The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120

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Abstract A major acute phase protein (pig-MAP) has been isolated from the sera of pigs after turpentine injection. The protein is the pig counterpart of a recently cloned human serum protein denominated PK-120, which is a putative substrate for kallikrein [Nishimura et al., 1995 FEBS Lett. 357, 207–211]. The protein exists in other mammalian species and it is also an acute phase protein, at least in the rat. Pig-MAP shows homology, as PK-120, with the heavy chain 2 (HC-2) of the inter-α-trypsin inhibitor superfamily but does not possess trypsin inhibitory activity.

Key words: Pig plasma protein; Acute phase response (pig, rat); Human PK-120; Inter-α-trypsin inhibitor

1. Introduction

Acute inflammation induces an important increase in the concentration of a subset of plasma proteins called acute phase reactants [1,2]. These proteins have been particularly well studied in man and rat. Though the roster of these proteins is probably not complete, in humans the concentrations of C reactive protein (CRP) and serum amyloid A increase more than a hundred times during the acute phase response. The plasma concentration of α_1-acid glycoprotein (AGP), haptoglobin, α_1-antitrypsin and fibrinogen can also increase several times after the onset of this process. In the rat, a type of α_2-macroglobulin, AGP, haptoglobin, fibrinogen and, particularly, a cysteine proteinase inhibitor, which is a kininogen, are the major acute phase proteins [3]. In other species the acute phase protein response is poorly defined [4].

In a recent work we have characterized the acute phase proteins in pigs after turpentine injection [5]. Besides CRP and haptoglobin we have observed a serum α_2-globulin, with an M_r about 115–120 kDa, which apparently is the major acute phase (MAP) protein in pigs. This protein, called pig-MAP, seemed to be a new mammalian plasma protein.

The present work describes the isolation of pig-MAP. Antibodies against pig-MAP, raised in rabbits, allow us to detect proteins analogous to it in other species, including rat and man. In the rat this protein is also an important acute phase reactant. Partial amino acid sequence of the pig protein reveals homology with the heavy chain 2 (HC-2) of the inter-α-trypsin inhibitor (ITI) family of serum proteins. A sample of the human counterpart has also been sequenced in its amino terminal region. Both human and pig proteins show a high homology. Recently, Nishimura et al. [6] have reported the cDNA cloning and the deduced amino acid sequence of a new human plasma protein denominated PK-120, which is a putative kallikrein substrate [6,7]. This human protein and pig-MAP are homologous.

2. Materials and methods

2.1 Materials

Acute phase response was induced in pigs by turpentine injection [5] or surgically after small bowel autotransplantation (Experimental Transplantation Programme of the Medicine Faculty, Zaragoza University). Blood samples were obtained just before the inflammatory treatment and daily afterwards [5]. Acute inflammation was also induced in Wistar rats by turpentine injection [3] and blood samples were obtained 48 h later. As a control, age and sex mated rats were used. Human plasma from healthy donors was provided by the University Hospital.

2.2. Pig-MAP isolation

Pig-MAP was isolated from 48 h acute phase pig serum containing the following protease inhibitors: 60 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 3 mg/ml Polybrene (hexadimetrine bromide), and 10 mM lysine-HCl. Aliquots of serum (total, 10 ml) were first chromatographied on a 100 × 2 cm Sephadex G-150 column equilibrated in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4 containing 0.1% NaN_3. Fractions enriched in pig-MAP containing low amounts of albumin were selected. The fractions were then dialysed against 0.05 M Tris-HCl, 0.02 M NaCl pH 7.5 buffer (Tris buffer) and applied to a 10 × 2 cm column of Sepharose 4B-immobilized Cibacron Blue [8] and equilibrated in the same buffer. A fraction, which contained pig-MAP and albumin as the major contaminant, was eluted with the Tris buffer containing up to 0.5 M NaCl. Finally, this fraction was dialysed against the Tris buffer and applied to a 10 × 2 cm DEAE-Sephadex A-50 column equilibrated in the same buffer. Proteins were eluted with a saline linear gradient up to 0.3 M NaCl in the Tris buffer (total volume 500 ml). In each step, fractions containing pig-MAP were monitored by SDS-PAGE (10%) under reductive conditions.

2.3 Purification of the human protein homologous to pig-MAP

A partial purification of the human serum protein homologous to pig-MAP was carried out as follows: a protein fraction was obtained by 40–60% saturation ammonium sulphate precipitation of 400 ml fresh human serum containing protease inhibitors (section 2.2). This fraction was the starting sample for further purification of the protein using a similar method to that described for pig-MAP.

2.4 Antiserum and antibodies

Antiserum to pig-MAP was raised in rabbits by subcutaneous injections of purified pig-MAP as previously reported for other pig serum proteins [9]. The antiserum was adsorbed with insolubilized normal pig plasma [10] previously selected by the very low concentration of pig-MAP. Antibodies against the protein were isolated using insolubilized acute phase pig serum [10] as the immunoadsorbent.

2.5 Electrophoresis and Western blotting

Protein samples were analysed by reductive SDS-PAGE (10%). The separated proteins were electroblotted from the gel to nitrocellulose membranes (Hybond-C super, Amersham) at 20 V, for 1 h in a semi-dry

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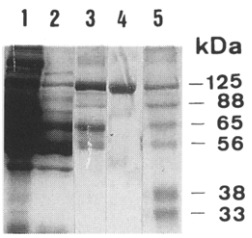

Fig. 1. Reductive SDS-PAGE (10%) of samples obtained during pig-MAP purification. Lane 1, starting acute phase pig serum; lane 2, fraction after Sephadex G-150; lane 3, pig-MAP fraction eluted from Sepharose 4B-immobilized Cibacron Blue; lane 4, purified pig-MAP after DEAE-Sephadex A-50 chromatography; lane 5, molecular weight markers.

transfer cell (BIO-RAD). After blotting, the membranes were incubated first with isolated antibodies to pig-MAP (1 μg/ml) and then with alkaline phosphatase-conjugated goat anti rabbit IgG, absorbed with human serum proteins (Sigma, diluted 1/10,000). The immunocomplexes were revealed with a fresh solution of 0.1 mg/ml Nitro-blue tetrazolium chloride and 0.06 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.2 M Tris, 1 mM $MgCl_2$ pH 9.6 buffer The MW-SDS-200 protein kit (Sigma) was used for the molecular weight markers.

2 6. Trypsin digestion and sequencing

Pig-MAP (500 μg) was digested with 1/50 amount (by weight) of sequencing grade trypsin (Boehringer-Mannheim) in a 0 1 M NH_4HCO_3, pH 8.3 buffer for 6 h at 37°C (total volume 700 μl). The native protein (100 μg) and the tryptic digestion sample were electrophoresed in reductive SDS-PAGE (8 to 20% gradient) electroblotted as before to polyvinilidene difluoride membranes (Immobilon P, Millipore) and stained with Coomassie brilliant blue-R [11]. Bands corresponding to the undigested protein and to a 23 kDa fragment were cut out and directly applied for NH_2-terminal amino acid sequentiation. This was carried out by custom charge in the Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK. The purified human protein was treated in a similar way.

2.7 Determination of pig-MAP

Serum concentration of pig-MAP was determined by radial immunodiffusion in 1% agarose gels containing the specific rabbit antiserum against the protein. Purified pig-MAP was used as a reference standard.

2.8. Protease inhibition

Trypsin and chymotrypsin inhibitors were detected by the method of Uriel and Berges [12] in gels after conventional or SDS-PAGE electrophoresis.

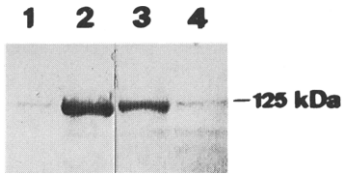

Fig. 3 Western blotting of 1 μl of 1/5 diluted sera from: a pig before (lane 1) and 48 h after turpentine injection (lane 2); a pig before (lane 4) and 48 h after small bowel autotransplantation (lane 3).

3. Results

Fig. 1 shows the protein patterns in reductive SDS-PAGE of samples collected at different steps in the pig-MAP purification. The purified protein fraction shows a major band of around 120 kDa (lane 4). Minor bands of around 80 and 50 kDa are fragments of the protein since they react with specific antibodies to pig-MAP by Western blotting. Using the specific antisera, the concentration of pig-MAP was determined in the sera of five pigs with turpentine-induced inflammation (Fig. 2a). A maximum in the concentration of pig-MAP was observed 24–48 h after turpentine injection. This concentration reaches values of 4–8 mg/ml, which are 10 to 30 times higher than before the inflammatory process. To confirm that the rise of pig-MAP is not the consequence of a toxic effect of turpentine, the protein was also determined in sera of pigs under small bowel autotransplantation (surgical trauma). The results (Fig. 2b) were similar to those obtained before. Western blotting analysis indicates that the intensity of the 120 kDa band dramatically increases in sera of inflammated pigs (Fig. 3). This 120 kDa polypeptide correspond to the M_r of the native protein as confirmed by gel filtration on a calibrated Sephadex G-150 column.

Proteins homologous to pig-MAP were detected in the sera of other animal species (sheep, bovine, human and rat) by Western blotting and revealed with anti pig-MAP antibodies (Fig. 4). In all these cases, a band of around 120 kDa was observed. Lanes 4 and 5 in Fig. 4 correspond to pooled sera from control and turpentine injected rats. The intensity of the 120 kDa band greatly increases in the sera of inflammated rats. Therefore, in the rat the homologous protein to pig-MAP is also an acute phase protein.

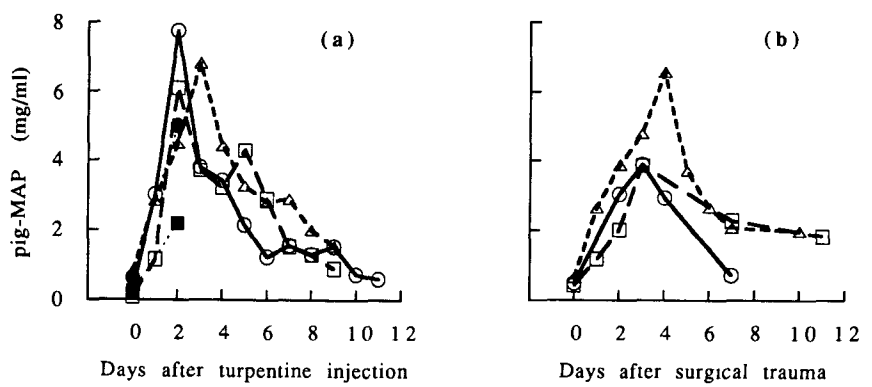

Fig. 2. Time-course of pig-MAP serum concentration (mg/ml) in pigs with acute phase processes: (a), values from five pigs, before (day 0) and on subsequent days after turpentine injection. In two pigs (closed circles and closed squares) only the samples corresponding to 0 and 2 days were analysed; (b) values from three pigs before (day 0) and on subsequent days after small bowel autotransplantation.

NH_2-terminal amino acid sequences from pig-MAP, from the 23 kDa tryptic fragment and from the homologous human protein, are shown in Fig. 5. Search in the SWISS-PROT Protein Database showed moderate homology with the HC-2 of the ITI [13]. However, the pig-MAP did not possess trypsin or chymotrypsin inhibitory activity detectable by the method of Uriel and Berges [12], which, by contrast, easily detects inhibitory activity of ITI components (data not shown). Recently, Nishimura et al. [6] have published the cDNA cloning of a new human serum protein denominated PK-120. The deduced amino acid sequence of this protein shows a high homology with the partial sequences of the pig-MAP and identity with the NH_2-terminal sequence of the human protein reported here (Fig. 5). Therefore, pig-MAP is homologous to human PK-120.

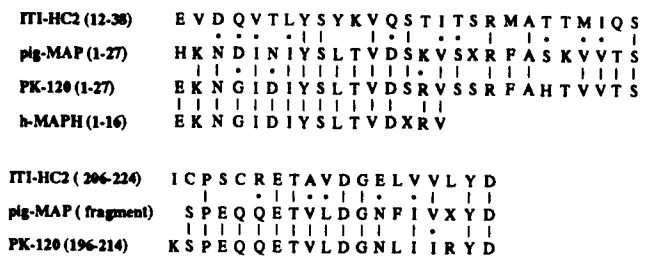

Fig. 5. Amino acid alignments of the NH_2-terminal sequences of: (top), pig-MAP and human homologous protein (h-MAPH) compared with the sequence (amino acids 12–38) from ITI-HC2 [13] and NH_2-terminal sequence from human PK-120 [6]; (bottom), a 23 kDa tryptic fragment of pig-MAP compared with the corresponding sequences of the ITI-HC2 (amino acids 206–224) and human PK-120 (amino acids 196–214) |, indicates amino acid identity and •, analogy.

4. Discussion

In a recent work [5] we have detected a major acute phase serum protein in pigs, which has not been previously described in this or in other species. The protein has been now isolated to homogeneity. Antibodies to it allow us to detect homologous proteins to it in several mammalian species, including man and the rat. In the latter the protein is also a major acute phase component, since the concentration of the protein increases several times 48 h after turpentine injection (Fig. 4). This fact is surprising because the rat has been widely used as a model in studies of acute inflammation. Probably, the human protein homologous to pig-MAP is also an acute phase reactant. Preliminary studies from our laboratory indicate that the intensity of the 120 kDa band significantly increases in the sera of 60% of patients after different surgical trauma. However, more systematic studies need to be performed to confirm that this human protein is also an acute phase protein.

The NH_2-terminal amino acid sequences from pig-MAP and from an internal tryptic fragment show a high homology (more than 85%) with the corresponding regions of a newly described human serum protein, denominated PK-120, which is a putative substrate for kallikrein [6]. This human protein was previously isolated from human plasma [7,14] and shows a notable susceptibility to proteolysis. We have observed the same effect during the isolation of the pig-MAP (see Fig. 1). Moreover, degradation products of this protein increase in serum samples stored for a few days at 4°C.

PK-120 and pig-MAP show homology with the ITI superfamily of serum trypsin inhibitors [6, this work]. These inhibitors are constituted by one or two heavy chains and one light chain denominated bikunin [15]. Heavy and light chains are bound together by glycosamino glycan bridges that are resistant to the action of reductive SDS buffers [16]. The glycosamino glycans can be broken down by hyaluronidase or chondroitinase [16,17]. Bikunin is formed by two Kunitz type domains that are responsible for the trypsin-like proteinase inhibition [15]. This bikunin chain seems to be absent in pig-MAP because this protein does not inhibit trypsin.

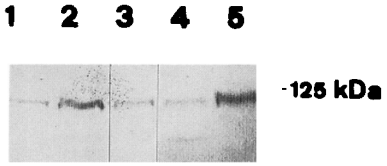

Fig. 4. Western blotting using specific antibodies anti pig-MAP of 1 μl of serum from the following species: sheep (lane 1); bovine (lane 2); human (lane 3), normal (lane 4) and turpentine injected (lane 5) rats.

Proteins of the ITI superfamily, besides their trypsin inhibitory activity, interact with components of extracellular matrix [18–22]. This property may be considered as a modulatory factor in cell migration and proliferation. The fact that kallikrein acting upon PK-120 could release peptides with kinin activity [6] and the fact that this protein is an acute phase protein, open a new gate to study such a complex process as inflammation.

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References

[1] Kushner, J. (1982) Ann. NY Acad. Sci. 389, 39–49.
[2] Hienrich, P.C., Castell, J V. and Andus, T. (1990) Biochem. J. 265, 621–636.
[3] Schreiber, G., Howlett, G., Nagashima, M., Millership, A., Martin, H , Urban, J and Kotler, L (1982) J. Biol. Chem. 257, 10271–10277
[4] Gruys, E , Obwolo, M J. and Toussaint, M.J.M. (1994) Vet. Bull. 64, 1009 1018.
[5] Lampreave, F , González-Ramón, N., Martínez-Ayensa, S., Hernández, M.A., Lorenzo, H K , García-Gil, A. and Piñeiro, A. (1994) Eelectrophoresis 15, 672–676
[6] Nishimura, H., Kakizaki, I., Muta, T., Sasaki, N., Pu, P.X , Yamashita, T and Nagasawa, S. (1995) FEBS Lett 357, 207–211
[7] Pu, X.P., Iwamoto, A., Nishimura, H and Nagasawa, S (1994) Biochim. Biophys Acta 1208, 338–343.
[8] Naval, J , Calvo, M , Lampreave, F and Piñeiro, A (1982) Comp. Biochem. Physiol 71B, 403–407
[9] Lampreave, F. and Piñeiro, A. (1992) J. Reprod Fert. 95, 441–449.
[10] Avrameas, S. and Ternynck, T (1969) Immunochemistry 6, 53–66
[11] Matsudaira, P (1987) J. Biol Chem. 262, 10035–10038
[12] Uriel, J. and Berges, J. (1968) Nature 218, 578–580.
[13] Gebhard, W., Schreitmüller, T., Hochstrasser, K and Wachter, E (1988) FEBS Lett. 229, 63–67.
[14] Hammer, C H., Jacobs, R M. and Frank, M.M. (1989) J Biol. Chem. 264, 2283–2291.
[15] Salier, J.P. (1990) Trends Biochem Sci. 15, 435–439.

[16] Enghild, J.J , Thøgersen, I.B., Pizzo, S.V. and Salvesen, G. (1989) J. Biol. Chem. 264, 15975–15981.
[17] Enghild, J.J., Salvesen, G., Hefta, S.A., Thøgersen, I.B., Rutherfurd, S. and Pizzo, S V. (1991) J. Biol. Chem. 266, 747–751.
[18] Yoneda, M., Suzuki, S. and Kimata, K. (1990) J. Biol Chem. 265, 5247–5257.
[19] Huang, L., Yoneda, M. and Kimata, K. (1993) J. Biol. Chem. 268, 26725–26730.
[20] Wisniewski, H.G., Burgess, W.H., Oppenheim, J.D. and Vilcek, J. (1994) Biochemistry 33, 7423–7429.
[21] Chen, L., Mao, S.J.T., McLean, L.R., Powers, R.W. and Larsen, W.J. (1994) J. Biol. Chem. 269, 28282–28287.
[22] Castillo, G.M. and Templeton, D.M. (1993) FEBS Lett. 318, 292–296.