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The growth suppressing *gas1* product is a GPI-linked protein

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Abstract Growth arrest specific (*gas*) 1 gene product is expressed in non-transformed fibroblasts in response to stimuli driving cells into Go phase. Gas1 has been demonstrated to inhibit cell proliferation when over-expressed in proliferating fibroblasts. This activity depends on a function of the p53 protein independent of its transactivating ability. To better define the pathway leading from Gas1, which is located on the plasma membrane, to p53, we have undertaken a detailed characterization of its topology. We demonstrate that the protein undergoes cotranslational modifications in the endoplasmic reticulum, consisting of signal peptide cleavage, N-linked glycosylation and glycosyl-phosphatidylinositol anchor addition. Immunoelectron microscopy shows that, in its mature form, Gas1 is randomly distributed over the outer leaflet of the plasma membrane and that upon antibody-induced clustering it relocalizes to caveolae.

Key words: *gas1* product; Growth inhibition; GPI-anchored protein; p53 protein; Signal peptide

1. Introduction

When deprived of growth factors or grown at high cell density, normal fibroblasts leave the cell cycle and enter Go. Under these conditions, an intrinsic program of gene expression is induced whereby a set of genes, called growth arrest specific (*gas*) genes, becomes highly expressed [1]. This pattern of gene expression is reversible since growth-arrested fibroblasts, when coordinately reentering the cell cycle, down-regulate the expression of *gas* genes.

Gas1 has previously been shown to exert a growth suppressing effect when ectopically expressed both during synchronous cell cycle reentry or during the exponential growth/division cycle [2,3]. It has been demonstrated that *gas1* growth inhibitory effect relies on a wt-p53 function independent of its transactivating functions [4]. Since Gas1 is a plasma membrane protein [2,3], these evidences point to the existence of a still uncharacterized pathway from the plasma membrane to p53.

Primary amino acid sequence analysis and comparison between mouse and human *gas1* cDNA predicted the existence of two hydrophobic amino acid stretches, one at each terminus of the protein [2,3]. Furthermore, a notable difference between the mouse and human sequence exists at the level of the initiating methionine. We thus wanted to better define the domains responsible for Gas1 interaction with the plasma membrane and the resulting topology of the protein with respect to the plane of the membrane.

Here we report that the N-terminal hydrophobic amino acid stretch represents a signal peptide, required to target the nascent peptide to the endoplasmic reticulum (ER) where it is cleaved off. Next we demonstrate that the C-terminal hydrophobic amino acid stretch represents a consensus sequence for the glycosyl-phosphatidylinositol (GPI) anchor substitution, tethering Gas1 to the plasma membrane. Immunoelectron microscopy (EM) analysis revealed a random distribution of Gas1 upon the plasma membrane, which becomes segregated into caveolae when anti-Gas1 antibodies are added before fixation.

2. Materials and methods

2.1. Plasmid construction

The pGDSV7S gas1-Met1 construct was obtained by cloning a *Bam*HI/*Eco*RI *gas1* cDNA 1.8 kb fragment into the *Bam*HI/*Eco*RI sites of the pGDSV7S vector [5]. The pGDSV7S gas1-Met42 construct was obtained by PCR amplification of a *Not*I/*Bam*HI 1.4 kb *gas1* cDNA fragment cloned into the *Not*I/*Bam*HI sites of pGDSV7S. The pGDSV7S gas1-Met65 construct was obtained by site-directed mutagenesis of the methionine at position 42. *gas1* therefore was amplified using two synthetic oligonucleotide primers both harboring an A→T substitution thus modifying the ATG codon to a TTG codon (Leu). The primers were used to amplify a 5′ and a 3′ fragment of *gas1*; the two amplified fragments were purified from agarose gel and used as templates to amplify the entire gas1-Met65 fragment which was cloned into the *Not*I/*Eco*RI sites of the pGDSV7S vector. For in vitro translation experiments, the same fragments were PCR-amplified so that the respective methionines could be cloned in frame substituting the viral methionine in position +5 of the pCITE-1 vector (Novagen, Madison, WI, USA) which corresponds to the *Nco*I site in the vectors polylinker.

2.2. Cell culture and transfections

NIH3T3 and COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (DMEM+10% FCS) and serum-starved as described [2]. Transfection of NIH3T3 cells was performed with 8 μg of DNA/8×10^4 cells using the calcium-phosphate transfection procedure [6]. Transfection of COS7 cells was performed with 2 μg of DNA/35 mm plate using the DEAE-dextran procedure [6].

2.3. In vitro translation

The in vitro translation experiments were performed using the TnT rabbit reticulocyte system (Promega) according to the manufacturer's instructions. Trypsin (Boehringer) digestion was performed at 0°C for 1 h at a final trypsin concentration of 1 mg/ml. Soybean α1 anti-trypsin (final concentration: 5 mg/ml, Boehringer) was added to stop the reaction. Deglycosylation with peptide-*N*-glycosidase F

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(PNGase F, Oxford GlycoSystems), at the concentration of 100 U/ml, was performed on the trypsin-treated product according to manufacturer's instructions, after trypsin inactivation. For membrane translocation analysis, translation was performed in the presence of dog pancreatic microsomes (DPM, New England Nuclear) [2]; microsomes were lysed by the detergent contained in the buffer supplied for PNGase F treatment finally performed.

2.4. Antibody preparation and Western blot analysis

Peptides were synthesized according to the Fmoc solid phase peptide synthesis [7], and were designed to span Gas1 amino acids: 2–14 ('Pep 1'); 337–356 ('337'). Peptides were purified by reverse-phase FPLC. The recombinant protein QE 229 was generated by cloning the *gas1* cDNA fragment spanning amino acids 229–342 into the expression vector pQE-11 (Qiagen) in frame with 5′ pQE $[His]_6$ tag. The recombinant protein was produced as suggested by the manufacturer and purified on Ni-NTA resin. The purified recombinant protein and the KLH crosslinked peptides were injected in rabbits to raise specific antisera. Antibodies were affinity-purified on columns coupled with peptides or with the recombinant protein using respectively SulfoLink Coupling Gel (Pierce) or Affi-Prep 10 (Bio-Rad) according to the manufacturer's instructions. Affinity-purified antibodies are referred to as 'anti-Pep 1', 'anti-337' and 'anti-QE 229'.

Mouse monoclonal 'anti-caveolin' antibody was purchased from Transduction Laboratories. Rabbit anti-human placental alkaline phosphatase ('anti-hPLAP') antibody was purchased from Dako, Denmark. Rabbit anti-cross reacting determinant ('anti-CRD') antibody was included in the 'GPI anchor detection kit' purchased from Oxford GlycoSystems. Western blot analysis was performed as described [2].

2.5. Cell labelling procedures and immunoprecipitations

[9,10-^{3}H]Palmitic acid (54 Ci/mmol, Amersham) dissolved in ethanol was dried in a Speed Vac concentrator (Savant) using silanized Eppendorf tubes and resuspended in DMEM+10% FCS.

Transfected COS cells were labelled with [9,10-^{3}H]palmitic acid (200 μCi/ml) for 10–12 h. Sodium pyruvate 5 mM was added to limit palmitic acid conversion to amino acids. Cells were lysed with TBS+0.8% sodium dodecyl sulfate (SDS) (Tris–HCl 50 mM, pH 7.5, NaCl 150 mM, SDS 0.8%) and diluted with an equal volume of TBS+2% Triton X-100.

Transfected COS cells were starved for methionine 1 h before addition of 150 μCi/ml of [^{35}S]methionine (Amersham), labelled for 5 h and lysed as above.

Preclearing of cell lysates was made with non-immune rabbit IgG and Immuno-Precipitine (Gibco BRL); immunoprecipitation was performed with anti-QE 229 antibody where not differently specified, at 1 μg/ml for 2–4 h at 4°C; immunoprecipitates were resolved on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) [8].

2.6. Phosphatidylinositol specific phospholipase C (PI-PLC) digestion

hPLAP-transfected NIH3T3 cells were growth-arrested by serum deprivation; cells were collected in phosphate-buffered saline (PBS) by scraping, pelleted and resuspended in 100 μl of lysis buffer (PBS containing: Triton X-114 0.1%, EDTA 5 mM, 20 μg/ml of leupeptin, pepstatin, antipain and chymostatin). To analyze cell lysates, the 'GPI anchor detection kit' was used according to the manufacturer's instructions. For PI-PLC digestion on plate, pGDSV7S gas1-Met42-transfected COS7 cells from one 35 mm dish were grown for an additional 24 h in DMEM+10% fetal bovine serum and then replated in 48-well tissue culture microtiter plates (Costar, 1 ml/well). 24 h later, 70 μl of PI-PLC buffer (Tris–HCl 25 mM, pH 7.5, sucrose 250 mM, glucose 10 mM, $CaCl_2$ 0.5 mM, $MgCl_2$ 0.5 mM) was added to each well, with or without PI-PLC (5 U/ml). Digestion was performed at 37°C for 1 h; the medium from seven wells was pooled and proteins were concentrated by ultrafiltration in Amicon Microcon (MWCO 10 000) up to the dead volume. Samples were then subjected to SDS–PAGE and Western blot.

2.7. Immunoelectron microscopy

NIH3T3 cells were grown on plastic coverslips in the presence of 10% FCS or serum-starved for 48 h in 0.5% FCS. Cells were surface-labelled with affinity-purified antibodies to Gas1 followed by 10 nm protein A-gold either after prefixation with 0.1% glutaraldehyde in 100 mM cacodylate buffer or at 4°C before fixation. Fixation and further processing were exactly as described previously [9,10].

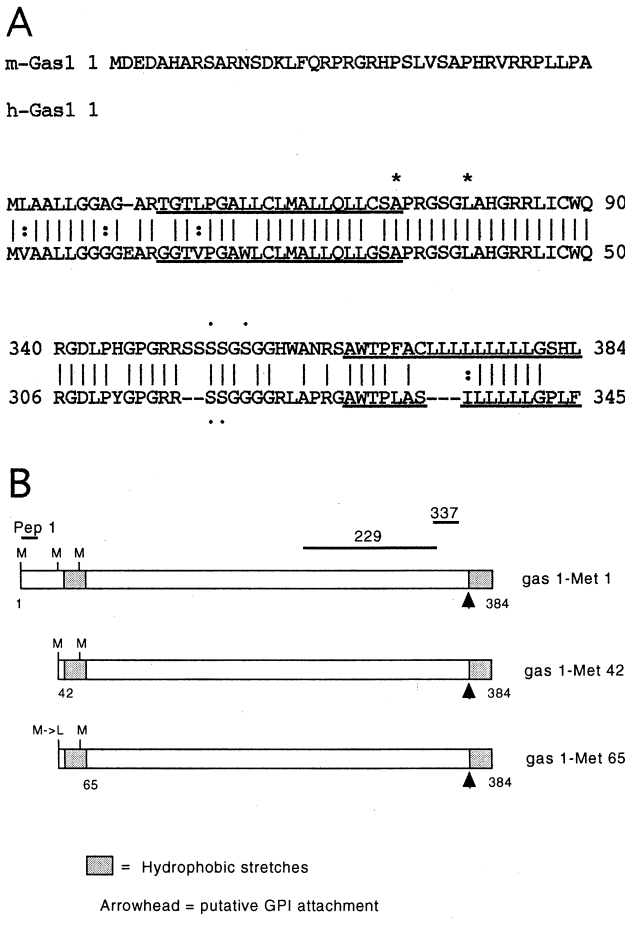

Fig. 1. Partial Gas1 protein sequence, construct maps and antibody epitopes are shown. (A) Alignment of murine and human Gas1 amino acid sequences restricted to the amino- and carboxy-terminal regions involved in cotranslational modification. Hydrophobic regions are underlined; asterisks represent candidate cleavage sites of the signal peptide; dots indicate candidate amino acids undergoing GPI anchor addition. (B) Wild type *gas1* cDNA (gas1-Met1) was cleaved before Met42 to produce the deletion gas1-Met42. A second construct was derived from gas1-Met42 by site-directed mutagenesis leading to a 42Met→Leu substitution giving raise to gas1-Met65. Bold bars indicate the epitopes against which antibodies were raised (see Section 2 for details).

3. Results and discussion

3.1. Analysis of Gas1 protein processing

The murine *gas1* cDNA (GenBank X65128-MMGASIMR) open reading frame contains at least three putative initiating methionines at positions 1, 42 and 65. Comparison with the human *gas1* sequence (GenBank L13698) (Fig. 1A) reveals that only methionines 42 and 65 are conserved [3].

To test if the two latter methionines represent initiation sites for translation, two constructs of murine *gas1* were produced (Fig. 1B): the first contained a deletion of the amino acids 1–41, thus starting with methionine 42 (gas1-Met42); the other construct derived from the former but with a 42Met→Leu substitution leaving only Met65 available for translation (gas1-Met65). COS7 cells, not expressing endogenous Gas1 (Fig. 2A, lane e), were transfected with the three pGDSV7S plasmids encoding gas1-Met1, gas1-Met42 and gas1-Met65, lysed and immunoprecipitated with anti-Gas1 antibody. Fig. 2A shows that the murine *gas1* cDNA starting

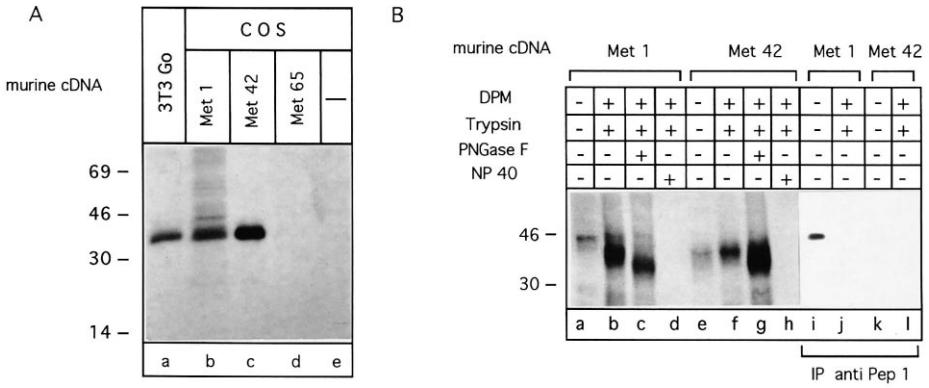

Fig. 2. Biochemical analysis of Gas1. (A) COS cells were transfected with either pGDSV7S gas1-Met1 (b), pGDSV7S gas1-Met42 (c) or pGDSV7S gas1-Met65 (d). Lysates from transfected and not transfected (e) COS cells and from resting NIH3T3 cells (a) were immunoprecipitated with 'anti-229' antibody and resolved on SDS–PAGE. (B) pCITE gas1-Met1 and pCITE gas1-Met42 were used for in vitro translation experiments. Translation was performed in the absence (a and e) or in the presence of DPM followed by trypsin digestion without (b and f) or with previous solubilization with NP40 (d and h). Translation products were solubilized and digested with PNGase F (c and g). (i–l) Immunoprecipitation with 'anti-Pep 1' antibody of in vitro translation products corresponding respectively to lanes a, b, e and f. See text for details.

with Met1 or Met42 can successfully initiate translation in vivo (lanes b and c), while Met65 is completely ineffective, either in vivo (lane d) or in vitro (not shown). The mature proteins as ectopically synthesized in COS7 cells had an apparent molecular weight similar to the endogenous Gas1 expressed by resting NIH3T3 cells (lane a), thus indicating that either the initiating methionine is Met42 in both cases or that, if Met1 is used, further processing leads to a product with similar electrophoretic mobility.

To analyze the N-terminal requirement in the initial processing steps, membrane insertion and topology of Gas1 was studied in an in vitro translation system using the pCITE gas1-Met1 and pCITE gas1-Met42 constructs, the pCITE vectors strictly allowing for the methionine placed after CITE sequence to initiate translation.

As shown in Fig. 2B, the pCITE-driven in vitro translation resulted in two primary products (lanes a and e) with an expected difference in relative mobility due to gas1-Met1 containing 41 amino acids more than gas1-Met42. In vitro translation in the presence of DPM with subsequent trypsin digestion is an established method to select for proteins fully translocated into the membranes, since they become protected from the action of the protease. Under these conditions, translation of both cDNAs resulted in a lower and similar sized band for both pCITE gas1-Met1 and pCITE gas1-Met42 (lanes b and f), with a molecular weight similar to that of the endogenous Gas1. We therefore hypothesize that such a band corresponds to the form of the protein as processed in the ER. To confirm that the resulting product was fully protected, trypsin digestion carried out in the presence of the detergent NP40 resulted in the complete disappearance of the respective products (lanes d and h). Treatment of the detergent-unmasked product with the PNGase F resulted in a faster migrating band, thus demonstrating that the Gas1 in vitro translated product is *N*-glycosylated (lanes c and g).

Immunoprecipitation was performed with 'anti-Pep 1' antibody on the in vitro translation products as obtained from gas1-Met1 and gas1-Met42 either in the absence of DPM or in the presence of DPM followed by trypsinization. This antibody recognizes only the product of gas1-Met1 translated in the absence of DPM (lane i), while it fails to immunoprecipitate either the gas1-Met42 product (lanes k and l) (not con-

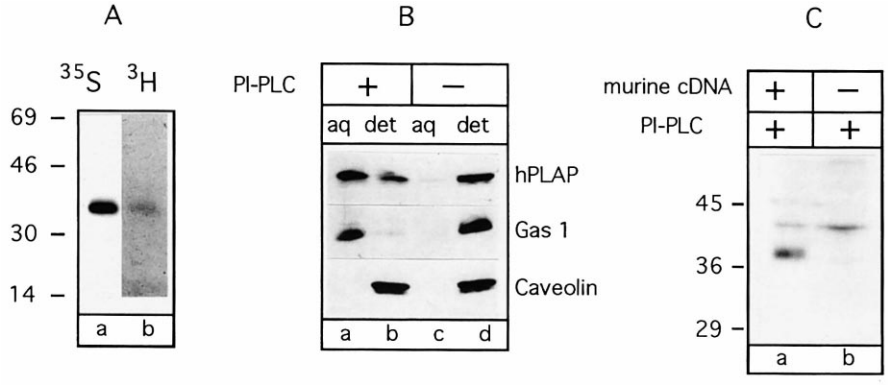

Fig. 3. Gas1 palmitoylation and PI-PLC digestion. (A) pGDSV7S gas1-Met42-transfected COS cells were labelled either with [^{35}S]methionine (a) or with [^{3}H]palmitic acid (b). Cells were lysed and immunoprecipitated with 'anti-229' antibody. Immunoprecipitates were resolved on SDS–PAGE. (B) hPLAP-transfected, serum-starved NIH3T3 cells were recovered in 100 μl of PI-PLC buffer. 50 μl aliquots were treated with (a and b) or without (c and d) PI-PLC and subjected to Triton X-114 phase separation (see text for details). The aqueous (a and c) and the detergent (b and d) phases were resolved on SDS–PAGE, blotted and probed with either 'anti-hPLAP', 'anti-229' or 'anti-caveolin' antibodies as indicated. (C) pGDSV7S gas1-Met42-transfected (a) and not transfected (b) COS cells were treated on plate with PI-PLC. Supernatant was collected, concentrated, electrophoresed and blotted. The membrane was probed with 'anti-CRD' antibody.

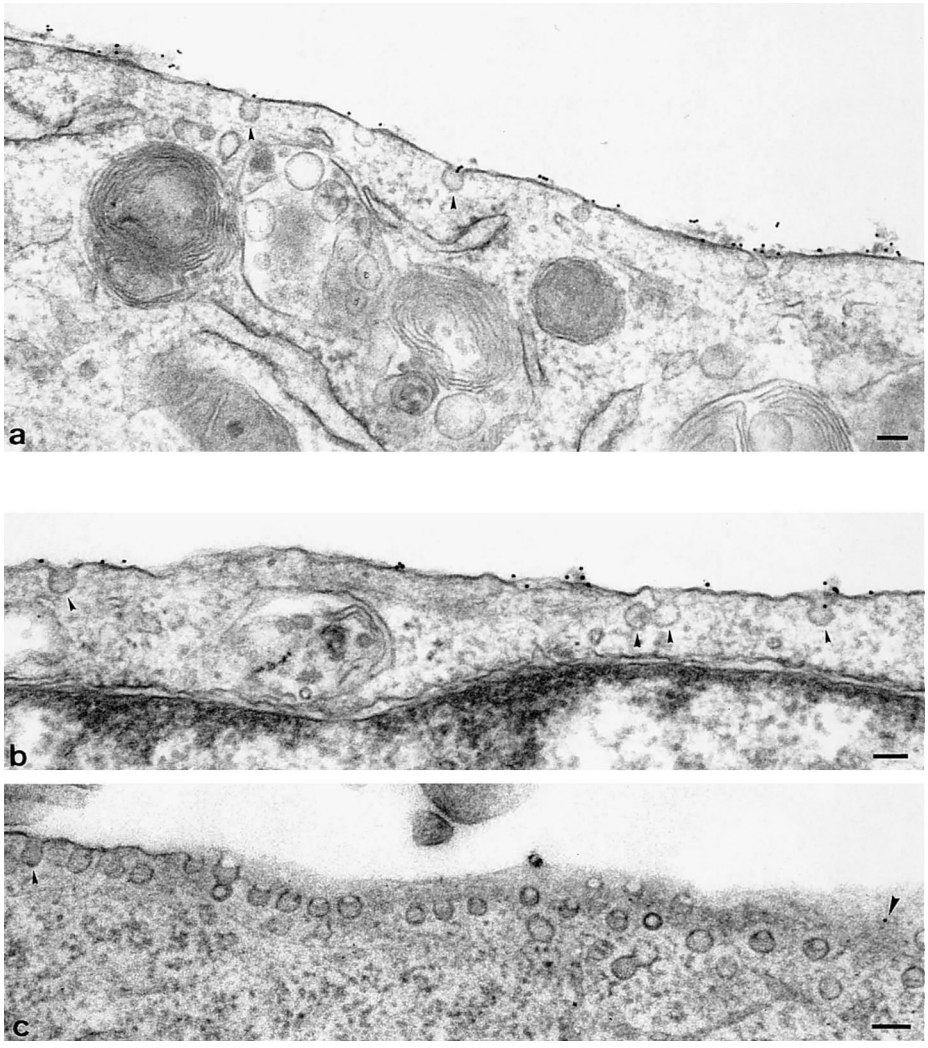

Fig. 4. EM analysis of surface labelling of Gas1 in resting or dividing NIH3T3 cells. Resting (a and b) or proliferating (c) NIH3T3 cells were fixed with 0.1% glutaraldehyde and labelled with 'anti-229' antibody followed by 10 nm protein A-gold. In the resting cells (a and b), heavy gold labelling is evident over the entire cell surface. In contrast, negligible labelling is observed on the surface of dividing cells labelled under identical conditions (c, gold particle indicated by large arrowhead). Caveolae (60 nm uncoated surface invaginations) are indicated by small arrowheads. Bar = 100 nm.

taining the epitope), or the gas1-Met1 protein after processing in the membranes (lane j). This antibody also fails to recognize the endogenous Gas1 from NIH3T3 as well (not shown).

From these data, we conclude that in vitro primary translation products starting either from the first or the second methionine are similarly processed by the microsomal fraction to a protein with similar mobility as the in vivo *gas1* product.

The predicted murine protein sequence from Arg52 to Arg85 was analyzed on the basis of the weight matrix calculated by von Heijne [11] to predict signal sequence cleavage sites. Pro75, Gly77, Ala74 and Leu80 were found as possible sites of cleavage. Proline is suggested to be absent from −3 through +1 relative to the mature form and Ala74 and Leu80 only fulfill this requirement, thus potentially representing the first amino acids of the mature Gas1. The results of this analysis are in agreement with the presented experimental data.

The data presented exclude the presence of a transmembrane domain at the N-terminus of the mature protein. It follows that Gas1 relies only on the C-terminal hydrophobic stretch to anchor to the plasma membrane. The absence of a hydrophilic sequence following the putative transmembrane domain hints at the presence of a GPI moiety substituting the amino acid stretch.

The GPI anchor contains ethanolamine, a short glycosidic chain, a myo-inositol and a diacyglycerolphosphate [12]. We first tested whether in vivo Gas1 could incorporate fatty acids. COS cells were transfected with pGDSV7S gas1-Met42 and separately labelled with [^{3}H]palmitate and [^{35}S]methionine. Gas1 was immunoprecipitated from cell lysates and separated on SDS–PAGE; Fig. 3A shows that it incorporated [^{3}H]palmitate (lane b).

GPI-anchored proteins are sensitive to PI-PLC digestion thus behaving as soluble proteins [12]. NIH3T3 cells were transfected with hPLAP, a well studied GPI-anchored protein [13], solubilized in a buffer containing Triton X-114 and digested with PI-PLC. Following addition of 2% final Triton X-114 and temperature-induced phase separation, aqueous and detergent phases were analyzed by immunoblotting (Fig. 3B). Compared to the undigested control, where Gas1

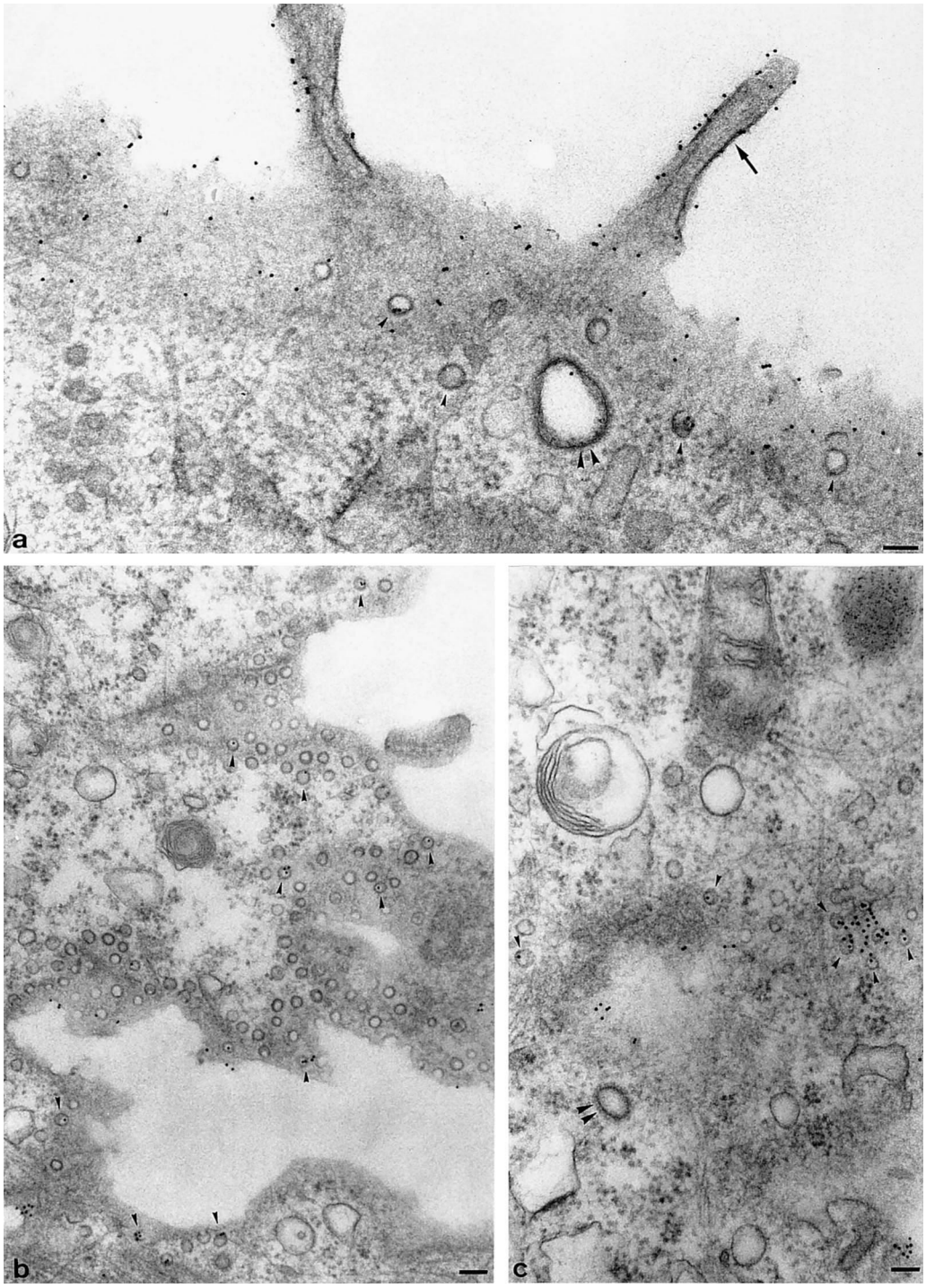

Fig. 5. EM analysis of surface labelling of Gas1 in resting NIH3T3 cells with or without prefixation. Resting NIH3T3 cells were labelled after prefixation with glutaraldehyde as in (a) or were labelled at 4°C with 'anti-229' antibody and 10 nm protein A-gold prior to fixation (b and c). (a) (prefixation) shows a section across the cell surface; labelling for Gas1 is distributed almost randomly over the entire cell surface including coated pits (double arrowheads) and caveolae (arrowheads). Surface projections (arrow) are particularly heavily labelled. Cells labelled before fixation (b and c) show a clear concentration of gold particle clusters within caveolae (arrowheads). Labelling is rarely observed in coated pits (e.g. see coated pit/vesicle indicated by double arrowheads in c). Bar = 100 nm.

is retained in the detergent phase (lane d), PI-PLC treatment totally released the Gas1 protein into the aqueous phase (lane a). hPLAP similarly proved sensitive to the digestion while caveolin, a transmembrane protein endogenously expressed by NIH3T3 cells [14], was totally retained in the detergent phase. Similar results were obtained for the human Gas1 protein when expressed in COS cells (not shown). The complete release of the Gas1 protein from the membrane fraction into the aqueous phase by PI-PLC treatment indicates that the whole of the mature protein is GPI-anchored.

Following PI-PLC digestion of GPI-anchored proteins, the remaining myo-inositol phosphate cyclizes, creating a new epi-

tope called CRD, against which a specific antibody is available.

pGDSV7S gas1-Met42-transfected COS cells and a non-transfected control were digested with PI-PLC on plate; the supernatant was concentrated, separated on SDS–PAGE and analyzed with an 'anti-CRD' antibody by Western blot. As shown in Fig. 3C, a positive band to this antibody was detected only in the supernatant corresponding to the transfected and PI-PLC-treated cells at the height of Gas1 (lane a), while the PI-PLC-treated untransfected control was negative (lane b). Stripping of the membrane and reprobing with 'anti-229' antibody resulted in a signal completely superimposable to the former band (not shown).

From these evidences, we conclude that Gas1 in its mature form is inserted in the plasma membrane through a GPI anchor at its carboxy-terminus.

Inspection of the primary amino acid sequence of the carboxy-terminus of the murine Gas1 (Fig. 1A) confirmed the presence of a consensus sequence for the GPI modification [15,16]; more specifically Ser353 and Ser356 represent the most probable candidate acceptor sites. Such consensus is also present in the human homologue at Ser317 and Ser318. Localization of the GPI consensus at this site agrees with the results obtained when using the 'anti-337' antibody, whose epitope ends at Ser356 of the murine protein. This antibody in fact recognizes the mature protein product (not shown), thus indicating that the protein cleavage for GPI substitution conserves such an epitope.

3.2. Plasma membrane localization

Immunoelectron microscopy was used to investigate the distribution of Gas1 on the plasma membrane and to quantitate the relative expression in growth-arrested versus proliferating fibroblasts.

NIH3T3 cells cultured under the two different conditions were fixed and immunolabelled with 'anti-229' antibody. Under resting conditions, a strong signal was present on the plasma membrane (Fig. 4a,b). In contrast, proliferating cells labelled in parallel showed extremely low labelling (Fig. 4c). A quantitative analysis of the gold labelling density showed a more than 20-fold higher level of Gas1 on the plasma membrane in growth-arrested cells (22 particles/μm^2) than in proliferating cells (0.9 particles/μm^2).

Previous studies have described a concentration of GPI-anchored proteins in surface caveolae [17]. In contrast, more recent studies suggest that GPI-anchored proteins normally show a random distribution over the cell surface but are redistributed into caveolae on crosslinking with antibodies before fixation [17,18]. We therefore examined the surface distribution of Gas1 with or without a glutaraldehyde prefixation step.

Fig. 5a (see also Fig. 4a,b) shows that labelling for Gas1 is almost randomly distributed over the plasma membrane, including caveolae and coated pits, when cells are fixed before labelling. We assume that no further mobility and/or clustering are induced by the antibody after glutaraldehyde fixation and therefore that this labelling pattern represents the natural distribution of Gas1.

Fig. 5b,c shows that the pattern is completely different when cells are immunolabelled at 4°C before fixation: gold particles are not randomly spread over the entire plasma membrane but are found as clusters within caveolae. Labelling was rarely seen within clathrin coated pits under these conditions. It therefore appears that clustering of Gas1 within caveolae is increased upon crosslinking with 'anti-229' antibodies.

Increasing evidence has demonstrated that GPI anchors target the proteins to plasma membrane 'rafts' rich in glycosphingolipids, sphingomyelin and cholesterol. Such a composition is only partially solubilized by common non-ionic detergents at 4°C, resulting in GPI-anchored proteins partitioning into detergent-insoluble glycosphingolipid-enriched complexes (DIG) [19]. This property also holds true for Gas1 (not shown). Similar detergent insolubility of these rafts and caveolae [14,20] has allowed the copurification of GPI-anchored proteins with caveolar markers, namely caveolin [20,21]. These biochemical evidences have led to the assumption that detergent-insoluble complexes correspond to plasma membrane caveolae [20]; however, this assumption has been questioned on the basis of (a) the presence of GPI-linked proteins in detergent-insoluble complexes in lymphocytes, which lack caveolae [22], and (b) a different biochemical procedure to isolate caveolae from the rest of the plasma membrane, whereby GPI-anchored proteins appear to reside only at the neck of, but not inside, caveolae [23].

Immunoelectron microscopy analysis, showing Gas1 randomly distributed all over the plasma membrane without either particular inclusion or association to the neck of caveolae, confirms the interpretation that DIGs and associated proteins are not necessarily localized to caveolae. Other, more specific, protein–protein interactions should account for GPI-anchored protein localization.

It has been demonstrated that antibodies against GPI-anchored proteins, when added to cells before fixation, induce clustering of the proteins and their relocalization into caveolae [11,18]; in lymphocytes, such an antibody-induced clustering of GPI-anchored proteins results in cell activation, correlated with Tyr phosphorylation of Src and Src-like kinases [24,25]. Although EM analysis confirms such an antibody-induced redistribution for Gas1 as well, we have not found any evidence indicating that treatment of growth-arrested NIH3T3 cells with anti-Gas1 antibody elicits a specific phosphorylation pattern.

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