Analysis of the domain requirement in Gas1 growth suppressing activity

Maria Elisabetta Ruaro[a,1], Marco Stebel[a,1], Paolo Vatta[a], Stefania Marzinotto[a], Claudio Schneider[b,*]

[a] *Laboratorio Nazionale Consorzio Interuniversitario per le Biotecnologie (L.N.C.I.B.), Area Science Park, Padriciano 99, 34012 Trieste, Italy*
[b] *Dipartimento di Scienze e Tecnologie Biomediche, Universita' degli Studi di Udine, Trieste, Italy*

Received 17 July 2000; revised 21 August 2000; accepted 23 August 2000

Edited by Veli-Pekka Lehto

Abstract The product of the growth arrest specific gene, *gas1*, is a membrane-associated protein which activates a p53-dependent growth suppression signalling pathway. We have shown that Gas1 is linked to the plasma membrane through a glycosyl-phosphatidylinositol (GPI) anchor. Several GPI-anchored protein have been identified as part of receptor complexes either as co-receptors or as membrane bound ligands. In this report, we characterize the Gas1 domains required for its growth suppression function and demonstrate the dispensability of Gas1 GPI anchor.

Key words: Gas1; p53 protein; GPI anchor; Domain requirement; Signal transduction

1. Introduction

Gas1 is a membrane-associated protein which is highly expressed at growth arrest and down-regulated under proliferative conditions. We have previously shown that this protein is able to interfere with cell proliferation when ectopically expressed in asynchronously growing cells or during the Go to S transition [1,2]. We have also shown that the inhibitory effect of Gas1 depends on the presence of p53 [3]. In particular the proline-rich domain of p53 [4] and not its transactivation function is required for the Gas1-induced growth suppression [3,4]. Since Gas1 is localized on the plasma membrane, it might be involved in the transduction of an inhibitory signal, at growth arrest, leading to p53.

We have demonstrated that Gas1 is tethered to the plasma membrane through a glycosyl-phosphatidylinositol (GPI) anchor [5]. A wide variety of proteins are attached to the cell surface by a GPI anchor. Such proteins include hydrolytic enzymes, cell adhesion molecules, receptors and cell surface antigens [6]. Several GPI-anchored protein have been identified as part of a receptor complex either as co-receptors [7] or as membrane bound ligands [8].

As it is the case for other GPI-anchored proteins [7], in this work we have demonstrated that the GPI anchor is dispensable for the growth inhibitory activity of Gas1. Moreover, by the use of chimeric constructs between Gas1 and the GPI-anchored human placental alkaline phosphatase (hPLAP), we have defined the minimal region endowed with the Gas1 growth arrest activity.

*Corresponding author. Fax: (39)-40-398990.
E-mail: schneide@sci.area.trieste.it

[1] These authors equally contributed to this work.

2. Materials and methods

2.1. Plasmids

Most of the constructs used in this work were subcloned in the pGDSV7S vector [9].

pGDSV7S-gas1Met42 is described in Stebel et al. [5].

pGDSV7S-hSEAP (human SEcreted Alkaline Phosphatase) encodes a secreted form of PLacental Alkaline Phosphatase (hPLAP), ending at the amino acid (aa) 489 and therefore lacking the GPI consensus sequence.

pGDSV7S-Gas1/hSEAP encodes a chimera containing Gas1 from aa 42 to 366 and hSEAP from aa 1 to 489.

pGDSV7S-Gas1-6His encodes a soluble form of Gas1, having substituted the GPI consensus sequence from aa 367 onwards with a six histidine tag.

pGas1-Fc encodes a Gas1 protein from aa 42 to 367 in fusion with the human Fc domain. To construct the chimera, a PCR fragment of Gas1 was amplified and subcloned in the *Hin*dIII–*Bam*HI sites of the pIg vector (R&D Systems).

pMuc-Fc is a fusion construct provided by R&D Systems containing the MUC18 glycoprotein [10] in fusion with the human Fc domain.

pGDSV7S-hPLAP encodes the full-length hPLAP, derived from subcloning of the *Eco*RI fragment of pSVT7hPLAP [11].

pGDSV7S-Gas1-Sma/hPLAP contains *gas1* up to the *Sma*I site and the hPLAP; it encodes a chimera containing Gas1 from aa 42 to 193 followed by hPLAP from aa 55 to the end.

pGDSV7S-Gas1-Hind/hPLAP contains *gas1* up to the *Hin*dIII site and encodes a chimera containing Gas1 from aa 42 to 229 followed by hPLAP from aa 26 to the end.

pGDSV7S-hPLAP/Hind-Gas1 contains the sequence of hPLAP deleted of the GPI addition signal and *gas1* from the *Hin*dIII site to the end; it encodes a chimera containing hPLAP from aa 22 to 383 followed by Gas1 from aa 230 to the end.

pGDSV7S-hPLAP/Gas1_Sma-Hind contains a fragment of Gas1 comprised between the *Sma*I and *Hin*dIII sites (corresponding to aa 182–234) inserted at the *Pst*I site of hPLAP.

2.2. Cell culture and transfections

NIH3T3 and COS7 cells were grown as described [2]. Transfection of COS7 cells was performed with 2 μg of DNA/35 mm diameter plate or 15 μg DNA/10 cm diameter plate, using the DEAE-dextran procedure as described [12].

2.3. [^{35}S]Met cell labelling procedure and immunoprecipitation

2 days after transfection, COS7 cells were starved for methionine for 1 h prior to addition of 150 μCi/ml of [^{35}S]methionine (Amersham). Cells were labelled for 5 h, lysed and processed as described [5]. The different protein products were immunoprecipitated by using affinity-purified anti-Gas1 antibodies. For immunoprecipitation of hPLAP and hSEAP, commercial anti-hPLAP antibodies (Dako) were used. Gas1-Fc and Muc-Fc were pulled-down from cell supernatants using protein A-Sepharose (Pharmacia).

2.4. Gas1-Fc production and inhibition assay

Gas1-Fc and Muc-Fc plasmids were transfected, as described, into COS7 cells. 24 h after transfection, the medium was changed to 0.5% fetal calf serum to reduce cell growth while allowing accumulation of the secreted protein. Cells were incubated for a further 48 h; medium

0014-5793/00/$20.00
PII: S0014-5793(00)02005-6

Fig. 1. Growth inhibitory analysis of soluble Gas1 constructs. A: Scheme of the chimeras. The numbers in the upper part of each construct refer to the aa sequence of Gas1 as predicted from the open reading frame (ORF); the numbers of the aa of the human Fc region are not reported. B: Immunoprecipitation of the soluble products of the constructs Gas1-6His and Gas1-SEAP from the culture medium of transfected, [^{35}S]Met-labelled COS7 cells. C: Relative inhibition of BrdU incorporation by expression of the Gas1 GPI minus constructs (Gas1-hSEAP and Gas1-6His) compared to the Gas1 GPI-linked (Gas1-Met42). Relative inhibition of DNA synthesis was calculated as follows: % relative inhibition = [% BrdU positive cells (uninjected) − % BrdU positive cells (Gas1/6His positive)/% BrdU positive cells (uninjected)] × 100. The mean of three independent experiments with at least 300 over-expressing scored cells is shown. D: The conditioned medium from the Gas1-Fc- and Muc-Fc-transfected and [^{35}S]Met-labelled COS7 cells was subjected to pull-down with protein A-Sepharose, separated on SDS–PAGE and the proteins were visualized by autoradiography. E: The conditioned medium from Gas1-Fc and Muc-Fc-transfected COS7 pulled-down with protein A-Sepharose, separated on SDS–PAGE and immunodecorated with anti-human Fc antibody. F: Relative inhibition of BrdU incorporation by conditioned medium of Gas1-Fc- or Muc-Fc- (negative control) transfected COS cells. Inhibition was calculated as relative to the BrdU incorporation percentage in untreated cells.

→

was collected and centrifuged at 2500 × *g* for 10 min to eliminate the cellular debris. To quantify the secreted recombinant protein, 1 μl of different dilutions of each conditioned medium were spotted on nitrocellulose together with a standard concentration of human IgG. After air drying, the membrane was blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and incubated with protein A-peroxidase (Sigma) (1:1000 in PBS–3% BSA) for 1 h at room temperature. Enzyme activity was detected using the enhanced chemiluminescence method (Amersham, UK). For the inhibition assays, NIH3T3 cells were plated on a coverslip in 35 mm Petri dishes as described [2]. After 24 h, 500 μl of Gas1-Fc transfected COS7 cells medium, containing 3 ng/μl of recombinant protein, was directly added to 1.5 ml of culture medium of growing NIH3T3 cells (750 ng/ml Gas1-Fc final concentration). As a control, medium from Muc-Fc-transfected COS cells, containing the same concentration of recombinant protein, and medium from mock-transfected cells were used. 18 h later, 50 μM bromo-deoxy-uridine (BrdU) was added and incubation was continued for a further 6 h. Cells were then fixed and processed for immunofluorescence. Incorporation of BrdU was revealed by anti-BrdU monoclonal antibody (IgG2a) (Amersham) followed by TRITC-conjugated goat anti-mouse IgG2a antibody (Southern Biotechnology). For visualizing the nuclei, the cells were incubated with 2 μg/ml Hoechst 33342 (Fluka) in PBS. Relative amounts of BrdU positive cells (indicative of S phase) in the sample treated with Gas1-Fc or with Muc-Fc were compared with that of untreated cells.

2.5. Microinjection and biological assays

All microinjection experiments were performed using an automated injection system, AIS (Zeiss, Oberkochen, Germany), as described [2]. 50 ng/μl of cDNA was microinjected into the nuclei of asynchronously growing NIH3T3 cells. After injection, cells were incubated for 18 h, then 50 μM BrdU was added and incubation was continued for a further 6 h. Cells were then fixed and processed for immunofluorescence as described [2,3]. Gas1 was revealed by affinity-purified QE 229 anti-Gas1 antibody [5] followed by FITC-conjugated goat anti-rabbit IgG (Sigma). hPLAP was revealed by polyclonal antibodies (Dako) followed by FITC-conjugated goat anti-rabbit IgG (Sigma). Incorporation of BrdU was revealed as described above.

3. Results

3.1. The GPI anchor is dispensable for the growth arrest activity of Gas1

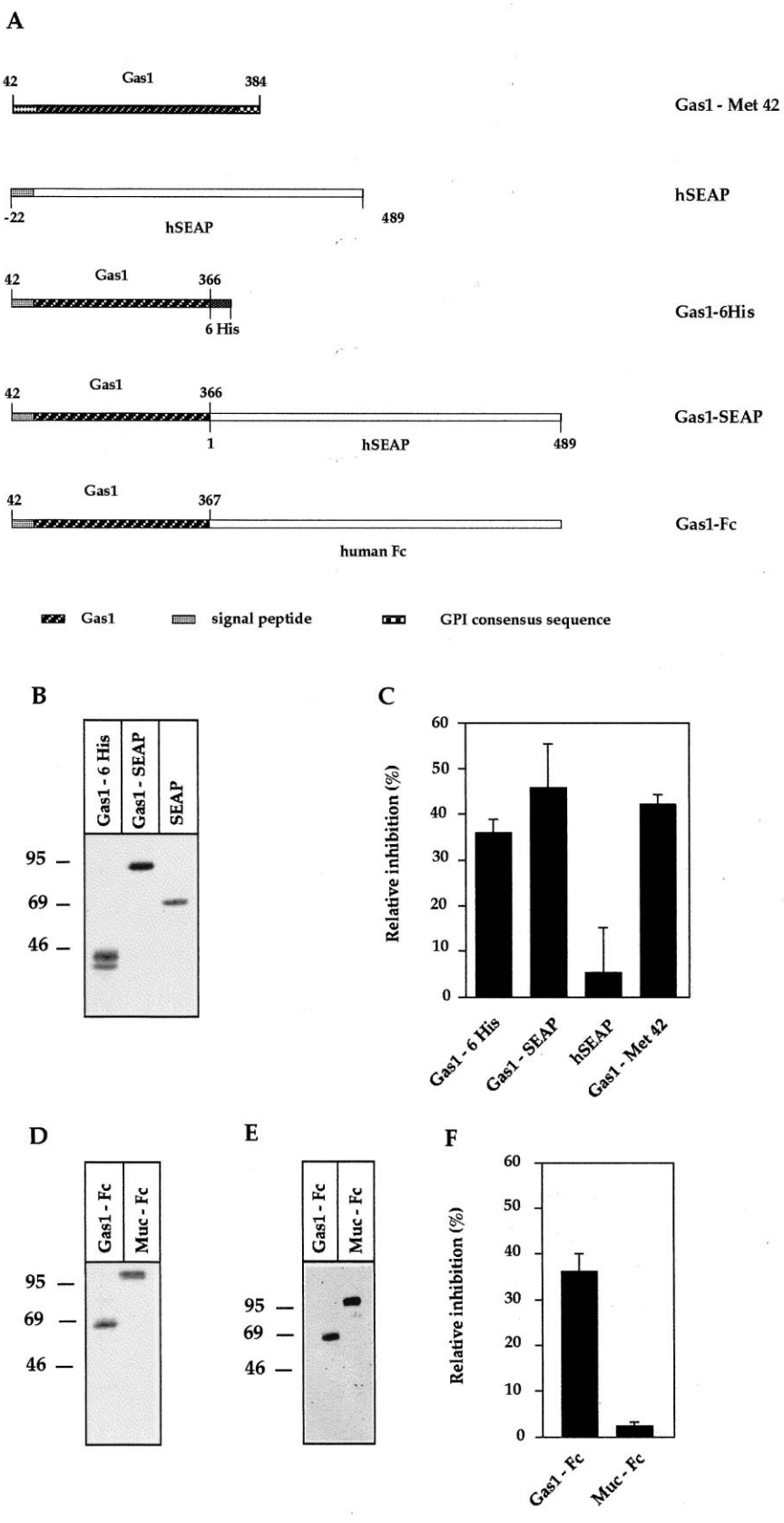
In the accompanying paper [5], we have demonstrated that Gas1 is a GPI-anchored protein. Among the several proteins with diverse physiological functions that are tethered to the surface of the cell by a GPI anchor, some are essential components of receptor complexes [13,14], and in some cases the presence of the soluble form of the co-receptor still allows the transmembrane protein component to transduce the signal. In order to elucidate whether Gas1 belongs to this category of proteins, we wanted to assess whether the GPI anchor was required for the Gas1 inhibitory activity.

Two constructs were generated in which the GPI consensus sequence of Gas1 was replaced by either a six His tag (Gas1-6His) or by the sequence of human secreted alkaline phosphatase (Gas1-SEAP) (Fig. 1A). Immunoprecipitation with anti-Gas1 antibody of the culture medium of COS7 cells transfected with either Gas1-6His or Gas1-SEAP expression vectors confirmed that both proteins were efficiently secreted (Fig. 1B). Next, the same constructs were analyzed for their ability to induce growth arrest in NIH3T3 fibroblasts. Ectopic expression of these constructs was performed by microinjection and its effects on cell cycle were evaluated by measuring the relative level of BrdU incorporation. As shown in Fig. 1C, Gas1-6His and Gas1-SEAP exhibited a growth inhibitory activity comparable to the full-length, GPI-anchored Gas1, while hSEAP (negative control) had no effect. To further confirm this result, another soluble construct was prepared in which the Gas1 sequence was fused with the Fc region of the human IgG (Gas1-Fc). The corresponding protein produced in COS7 cells was efficiently secreted as shown by protein A-Sepharose pull-down of culture medium from radiolabelled cells (Fig. 1D) or Western blot with anti-human IgG antibodies (Fig. 1E). The growth arrest ability of this soluble construct was tested by adding the medium of COS7 transfected cells to the culture medium of asynchronously growing NIH3T3 fibroblasts. As shown in Fig. 1F, the Gas1-Fc fusion protein exogenously added to cells inhibited S phase entry as effectively as the other soluble forms of Gas1 expressed by microinjection (compare Fig. 1C with Fig. 1F). Muc-Fc (negative control) had no effect. These results demonstrate that anchoring to the plasma membrane is not required for Gas1 to exert its growth inhibitory activity and that the GPI moiety does not seem to participate in the signalling cascade.

3.2. Mapping the functional region of Gas1

In order to determine the domains of the Gas1 protein responsible for the growth inhibitory activity, a series of chimeras were constructed containing various fragments of *gas1* fused to the hPLAP cDNA using either the GPI anchor consensus sequence from hPLAP or from Gas1 (Fig. 2A). The protein products of the chimeras were recognized both by the anti-hPLAP antibody and by the anti-Gas1 antibody specific for the cloned regions, as assessed by immunoprecipitation (Fig. 2B) and by immunofluorescence microscopy (not shown).

To test the ability of the Gas1/hPLAP chimeras to induce growth arrest in NIH3T3 fibroblasts, ectopic expression was performed by microinjecting the individual expression plasmids and the effect on cell cycle was evaluated by measuring the level of BrdU incorporation. As shown in Fig. 2C, the Gas1-Hind/hPLAP hybrid consisting of the N-terminal half of Gas1 up to aa 229 showed a significant inhibitory effect which



was comparable to the 'wild type' Gas1. In contrast, the Gas1 C-terminal moiety from aa 230 to aa 384 (hPLAP/HindGas1) had no significant effect, similar to the negative control hPLAP.

In order to further delineate the inhibitory region, two other chimeras were constructed: Gas1-Sma/hPLAP which contains the sequence encoding Gas1 up to aa 193, and which contains the sequence encoding Gas1 from aa 182 to 234,

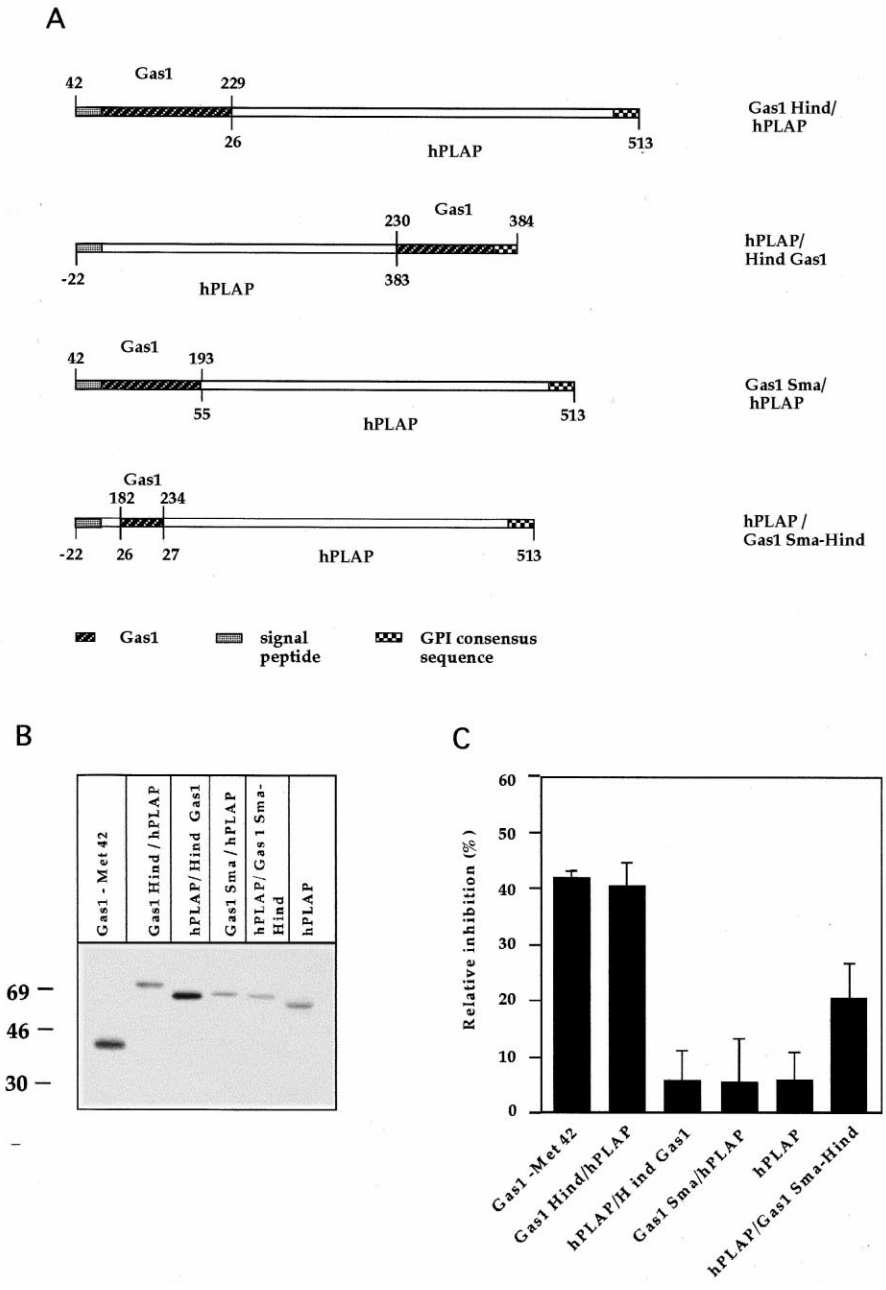


Fig. 2. Growth inhibitory analysis of Gas1-hPLAP constructs in NIH3T3 fibroblasts. A: Scheme of the chimeras. The numbers in the upper part of each construct refer to the aa sequence of Gas1 as predicted from the ORF; those in the lower part to the aa sequence of hPLAP. As for hPLAP, the numeration starts from −22 corresponding to the first translated aa which however belongs to the signal peptide and is cleaved off the mature protein. In each construct, the signal peptide traces to the protein at the amino-terminus, while the GPI consensus sequence traces to the protein at the carboxy-terminus. B: Immunoprecipitation of the different anchored constructs expressed in transfected, [^{35}S]Met-labelled COS cells. C: Relative inhibition of BrdU incorporation after expression of Gas1 or the different Gas1-hPLAP mutants. Inhibition was calculated as in Fig. 1C. The mean of three independent experiments with at least 300 over-expressing scored cells is shown.

both inserted into the hPLAP backbone (Fig. 2A). When similarly over-expressed in growing NIH3T3 cells, these chimeras did not show an inhibitory activity comparable to the full-length Gas1, although hPLAP/Gas1Sma-Hind retained significant activity. These results indicate that the portion of Gas1 from aa 182 to aa 234 is required, although not sufficient, to confer inhibitory function. This suggested that either the surrounding aa in the Gas1 sequence were important to provide the conformation required for Gas1 function or that another domain in the amino-terminal region although ineffective alone, was required to confer a full inhibitory activity to Gas1. To verify the latter possibility, the two constructs Gas1-Sma/hPLAP and the hPLAP/Gas1Sma-Hind were co-microinjected. The percentage of inhibition obtained did not differ from that of the single construct, thus indicating that the two chimeras are not able to cooperate (not shown). We can therefore conclude that the most critical domain resides between aa 182 and aa 234 but that this domain is influenced by the surrounding aa sequence to exert growth suppressing activity efficiently.

4. Discussion

Gas1 exerts growth suppression activity in various cell types. We have demonstrated, in the accompanying paper [5], that the Gas1 protein is associated to the plasma membrane via a GPI anchor. Several GPI proteins have been identified as components of receptor complexes. In the case of GDNF signalling, a family of soluble factors – glial cell-derived neurotrophic factor (GDNF) [15], neurturin (NTN) [16,17], persephin [18] and artemin [19] – has been characterized for supporting survival in different populations of neurons. GDNF and NTN interact on the plasma membrane with their specific GPI-linked co-receptor, respectively called GFRα-1 and GFRα-2. Once formed, the complex ligand–co-receptor binds to the transmembrane protein kinase Ret which is responsible for transducing the signal. Therefore the signalling protein, Ret, is shared while the GPI-linked co-receptor determines ligand specificity [15–18,20]. Another example is that of lipopolysacharide (LPS)-induced cellular signalling in myeloid cells. The response to LPS requires a plasma protein (LPS-binding protein, LBP) that forms a complex with LPS, which then is able to interact with the GPI-linked CD14 receptor. Most importantly, the soluble form of CD14 has been shown to be sufficient to mediate LPS-induced signalling [21], thus prompting the search for the supposed transmembrane signalling receptor. This has led to the discovery of the Toll-like receptor 2 with which the complex LPS–LBP–CD14 interacts [14]. We therefore wished to verify whether this could be true also in the case of Gas1. By the use of different soluble constructs, we have demonstrated the dispensability of the GPI anchor. The use of Gas1-Fc exogenously added to the cells was particularly instructive since it clearly demonstrated that Gas1 can induce growth arrest from outside, excluding the possibility that the soluble constructs expressed by microinjection could function along the secretion pathway.

Moreover, by the use of chimeric constructs, we were able to restrict the 'minimal' functional domain of Gas1 to the region comprised between the *Sma–Hin*d sites (aa 182–234). This domain still retains some inhibitory activity and antibodies raised against this epitope are able to block Gas1 function (data not shown).

Altogether, the results presented support the hypothesis of the existence of a complex, containing Gas1, involved in the transduction of a growth suppression signal during Go. Further investigation is required to try and identify the other component(s) of this complex.

Acknowledgements: We are grateful to Dr. J.L. Millan (La Jolla Cancer Center, CA, USA) for kindly providing the pSVT7hPLAP plasmid encoding hPLAP. We thank Dr. J.G. Flanagan (Harvard Medical School, Boston, MA) for the APtag-1 plasmid encoding the secreted form of hPLAP. This work was supported by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC) to C.S.

References

[1] Del Sal, G., Ruaro, M.E., Philipson, L. and Schneider, C. (1992) Cell 70, 595–607.
[2] Del Sal, G., Collavin, L., Ruaro, M.E., Edomi, P., Saccone, S., Della Valle, G. and Schneider, C. (1994) Proc. Natl. Acad. Sci. USA 91, 1848–1852.
[3] Del Sal, G., Ruaro, M.E., Utrera, R., Cole, C.N., Levine, A.J. and Schneider, C. (1995) Mol. Cell. Biol. 15, 7152–7160.
[4] Ruaro, E.M., Collavin, L., DelSal, G., Haffner, R., Oren, M., Levine, A.J. and Schneider, C. (1997) Proc. Natl. Acad. Sci. USA 94, 4675–4680.
[5] Stebel, M., Vatta, P., Ruaro, E.M., Del Sal, G., Parton, R. and Schneider, C. (2000) FEBS Lett. 481, 152–158.
[6] Wang, J., Mariarz, K. and Ratnam, M. (1999) J. Mol. Biol. 286, 1303–1310.
[7] Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.-C., Hu, S., Altrock, B.W. and Fox, G.M. (1996) Cell 85, 1113–1124.
[8] Davis, S., Gale, N.W., Aldrich, T.H., Maisonpierre, P.C., Lhotak, V., Pawson, T., Goldfarb, M. and Yancopoulos, G.D. (1994) Science 266, 816–819.
[9] Del Sal, G., Manfioletti, G., Gustincich, S., Ruaro, M.E. and Schneider, C. (1994) BioTechniques 16, 134–138.
[10] Gershenwald, J.D., Huang, J.E., Luca, S., Hudson, M., Tainsky, M.J. and Bar-Eli, M.A. (1998) J. Biol. Chem. 273, 16501–16508.
[11] Millan, J.L. (1986) J. Biol. Chem. 261, 3112–3115.
[12] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Current protocols in Molecular Biology, Vol. 1, sect. 9.
[13] Davis, S. and Yancopoulos, G.D. (1993) Curr. Biol. 3, 20–24.
[14] Yang, R.B., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L. and Godowski, P.J. (1998) Nature 395, 284–288.
[15] Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.-C., Hu, S., Altrock, B.W. and Fox, G.M. (1996) Cell 85, 1113–1124.
[16] Klein, R.D., Sherman, D., Ho, W.-H., Stone, D., Bennett, G.L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J.-A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A., Phillips, H., Henderson, C.E., Takahashi, M. and Rosenthal, A. (1997) Nature 387, 717–721.
[17] Bello, A.B., Adu, J., Pinon, L.G.P., Horton, A. and Thompson, J. (1997) Nature 387, 721–724.
[18] Milbrandt, J., deSauvage, F.J., Fahrner, T.J., Baloh, R.H., Leitner, M.L., Tansey, M.G., Lampe, P.A., Heuckeroth, R.O., Kotzbauer, P.T., Simburger, K.S., Golden, J.P., Davies, J.A., Vejsada, R., Kato, A.C., Hynes, M., Sherman, D., Nishimura, M., Wang, L.C., Vandlen, R., Moffat, B., Klein, R.D., Poulsen, K., Gray, C., Garces, A. and Johnson Jr., E.M. et al. (1998) Neuron 20, 245–253.
[19] Baloh, R.H., Tansey, M.G., Lampe, P.A., Fahrner, T.J., Enomoto, H., Simburger, K.S., Leitner, M.L., Araki, T., Johnson Jr., E.M. and Milbrandt, J. (1998) Neuron 21, 1291–1302.
[20] Treanor, J.J., Goodman, L., deSauvage, F., Stone, D.M., Poulsen, K.T., Beck, C.D., Gray, C., Armanini, M.P., Pollock, R.A., Hefti, F., Phillips, H.S., Goddard, A., Moore, M.W., Buj-Bello, A., Davies, A.M., Asai, N., Takahashi, M., Vandlen, R., Henderson, C.E. and Rosenthal, A. (1996) Nature 382, 80–83.
[21] Frey, E.A., Miller, D.S., Jahr, T.G., Sundan, A., Bazil, V., Espevik, T., Finlay, B.B. and Wright, S.D. (1992) J. Exp. Med. 176, 1665–1671.