Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3987–3991, May 1990
Medical Sciences

Reactivity of primary biliary cirrhosis sera with *Escherichia coli* dihydrolipoamide acetyltransferase (E2p): Characterization of the main immunogenic region

(2-oxo acid dehydrogenase complexes/lipoic acid/autoimmunity)

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Communicated by Lester J. Reed, March 12, 1990

ABSTRACT **Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by the presence of antimitochondrial autoantibodies in the serum. The major antigens recognized by the antibodies are the E2 components of the 2-oxo acid dehydrogenase complexes, all of which possess covalently attached lipoic acid cofactors. A bacterial etiology has been proposed for the disease, and patients' antibodies are known to recognize the E2 subunits (E2p) of both mammalian and bacterial pyruvate dehydrogenase complexes. Immunoblotting and ELISA inhibition techniques using extracts of *Escherichia coli* deletion strains, genetically restructured E2 polypeptides, and isolated lipoyl domains demonstrate that (*i*) the E2o subunit of the *E. coli* 2-oxoglutarate dehydrogenase complex is recognized by patients' antibodies; (*ii*) the main immunogenic region of E2p lies within the lipoyl domains; (*iii*) the presence of a lipoyl residue within the domain is crucial for effective recognition by the antibodies; and (*iv*) octanoylated E2p, octanoylated E2o, and octanoylated lipoyl domain, produced by a mutant deficient in lipoate biosynthesis, are recognized by patients' antibodies but not as effectively as their lipoylated counterparts. These findings indicate that antibodies in PBC patients' sera bind to a unique peptide-cofactor conformation within the lipoyl domains of the E2 polypeptides and that this epitope is partially mimicked by substituting the lipoyl cofactor with an octanoyl group.**

Primary biliary cirrhosis (PBC) is a chronic liver disease characterized by progressive inflammatory destruction of intrahepatic bile ducts, leading to cirrhosis and liver failure (1). The etiology of PBC remains unclear, but a variety of evidence supports an autoimmune process and a serological marker for the diagnosis of PBC is the presence of antimitochondrial autoantibodies directed against a family of antigens termed M2 (2). We and others have recently identified several constituents of the M2 antigens as components of the mitochondrial 2-oxo acid dehydrogenase complexes: pyruvate, 2-oxoglutarate, and branched-chain 2-oxo acid dehydrogenase complexes (PDC, OGDC, and BCOADC, respectively) (3–7). Each complex consists of multiple copies of three component enzymes termed E1, E2, and E3 (8). The major antigenic species are the E2 components [dihydrolipoamide acetyltransferases (acetyl-CoA:dihydrolipoamide *S*-acetyltransferase, EC 2.3.1.12)] of each complex and protein X of PDC (4, 9). The E2 components of the 2-oxo acid dehydrogenase complexes have highly segmented structures consisting of up to three lipoyl domains in which the lipoic acid cofactor is covalently attached to a lysine residue, an E3-binding domain, and a carboxyl-terminal catalytic inner-core domain (ref. 8 and Fig. 1). The domains are connected by conformationally mobile linker sequences, many of which are rich in alanine and proline residues.

Lipoic acid is known to stimulate antibody responses both *in vitro* (10) and in immunosuppressed mice (11). The presence of at least one lipoyl moiety in all E2 components (and protein X) suggests that lipoate may have a role to play in the "autoimmune" response to these proteins. Although lipoate alone is not responsible for antibody binding (12, 13), there are two lines of evidence implying that the unique peptide environment surrounding lipoic acid is important with respect to antigenicity. Fussey *et al.* showed that, following tryptic cleavage of bovine E2p, antibodies bind exclusively to the lipoyl-containing region of the polypeptide (12). Using recombinant proteins, Van de Water *et al.* (14) presented evidence localizing the main immunogenic region of rat E2p to a 200-residue segment of polypeptide and found that a peptide corresponding to the 20 amino acids flanking the lipoic acid attachment site was able to absorb reactivity from PBC sera, albeit only at sera dilutions of 1:80,000.

Although the cause remains unknown, it has been hypothesized that PBC may have a bacterial etiology. Antibodies with the same apparent specificity as the anti-mitochondrial autoantibodies found in PBC sera can be induced in rabbits by inoculation with rough (R) mutants of Gram-negative bacteria (15). Furthermore, intestinal colonization by R forms of *Escherichia coli* has recently been reported in PBC patients, and this may be important in disease pathogenesis (16). Another group has proposed a specific association between recurrent urinary tract infection and PBC (17) and reported that patients with recurrent bacteriuria, but not PBC, have low-titer anti-M2 anti-mitochondrial autoantibody (18).

It has been shown that antibodies in sera from PBC patients also react with certain bacterial proteins (15, 19, 20). One of these antigenic polypeptides has been shown to be *E. coli* PDC E2 polypeptide (E2p) (12, 21), which, like other bacterial E2 components, exhibits a high degree of structural homology with its mammalian counterparts (22–24), although the number of lipoyl domains varies with the source and type of complex. In *E. coli*, for example, the E2 component of PDC (E2p) has three lipoyl domains, whereas that of OGDC (E2o) has only one lipoyl domain.

A variety of reconstructed E2p components and subcomponents have been created by site-specific mutagenesis (22, 25). Recently it has been shown that overexpression of a lipoyl domain subgene produces both lipoylated and unlipoyl-

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Abbreviations: PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex.

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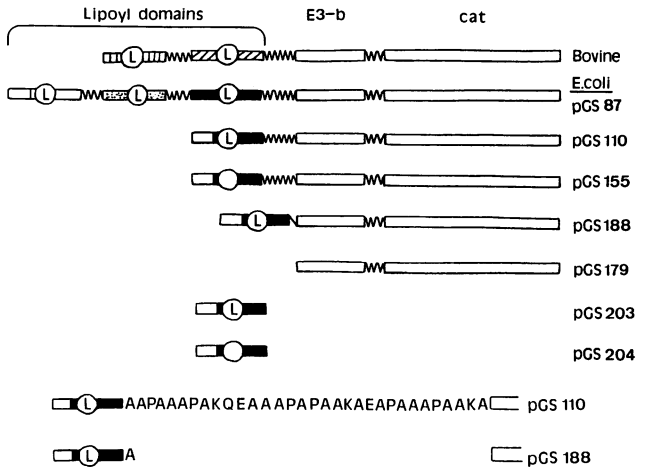

FIG. 1. Schematic representation of PDC E2 polypeptide (E2p) and genetically restructured E2p variants encoded by different plasmids. In lipoyl domains, L denotes the presence of a lipoic acid cofactor; E3-b designates the domain required for binding the E3 component; and cat designates the catalytic domain containing acetyltransferase activity. The differential shading of the lipoyl domains denotes that they are homologous but not identical. The zig-zag lines represent the alanine/proline-rich interdomain linkers. The amino acid sequence of the linker region that is largely deleted in pGS188 is shown.

ated forms of the domain (26, 27), and under lipoyl-deficient conditions, an octanoylated form of the domain accumulates (27). In the present study, genetically reconstructed components and subcomponents have been used to localize the main immunogenic region on *E. coli* E2p; and lipoylated, unlipoylated, and octanoylated variants of the lipoyl domain have been used to investigate the role of lipoic acid within the B-cell epitope.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The following strains of *E. coli* K-12 were used (relevant genotypes only): HfrH, wild-type strain (27); JRG1342 (*aceEF-lpd*Δ18), a PDC deletion strain (28); TK3D18 (*kdp-sucAB*Δ18), an OGDC deletion mutant (29), and JRG26 (*lip-2*), a lipoic acid biosynthesis mutant, formerly W1485*lip2* (28).

A series of plasmids expressing *E. coli* PDC and variants containing restructured E2p components were used; Fig. 1 represents the structures of the engineered polypeptides aligned with mammalian and *E. coli* E2p components. Mammalian E2p has two lipoyl domains, whereas wild-type *E. coli* (pGS87) has a "three-lip" E2p (29); pGS110 encodes a "one-lip" E2p (30); pGS155 has a "one-lip" E2p in which the lipoylatable lysine residue is replaced by glutamine, thus precluding modification (25, 31); pGS179 encodes a "no-lip" E2p (32); and pGS188 expresses a "one-lip" E2p that lacks most of the alanine/proline-rich linker region between the lipoyl domain and the E3 binding domain (33). The PDCs encoded by these plasmids are expressed from the natural promoters. Two plasmids that express independently folded hybrid lipoyl domains from *aceF* subgenes via thermoinducible phage λ promoters were also used: pGS203 and pGS204, which are identical except for a glutamine codon that replaces that of the lipoylatable lysine residue in pGS204 (26).

Transformed strains were selected by their ampicillin resistance, and the presence of the plasmids was confirmed by agarose gel electrophoresis of cleared cell lysates.

Microbiological and Enzymological Techniques. Cultures (50 ml) were grown to stationary phase at 37°C in L broth (34) containing ampicillin (50 μg/ml) as required. The lipoate biosynthesis mutant, JRG26, and its derivatives were grown in minimal medium (35) containing 0.2% glucose and supplements either of 2 mM acetate/2 mM succinate to produce unlipoylated (inactive) PDC and OGDC or of lipoic acid at 5 ng/ml to produce lipoylated (active) complexes (28). Ultrasonic extracts were prepared and assayed to confirm the presence or absence of PDC and OGDC activities (36). Plate tests were used to verify the nutritional phenotypes of PDC- and OGDC-deficient strains before and after growth in liquid media.

Samples of purified PDC with wild-type or reconstructed E2p components were provided by S. J. Angier and R. N. Perham. Lipoyl domains were isolated after thermoinduction either from pGS203 and pGS204 in a wild-type strain to give lipoylated (L203), unlipoylated (U203), and mutant (204) domains or from pGS203 in a *lip* mutant (JRG26) grown under lipoate-deficient conditions as described (27) to give an octanoylated domain ($L203_{26}$) and then isolated by the methods of Packman *et al.* (37) and Ali *et al.* (27).

Human Sera. Sera were obtained (and stored at −20°C) from 129 patients with documented PBC as detailed (9), and a pooled PBC sera stock was created by using equal aliquots of these individual samples.

Immunoblotting. Purified proteins and/or *E. coli* extracts were subjected to $NaDodSO_4$/PAGE on 10% gels (38) and then transferred electrophoretically to nitrocellulose (39). After blocking and washing, the nitrocellulose was incubated for 2 hr with human sera at a dilution between 1:50 and 1:1000. Detection of bound antibodies was by use of secondary goat anti-human IgG or IgM (γ or μ chain-specific, respectively) peroxidase-conjugated antibodies (Sigma) with 4-chloro-1-naphthol as substrate (3). All immunoblotting experiments were carried out with pooled PBC sera and with sera from three individual PBC patients. All sera tested gave identical blotting profiles for both IgG and IgM antibodies.

Immunoabsorption of PBC Sera. An ELISA to detect and quantitate antibodies directed against *E. coli* E2p was established. Wells (88) of a microtiter plate were coated overnight at 4°C with 1.5 μg of purified *E. coli* PDC (containing 0.48 μg of E2p). After blocking and washing, each well was incubated with duplicate aliquots (100 μl) of absorbed or nonabsorbed diluted human sera, and the method of Heseltine *et al.* (40) was used to visualize reactivity. The remaining eight wells of each plate were coated with purified bovine E2p + X (0.25 μg of total protein) and used to assay positive and negative quality-control samples and an ELISA standard. The same standard sample as in ref. 40 was assayed in quadruplicate, allowing calculation of the ELISA index value and providing a means of interassay standardization.

Sera from individual PBC patients or pooled PBC sera were diluted in 0.9% NaCl/0.5% bovine serum albumin (BSA) to a final concentration of 1:2000 and were incubated overnight at 4°C with test compounds at a range of concentrations (0.027 μM–1 mM). Duplicate samples (100 μl) were tested subsequently in the ELISA to determine the remaining reactivity. Results were expressed as a percentage of the index value for nonabsorbed sera assayed in tandem.

RESULTS

Antibodies in PBC sera reacted strongly with two polypeptides of apparent molecular masses 83 and 54 kDa in crude extracts of wild-type *E. coli* (Fig. 2*A*, lane 1). Immunoblotting of PBC sera against *E. coli* deletion strains lacking the E2 components of either PDC (JRG1342) or OGDC (TK3D18), showed that reactivity to the respective 83-kDa or 54-kDa bands was lost (Fig. 2*A*, lanes 2 and 3), thus identifying these immunoreactive polypeptides as the E2p and E2o components of bacterial 2-oxo acid dehydrogenase complexes.

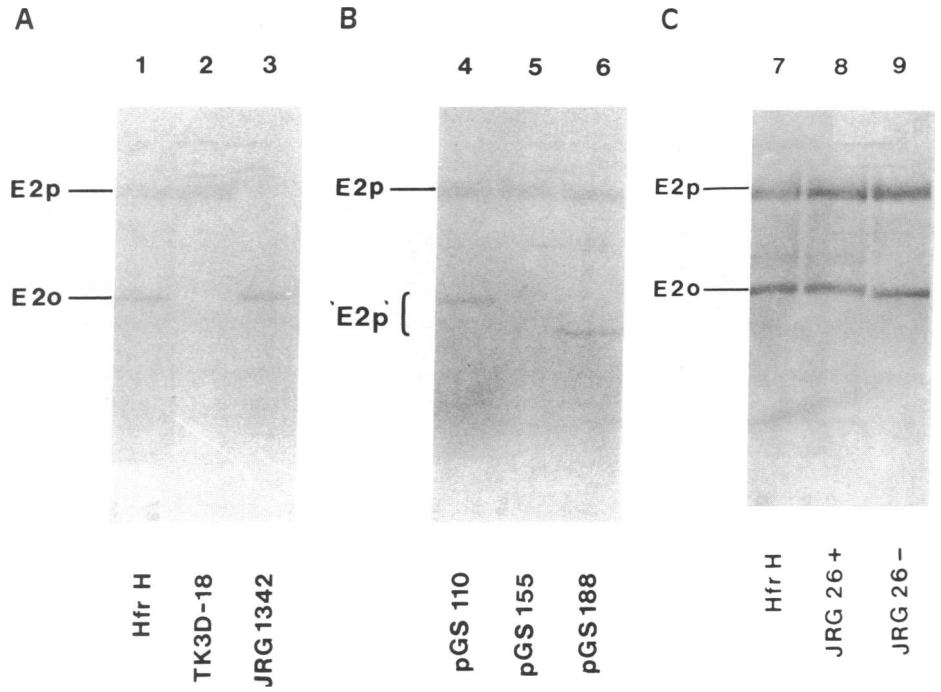

FIG. 2. Reactivity of pooled PBC sera against lipoylated or octanoylated E2p and E2o and against genetically engineered E2p variants as assessed by immunoblotting against *E. coli* extracts. Crude *E. coli* extracts (80 μg of total protein) were subjected to $NaDodSO_4$/PAGE and blotted against pooled PBC sera at a dilution of 1:500. (*A*) Lanes: 1, wild-type *E. coli* (HfrH); 2, strain TK3D18 (E2o -ve); 3, strain JRG 1342 (E2p -ve). (*B*) Extracts of strain TK3D18 transformed with different plasmids expressing "one-lip" E2p variants ('E2p'). Lanes: 4, pGS110; 5, pGS155 (lysine → glutamine); 6, pGS188 (linker deletion). (*C*) Lanes: 7, wild-type *E. coli* (HfrH); 8 and 9, extracts from the lipoic acid biosynthesis mutant JRG26 grown with either lipoate (lane 8) to produce lipoylated E2p and E2o proteins or with acetate plus succinate (lane 9) to produce octanoylated E2 proteins. Identical results were obtained with pooled PBC sera and serum from three different PBC patients, with either IgG-specific or IgM-specific secondary antibodies. No reactivity was observed against either E2p or E2o when control sera were used at a dilution of 1:500.

The ability to visualize reactivity against the E2 polypeptides in crude extracts of *E. coli* facilitated studies on the antigenicity of the engineered forms of the E2 components. The E2o polypeptide has a single lipoyl domain, and because it has a similar mobility in $NaDodSO_4$/PAGE to the "one-lip" E2p variants, the plasmids encoding these altered complexes were transferred to a strain lacking E2o (TK3D18) prior to analysis by immunoblotting. Fig. 2*B* shows that the pGS110-encoded "one-lip" E2p is recognized by patients' sera (lane 4), as is the linker-deficient derivative expressed from pGS188 (lane 6). However, the unlipoylatable (lysine → glutamine mutant) E2p encoded by pGS155 is not detected (lane 5), nor is the "no-lip" E2p encoded by pGS179 (data not shown).

When PBC serum samples were immunoblotted against purified *E. coli* PDC containing wild-type or genetically engineered E2p, it was again found that antibodies bound only to E2 polypeptides containing lipoyl domains (Fig. 3). Wild-type E2p (pGS87; lanes 1, 5, and 9) and "one-lip" E2p (pGS110; lanes 2, 6, and 10) gave positive responses, but antigenicity was lost with the pGS179-encoded "no-lip" E2p (lanes 4, 8, and 12). However, the E2p lacking most of the linker region between the lipoyl and E3-binding domains (pGS188; lanes 3, 7, and 11) was recognized by antibodies in PBC sera equally well as the corresponding protein containing the intact flexible linker sequence (pGS110; lanes 2, 6, and 10). The small amount of reactivity observed with a 54-kDa polypeptide in lanes 5, 7, and 8 of Fig. 3 reflects a minor contamination of the purified complexes with E2o. Blotting with a serum known to lack reactivity against E2o failed to detect this band (lanes 9–12). These findings indicate that the linear epitopes recognized by the PBC-specific antibodies are contained within the lipoyl domains of the E2 polypeptide.

The importance of the lipoyl substituent was investigated with a *lip* mutant (JRG26), which could be grown under lipoyl-deficient conditions by using glucose minimal medium supplemented with acetate and succinate instead of lipoate. Somewhat surprisingly, the immunoblots of crude extracts of lipoyl-deficient bacteria were essentially the same as those obtained after growth in the presence of lipoate, a positive reaction against E2p and E2o being observed in each case (Fig. 2*C*, lanes 8 and 9). This was not due to reversion of the *lip* mutant, as judged by enzymological and nutritional tests. It is now known that, in the absence of lipoate, these subunits are modified at the lipoylation site by attachment of octanoate (27). Therefore, it appears that octanoylation resembles lipoylation in conferring reactivity towards the PBC antibodies.

To further investigate the role of lipoate in antibody recognition, different types of purified lipoyl domain were tested for their ability to absorb reactivity against E2p in an ELISA system. Preliminary experiments established that preincubating pooled PBC sera with *E. coli* PDC absorbed reactivity against E2p (Fig. 4). Reactivity was completely removed by preincubation with PDC at a concentration containing approximately 0.66 μM lipoyl domains. When a corresponding concentration of free lipoylated domains (L203) was preincubated with PBC sera, 52% of the reactivity against E2p was absorbed. The octanoylated domain ($L203_{26}$) absorbed approximately 35% of the reactivity when preincubated at the same protein concentration. In contrast, the unlipoylated domain (U203) and the domain containing the lysine → glutamine substitution (204) were ineffective, equivalent amounts absorbing only approximately 6% of the reactivity against E2p.

Preincubation with free lipoic acid or lipoamide (1 mM) did not reduce reactivity against E2p below control levels. Pre-

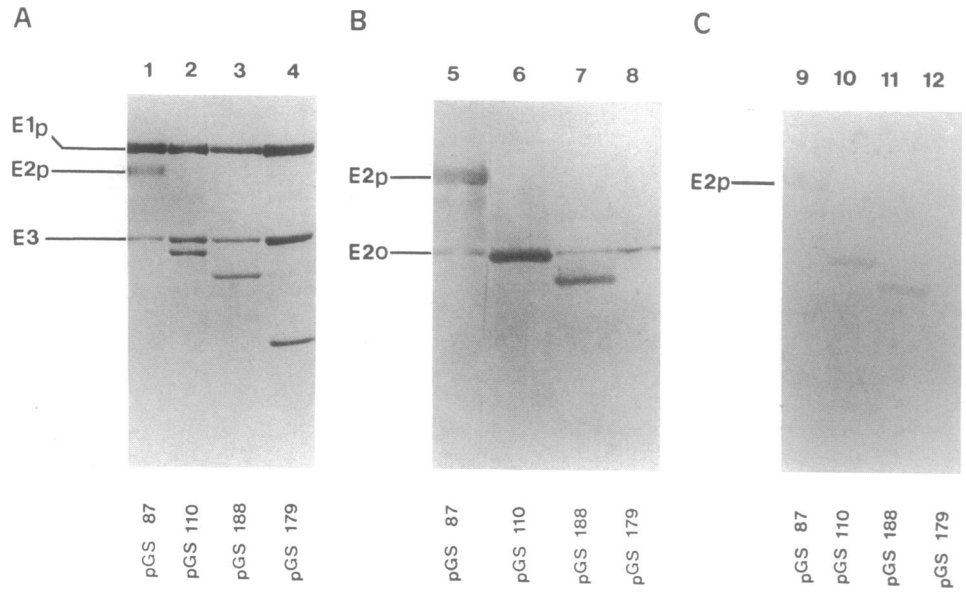

FIG. 3. Immunoblotting of PBC sera against purified *E. coli* PDC containing wild-type and genetically engineered E2p variants. Different *E. coli* PDC preparations were subjected to $NaDodSO_4$/PAGE and stained with Coomassie brilliant blue R (*A*) or were transferred to nitrocellulose and immunoblotted against either pooled PBC sera (*B*) or an individual E2o-ve PBC serum (*C*), both at a dilution of 1:500. Lanes: 1, 5, and 9, PDC with wild-type E2p (pGS87); 2, 6, and 10, PDC with "one-lip" E2p (pGS110); 3, 7, and 11: PDC with linker-deleted "one-lip" E2p (pGS188); 4, 8, and 12, PDC with "no-lip" E2p (pGS179). Approximately 15 μg of total protein was used in lanes 1–4 (each) and 6 μg of total protein in lanes 5–12 (each).

liminary studies with a 10-residue synthetic peptide containing the potential lipoylation site have shown that lipoylation of the peptide improves its ability to bind to PBC antibodies, but both peptides are considerably less effective than the intact lipoylated domains (unpublished data). These findings indicate that the lipoyl domains contain the main immunogenic region recognized by antibodies in the sera of PBC patients and that a substituent, the lipoyl cofactor or even an octanoyl group, is necessary for effective recognition by the antibodies.

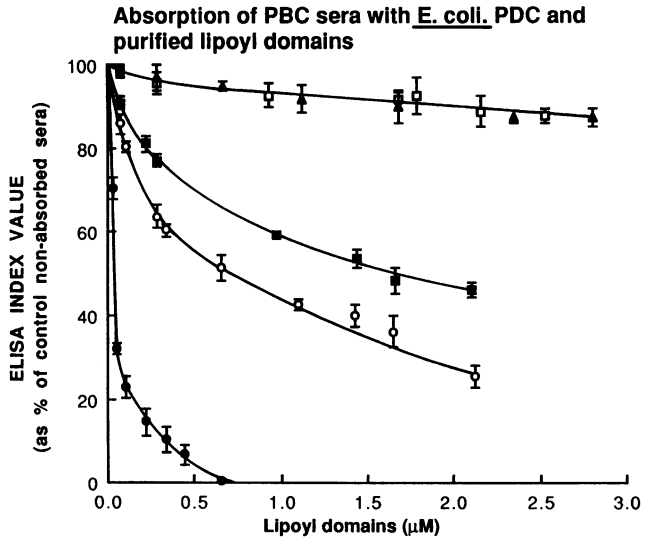

FIG. 4. Absorption of PBC sera with *E. coli* PDC and purified lipoyl domains. Aliquots (250 μl) of pooled PBC sera (dilution, 1:2000) were incubated overnight at 4°C with increasing amounts of *E. coli* PDC (●) or one of four purified lipoyl domains: L203, lipoylated (○); U203, unlipoylated (□); $L203_{26}$, octanoylated (■); and L204, unlipoylated lysine → glutamine mutant (▲). The degree of reactivity remaining was determined by assaying duplicate samples (100 μl) in an ELISA against 1.5 μg of *E. coli* PDC (containing 0.48 μg of E2p). The results were expressed as a percentage of the value for nonabsorbed sera (means ± SD) and were quoted as the concentration of lipoyl domains (μM). Each point is the average of between 4 and 20 observations. Similar results were obtained with pooled PBC sera and serum from an individual patient.

DISCUSSION

Progress recently has been made in identifying the major M2 autoantigens in PBC as components of the mitochondrial 2-oxo acid dehydrogenase complexes (3–7). In view of the possible bacterial etiology of the disease, attention also has focused on the observed reaction of PBC-specific antibodies with bacterial proteins (15, 19, 20). In this communication evidence is presented that the two major *E. coli* polypeptides recognized by PBC patients' antibodies are the E2 components of the PDC (E2p) and the OGDC (E2o), emphasizing the central role of the 2-oxo acid dehydrogenase complexes in this disease.

By using variants of *E. coli* E2p, the main immunogenic region of this antigenic protein has been located in the lipoyl-containing domains that form the amino-terminal portion of the polypeptide. Deletion of the lipoyl domains prevents binding between PBC patients' antibodies and E2p. The folded lipoyl domains of E2p move between the active sites of this large multienzyme complex during catalysis, the inferred mobility being thought to derive from the conformationally mobile linker sequences (33). Synthetic peptides corresponding to these regions are highly immunogenic when injected into rabbits (41), consistent with reported correlation between flexibility and antigenicity (42). However, evidence presented here indicates that these flexible regions of polypeptide do not play a significant role in antibody binding in PBC (Fig. 2). Furthermore, synthetic peptides corresponding to the primary sequence of the highly flexible linkers within *E. coli* E2p failed to exhibit reactivity when either immunoblotted against PBC sera after $NaDodSO_4$/PAGE on 20% gels or tested in an ELISA inhibition assay (unpublished data).

The results presented here with bacterial E2p extend conclusions drawn from earlier work on the antigenicity of mammalian E2p. Previous indications that the main immunogenic regions of bovine and rat E2p are located in the

lipoyl-containing regions were obtained by limited proteolysis of mature proteins (12) and by the combined use of synthetic peptides and genetic engineering (14). The role of the lipoic acid cofactor in antibody binding was not studied with mammalian E2p, but the availability of various restructured bacterial E2p components and subcomponents with well-defined lipoate contents has now facilitated a detailed investigation.

Immunoblotting experiments indicated that lipoate residues are essential for the effective recognition of E2p by PBC sera because unlipoylated E2p, in which the critical lysine residue is replaced by glutamine, is no longer recognized by the PBC antibodies. Data from ELISA inhibition studies support the notion that the lipoic acid moiety is an integral part of the antigenic site. Lipoylated domains reduced the ELISA index value significantly, whereas domains lacking the cofactor are essentially ineffective. Interestingly, adding another bulky substituent, octanoic acid, to the critical lysine residue restores the antibody-binding properties. Octanoylation of E2p (and E2o) in the lipoic acid biosynthesis mutant (JRG26) occurs under conditions of lipoate deprivation (27), and immunoblotting of extracts from this mutant against PBC sera revealed two antigenic polypeptides of 83 and 54 kDa, corresponding to the octanoylated versions of E2p and E2o, respectively. The antibody binding was quantitated by testing an octanoylated E2p domain in an ELISA inhibition assay, where it was found to reduce the index value significantly but not quite as effectively as the analogous lipoylated domain. Therefore, octanoylation must recreate a local environment sufficiently similar to that provided by lipoate to allow the resultant protein to be recognized by PBC antibodies, albeit to a lesser degree. It has been reported that a synthetic peptide corresponding to the primary sequence surrounding the lipoic acid attachment site in rat E2p can absorb antibodies from PBC sera, but only at high sera dilutions (14). Unfortunately the effect of lipoylation of that peptide was not examined.

It is concluded that the main immunogenic region of *E. coli* E2p, recognized by anti-mitochondrial autoantibodies in sera from PBC patients, lies within its lipoyl-containing domains. Although lipoic acid alone does not absorb reactivity from PBC sera, the evidence strongly suggests that the lipoyl moiety forms an integral part of the major B-cell epitope. In the absence of the lipoyl group, substantial antibody binding is possible only if the cofactor is replaced by another group, such as octanoic acid, which can mimic the unique peptide–cofactor conformation.

We thank Professor R. N. Perham (Department of Biochemistry, University of Cambridge) and Dr. S. J. Angier (Department of Molecular Biology and Biotechnology, University of Sheffield) for supplying some of the wild-type and mutant PDC used in this study. We are grateful to the Science and Engineering Research Council, United Kingdom, for supporting this work with research studentships (to S.P.M.F. and S.T.A.) and a project grant (to J.R.G.).

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