Characterization of antibodies to the glycosyl-phosphatidylinositol membrane anchors of mammalian proteins

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Two polyclonal antisera were raised in rabbits to the phospholipase C-solubilized forms of pig renal dipeptidase (EC 3.4.13.11) and pig aminopeptidase P (EC 3.4.11.9). These antisera were purified and shown to cross-react with other glycosyl-phosphatidylinositol (G-PI)-anchored proteins isolated from pig, human and trypanosomes. The epitopes involved in this cross-reactivity were characterized by Western-blot analysis after mild acid or nitrous acid treatment of the G-PI-anchored proteins and by a competitive e.l.i.s.a. with other G-PI-anchored proteins and individual components of the anchor structure. These studies revealed that the primary epitope for both antisera is the inositol 1,2-(cyclic)monophosphate that is formed on phospholipase C cleavage of the intact G-PI anchor. Other minor epitopes, such as phosphoethanolamine, probably involve side-chain modifications to the core anchor structure that may be species-specific.

INTRODUCTION

Numerous cell-surface proteins have been identified as possessing a glycosyl-phosphatidylinositol (G-PI) membrane anchor (Low, 1989; Turner, 1990). The C-terminal amino acid of such proteins is covalently attached via phosphoethanolamine to a sequence of three mannose residues. The reducing end of the mannose chain is in turn linked via glucosamine to the inositol head-group of a phosphatidylinositol molecule. A characteristic feature of G-PI-anchored proteins is the presence in the anchor structure of the so-called cross-reacting determinant (CRD). Polyclonal antisera raised in rabbits to the soluble form of the variant surface glycoprotein (sVSG) of trypanosomes cross-react with other G-PI-anchored proteins (Cardoso de Almeida & Turner, 1983; Low, 1987). In the G-PI membrane anchor of VSG the epitopes involved in the CRD have been characterized (Shak et al., 1988; Zamze et al., 1988). They are the inositol 1,2-(cyclic)monophosphate that is formed on cleavage of the anchor by phosphatidylinositol-specific phospholipase C (PI-PLC), the non-N-acetylated glucosamine residue and the galactose side chain. The first two of these epitopes are common to all G-PI-anchored proteins, being present in the conserved core structure, whereas the galactose side chain appears to be specific for VSGs (Doering et al., 1990; Thomas et al., 1990).

We have extensively characterized the G-PI membrane anchors of the two mammalian cell-surface peptides renal dipeptidase (DP; dehydropeptidase I, EC 3.4.13.11) and aminopeptidase P (AP-P; EC 3.4.11.9) (Hooper et al., 1987, 1990a,b; Hooper & Turner, 1988, 1989; Littlewood et al., 1989). Both DP and AP-P are recognized by an antiserum to sVSG, the cross-reactivity being due predominantly to the inositol 1,2-(cyclic)monophosphate moiety (Hooper & Turner, 1989; Hooper et al., 1990a,b). In the present study we show that polyclonal antisera raised in rabbits to both pig DP and AP-P recognize other G-PI-anchored proteins from the same and different species. These antisera have been purified and the epitopes involved in their cross-reactivity determined by Western-blot analysis and by a competitive e.l.i.s.a. These studies reveal that the primary epitope for both antisera is the inositol 1,2-(cyclic)monophosphate formed on PI-PLC cleavage of the intact G-PI anchor. Other minor epitopes, such as the phosphoethanolamine residue, may also be involved in the cross-reactivity, as well as uncharacterized species-specific epitopes.

EXPERIMENTAL

Materials

Variant surface glycoprotein 117 (sVSG) was a gift from Dr. M. A. J. Ferguson, Department of Biochemistry, University of Dundee, Dundee, U.K. Biotinylated rabbit immunoglobulin from donkey and streptavidin–biotinylated horseradish peroxidase complex were purchased from Amersham International, Aylesbury, Bucks., U.K. 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), myo-inositol, myo-inositol 1,2-(cyclic)monophosphate, myo-inositol 1-monophosphate, myo-inositol 2-monophosphate, L-α-phosphatidylinositol, ethanolamine, O-phosphoethanolamine, d(+)–galactose, N-acetyl-D-galactosamine, d(+)-glucosamine, N-acetyl-D-glucosamine, d(+)-mannose and CNBr-activated Sepharose 4B were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Titrtekt 96-well poly(vinyl chloride) microtitre plates were from Flow Laboratories, Irvine, Ayrshire, U.K. All other materials were obtained from sources previously noted.

DP was purified from pig and human kidney cortex by affinity chromatography on cilastatin–Sepharose after solubilization with either bacterial PI-PLC (phospholipase-solubilized dipeptidase; pDP) or n-octyl β-D-glucopyranoside (detergent-solubilized membrane-form dipeptidase; mDP) (Hooper & Turner, 1989; Littlewood et al., 1989; Hooper et al., 1990a).
AP-P was purified from pig kidney cortex after solubilization with bacterial PI-PLC (pAP-P) as described in Hooper et al. (1990b).

**Production and purification of antibodies**

Antibodies were raised against purified pDP (Littlewood et al., 1989) and pAP-P in New Zealand White rabbits. An IgG fraction was prepared from the serum by affinity chromatography on a column of Protein A-Sepharose. The cross-reacting determinant antibodies recognizing the CRD component were purified from the IgG fraction by affinity chromatography on a column of the appropriate peptidase (see Table 1) immobilized on CNBr-activated Sepharose as described in Matsas et al. (1986).

**SDS/PAGE and immunoelectrophoretic blot analysis**

SDS/PAGE was performed with a 7-17\% polycrylamide gradient as described previously (Relton et al., 1983). Immunoelectrophoretic (Western) blot analysis was carried out with Immobilon P [poly(vinylidene difluoride)] membranes and a 125I-labelled second antibody as detailed previously (Hooper & Turner, 1987; Hooper et al., 1990a).

**Mild acid and nitrous acid treatment**

The inositol 1,2-(cyclic)phosphate ring, formed by PI-PLC cleavage, was selectively decyclized by incubation of the protein with 1 M HCl for 30 min at 23 °C (Zamze et al., 1988). After neutralization with NaOH, samples were subjected to Western-blot analysis. Proteins were deaminated by treatment with 0.25 M-sodium acetate/0.25 M-NaNO_3, pH 5.0, for 3 h at 23 °C (Zamze et al., 1988). Control samples were treated with 0.25 M-sodium acetate/0.25 M-NaCl, pH 4.0. After neutralization, samples were acetone-precipitated and then subjected to Western-blot analysis.

**E.L.I.S.A**

The appropriate peptidase was diluted in 0.05 M-sodium carbonate, pH 9.6, and added to each well of a microtitre plate. Plates were rocked for 10 min and then left at 4 °C for 16-20 h. The wells were then washed twice with phosphate-buffered saline (PBS) (20 mM-Na_2HPO_4/2 mM-NaH_2PO_4/0.25 M-NaCl, pH 7.4), filled with 1% (w/v) BSA in PBS and incubated at 23 °C for 3 h. Finally, the wells were washed four times with PBS, then filled with PBS and stored at 4 °C.

The CRD was characterized by measuring the ability of compounds to inhibit the binding of the appropriate antibody to the corresponding peptidase (see Table 1) in the microtitre plate wells. Dilutions of the test compound in 0.1% (w/v) BSA in PBS were pre-incubated with antibody for 2 h at 23 °C. Samples were centrifuged at 8800 g for 10 min, then triplicate 50 µl portions were transferred to coated microtitre plate wells and incubated for 16-20 h at 4 °C. The plates were washed twice with PBS, twice with 0.05% (v/v) Tween 20 in PBS and finally twice with PBS. Then biotinylated rabbit immunoglobulin (1:750 dilution in 0.1% BSA in PBS) was added to each well and incubated for 1 h at 37 °C. After washing as before, bound antibodies were detected with streptavidin–biotinylated horseradish peroxidase complex (1:1500 dilution in 0.1% BSA in PBS) and incubated for 1 h at 37 °C. The plates were washed as before and the assay developed with 1 mM-ABTS in 0.2 M-citrate/phosphate buffer, pH 4.3, containing 0.05% (v/v) H_2O_2. After incubation at 23 °C in the dark, the reaction was terminated by the addition of 0.01% (w/v) NaNO_2 in 0.1 M-citric acid and the absorbance was measured at 405 nm in an Anthos 2001 microtitre plate reader.

**RESULTS**

**Cross-reactivity of antibodies with other G-PI-anchored proteins**

A polyclonal antiserum (crude RP147) raised to phospholipase-solubilized and affinity-purified pig kidney dipeptidase (pDP) (Littlewood et al., 1989) was found on Western-blot analysis to recognize other G-PI-anchored proteins that had been solubilized with phospholipase C (Fig. 1). These included pig kidney AP-P, human kidney DP and trypanosome sVSG. In all cases this cross-reactivity was selectively abolished by prior treatment of the proteins with either 1 M-HCl or nitrous acid (Fig. 1). Neither of these two treatments prevented the binding of the crude RP147 antiserum to pig DP. Similarly, a polyclonal

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen injected into rabbit</th>
<th>Chromatography used to purify antibody</th>
<th>Dilution used in E.L.I.S.A. and Western blot</th>
<th>Peptidase immobilized on microtitre plate in E.L.I.S.A.</th>
</tr>
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<tr>
<td>Crude RP147</td>
<td>pDP</td>
<td>Protein A-Sepharose</td>
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<td>pAP-P (0.25 µg/well)</td>
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<td>Purified RP147</td>
<td>pDP</td>
<td>Immobilized pAP-P</td>
<td>1:50</td>
<td>pDP (1.0 µg/well)</td>
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<td>Purified RP171</td>
<td>pAP-P</td>
<td>Immobilized pDP</td>
<td>1:25</td>
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![Fig. 1. Western blot of G-PI-anchored proteins with crude RP147 antibody](image-url)
Antibodies to glycosyl-phosphatidylinositol membrane anchors of proteins

Fig. 2. Western blot of aminopeptidase P with crude RP147 antibody

Samples of pAP-P (5 μg of protein per track) were transferred to Immobilon P membranes as described in the Experimental section. The tracks were then incubated with crude RP147 antiserum as indicated below, and bound antiserum was detected with a 125I-labelled second antibody, followed by autoradiography. Lane 1, untreated antibody; lane 2, antibody pre-incubated for 4 h at 4 °C with 1 mM-myo-inositol 1,2-(cyclic)monophosphate; lane 3, antibody pre-incubated with 1 mM-myo-inositol 1-monophosphate; lane 4, antibody pre-incubated with 1 mM-myo-inositol 2-monophosphate.

Fig. 3. Western blot of G-PI-anchored proteins with purified RP147 antibody

Samples of purified proteins (pig pDP, 5 μg of protein per track; pig pAP-P, 5 μg; human pDP, 10 μg; pig mDP, 5 μg; sVSG, 3 μg) were prepared and analysed as described in the Experimental section. After electrophoretic transfer to Immobilon P membranes, the tracks were blotted with purified RP147 antiserum. Bound antiserum was detected with a 125I-labelled second antibody, followed by autoradiography.

Fig. 4. Western blot of G-PI-anchored proteins with purified RP171 antibody

Samples of purified proteins (pig pDP, 5 μg of protein per track; pig pAP-P, 5 μg; human pDP, 10 μg; pig mDP, 5 μg; sVSG, 3 μg) were prepared and analysed as described in the Experimental section. After electrophoretic transfer to Immobilon P membranes, the tracks were blotted with purified RP171 antiserum. Bound antiserum was detected with a 125I-labelled second antibody, followed by autoradiography.

Fig. 5. E.L.I.S.A. with G-PI-anchored proteins

The ability of G-PI-anchored proteins to inhibit the binding of either (a) RP147 or (b) RP171 to the corresponding immobilized peptidase was determined as described in the Experimental section. Pre-immune rabbit serum showed no binding to either immobilized pAP-P or pDP. ○—○, pig pDP; ■—■, pig pAP-P; □—□, pig mDP; ○—○, human pDP; △—△, sVSG.
antiserum (crude RP171) raised to phospholipase-solubilized pig kidney aminopeptidase P (pAP-P) recognized pig kidney DP and trypanosome sVSG, and this recognition was abolished by treatment of the proteins with 1 M-HCl or nitrous acid (results not shown).

As analysed by Western blotting, the binding of the crude RP147 antiserum to pig AP-P was also abolished by pre-incubation of the antiserum with inositol 1,2-(cyclic)monophosphate (Fig. 2). No inhibition of binding was observed with inositol 1-monophosphate or inositol 2-monophosphate (Fig. 2).

**Purification of the antibodies recognizing the CRD component**

The crude Protein A-Sepharose-fractionated antisera were further purified on columns of immobilized peptidase before characterization of the epitopes involved in the CRD. RP147, which was raised to pig pDP, was passed down a column of immobilized pig pAP-P (see the Experimental section for details). The population of antibodies that bound to the immobilized AP-P were eluted with 0.2 m-glycine, pH 2.3, and are referred to as purified RP147 antibodies (Table 1). Likewise, RP171, which was raised to pAP-P, was purified on a column of immobilized pig pDP (Table 1). In both cases the run-through fractions from the columns failed to recognize phospholipase C-solubilized G-PI-anchored proteins either on Western-blot analysis or by e.i.s.a. (results not shown).

**Characterization of the epitopes involved in the CRD by Western-blot analyses**

On Western-blot analysis purified RP147 antibody recognized the phospholipase-solubilized forms of pig and human DP, pig AP-P and sVSG (Fig. 3). With all four proteins this recognition was abolished by prior treatment of the proteins with either 1 M-HCl or nitrous acid. Purified RP147 also cross-reacted weakly with the detergent-solubilized amphipathic form of pig di-peptidase (mDP), and this recognition was abolished after nitrous acid treatment of the protein (Fig. 3).

Similarly, purified RP171 recognized the phospholipase-solubilized forms of pig and human DP, pig AP-P and sVSG on Western-blot analysis (Fig. 4). However, the recognition of pig DP and pig AP-P was not abolished with 1 M-HCl, only with nitrous acid. HCl (1 M) and nitrous acid did abolish the cross-reaction of purified RP171 with sVSG and human DP. Purified RP171 also recognized pig mDP, and this recognition was abolished by nitrous acid treatment.

**Characterization of the epitopes involved in the CRD by e.i.s.a.**

A competitive e.i.s.a. was developed in order to quantify the ability of G-PI-anchored proteins (Fig. 5) and components of the G-PI anchor (Fig. 6) to inhibit binding of the antibodies recognizing the CRD component. The binding of purified RP147 to immobilized pig AP-P was inhibited in a dose-dependent manner by pig pDP, rising to a maximum of 100% (Fig. 5a). Pig pAP-P caused maximally 85% inhibition of binding of RP147, whereas human pDP and sVSG inhibited binding by 62% and 55% maximally respectively (Fig. 5a). Essentially no inhibition of binding of RP147 was observed with pig mDP. Both pig pDP and pig pAP-P completely inhibited the binding of purified RP171 to immobilized pig DP in a dose-dependent manner (Fig.

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Fig. 6. E.I.I.S.a. with components of the G-PI anchor

The ability of individual components of the G-PI membrane anchor to inhibit the binding of either RP147 (-----) or RP171 (○○○) to the corresponding immobilized peptidase was determined as described in the Experimental section. No inhibition of binding of either antibody was observed with inositol 1-monophosphate.
Table 2. E.I.i.s.a. inhibition of binding of antibodies to the CRD component

The ability of G-PI-anchored proteins or components of the G-PI anchor to inhibit the binding of the antibodies to the CRD component on the corresponding immobilized peptidase was determined as described in the Experimental section. The \( I_{50} \) is the concentration of inhibitor giving 50% inhibition of antibody binding. Where indicated the results are the means ± S.E.M. for three determinations.

<table>
<thead>
<tr>
<th>Inhibitor</th>
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<tr>
<td>Pig pDP</td>
<td>0.5 ± 0.2 nM</td>
</tr>
<tr>
<td>Pig pAP-P</td>
<td>4.9 ± 2.5 nM</td>
</tr>
<tr>
<td>Human pDP</td>
<td>15.9 nM</td>
</tr>
<tr>
<td>sVSG</td>
<td>39.8 nM</td>
</tr>
<tr>
<td>Inositol 1,2-(cyclic)monophosphate</td>
<td>1.0 ± 1.1 ( \mu ) M</td>
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5b). Human pDP and sVSG caused a maximum of 22% and 18% inhibition of binding respectively, whereas detergent-solubilized pig mDP inhibited binding to RP171 by up to 39% (Fig. 5b). The ability of the various G-PI-anchored proteins to inhibit binding of purified RP147 and RP171 is compared in Table 2. All of the phospholipase-solubilized proteins were more effective at inhibiting the binding of RP147 than the binding of RP171.

The ability of individual components of the G-PI anchor structure to inhibit binding of the antibodies to the CRD component was examined (Fig. 6). Inositol 1,2-(cyclic)monophosphate caused dose-dependent inhibition of binding of purified RP147 and RP171, rising to 98% and 75% inhibition respectively. In contrast, inositol 1-monophosphate, inositol 2-monophosphate and myo-inositol maximally inhibited binding of RP147 and RP171 by 17%. Significant inhibition of binding of RP147 (43%) and RP171 (26%) was observed with phosphatidylinositol. Negligible inhibition of binding (maximally 15%) of RP147 and RP171 was observed with mannose, glucosamine, N-acetylgalactosamine, galactose, N-acetylgalactosamine and ethanolamine. The binding of RP147 was inhibited by 32% by phosphoethanolamine, whereas the binding of RP171 was not inhibited.

**DISCUSSION**

We have previously purified DP and AP-P from pig kidney cortex after release from the membrane by bacterial PI-PLC (Littlewood et al., 1989; Hooper et al., 1990b). Serendipitously, we observed that the polyclonal antiserum raised in rabbits to pig DP (Littlewood et al., 1989) cross-reacted with the phospholipase C-solubilized hydrophilic forms of pig AP-P, human DP and trypanosome VSG. In contrast, the detergent-solubilized amphipathic form of human DP was not recognized by the same antiserum on Western-blot analysis (Hooper et al., 1990a), thus suggesting that the site of cross-reactivity in these four distinct proteins resided in the common G-PI anchor moiety and was cryptic in the membrane-bound amphipathic form, becoming exposed on release by phospholipase C (see Fig. 7).

To confirm this hypothesis and to characterize the epitopes involved in this cross-reactivity, the antisera were first purified by affinity chromatography on columns of immobilized peptidase. Secondly, as well as using Western-blot analyses, a competitive e.i.s.a. was developed in order to quantify the ability of various compounds to inhibit the binding of the antibodies to immobilized peptidase.

On Western-blot analysis, the binding of RP147 antibody to all the G-PI-anchored proteins examined was abolished by prior treatment of the proteins with either mild acid (1 M HCl) or nitrous acid. Mild acid selectively decylizes the inositol 1,2-(cyclic)monophosphate, which is formed on phospholipase C cleavage of the anchor, yielding approx. 80% and 20% of inositol 1-monophosphate and inositol 2-monophosphate respectively (see Fig. 7) (Ferguson et al., 1985). Nitrous acid deaminates the glucosamine residue, hydrolysing the glucosamine–inositol bond (see Fig. 7) (Ferguson et al., 1985). It should be noted that nitrous acid is effective on both the phospholipase C-solubilized form of the protein and the intact membrane-bound or amphipathic form. These results therefore indicate that the site of cross-reactivity for RP147 antibody resides primarily in the inositol 1,2-(cyclic)monophosphate. In contrast, the binding of RP171 antibody to pig DP and pig AP-P was only abolished by nitric acid treatment, not by mild acid. The cross-reactivity with sVSG and human DP was abolished by either mild acid or nitrous acid. This suggests that on the pig proteins, at least, as well as the inositol 1,2-(cyclic)monophosphate epitope there is also a minor mild-acid-resistant nitric acid-sensitive epitope (possibly a substituent on the inositol ring) involved in the cross-reactivity with RP171 antibody.

A competitive e.i.s.a. was used to assess the ability of various components of the G-PI anchor to inhibit binding of the antibodies. The most effective inhibitor was inositol 1,2-(cyclic)monophosphate, whereas inositol 1-monophosphate and inositol 2-monophosphate had negligible inhibitory effect. This observation was also confirmed by Western-blot analysis, where the recognition of pig AP-P by RP147 was abolished upon preincubation of the antibody with inositol 1,2-(cyclic)monophosphate but not with inositol 1-monophosphate or inositol 2-monophosphate. Other components of the conserved core structure of the G-PI anchor, namely inositol, mannose, ethanolamine and glucosamine (see Fig. 7) (Doering et al., 1990; Thomas et al., 1990), did not significantly inhibit binding of either antibody in the e.i.s.a. Galactose and N-acetylgalactosamine are side-chain
modifications on the core G-PI anchor structure in VSG and rat brain Thy-1 respectively (Ferguson et al., 1988; Homans et al., 1988), but N-acetylglucosamine is not known to be a component of any G-PI anchor and thus served as a negative control. None of these three compounds significantly inhibited the binding of either RP147 or RP171 antibodies. The binding of both antibodies was partially inhibited by phosphtidylinositol. The only significant difference between the two antibodies was displayed with phosphoethanolamine, which partially inhibited the binding of RP147 antibody but had negligible effect on RP171 binding. Thus in the case of RP147 the phosphoethanolamine residue may be a minor epitope.

The cross-reactivity of other G-PI-anchored proteins was assessed by both Western blotting and the e.l.i.s.a. The phospholipase C-solubilized forms of pig and human DP, pig AP-P and trypanosome VSG were all recognized on Western-blot analysis by both RP147 and RP171 antibodies. However, only with the e.l.i.s.a. was it apparent that there were quantitative differences in the binding of the antibodies to these four G-PI-anchored proteins. For both RP147 and RP171 the rank order of potency of the G-PI-anchored proteins was pig DP > pig AP-P > human DP = trypanosome VSG. Although inositol 1,2-(cyclic)monophosphate inhibited the binding of both RP147 and RP171 antibodies, it was clearly a less efficient inhibitor than the phospholipase C-solubilized forms of the proteins. A similar result was observed with antisera raised to sVSG (Shak et al., 1988). These results imply that there is also species-specific recognition, possibly as a result of species-specific side-chain modifications on the conserved core structure of the G-PI anchor, as seen with trypanosome VSG, rat brain Thy-1 and human erythrocyte acetylcholinesterase (Doering et al., 1990; Thomas et al., 1990). The presence of such side-chain modifications on pig and human DP and pig AP-P will require the complete structural determination of their G-PI membrane anchors.

The detergent-solubilized amphipathic form of pig DP, which retains the complete lipid anchor, cross-reacted with RP171 antibody and, to a much lesser extent, with RP147. In both cases the recognition was abolished by nitrous acid treatment, indicating the possible involvement of the phosphorylinoisitol moiety or some other substituent on the inositol ring. This is partly confirmed by the observation that phosphorylinoisitol partially inhibited the binding of both RP147 and RP171 antibodies in the e.l.i.s.a.

In conclusion, the two antibodies RP147 and RP171 raised in rabbits against phospholipase C-solubilized forms of pig DP and pig AP-P respectively have been shown to cross-react with other G-PI-anchored proteins. In both cases the primary epitope involved in this cross-reactivity is inositol 1,2-(cyclic)mono-phosphate. This epitope is cryptic in the membrane-bound form of such proteins and is only generated on cleavage by phospholipase C. Other minor epitopes probably involve side-chain modifications to the core anchor structure that appear to be species-specific. The antibodies to the mammalian G-PI anchors that have been characterized in this study may prove to be more useful than the trypanosome antibodies in structural and bio-synthetic studies on mammalian G-PI-anchored proteins, and on elucidating the mechanism by which the G-PI-derived mediators are generated and act in response to insulin and other hormones (Low & Saltiel, 1988; Romero et al., 1988; Low, 1989).

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REFERENCES


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