Biochemical Pharmacology, Vol. 44, No. 4, pp. 621–629, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

THE TOPOGRAPHY OF TRIFLUOROACETYLATED PROTEIN ANTIGENS IN LIVER MICROSOMAL FRACTIONS FROM HALOTHANE TREATED RATS

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(*Received* 9 *April* 1992; *accepted* 9 *June* 1992)

Abstract—Sera from patients with halothane hepatitis contain immunoglobulin G (IgG) antibodies to trifluoroacetylated liver microsomal proteins of 100, 76, 59, 57 and 54 kDa, which are produced as a consequence of metabolism of halothane to trifluoroacetyl halide by cytochrome(s) P450. In the present study, the membrane topographies of the various antigens in rat liver microsomal fractions were investigated. Liver microsomal fractions from rats treated with halothane *in vivo*, and rat liver microsomal fractions which had been incubated with halothane *in vitro*, were used as the source of trifluoroacetyl antigens. The antigens were detected by immunoblotting. Whereas the 100, 76, 59 and 57 kDa antigens were solubilized from the microsomal membrane by either 0.1 M sodium carbonate or 0.1% (w/v) sodium deoxycholate, the 54 kDa antigen was not solubilized by 0.1% (w/v) sodium deoxycholate. In intact microsomal fractions, the 100, 76, 59 and 57 kDa antigens were not degraded appreciably by trypsin unless detergent was added to permeabilize the microsomal membrane. These results indicate that the 54 kDa antigen is an integral membrane protein, whereas the 100, 76, 59 and 57 kDa antigens are peripheral membrane proteins situated within the lumen of microsomal vesicles, and hence presumably located within the lumen of the endoplasmic reticulum *in vivo*.

A very small fraction of patients exposed to the inhalational anaesthetic halothane ($CF_3CHClBr$) develop severe liver damage, termed commonly "halothane hepatitis" [1, 2]. The mechanisms involved are of particular interest because a series of investigations have implicated an immune response to halothane metabolite-modified liver antigens (reviewed in Refs 3 and 4). Initially, the halothane-induced antigens were detected by indirect immunofluorescence and by induced cellular cytotoxicity testing, and were shown to be present on the surface of hepatocytes isolated from livers of rabbits which had been anaesthetized with halothane *in vivo* [5]. Subsequently, experiments performed using the techniques of SDS–PAGE and immunoblotting revealed that the antigens comprise a group of at least five distinct polypeptides, having molecular masses of 100, 76, 59, 57 and 54 kDa, which are expressed in livers from halothane-exposed rabbits, rats and humans [6–8].

Investigation of the subcellular locations of the halothane-induced polypeptide antigens has shown that they are concentrated in the hepatic microsomal fraction, as prepared by differential centrifugation and sucrose density gradient ultracentrifugation [6]. Generation of the antigens has been shown to involve covalent binding to the polypeptides of trifluoroacetyl halide (TFA halide‖; CF_3COX) [7], which is produced from halothane ($CF_3CHClBr$) by cytochrome P450-mediated metabolism [9, 10]. Experiments in which TFA groups were removed from the antigens using 1 M piperidine have demonstrated that the epitopes recognized by the patients' antibodies contain the TFA hapten [7]. However, antibody binding was inhibited poorly in the presence of a large excess of the haptenic inhibitor *N*-ε-TFA-L-lysine [7]. This suggests that the epitopes recognized by patients' antibodies consist of the TFA group together with structural features present on the various polypeptides [7].

Recently, one of the antigens (59 kDa) was purified to apparent homogeneity and was identified as a TFA-labelled liver microsomal carboxylesterase isozyme (EC 3.1.1.1) in view of its NH_2-terminal amino acid sequence, its catalytic activity and other biochemical properties [11]. Microsomal carboxylesterases are believed to be water-soluble proteins which are resident within the lumen of the endoplasmic reticulum [12–14]. However, the active site of cytochromes P450, which is the site of generation of TFA halide [7], is located on the cytoplasmic face of the membrane [15, 16]. This raises the question of whether the 59 kDa TFA-labelled carboxylesterase is located within the lumen of the endoplasmic reticulum, and whether the other TFA–protein antigens share the same subcellular location. If so, this would have important implications as regards the mechanism of antigen generation, and also as regards the processes involved in expression of the antigens on the hepatocyte surface membrane.

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‖ Abbreviations: TFA, trifluoroacetyl; DOC, sodium deoxycholate; IgG, immunoglobulin G; PBS, phosphate-buffered saline.

With this in mind, in the present study we have used biochemical techniques to investigate the membrane topography of the various antigens in rat liver microsomal fractions. Liver microsomal fractions were studied because they primarily consist of sealed vesicles derived from the endoplasmic reticulum, in which the intravesicular contents are equivalent to the intra-lumenal contents of the endoplasmic reticulum [17–19].

MATERIALS AND METHODS

Animals, drug treatments and preparation of liver microsomal fractions. Male Sprague–Dawley rats (180–200 g, from Harlan Olac, Oxfordshire, U.K.) were treated i.p. with halothane, as a 21.5% solution in sesame oil, at a dose of 10 mmol/kg. "Control" animals received an equivalent dose of sesame oil alone and "untreated" rats received no treatment. Animals were fasted and after 18 hr were killed by cervical dislocation. Livers were removed and homogenized in ice-cold 15 mM Tris–HCl pH 6.8 containing 0.25 M sucrose and 0.1 mM EDTA. Microsomal fractions were prepared by differential centrifugation [20] and were stored at −70°.

In vitro *microsomal incubations.* Microsomal fractions from untreated rats were diluted in buffer (20 mM Hepes, 0.1 mM EDTA, adjusted to pH 7.5 with KOH) and incubated with vigorous shaking for 2 hr at 37°, in the presence of 20 mM halothane, 10 mM NADH and 10 mM NADPH, under oxygen in sealed reaction containers at a protein concentration of 9.8 mg/mL. The samples were cooled on ice and residual halothane was removed under vacuum. Samples were then analysed by SDS–PAGE and immunoblotting, or were treated with protease.

Solubilization of microsomal fractions. Microsomal fractions were diluted to 8 mg protein/mL in ice-cold phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM sodium phosphate, 8.1 mM potassium phosphate pH 7.4) and were centrifuged for 80 min at 4° and 160,000 *g* (r_{av} 5.73 cm) in a Beckman Type 65 rotor. The supernatants were discarded and the washed microsomal pellets were taken for solubilization.

For sodium deoxycholate (DOC) solubilization, microsomal pellets were resuspended to 8 mg protein/mL in 0.1% (w/v) DOC, 10 mM Tris–HCl pH 7.4, and were incubated on ice for 45 min before centrifugation, which was performed as above. Supernatants (proteins soluble in 0.1% DOC) were removed by aspiration. Pellets were resuspended in an equivalent volume of 2% (w/v) DOC, 10 mM Tris–HCl pH 7.4, then incubated 45 min on ice and centrifuged once more. The final supernatants (proteins soluble in 2% DOC) were removed by aspiration and the pellets were resuspended in PBS.

For sodium carbonate solubilization, microsomal pellets were resuspended to 8 mg protein/mL in 0.1 M sodium carbonate (pH 11), incubated 45 min on ice and then centrifuged. Supernatants were removed and pellets were resuspended in PBS; both supernatants and resuspended pellets were adjusted to approx. pH 7.0 by dropwise addition of conc. HCl.

Protease digestion. Microsomal fractions (5 mg protein/mL) were incubated for 1 hr at 37° in digestion buffer (50 mM Tris adjusted to pH 7.5 with HCl, containing 0.25 M sucrose, 50 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$), in the presence of either 1 mg/mL trypsin (EC 3.4.21.4) or 0.5 mg/mL trypsin plus 0.5 mg/mL chymotrypsin (EC 3.4.21.1), and in the presence or absence of 0.4% (w/v) Triton X-100. Digestion was terminated by addition of one-third volume of an ice-cold 6 mg/mL solution of either soybean trypsin inhibitor (for trypsin digestion) or aprotinin (for trypsin–chymotrypsin digestion), which were dissolved in digestion buffer. After incubation for 10 min on ice, samples were analysed by SDS–PAGE and immunoblotting.

SDS–PAGE and immunoblotting. The procedures used for SDS–PAGE, electrophoretic transfers to nitrocellulose and development of nitrocellulose blots with antibodies have been described elsewhere [6, 7, 21]. SDS–PAGE was performed on conventional large gels (12 cm resolving gel) or on minigels (5.5 cm resolving gels), which gave slightly poorer electrophoretic resolution but which could be run much more conveniently. Antibody development involved incubation of the nitrocellulose with primary antiserum, then incubation with horseradish peroxidase-conjugated secondary antiserum, followed by peroxidase colour development with 4-chloro-1-naphthol. Gel sample loadings and antibody incubation conditions are given in the figure legends. For the experiments shown in Figs 2 and 3, the patient's serum used for immunoblot development was pre-absorbed to minimize antibody reactivity towards normal liver proteins. This was achieved by incubating the diluted serum (volume 10 mL, dilution 1:50) for 24 hr at 4° with a nitrocellulose sheet which contained microsomal protein from control rat liver, that had been run on a minigel (840 μg protein in total).

Other methods. Protein was determined according to Lowry *et al.* [22] with bovine serum albumin as standard. Activity of NADPH–cytochrome P450 (cytochrome *c*) reductase (EC 1.6.2.4) was assayed as described by Gibson and Skett [23].

Reagents. Sodium deoxycholate, sesame oil, Triton X-100, β-NADPH (type X), β-NADH (grade III), bovine pancreatic chymotrypsin (type I-S), bovine pancreatic trypsin (type III), soybean trypsin inhibitor (type I-S) and aprotinin were from the Sigma Chemical Co. (Poole, U.K.). Halothane was from the Aldrich Chemical Co. (Poole, U.K.) and was distilled before use. Other chemicals were from standard commercial sources, as described previously [6, 7]. Human sera, which contained antibodies to the TFA–liver protein antigens, were obtained from three patients with halothane hepatitis. Anti-TFA antiserum and antiserum to NADPH–cytochrome P450 reductase were prepared as described elsewhere [20, 24]. Horseradish peroxidase conjugated antisera to human (immunoglobulin G) IgG and rabbit IgG were from Tissue Culture Services Ltd. (Buckingham, U.K., codes 6400 and 2390, respectively).

RESULTS

Detection of TFA antigens by immunoblotting

Immunoblotting experiments were performed

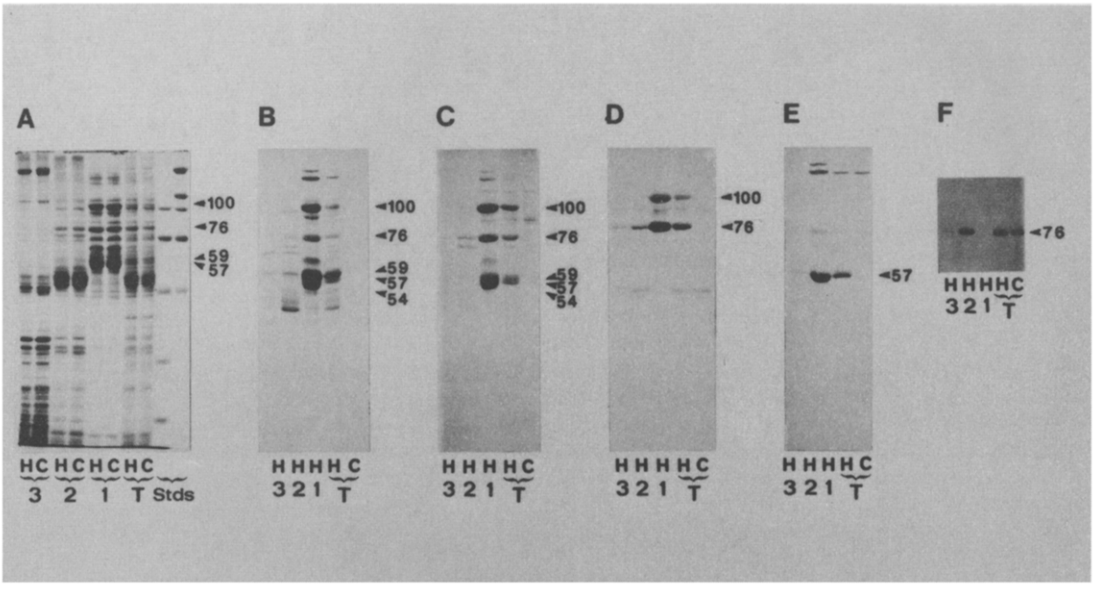

Fig. 1. Solubility in DOC of TFA antigens generated *in vivo*. Liver microsomal fractions from halothane-treated (H) or control (C) rats were solubilized by sequential incubation with 0.1% DOC buffer, then 2% DOC buffer. Samples were: total membranes (T); proteins soluble in 0.1% DOC (1); proteins soluble in 2% DOC (2); proteins insoluble in DOC (3); molecular mass markers (Stds). The molecular masses of the markers used were: 200, 116.25, 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa. (A) Gel stained for protein; (B–F) immunoblots. Immunoblots were developed with: anti-TFA antiserum dilution 1:1000 (B); sera from three patients with halothane hepatitis, each dilution 1:100 (C, D and E); and anti-reductase antiserum dilution 1:500 (F). Primary antibody incubations were for 3 hr at room temperature. Second antibody incubations were for 2 hr at room temperature, with reagents dilution 1:500. Panels A–E were from large gels (36.5 μg protein/track) and panel F was from a minigel (12.6 μg protein/track).

using sera from three patients with halothane hepatitis and also anti-TFA antiserum. The specificities of the sera have been characterized previously [6, 7, 24]. The anti-TFA antiserum, which was from a rabbit immunized with TFA–rabbit serum albumin and was specific for the TFA hapten [24], recognized a range of TFA-labelled polypeptides expressed in livers of halothane-treated rats [Fig. 1B: compare antibody reactivity towards halothane treated fractions (H) with reactivity towards control fractions (C)]. The patients' sera recognized five distinct TFA antigens which were resolved by SDS–PAGE. These corresponded in molecular masses to major TFA-labelled polypeptides recognized by the anti-TFA antiserum (Fig. 1: compare C, D and E with B). One of the patients' sera recognized each of the TFA antigens (100, 76, 59, 57 and 54 kDa) (Fig. 1C and Fig. 2B). Another serum recognized only the 100 and 76 kDa antigens (Fig. 1D) and the third serum recognized only the 57 kDa antigen (Fig. 1E).

Two different methods were adopted for generation of the TFA antigens. Firstly, rats were treated with halothane *in vivo*, then left for 18 hr to metabolize the drug before being killed. This resulted in expression of relatively high levels of the 100, 76, 59 and 57 kDa antigens, and expression of extremely low levels of the 54 kDa antigen (Fig. 1B–E). Secondly, liver microsomal fractions from untreated rats were incubated with halothane *in vitro*, in the presence of NADPH and oxygen. This resulted in preferential expression of the 54 kDa antigen (Figs 2 and 3). Overall, the levels of TFA antigens expressed *in vitro* were much lower than the levels expressed *in vivo*. These results are consistent with studies reported previously, which have shown that in rats treated with halothane *in vivo*, the 54 kDa antigen is generated and degraded much more rapidly than are the other antigens, and so is scarcely detectable by 18 hr [21], and that the 54 kDa antigen is generated preferentially when liver microsomal fractions are incubated with halothane *in vitro* [7].

In addition to containing antibodies to TFA antigens, the patients' sera contained antibodies which recognized a number of protein antigens which were not TFA-labelled, and were expressed in microsomal fractions prepared from livers of rats not treated with halothane. Detection of the relatively low levels of TFA antigens generated *in vitro* necessitated the use of quite extreme antibody incubation conditions. This resulted in a marked increase in the intensity of recognition of several of the non-TFA antigens (compare Figs 2B and 3B, with Fig. 1C).

Topographies of antigens generated in vivo

Extraction of liver microsomal fractions from

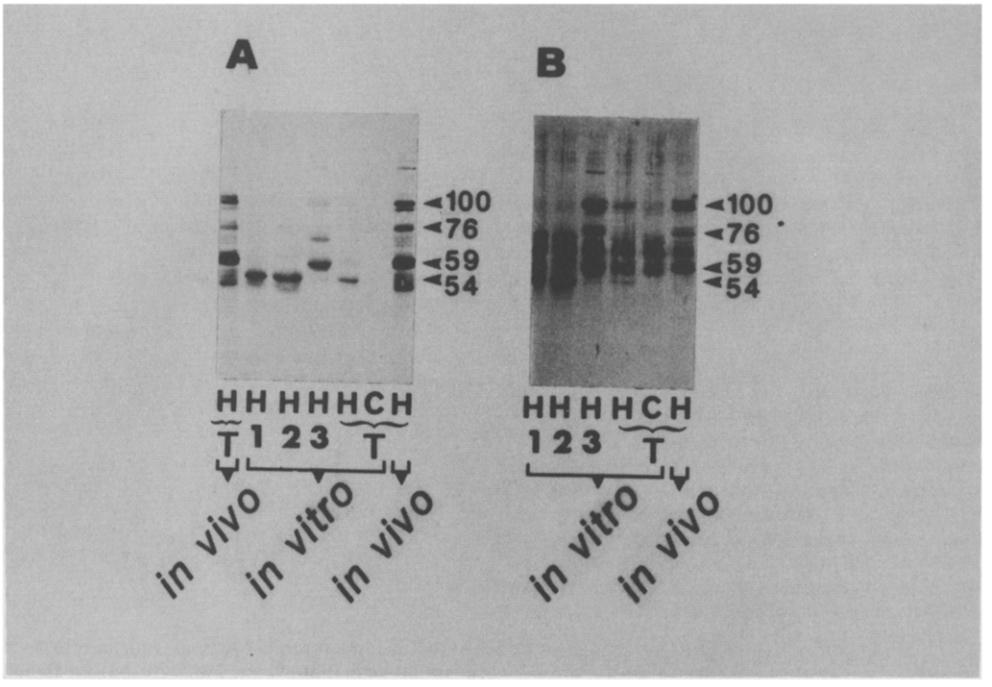

Fig. 2. Solubility in DOC of TFA antigens generated *in vitro*. The *in vivo* sample was a liver microsomal fraction from a rat treated with halothane *in vivo*. *In vitro* samples were liver microsomal fractions from untreated rats which were incubated either with (H) or without (C) halothane, then solubilized with DOC (see Fig. 1 and text) to yield proteins soluble in 0.1% DOC (3), proteins soluble in 2% DOC (2) and proteins insoluble in DOC (1). (T) represents total microsomal fraction. Minigels were run and blots were developed with: anti-TFA antiserum dilution 1:250 (A); or serum from a patient with halothane hepatitis dilution 1:50, which had been pre-absorbed against control microsomal fraction (B). Serum B corresponds to serum C of Fig. 1. Primary and secondary antibody incubations were for 24 hr at 4°; second antibodies were dilution 1:250. Sample loading was 17.6 μg protein/track, except for the *in vivo* fraction which was 5 μg protein/track.

halothane treated rats with buffer containing 0.1% DOC resulted in solubilization of the 100, 76, 59 and 57 kDa TFA antigens (Fig. 1B, C, D and E). The antigens were also solubilized when the microsomal fractions were extracted with 0.1 M sodium carbonate (Fig. 4B, C and D). More efficient solubilization was achieved with 0.1% DOC than with 0.1 M sodium carbonate (compare Fig. 4 with Fig. 1). Protein estimations revealed that 23% of total microsomal protein was solubilized with 0.1% DOC and 40.7% was solubilized with 0.1 M sodium carbonate. To investigate whether 0.1% DOC or 0.1 M sodium carbonate caused appreciable solubilization of integral membrane proteins, their ability to solubilize the enzyme NADPH–cytochrome P450 reductase was investigated. As determined by immunoblotting, the enzyme was not solubilized from the microsomal membrane by either treatment, but was solubilized when membranes were extracted with 2% DOC (Fig. 1F and Fig. 4E). Assays of the activity of the enzyme, which were performed using oxidized cytochrome *c* as substrate [23], confirmed that only 3.1% of reductase activity was soluble in 0.1% DOC, whereas 96.6% of activity was soluble in 2% DOC (Table 1).

When the liver microsomal fractions were incubated with trypsin in the presence of detergent, the 100, 76, 59 and 57 kDa TFA antigens were completely degraded (Fig. 5B–D). The antigens were degraded only to a slight extent when the microsomal fractions were incubated with trypsin without detergent (Fig. 5B–D). In contrast, NADPH–cytochrome P450 reductase was degraded extensively by trypsin in the absence of detergent (Fig. 5E; note the increased electrophoretic mobility and greatly decreased antibody staining seen after trypsin digestion), as were many other polypeptides (Fig. 5A). Quite harsh incubation conditions were employed in the digestion experiments (1 hr incubation at 37°, with 1 mg/mL trypsin) because preliminary experiments had shown that milder conditions resulted in incomplete degradation of the TFA antigens, even when detergent was present. Control experiments, in which microsomal fractions were incubated without added trypsin, in the presence or absence of detergent, resulted in no detectable degradation of the antigens (Fig. 5B–D). Thus, the results obtained are not attributable simply to activation of endogenous microsomal proteases by detergent. A further series of proteolytic digestion experiments, in which a mixture of trypsin plus chymotrypsin was used in place of trypsin, yielded

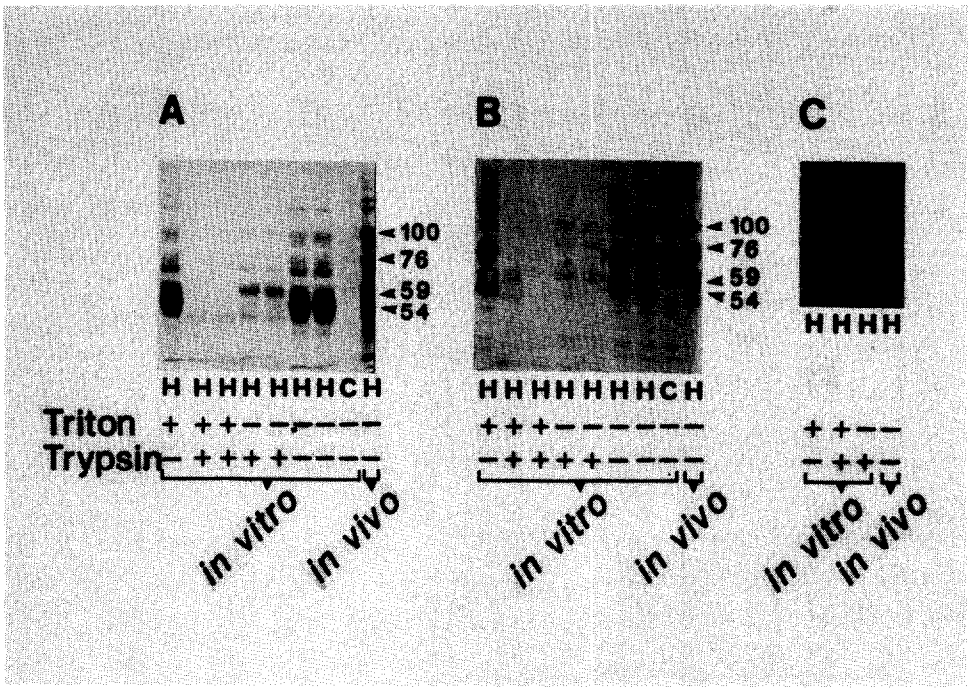

Fig. 3. Trypsin digestion of TFA antigens generated *in vitro*. *In vivo* and *in vitro* samples were prepared as described in the legend to Fig. 2. The *in vitro* samples were incubated for 1 hr at 37°, with (+) or without (−) trypsin and/or Triton X-100. Minigels were run and immunoblots were developed with: anti-TFA antiserum (A); serum from a patient with halothane hepatitis, which had been pre-absorbed against control microsomal fraction (B); and anti-reductase antiserum dilution 1:250 (C). Antibody incubation conditions were as described for Fig. 2. Serum B corresponds to serum C of Fig. 1. Sample loading was 33 μg protein/track, except for the *in vivo* fraction which was 10 μg protein/track.

results which were very similar to those obtained with trypsin alone (results not shown).

Topography of antigens generated in vitro.

In agreement with the results described above, the 100, 76 and 59/57 kDa antigens generated when microsomal fractions from untreated rats were incubated with halothane *in vitro* were solubilized when the fractions were extracted with 0.1% DOC (Fig. 2A and B). Furthermore, although appreciable tryptic degradation of the 100, 76 and 59–57 kDa TFA antigens produced *in vitro* was observed when microsomal fractions were incubated with trypsin in the absence of detergent, complete degradation of the antigens was evident only when detergent was present (Fig. 3A and B). In contrast, the 54 kDa TFA antigen was insoluble in 0.1% DOC (Fig. 2A and B) and was completely degraded when microsomal fractions were incubated with trypsin in the absence of detergent (Fig. 3A and B), as was NADPH–cytochrome P450 reductase (Fig. 3C).

DISCUSSION

Extraction with low concentrations of DOC or with 0.1 M sodium carbonate have been reported to permeabilize microsomal vesicles and to release peripheral proteins, without dissolving the membrane and solubilizing integral proteins [25, 26]. In addition, it has been shown that proteins exposed on the outer (cytosolic) surface of microsomal vesicles are readily accessible to proteases in the absence of detergent, while proteins located within the vesicle lumen are inaccessible to proteases unless the membrane is permeabilized, for example by addition of detergent [19, 27]. We found that the 100, 76, 59 and 57 kDa TFA antigens were solubilized when liver microsomal fractions from rats treated with halothane *in vivo* were extracted with 0.1% DOC or with 0.1 M sodium carbonate, and were resistant to degradation by proteases unless detergent was present. These results indicate that the 100, 76, 59 and 57 kDa antigens are peripheral membrane proteins which are situated within the lumen of the endoplasmic reticulum. This interpretation is supported by our investigation of the apparent topography of the enzyme NADPH–cytochrome P450 reductase, which is an integral membrane protein exposed on the cytoplasmic face of the endoplasmic reticulum [28]: the protein was not solubilized by either 0.1% DOC or 0.1 M sodium carbonate and was degraded by trypsin in the absence of detergent.

The 54 kDa antigen, which was generated *in vitro* by incubation of rat liver microsomal fractions with halothane, was not solubilized when the fractions were extracted with 0.1% DOC. Thus, it appears

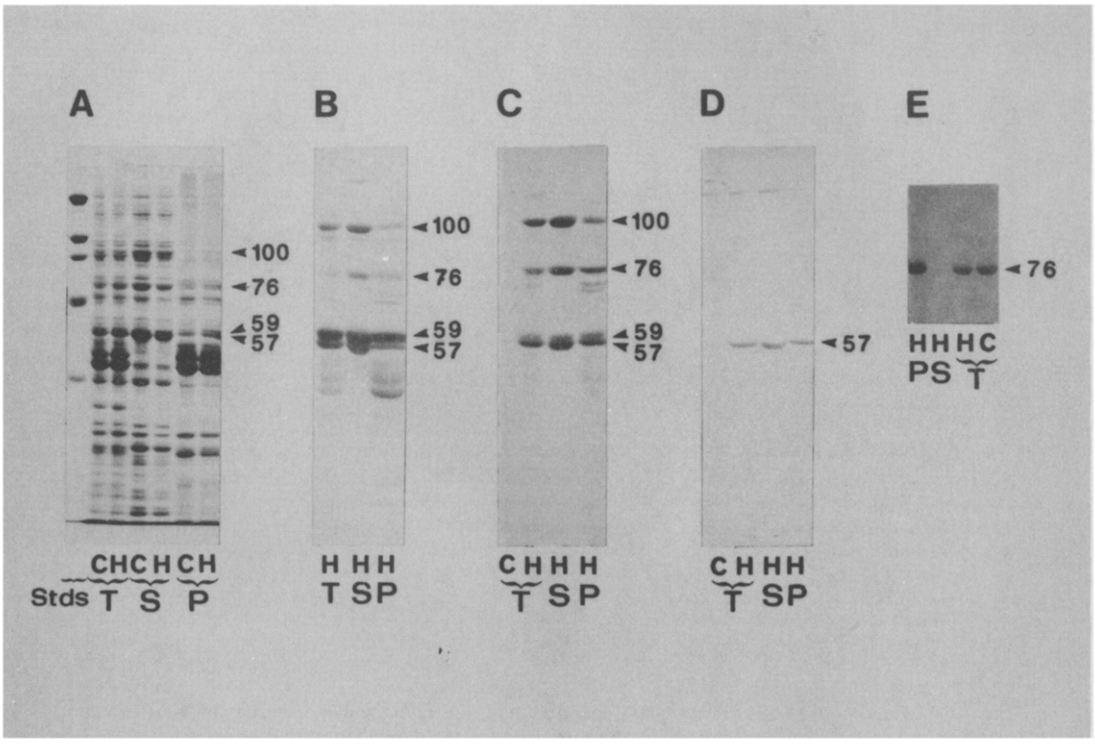

Fig. 4. Solubility in sodium carbonate of TFA antigens generated *in vivo*. Liver microsomal fractions from halothane treated (H) or control (C) rats were extracted with 0.1 M sodium carbonate. Samples were: total membranes (T); proteins soluble in carbonate (S); proteins insoluble in carbonate (P); molecular mass markers (Stds) (see Fig. 1). (A) Gel stained for protein; (B–E) immunoblots. Immunoblots were developed with: anti-TFA antiserum (B), sera from patients with halothane hepatitis (C and D) and anti-reductase antiserum (E). Sera C and D correspond to sera C and E of Fig. 1. Antibody incubation conditions were as described for Fig. 1. Panels A–D were from large gels (50 μg protein/track) and panel E was from a minigel (12.5 μg protein/track).

Table 1. Distribution of NADPH–cytochrome *c* reductase activity in subfractions obtained by extraction of rat liver microsomal fractions with sodium deoxycholate

	Halothane			Control		
Subfraction	Protein content (% of total)	Specific activity*	Activity as % of total	Protein content (% of total)	Specific activity*	Activity as % of total
0.1% DOC	23.0	0.012 ± 0.002	2.2	20.7	0.017 ± 0.001	3.1
2% DOC	71.4	0.169 ± 0.012	97.6	73.4	0.148 ± 0.013	96.6
Insoluble	5.6	0.004	0.2	5.9	0.006	0.3

Microsomal fractions were from livers of rats treated *in vivo* with halothane in sesame oil ("halothane"), or sesame oil alone ("control"), 18 hr prior to being killed. These were extracted sequentially with 0.1% DOC, then 2% DOC.
* μmol/min/mg protein (mean ± SE, N = 3).

that the 54 kDa antigen is an integral membrane protein. Complete degradation of the 54 kDa antigen occurred when the microsomal fractions were incubated with protease in the absence of detergent, suggesting that the protein is exposed on the outer, cytosolic face of microsomal vesicles. However, since appreciable proteolytic degradation of the 100, 76 and 59/57 kDa antigens generated *in vitro* also occurred in the absence of detergent, marked membrane permeabilization must have occurred during incubation of the membrane fractions with halothane, or during the subsequent incubation with protease. In view of this, at present we are unable to conclude unequivocally that the 54 kDa antigen

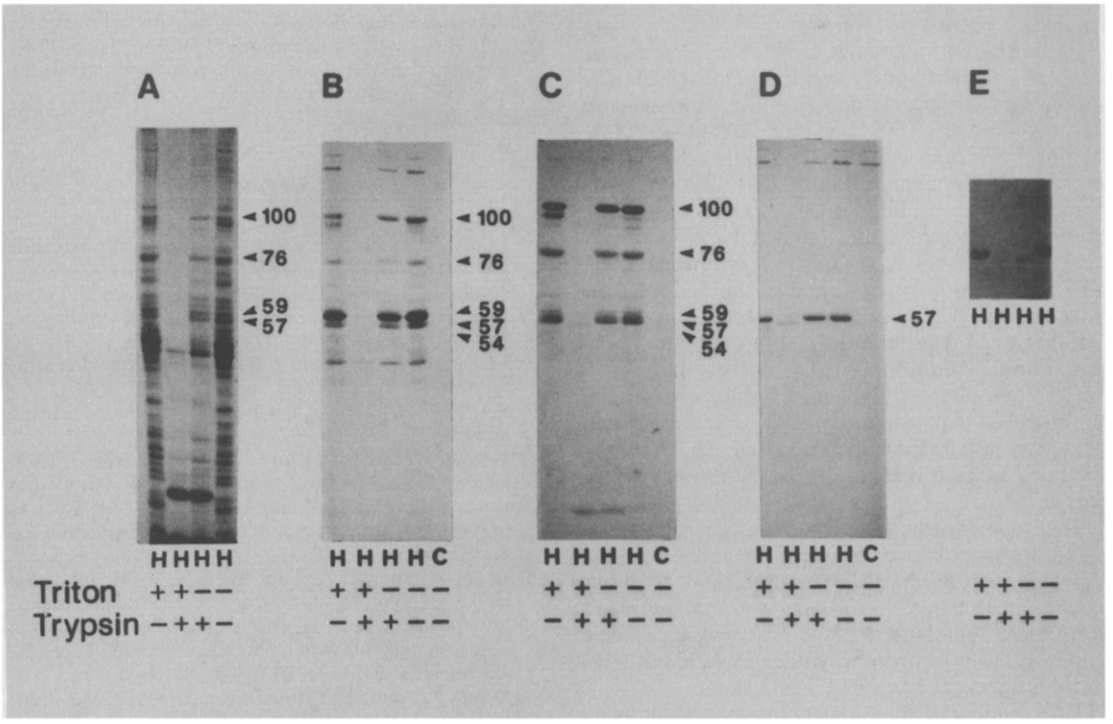

Fig. 5. Trypsin digestion of TFA antigens generated *in vivo*. Liver microsomal fractions from halothane treated (H) or control (C) rats were incubated for 1 hr at 37° with (+) or without (−) trypsin and/or Triton X-100, as indicated. (A) Gel stained for protein; (B–E) immunoblots. Immunoblots were developed with: anti-TFA antiserum (B); sera from patients with halothane hepatitis (C and D); and anti-reductase antiserum (E). Sera C and D correspond to sera C and E of Fig. 1. Antibody incubation conditions were as described for Fig. 1. Panels A–D were from large gels (37 μg protein/track) and panel E was from a minigel (33 protein μg/track).

is exposed on the cytosolic face of the endoplasmic reticulum.

In a previous study, one of the TFA antigens (59 kDa) was purified and was shown unequivocally to be a TFA-modified liver microsomal carboxylesterase isozyme [11]. The method described for purification of this antigen was based upon affinity chromatography on an anti-TFA antibody column, which resulted in a relatively low yield of purified antigen [11]. Recently we have developed an alternative strategy, which has taken advantage of the solubility of the antigens in 0.1% DOC and which appears to be of general utility for purification of TFA antigens. Using this approach, antigens of 58, 57, 63 and 100 kDa have been purified. The 58 and 63 kDa antigens were not identified in previous immunoblotting studies because their electrophoretic mobilities are very similar to those of the 57 and 59 kDa antigens. The 58 kDa antigen has been shown to correspond in amino acid sequence to a TFA-labelled form of a protein which previously had been postulated to be a phosphatidylinositol-specific phospholipase C-α [29], while the 63 kDa antigen has been shown to correspond to a TFA-modified form of the calcium-binding protein calreticulin [30, 31]. Preliminary analysis of the 57 kDa antigen has indicated that it bears a very close amino acid sequence similarity to the enzyme protein disulphide isomerase [32, 33]. In addition, amino acid sequence analysis of the 100 kDa antigen has suggested that it may be a TFA-labelled form of a stress-inducible protein variously termed endoplasmin, ERp99 and/or GRP94 [34–37].

Studies undertaken by other workers have indicated that microsomal carboxylesterase, protein disulphide isomerase, calreticulin and endoplasmin are all members of a class of peripheral membrane proteins which are resident within the lumen of the endoplasmic reticulum [14, 30, 33, 38, 39]. The results obtained in the present study demonstrate that the TFA-modified forms of the proteins, which are produced in livers of halothane-treated rats, exhibit the same membrane topographies as the unmodified proteins. Why proteins resident within the lumen of the endoplasmic reticulum should be major targets of the TFA halide metabolite of halothane is unknown. This issue is of particular interest since the active site of the cytochrome P450 molecule, which is responsible for generation of the TFA halide reactive species, is contained within a large carboxyl terminal domain situated on the cytoplasmic face of the endoplasmic reticulum

[15, 16]. How does interaction of the proteins with TFA halide occur?

The most likely explanation is that TFA halide partitions readily into the membrane of the endoplasmic reticulum, due to its hydrophobicity, and traverses the lipid bilayer, enabling TFA-labelling to occur on the lumenal side of the membrane. It is highly unlikely that TFA-labelling occurs on the cytosolic face of the membrane since proteins resident within the lumen of the endoplasmic reticulum are believed to be synthesized on polysomes bound to membrane of the endoplasmic reticulum, and to be translocated through the membrane co-translationally [40]. Indeed, co-translational transport of microsomal carboxylesterases into microsomal vesicles has been demonstrated experimentally [14]. Co-translational interaction of nascent polypeptide chains with TFA halide, on the cytosolic face of the membrane, might occur prior to membrane insertion. However, this possibility is inconsistent with our observation that substantial proportions of the 100, 76 and 59/57 kDa TFA antigens produced by microsomal fractions incubated with halothane *in vitro* were not degraded by protease unless detergent was present, since significant levels of protein synthesis would not have occurred during the *in vitro* incubations.

TFA-labelling of the 54 kDa antigen, which is an integral membrane protein and which may well be exposed appreciably on the cytosolic face of the membrane, is more likely to occur on the cytosolic face of the membrane. Indeed, in view of its apparent molecular mass, subcellular location, membrane topography and relatively rapid rate of generation *in vivo* and *in vitro* ([7, 21]; the present results), the 54 kDa antigen might well be a TFA-labelled cytochrome P450 isozyme. In this regard, a phenobarbitone-inducible isozyme of cytochrome P450 has been shown to be the major TFA-labelled protein produced in livers of rats treated with halothane after induction with phenobarbitone [20].

Experiments performed using the techniques of indirect immunofluorescence and antibody-dependent cell-mediated cytotoxicity have demonstrated that TFA antigens are expressed on the surface of hepatocytes isolated from livers of halothane-treated rabbits and rats [5, 24, 41]. The mechanisms involved in expression of the antigens on the hepatocyte surface are of particular importance because only antigens which are expressed on the surface membrane should be accessible to immune effector mechanisms in intact hepatocytes *in vivo*. In principle, translocation of TFA antigens from the lumen of the endoplasmic reticulum to the cell surface could occur via the normal processes of membrane flow [40]. For resident lumenal proteins, including microsomal carboxylesterases, protein disulphide isomerase and calreticulin, this would have to involve circumvention of the highly efficient retention mechanisms which operate under normal circumstances to restrict the proteins to this compartment [42, 43]. Perhaps this occurs because TFA-labelling of the proteins interferes with the structural features of the proteins which are responsible for their retention within the endoplasmic reticulum.

Acknowledgements—J.G.K. was supported by an Advanced Training Fellowship in Toxicology and by a Wellcome Trust project grant, both from the Wellcome Trust, U.K. J.L.M. was supported by a Pharmacology Research Associate Fellowship from the National Institute of General Medical Sciences, U.S.A.

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