cDNA Sequence Coding for a Rat Glia-Derived Nexin and Its Homology to Members of the Serpin Superfamily

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ABSTRACT: Rat glial cells release a neurite-promoting factor with serine protease inhibitory activity. By using a rat glioma cDNA clone as a probe, it was possible to isolate rat cDNAs containing the entire sequence coding for this neurite-promoting factor. The largest rat cDNA (approximately 2100 bp) was characterized by DNA sequencing. It contained the entire coding region, 135 bp of the 5′ nontranslated region, and about 750 bp of the 3′ nontranslated region. The open reading frame coded for 397 amino acids including a putative signal peptide of 19 amino acids. The correct identity of the coding sequence was substantiated by the fact that the sequence of tryptic peptides, derived from the purified rat factor, matched exactly with the deduced amino acid sequence. The rat protein sequence had 84% homology with the corresponding protein from human glioma cells. Both amino acid sequences indicated that the proteins belong to the protease nexins [Baker, B. J., Low, D. A., Simmer, R. L., & Cunningham, D. D. (1980) *Cell* (*Cambridge, Mass.*) *21*, 37–45] and therefore can be defined as glia-derived nexins (GDNs). Further analysis showed that both rat and human GDN belong to the serpin superfamily and share 41%, 32%, and 25% homology with human endothelial-cell-type plasminogen activator inhibitor, antithrombin III, and α-1 proteinase inhibitor, respectively.

The rat glia-derived neurite-promoting factor is a glycoprotein with a M_r of 43 000 (Guenther et al., 1985). It is secreted both from cultured C6 glioma cells (Monard et al., 1973) and from rat brain primary cultures (Schuerch & Monard, 1978). The purified protein has been shown to promote neurite extension in neuroblastoma cells and to inhibit the activity of urokinase by forming SDS[1]-resistant complexes (Guenther et al., 1985). Other serine proteases such as thrombin, trypsin, and tissue-type plasminogen activator are also inhibited by this factor (Monard et al., 1983; Stone et al., 1987). The inhibition of thrombin is accelerated over 40-fold by heparin (Stone et al., 1987). Furthermore, the rat protein has been shown to reduce the rate of migration of granule cell neurons in cultured explants from early postnatal mouse cerebellum (Lindner et al., 1986). Such biological data suggest that the factor is a molecule involved in neuronal development (Lindner et al., 1986; Monard, 1985) and maybe even in regeneration (Patterson, 1985). Its physiological function has not yet been clearly defined, but it could contribute to a balance between proteases and protease inhibitors during neurite outgrowth (Monard, 1985).

Our previous data, showing that the first 28 amino acids from the N-terminus of protease nexin I were identical with those of the factor derived from human glioma cells (Gloor et al., 1986), suggest that the two glia-derived proteins and protease nexin I are very similar. Their close relationship, based on sequencing data and biochemical properties, allows us to name our factor glia-derived nexin (GDN), and it implies a possible biological function of nexins in the nervous system.

The biological relevance and the mode of action of GDN need further investigation. Such studies require appropriate molecular tools, and it is obvious that animal models (e.g., rat neuronal cells and tissues) will have to be used. The nucleotide and amino acid sequences of rat GDN shown in this paper therefore represent the basic key to establish onleading experiments.

Because rat GDN is an efficient inhibitor of serine proteases, we compared the amino acid sequence of both rat and human GDN[2] with three members of the serpin superfamily (Carell & Travis, 1985), namely, human endothelial-cell-type plasminogen activator inhibitor (Ny et al., 1986; Pannekoek et al., 1986), antithrombin III (Bock et al., 1982), and α-1 proteinase inhibitor (Kurachi et al., 1981). We show here that at the level of primary structure both rat and human GDNs are homologous to these protease inhibitors and therefore belong to the serpin superfamily.

Experimental Procedures

Materials

Restriction enzymes and DNA-modifying enzymes were from Boehringer; deoxy- and dideoxynucleotides for cDNA sequencing were exclusively from P-L Biochemicals.

Methods

cDNA Cloning. The cDNA was constructed following the method described by Gubler and Hoffmann (1983), including a few modifications (Gloor et al., 1986). dC-tailed cDNA was annealed to the *Pst*I cut, dG-tailed pUC8 vector and used to transform *Escherichia coli* HB 101. Double-stranded cDNA was labeled by nick translation (Rigby et al., 1977) and used for colony hybridization as described (Gloor et al., 1986) and for "Southern blots" (Southern, 1975; Maniatis et al., 1982).

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; PTH, phenylthiohydantoin; GDN, glia-derived nexin; h-ePAI, human endothelial-cell-type plasminogen activator inhibitor; h-AT III, human antithrombin III; h-α1PI, human α-1 proteinase inhibitor; bp, base pair.

[2] We have two different sequences for the human GDN because two subclones were isolated, which coded either for Arg or for Thr-Gly (Gloor et al., 1986) at position 329 (Figure 2). In this paper we show the first possibility (Arg), because it matches exactly with the rat protein sequence at this position. Note that a mistake has been discovered in the already published human GDN amino acid sequence (Gloor et al., 1986): at position 260 a Ser must be replaced by a Glu.

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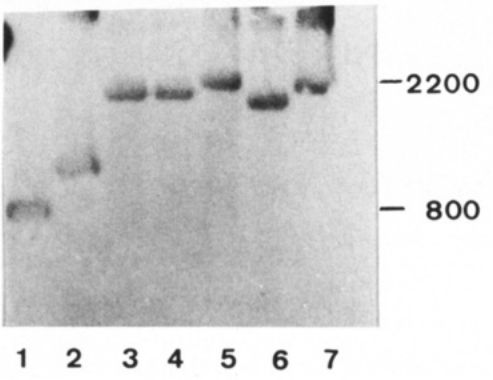

FIGURE 1: Cross-hybridization of nick-translated clone 15.H5 (lane 1) with the inserts of six recombinant clones (lanes 2–7) isolated from the rat cDNA library. The inserts have been excised from the vector pUC8 by digestion with *Hin*dIII and *Eco*RI. Marker length is indicated in base pairs.

The cDNA was sequenced on both strands by the method of Sanger et al. (1977).

Peptides. To obtain tryptic fragments, the reduced and carboxymethylated (Ruegg & Rudinger, 1977) rat GDN was dialyzed against 0.1% TFA (GDN is very soluble under acidic conditions). The pH was then adjusted to 8.0 with a concentrated solution of ammonium bicarbonate. Trypsin was immediately added to obtain a protein/enzyme ratio of 80/1, and the reaction mixture was incubated for 24 h at 37 °C. The resulting tryptic fragments of GDN were separated by RP-HPLC using a wide-pore 0.46 × 25 cm C8 column (Baker-bond, J. T. Baker, RP 7105-0). The peptides were eluted with linear gradients of acetonitrile in 0.1% TFA in water. Standard gradients were commenced 5 min after injection of the peptide solution and run for 90 min from 0% to 100% buffer B (aqueous 50% acetonitrile with 0.1% TFA; buffer A was 0.1% TFA in water). The amino acid sequence analysis of the peptides was performed as described by Hewick et al. (1981). PTH-amino acids were analyzed as described by Hunkapiller and Hood (1983) with 5% tetrahydrofuran added to buffer A.

Computer Alignment. The five protein sequences (r-GDN, h-GDN, h-ePAI, h-AT III, and h-α1PI) were compared by using an alignment program (Dayhoff, 1979) that calculates the best alignment between a pair of sequences. The resulting alignments of pairs of sequences were then used to build a comparative alignment of these inhibitors. Only a minimum number of gaps was allowed, and the relative homologies were calculated by dividing the number of identical residues by the number of total residues.

Results and Discussion

Our previously isolated clone 15.H5 (Gloor et al., 1986) coding for a part of rat GDN was used to screen another rat

Sequence of human GDN

```
                                                                    20
ATGAACTGGCATCTCCCCCTCTTCCTCTTGGCCTCTGTGACGCTGCCTTCCATCTGCTCC
MetAsnTrpHisLeuProLeuPheLeuLeuAlaSerValThrLeuProSerIleCysSer
                                                                    40
CACTTCAATCCTCTGTCTCTCGAGGAACTAGGCTCCAACACGGGGATCCAGGTTTTCAAT
HisPheAsnProLeuSerLeuGluGluLeuGlySerAsnThrGlyIleGlnValPheAsn
                                                                    60
CAGATTGTGAAGTCGAGGCCTCATGACAACATCGTGATCTCTCCCCATGGGATTGCGTCG
GlnIleValLysSerArgProHisAspAsnIleValIleSerProHisGlyIleAlaSer
                                                                    80
GTCCTGGGGATGCTTCAGCTGGGGGCGGACGGCAGGACCAAGAAGCAGCTCGCCATGGTG
ValLeuGlyMetLeuGlnLeuGlyAlaAspGlyArgThrLysLysGlnLeuAlaMetVal
                                                                   100
ATGAGATACGGCGTAAATGGAGTTGGTAAAATATTAAAGAAGATCAACAAGGCCATCGTC
MetArgTyrGlyValAsnGlyValGlyLysIleLeuLysLysIleAsnLysAlaIleVal
                                                                   120
TCCAAGAAGAATAAAGACATTGTGACAGTGGCTAACGCCGTGTTTGTTAAGAATGCCTCT
SerLysLysAsnLysAspIleValThrValAlaAsnAlaValPheValLysAsnAlaSer
                                                                   140
GAAATTGAAGTGCCTTTTGTTACAAGGAACAAAGATGTGTTCCAGTGTGAGGTCCGGAAT
GluIleGluValProPheValThrArgAsnLysAspValPheGlnCysGluValArgAsn
                                                                   160
GTGAACTTTGAGGATCCAGCCTCTGCCTGTGATTCCATCAATGCATGGGTTAAAAACGAA
ValAsnPheGluAspProAlaSerAlaCysAspSerIleAsnAlaTrpValLysAsnGlu
                                                                   180
ACCAGGGATATGATTGACAATCTGCTGTCCCCAGATCTTATTGATGGTGTGCTCACCAGA
ThrArgAspMetIleAspAsnLeuLeuSerProAspLeuIleAspGlyValLeuThrArg
                                                                   200
CTGGTCCTCGTCAACGCAGTGTATTTCAAGGGTCTGTGGAAATCACGGTTCCAACCCGAG
LeuValLeuValAsnAlaValTyrPheLysGlyLeuTrpLysSerArgPheGlnProGlu
                                                                   220
AACACAAAGAAACGCACTTTCGTGGCAGCCGACGGGAAATCCTATCAAGTGCCAATGCTG
AsnThrLysLysArgThrPheValAlaAlaAspGlyLysSerTyrGlnValProMetLeu
                                                                   240
GCCCAGCTCTCCGTGTTCCGGTGTGGGTCGACAAGTGCCCCCAATGATTTATGGTACAAC
AlaGlnLeuSerValPheArgCysGlySerThrSerAlaProAsnAspLeuTrpTyrAsn
                                                                   260
TTCATTGAACTGCCCTACCACGGGGAAAGCATCAGCATGCTGATTGCACTGCCGACTGAG
PheIleGluLeuProTyrHisGlyGluSerILeSerMetLeuIleAlaLeuProThrGlu
                                                                   280
AGCTCCACTCCGCTGTCTGCCATCATCCCACACATCAGCACCAAGACCATAGACAGCTGG
SerSerThrProLeuSerAlaIleIleProHisIleSerThrLysThrIleAspSerTrp
                                                                   300
ATGAGCATCATGGTCCCCAAGAGGGTGCAGGTGATCCTGCCCAAGTTCACAGCTGTAGCA
MetSerIleMetValProLysArgValGlnValIleLeuProLysPheThrAlaValAla
                                                                   320
CAAACAGATTTGAAGGAGCCGCTGAAAGTTCTTGGCATTACTGACATGTTTGATTCATCA
GlnThrAspLeuLysGluProLeuLysValLeuGlyIleThrAspMetPheAspSerSer
                                                                   340
AAGGCAAATTTTGCAAAAATAACAAGGTCAGAAAACCTCCATGTTTCTCATATCTTGCAA
LysAlaAsnPheAlaLysIleThrArgSerGluAsnLeuHisValSerHisIleLeuGln
                                                                   360
AAAGCAAAAATTGAAGTCAGTGAAGATGGAACCAAAGCTTCAGCAGCAACAACTGCAATT
LysAlaLysIleGluValSerGluAspGlyThrLysAlaSerAlaAlaThrThrAlaIle
                                                                   380
CTCATTGCAAGATCATCGCCTCCCTGGTTTATAGTAGACAGACCTTTTCTGTTTTTCATC
LeuIleAlaArgSerSerProProTrpPheIleValAspArgProPheLeuPhePheIle
                                                                   397
CGACATAATCCTACAGGTGCTGTGTTATTCATGGGGCAGATAAACAAACCC
ArgHisAsnProThrGlyAlaValLeuPheMetGlyGlnIleAsnLysPro
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Sequence of rat GDN

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                                                                    20
ATGAATTGGCATTTTCCCTTCTTCATCTTGACCACAGTGACTTTATCCTCTGTGTACTCC
MetAsnTrpHisPheProPhePheIleLeuThrThrValThrLeuSerSerValTyrSer
                                                                    40
CAGCTCAACTCTCTGTCACTCGAGGAACTAGGCTCTGACACAGGGATCCAGGTTTTCAAT
GlnLeuAsnSerLeuSerLeuGluGluLeuGlySerAspThrGlyIleGlnValPheAsn
                                                                    60
CAGATCATCAAATCACAGCCTCATGAGAACGTTGTCATTTCTCCGCACGGGATTGCGTCC
GlnIleIleLysSerGlnProHisGluAsnValValIleSerProHisGlyIleAlaSer
                                                                    80
ATCTTGGGCATGCTGCAGCTGGGGGCTGACGGCAGGACGAAGAAGCAGCTCTCAACGGTG
IleLeuGlyMetLeuGlnLeuGlyAlaAspGlyArgThrLysLysGlnLeuSerThrVal
                                                                   100
ATGCGATACAATGTGAACGGAGTCGGAAAAGTGCTGAAGAAGATCAACAAGGCTATAGTC
MetArgTyrAsnValAsnGlyValGlyLysValLeuLysLysIleAsnLysAlaIleVal
                                                                   120
TCCAAAAAGAATAAAGACATAGTGACCGTGGCCAATGCTGTGTTTGTCAGGAATGGCTTT
SerLysLysAsnLysAspIleValThrValAlaAsnAlaValPheValArgAsnGlyPhe
                                                                   140
AAAGTGGAAGTGCCTTTTGCAGCAAGGAACAAAGAGGTGTTTCAGTGTGAAGTACAGAGT
LysValGluValProPheAlaAlaArgAsnLysGluValPheGlnCysGluValGlnSer
                                                                   160
GTGAACTTCCAGGACCCGGCCTCTGCTTGTGATGCCATCAATTTTTGGGTCAAAAATGAG
ValAsnPheGlnAspProAlaSerAlaCysAspAlaIleAsnPheThrValLysAsnGlu
                                                                   180
ACGAGGGGCATGATTGACAACCTACTTTCCCCAAATCTGATCGATAGTGCTCTTACCAAA
ThrArgGlyMetIleAspAsnLeuLeuSerProAsnLeuIleAspSerAlaLeuThrLys
                                                                   200
CTGGTCCTCGTTAACGCAGTGTATTTCAAGGGTTTGTGGAAATCCCGGTTTCAACCTGAG
LeuValLeuValAsnAlaValTyrPheLysGlyLeuTrpLysSerArgPheGlnProGlu
                                                                   220
AACACGAAGAAACGGACCTTCGTGGCAGGTGATGGAAAATCCTACCAAGTACCCATGCTA
AsnThrLysLysArgThrPheValAlaGlyAspGlyLysSerTyrGlnValProMetLeu
                                                                   240
GCCCAGCTCTCCGTGTTCCGCTCTGGGTCTACCAAAACCCCAAATGGCTTATGGTACAAC
AlaGlnLeuSerValPheArgSerGlySerThrLysThrProAsnGlyLeuTrpTyrAsn
                                                                   260
TTCATTGAGCTACCCTACCATGGTGAGAGCATCAGCATGTTGATCGCCCTGCCAACAGAG
PheIleGluLeuProTyrHisGlyGluSerIleSerMetLeuIleAlaLeuProThrGlu
                                                                   280
AGCTCCACCCCACTGTCCGCCATCATCCCTCACATCAGTACCAAGACCATCAATAGCTGG
SerSerThrProLeuSerAlaIleIleProHisIleSerThrLysThrIleAsnSerTrp
                                                                   300
ATGAACACCATGGTACCCAAGAGGATGCAGCTGGTCCTGCCCAAGTTCACAGCTCTGGCA
MetAsnThrMetValProLysArgMetGlnLeuValLeuProLysPheThrAlaLeuAla
                                                                   320
CAAACAGATCTGAAGGAGCCACTGAAAGCCCTTGGCATTACTGAGATGTTTGAACCGTCA
GlnThrAspLeuLysGluProLeuLysAlaLeuGlyIleThrGluMetPheGluProSer
                                                                   340
AAGGCAAATTTTGCAAAAATAACAAGGTCAGAGAGCCTTCACGTCTCTCACATCTTGCAG
LysAlaAsnPheAlaLysIleThrArgSerGluSerLeuHisValSerHisIleLeuGln
                                                                   360
AAAGCAAAAATTGAAGTCAGCGAAGATGGGACCAAAGCCGCAGTAGTGACAACTGCAATC
LysAlaLysIleGluValSerGluAspGlyThrLysAlaAlaValValThrThrAlaIle
                                                                   380
CTAATTGCAAGGTCATCGCCTCCCTGGTTTATAGTAGACAGGCCTTTCCTGTTCTGCATC
LeuIleAlaArgSerSerProProTrpPheIleValAspArgProPheLeuPheCysIle
                                                                   397
CGACACAATCCCACAGGTGCCATCTTGTTCCTGGGGCAGGTGAACAAGCCC
ArgHisAsnProThrGlyAlaIleLeuPheLeuGlyGlnValAsnLysPro
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FIGURE 2: Nucleotide and amino acid sequences of rat and human GDN. The numbering refers to the amino acid sequence. The signal peptide is shaded. Also shaded are the possible glycosylation sites (Asn-X-Thr/Ser) and the reactive centers (Arg-Ser). The amino acid sequences that were also obtained from sequence analysis of tryptic peptides are underlined.

glioma cDNA library. The identity of six isolated rat clones to clone 15.H5 was demonstrated by restriction mapping (not shown) and cross-hybridization (Figure 1). The longest clone (G2, 2100 bp, lane 5 in Figure 1) was sequenced in both directions by dideoxy sequencing. G2 shares 83% homology with the coding sequence of human GDN (Gloor et al., 1986) and contains 135 bp of the 5′ nontranslated region, the entire coding region (1191 bp), and approximately 750 bp of the 3′ nontranslated part. The open reading frame codes for 397 amino acids (Figure 2, right). To show that the amino acid sequence deduced from the cDNA sequence of clone G2 corresponds to that of rat GDN, tryptic peptides from the purified protein were isolated and sequenced. These sequences (underlined in Figure 2) match exactly with the deduced amino acid sequence of rat GDN. An agreement was also obtained between the experimentally determined (total hydrolysis) and the deduced amino acid composition (data not shown). GDN isolated from rat C6 glioma cells was not susceptible to Edman degradation. We therefore must assume that the native protein has a blocked N-terminus. On the basis of the homology with human GDN (Figure 3) and the identity of the N-termini of human GDN and protease nexin I (Gloor et al., 1986), we believe that rat GDN starts at amino acid position 20 (Ser, Figure 2). The first 19 amino acids thus seem to be a signal peptide (shaded, Figure 2) that is cleaved off during post-translational modification.

The rat GDN protein has a slightly higher apparent molecular weight on SDS–PAGE [43 000 (Guenther et al., 1985)] than that calculated from the amino acid sequence (41 700). This and the periodic acid staining on SDS–PAGE (Guenther et al., 1985) indicated the glycoprotein nature of GDN. Analysis of both rat and human sequences revealed two putative glycosylation sites (Asn-X-Thr/Ser) in the rat sequence (positions 159–161 and 383–385, Figure 2, right panel) and three in the human sequence (positions 159–161, 181–183, and 383–385, Figure 2, left panel). The positions of the two putative glycosylation sites in the rat protein have been conserved in the human GDN.

Three Cys residues are found in both rat (residue numbers 136, 150, and 379, Figure 2, right panel) and human (residue numbers 136, 150, and 228, Figure 2, left panel) amino acid sequences. Two of these Cys are at the same position in both proteins. The third, however, is located closer to the C-terminal end in the rat protein. It remains to be demonstrated if these three Cys are involved in the possible formation of S–S bridges.

Because rat GDN is an efficient inhibitor of serine proteases (Guenther et al., 1985; Stone et al., 1987), we looked for homologies of GDN with members of a family of protease inhibitors, the serpins, introduced by Carell and Travis (1985) [see also Hunt and Dayhoff (1980)].

In Figure 3 the alignment of rat GDN, human GDN, human endothelial-cell-type plasminogen activator inhibitor [h-ePAI (Ny et al., 1986; Pannekoek et al., 1986)], human antithrombin III [h-AT III (Bock et al., 1982)], and human α-1 proteinase inhibitor [h-α1PI (Kurachi et al., 1981)] is shown. The overall homology was 84%, 41%, 32%, and 25%, respectively. These values are in the range of those within the serpin superfamily (Carell et al., 1982; Chandra et al., 1983), showing that GDN is a member of this family too. Furthermore, the alignment suggests Arg^{364}-Ser^{365} to be at the reactive center (P_1–P'_1) of this inhibitor (shaded in Figure 2). This coincides with the specificity of GDN for thrombin, trypsin, and urokinase (Guenther et al., 1985; Stone et al., 1987), proteases that can cleave at the carboxyl site of arginyl residues.

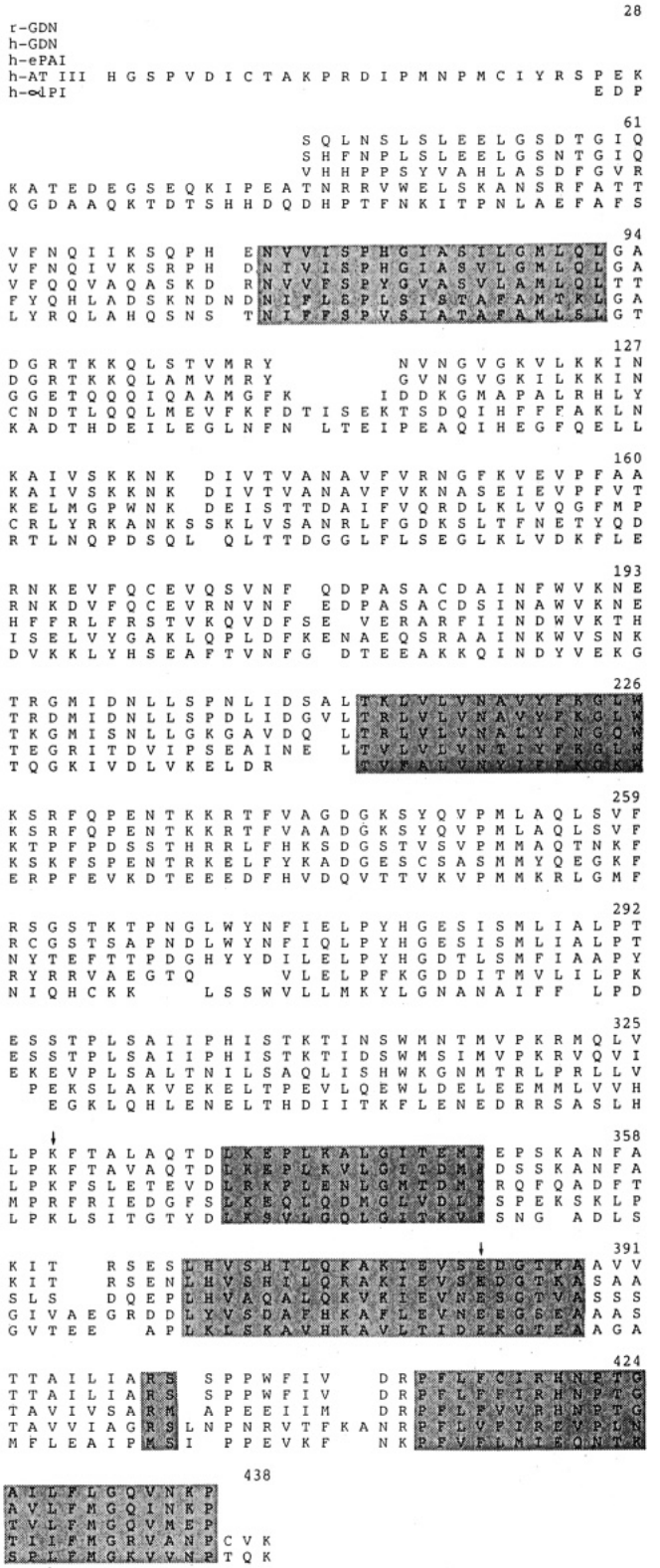

FIGURE 3: Alignment of the amino acid sequence of rat GDN with human GDN, endothelial-cell-type plasminogen activator inhibitor, antithrombin III, and α-1 proteinase inhibitor. The numbering refers to the overall length of the five aligned proteins, starting with His of antithrombin III and ending with Lys of antithrombin III and α-1 proteinase inhibitor. The five boxes at positions 75–92, 212–226, 337–350, 368–388, and 413–435 represent regions of high homology between all proteins. The reactive centers (positions 399, 400) are also shaded. The conserved Glu residue 17 amino acids N-terminal of the reactive center (position 383) and the site to which it forms a salt bridge (position 328) are marked by arrows.

The homology between these protease inhibitors is not completely randomly distributed along the entire sequence. Two features are obvious: (1) the homology increases toward the C-terminus and (2) short regions are highly conserved. In Figure 3 we have labeled five such regions (positions 75–92, 212–226, 337–350, 368–388, and 413–435) in which the overall homology rises to 64% (positions 212–226). Because the sequences in these regions match without introduction of any gaps, we believe them to represent sites with related structural or biochemical properties.

Besides these homologous regions GDN has a higher sequence similarity to h-ePAI than to the other serpins analyzed (41% vs. 32% and 25%). This implies a close relationship between these two proteins. It is, however, important to stress that even such conserved inhibitors have different target proteases, mainly due to the different sequences at their reactive centers [see also Carell and Travis (1985)]. h-ePAI, the reactive center of which is Arg-Met, is reported to be an inhibitor specific for plasminogen activator (Ny et al., 1986; Pannekoek et al., 1986). On the other hand, we consider GDN to be an inhibitor with high specificity for thrombin because its association rate is the fastest with thrombin compared to the rates of other proteases (Stone et al., 1987). Furthermore, its reactive center is identical with the one of antithrombin III (Arg-Ser).

Another argument verifying Arg-Ser to be the reactive center of GDN is the following. One of the mutants of α-1 proteinase inhibitor (the Z variant) results from a single amino acid exchange at position 342 (Glu to Lys). This Glu residue is reported to be involved in the formation of the salt bridge Glu^{342} to Lys^{290}, which stabilizes the three-dimensional structure of α-1 proteinase inhibitor (Loebermann et al., 1984). We have analyzed whether the sequences mentioned in this report are conserved in the Glu^{342} position, which is affected by the Z-variant deficiency. This Glu, which we refer to as P_{17} with respect to the P_1 position of the reactive center, is present in all protease inhibitors shown in Figure 3 and, furthermore, in other members of the serpin superfamily, the sequences of which are not shown in this paper, namely, human protein C inhibitor (Suzuki et al., 1987), α-2 antiplasmin (Holmes et al., 1987), α-1 antichymotrypsin, and chicken ovalbumin (Chandra et al., 1983). The position of the basic Lys^{290} residue, which we refer to as P_{69}, is also conserved in all these serpins with the exception of chicken ovalbumin (Arg at P_{69}), antithrombin III (Arg at P_{71}), and α-2 antiplasmin (Lys at P_{67}). The fact that the distance between the reactive center (P_1) and the P_{17} and the P_{69} residues is constant in seven (including GDN) out of nine serpins suggests that Arg-Ser is the reactive center of GDN and indicates a close structural relationship within this superfamily.

The information we have obtained from the sequence of rat GDN and from the comparison with other serine protease inhibitors will now allow us to further investigate structure–function relationship of this inhibitor.

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