FEBS Letters 380 (1996) 246–250 FEBS 16644

SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and to a rodent sperm-coating glycoprotein

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Received 21 December 1995

Abstract A novel 28 kDa glycoprotein was purified from exocytosed material from human neutrophils and its primary structure partially determined. Degenerate oligonucleotide primers were used to amplify cDNA clones from a human bone marrow cDNA library. The deduced 245 amino acid sequence of the 2124 bp full-length cDNA showed high degrees of similarity to the deduced sequences of the human gene TPX-1 and of sperm coating glycoprotein from rat and mouse. Subcellular fractionation of human neutrophils indicated that the protein is localized in specific granules. The protein was named SGP28 (specific granule protein of 28 kDa).

Key words: TPX-1; Acidic epididymal glycoprotein; Pathogenesis related protein 1; Subcellular fractionation

1. Introduction

The neutrophil is equipped with a huge number of granules in addition to the highly mobilizable secretory vesicles (for review see [1]). Granules can be divided into peroxidase positive, azurophil granules and peroxidase negative granules, of which the latter encompass specific granules and gelatinase granules [2,3]. These granules are mobilized in a sequential order [4]. Exocytosis is an important aspect of neutrophil function. Peroxidase negative granule proteins like collagenase and gelatinase indirectly contribute to the microbicidal potential of the neutrophil by degrading extracellular matrix, thus allowing migration of the cell in tissues [2,3,5]. Other matrix proteins are directly involved in the killing of microorganisms, exemplified by cathepsin G, azurozidin, defensins, and bactericidal/permeability increasing protein, all of which are located in azurophil granules [1,6]. A novel microbicidal protein hCAP-18 has recently been identified in specific granules [7]. The identification and characterization of novel matrix proteins and the determination of their subcellular localization is therefore crucial for the understanding of the diversity of neutrophil function.

We here describe the isolation and partial amino acid sequence of a novel 28 kDa protein (SGP28), its cDNA sequence, and its subcellular localization in specific granules of human neutrophils.

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The cDNA sequence has been submitted to EMBL and has been given the accesion number X94323.

2. Materials and methods

2.1. Purification of SGP28

Isolated neutrophils were resuspended in Krebs-Ringer phosphate at approximately 5×10^8 cells/ml and preincubated at 37°C for 5 min. Phorbol myristate-acetate (PMA, Sigma) was added at 5 μg/ml and the neutrophils stimulated for 20 min. Protease inhibitors were added (phenylmethylsulfonylfluoride (1 mM), aprotinin (200 KIE/ml), pepstatin (1 mM)) and the cells pelleted by centrifugation. After precipitation in 18% PEG, removal of PEG from non-precipitated proteins by addition of 0.5 M K_2HPO_4, and a change of buffer to 0.05 M sodium acetate, the PEG supernatant was subjected to cation exchange-chromatography on Mono S using FPLC (Pharmacia) (all procedures as described in [8]). Fractions containing SGP28, as evaluated by SDS-PAGE, were pooled and re-chromatographed on MonoS and the eluate subjected to gel filtration on Superose 12 (Pharmacia), using 25 mM Tris-HCl, pH 8.0, 100 mM NaCl as buffer.

2.2. Endoglucosidase treatment

SGP28 was incubated with either *N*-glycanase (10 U/ml), *O*-glycanase (50 mU/ml) or endoglycosidase H (0.25 U/ml) as previously described [9]. Glucosidases were obtained from Genzyme.

2.3. Structural analysis

Tryptic peptides were obtained by incubation of the reduced and carboxyamidomethylated protein with trypsin as described [8]. The peptides were purified on reversed phase HPLC using a Vydac C_8 column (2.1 × 150 mm) and eluted at 0.2 ml/min with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile (0.5%/min). Molecular masses of individual fragments were determined by laser desorption mass spectrometry performed in a Biflex instrument (Bruker-Franzen) using α-cyano-4-hydroxy-cinnamic acid as matrix. The intact protein and the tryptic peptides were subjected to amino acid sequence analysis using an automatic protein sequencer (Procise, Applied Biosystems).

2.4. Cloning and sequencing of SGP28 cDNA

Two degenerate primers were designed from the partial amino acid sequence of SGP28 to fit the regions coding for amino acids 55–60 (PPARNM: 5′-CCICCIGCI(A/C)GIAA(C/T)ATG-3′) and 171–178 (YCPAGNWA: 5′-GCCCA(A/G)TTICCIGCIGG(A/G)CA(A/G)TA-3′). The two primers were used for PCR amplification from a human bone marrow cDNA library (Clontech) with Taq polymerase (Promega). The amplified fragment was cloned in the plasmid vector pCR II using the TA cloning kit (Invitrogen) and the insert of one clone sequenced with the Sequenase kit (Amersham). From this sequence, two specific primers were designed to amplify the 5′ and 3′ region of SGP28. The 5′ region of SGP28 was amplified from a human bone marrow cDNA library with the thermostable proofreading polymerase Pwo (Boehringer-Mannheim) using a primer covering nucleotides 279–299 (5′-GGTTACTGTGTCTGTAATTGC-3′) and a vector specific primer. The amplification fragment was cloned in pCR II and two clones sequenced. For amplification of the 3′-region, a primer covering nucleotides 226–246 (5′-GATGGAATGGAACAAAGAGGC-3′) and a vector specific primer was used. Taq polymerase was used (as Pwo failed) for amplification from the human bone marrow cDNA library. The amplification fragment was cloned

SSDI S0014-5793(96)00030-0

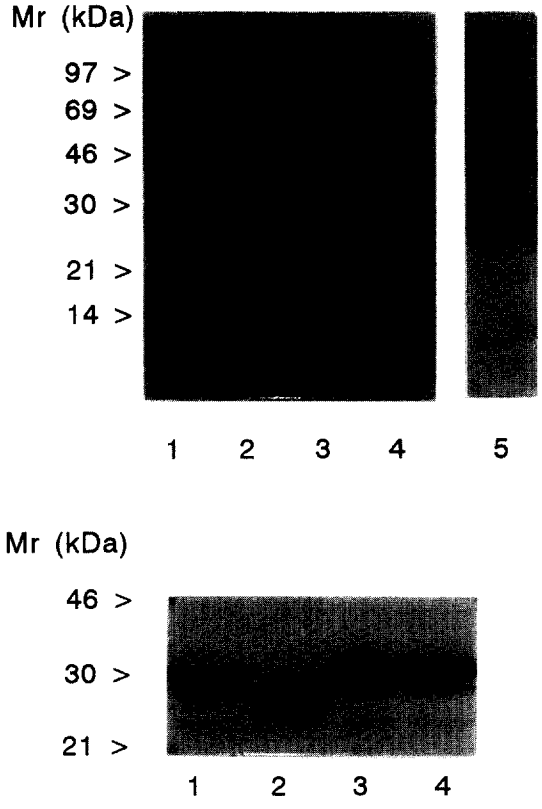

Fig. 1. SDS-PAGE protein profiles during purification and deglycosylation of SGP28. Upper panel (non-reducing conditions): material exocytosed by PMA (lane 1); PEG-supernatant (lane 2); SGP28-containing fractions after chromatography on MonoS (lane 3); pure SGP28 after gel filtration on Superose 12 (lane 4); Western blotting of purified SGP28 with antibodies against a synthetic SGP28 dodecapeptide (lane 5). Lower panel (reducing conditions): samples of purified SGP28 were incubated with either buffer (lane 1), *N*-glycanase (lane 2), *O*-glycanase (lane 3) or endoglycosidase H (lane 4).

in pCR II and three clones sequenced. All clones were sequenced in both directions.

2.5. Generation of anti-peptide antibodies

A peptide (ELRRAVSPPARNC) corresponding to a part of the amino-terminal sequence extended with Cys and covalently coupled to BSA, was purchased from Kem-En-Tec (Copenhagen, Denmark). Immunization of rabbits was carried out by Dako (Glostrup, Denmark).

2.6. Subcellular fractionation

Fractionation was performed as described [3]. In short, diisopropylfluorophosphate (5 mM) treated neutrophils were disrupted by nitrogen cavitation at $0.5–1 \times 10^8$/ml. The postnuclear supernatant (10 ml) was applied on a three-layer Percoll density gradient and centrifuged at 37,600×*g* for 30 min. The gradient was fractionated and fractions assayed for myeloperoxidase, lactoferrin, gelatinase, latent alkaline phosphatase, and HLA. Except for the enzymatic assay for alkaline phosphatase, all markers were measured by ELISA [3]. For Western blotting of subcellular fractions, Percoll was removed from 800 µl of each fraction by ultracentrifugation, and the biological material resuspended in 200 µl PBS.

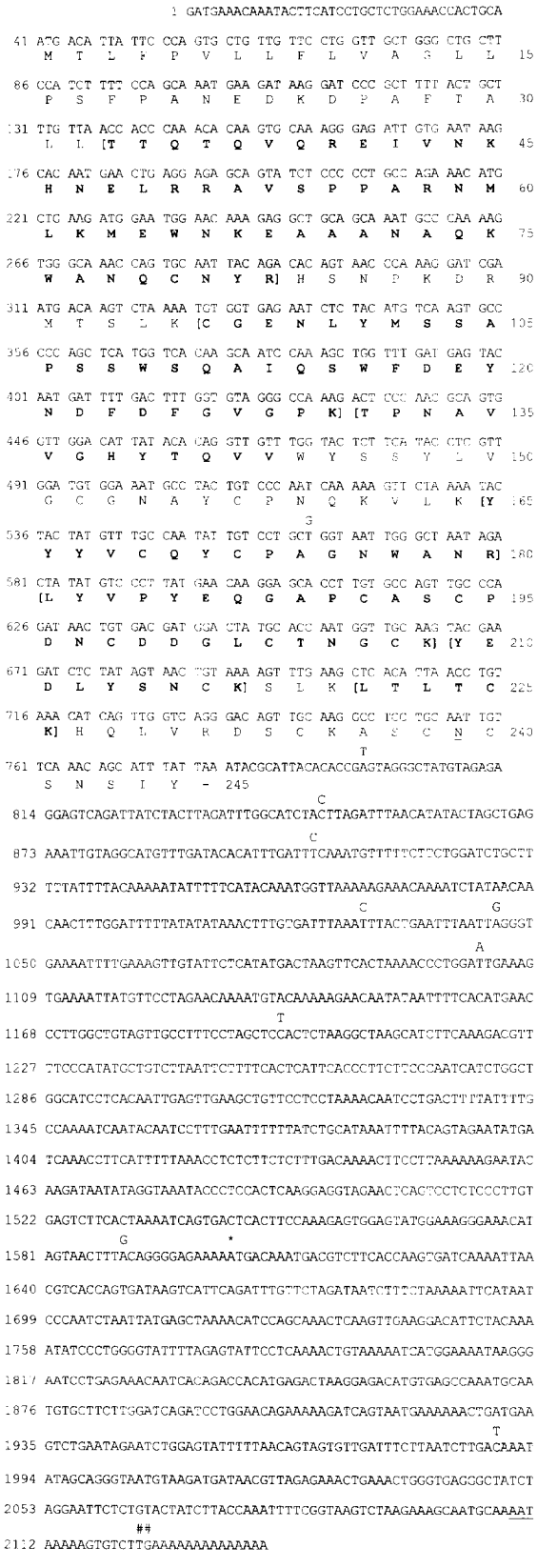

Fig. 2. cDNA and amino acid sequence of SGP28. The cDNA sequence is given as determined from clones with overlapping sequence. The deduced amino acid sequence is shown below the nucleotide sequence. The amino acids identified by sequencing of the N-terminus and of tryptic peptides of the protein are given in bold and individual fragments marked by brackets. Nucleotide sequence variation was observed at 10 positions. The nucleotide only observed in one of the three clones is shown above the sequence. One of the three clones had an additional A at position 1603 (*) and a C at the very 3′-end instead of TG (##). The poly(A) signal and the asparagine of the *N*-glycosylation site (position 239) are underlined.

2.7. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed largely as described by Laemmli and Towbin, respectively [10,11]. The nitrocellulose sheets were blocked for 1 h in 5% skim milk. The antiserum against the SGP28-peptide-BSA was preincubated for at least 2 h in PBS containing 0.5% BSA. Color was developed by the metal-enhanced DAB-chromogen (Pierce).

3. Results and discussion

SGP28 was purified from exocytosed material from PMA-stimulated human neutrophils. The purification involved PEG-precipitation of exocytosed proteins followed by cation-exchange chromatography of non-precipitated protein. Eluates containing SGP28 were finally subjected to gel-filtration on Superose 12. SDS-PAGE of the different purification steps is shown in Fig. 1. The purified protein was reduced and denaturated by boiling in SDS and β-mercaptoethanol followed by incubation in the presence of buffer or either one of the endoglycosidases *N*-glycanase, *O*-glycanase, or Endoglucosidase H (Fig. 1). The reduction of SGP28 resulted in a shift in mobility from 28 kDa to approximately 30 kDa. Furthermore, treatment with N-glycanase resulted in reduction of the apparent molecular mass of the protein from 30 kDa to approximately 27 kDa, whereas the other glucosidases had no significant effect (Fig. 1). This indicates the presence of one or more complex N-linked oligosaccharide side chains in SGP28.

In order to identify the protein, the amino acid sequences of the amino-terminus and of several tryptic fragments were determined as shown in brackets in Fig. 2. Based on these amino acid sequences two degenerate oligonucleotide primers were used for PCR amplification of a human bone marrow cDNA library resulting in a 350 bp product. From its sequence, two specific primers were designed to amplify the 5′ and 3′ region of SGP28. Two clones covering the 5′ region (nt 1-299 and 3-299) showed complete identity in the overlapping

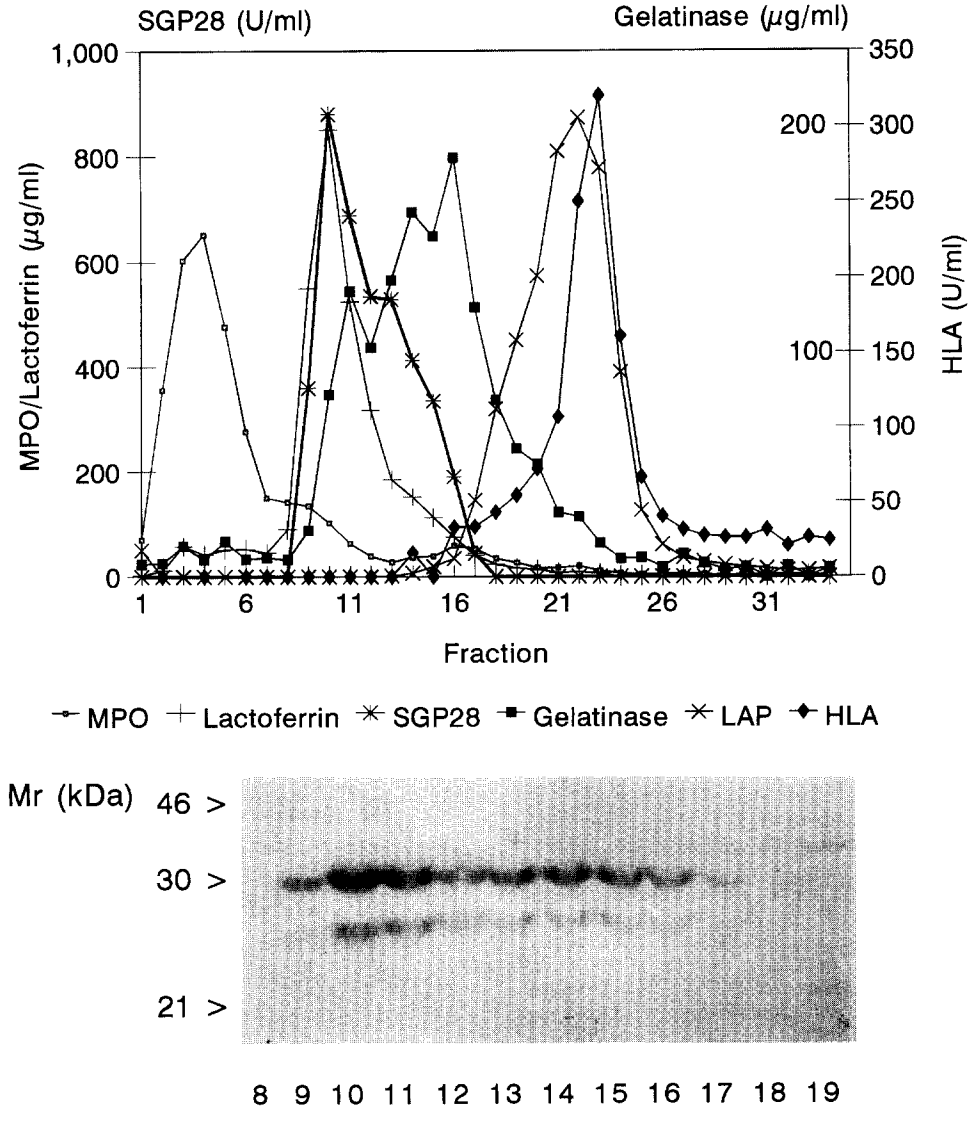

Fig. 3. Subcellular localization of SGP28. Isolated, disrupted neutrophils were applied on a three layer Percoll-density gradient, which was centrifuged and fractionated. Fractions were assayed for the following marker proteins as shown in the upper panel: myeloperoxidase (MPO, azurophil granules), lactoferrin (specific granules), gelatinase (gelatinase granules), latent alkaline phosphatase (secretory vesicles), and HLA (plasma membranes). The lower panel shows Western blotting of subcellular fractions with the antibodies against the synthetic SGP28-peptide. No reactivity was observed in fractions 1 through 7 and 20 through 27 (not shown). The distribution profile of values for densitometric scans of each fraction are shown in the upper panel (SGP28).

sequence. Comparison of three clones of the 3′-region (all three from nt 271-2124) showed sequence variations at 10 positions probably due to misincorporated nucleotides during the PCR reaction (Fig. 2). Furthermore, one of the clones differed by both having an additional A at position 1603 and by having a C at the very 3′-end of the transcript (before the poly(A) tail) instead of TG. The full-length cDNA sequence of 2124 bp is shown in Fig. 2. It has an open reading frame of 735 bp encoding a protein of 245 amino acid residues. One potential *N*-glycosylation site was identified at asparagine residue 239 (underlined in Fig. 2), in accordance with the deglycosylation data presented above. The first 19 amino acids fulfill the criteria for a consensus sequence of a signal peptide according to von Heijne [12]. According to this, the amino-terminal (residue 20 in Fig. 2) of the 226 amino acid mature protein of 27.6 kDa is alanine. However, the amino-terminal amino acid of the purified protein is threonine (residue 33 in Fig. 2), but the deduced amino acid sequence preceding this threonine contains several charged residues (the sequence NEDKDP, amino acids 21–26 in Fig. 2). Thus, the amino acids at position 32/33 are unlikely as a cleavage site for a signal peptide, according to the rules of von Heijne [12]. This indicates a post-translational N-terminal trimming of the protein by 13 amino acids, either by proteolysis during purification, or prior to packaging in granules. The identical mobility of the purified SGP28 and SGP28 present in subcellular fractions of specific granules (both migrate at 30 kDa under reducing conditions as detected by Western blotting, see below), suggests N-terminal trimming prior to arrival in specific granules as the most likely event. To the authors knowledge, the existence of proforms of specific granule proteins has not been described previously, whereas this is a well known phenomenon for many azurophil granule proteins [6].

For subsequent determination of the subcellular localization in human neutrophils of SGP28, antibodies were raised against a synthetic dodecapeptide (ELRRAVSPPARNC) corresponding to amino acids 48–59 of SGP28 (Fig. 2) extended with Cys. The anti-peptide antibodies recognized the purified SGP28 as evidenced by Western blotting (Fig. 1, upper panel). Subcellular fractionation of isolated, disrupted neutrophils was performed on a three-layer Percoll density gradient, which resolves all known mobilizable organelles, including azurophil, specific, and gelatinase granules in addition to secretory vesicles. Fig. 3 shows the distribution of marker enzymes for these subsets. Western blotting of subcellular fractions with anti-SGP28 antibody (under reducing conditions) revealed a dominating band at 30 kDa and a minor 28 kDa in fractions 8 through 16, peaking in fractions 10 and 11. This distribution profile is similar to that of lactoferrin as demonstrated by the colocalization of the profiles of lactoferrin determined by ELISA and the values of the densitometric scan

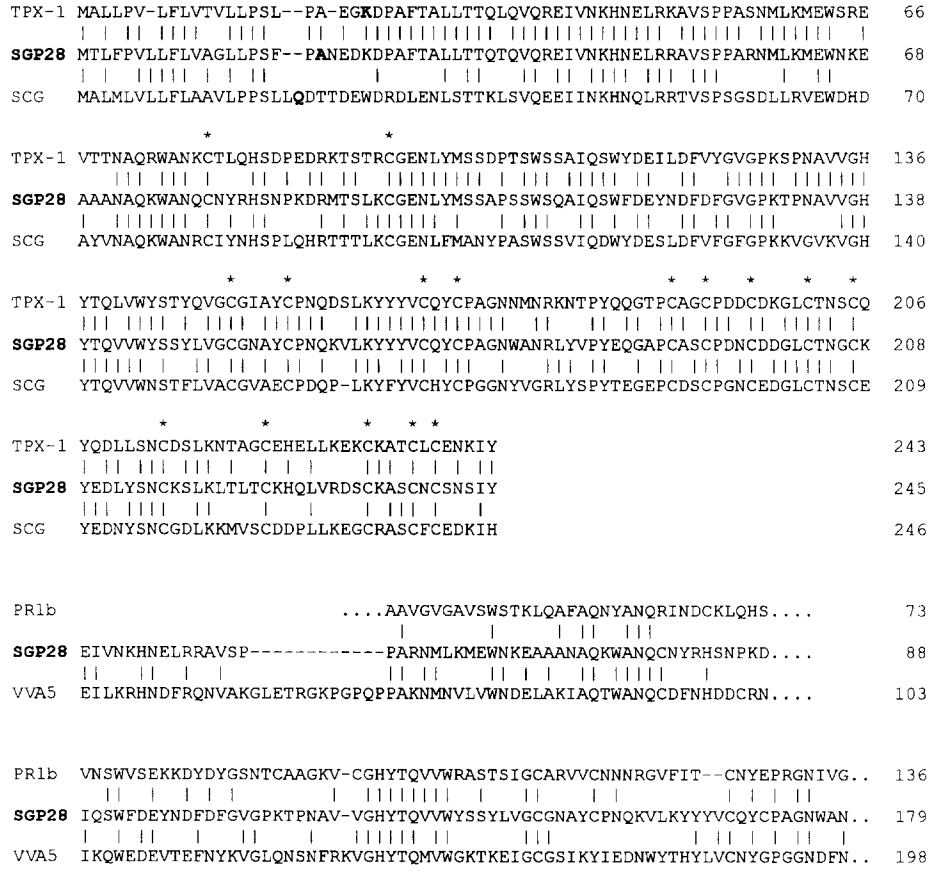

Fig. 4. Alignment of SGP28 with TPX-1 (human), sperm coating glycoprotein (SCG, rat), vespid venom antigen 5 (VVA5), and pathogenesis related protein 1b (barley) (PR1b). The aligned sequences are all deduced from cDNA sequences [13,14,18,19]. Vertical lines mark identical amino acids. Alignment of part of SGP28 with PR1b and VAA5 is shown in the lowest part of the figure. Conserved cysteine residues are marked by asterisks. The N-terminal amino acids, following the putative cleavage sites of the signal peptides (as suggested in the references given above) are shown in bold.

of the immunoblotting for SGP28 (Fig. 3). This indicates SGP28 to be a constituent of specific granules. The presence of the 28 kDa band could be caused by limited proteolysis of the major 30 kDa band. Since the protein was isolated from material exocytosed by PMA it is most likely a matrix protein. This is further corroborated by the lack of membrane spanning sequences in the deduced sequence of the protein. We suggest the protein be named SGP28 (specific granule protein of 28 kDa).

In the sequence similarity search, SGP28 was found to be highly similar to the deduced amino acid sequences of the cDNAs of the testis-specific human gene TPX-1 [13] and of an androgen-dependent sperm coating glycoprotein (also known as protein DE and acidic epididymal glycoprotein (AEG)) from mouse and rat epididymis [14,15], with 72.4 and 55% identical amino acids, respectively (Fig. 4). TPX-1 and sperm coating glycoprotein were previously concluded to be highly similar but distinct proteins belonging to a novel gene-family [13]. Our findings indicate that SGP28 is a new member of this gene family. By Northern blotting, AEG and a highly similar AEG-2 have been demonstrated in the mouse submandibular gland, where the transcription is androgen-dependent [16]. It remains to be established whether the expression of SGP28 is influenced by androgens.

SGP28, TPX-1 and sperm coating glycoprotein are very rich in cysteines, especially in the carboxy-terminal third of the proteins (marked by asterisks in Fig. 4). This is a characteristic feature of several natural antibiotic peptides [17]. All of these residues have been conserved from the rodent sperm coating glycoprotein and Tpx-1 to the human proteins TPX-1 and SGP28. These cysteines could potentially form intramolecular disulfide bridges, which is in accordance with the diminished mobility of reduced SGP28 compared to the unreduced form, when visualized by SDS-PAGE (Fig. 1).

In the alignment studies, pathogenesis related protein 1 from various plants and the vespid venom antigen 5 showed significant similarity to part of SGP28 (Fig. 4). From the alignment of SGP28 with pathogenesis related protein 1b from barley [18], venom antigen 5 from the white face hornet [19], TPX-1 and sperm coating glycoprotein, it is observed that the amino acid sequence GHYTQVVW (residues 137–144 in SGP28, Fig. 4) is a particularly well preserved region in all the identified proteins, indicating an important functional role of this domain. While the functions of TPX-1 (not yet isolated as a protein) and vespid venom antigen 5 are unknown, pathogenesis related proteins constitute a large family of plant proteins, whose synthesis is known to be induced upon infection. This suggests a role for these proteins in the host defense of plants [20]. In support of this notion, the pathogenesis related protein P14 from tomato exhibited antifungal activity in vitro [21]. Sperm coating glycoprotein is known to associate with the acrosomal region of spermatozoa after secretion from the epididymis [22]. Antibodies against the protein were able to block fertilization of an ovum [23], indicating a role for sperm coating glycoprotein in the penetration by spermatozoa of the extracellular matrix-like zona pellucida, which surrounds the ovum. A role of SGP28 in degradation of the extracellular matrix during neutrophil migration is possible and suggested by its localization in specific granules, where other matrix degradative enzymes are stored [1–3,5]. The elucidation of the potential anti-microbial and matrix-degradative actions of SGP28 awaits its recombinant production.

Acknowledgements: Dr. Jakob Ramlau is kindly acknowledged for advice regarding anti-peptide antibodies. The expert technical assistance of Charlotte Horn, Pia L. Olsen, Sanne Juul Christiansen, Lone Blom, and Allan Kastrup is greatly appreciated.

References

[1] Borregaard, N., Lollike, K., Kjeldsen, L., Sengeløv, H., Bastholm, L., Nielsen, M.H. and Bainton, D.F. (1993) Eur. J. Haematol. 51, 187–198.
[2] Dewald, B., Bretz, U., and Baggiolini, M. (1982) J. Clin. Invest. 70, 518–525.
[3] Kjeldsen, L., Sengeløv, H., Lollike, K., Nielsen, M.H. and Borregaard, N. (1994) Blood 83, 1640–1649.
[4] Sengeløv, H., Kjeldsen, L., and Borregaard, N. (1993) J. Immunol. 150, 1535–1543.
[5] Weiss, S.J. and Peppin, G.J. (1986) Biochem. Pharmacol. 35, 3189–3197.
[6] Spitznagel, J.K. (1990) J. Clin. Invest. 86, 1381–1386.
[7] Cowland, J.B., Johnsen, A.H. and Borregaard, N. (1995) FEBS Lett. 368, 173–176.
[8] Kjeldsen, L., Johnsen, A.H., Sengeløv, H. and Borregaard, N. (1993) J. Biol. Chem. 268, 10425–10432.
[9] Kjeldsen, L., Bjerrum, O.W., Hovgaard, D., Johnsen, A.H., Sehested, M. and Borregaard, N. (1992) Eur. J. Haematol. 49, 180–191.
[10] Laemmli, U.K. (1970) Nature 227, 680–695.
[11] Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[12] Von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690.
[13] Kasahara, M., Gutknecht, J., Brew, K., Spurr, N. and Goodfellow, P.N. (1989) Genomics 5, 527–534.
[14] Brooks, D.E., Means, A.R., Wright, E.J., Singh, S.P. and Tiver, K.K. (1986) Eur. J. Biochem. 161, 13–18.
[15] Charest, N.J., Joseph, D.R., Wilson, E.M. and French, F.S. (1988) Mol. Endocrinol. 2, 999–1004.
[16] Mizuki, N. and Kasahara, M. (1992) Mol. Cell. Endocrinol. 89, 25–32.
[17] Gabay, J.E. (1994) Science 254, 373–374.
[18] Bryngelsson, T., Sommer-Knudsen, J., Gregersen, P.L., Collinge, D.B., Ek, B. and Thordal-Christensen, H. (1994) Mol. Plant Microbe Interact. 7, 267–275.
[19] Fang, K.S.Y., Vitale, M., Fehlner, P. and King, T.P. (1988) Proc. Natl. Acad. Sci. USA 85, 895–899.
[20] Rigden, J. and Coutts, R. (1988) Trends Genet. 4, 87–89.
[21] Niderman, T., Bruyère, T., Gügler, K. and Mösinger, E. (1993) in: Mechanisms of Plant Defense Responses (Fritig, B. and Legrand, M. eds.) p. 450, Kluwer Academic Publishers, The Netherlands.
[22] Brooks, D.E. (1982) Int. J. Androl. 5, 513–524.
[23] Cuasnicu, P.S., Escheverria, F.G., Piazza, A.D., Cameo, M.S. and Blaquier, J.A. (1984) J. Reprod. Fertil. 72, 467–471.