Lack of Replicative Senescence in Normal Rodent Glia

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Replicative senescence is thought to be an intrinsic mechanism for limiting the proliferative life-span of normal somatic cells. We show here that rat Schwann cells can be expanded indefinitely in culture while maintaining checkpoints normally lost during the immortalization process. These findings demonstrate that senescence is not an inevitable consequence of extended proliferation in culture.

In contrast to germ cells and certain stem cells, most somatic cell types are thought to have a limited proliferative life-span. This limit may have evolved as a protective mechanism against cancer, although it may also cause accumulation of cells at the end of their proliferative life-span that may be responsible for certain aspects of the aging process (*1*).

Limitation of proliferative life-span is thought to be controlled by intrinsic mechanisms that dictate the number of times a particular cell can divide. In some human cells, such as fibroblasts, the mitotic counting mechanism that determines life-span appears to be the progressive shortening of telomeres, which occurs upon each division in cells lacking telomerase activity (*2*). In contrast, telomere-independent mechanisms involving the induction of the tumor suppressor protein p53 and the cyclin-dependent kinase inhibitors (CDKIs) $p16^{INK4A}$, $p19^{ARF}$, and $p21^{Cip1}$ appear to be involved in triggering a premature growth arrest in rodent cells and in some other human cell types (*3, 4*).

Schwann cells are the glial cells of the peripheral nervous system. Upon dissociation from the nerve and explantation into culture, the cells reenter the cell cycle and can be expanded in culture with specific mitogens (*5*). To study the proliferative life-span of Schwann cells, we used cells purified from the sciatic nerves of 7-day-old rats. In addition to the Schwann cells, we simultaneously isolated the other major cell type of the sciatic nerve, perineural fibroblasts, as a control cell type with a predicted limited proliferative life-span (Fig. 1). To characterize the long-term proliferative potential of Schwann cells and fibroblasts, we measured the proliferation of both cell types in culture. The proliferation rate of the Schwann cells remained constant with continual passaging (Fig. 1). At no point did any batch of Schwann cells enter a period of slow growth characteristic of entry into senescence. Five batches of Schwann cells have been maintained in culture for at least 50 passages [75 population doublings (PDs)]. This is equivalent to each cell giving rise to $>10^{20}$ cells. Late-passage Schwann cells were morphologically indistinguishable from early-passage cells, exhibited a similar requirement for mitogens, and maintained a diploid status, as measured by fluorescence-activated cell sorting analysis (*6*).

In contrast, the fibroblasts, which initially proliferated at a faster rate than the Schwann cells, ceased proliferating by passage 3 or 4 (~8 PDs) (Fig. 1). Coincident with the cell cycle arrest, the cells developed the large, flattened morphological phenotype characteristic of replicative senescence (Fig. 2A). The continual passaging of each batch of fibroblasts tested (seven in total) resulted in the eventual outgrowth of immortalized cells. Three of these immortalized batches (Imm1, Imm2, and Imm3) were further analyzed and have undergone more than 100 PDs. Each immortalized cell line had a distinctive morphology and proliferative rate, consistent with a clonal origin.

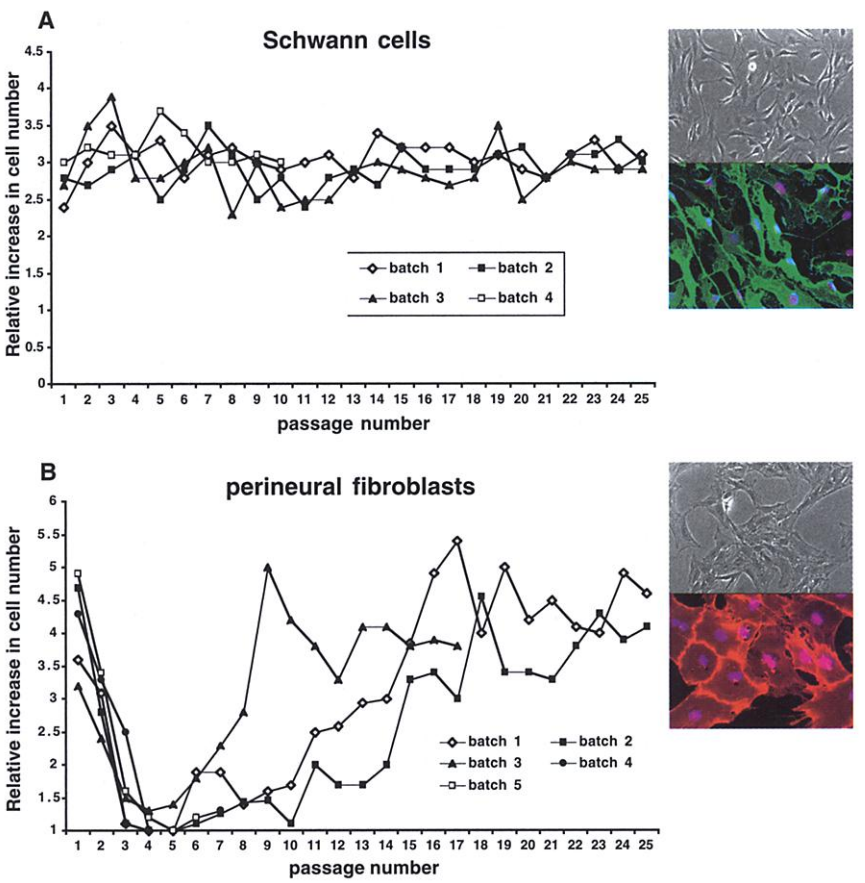

Fig. 1. Schwann cells maintain a constant proliferative rate in culture. Fibroblasts were separated from the Schwann cells by sequential immunopanning with an antibody recognizing Thy1.1, a marker of rat fibroblasts, as described (*18*). (**A**) The unbound population was shown to be 99.9% Schwann cells by immunostaining for S100 (*6*) and for the low-affinity nerve growth factor receptor. (**B**) The purified fibroblasts appeared homogeneous, had a morphology distinct from that of the Schwann cells, and were 99.6% Thy1.1-positive. Fibroblasts were cultured in Dulbecco's minimum essential medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Autogen Bioclear). Schwann cells were grown on poly-L-lysine (Sigma)–coated dishes in DMEM containing glucose (1.5 mg/ml; Gibco), supplemented with 3% FBS, forskolin (1 μM; Calbiochem), and β-neuregulin (20 ng/ml, R&D) at 10% CO_2 and 20% O_2. A modified 3T3 assay was carried out on Schwann cells (A) and fibroblasts (B). Schwann cells (4.8×10^5) and fibroblasts (5×10^5) were plated in triplicate onto 9-cm dishes under the conditions detailed above. Every 3 days, cells were trypsinized, counted with a Coulter counter, and reseeded at constant density. Each point is the mean of triplicate counts.

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The senescent phenotypes seen in rodent and human cells are associated with the induction of a particular isoform of β-galactosidase (SA–β-Gal) and with defects in mitosis that result in multinucleated and giant cells (*7*). The cell cycle–arrested fibroblasts (passages 4 to 10), but not earlier-passage cells, stained strongly for SA–β-Gal, consistent with the cells being in a senescent-like state (Fig. 2A). In addition, these cells had enlarged nuclei and were frequently multinucleated (30 to 40%). In contrast, SA–β-Gal staining was not detectable in Schwann cell cultures, even after extended passaging (Fig. 2A). The incidence of multinucleated and giant cells was consistently very low (<1%).

Primary cells exhibit a senescent-like phenotype after prolonged inhibition of the cell cycle by DNA damage or by overexpression of CDKIs (*8, 9*). Treatment of Schwann cells with aphidicolin, an inhibitor of DNA polymerase that blocks cells in or at S phase, resulted in a senescent-like morphology and positive SA–β-Gal staining (Fig. 2A). Thus, Schwann cells are capable of acquiring a senescence-like phenotype upon prolonged activation of a cell cycle checkpoint.

It has recently been suggested that senescence is triggered in rodent cells and some human cells as a result of "culture shock" rather than by an intrinsic self-limiting mechanism (*4, 10*). To test this hypothesis, we cultured the Schwann cells in different culture conditions. When cultured in high serum (20%), Schwann cells initially proliferated at a faster rate than Schwann cells in low serum (*6*). However, after 2 weeks of continual passaging, senescent-like cells started to appear in the cultures. These cells were frequently multinucleated and stained positively for SA–β-Gal (Fig. 2A) (*11*). These results show that culture conditions can differentially trigger a premature growth arrest, and thus senescence is not per se the result of long-term proliferation in culture.

Plating cells at low density resulted in the outgrowth of clones at very high efficiency (>50%) (*12*). Moreover, the cloning efficiency did not vary with the passage number of the seeded cells. Six of these clones were expanded (>50 PDs) and analyzed further. The clones appeared homogeneous, and their morphology, proliferation rate, and requirement for mitogens were indistinguishable from those of the pooled Schwann cells (*6*). This observation, in addition to the constant-growth kinetics of the pooled cells, is consistent with Schwann cells not requiring a genetic event to acquire an unlimited proliferative life-span.

In some human cells, telomere length is critical for determining proliferative life-span. This is not thought to be true in mouse cells because they express active telomerase, the enzyme responsible for maintaining telomeres. Telomeric repeat amplification protocol (TRAP) assays carried out on early- and late-passage rat Schwann cells and early, senescent, and immortalized fibroblasts showed that both cell types maintained a high level of telomerase activity, comparable to the levels in human tumor cell lines (Fig. 2B) (*6*). In addition, measurement of telomere length in the two cell types showed that they had similar-length telomeres that were maintained in long-term culture (*6*). Thus, different capacities for telomere maintenance cannot account for the proliferative capacities of the two cell types.

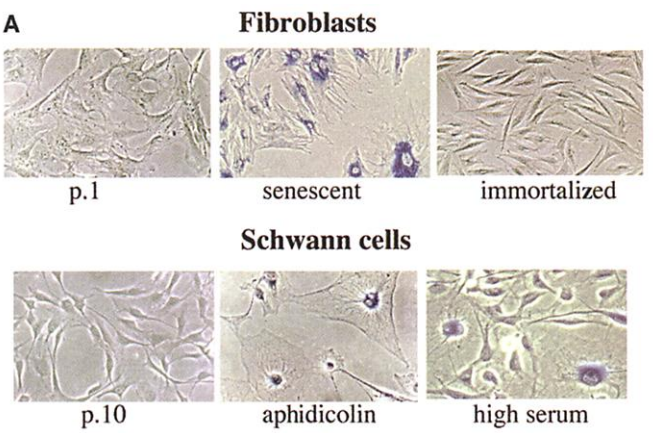

Fig. 2. Schwann cells do not exhibit a senescent phenotype in culture. (**A**) Expression of the senescence marker SA–β-Gal was detected by X-Gal staining at pH 6 (*7*). Early-passage fibroblasts [upper panel, passage 1 (p.1)] were negative. Fibroblasts were positive between passages 4 and 10 (upper panel, center). Immortalized fibroblasts (upper panel, right) did not stain. Schwann cells at any passage (represented by p.10, lower panel) did not express detectable SA–β-Gal activity. Schwann cells were treated with aphidicolin (1 μg/ml) for 5 days or cultured continuously for 2 to 3 weeks in Schwann cell medium supplemented with 20% FBS. The cells developed a senescence-like morphology and stained positive for SA–β-Gal [see also (*11*)]. (**B**) Schwann cells and fibroblasts express active telomerase. Extracts, equivalent to 5000 and 500 cells, from early-passage fibroblasts or Schwann cells were assayed for telomerase activity using a standard TRAP assay (*19*). H, heat-inactivated control.

The induction of p53 and the CDKIs $p16^{INK4A}$, $p19^{ARF}$, and $p21^{Cip1}$ is associated with the onset of senescence and is considered to be responsible for the resulting cell cycle arrest. $p16^{INK4A}$ and $p19^{ARF}$ were induced to similar levels in Schwann cells and fibroblasts upon passaging in culture (Fig. 3, A and B). No changes in p21, p27, or p53 levels were observed in either cell type (*6*). In the fibroblasts, expression of $p16^{INK4A}$ was detectable at passage 3, concurrent with the onset of a growth arrest consistent with a role for $p16^{INK4A}$ in cell cycle withdrawal. In the Schwann cells, $p16^{INK4A}$ was induced at a later stage in culture (passage 8, 12 PDs) but was not sufficient to cause a cell cycle arrest, as no change in proliferation rate was observed at this point. In both cell types, the levels of $p16^{INK4A}$ remained fairly constant once induced. In both Schwann cells and fibroblasts, $p19^{ARF}$ expression was induced rapidly and to similar levels after transfer into culture (Fig. 3B). In the fibroblasts, detectable levels were evident after only 3 days in culture, when cells were proliferat-

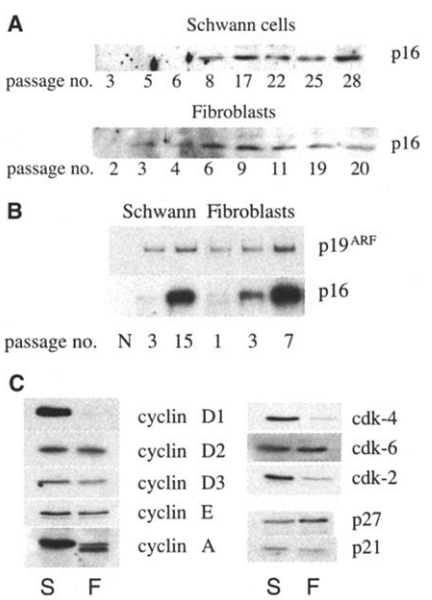

Fig. 3. The cyclin-dependent kinase inhibitors $p16^{INK4A}$ and $p19^{ARF}$ are induced in fibroblasts and Schwann cells upon in vitro culture. (**A**) $p16^{INK4A}$ protein levels were analyzed by Western blotting in Schwann cells and fibroblasts. (**B**) $p19^{ARF}$ and $p16^{INK4A}$ RNA levels were analyzed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). RNA extracts, made from cells at various stages in culture and from freshly dissected rat sciatic nerves (containing both Schwann cells and fibroblasts) (N), were used to make cDNA. Levels were equalized for glyceraldehyde phosphate dehydrogenase (GAPDH) expression. (**C**) Lysates prepared from proliferating, early-passage Schwann cells (S) and fibroblasts (F) were analyzed by Western blotting for cyclin, CDK, and CDKI expression levels using the antibodies described (*11*).

ing rapidly, consistent with previous reports for mouse embryo fibroblasts (*13*). A comparison of the expression levels of cyclins and CDKs in low-passage, proliferating Schwann cells and fibroblasts revealed differences between the two cell types. In particular, Schwann cells expressed much higher levels of cyclin D1, Cdk-4, and Cdk-2 than did the fibroblasts (Fig. 3C). The higher levels of cyclins and CDKs may explain the insensitivity of the Schwann cells to $p16^{INK4A}$ and $p19^{ARF}$ expression.

The genetic changes responsible for spontaneous immortalization in culture have been well characterized. A study of spontaneously immortalized mouse embryo fibroblast cell lines revealed that 75% had acquired p53 mutations, whereas the remaining 25% had lost $p16^{INK4A}$ and $p19^{ARF}$ expression (*14*). We tested whether the late-passage Schwann cells retained these checkpoints; this would provide further evidence that the cells have not been genetically selected for passage through senescence. All six Schwann cell clones expressed $p16^{INK4A}$ and $p19^{ARF}$ at similar levels to those of late-passage cells (Fig. 4A). However, one of the three immortalized batches of fibroblasts (Imm1) had lost both $p16^{INK4A}$ and $p19^{ARF}$ expression (Fig. 4A).

Western blotting of the six Schwann cell clones and late-passage pooled cells revealed low, barely detectable levels of p53 (*6*). This result suggested that the Schwann cells express the normal, active form of the protein, because the mutant forms of p53 are expressed at higher levels than the wild-type protein. To verify the expression of normal, active p53, we determined whether the cells maintained intact p53-dependent checkpoints. X-ray irradiation, which results in a p53-dependent cell cycle arrest of primary cells, caused cell cycle arrest in the six Schwann cell clones and early- and late-passage pools (Fig. 4B). As expected, Schwann cells expressing dominant negative p53 (dn-p53) were completely refractory (*15*). Two of the three immortalized fibroblast cell lines (Imm1 and Imm3) arrested normally in response to x-ray exposure, whereas Imm2 fibroblasts had an attenuated response, indicating a defect in a p53 signaling pathway in these cells (Fig. 4B).

Expression of oncogenic Ras in most primary cells results in a G_1 cell cycle arrest, whereas this arrest is not observed in most immortalized cells, which are efficiently transformed by activated Ras (*15–17*). Using retroviral vectors, we expressed H-*ras*V12 in early- and late-passage Schwann cells and in Schwann cells expressing dn-p53. Ras inhibited DNA synthesis to similar extents in early- and late-passage Schwann cells (Fig. 4B) and in all six Schwann cell clones (*6*) but did not inhibit Schwann cells expressing dn-p53. Thus, late-passage cells have not acquired any of the mutations that commonly result in immortalization and that cooperate with activated Ras. Similar experiments with the immortalized fibroblasts showed that two of the three cell lines (Imm1 and Imm3) proliferated at a faster rate after Ras expression (Fig. 4B) and therefore have undergone genetic changes that permit Ras transformation. Imm1 fibroblasts have lost $p16^{INK4A}$ and $p19^{ARF}$ and hence would be predicted to be transformable by Ras. Imm3 fibroblasts, however, maintained $p16^{INK4A}$ and $p19^{ARF}$ expression and had an intact p53-dependent checkpoint. We have not as yet been able to determine the genetic lesion responsible for bypassing senescence and permitting Ras transformation in these cells. Imm2 cells undergo cell cycle arrest in response to Ras despite having a defective p53 response to DNA damage, which suggests a mutation in the damage checkpoint pathways independent of Ras signaling.

Our data show that rat Schwann cells appear to have the capacity for unlimited proliferation, whereas fibroblasts isolated from the same nerves undergo the classical replicative senescence seen in rodent fibroblasts. In keeping with previous studies, the fibroblasts that emerge from this premature growth arrest have undergone alterations in their normal checkpoint controls, whereas Schwann cells expanded extensively in culture retain all the checkpoints commonly lost upon immortalization.

If it is important to limit the proliferative life-span of cells, it is surprising that two cell types from the same tissue have such disparate proliferative capacities. This difference may reflect distinct functions of the two cell types in vivo. Alternatively, it is possible that culture conditions determine proliferative life-span and that any cell (expressing telomerase activity) can be induced to proliferate indefinitely if the appropriate culture conditions are found. Our findings that Schwann cells can be induced to enter a senescence-like proliferative arrest when cultured in high serum are consistent with this idea. Studies of human cells suggest that if telomerase expression is induced, certain cell types can also proliferate indefinitely. It is possible that human cell types that require genetic changes in addition to telomerase activation for sustained proliferation are behaving like rodent fibroblasts, and that they would behave differently in altered culture conditions. Whether cell life-span, excluding the role played by telomere shortening, is controlled by intrinsic mechanisms or by culture conditions will be critical in

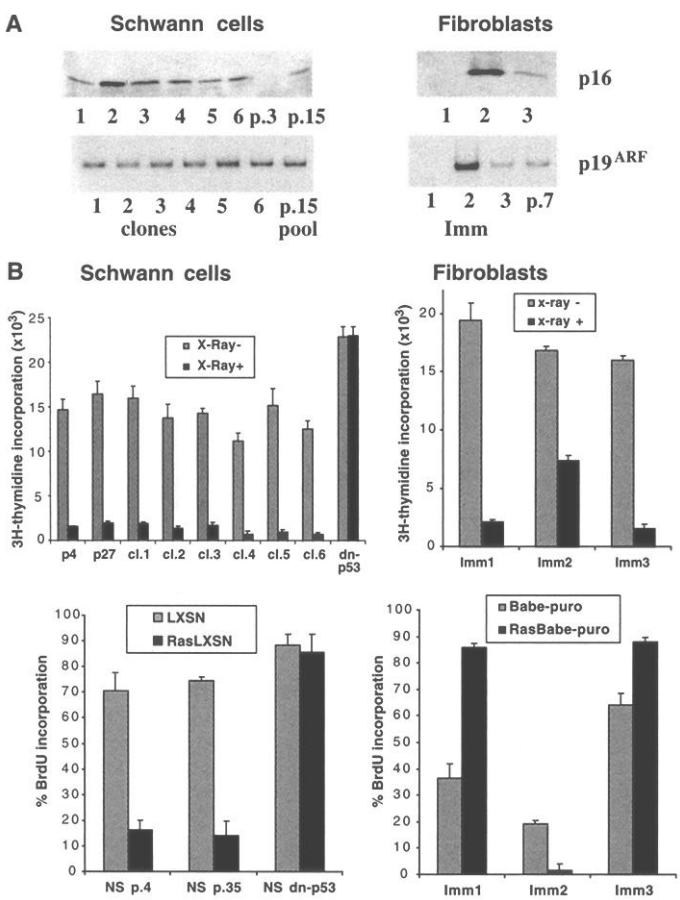

Fig. 4. Schwann cells maintain cell cycle checkpoints throughout long-term culture. (**A**) Six Schwann cell clones and early- and late-passage pooled cells were analyzed for expression of $p16^{INK4A}$ by Western blotting and for $p19^{ARF}$ mRNA by semiquantitative RT-PCR. The immortalized fibroblasts (Imm1 to 3) were similarly analyzed. (**B**) Schwann cells and fibroblasts were treated with x-rays (12 Gy). Twenty hours after irradiation, [^{3}H]thymidine (0.5 μCi/ml) was added for 4 hours. Schwann cells and fibroblasts were infected with retroviruses constructed to express activated Ras or empty vector. After drug selection, bromodeoxyuridine (BrdU) incorporation was assessed by immunofluorescence after a pulse of 16 hours (Schwann cells) or 9 hours (fibroblasts). Each experiment was carried out in triplicate, with a minimum of 600 cells counted from each experiment.

determining our ability to culture these cell types in the future.

A finite proliferative capacity has been proposed to be an intrinsic property of normal cells, acting as a key regulatory mechanism for controlling inappropriate proliferation. Our results indicate that a reassessment of these ideas is required.

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Control of Fusion Pore Dynamics During Exocytosis by Munc18

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Intracellular membrane fusion is mediated by the SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) proteins. All vesicle transport steps also have an essential requirement for a member of the Sec1 protein family, including the neuronal Munc18-1 (also known as nSec1) in regulated exocytosis. Here, in adrenal chromaffin cells, we expressed a Munc18 mutant with reduced affinity for syntaxin, which specifically modified the kinetics of single-granule exocytotic release events, consistent with an acceleration of fusion pore expansion. Thus, Munc18 functions in a late stage in the fusion process, where its dissociation from syntaxin determines the kinetics of postfusion events.

Fusion of vesicles with target membranes is a key aspect of vesicular traffic and neurotransmitter release and is mediated by a core machinery of SNARE proteins (*1–3*). The three synaptic SNAREs (syntaxin 1, SNAP-25, and VAMP) are sufficient for bilayer fusion in vitro (*4*). This is, however, orders of magnitude slower than synaptic vesicle fusion, suggesting a role for additional factors (*5*). Members of the Sec1 family of proteins are required for all intracellular vesicular traffic steps (*6–10*), and the neuronal Munc18-1 protein (*11, 12*) is essential for synaptic vesicle exocytosis but not for constitutive exocytosis (*6, 8*). It binds to a closed conformation of syntaxin 1, which is unable to participate in the SNARE complex (*13–15*) and thus may control the addition of syntaxin into the complex. In current models, syntaxin assembles into a "loose" SNARE complex with Munc18 dissociation and the subsequent zippering of SNAREs into a "tight" complex immediately preceding membrane fusion (*2, 16*). Although SNARE complex assembly is crucial for membrane fusion, it is not known whether the complete assembly of the SNARE complex occurs before, during, or after fusion. Fusion proceeds via the formation of a reversible fusion pore (*17*), but the relation of the function of the SNARE proteins or Sec1 proteins to fusion pore opening and expansion is unknown.

We examined the effect of overexpression of wild-type Munc18 or a mutant form, $Arg^{39} \rightarrow Cys^{39}$ (R39C), on exocytosis using single-cell amperometry to resolve the frequency and kinetics of individual secretory granule release events. The R39C mutation was investigated for two reasons. First, the equivalent mutation in the *Drosophila* ortholog Rop, $Arg^{50} \rightarrow Cys^{50}$ in the F3 mutant (*9*), produces flies showing an increase in evoked neurotransmission (*8*). Second, the crystal structure of the Munc18–syntaxin 1 complex has revealed that Arg^{39} makes direct contact with Glu^{234} of syntaxin, leading to the prediction that the R39C mutation should weaken the binding interaction between the two proteins (*18*). A reduction of high-affinity binding of Munc18 containing the R39C mutation to syntaxin 1 was confirmed with an in vitro binding assay (*11, 19*) (Fig. 1A). When binding was assayed over a range of Munc18 protein concentrations (2 to 109 nM), the affinity of R39C for syntaxin was reduced by about fivefold in comparison to the wild type, from 6.5 to 35 nM, respectively. The R39C mutant (Fig. 1B) still bound the other Munc18 binding proteins Doc2 (*20*) and Mint1 (*21*) from a rat brain extract (*22*).

The effect of wild-type or R39C Munc18 on overall dense-core granule exocytosis was first assayed in PC12 cells by using transfection and coexpression of growth hormone (GH) (*23–25*). Transfection resulted in an ~10-fold overexpression of wild-type Munc18 (*26*). Overexpression of wild-type protein had no statistically significant effect on the extent of evoked exocytosis due to 10 μM free Ca^{2+} in permeabilized cells, but the R39C construct produced a significant (55%) inhibition (Fig. 1D). The difference in effect between the two proteins was not due to differences in expression, as both were expressed in virtually all transfected cells (Fig. 1C). We analyzed the effect of overexpression of the proteins in adrenal chromaffin cells using carbon-fiber amperometry to allow direct analysis of single-granule release events (*27–30*). Transfected chromaffin cells were detected by coexpression of green fluorescent protein (GFP), allowing untransfected cells in the same dishes to be used as controls. This ensured that all cells had been through the transfection protocol, and the same carbon fibers were used to record from transfected and control cells in each series of experiments. The cells were stimulated by local application of digitonin and Ca^{2+} to permeabilize the cells and allow Ca^{2+} to directly activate exocytosis (*24, 31*). Because the granules in these cells have a half-life of >15 days (*32*), this assay measured release

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