Apoptotic phenotype induced by overexpression of wild-type *gas3/PMP22*: its relation to the demyelinating peripheral neuropathy CMT1A

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Although the Gas3/PMP22 protein is expressed at highest levels in differentiated Schwann cells, its presence, albeit at lower levels, in non-neuronal tissues and in NIH-3T3 growth-arrested fibroblasts argues for a more general function of this protein that is uncoupled to myelin structure. We show that *gas3/PMP22* overexpression in NIH-3T3 growing cells leads to an apoptotic-like phenotype, which is suppressed by antioxidants and characterized by typical membrane blebbing, rounding up, and chromatin condensation, but with no evidence of DNA fragmentation. REF-52 fibroblasts seem to be completely refractive to *gas3/PMP22* overexpression. Recently, several point mutations of the human *gas3/PMP22* gene have been associated with Charcot-Marie-Tooth type 1A (CMT1A), a common hereditary demyelinating neuropathy. When *gas3/PMP22* point mutations (*L16P*, *S79C*, *T118M*, and *G150D*) are similarly overexpressed in NIH-3T3 cells, the induced apoptotic-like phenotype as compared to the wild-type is significantly reduced. Both of the dominant mutations (*L16P*, *S79C*) for CMT1A behave as dominant negatives with respect to the wild type, whereas *T118M*, the only recessive mutant described, behaves as recessive under the same coexpression experiments. These data suggest a role for altered Schwann cell apoptosis in the pathogenesis of CMT1A.

[Key Words: Apoptosis; Charcot-Marie-Tooth 1A; *gas3*; *PMP22*]

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gas3, originally isolated from NIH-3T3 cells for its growth arrest-dependent expression (Schneider et al. 1988; Manfioletti et al. 1990), has been subsequently characterized as a major component of the peripheral nerve myelin and named accordingly as peripheral myelin protein PMP22 (Spreyer et al. 1991; Welcher et al. 1991; Snipes et al. 1992). *gas3/PMP22* is abundantly expressed in differentiated myelin-forming Schwann cells, but it is dramatically down-regulated after nerve injury when Schwann cell proliferation starts (Spreyer et al. 1991; Welcher et al. 1991), thus indicating that its G₀-dependent expression also holds true in vivo. It encodes for an integral membrane protein presenting four putative transmembrane domains with an apparent molecular mass of 22 kD (Snipes et al. 1992).

Genetic studies have revealed that the *gas3/PMP22* gene is responsible for a set of inherited peripheral neuropathies in mice and humans. Myelin deficiency in peripheral nerve and continued Schwann cell proliferation

are the main histological characteristics of the mutated phenotypes, whereas impaired conduction of nerve impulses is the major pathological symptom (Suter et al. 1993).

In rodents, point mutations of *gas3/PMP22* are associated with the peripheral neuropathy phenotype Trembler and Trembler⁺ (Suter et al. 1992a,b). Point mutations in *gas3/PMP22* have been reported in ~30% of Charcot-Marie-Tooth 1A (CMT1A) patients (Valentijn et al. 1992a; Ionasescu et al. 1993; Roa et al. 1993a,b), the other cases being accounted for by a large duplication of the short arm of chromosome 17 containing the *gas3/PMP22* locus (Lupski et al. 1991; Matsunami et al. 1992; Timmerman et al. 1992; Valentijn et al. 1992b; Roa et al. 1993a,b).

Further studies have confirmed the crucial role of Gas3/PMP22 in the demyelinating neuropathies. Dejerine-Sottas syndrome, showing similar but more severe symptoms than CMT1A, is also characterized by point mutations in *gas3/PMP22* (Roa et al. 1993c). Furthermore *gas3/PMP22* has also been implicated in other forms of peripheral neuropathies: Hereditary neuropathy

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with liability to pressure palsies (HNPP–tomaculous neuropathy) is associated with a deletion of the same chromosomal region containing *gas3/PMP22* duplicated in CMT1A patients (Chance et al. 1993).

These studies have revealed two surprising findings: (1) that a variety of genetic mechanisms involving *gas3/PMP22*, including point mutations, duplication, and deletions, may give rise to distinct hereditary peripheral neuropathies; and (2) two different genetic mechanisms (duplication and point mutations) can result in similar clinical phenotypes of CMT1A (Patel and Lupski 1994).

Identification of functional or phenotypic differences between *gas3/PMP22* wild-type and point mutation derivatives should thus represent a first step toward the understanding of its involvement in CMT1A. To date, however, no phenotype and thus no biological function for Gas3/PMP22 has been established. Our initial observation of Gas3/PMP22 suggested a possible function in regulating some general aspects of cell proliferation. Its expression is increased at growth arrest in NIH-3T3 cells and is down-regulated in association with carcinogenesis in non-neuronal tissues such as lung, where it is also expressed (Re et al. 1992).

In this report we have extended the findings of our initial model system and demonstrate that expression of *gas3/PMP22* in NIH-3T3 cells leads to a phenotype similar to apoptosis. Furthermore, we show a correlation between phenotypic dominant or recessive behaviors, and dominant and recessive modes of inheritance for the various CMT1A and Trembler point mutation alleles analyzed.

Results

Expression of *gas3/PMP22* product in NIH-3T3 cells and in mouse PNS tissues

Antiserum against mouse Gas3/PMP22 protein, raised in rabbit against the putative extracellular domain of mGas3 (amino acids 16–63, presenting 98% identity between mouse and rat), was affinity purified as described in Materials and methods. Western immunoblot analysis of total protein isolated from rat sciatic nerves (Fig. 1A, lane 1) shows that the antibody recognizes a major band of 22 kD and a minor band of 18 kD, in agreement with previous studies (Welcher et al. 1991).

Lysates were treated with *N*-glycosylase F (PNGase F), which removes Asn-linked oligosaccharide chains, to identify the 18-kD protein as the Gas3/PMP22 precursor. After this treatment the anti-mGas3/PMP22 antibody recognized only one molecular species of 18 kD apparent size, with the same mobility as the lower band in untreated rat sciatic nerve lysates (Fig. 1A, lane 2). Interestingly pretreatment of serum-starved NIH-3T3 extracts with PNGase F allowed detection of a band comigrating with the unglycosylated form of Gas3/PMP22 in rat sciatic nerve (Fig. 1A, lane 4). Conversely we could not detect Gas3/PMP22 protein with anti-mGas3/PMP22 antibody in the untreated cell lysates (Fig. 1A, lane 3). This is probably attributable to epitope

Figure 1. Western blot analysis of Gas3/PMP22 expression in PNS and NIH-3T3 cells. (A) Tissue and cellular extracts were treated with or without PNGase F as described in Materials and methods. As a positive control, rat sciatic nerve extract, untreated (–) or treated (+) with PNGase F, was used. The amount of rat sciatic nerve protein loaded (lane 1,2) was 40× less than the resting NIH-3T3 lysates (lane 3,4). (B) PNGase F-treated lysates from actively growing NIH-3T3 cells and various times after serum starvation. (C) PNGase F-treated lysates from exponentially growing NIH-3T3 cells and various days after cell seeding. Western blot analysis was performed using affinity-purified anti-mGas3/PMP22 antibody.

masking because the consensus site of *N*-glycosylation lies at position 41 within the region of mGas3/PMP22 used as immunogen. Recognition of the mGas3/PMP22 glycosylated form as expressed in peripheral nerves by the same anti-mGas3/PMP22 could depend on Schwann cell-specific processing of the mature carbohydrate moiety. Similar behavior of anti-mGas3/PMP22 antibodies has already been reported for antibodies raised against a synthetic peptide of rat Gas3/PMP22 comprising the consensus site for *N*-glycosylation (Pareek et al. 1993). We conclude that anti-mGas3/PMP22 antibody reacts

with mature and precursor forms of Gas3/PMP22 protein expressed in rat sciatic nerve and with only the unglycosylated form in NIH-3T3 mouse fibroblasts. Moreover, in peripheral nervous system (PNS) tissues Gas3/PMP22 protein is expressed ~200 times more compared with that in growth-arrested NIH-3T3 cells.

Because expression of *gas3/PMP22* mRNA is induced at growth arrest by either serum starvation or saturation density in NIH-3T3 cells (Manfioletti et al. 1990), we examined whether the protein was similarly expressed under both conditions. Expression of mGas3/PMP22 was thus analyzed during growth arrest induced by both serum starvation and saturation density by Western immunoblot analysis. Equal protein amounts of PNGase F-treated extracts were loaded for each time point (as assessed by Coomassie blue staining of separate gels). Figure 1B shows the kinetics of the appearance of Gas3/PMP22 protein after serum depletion from exponentially growing NIH-3T3 cells. As expected, Gas3/PMP22 protein appears as a band with an apparent molecular mass of 18 kD in all lanes: Its level is significantly increased 48 hr after serum starvation. A similar increase in expression of Gas3/PMP22 is observed during density-dependent inhibition (Fig. 1C). These results are thus in agreement with the previous analysis of mRNA expression (Manfioletti et al. 1990) indicating that Gas3/PMP22 protein levels are increased under different growth arrest conditions.

Expression of Gas3/PMP22 protein in non-neuronal rat and human tissues

Because we were able to show that Gas3/PMP22 protein is expressed in NIH-3T3 mouse fibroblasts, we investigated whether it is present in rat and human non-neuronal tissues. Figure 2 shows the results of Western analysis on rat tissue lysates pretreated with PNGase F be-

fore SDS-PAGE. An 18-kD band of the same relative mobility as the one in rat sciatic nerve is present in lung and intestine, and a faint band can also be seen in kidney (Fig. 2A). Thus, Gas3/PMP22 protein is expressed abundantly in sciatic nerve but is also present, albeit at much lower relative levels, in non-neuronal rat tissues in agreement with previous Northern analysis data (Welcher et al. 1991; Spreyer et al. 1991).

We decided to confirm this pattern of Gas3/PMP22 expression in different human tissues. For this purpose, antiserum against human Gas3/PMP22 was raised in rabbit against the putative extracellular domain of hGas3 (from amino acids 16 to 63), which presents 79% amino acid identity with the same region of mouse protein. Affinity-purified antibodies specific for hGas3/PMP22 were used in Western analysis on different human tissues. The anti-hGas3/PMP22 antibody recognizes a molecular species of 22 kD and additional lower size bands in human phrenic nerve (Fig. 2B). The pattern in human phrenic nerve is similar to the rat sciatic nerve with the notable difference of a third lowest size band that probably represents a proteolytic fragment generated by postmortem autolysis. When the lysates were treated with PNGase F before Western analysis, the mobility of the 22-kD band relative to the mature Gas3/PMP22 protein shifted to 18 kD (Fig. 2B).

Furthermore after PNGase F treatment, 18-kD bands were detectable in lanes corresponding to human lung and intestine, as shown in Figure 2B; a faint band was also detected in heart.

No detectable expression of another myelin protein, P0, in the non-neuronal tissues examined was found by Western analysis (data not shown) using monoclonal antibody P07 (Archelos et al. 1993), thus suggesting a lack of contamination from peripheral nerves.

We conclude that Gas3/PMP22 protein is abundantly expressed in the PNS and at reduced levels in non-neu-

Figure 2. Western blot analysis of Gas3/PMP22 expression in different rat and human tissues. (A) Lysates from different rat tissues were treated with PNGase F before Western analysis. As a positive control rat sciatic nerve extract, untreated (–) or treated (+) with PNGase F, was used. Affinity-purified anti-mGas3/PMP22 was used for Western analysis. Rat peripheral nerve lysate was 40× lower than the other lysates. (B) Lysates from different human tissues were treated with PNGase F before Western analysis. As a positive control phrenic nerve extract, untreated (–) or treated (+) with PNGase F, was used. Affinity-purified anti-hGas3/PMP22 antibody was used for Western analysis. Human phrenic nerve lysate was 25× lower than the other tissue lysates.

Figure 3. Confocal generated overlay showing cellular and nuclear phenotype in *gas3*/*PMP22*-microinjected cells. (A) NIH-3T3 cells 24 hr after seeding were microinjected with pGDSV7-*hgas3*/*PMP22* (50 ng/ μ l). After 24 hr, cells were fixed and processed for immunofluorescence analysis to visualize hGas3/*PMP22* (green) with a specific anti-hGas3/*PMP22* antibody and propidium iodide to visualize nuclei (red). Arrows indicate cells detaching from substratum. (B) NIH-3T3 cells 24 hr after seeding were microinjected with pGDSV7-*hgas3*/*PMP22* (50 ng/ μ l). Immediately after microinjection *N*-acetyl-L-cysteine was added to the culture medium at a final concentration of 30 mM. After 24 hr cells were fixed and processed for immunofluorescence to visualize hGas3/*PMP22* and nuclei. (C) REF-52 cells 24 hr after seeding were microinjected with pGDSV7-*hgas3*/*PMP22* (50 ng/ μ l). After 30 hr cells were fixed and processed for immunofluorescence to visualize hGas3/*PMP22* and nuclei. Bar, 7.5 μ m.

tially growing NIH-3T3 cells. Human *gas3*/*PMP22* cDNA [Edomi et al. 1993] was cloned in an SV40 expression vector [Del Sal et al. 1994], and nuclear microinjection was used to drive its overexpression.

NIH-3T3 cells growing on a coverslip were microinjected with pGDSV7-*hgas3*/*PMP22* (50 ng/ μ l), fixed after 24 hr, and processed for immunofluorescence analysis to detect hGas3/*PMP22* as described in Materials and methods. Because anti-hGas3/*PMP22* antibody cross-reacts with mGas3/*PMP22* only weakly (data not shown), cells overexpressing hGas3/*PMP22* were specifically scored. Figure 3A shows confocal microscopic analysis of a representative field of microinjected NIH-3T3 cells, double stained for Gas3/*PMP22* (green) and nuclei (red). NIH-3T3 cells overexpressing Gas3/*PMP22* show a dramatic morphological alteration. Cells are loosely attached to the coverslip and are detected on the surface of other cells with a markedly collapsed cellular body and condensed nuclei (Fig. 3A, arrows). Overexpression of various other *gas* or control genes cloned in the same vector in the same cells under identical experimental conditions never resulted in such effects.

The altered phenotype induced by *gas3*/*PMP22* overexpression was completely abrogated by addition of the reducing agent *N*-acetyl-L-cysteine (NAC) (30 mM) to the culture medium (Fig. 3B). The same protecting effect could also be obtained with ascorbic acid (250 μ M) (data not shown), thus giving evidence for a role of reactive oxygen intermediates (ROI) in the generation of *gas3*/*PMP22*-dependent phenotype.

The effect observed in NIH-3T3 fibroblasts after *gas3*/*PMP22* overexpression is not a universal phenomenon. REF-52 cells that do not express endogenous *gas3*/*PMP22* (data not shown) appear perfectly normal after ectopic overexpression of *gas3*/*PMP22* under the same experimental conditions used for NIH-3T3 cells (Fig. 3C). To confirm that Gas3/*PMP22* protein was produced in REF-52 at a level similar to NIH-3T3 cells, increasing amounts of pGDSV7-*hgas3*/*PMP22* plasmid (50, 100, 150 ng/ μ l) were microinjected in REF-52 and immunofluorescence analysis was performed 30 hr after microinjection. In all cases, no obvious alteration of cell mor-

ronal tissues, such as lung and intestine in rat as well as in man.

Overexpression of *gas3*/*PMP22* in cultured cells

Because both *gas3*/*PMP22* mRNA and protein levels are increased after cell cycle exit, we decided to investigate the effect of ectopic *gas3*/*PMP22* expression on exponen-

phology was ever discerned in REF-52 cells overexpressing *gas3/PMP22*. We conclude that the altered phenotype induced by *gas3/PMP22* overexpression is cell type specific and shows some characteristics of apoptosis.

Detailed analysis of *gas3/PMP22*-induced phenotype in NIH-3T3 fibroblasts

The term apoptosis was originally proposed to describe a common series of morphological changes that accompany cell death. Plasma membrane blebbing and alteration of the nuclear architecture represent two main features of this process (Kerr et al. 1971; Wyllie et al. 1980). We thus decided to analyze whether these same features could be observed in NIH-3T3 cells overexpressing *hgas3/PMP22*.

To better define the formation of membrane blebbing we decided to uniformly stain the plasma membrane border. This was achieved by coexpressing *gas3* and *gas2*, a component of the microfilament system that localizes at the cell border (Brancolini et al. 1992). NIH-3T3 cells were coinjected with pGDSV7-*hgas3/PMP22* (50 ng/ μ l) and pGDSV7-*gas2* (10 ng/ μ l), and immunofluorescence analysis was performed by double staining for Gas2 and actin. The results of such analyses are shown in Figure 4, A and B, which represents a sequence of horizontal sections from the basal through the apical regions (from left to right) of a coinjected cell stained for Gas2 (Fig. 4A) and actin (Fig. 4B). The features of the plasma membrane of this cell are consistent with structures known as blebs, which are characteristic of the apoptotic phenotype. Membrane blebbing is only noticed in the initial phases of the apoptotic process (Evan et al. 1992) and found in

only 2%–6% of the *gas3/PMP22*-overexpressing cells. This could represent the rapid kinetics of this process. Parallel analysis of microfilament distribution (Fig. 4B) also shows evident changes in microfilament organization. Confocal analysis showed that increased cell rounding accompanies overexpression of *gas3/PMP22*. NIH-3T3 cells overexpressing *gas3/PMP22* present a measured cell height of $14.05 \pm 2.05 \mu\text{m}$ when compared with a normal height of $5.73 \mu\text{m} \pm 0.38$, as measured in cell overexpressing *gas2* as control gene (data not shown).

We next examined nuclear morphology by confocal microscopy in cells overexpressing Gas3/PMP22 protein by immunofluorescence and propidium iodide staining 24 hr after microinjection. Figure 4, C and D, shows a representative field in which the cells overexpressing *gas3/PMP22* (Fig. 4C) present altered and highly condensed nuclei (Fig. 4D), one of the characteristics of cells undergoing apoptosis.

Another feature of apoptosis, but one that is not universal (Jacobson et al. 1994; Schulze-Osthoff et al. 1994), is DNA fragmentation, which can be detected in situ by the TUNEL technique (Gravieli et al. 1992). Using this method we have not been able to find evidence of DNA fragmentation in cells overexpressing Gas3/PMP22 protein with respect to a positive control (DNase-induced fragmentation).

To quantitate the apoptotic-like phenomenon induced by *gas3/PMP22* overexpression, we correlated the end results of such a process, that is, evident nuclear alteration and decreased cell number. The latter effect should be an expected consequence of the described increased cell rounding preceding complete detachment from adhesion substratum. As a control allowing the quantita-

Figure 4. Detailed analysis of phenotype induced by *gas3/PMP22* overexpression in NIH-3T3 fibroblasts. (A,B) Confocal analysis showing membrane blebbing in NIH-3T3 cell overexpressing *hgas3/PMP22*. NIH-3T3 cells 24 hr after seeding were microinjected with pGDSV7-*hgas3/PMP22* (50 ng/ μ l) and pGDSV7-*gas2* (10 ng/ μ l). After 24 hr cells were fixed and processed for immunofluorescence analysis to visualize Gas2 (A) and stained with phalloidin-TRITC conjugated to visualize actin filaments (B). Each image represents a section from the basal through the apical regions of the cells. Numbers represent distance from the basal plane. Bar, 7.5 μm . (C,D) Confocal analysis showing altered cellular and nuclear morphology in NIH-3T3 cells overexpressing *hgas3/PMP22*. NIH-3T3 cells 24 hr after seeding were microinjected with pGDSV7-*hgas3/PMP22* (50 ng/ μ l). After 24 hr cells were fixed and processed for immunofluorescence analysis to visualize hGas3/PMP22 (C) and stained with propidium iodide to visualize nuclei (D). The arrow indicates altered nuclei with condensed chromatin. Bar, 7.5 μm .

tion of total cells recovered under the same experimental procedure, pGDSV7-*gas2* was microinjected in parallel. As reported in Table 1, overexpression of *gas3/PMP22* drastically decreases the number of injected cells that are recovered for analysis as compared to the control *gas2*. A significant fraction of recovered Gas3/PMP22-positive cells presented altered nuclear morphology. Both the described effects and quantitative results are consistent with an apoptotic process induced by *gas3/PMP22*. Alteration in nuclear morphology was thus selected as the parameter to quantitate the phenomenon.

Overexpression of gas3/PMP22 CMT1A point mutations shows a significantly reduced apoptotic-like phenotype

Different point mutations in the human *gas3/PMP22* gene have been described in CMT1A patients (Patel and Lupski 1994). The mutations described code for single amino acid substitutions within one of the four putative transmembrane domains of Gas3/PMP22. We evaluated the possibility that such point mutations behave differently from the wild type in their potency to confer the described apoptotic-like phenotype, as quantitated by the degree of nuclear alteration. Mutant human *gas3/PMP22* cDNAs were constructed by introducing point mutations as found in human CMT1A (*L16P*, *S79C*, *T118M*) and one mutation carried by mice with the Trembler phenotype (*G150D*) (Suter et al. 1992a), all cloned in the same expression vector.

We first analyzed the expression of the various mutated products by Western analysis of COS-7 cells transfected with the respective constructs (Fig. 5A). After treatment with PNGase F, a band of 18 kD with the same relative size as found in human phrenic nerve was detected in COS-7 cells transfected with the different *gas3/PMP22* constructs but not in mock-transfected

cells. The relative expression levels of the different point mutations were comparable with the wild type.

NIH-3T3 cells overexpressing point mutation *L16P*, as an example of the different point mutations, show both normal phenotype (Fig. 5B) and normal nuclear morphology (Fig. 5C). In contrast, cells overexpressing wild-type *gas3/PMP22*, under the same experimental conditions show the previously described altered phenotype (rounded cells) (Fig. 5D), with highly condensed and altered nuclei (Fig. 5E).

We next performed a microinjection analysis using all of the different point mutations. Wild-type or mutant cDNA cloned in the pGDSV7 expression vector was microinjected at a concentration of 50 ng/μl in growing NIH-3T3 cells. At various times after microinjection, cells were fixed and processed for immunofluorescence to detect Gas3/PMP22. A change in nuclear morphology, as revealed by Hoechst 33342 staining (Fig. 5E), was used to quantitate the apoptotic-like phenotype.

Figure 5F shows the relative ability of the mutants to induce the apoptotic-like phenotype in NIH-3T3 cells, as compared to the wild type. All of the point mutations analyzed show significantly decreased ability to induce nuclear condensation with respect to the wild type.

Coexpression of the wild-type and mutants gas3/PMP22 in NIH-3T3 cells correlates with the apoptotic-like phenotype and Mendelian inheritance

The described point mutations of *gas3/PMP22* fall into two distinct groups according to the mode of inheritance: *L16P*, *S79C*, *G150D* are dominant, whereas *T118M* is the only recessive point mutation described (Roa et al. 1993c). All of the point mutations tested by overexpression have significantly reduced potency in inducing the apoptotic-like phenotype with respect to the wild type. We thus asked whether a correlation could exist between the Mendelian segregation of the mutations and their ability to be dominant or recessive with respect to the apoptotic-like phenotype induced by the wild-type product.

The different mutant plasmids (25 ng/μl) were injected into the nucleus of NIH-3T3 cells together with the same concentration of wild-type plasmid. An immunofluorescence assay was performed 24 hr later, and the results were compared to overexpression of wild-type or the single mutants alone (at a concentration of 50 ng/μl) performed in parallel. As shown in Figure 6, only coexpression of the recessive *T118M* mutation with a *gas3/PMP22* wild-type construct is as efficient as the wild type alone in inducing the apoptotic-like phenotype. *L16P*, *S79C*, and *G150D* mutations, dominant in the CMT1A disease, are also dominant with respect to the ability of the wild type to induce the apoptotic-like phenotype. These mutated products thus behave dominant negatively.

Discussion

The biological function of Gas3/PMP22 protein is still

Table 1. Relationship between cell recovery and altered nuclear morphology after *gas3/PMP22* overexpression in NIH-3T3 cells

Injected plasmid	Recovered cells (%)	Cells showing altered nuclear morphology (%)
pGDSV7- <i>gas3</i>	16.8 ± 1.0	54.2 ± 5.4
pGDSV7- <i>gas2</i>	82.0 ± 4.3	0

Nuclear microinjection of pGDSV7-*gas3/PMP22* (50 ng/μl) and pGDSV7-*gas2* (50 ng/μl) was performed in NIH-3T3 cells. After 24 hr, cells were fixed and analyzed by double immunofluorescence using specific antibodies and FITC-conjugated secondary antibody. The data (mean ± s.d.) shown are percentage of FITC-positive cells among the number of injected cells. The altered nuclear morphology as detected by Hoechst 33342 staining is reported as percentage with respect to the recovered cells for both Gas3/PMP22 and Gas-2-positive cells. Cells (250) were injected in each experiment, and data were collected from three independent experiments.

Figure 5. Overexpression of *gas3/PMP22* CMT1A point mutations in NIH-3T3 cells. (A) Western blot analysis of *hgas3/PMP22* wild-type and different point mutations as expressed by transfected COS-7 cells. (B,C) Immunofluorescence analysis of NIH-3T3 cells overexpressing *hgas3/PMP22* point mutations. Exponentially growing NIH-3T3 cells were microinjected with pGDSV7-*L16P* (50 ng/ μ l). After 24 hr cells were fixed and processed for immunofluorescence analysis to visualize *hgas3/PMP22/L16P* mutant (B) and stained with Hoechst 33342 to visualize nuclei (C). (D,E) Exponentially growing NIH-3T3 cells were microinjected with pGDSV7-*hgas3/PMP22* (50 ng/ml). After 24 hr cells were fixed and processed for immunofluorescence analysis to visualize hGas3/PMP22 (D) and stained with Hoechst 33342 to visualize nuclei (E). (F) Comparative analysis of the ability to induce an apoptotic-like phenotype in NIH-3T3 cells by the different *hgas3/PMP22* point mutations. Exponentially growing NIH-3T3 cells were microinjected with pGDSV7-*hgas3/PMP22* wild type and with different point mutations (50 ng/ μ l). After various times from microinjection cells were fixed and processed for immunofluorescence analysis to visualize hGas3/PMP22 and stained with Hoechst 33342 to visualize nuclei. Percentages of cells overexpressing hGas3/PMP22 wild-type or the different point mutations and presenting condensed nuclei (apoptotic-like phenotype) at different times from microinjection are shown in the histogram analysis. Results represented were obtained from at least three independent experiments and a minimum of 150 cells were scored for each time point. Bar, 7.5 μ m.

unknown. Its high expression level in myelin-forming Schwann cells has suggested a major structural role as a myelin protein involved in membrane compaction. However, its expression has been originally linked with growth arrest in NIH-3T3 cells (Manfioletti et al. 1990), thus suggesting a more general regulatory role.

Here we report that ectopic expression of *gas3/PMP22* cDNA in NIH-3T3 fibroblasts is associated with a general alteration in cellular shape involving membrane blebbing, nuclear condensation, and cellular rounding, which are all features accompanying the apoptotic process (Kerr et al. 1971; Wyllie et al. 1980). This apoptotic-like phenotype is efficiently inhibited by addition of reducing agents in the medium, thus, indicating that the process depends on the presence of reactive oxygen intermediates, which are well known inducers of apoptosis (Hockenbery et al. 1993). Moreover, the lack of the apoptotic-like phenotype by *gas3/PMP22* overexpression in

REF-52 cells strongly suggests a cell type-specific responsiveness. This evidence could suggest that Gas3/PMP22 does not induce apoptosis directly, but permits cellular entry to a state in which apoptosis becomes more readily accessible.

When the *gas3/PMP22* point mutations associated with CMT1A and Trembler were similarly analyzed by overexpression in NIH-3T3 cells, all of the mutants showed a significantly reduced ability to trigger the apoptotic-like phenotype as compared to the wild type.

Mutations *L16P*, *S79C* (human), and *G150D* (murine), display a pathological phenotype evident in heterozygotes (dominant phenotype) (for review, see Patel and Lupski 1994). The *T118M* substitution in human Gas3/PMP22 protein is the only identified mutant allele that segregates as a recessive trait (Roa et al. 1993c). CMT1A symptoms in patients with the *T118M* substitution become evident only in the pseudodominant condition as-

Figure 6. Coexpression of wild-type and mutant *hgas3/PMP22* in NIH-3T3 cells. NIH-3T3 cells 24 hr after seeding were microinjected with pGDSV7-*hgas3/PMP22* wild-type, pGDSV7-*L16P*, pGDSV7-*S79C*, pGDSV7-*T118M*, and pGDSV7-*G150D* (50 ng/ μ l). For coexpression experiments wild-type and mutant constructs were coinjected at a concentration of 25 ng/ μ l each. Cells were fixed 24 hr after microinjection and processed for immunofluorescence analysis to visualize hGas3/PMP22 and stained with Hoechst 33342 to visualize nuclei. Percentages of cells overexpressing hGas3/PMP22 wild type together with the different point mutations and presenting condensed nuclei (apoptotic-like phenotype) are shown in the diagram analysis. Results were obtained from at least three independent experiments with a minimum of 150 cells scored for each experiment.

sociated with HNPP disease, where one copy of the *gas3/PMP22* locus on a homologous chromosome 17 is deleted (Nicholson et al. 1994). On the contrary, in the heterozygous condition *T118M*-carrying individuals are asymptomatic (Roa et al. 1993b). Given this genetic basis we have coexpressed the different mutants with the wild-type *gas3/PMP22* cDNA, thus reproducing a heterozygous condition. All dominant mutations (*L16P*, *S79C*, and *G150D*) when coexpressed with the wild type significantly suppress the typical wild-type apoptotic-like phenotype. On the contrary, coexpression of *T118M* with wild type is unable to suppress the effect of wild type, thus confirming the recessive nature of the *T118M* *gas3/PMP22* mutation. Therefore the apoptotic-like phenotype associated with wild-type *gas3/PMP22* gene product overexpression is strictly linked with the genetics of the disease suggesting that mutations *L16P*, *S79C*, and *G150D* act in a dominant negative fashion.

We can thus provocatively speculate that regulation of susceptibility to apoptosis by Gas3/PMP22 could play a relevant role in the control of Schwann cell-differentiation. Control of Schwann cell renewal *in vivo* should reflect the balance of the various alternative fates, namely continued cellular division, growth arrest, differentiation into myelin-forming Schwann cells, or lastly apoptosis. Defects in modulating cellular responsiveness to apoptosis, evidenced in the Gas3/PMP22 mutants, could thus explain continued proliferation and a consequent lack of correct differentiation as found in CMT1A. However, this function of Gas3/PMP22 does not exclude a second structural role that it may play during myelin formation or maintenance given the extremely high

level of expression of this product. This second function should only be compatible with suppression of its apoptotic modulatory function, which in turn depends on the cellular responsiveness. It is common knowledge that the capacity of an individual cell to execute a suicidal response following a death stimulus can vary markedly within a given differentiation pathway.

Possessing a dual function, both as a regulator of apoptosis and as a structural component in a given differentiation pathway, could be evolutionarily economical and logical. It is intriguing that a similar dual function in controlling apoptosis and myelin formation has been invoked for myelin proteolipid protein (PLP), which is structurally related to Gas3/PMP22 and a major component of the myelin in the central nervous system (CNS). In this case, a particular point mutation (*rumpshaker*) has allowed the dissection of these two functions of PLP (Schneider et al. 1992; Pham-Dinh et al. 1993; Kagawa et al. 1994).

As shown previously, Gas3/PMP22 protein is expressed at much lower levels in nonmyelin-forming cells or tissues. We should thus exclude here that Gas3/PMP22 exerts a myelin-specific structural role, leaving the apoptotic regulatory function as the most conceivable one.

Apoptosis should be considered as part of the homeostatic balance that regulates G_0 where Gas3/PMP22 expression is increased. It has been established that withdrawal from the cell cycle after growth factor deprivation increases cellular sensitivity toward apoptosis (Raff 1992; Hoffman and Liebermann 1994). Increased expression of Gas3/PMP22 should thus represent an obvious consequence.

Materials and methods

Gas3 expression in *Escherichia coli*

The glutathione *S*-transferase (GST)-mGas3 and GST-hGas3 fusion proteins, both comprising the region from amino acids 16 to 63 of mouse and human Gas3, were constructed by PCR using pBluescript-*mgas3* (Manfioletti et al. 1990) or pBluescript-*hgas3* (Edomi et al. 1993) as template. The sense primers oligonucleotide 16 (5'-ACTAGGATCCTGGTGCTGCTGTTCGTCTCC-3'), containing a *Bam*HI cloning site, and the reverse primer oligonucleotide 63 (5'-GCAAGAATTCTGCAGCCATTCGTTTGGTGAT-3'), containing an *Eco*RI cloning site, were used. Amplified fragments were gel purified, digested with *Bam*HI and *Eco*RI, and then subcloned in the pGEX-3X vector (Pharmacia Biotech). Fusion proteins were expressed in *E. coli* after induction with 1 mM IPTG for 3 hr, after which bacteria were pelleted and lysed. The resulting inclusion bodies were solubilized in SDS-sample buffer (2% SDS, 60 mM Tris at pH 6.8, 10% glycerol, 2% β ME) by boiling for 10 min. Proteins were separated on a preparative SDS-polyacrylamide gel, and the band corresponding to the fusion protein was electroeluted as described previously (Brancolini et al. 1992).

To obtain the maltose-binding protein (MBP)-Gas3 fusion proteins MBP-mGas3 and MBP-hGas3, the amplified fragments described above were filled in with Klenow and blunt ligated into pMALc2 vector after digestion with *Bam*HI and *Eco*RI (New England Biolabs). Expression of the fusion proteins in *E. coli* was achieved by induction with 1 mM IPTG for 1 hr.

MBP-Gas3 fusion proteins were purified from bacteria as recommended by the vendor (New England Biolabs).

Preparation of the antisera and affinity purification of antibodies

Rabbits were injected with 300 μ g of purified bacterial GST-mGas3 or GST-hGas3 fusion proteins mixed 1:1 with complete Freund's adjuvant. Subsequently they were injected with the same amount of protein in incomplete Freund's adjuvant every 3 weeks. Anti-hGas3 and anti-mGas3 antibodies were affinity purified, by means of Affi-Prep 10 support (Bio-Rad Laboratories) coupled to fusion proteins MBP-hGas3 and MBP-mGas3. The respective fusion protein (1 mg) was coupled to Affi-Prep 10 support, and the affinity matrix was used to obtain anti-hGas3 and anti-mGas3 antibodies. Immune serum (2 ml) was incubated batchwise with the matrix, and after extensive PBS washing in the column, the antibodies were eluted with 0.25 M glycine at pH 2.8. Fractions of 400 μ l were collected and neutralized with 100 μ l of 2 M Tris at pH 8.

Construction of gas3/PMP22 wild type and mutants for expression

For expression in eukaryotic cells the *hgas3* open reading frame (ORF) was subcloned in the pGDSV7 vector (Del Sal et al. 1994). ORF *hgas3* was constructed by PCR using pBluescript-*hgas3* (Edomi et al. 1993) as template. The sense primer oligonucleotides 5' (5'-GAGTGAATTCAACTCCGCTGAGCAGAAC-TT-3'), containing an *EcoRI* cloning site, and the reverse primer oligonucleotide 3' (5'-GAGTAAGCTTTCATCGCGT TTCCG-CAAGA-3'), containing a *HindIII* cloning site were used. The amplified fragment was gel purified, digested with *EcoRI* and *HindIII*, and subcloned in the pGDSV7 vector (Del Sal et al. 1994).

A site-directed mutagenesis method by overlap extension through PCR (Ho et al. 1989) was used to produce the point mutation forms of ORF *hgas3*. As external primers the oligonucleotide 5' and 3' were used. The pair of complementary inverse oligonucleotides containing the mutated nucleotide were the following:

for the mutant L $\rightarrow$ P at position 16,

ol 1 (5'-TCGCGGTGCCGGTGCTGCT-3')
ol 1' (5'-AGCAGCACCGGCACCGCA-3')

for the mutant S $\rightarrow$ C at position 79,

ol 2 (5'-GCATTCTGTGTCTGTTCT-3')
ol 2' (5'-AGGAACAGACACAGAATGC-3')

for the mutant T $\rightarrow$ M at position 118,

ol 3 (5'-CCATCTACATGGTGAGGCA-3')
ol 3' (5'-TGCCTCACCATGTAGATGG-3')

for the mutant G $\rightarrow$ D at position 150,

ol 4 (5'-TTCTCAGCGATGTCATCTA-3')
ol 4' (5'-TAGATGACATCGCTGAGAA-3')

All constructs generated were sequenced using an automated (ALF) system to check for the respective mutations introduced and the translating fidelity of the inserted PCR fragments.

Cell lines and culture conditions

NIH-3T3, REF-52, and COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal

calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μ g/ml). In each experiment 4×10^4 cells/ml were seeded in 35-mm petri dishes.

For serum starvation, medium was changed to 0.5% FCS when NIH-3T3 cells were subconfluent; cells were then left in this medium for 48 hr. After incubation with 50 μ M bromodeoxyuridine (BrdU) for an additional 1 hr, <3% of the nuclei was labeled.

For density-dependent inhibition 24 hr after plating (considered as the starting point for growing cells) the medium was changed every 2 days. After 4 days in culture, incubation with BrdU for 2 hr resulted in 5% incorporation in the nuclei.

Transfection experiments in COS-7 cells were performed using the DEAE-dextran method. Cells were grown in 35-mm petri dishes, washed twice with DMEM, and added with 400 μ l of DMEM containing 0.5 mg/ml of DEAE-dextran and 1.2 μ g of DNA. After incubation at 37°C for 30 min, DMEM containing 10% FCS and chloroquine (100 μ M) was added and incubation was prolonged for 4 hr. Cells were then washed twice with DMEM and cultured for 2 days in DMEM containing 10% FCS.

Immunoblotting

Preparation of cellular or organ extracts was performed as described previously (Brancolini et al. 1992). PNGase F treatment lysates were prepared in 0.5% SDS, 1% β ME, and boiled for 10 min. Samples were then made 50 mM sodium phosphate at pH 7.5, 1% NP-40, and 500 NEB (New England Biolabs) units of N-glycosidase F (PNGase F) (New England Biolabs) was added. Incubation was performed for 2 hr at 37°C. After adding SDS-sample buffer, protein samples were separated by SDS-PAGE using 10% polyacrylamide gels and Tris/tricine buffer as described (Schagger and von Jagow 1987).

For Western blotting proteins were transferred to 0.2- μ m-pore-sized nitrocellulose (S&S) using a semidry blotting apparatus (Bio-Rad) (transfer buffer, 20% methanol, 48 mM Tris, 39 mM glycine, 0.0375% SDS). After staining with Ponceau S, the nitrocellulose sheets were saturated for 2 hr in PBS, 0.5% casein, and 0.3% Tween 20 and incubated overnight at room temperature in the same buffer with the anti-Gas3 antibody. Blots were then rinsed three times with PBS, 0.3% Tween 20, reacted with alkaline phosphatase-conjugated goat anti-rabbit antibody for 2 hr at room temperature, and treated for chemiluminescent detection as recommended by the vendor (Tropix, Bedford, MA). Mouse mAb P07 (Archelos et al. 1993) against PNS myelin protein P0 was kindly provided from L. Wrabetz (Istituto Scientifico S. Raffaele, Milan, Italy).

Immunofluorescence microscopy

For indirect immunofluorescence microscopy, NIH-3T3 or REF-52 cells were grown under the described conditions and then fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were washed with PBS/0.1 M glycine at pH 7.5 and then permeabilized with cold acetone for 1 min at -20°C . The coverslips were treated with the first antibody (anti-hGas3/PMP22 diluted in PBS 3% BSA) for 1 hr in a moist chamber at 37°C. To detect Gas2 protein, NIH-3T3 cells were incubated with a rabbit anti-Gas2 antibody (Brancolini et al. 1992). They were then washed with PBS three times, followed by incubation with FITC-conjugated anti-rabbit second antibody (Dako) for 1 hr at 37°C. For detection of actin filaments, TRITC-phalloidin (Sigma) was used. Nuclei were labeled with Hoechst 33342 dye or propidium iodide (5 μ M) (Sigma). Finally, cells were examined by epifluorescence with a Zeiss Axiovert 35 microscope or a Zeiss laser scan microscope (LSM 410)

equipped with a 488 λ argon laser and a 543 λ helium neon laser. The following set of filters were used: rhodamine (BP546, FT580, LP 590), fluorescein (450–490, FT 510, LP520), and Hoechst 33342 (BP 365, FT 395, LP 397).

Microinjection

Microinjection was performed using the Automated Injection System (Zeiss Oberkochen, Germany) as described previously (Del Sal et al. 1992). Cells were injected with 50 ng/ μ l of expression vector (Del Sal et al. 1994). Each cell was injected for 0.3 sec at a constant pressure of 150 hecto-Pascal. When used, *N*-acetyl-cysteine 30 mM (Sigma) or ascorbic acid 250 μ M (Sigma) was added to the culture medium immediately after microinjection.

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