. This report also describes the first monoclonal antibody directed against a chemically synthesized peptide sequence which is unique for a given P-450. This approach is especially useful when the desired antibody specificity cannot be obtained using the entire enzyme molecule. In addition, this method will also be useful in generating antibodies to P-450s that are not available for immunization and screening, but for which peptides can be synthesized on the basis of a known DNA sequence.

The anti-peptide antisera are specific for P450 IA2 relative to P450 IA1 by the criteria of EIA using purified P-450s. However, Western blots revealed binding to IA1, although to a significantly lesser extent, and then only in situations in which IA1 was in excess of IA2 by 5-10 fold. In addition binding by both antisera was observed to another microsomal protein.

Thus, our studies demonstrate the utility of synthetic peptides designed from the known structures of two highly related cytochrome P450 proteins (IA1 and IA2) for the generation of antibodies of relative high specificity for one of these P450s (IA2). This technique will be useful for the production of both polyclonal and monoclonal antibodies with high specificity for single forms of cytochrome P450.

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