Neurosecretion Competence, an Independently Regulated Trait of the Neurosecretory Cell Phenotype*

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Neurosecretion competence is intended as the ability of neurosecretory cells to express dense and clear vesicles discharged by regulated exocytosis (neurotransmitter release). Such a property, which so far has never been studied independently, is investigated here by a heterotypic cell fusion approach, using a clone of rat pheochromocytoma PC12 cells totally incompetent for neurosecretion that still largely maintains its typical molecular and cellular phenotype. When fused with wild-type partners of various species (rat, human) and specialization (PC12, neuroblastoma SH-SY5Y, HeLa), the defective cells reacquire their competence as revealed by the expression of their secretion-specific proteins. Fused wild-type cells therefore appear able to complement defective cells by providing them with factor(s) inducing the reactivation of their secretory program. The mechanism of action of these factors may consist not in a coordinate unblocking of transcription but in the prevention of a rapid post-transcriptional degradation of the mRNAs for secretion-specific genes.

Regulated exocytosis, a fundamental activity of neurosecretory cells, is among the processes most intensively investigated. So far, however, the studies have focussed primarily on the identification of the proteins involved and on the mechanistic interactions that underly vesicle discharge (1–4). In contrast, the way by which neurosecretory cells become competent for neurosecretion has received much less attention. Competence acquisition requires the appearance of specific organelles, dense-core (DV) and clear or synaptic-like (SLV) vesicles, an event that takes place during phenotype development. Because of this temporal coincidence, the first process is widely envisaged as a step of the second (5, 6).

To investigate the relationships between competence and phenotype, we used clones isolated from a neurosecretory cell model, the rat pheochromocytoma PC12 line (7, 8). Most PC12 clones, indicated here as wild-type clones, exhibit DVs from which exocytic release of secretory proteins and catecholamines occurs after appropriate stimulation. Moreover, Western blots, immunocytochemistry, and patch clamping document in these clones the expression and discharge of acetylcholine-containing SLVs (8–10). An additional clone has been found to lack en block both types of secretory vesicles, i.e. to be neurosecretion incompetent, no matter whether analyzed at rest or after a variety of stimulatory treatments, while maintaining numerous markers typical of PC12 and other neurosecretory cells (7, 8). These results suggest the lack in the defective clone of one or more factors that impart to the cells their neurosecretion competence, acting independently, at least in part, from the factors governing the rest of the phenotype (8). Here we report direct evidence supporting this hypothesis, showing that defective PC12 cells reacquire their competence when fused to various types of wild-type cells, regardless of their species of origin and of their specialization. Moreover, molecular results suggest competence to be conferred not at the transcriptional but at a post-transcriptional level.

EXPERIMENTAL PROCEDURES

Control (clones 15 and 251) and defective (clone 27) rat pheochromocytoma PC12 clones, all resistant to G418 due to their stable transfection with an appropriate construct, and the PC12-27 subclone (c7) resistant also to hygromycin-B and expressing the human isoform of chromogranin B (CgB) have been described (7, 8). Properties of the second defective clone (PC12-Trk) are given in Refs. 11 and 12. The anti-rat CgB antibodies were purified rabbit IgGs (13). The anti-human CgB was a mouse monoclonal antibody (14). The anti-rat synaptophysin (Sph) (monoclonal antibody) was purchased from Boehringer Mannheim, the anti-TrkA was from Santa Cruz, and the anti-synaptotagmin (Syt) and the anti-human β1-integrin subunit were purified rabbit IgGs (15, 16).

Microscopy—Cell fixation (with mixtures of formaldehyde and glutaraldehyde), permeabilization, immunolabeling, and confocal microscopy are specified in Ref. 17. For electron microscopy the cells detached from the dishes and centrifuged were fixed with the aldehyde mixture (15, 16).

Cell Fusions—For fusions between PC12 clones (clones 15 and 27, homotypic and heterotypic), cells were detached with a gentle stream of medium, resuspended at the concentration of 10⁶/ml, and loaded with either 5 μM Orange CMTMR or 2 μM Green CMFDA (Molecular Probes) (18). After three washes in Hanks’ balanced salt solution and mixing 50:50 of the two preparations, the cells were fused with PEG 4000 (Merek) (19). Sorting of labeled cells was in a FACS Plus (Becton Dickinson).

DNA Extraction, Northern Blotting, and RNase Protection—DNA extraction was performed according to the single-step method (20).

The abbreviations used are: DV, dense-core vesicle(s); SLV, clear or synaptic-like vesicle(s); CgB, chromogranin B; Sph, synaptophysin; Syt, synaptotagmin; FACS, fluorescence-activated cell sorter; SgII, secretogranin II.
secretory markers (7, 8), the differences do not go beyond general interclone variability (7). PC12-27 cells, however, lack not only DVs and SLVs (7) but also their cargo and membrane proteins (including CgB, Fig. 1B; SgII; VAMP-2; Syt; Sph) (7, 8), whereas in wild-type clones 15 these proteins are abundant and exhibit prominent punctate patterns (Fig. 1A). The defect of PC12-27 is not restricted to the secretory organelles but includes many (if not all) of the components participating in the regulated exocytosis/endocytosis cycle, such as the G protein, rab3a, and the tSNAREs present in the plasma membrane, syntaxin-1, and SNAP-25 (7, 8). From here on the proteins mentioned so far will be referred to as secretion-specific. In conclusion, PC12-27 do not appear as undifferentiated cells. Rather, they are PC12 that, although possessing properties typical of the neurosecretory phenotype, are totally incompetent for regulated neurosecretion.

Fusions between PC12 Cells—To establish whether neurosecretion competence operates under positive or negative control, parallel cell aliquots of the defective 27 and the control 15 PC12 clones were loaded with either orange or green fluorescent vital dyes (18), fused with PEG 4000 (19) and sorted by FACS. The cells exhibiting double fluorescence (Fig. 1F), were processed by immunolabeling (confocal microscopy) and for electron microscopy. Of these fused cells, the homotypic 27/27 never showed appearance of DVs and secretion-specific proteins (Fig. 1C and not shown), whereas the 15/15 never showed any loss of them (not shown). In contrast, over 90% of the 27/15 hybrids resembled controls in their punctate immunolabeling for DV and SLV proteins as seen already 48 h after fusion until weeks later (Fig. 1, D and E for CgB and Fig. 1F for Sph). At the electron microscope, numerous DVs were seen in the hybrids, localized preferentially below the plasma membrane (Fig. 1, G and H) as in control PC12 (23). Moreover, when a hybrid population was pulse-labeled with [35S]sulfate, release of [35S]-CgB was found to take place by typical regulated exocytosis. Such a release, in fact, required a period of chase after the pulse, necessary for the transport of labeled protein to DVs, and depolarization (with high K+) in a Ca2+-dependent manner (Fig. 2) (8). We conclude that in the hybrids the defect of PC12-27 cells had been fully complemented. This suggests neurosecretion competence to be controlled positively and not negatively.

Further information was obtained by additional inter-PC12 fusions, carried out with two defective PC12 clones, both incompetent for neurosecretion, PC12-27 (8) and PC12-Trk (11, 12). For convenience, the PC12-27 cells employed in these experiments were from a subclone (c7) stably transfected with the human CgB (localized primarily in the Golgi complex) and resistant not only to G418 but also to hygromycin-B (8). The Trk clone overexpressing the nerve growth factor receptor, TrkA, isolated from another parental population, differs from clone 27 in general morphology, responsiveness to nerve growth factor, and rate of growth (11, 12). The 27/Trk hybrids, resistant to hygromycin-B, were identified by double immunostaining for TrkA and human CgB. Their neurosecretion com-
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petence was investigated by an antibody specific for the rat isof orm of CgB, with consistently negative results. In fact, neither expression of the rat secretory protein nor changes in the distribution of the human CgB from Golgi to punctate were observed (not shown), suggesting that no complementation had taken place. The results therefore appear compatible with the possibility that the same regulatory factor(s) lacking in PC12-27 is (are) responsible also for the defect in the Trk clone.

Fusions with Human Cells—An important point that could not be established with the inter-PC12 fusion experiments is whether the competent phenotype of the hybrids resulting from the PC12-27 and PC12-15 fusions was due to the contribution of control cells, whose competence was unaffected by the fusion with defective cells, or also to competence reacquisition by the latter. In fact, no way was available to distinguish the products of control cells, whose competence was unaffected by the fusion PC12-27 and PC12-15 fusions was due to the contribution of control cells, whose competence was unaffected by the fusion with defective cells.

In the unfused PC12-27, SH-SY5Y, and HeLa cells, as well as in all homotypic fusions, no signal for DV and SLV proteins could ever be appreciated (Fig. 3A’ and not shown). In contrast, clear positivity was recorded in many but not all hybrids of both the PC12-27/SH-SY5Y (Fig. 3, B’ and C’) and the PC12-27/HeLa (Fig. 3, D’ and E’) type. Even more important, the CgB expressed by the hybrids was recognized specifically by the anti-rat antibody (Fig. 3, B’ and D’), for which human cells are negative (Fig. 3A’), with a distribution coinciding with that of the other secretion-specific proteins, including Syt (Fig. 3, C’ and E’).

In contrast with the anti-human CgB antibody, no immunostaining was appreciated. In terms of intensity and distribution, the signal for the rat CgB and the other vesicle proteins in PC12-27/SH-SY5Y hybrids resembled that of control PC12-15, whereas in PC12-27/HeLa it was less prominent and more clustered in areas that might correspond to the Golgi complex (Fig. 3, D’ and E’). In the latter, therefore, the intracellular traffic of secretion-specific proteins was possibly delayed. Still, a punctate distribution of the label was also visible (cf. Fig. 3, B’–E’ to Fig. 3, A and D–F), and typical DVs were recognized by electron microscopy (not shown). We conclude, therefore, that the neurosecretion competence established in the hybrids, no matter whether by fusion with SH-SY5Y or HeLa cells, was sustained by the expression of secretion-specific proteins encoded by genes of PC12-27 cells.

Molecular Mechanism(s) Underlying the Competence Defect—In previous studies, Northern blot assays of PC12-27 cells had revealed the mRNAs levels for several missing cargo and membrane vesicle proteins to be much lower (0–8%) than in controls, with no major changes of their long term turnover (8). These results suggested that the defect of clone 27 consists of a blockade of transcription of the secretion-specific genes. When, however, run-on experiments were carried out (Fig. 4, left panel), the results appeared incompatible with the above conclusion, because of the three transcripts investigated, the PC12-27 signal was decreased (moderately) for only one (the vesicle membrane vSNARE, VAMP-2) and unchanged or even increased by 2-fold for the others (the secretory proteins, CgB and SgII) (Fig. 4, left panel). These major discrepancies between Northern blots and run-on results appeared specific to PC12-27 cells. When in fact a nonsecretory cell type (Fisher rat fibroblasts) was analyzed, a good match between the very low or negative Northern blots for the three mRNAs and the hardly appreciable (SgII) or completely negative (CgB and VAMP-2) run-on signals was observed (not shown).

To explain the PC12-27 transcription results, a variety of control experiments were carried out. The possibility that the excess SgII run-on signal was due to transcriptional atte

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**Fig. 3.** Human neuroblastoma SH-SY5Y and epithelial HeLa cells; neurosecretion competence of hybrids obtained by their fusion with PC12-27. Top panels refer to unfused cells of the human neuroblastoma SH-SY5Y line, shown in phase contrast (a) and after immunolabeling for the human cell marker (β1-integrin, A) and the rat CgB (A’). B and C show PC12-27/SH-SY5Y hybrids, identified by their β1-integrin positivity (B and C), showing immunolabeling for rat CgB (B’) and Syt (C’). D and E, PC12-27/HeLa hybrids immunolabeled for integrins (D and E), rat CgB (D’), and Syt (E’).
tion (25) could be excluded because (a) the results obtained with the whole cDNA were duplicated using probes containing either the 5’ or the 3’ sequences (Fig. 4, left panel) and (b) no additional mRNA fragments (including unspliced or partially spliced transcripts) but only the processed bands (accounting for −5% of controls) were revealed by RNase protection (Fig. 4, right panel). Moreover, when the latter assay was carried out in PC12-27 by using a sense RNA probe, no signal appeared, thus excluding transcription of the noncoding strand (26) in the SgII gene (Fig. 4, right panel). Also unlikely appears a blockade of mRNA transport across the nuclear envelope, with ensuing retention into the nucleus. In fact, when the nuclear and cytoplasmic SgII and VAMP-2 mRNAs were assayed separately by Northern blot, their ratios were found to be the same in PC12-27 and PC12-15 clones (not shown), despite the huge differences of the absolute values.

**DISCUSSION**

Taken as a whole, our fusion results provide information about neurosecretion competence. Only when the fusion of PC12-27 cells was homotypic or with the cells of another similarly defective PC12 clone, Trk, did the resulting cells remain incompetent; otherwise they were mostly competent for neurosecretion. Moreover, the results with rat/human hybrids (fusions of PC12-27 with either SH-SY5Y or HeLa) demonstrate that the competence was sustained by the reactivation of the PC12 program with expression of gene products that, before fusion, were not expressed in the defective clone. The simplest hypothesis that emerges from our data is that one or more factor(s) that PC12-27 cells are unable to express. Our findings may ultimately open a new chapter in the field of neurosecretory cell differentiation. In addition, the discovery of new mechanisms could have impact also in applicative fields, such as cell engineering and cell transplantation where the role of neurosecretory cells has remained so far only marginal.

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