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Identification and tissue expression of a splice variant for the growth arrest-specific gene *gas6*

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Abstract The growth arrest-specific gene *gas6* encodes a secreted protein (Gas6) which is a member of the vitamin K-dependent protein family and was identified as a ligand for the Axl tyrosine kinase receptor family. Gas6 shares significant similarity with protein S and a similar domain organisation: an extensively γ-carboxylated amino-terminal, four epidermal growth factor-like motifs and a large carboxy-terminal region, known as the D domain. Here we report on the isolation of a splice variant (*gas6SV*) characterised by an in-frame 129 bp insertion between the fourth EGF domain and the D domain. The gene *gas6* was previously mapped on chromosome 13. The genomic organisation of *gas6* has been investigated demonstrating the presence of alternative splicing consensus sites. Expression of *gas6SV* has been investigated in various human tissues and found to have a similar distribution pattern as *gas6*, with the exception of the spleen where *gas6SV* seems to be the predominant form.

Key words: Growth arrest; Growth factor

1. Introduction

The *gas6* product (Gas6) has been identified as a new member of the vitamin K-dependent family of proteins [1], with a significant degree of amino acid identity to human protein S, a negative co-regulator in the coagulation pathway [2]. Both human and murine *gas6* have been characterised and revealed a high degree of amino acid conservation (81% amino acid identity) [1]. A rat *gas6* homologue was identified as a growth-potentiating factor for smooth muscle cells [3]. Similarly to protein S, Gas6 shows several defined structural motifs: a γ-carboxylated amino-terminus (Gla domain), four epidermal growth factor (EGF)-like repeats and a large carboxy-terminal region with similarity to steroid-binding globulin. Gas6 lacks the consensus domain recognised by thrombin which is involved in the regulation of the biological activity in protein S [1,2].

Recently, the human *gas6* gene has been assigned to chromosome 13, region q34 [4], where also the vitamin K-dependent proteins factor VII and X have been previously mapped [5], thus defining a distinct localisation with respect to *protein S* gene that has been mapped in the centromeric region (p11.1–q11.2) of chromosome 3. *gas6* is widely expressed in many tissues and quite abundantly in lung, intestine and endothelium, while it is almost undetectable in the liver, where protein S can be detected at high levels [1,6]. Human Gas6 was shown to act as the ligand for a family of tyrosine kinase receptors with transforming activities: Axl [7], Rse [8] and Mer [9]. We have previously reported that Gas6 is able to induce cell cycle re-entry and protect serum-starved NIH3T3 cells from cell death by apoptosis and that such activities require the activation of a PI3K-dependent pathway [10,11]. Here we describe the identification and expression pattern of a *gas6* splice variant (*gas6SV*) potentially encoding a protein containing an additional 43 amino acid peptide linking the fourth EGF domain and the C-terminal D domain.

2. Materials and methods

2.1. Isolation of cDNA clones for human gas6

A HeLa cDNA library generated in λ vector 1149 was screened as reported [1]. Of the two cDNA clones isolated, one represented the reported cDNA [1], while the other represents the splice variant described here.

2.2. Isolation of human gas6 genomic clones

Filters of a cosmid human genomic library specific for chromosome 13 [12] were kindly provided from Reference Library Data Base (RLDB), Imperial Cancer Research Foundation (ICRF).

gas6 cDNA restriction fragments were labelled with [α^{32}P]dCTP using random priming at a specific activity of 1×10^8 cpm/μg and hybridisation was carried out at 42°C for 18 h, with 1×10^6 cpm/ml in 50% formamide, 4×SSC (standard sodium citrate), 10% dextran sulphate, 1% SDS (sodium dodecyl sulphate), 10×Denhardt's solution, 50 mg/ml denatured salmon sperm DNA, 1 mM EDTA (pH 8) and 50 mM sodium phosphate (pH 7). Washes were performed at high stringency, once for 15 min in 2×SSC, 1% SDS at 55°C and twice for 30 min in 0.2×SSC, 0.1% SDS at 60°C.

2.3. PCR amplification

The reaction was carried out in an Air Thermal Cycler (Idaho) using two primers mapping respectively at nt 924–941 and nt 1139–1156, as shown in Fig. 1. After 3 min of denaturation (94°C), 30 cycles were performed under these conditions and order: 10 s at 94°C, 10 s at 60°C, 20 s at 72°C. In the last cycle, elongation time was prolonged to 5 min. PCR product was subcloned in pBluescript (Stratagene) and sequenced.

2.4. DNA sequence and analysis

The nucleotide sequence was determined by the dideoxy-chain termination method [13], with the T7 sequencing kit (Pharmacia). Specific synthetic oligonucleotides were used as primers for the sequencing reactions.

Sequences of cDNA clones was obtained using the EMBL-ALF sequencer, whereas genomic clones and radioactive sequencing reactions were run on ultra-thin polyacrylamide gels [14]. Sequence analysis was performed using the IntelliGenetics software package.

2.5. RT-PCR expression analysis

Reverse transcription was performed on 6 μg of total RNA extracted from different tissues [15], using oligodT [16–18] as a primer

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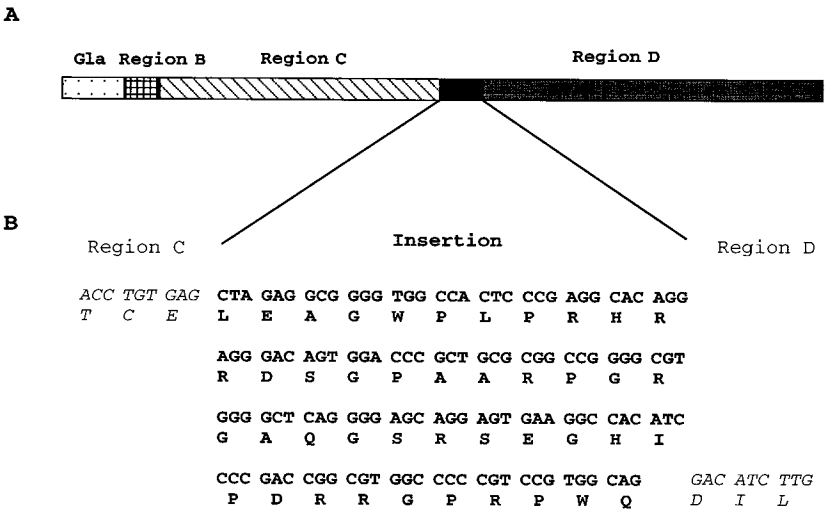

Fig. 1. Sequence analysis of the *Gas6SV* insertion. A: Gas6SV cDNA organisation after Manfioletti et al. [1]. The dotted white line indicates the Gas6SV γ-carboxylated domain (Gla) which is followed by the short linker region B (square-hatched) and the four EGF repeats containing domain C (hatched). The black line indicates the 129 bp sequence of the Gas6SV insertion, while the grey line represent the large C-terminal D domain. B: Sequence of the 129 bp in-frame insertion is shown in bold. Both nucleotide (upper lines) and amino acid (lower lines) sequences are shown. The last codons of region C and first codons of region D common to both *gas6* and *gas6SV* are shown.

and M-MLV Reverse Transcriptase (BRL). One tenth of each reaction was then used for amplifications with two different primer pairs, the first one specific for the insertion (nt 1–18 and nt 112–129) and the second one mapping in the D region (nt 1100–1116 and nt 1255–1272), as shown in Fig. 2. As a negative control for PCR, the same reaction mixture with no M-MLV Reverse Transcriptase added was used. After 2 min of denaturation (94°C), 35 cycles were performed in the following conditions: 8 s at 94°C, 10 s at 60°C for the first primers pair and 57°C for the second one, 10 s at 72°C. In the last cycle, the elongation time was prolonged to 5 min. Samples were run on a 2.5% agarose gel, blotted [15] and filters were hybridised with the same probe, constituted by a fragment of the splice variant cDNA spanning from the *Sac*I site in position 698 to the whole 3′ UTTER. This fragment was labelled as described above and hybridised for 18 h at 65°C in 1 M NaCl, 1% SDS, 100 mg/ml denatured salmon sperm DNA and 5×Denhardt's solution. Filters were washed at 65°C, twice in 2×SSC, 0.1% SDS for 15 min and once in 0.2×SSC, 0.1% SDS for 30 min.

3. Results and discussion

The original screening that allowed isolation of *gas6* [1] also revealed the existence of an additional cDNA encoding a splicing variant for human *gas6* (*gas6SV*). Sequence analysis indicated that this cDNA is 2627 bp long and encodes a 716 amino acid protein. This splicing variant form shows a cDNA organisation similar to that of the previously characterised *gas6* [1] except for an in-frame 129 bp insertion between the regions encoding the fourth EGF-like and the D domain (Fig. 1). This insertion encodes a 43 amino acid sequence that does not show significant similarity to any known sequence stored in the SwissProt data bank. In order to assess the presence of the respective splice junction consensus sequences the genomic organisation of the region surrounding this insertion was analysed. A human cosmid library specific for chromosome 13, where the human *gas6* gene had previously been mapped [4], was screened with two different probes, corresponding respectively to the 5′ (nt 1–1000) and the 3′ (nt 1001–2461) portions of the *gas6* cDNA sequence, as reported in Gene Bank 84. A cosmid clone (ICRFc108P1847) positive both for the 5′ and the 3′ *gas6* cDNA probes was selected for further characterisation. In order to isolate only the region of interest, PCR was performed using two primers based on the fourth EGF-like repeat and the D region of the *gas6* sequence respectively, as reported in Fig. 2. Amplified products were run on agarose gel and transferred onto nylon membrane. After hybridisation with an insertion-specific probe, a single band of about 2000 bp was recognised. The same pattern was also obtained with two other independent clones isolated from the same screening (ICRFc108L0128 and ICRFc108G072). The amplified genomic fragment was subcloned and sequenced. The intron/exon organisation of this region indicates that the 129 bp insertion present in the *gas6SV* cDNA is included in the exon-containing 5′ sequence of region D (Fig. 2). This exon is separated from the upstream exon, encoding the fourth

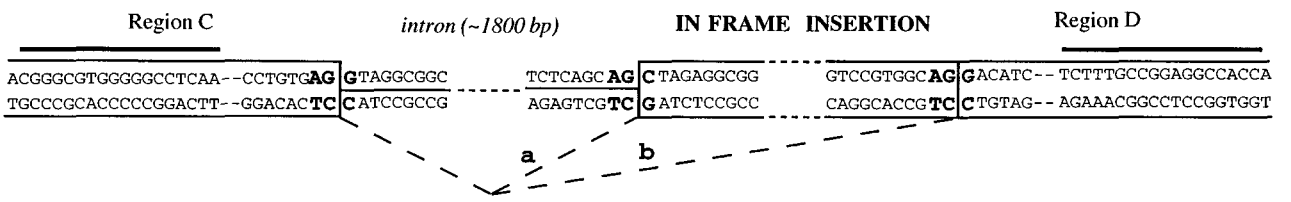

Fig. 2. Genomic organisation of the *gas6* gene region surrounding the insertion. Two different transcripts originate from an alternative splicing that either comprises or excludes the 129 bp insertion (as shown by dashed lines): a: excision of the 1800 bp intron generates the *gas6SV* form; b: when the insertion is also spliced as part of the intron, the previously characterised *gas6* shorter form is obtained [1]. Splice donor and acceptor site sequences are indicated in bold. Position of the oligonucleotides used for the region amplification is marked by bold lines.

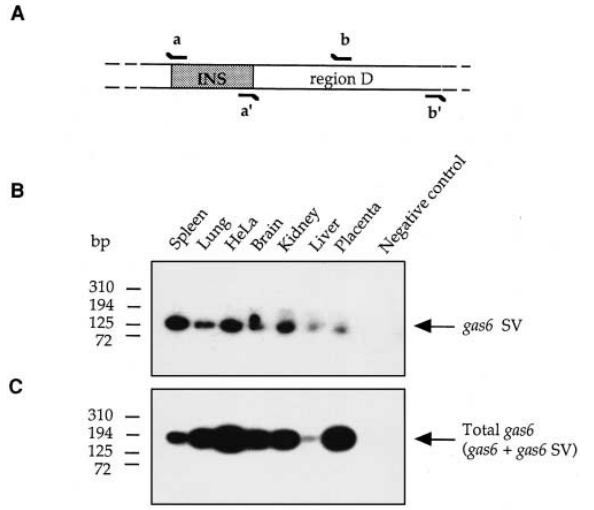

Fig. 3. Expression analysis of *gas6SV*. A: Position of the two primer pairs used for reverse transcription PCR (RT-PCR). The first pair (a and a′) maps within the insertion, allowing specific amplification of *gas6SV* mRNA. The second pair (b and b′) maps in a region present both in *gas6* and *Gas6SV* transcripts, and has been used to asses the total *gas6* mRNA content in each tissue. B: Southern blotting of RT-PCR performed on equal amounts of total RNA from the indicated human tissues, using primers a and a′. A single band of 129 bp was obtained. C: The same RT reaction mixtures were used for PCR with primers b and b′ and analysed by Southern blot as above. A single band of 172 bp representative of both *gas6* and *gas6SV* was obtained. In both filters a negative control (PCR performed without adding template cDNA) was included.

EGF-like domain of region C, by an intron of about 1800 bp. The 5′ splice junction matches the canonical consensus sequence [17], while two different putative splice sites are present at the 3′. The first one is located in the 5′ region of the insertion and has a weaker consensus than the following one, as shown in Fig. 2. This genomic organisation therefore allows two alternative transcription products, in which the 129 bp insertion can be either comprised (Gas6SV) or excluded (Gas6). The tissue distribution of Gas6 has already been reported. This transcript is expressed in lung, intestine and bone marrow [1]. In order to determine the expression of the *gas6SV* mRNA with respect to the total *gas6* mRNA, several human tissues were analysed by RT-PCR using two different sets of primers. The first one is insertion-specific (nt 1–18 and nt 112–129, relative to insertion sequence), while the second one, mapping in the D domain (nt 1099–1115 and nt 1254–1271) is common to both transcripts (Fig. 3). Consistent with the previous Northern blot analysis [1], *gas6* mRNA was found to be expressed at rather high levels in most of the tissues investigated with the exception of the liver (see Fig. 3B). A similar pattern of expression, albeit at significantly lower levels, was shown for the *gas6SV* (Fig. 3C). In spleen, however, the signal detected after amplification of the sole splicing variant form was comparable to that obtained from the total *gas6*, indicating that the *gas6SV* form predominates in this tissue. Placenta (and colon, not shown) showed a low level of *gas6SV* expression; conversely, significant levels of *gas6* could be detected in these tissues.

We have previously reported the isolation and characterisation of the growth arrest-specific gene *gas6* [1]. The protein encoded by *gas6*, Gas6, was found to be a growth factor with mitogenic and antiapoptotic activities [10,11]. Here we report the isolation and the expression pattern of a *gas6* splice variant containing an additional 129 bp in the protein open reading frame. Interestingly, *gas6* and its receptors are widely expressed while the splice variant seems to have a more restricted pattern of expression. The Gla domain of Gas6, similarly to other Gla domains, should be involved in membrane interaction [2], while the C-terminal D domain was found to activates both Rse [18] and Axl receptor phosphorylation (see accompanying manuscript). We can therefore hypothesise that the insertion of an additional hinge region could be involved in the regulation of growth factor presentation. Finally, since the inserted sequence present in Gas6SV is the target of a protease that cleaves the active D domain from Gas6SV (see accompanying manuscript), the existence of splice variants could represent a role for modulation of both growth factor activity and availability.

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