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MOLECULAR CLONING AND CHARACTERIZATION OF NOVEL PROSTATE ANTIGEN cDNA's

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Three different prostate antigen cDNA's were isolated from a PC 82 prostate tumor cDNA library. PA 7S has a size of 1.4 kb and contains the almost complete information for the 3S kD prostate antigen preproprotein. The 1.6 kb PA 525 cDNA lacks about 0.2 kb of the 3'-non coding region and contains an additional internal 0.4 kb fragment as a result of alternative splicing. PA 424 represents a 0.6 kb variant of PA 7S. It contains a 0.1S kb internal fragment and a poly(A) tail preceded by an AAGAAA motive at the 3'-end. The predicted protein products of PA 525 and PA 424 will be different from PA 7S at the C-terminal end. In RNA preparations of two human prostate tumors (PC 82 and PC EW) seven different prostate antigen transcripts can be detected ranging in size from 0.5 kb to 5.6 kb. PA 7S cDNA represents the major 1.5 kb mRNA. PA 424 correlates with a 0.9 kb transcript and PA 525 with a 1.9 kb mRNA species.

Prostate antigen (PA) or prostate specific antigen (PSA) is a glycoprotein with a molecular weight of approximately 3S kD (1). The complete amino acid and cDNA sequence have recently been determined (2,3). The structure shows strong homology with kallikrein-like proteins. The highest homology is found with the human kallikrein hGK-1 (80%) and human pancreatic kallikrein (62%) (4,5). Similar to PA these proteins are serine proteases. The PA substrate probably is high molecular weight seminal vesicle protein also known as seminogelin, which is secreted by the seminal vesicles and causes the gel-like structure of the semen (6,7). PA is presumed to play a role in "dissolving" the seminal coagulum by digesting this protein into various

Abbreviations: PA, prostatic antigen; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; bp, basepairs; kb, kilobases; kD, kilodaltons.

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fragments. PA is exclusively synthesized by the epithelial cells of the prostate gland (8,9). Normally only small amounts of PA can be found in the serum. However, in the case of invasive growth of prostate tumors significantly higher PA levels are detected (10-14). Therefore, measuring of serum PA concentration is presumed to be of high value in diagnosis of prostatic cancer. Because of its tissue specific expression and its clinical importance, knowledge of the properties and expression of PA are of high interest. As part of our study of PA gene expression we molecularly cloned PA cDNA's. In this paper the characterization of two novel PA cDNA's is described.

Methods

Isolation of total cellular RNA from the different cell lines was done by the guanidinium thiocyanate method (15). Poly A⁺RNA was isolated by oligo dT cellulose chromatography. In collaboration with Dr. R. Dijkema (Organon, Oss) a lambda gt 10 cDNA library of 1.5 x 10^6 independent clones was prepared from PC 82 prostate tumor poly A⁺RNA using standard conditions (16). Screening was carried out with an in vitro synthesized oligonucleotide (TTACTACACACACGGGTTCACGTGGGGGTTTTTCACTGGTTTAAGTACGA), which was deduced from amino acids 160 - 176 of the published PA sequence (3). Duplicate nitrocellulose filters were hybridized overnight at 42°C in 6xSSC containing 10x Denhardt, 0.1% SDS, and 100 µg/ml salmon sperm DNA. The filters were washed twice in 3x SSC, 0.1% SDS for 20 minutes at 37°C and once in 1x SSC for 20 minutes at room temperature. Filters were exposed to X-ray films for 16 to 48 hours at -70°C using intensifier screens. To obtain additional PA cDNA clones the library was rescreened under stringent conditions with the Eco RI - Sac I fragment of PA 7S (see figure 1). Positive clones were isolated by two cycles of purification.

DNA was isolated from these clones and cDNA fragments were subcloned into pUC9 for further analysis (17). Nucleotide sequences of the appropriate fragments subcloned into M13mp18/19 were determined by the dideoxy chain termination method (18).
Northern blot analysis of poly A⁺RNA was performed with glyoxal denatured RNA, separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane. The probe was labeled as described (19). Hybridization was at stringent conditions in the presence of 50% formamide at 42°C. After extensive washing filters were exposed to X-ray film at -70°C using intensifier screens.

PC 82 and PC EW prostate tumors were propagated as transplants on male nude mice (20-22).

Results

We used a specific oligonucleotide probe to screen a PC 82 prostate tumor cDNA library for PA cDNA's (see methods section). Two PA cDNA's were isolated, PA 133 (approximately 250 bp, data not shown) and PA 525 (approximately 1600 bp). Using the 5'

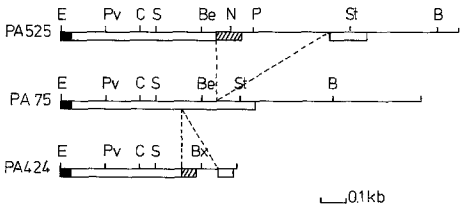

Figure 1: Comparison of the restriction maps of PA 75, PA 525 and PA 424. The open boxes correspond to the open reading frame of PA 75 encoding the mature protein. Closed boxes represent the sequences encoding (part of) the signal peptide and the propeptide sequence. The hatched areas represent remaining parts of the open reading frame of PA 424 and PA 525. Ba=Bam HI, Be=Bst EII, Bx=Bst XI, C=Cla I, E=Eco RI, N=Nco I, P=Pst I, Pu=Pvu II, S=Sac I and St=Stu I.

Eco RI -Sac I fragment of PA 525 (see figure 1) as a probe the library was rescreened. Two clones, PA 75 and PA 424, with a restriction map different from PA 525 were isolated (see figure 1). The size of PA 75 was 1400 bp. This cDNA lacked the Nco I and Pst I sites present in PA 525. PA 424 was about 600 bp, and contained a Bst EII site which was absent in PA 525 and PA 75.

Sequence analysis demonstrated that, beginning directly at the 5'-end, PA 75 contains an open reading frame of 771 bp encoding 13 amino acids of the signal peptide and the complete PA proprotein, that consists of 244 amino acids. PA 75 differs from the earlier reported cDNA clone (2) at two positions (790 and 1033) in the 3'-noncoding region. Discrepancies observed between the PA amino acid sequence as determined by direct analysis of peptide fragments of the purified protein and as deduced from the cDNA structure (2,3) were also found in PA 75. PA 75, 424 and 525 begin at exactly the same position as the PA cDNA isolated by Lundwall and Lilja (2). This implies that the 5'-Eco RI site is a natural occurring sequence and not derived from the linkers used to prepare the cDNA library.

Structural analysis of PA 424 revealed, starting at position 483, the presence of a short stretch of 145 bp, which was absent in PA 75. This sequence is followed by a 32 bp fragment, which is identical to PA 75 beginning at position 484 (figure 2). PA 424 ends in 28 A-residues. The internal 145 bp fragment seems to be a retained intron. At the boundaries the consensus GT...AG sequence is found. The human kallikrein gene hGK-1, which has recently been sequenced, and which shows a homology to PA of about 80%,

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                            30       ▽               60▼                           90
PA  75  GGTTGTCTTCCTCACCCTGTCCGTGACGTGGATTGGTGCTGCACCCCTCATCCTGTCTCGGATTGTGGGAGGCTGGGAGTGCGAGAAGCA
PA 525  GGTTGTCTTCCTCACCCTGTCCGTGACGTGGATTGGTGCTGCACCCCTCATCCTGTCTCGGATTGTGGGAGGCTGGGAGTGCGAGAAGCA
PA 424  GGTTGTCTTCCTCACCCTGTCCGTGACGTGGATTGGTGCTGCACCCCTCATCCTGTCTCGGATTGTGGGAGGCTGGGAGTGCGAGAAGCA
                           120                           150                           180
PA  75  TTCCCAACCCTGGCAGGTGCTTGTGGCCTCTCGTGGCAGGGCAGTCTGCGGCGGTGTTCTGGTGCACCCCCAGTGGGTCCTCACAGCTGC
PA 525  TTCCCAACCCTGGCAGGTGCTTGTGGCCTCTCGTGGCAGGGCAGTCTGCGGCGGTGTTCTGGTGCACCCCCAGTGGGTCCTCACAGCTGC
PA 424  TTCCCAACCCTGGCAGGTGCTTGTGGCCTCTCGTGGCAGGGCAGTCTGCGGCGGTGTTCTGGTGCACCCCCAGTGGGTCCTCACAGCTGC
                           210                           240                           270
PA  75  CCACTGCATCAGGAACAAAAGCGTGATCTTGCTGGGTCGGCACAGCCTGTTTCATCCTGAAGACACAGGCCAGGTATTTCAGGTCAGCCA
PA 525  CCACTGCATCAGGAACAAAAGCGTGATCTTGCTGGGTCGGCACAGCCTGTTTCATCCTGAAGACACAGGCCAGGTATTTCAGGTCAGCCA
PA 424  CCACTGCATCAGGAACAAAAGCGTGATCTTGCTGGGTCGGCACAGCCTGTTTCATCCTGAAGACACAGGCCAGGTATTTCAGGTCAGCCA
                           300                           330                           360
PA  75  CAGCTTCCCACACCCGCTCTACGATATGAGCCTCCTGAAGAATCGATTCCTCAGGCCAGGTGATGACTCCAGCCACGACCTCATGCTGCT
PA 525  CAGCTTCCCACACCCGCTCTACGATATGAGCCTCCTGAAGAATCGATTCCTCAGGCCAGGTGATGACTCCAGCCACGACCTCATGCTGCT
PA 424  CAGCTTCCCACACCCGCTCTACGATATGAGCCTCCTGAAGAATCGATTCCTCAGGCCAGGTGATGACTCCAGCCACGACCTCATGCTGCT
                           390                           420                           450
PA  75  CCGCCTGTCAGAGCCTGCCGAGCTCACGGATGCTGTGAAGGTCATGGACCTGCCCACCCAGGAGCCAGCACTGGGGACCACCTGCTACGC
PA 525  CCGCCTGTCAGAGCCTGCCGAGCTCACGGATGCTGTGAAGGTCATGGACCTGCCCACCCAGGAGCCAGCACTGGGGACCACCTGCTACGC
PA 424  CCGCCTGTCAGAGCCTGCCGAGCTCACGGATGCTGTGAAGGTCATGGACCTGCCCACCCAGGAGCCAGCACTGGGGACCACCTGCTACGC
                           480
PA  75  CTCAGGCTGGGGCAGCATTGAACCAGAGGAGT............................................................
PA 525  CTCAGGCTGGGGCAGCATTGAACCAGAGGAGT............................................................
PA 424  CTCAGGCTGGGGCAGCATTGAACCAGAGGAGTGTACGCCTGGGCCAGATGGTGCAGCCGGGAGCCCAGATGCCTGGGTCTGAGGGAGGAG

PA  75  ......................................................................................TCTT
PA 525  ..........................................(145).......................................TCTT
PA 424  GGGACAGGACTCCTGGGTCTGAGGGAGGAGGGCCAAGGAACCAGGTGGGGTCCAGCCCACAACAGTGTTTTTGCCTGGCCCGTAGTCTT
                           510                           540                           570
PA  75  GACCCCAAAGAAACTTCAGTGTGTGGACCTCCATGTTATTTCCAATGACGTGTGTGCGCAAGTTCACCCTCAGAAGGTGACCAAGTTCAT
PA 525  GACCCCAAAGAAACTTCAGTGTGTGGACCTCCATGTTATTTCCAATGACGTGTGTGCGCAAGTTCACCCTCAGAAGGTGACCAAGTTCAT
PA 424  GACCCCAAAGAAACTTCAGTGTGTGGAC(A)N
                           600
PA  75  GCTGTGTGCTGGACGCTGGACAGGGGGCAAAAGCACCTGCTCG.................................................
PA 525  GCTGTGTGCTGGACGCTGGACAGGGGGCAAAAGCACCTGCTCGTGGGTCATTCTGATCACCGAACTGACCATGCCAGCCCTGCCGATGGT

PA  75  ............................................................................................
PA 525  CCTCCATGGCTCCCTAGTGCCCTGGAGAGGAGGTGTCTAGTCAGAGAGTAGTCCTGGAAGGTGGCCTCTGTGAGGAGCCACGGGGACAGC

PA  75  ........................................(442)...............................................
PA 525  ATCCTGCAGATGGTCCTGGCCCTTGTCCCACCGACCTGTCTACAAGGACTGTCCTCGTGGACCCTCCCCTCTGCACAGGAGCTGGACCCT

PA  75  ............................................................................................
PA 525  GAAGTCCCTTCCCTACCGGCCAGGACTGGAGCCCCTACCCCTCTGTTGGAATCCCTGCCCACCTTCTTCTGGAAGTCGGCTCTGGAGACA

PA  75  ............................................................................................
PA 525  TTTCTCTCTTCTTCCAAAGCTGGGAACTGCTATCTGTTATCTGCCTGTCCAGGTCTGAAAGATAGGATTGCCCAGGCAGAAACTGGGACT
                                                        630                           660
PA  75  ....................................GGTGATTCTGGGGGCCCACTTGTCTGTAATGGTGTGCTTCAAGGTATCACGTCAT
PA 525  GACCTATCTCACTCTCTCCCTGCTTTTACCCTTAGGGTGATTCTGGGGGCCCACTTGTCTGTAATGGTGTGCTTCAAGGTATCACGTCAT
                           690                           720                           750
PA  75  GGGGCAGTGAACCATGTGCCCTGCCCGAAAGGCCTTCCCTGTACACCAAGGTGGTGCATTACCGGAAGTGGATCAAGGACACCATCGTGG
PA 525  GGGGCAGTGAACCATGTGCCCTGCCCGAAAGGCCTTCCCTGTACACCAAGGTGGTGCATTACCGGAAGTGGATCAAGGACACCATCGTGG
                           780                           810                           840
PA  75  CCAACCCCTGAGCACCCCTATCAACTCCCTATTGTAGTAAACTTGGAACCTTGGAAATGACCAGGCCAAGACTCAAGCCTCCCCAGTTCT
PA 525  CCAACCCCTGAGCACCCCTATCAACTCCCTATTGTAGTAAACTTGGAACCTTGGAAATGACCAGGCCAAGACTCAAGCCTCCCCAGTTCT
                           870                           900                           930
PA  75  ACTGACCTTTGTCCTTAGGTGTGAGGTCCAGGGTTGCTAGGAAAAGAAATCAGCAGACACAGGTGTAGACCAGAGTGTTTCTTAAATGGT
PA 525  ACTGACCTTTGTCCTTAGGTGTGAGGTCCAGGGTTGCTAGGAAAAGAAATCAGCAGACACAGGTGTAGACCAGAGTGTTTCTTAAATGGT
                           960                           990                          1020
PA  75  GTAATTTTGTCCTCTCTGTGTCCTGGGGAATACTGGCCATGCCTGGAGACATATCACTCAATTTCTCTGAGGACACAGATAGGATGGGTT
PA 525  GTAATTTTGTCCTCTCTGTGTCCTGGGGAATACTGGCCATGCCTGGAGACATATCACTCAATTTCTCTGAGGACACAGATAGGATGGGTT
                          1050                          1080                          1110
PA  75  GTCTGTGTTATTTGTGGGGTACAGAGATGAAAGAGGGGTGGGGATCCACACTGAGAGAGTGGAGAGTGACATGTGCTGGACACTGTCCAT
PA 525  GTCTGTGTTATTTGTGGGGTACAGAGATGAAAGAGGGGTGGGGATCCACACTGAGAGAGTGGAGAGTGACATGTGCTGGACACTGTCCAT
                          1140                          1170                          1200
PA  75  GAAGCACTGAGCAGAAGCTGGAGGCACAACGCACCAGACACTCACAGCAAGGATGGAGCTGAAAACATAACCCACTCTGTCCTGGAGGCA
PA 525  GAAGCACTGAGCAGAAGCTGGAGGCACAACGCACCAGACACTCACAGCAAGGATGGAGCTGAAAACATAACCCACTCTGTCCTGGAGG
                          1230                          1260                          1290
PA  75  CTGGGAAGCCTAGAGAAGGCTGTGAGCCAAGGAGGGAGGGTCTTCCTTTGGCATGGGATGGGGATGAAGTAAGGAGAGGGACTGGACCCC
                          1320                          1350                          1380
PA  75  CTGGAAGCTGATTCACTATGGGGGGAGGTGTATTGAAGTCCTCCAGACAACCCTCAGAGTTTGATGAGTTTCCTAGTAGAACTCACAGAA

PA  75  ATAAAGAGCTGTTATACTGTG(A)N
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Figure 2: Comparison of the nucleotide sequences of PA 75, PA 525 and PA 424. The dotted lines represent open spaces. The open arrow head represents the end of the signal peptide and the beginning of the propeptide. The solid arrow head represents the sequence encoding the end of the propeptide and the beginning of the mature protein. The sequences which code for a stop codon and the poly(A) signal sequences are underlined. Poly(A) tails are given by the symbol: $(A)_n$.

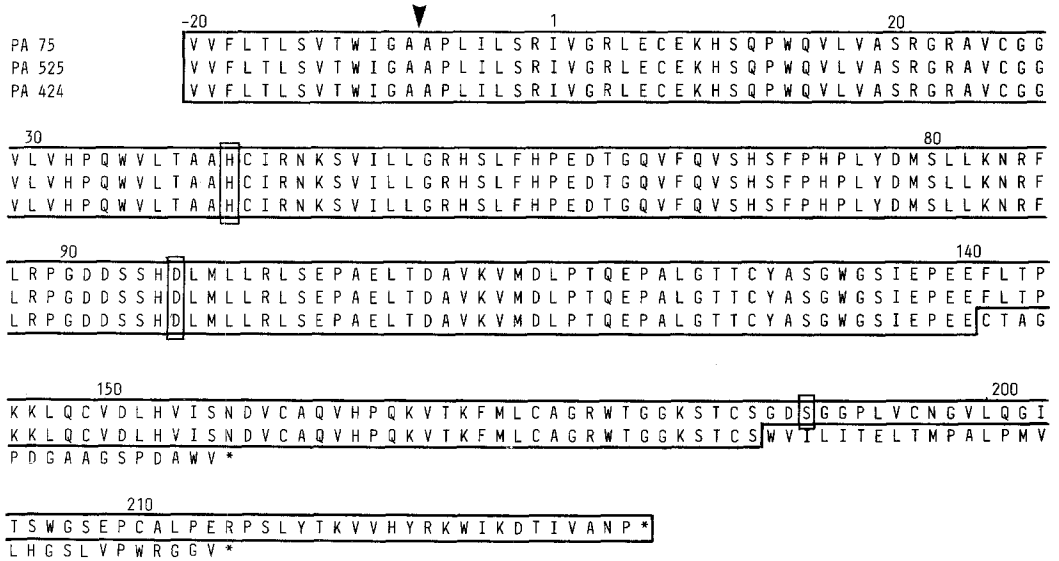

FIGURE 3: Comparison of the predicted amino acid sequences of PA 75, PA 525 and PA 424 proteins. The identical sequences are boxed. The small boxes contain the amino acids that are thought to be responsible for the serine protease activity of the protein.

contains at exactly the same position a 113 bp intron (4). Especially the first 61 bp of this sequence are highly conserved between PA 424 and hGK-1. The poly(A) tail of PA 424 is preceded by an AAGAAA motive which presumably serves as the polyadenylation signal. The AAGAAA sequence is situated within the coding region of PA 75. A possible mature protein product of PA 424 will have a size of 156 amino acids of which the first 140 amino acids identical to PA 75 (figure 3).

Sequence analysis of PA 525 showed that it contains a fragment of 442 bp, in which the Nco I and Pst I site are situated, which is not present in PA 75. The diversion from the PA 75 sequence begins at position 620 (figure 2). Starting at position 1062 of PA 525 the sequence further parallels that of PA 75. PA 75 lacks the 3'-203 bp of PA 75, including the polyadenylation motive. For several reasons it is likely that PA 525 results from alternative splicing. The structure reveals it to contain a splice acceptor site, but not the GT donor consensus sequence (figure 2). Comparison of PA 525 with the structure of the hGK-1 gene shows that hGK-1 contains an intervening sequence at an identical position (4), however, this intron is much larger (1337 bp). The PA 525 internal fragment is highly homologous to the last 446 bp

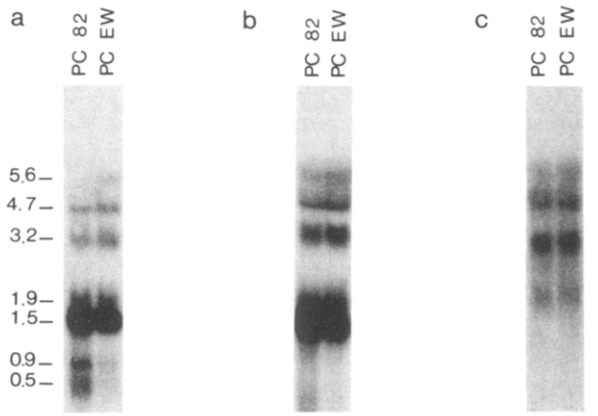

Figure 4: Northern blots of PC 82 and PC EW poly A+RNA hybridized with different PA probes. a; 5'-Eco RI-Sac I fragment. b; 3'-Bam HI-Eco RI fragment.c; Nco I-Pst I fragment of PA 525.

of the hGK-1 intron. Preliminary data from a recently isolated genomic PA clone confirm that the 442 bp fragment of PA 525 is part of a larger intervening sequence (data not shown). Translation of PA 525 mRNA will result into a mature PA protein of 214 amino acids. The terminal 28 amino acids of this protein will be different from the PA 75 protein (figure 3).

The 380 bp Eco RI - Sac I fragment common for the various PA cDNA clones was used to analyze by Northern blotting PA mRNA expression in the PC 82 and PC EW human prostate tumors (figure 4). At least seven different hybridizing transcripts were observed ranging in size from 0.5 to 5.6 kb. The 1.5 kb band represents the major mRNA species. To obtain additional information identical blots were hybridized with the 3'-Bam HI - Eco RI fragment of PA 525 and the Nco I - Pst I PA 525 fragment as probes (figure 4b,c). The 0.5 kb, 0.9 kb and the abundant 1.5 kb mRNA transcripts could not be detected by the Nco I - Pst I probe. The Bam HI - Eco RI fragment specifically hybridized with the five larger mRNA's. These data can be explained by assuming that PA 75 corresponds to the major 1.5 kb mRNA species and PA 424 to the 0.9 kb mRNA. PA 525 represents the major part of the 1.9 kb transcript.

Discussion

In this study the characterization of two novel PA cDNA clones, PA 525 and PA 424, is described and evidence is presented showing

that these cDNA's correspond to a 1.9 and a 0.9 kb PA mRNA species, respectively. PA 525 results from alternative splicing, PA 424 from intron retention and alternative polyadenylation as compared to PA 75, which represents the major PA 1.5 kb transcript. The AAGAAA polyadenylation motive of PA 424 is remarkable. As compared to the AATAAA consensus sequence, the AAGAAA signal has been reported to be very weak (23).

It is presumed that PA 525 and PA 424 encode variant forms of the major 35 kD PA protein. Even if the 145 bp intervening sequence of PA 424 is spliced out, the resulting protein will be different from PA 75. PA 424 contains the information for a protein of 156 amino acids, PA 525 will be able to encode a 218 amino acid protein. There are data available indicating that alternative forms of the PA protein exist. Purified PA protein preparations contain, in addition to the 35 kD protein, at least one or two 20 - 25 kD proteins, which specifically interact with monoclonal antibodies against PA (8). These proteins are likely candidates as products of different PA mRNA species. Alternatively they can be the result of proteolytic degradation of the 35 kD protein species.

The histidine residue at position 41, the asparagine at 96 and the serine at position 189, which are important for the proteolytic activity of kallikreins (24), are also found in PA 75 (see figure 3). Histidine 41 and asparagine 96 will be present in the protein products of PA 525 and 424. However, the C-terminal part will be completely different and serine 189 will be lacking. No doubt, this will strongly influence the enzymatic properties of these proteins (24). At present, it is not yet possible to predict a cellular function of the PA 525 and 424 proteins. Expression of PA 525 and 424 and characterization of purified PA 525 and 424 protein can establish whether these proteins show (modified) enzymatic activity.

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