Purification and Molecular Characterization of FAP, a Feto-acinar Protein Associated with the Differentiation of Human Pancreas*

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This work describes the purification of FAP, a feto-acinar pancreatic protein associated with the ontogenesis, differentiation, and transformation of the human exocrine pancreas. The protein was purified to homogeneity from pancreatic juices of patients with pancreatic pathology by a two-step chromatographic procedure which consisted of size exclusion on Sephacryl S-200 and affinity on heparin-Sepharose. The final purification gave a single band at Mₐ 110,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after Coomassie stain or autoradiography of the ³²P-labeled protein. Immunodetection with the murine monoclonal antibody mAb J28 in nitrocellulose replica demonstrated a main band, M, 110,000 component and trace components in the M, 100,000-80,000 range. The immunopattern was identical to that in the original crude pancreatic secretion, therefore showing that the molecular characteristics of the protein, i.e., molecular mass, microheterogeneity, and immunoreactivity, were not altered along the purification procedure. FAP was identified as an acidic protein (isoelectric point 4.2-4.8) consisting of a single polypeptide chain having no free SH residues. Analysis of the amino acid composition showed a high proline content. Twenty-two residues of the N-terminal sequence were determined. No significant homology between this peptide and other proteins was found following a search of the NBRF-18 data bank.

Sugar analysis showed the presence of mannoside which is consistent with N-linked carbohydrate chains and an unusual high ratio in N-acetylgalactosamine residues suggesting the presence of many O-linked carbohydrate chains. Sequential deglycosylation with neuraminidase, heparinase, and O-glycanase yielded a single M, 58,000 peptide showing that, relative to a molecular mass of 110,000, the carbohydrate moiety of FAP accounts for at least 47% of its apparent M, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The fetoacinar pancreatic protein (FAP) is associated with the ontogenesis and the development of the human pancreas and may have an important role in the development of pancreatic acinar cells. It is a member of the oncodevelopment-associated pancreatic antigens that were first identified using polyclonal antisera, in the Syrian golden hamster (1-3) as well as in human pancreas (4). FAP was further characterized with a murine monoclonal antibody (mAb J28) (5). By immunohistology on human embryonic and fetal pancreas, the earliest expression of FAP was seen in undifferentiated mesenchymal cells and in nascent acini at the beginning of the morphological differentiation of the pancreas (9-10 weeks gestation). Maximal synthesis occurs at the time of intense acinar proliferation (15-25 weeks gestation) and declines progressively thereafter (6). In the adult pancreas the protein is still present though at levels far lower than in the fetal organ (6, 7). The protein exhibits a strict tissue specificity as it was found exclusively in acinar cells of the pancreas (6).

FAP synthesis is enhanced in case of pathology of the pancreas, especially in exocrine cancer where FAP is mainly localized in residual acinar cells in the proximity of adenocarcinomas (7). Elevated concentrations of this protein were found in the serum of most patients with diseases of the pancreas (pancreatitis and adenocarcinoma), whereas patients suffering from extrapancreatic pathology had FAP serum concentrations comparable to those in healthy individuals. The serum concentration of FAP provides therefore a good test for pancreatic pathology and it is almost specific for cancer when combined with another serum marker (8).

FAP, like other members of the oncodevelopmental pancreatic antigens defined with polyclonal antisera, is able to bind to concanavalin A (9). This property allowed the partial purification of the protein from fetal pancreas homogenates by concanavalin A-Sepharose chromatography (10, 11). The protein is also contained in amniotic fluids and in relatively high concentrations in pathological pancreatic juices (11). However, FAP in these fluids was only poorly retained in concanavalin A-Sepharose columns.

FAP from all these sources exhibits molecular heterogeneity on SDS-polyacrylamide gel electrophoresis. The main component in all fluids is a M, 110,000 polypeptide that can be visualized by Coomassie staining. Immunostain with mAb J28 allows the detection of a second component in the M, 100,000 position and trace components in the M, 90,000-80,000 range. Occasionally, a band at M, 200,000 is found, compatible with dimerization of the protein (11).

In this study we describe the rapid purification to homogeneity of human FAP from pathological pancreatic juices, using a two-step purification procedure which consisted of size exclusion on Sephacryl S-200 and affinity on heparin-Sepharose. The final purification gave a single band at M, 110,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No significant homology between this peptide and other proteins was found following a search of the NBRF-18 data bank. Sugar analysis showed the presence of mannoside which is consistent with N-linked carbohydrate chains and an unusual high ratio in N-acetylgalactosamine residues suggesting the presence of many O-linked carbohydrate chains. Sequential deglycosylation with neuraminidase, heparinase, and O-glycanase yielded a single M, 58,000 peptide showing that, relative to a molecular mass of 110,000, the carbohydrate moiety of FAP accounts for at least 47% of its apparent M, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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taking advantage of its molecular weight and of its recently discovered ability to bind to heparin.\(^2\)

The method described is easy to perform and allows one to obtain the protein at milligram levels. The amino acid composition, sugar composition, structural features, and N-terminal sequence of FAP are also reported.

### Experimental Procedures

**Reagents**—Bovine serum albumin, diisopropyl fluorophosphate, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were from Sigma. Nitrocellulose membranes (BA-85 type) were from Schleicher & Schuell (Dassel, Federal Republic of Germany). Alkaline phosphatase-labeled rabbit anti-mouse IgG was from Promega Biotec. Phosphate-buffered saline (PBS) was from Biological Materials. Pancreatic juices were kindly provided by Dr. C. Figarella, Groupe de Recherches sur les Glândes Exocrines, Marseille, by Pr. M. Arsac, Hôpital Laennec, Paris, and by Drs. Y. Fujii and M. Eriuchi, the Institute of Medical Science Hospital, University of Tokyo, Japan. They were collected by catheterization of the ductus of Dr. S. Imperial and M. J. Escriberno, unpublished results.

**Biological Materials**—Pancreatic juices were then lyophilized and stored at -70 °C. Immediately after use, the powder was weighed and taken up in 5 X 10^-4 M DFP. SDS-PAGE and Western Blotting—A semi-quantitative dot-blot assay of FAP in pancreatic secretions was performed by serial dilutions applied to nitrocellulose membranes as described previously (6, 8).

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Purification of FAP, a Feto-acinar Protein from Human Pancreas

RESULTS

Selection of Pancreatic Juices—A panel of 27 pancreatic juices obtained from 26 patients with chronic pancreatitis and one patient with cancer of the duodenum invading the pancreas was screened for FAP content by semi-quantitative immunodot-blot assay. Seven representative juices giving different immunoreactivities with the monoclonal antibody 528 are shown in Fig. 1, top. Significant variations in FAP content from one juice to another were observed. In SDS-PAGE nitrocellulose replicas, dot-blot-positive juices (Fig. 1, bottom left) showed the typical pattern of FAP, i.e. an immunostained band at approximately M, 110,000 and a more or less pronounced polymorphism at lower M, (11). The M, 110,000 protein is visible in the Coomassie-stained counterpart (Fig. 1, bottom right). In this experiment, equal amounts of total protein in terms of optical absorbance were loaded. However, clear differences in the protein amount and composition were shown after Coomassie staining. FAP-negative juices were enriched in low M, peptides, suggesting proteolysis.

Sephacryl S-200 Chromatography—Lyophilized, FAP-rich juices were used for purification purposes. Each was re-dissolved in PBS and separated by chromatography on Sephacyl S-200. Triton X-100 (0.05%, v/v) was added to increase solubility of the sample and to improve the chromatographic separation. To minimize proteolytic degradation during the purification procedure, all operations were carried out at 4 °C and DFP (5 × 10−5 M final concentration) was added to all buffers and solutions. Fig. 2 shows the elution pattern corresponding to pancreatic juices 6 (Fig. 2A) and 3 (Fig. 2B) in Fig. 1. Proteins from pancreatic juices eluted as two well-resolved peaks. FAP immunoreactivity was found in the first peak. Intensity of dot-bLOTS (Fig. 2B, inset) correlated well with the absorbance at 280 nm of this peak. The amount of total protein in the peak varied from one sample to another. In experiment A it accounted for up to 80% of the total protein in the juice and in experiment B for only 15%. Intermediate situations were found in other juices. In all instances, however, FAP immunoreactivity was found in the first peak. Fractions with high FAP content, corresponding to a molecular mass higher than 90,000, were pooled and stored at −30 °C. Four such preparations were carried out before continuing purification.

Heparin-Sepharose Chromatography—FAP-containing fractions from the previous purification step were thawed in an ice-cooled bath and recirculated at 4 °C through a heparin-Sepharose column as described under “Experimental Procedures.” Heparin-bound proteins were eluted with 1 M NaCl, dialyzed against distilled water containing 5 × 10−5 M DFP, and freeze-dried. The heparin-bound fraction accounted for the average for 25% of the total protein loaded to the column. Recycling of the effluent allowed a supplementary 10% recovery relative to the first passage. The heparin-bound fraction (1 M NaCl eluates) reacted strongly with mAb J28 by immunodot-blot, while the effluent of the second passage was almost devoid of FAP.

Analysis of the Heparin-Bound Fraction—The heparin-bound fraction consisted of a single major protein band of 110,000 M, when analyzed by SDS-PAGE and stained with Coomassie (Fig. 3, lane 2). A single component was also shown by autoradiography of the radiolabeled protein (Fig. 3, lane 3). The pattern of the purified protein in Western blots (Fig. 3, lane 4) was the same as in the crude pancreatic secretion (Fig. 3, lane 5), consisting of a main component at M, 110,000 and weakly stained bands at M, 100,000 and 100,000–80,000. Recovery of FAP after purification was 5 and 1.8 mg for the juices 6 and 3, respectively. In both, the yield refers to a total of 100 mg of protein as starting material which corresponds to approximately 50 ml of pancreatic secretions containing on the average 2 mg of protein/ml. An estimate of 85 and 21 µg of FAP/mg of total protein was obtained by semiquantitative dot-blot in secretions 6 and 3, respectively. Thus, recovery after the two chromatographic steps, dialysis and lyophilization, is on average 65%.

In general, the purified FAP was stable and no modification after freezing and thawing procedures was observed. However, in repeatedly frozen and thawed samples of some preparations, low M, peptides appeared, compatible with proteolysis. This suggests that, despite an apparent high degree of purity,
Purification of FAP, a Feto-acinar Protein from Human Pancreas

Fig. 2. Sephacryl S-200 chromatography of human pancreatic secretions. Lyophilized pancreatic secretions were loaded onto a column (2.6 x 100 cm) of Sephacryl S-200 Superfine equilibrated in PBS containing 0.05% (v/v) Triton X-100 and 5 x 10^-6 M DFP. Elution was conducted at a flow rate of 21 ml/h. Fractions of 4.8 ml were collected. Each fraction was assayed for FAP by immunodot-blot using mAb 528. A and B show elution profiles of pancreatic secretions 6 and 3 in Fig. 1, respectively. 180 mg of dry powder corresponding to about 50 mg of total protein in A and B were processed. First peak in A accounts for 50% of the total protein content and in B for only 15%. In both, FAP eluted in fractions 37-57 with highest immunoreactivity being coincident with the maximum of the first peak (fractions 43-47). Markers for elution were dextran blue for void volume (Vo), human immunoglobulin G (IgG) (M, 150,000), bovine serum albumin (BSA) (M, 68,000), and cytochrome (CC) (M, 12,500).

contaminating proteases in trace amounts may persist and emphasizes that special care must be taken during purification and storage. Protease inhibitors should be included in the preparations and unnecessary freezing and thawing avoided.

FAP was considered nearly pure by the above mentioned criteria and used for the structural studies described below.

Molecular Characterization

Amino Acid Composition—The amino acid compositions of FAP purified from pancreatic juices 6 and 3 in Fig. 1 are given in Table I. The main feature is a high content in proline (16% in 6, 14% in 3). In addition to Pro, slight differences were noted in the percentages of Gly, Glx, and Ser. Otherwise, the amino acid compositions of both preparations were almost identical.

TABLE I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>A</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td>Ala</td>
<td>8.90</td>
<td>9.34</td>
</tr>
<tr>
<td>Asx</td>
<td>9.81</td>
<td>10.58</td>
</tr>
<tr>
<td>Arg</td>
<td>3.37</td>
<td>2.92</td>
</tr>
<tr>
<td>Cys</td>
<td>0.66</td>
<td>0.49</td>
</tr>
<tr>
<td>Gly</td>
<td>8.33</td>
<td>10.87</td>
</tr>
<tr>
<td>Glx</td>
<td>7.53</td>
<td>6.28</td>
</tr>
<tr>
<td>His</td>
<td>1.53</td>
<td>1.43</td>
</tr>
<tr>
<td>Ile</td>
<td>3.74</td>
<td>3.21</td>
</tr>
<tr>
<td>Leu</td>
<td>6.10</td>
<td>6.33</td>
</tr>
<tr>
<td>Lys</td>
<td>5.34</td>
<td>5.88</td>
</tr>
<tr>
<td>Met</td>
<td>1.73</td>
<td>1.76</td>
</tr>
<tr>
<td>Phe</td>
<td>3.62</td>
<td>3.76</td>
</tr>
<tr>
<td>Pro</td>
<td>16.32</td>
<td>14.24</td>
</tr>
<tr>
<td>Ser</td>
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<td>6.52</td>
</tr>
<tr>
<td>Thr</td>
<td>8.15</td>
<td>8.21</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.01</td>
<td>3.02</td>
</tr>
<tr>
<td>Trp</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Val</td>
<td>6.14</td>
<td>6.67</td>
</tr>
<tr>
<td>Total</td>
<td>99.83</td>
<td>101.45</td>
</tr>
</tbody>
</table>

Fig. 3. SDS-PAGE analysis of purified FAP. Purified FAP preparations were analyzed on SDS-PAGE as in Fig. 1. Lane i, molecular weight markers, Coomassie-stained. Lane 2, purified FAP, Coomassie-stained. Lane 3 is an autoradiography of 125I-labeled FAP. Lanes 4 and 5 are the Western blots of purified FAP of the original pancreatic secretion, respectively.

N-terminal Sequence Analysis—The results of the N-terminal sequence analysis of purified FAP are shown in Fig. 4. Clear assignments were made for 22 out of 23 residues. No PTH-derivative could be detected at the 8th cycle. Quantities and assignments were reproducible in two determinations. A single PTH-derivative was detected in every cycle confirming the purity of FAP and indicating that it may be composed of a single chain polypeptide. Undeglycosylated FAP was used in these experiments, which suggests that sugar attachment...
sites may be located beyond this sequence.

Sugar Composition—Purified FAP was shown to contain 12.5% neutral sugars following estimation by the phenolsulfuric acid technique.

The carbohydrate composition as determined by gas chromatography analysis after methanolysis is given in Table II. Calculations were based on 3 mannose residues. The protein contains a very high ratio of substituted hexoses (GalNAc and sialic acid residues) compared to neutral hexoses and a few fucose and sialic acid residues.

Enzymatic Deglycosylation—Presence of Man and a high GalNAc content strongly suggested mixed N- and O-linkages in FAP, with probably most of them in the latter configuration. In order to verify this hypothesis, purified FAP was subjected to various glycosidase digestions and analyzed by SDS-PAGE. No significant changes in the Mr of FAP were observed after incubation with either neuraminidase, N-endoglycosidase F or O-glycanase alone, or by using mixtures of neuraminidase and O-glycanase or neuraminidase and N-endoglycosidase F.

Hexosaminidase treatment of desialylated FAP yielded a peptide of apparent Mr of 66,000 (Fig. 5, lane 2) or Mr, 64,000 (Fig. 5, lane 3). Subsequent treatment with O-glycanase resulted in the formation of a single polypeptide of apparent Mr of 58,000 (Fig. 5, lane 5). No modification in the molecular mass of bovine serum albumin was observed in experiments run in parallel (data not shown). Any eventual digestion by contaminant proteolytic enzymes can thus be ruled out.

Immunoreactivity with mAb J28 was lost after digestion with hexosaminidase and O-glycanase (Western blot in Fig. 5, lane 5). These results suggest that mAb J28 recognizes a carbohydrate-dependent epitope.

Other Structural Characteristics—After isoelectric focusing of pancreatic secretions in the pH range 3–9, followed by immunodetection with mAb J28 in nitrocellulose transfers, two heavily stained bands at isoelectric points 4.2 and 4.8 were observed (Fig. 6).

Incubation of human pancreatic juices with 0.35 M β-mercaptoethanol for 5 min at 100 °C prior to SDS-PAGE did not affect either the Mr of the protein or its immunoreactivity with mAb J28.

Incubation of pure FAP with iodo-[1-14C]acetamide resulted in no fixation of radioactivity to the protein. In a parallel experiment performed with 1 nmol of papain, which contains a free SH group, the radioactivity incorporated in the protein fraction corresponded to about 1 nmol of iodoacetamide (data not shown).

According to these results, FAP has a single polypeptide chain structure containing no free Cys residues.

### DISCUSSION

FAP was initially identified in human fetal pancreas homogenates and was described as a concanavalin A binding protein. FAP behavior in differentiation and proliferation of exocrine pancreas as well as in inflammation and transformation processes prompted us to carry out further biochemical characterizations. The scarcity of fetal human pancreas hampered purification in the past. The recent observation that FAP is contained in relative high concentration in pancreatic juices from individuals with diseases of the pancreas (11) allowed us to reconsider its purification. The purification procedure described here is based on two observations. First, FAP binds to heparin. Second, among the various proteins in pancreatic juices showing heparin affinity, FAP is the only protein with Mr greater than 60,000 on SDS-PAGE. Isolation was readily and quickly achieved by two chromatographic steps: size exclusion on Sephacryl S-200 and affinity on heparin-Sepharose. The final preparation gave a single band at Mr, 110,000 by SDS-PAGE after Coomassie stain or autoradiography of the 125I-labeled preparation. Immunodetection with the murine monoclonal antibody mAb J28 in nitrocellulose replicas, demonstrated a main Mr, 110,000 component and trace components in the Mr, 100,000–80,000 range. The immunopattern was identical to that in the original crude pancreatic secretion, therefore showing that the structural characteristics of the protein, i.e. molecular mass, microheterogeneity, and immunoreactivity, were not altered along the purification procedure. Results were reproduced in four individual secretions, and the average recovery of FAP relative to the initial concentration of the protein in crude secretion was 65%.

High recovery relies mainly on the fact that FAP is almost

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**TABLE II**

**Sugar composition of FAP**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Molar ratio</th>
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<tbody>
<tr>
<td>Fuc</td>
<td>1.5</td>
</tr>
<tr>
<td>Man</td>
<td>3.0</td>
</tr>
<tr>
<td>Gal</td>
<td>9.5</td>
</tr>
<tr>
<td>GalNAc</td>
<td>12.3</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>15.4</td>
</tr>
<tr>
<td>NeuAc</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Molar ratios are given for 3 mannose residues. Calculated on the basis of a tri-antennary Man 3-Man 4-Man — minimal configuration, present in typical N-linked glycoproteins (25).*

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**Fig. 5. Enzymatic deglycosylation of FAP protein.** SDS-PAGE analysis of FAP after incubation with neuraminidase + hexosaminidase (lanes 2 and 3) or neuraminidase + hexosaminidase + O-glycanase (lane 5). Untreated FAP is in lane 4 and molecular weight markers are in lane 1. Left, Coomassie-stained samples. Right, mAb J28 immunostained replicas. Purified FAP concentration in all samples was 1 mg/ml.

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**Fig. 6. Isoelectric point of FAP.** A crude pancreatic juice (4 mg of protein/ml) was submitted to isoelectric focusing on IEF Phastgel plates at pH gradient 3–9. Proteins were transferred to a nitrocellulose membrane and immunostained with mAb J28. Plot shows the positions of pl standard proteins (isoelectric focusing calibration kit pH 3–10, Pharmacia) run in parallel and stained with Coomassie.
Purification of FAP, a Feto-acinar Protein from Human Pancreas

quantitatively bound to heparin. This contrasts with the small percentage of FAP retained by concanavalin A (11). Heparin is well known as an anticoagulant although it shows other interesting properties like the control of cell growth and regulation in angiogenesis (20, 21). Since FAP synthesis is associated with the differentiation and proliferation of pancreatic acinar cells fixation to heparin might be related to its biological function.

The availability of pure protein at milligram levels allowed chemical and structural studies. Amino acid composition was determined on preparations from two individual secretions obtained from one patient with adenocarcinoma of the duodenum invading the pancreas and one patient with chronic pancreatitis. Small, yet significant differences were seen in four amino acids: Pro, Ser, Gly, and Glx. Contaminants accounting for these differences seem unlikely because variations in the percents of remaining amino acids were small or within the error limits of the amino acid analysis. Protein sequence comparisons may determine whether this variability in the amino acid compositions is individually related. The most important feature of FAP is its very high proline content (14-16%). The best known proline-rich proteins are those from parotid glands. These glycoproteins are tissue-specific, like FAP, but their proline content is higher (25-40%) (22, 23) and they have less carbohydrate residues (19%) (24). The sequence of the 23 N-terminal amino acid residues of FAP was unambiguously established except for position 8. A single residue was obtained in every degradation step confirming the purity of FAP. This is consistent with a single-chain polypeptide structure and in good agreement with the absence of changes in the FAP M., under reducing or not reducing conditions. The single-chain polypeptide must contain only a small number of intrachain S-S bonds as the cysteic acid content was very low. No Cys residue appeared to be free as demonstrated by the absence of carboxymethylation by iodoacetamide.

Sequence data were used for computer sequence comparison. No significant homology could be established between the N-terminal sequence and any of the 9137 sequences compiled in the NBRF-18 data bank (16) showing that FAP is a new, not yet sequenced, protein.

FAP is heavily glycosylated. Neutral hexose content as determined by the phenol/sulfuric acid method is 12.5% by weight, that is, 13,750 g/mol for a configuration present in typical N-linked glycoproteins (25). A further M., 8000 decrease was obtained with O-glycosidase digestion. The enzyme removes the unsubstituted disaccharide Gal-GalNAc from Thr/Ser residues (27). O-glycosidase was effective only in hexosaminidase-treated FAP, which is in agreement with its substrate specificity. Twenty Gal-GalNAc residues are compatible with a M., 8000 decrease.

Reductions in M., caused by the three enzymes (52,000) fully agree with the amount of carbohydrates deduced from sugar analysis and neutral hexose quantification. Invariance in the FAP M., after treatment with N-endoglycosidase F is in agreement with the relative low content in Man residues as this enzyme cleaves only high Man complex structures (28, 29). Similarly, the small number of NeuAc can explain why the M., of native FAP was not modified by neuraminidase. Deglycosylation results suggest that FAP is substituted by many short O-linked carbohydrate chains and by a few N-linked, probably complex-type oligosaccharides. Comparable structures have been described in human immunoglobulins IgD (30) and IgA1 (31) as well as in human lysosomal membrane proteins 1 and 2 (1). In all of them, O-linked carbohydrate chains are bound to either Thr or Ser residues located in proline-rich sequences (hinge regions). These glycoproteins showed roughly half Pro/Ser or Pro/Thr linkages in their hinge regions. The average number of Pro, Ser, and Thr residues in FAP is consistent with O-glycosylation in hinge-like sequences.

FAP molecular polymorphism in SDS-PAGE, evidenced by mAb J28 immunostaining in nitrocellulose replicas, is due to differences in carbohydrate chains. This was demonstrated by obtaining a single deglycosylated polypeptide and by the loss of immunoreactivity in all variants by enzymatic deglycosylation. The loss in immunoreactivity indicates also that a minimal carbohydrate structure on FAP is required for mAb J28 antibody recognition. Therefore, heterogeneity due to sugar variability may be even underestimated.

In summary, the main conclusions of our structural study are: FAP is an acidic, proline-rich protein, which is heavily glycosylated and contains both O- and N-linked glycans most of them in the former configuration. FAP appears to be an unprecedentedly sequenced protein not showing any significant homology to the protein sequences reported so far.

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