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The specification of sympathetic neurotransmitter phenotype depends on gp130 cytokine receptor signaling

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SUMMARY

Sympathetic ganglia are composed of noradrenergic and cholinergic neurons. The differentiation of cholinergic sympathetic neurons is characterized by the expression of choline acetyltransferase (ChAT) and vasoactive intestinal peptide (VIP), induced in vitro by a subfamily of cytokines, including LIF, CNTF, GPA, OSM and cardiotrophin-1 (CT-1). To interfere with the function of these neuropoietic cytokines in vivo, antisense RNA for gp130, the common signal-transducing receptor subunit for neuropoietic cytokines, was expressed in chick sympathetic neurons, using retroviral vectors. A strong reduction in the number of VIP-expressing cells, but not of cells expressing ChAT or the adrenergic marker tyrosine hydroxylase (TH), was observed. These results reveal a physiological role of neuropoietic cytokines for the control of VIP expression during the development of cholinergic sympathetic neurons.

Key words: Cytokine, Retrovirus, Antisense, Choline acetyltransferase, Neuropeptide, VIP

INTRODUCTION

The generation of different types of neurons and their connections in specific circuits is a prerequisite for the function of the nervous system. Genetic, cell biological and biochemical approaches in both vertebrate and invertebrate systems have brought considerable progress in the understanding of the mechanisms involved in specifying the identity of neurons. A basic neurogenic program, involving proneural and neurogenic genes, together with axial patterning mechanisms, was shown to be essential for the generation of specific neuronal lineages (Calof, 1995; Tanabe and Jessell, 1996). In addition, specific neuronal phenotypes are characterized by the expression of specific classes or combinations of transcription factors (Tsuchida et al., 1994; Pfaff et al., 1996; Bengston et al., 1996; Valarché et al., 1993). Elimination of these genes leads to the selective loss of such cells or to a change in the phenotype (Pfaff et al., 1996; Morin et al., 1997). Most of these mechanisms act during early stages of neuron generation, before or around the last mitosis of neuron precursor cells. The properties of neurons are, however, not irreversibly determined by these early processes, but may be modified during later development by interactions with other cells. A particularly well-studied example for the environmental control of neuron differentiation is the transmitter phenotype specification of sympathetic neurons (Schotzinger et al., 1994).

Classical work established the presence of functionally distinct neuronal subpopulations in vertebrate sympathetic ganglia, cholinergic and noradrenergic neurons (reviewed in Elfvin et al., 1993). The expression of particular sets of transmitters and cotransmitters could be correlated with different functions and peripheral targets (Lindh and Hökfelt, 1990; Gibbins, 1992). The cholinergic sympathetic phenotype is characterized by the expression of choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), acetylcholinesterase (AChE) and the neuropeptide vasoactive intestinal peptide (VIP) (Landis and Keefe, 1983; Elfvin et al., 1993; Lundberg and Hökfelt, 1986; Ernsberger et al., 1997; Schäfer et al., 1997). The major peripheral targets of cholinergic sympathetic neurons, identified in different mammalian species, are sweat glands in the footpads and blood vessels in limb muscle (Lundberg et al., 1979; Dehal et al., 1992; Schotzinger et al., 1994; Anderson et al., 1996; Guidry and Landis, 1998). Vasodilatory cholinergic sympathetic neurons are characterized by VIP expression, the lack of CGRP and a clustered distribution in sympathetic ganglia, which distinguishes them from the uniformly distributed, VIP- and CGRP-positive neurons innervating sweat glands (Lindh et al., 1989; Lindh and Hökfelt, 1990; Gibbins, 1992; Morris et al., 1998). As avian species lack sweat glands, most if not all chick cholinergic sympathetic neurons may have vasodilatory functions. In chick paravertebral sympathetic ganglia cholinergic neurons are mostly clustered, express VIP and lack CGRP (New and Mudge, 1986; Ernsberger et al., 1997) and innervate arteries in limb muscle and skin (U. Zechbauer and H. Rohrer, unpublished).

During embryonic development in rodents, cholinergic marker genes (ChAT, VIP, VAChT) are first expressed during

or shortly after ganglion formation, when sympathetic neuroblasts still proliferate (Tyrrell and Landis, 1994; Guidry and Landis, 1998; Schäfer et al., 1997, 1998). In the chick embryo, a similar early phase of cholinergic differentiation is observed, only with the difference that VIP is not expressed during this developmental period (Ernsberger et al., 1997; Rohrer and Ernsberger, 1998). In both mammalian and avian species, cholinergic genes are, at least partially, co-expressed with adrenergic genes at this early stage of development (Tyrrell and Landis, 1994; Ernsberger et al., 1997; Schäfer et al., 1998). The early phase of target-independent cholinergic gene expression is followed by the innervation of different peripheral targets. The timing and developmental changes in the properties of the target innervation have been studied in sufficient detail only for sweat glands (reviewed in Schotzinger et al., 1994). For these neurons it has been clearly shown that they initially express noradrenergic properties and change their phenotype to cholinergic under the influence of the target (Landis and Keefe, 1983; Schotzinger and Landis, 1990; Guidry and Landis, 1998). This suggests that the sweat gland innervating neurons do not, or only transiently, express cholinergic properties before target innervation. Target-dependent signals induce the expression of cholinergic genes ChAT, VAChT, neuropeptides VIP and CGRP, and reduce the expression of noradrenergic properties (Schotzinger et al., 1994). The neurotransmitter properties of the blood vessel innervation in developing avian and mammalian species are largely unknown. From the early onset and maintenance of ChAT expression in chick sympathetic neurons (Ernsberger et al., 1997) it seems possible that blood vessels may be innervated by neurons that have initiated ChAT expression before target contact and maintain this expression during innervation. In contrast to ChAT, VIP expression in cholinergic chick sympathetic neurons correlates with target innervation and may be elicited by signals from the innervated target (New and Mudge, 1986; Ernsberger et al., 1997).

The molecular nature of the signals involved in cholinergic differentiation is not known. In cultures of rat and chick sympathetic neurons, several growth factors have been identified that induce ChAT and VIP and reduce the expression of noradrenergic markers. This group of factors includes cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) (Yamamori et al., 1989), ciliary neurotrophic factor (CNTF) (Ernsberger et al., 1989; Saadat et al., 1989), growth promoting activity (GPA) (Heller et al., 1993), oncostatin M (OSM) (Rao et al., 1992) and the recently discovered cardiotrophin-1 (CT-1) (Pennica et al., 1995b). These factors are structurally related and belong to the cytokine superfamily of growth factors (Bazan, 1991). The action of these factors is mediated through heteromeric cytokine receptor complexes (Bazan, 1990). The LIF receptor complex is composed of the ligand-binding subunit LIFRβ and the signal-transducing subunit gp130. The CNTF receptor complex consists of three subunits, the ligand-binding, GPI-linked CNTFRα, LIFRβ and gp130 (reviewed in Stahl and Yancopoulos, 1994). The action of cardiotrophin-1 also involves a GPI-linked α-receptor that is different from CNTFRα (Robledo et al., 1997), in addition to LIFRβ and gp130, whereas the receptor of OSM consists of gp130 and an OSM-specific OSM type II receptor (Mosley et al., 1996).

The cholinergic differentiation activities that induce the expression of ChAT and VIP in cultured sympathetic neurons and are detectable in footpad extract and sweat gland-conditioned medium are inhibited by function-blocking antibodies against LIFRβ (Habecker et al., 1997). Although this result shows that the activities detectable in vitro are due to neuropoetic cytokines, all known members of the family have been excluded as possible candidates (Habecker et al., 1995, 1997). To demonstrate the physiological importance of neuropoietic cytokines for the specification of sympathetic neuron transmitter phenotype, several attempts were made to interfere with their function in vivo. Mice with targeted deletions of genes for CNTF (Masu et al., 1993), LIF (Rao et al., 1993) or both LIF and CNTF (Francis et al., 1997) are viable and displayed normal cholinergic sympathetic innervation of both sweat glands and periosteum. It was observed, however, that in LIF(–/–) mice the lesion-induced upregulation of neuropeptide expression in sympathetic and sensory neurons is strongly impaired (Rao et al., 1993). This finding raised the possibility that LIF and other neuropoietic cytokines may be involved in lesion-induced processes rather than being important during development. Another approach to interfere with the function of these factors is to eliminate their receptors. However, transgenic mice deficient for CNTFRα (DeChiara et al., 1995), LIFRβ (Ware et al., 1995) and gp130 (Yoshida et al., 1996) die either perinatally or during embryonic development (gp130) and thus cannot be used to study target-dependent cholinergic sympathetic differentiation that proceeds between the first and third postnatal week.

To analyse the importance of cytokine receptor signaling for cholinergic differentiation in sympathetic neurons in vivo, we have cloned the chick orthologue of the signal transducing subunit of cytokine receptors, gp130, and developed an antisense RNA approach to interfere with gp130 expression in sympathetic neurons. It is demonstrated that by expression of antisense RNA for gp130 the effects of CNTF, GPA and CT-1 on VIP expression in cultured sympathetic neurons were strongly reduced. Expression of gp130 antisense RNA in vivo, using replication-competent retroviral vectors, resulted in a strong reduction of the number of VIP-expressing cells in sympathetic ganglia, but did not affect the expression of ChAT and TH. The results reveal the physiological importance of cytokine receptor signaling for the specification of the sympathetic neuron transmitter phenotype.

MATERIALS AND METHODS

Cloning of chick gp130

To obtain a gp130 cDNA-sequence from chicken, RT-PCR with the degenerate oligonucleotides sense AAGTTCGCTCA(G/A)GG(C/A)-GA(G/A)AT(C/T)GA and antisense TCGGGCAC(A/G)TT(A/G)-GGCCAGATGTG were used. These primers included the highly conserved transmembrane region of the known mammalian homologues (rat, human, mouse) (Wang et al., 1992; Saito et al., 1992; Hibi et al., 1990) using the chicken-codon usage table (HUSAR software package, German Cancer Research Center, Heidelberg). After PCR amplification (94°C, 20 seconds/50°C, 30 seconds/72°C, 45 seconds/35 cycles) the fragment was subcloned and sequenced. The sequence showed a high homology to the known gp130 transmembrane regions but was not identical to any of them. The fragment was used as a probe to screen a cDNA-library from cultured chick heart cells. A cDNA clone was isolated, containing a poly(A)-

tail and most of the coding sequence, but missing about 312 bp of the 5′ coding region, including the ATG, when compared to the known mammalian cDNA sequences. To complete the missing 5′ end of the chick gp130, RACE was used (5′-AmpliFINDER^{TM} cDNA Amplification Kit, Clonetech, Palo Alto) with the antisense primers GTATGCCTTCCAGGGTTCCAGGTACAG (cDNA synthesis) and GGAGGAATTCGGGCAAGCCTACTGTAACTG (nested PCR-amplification), including an *Eco*RI site for cloning. An identical 371 bp fragment was obtained in two independent RACE approaches. Sequence analysis showed an identical sequence when compared to the overlapping region of the chick gp130 lambda clone and a high homology (66-73%) to the known mammalian gp130 protein sequences. Sequence analysis was done using the HUSAR software package (German Cancer Research Center, Heidelberg). The chick gp130 nucleotide sequence will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number(s) AJ011688, GGA011688.

Transient transfection of sympathetic neurons in culture

Cultures of embryonic day 7 (E7) chick sympathetic neurons were prepared and maintained as described previously (Ernsberger et al., 1989). For the transfection of the sympathetic neurons, two non-overlapping fragments from the extracellular domain of chick gp130 were amplified via PCR (Pwo-polymerase/Boehringer) and cloned in both orientations into pCMX (Davis et al., 1991). Fragment 1 includes sequences in the 5′ region (bp –29 to +341; sense1, antisense1), amplified using the sense primer TGGGTACCGCGTGGCAGG and the antisense primer ATCTGTCCATCTGCTAAAACG. Fragment 2 (bp 342-1874; sense2, antisense2) was amplified using the sense primer TGAGCAGAATATTTATGGAATTTC and antisense primer ATGGCTTCAATTTCTCCTC. 20 μg of plasmid DNA was used to transfect 1.2×10^5 neurons in a 3.5 cm culture dish (Heller et al., 1995). 24 hours after transfection, factors were added at concentrations just sufficient to induce maximal numbers of VIP-positive cells (GPA, 0.5 ng/ml; CT-1, 300 ng/ml; chick HCM was added at comparable amounts of biological activity). After 4 days, the cells were double-stained for VIP and β-galactosidase as described previously (Heller et al., 1995). The proportion of transfected, β-gal-positive cells expressing VIP-IR was determined. The significance of the difference between sense and antisense transfections was determined by Student's *t*-test (pairwise comparison).

Retroviral constructs and in vitro infections

For the construction of retroviral vectors, a fragment from the extracellular domain of gp130 was amplified by PCR using primers containing *Cla*1 sites (sense primer GAGAGATCGATCTGTCCA-ACATGTTTTCTGGG, antisense primer GAGAGATCGATTCAG-CCTTCCCAAGAG). The resulting fragment includes virtually all of fragment A and an additional 300 bp of gp130 sequence (bp –10 to +641). The fragment was cloned in sense (sense-RCAS) and antisense (antisense-RCAS) orientation into the RCAS(B) (Hughes and Kosik, 1984; Morgan and Fekete, 1996) and verified via sequence analysis. SPF chick embryo fibroblasts were transfected with the retroviral DNA and maintained until complete viral infection of the cultured cells. To test the efficiency of the viral vectors to interfere with cytokine receptor signaling, cultured E7 sympathetic neurons (3×10^4 neurons in a 3.5 cm culture dish; Greiner) were infected by adding supernatants of confluent, virus-producing fibroblast cultures. 500 μl supernatant was added to freshly plated sympathetic neurons (1/3 of total volume). The cells were kept in MEM supplemented with 5% fetal calf serum, 10% horse serum, NGF (10 ng/ml) and retinoic acid (5×10^{-9}M) to support long-term survival of sympathetic neurons (Rodriguez-Tébar and Rohrer, 1991). After 24 hours, the cells were supplemented with fresh medium. 96 hours after infection GPA (0.5 ng/ml) was added to the medium. Medium including GPA was changed daily for an additional 7 days. The cultures were then double-stained with an antibody against the viral protein gag (mAb AMV-3C2) and VIP (Incstar) using essentially the same protocol as for double-staining of β-galactosidase and VIP. mAb AMV-3C2, developed by D. Boettiger, was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City. The proportion of gag-positive cells that expressed VIP-IR was determined. The significance of the difference between sense and antisense transfections was determined by Student's *t*-test (pairwise comparison).

Expression of antisense gp130 mRNA in sympathetic ganglia in vivo

Fertilized virus-free chicken eggs were obtained from Lohmann, Cuxhaven, Germany and incubated for 2 days. After opening the egg shell and staging the embryos, aggregates of infected, virus-producing (antisense-RCAS or sense-RCAS) chick embryo fibroblasts were implanted into the embryos at brachial level between the neural tube and the last somite formed (Reissmann et al., 1996). The eggs were sealed with tape and incubated until E14. According to morphological criteria (Hamburger and Hamilton, 1951), the development of antisense-RCAS infected embryos was not retarded as compared to embryos infected with control virus or uninfected embryos. Embryos were killed by decapitation. After removal of internal organs, the trunk and cervical region of the embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 9 hours, kept in 15% sucrose (in 0.1 M phospate buffer, pH 7.3) overnight, embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude Netherlands) and sectioned. Consecutive 14 μm cryostat cross-sections were seperately collected and analysed for expression of VIP protein and gag protein by immunohistochemistry, for VIP mRNA, TH mRNA, SCG10 mRNA, ChAT mRNA and gp130 mRNA by in situ hybridization. Numbers of embryos analysed for the different markers: VIP 7 sense-treated, 11 antisense-treated embryos; SCG10 and TH, 3 sense-treated and 3 antisense-treated embryos; ChAT, 4 sense-treated and 7 antisense-treated embryos.

Immunohistochemistry

Alternating sections were equilibrated to room temperature (RT), blocked for 1 hour with buffer 1 (PBS, supplemented with 1.7% NaCl and 0.2% (v/v) Triton X-100) containing 10% donkey serum and 10% FCS. Sections were then incubated overnight at room temperature with rabbit antibodies against VIP (1:200; Incstar; Sorin Diagnostics, Düsseldorf) or mouse monoclonal anti-gag antibodies (AMV-3C2; ascites, 1:200), diluted in buffer 1. After three washing steps of 15 minutes each, the sections were incubated with secondary antibodies (Cy3-coupled anti-rabbit and anti-mouse Ig, respectively) for 2 hours. After three additional washing steps (twice with buffer 1, once with PBS), the sections were mounted in glycerol (50% (v/v) in PBS) and analysed by fluorescence microscopy. The number of VIP-IR cells was counted on all sections infected by the virus (gag-IR or positive for viral RNA). The area of sympathetic ganglia was acquired by confocal microscopy (SARASTRO 2000, Molecular Dynamics) and analysed with a Silicon Graphics Workstation running the Image Space software program (Version 3.10, Molecular Dynamics).

In situ hybridization

Non-radioactive in situ hybridization on cryosections and preparation of digoxygenin-labeled probes for chick TH, VIP and SCG10 were conducted as previously described (Ernsberger et al., 1997). The probe to detect endogenous gp130 mRNA and sense-RCAS was obtained using the unique *Hin*dIII restriction site at position 733 for linearization of the vector previous to cRNA synthesis and thus covers all sequences COOH-terminal to position 733 (endgp130 probe). The RCAS vectors contain non-overlapping sequences (bp –10 to +641). When the endgp130 probe was used on sections of sense-RCAS infected embryos, no increased signal was obtained in the infected areas. In contrast, using a full-length cRNA-probe sense-RCAS infected areas could be detected by an increased signal intensity. The

endgp130 probe was used to analyse the effect of antisense gp130 RNA expression on endogenous gp130 mRNA levels. As it does not overlap with antisense gp130 RNA produced by antisense-RCAS, the absence of endogenous gp130 mRNA signal in the infected areas cannot be due to competition between viral RNA and the probe for endogenous RNA.

RT-PCR analysis

Total RNA was extracted from E7, E8, E9, E10, E11 and E14 sympathetic ganglia of chick embryos (RNeasy Kit, Qiagen), treated with RNase-free DNaseI and then reverse transcribed (RT-PCR Kit, Stratagene). PCR reactions were performed in the linear range. PCR primers for SCG10 have been previously described (v. Holst et al., 1997b). For gp130 PCR the primers used to generate the retroviral constructs were used. The identity of the amplified gp130 DNA fragment was verified by sequencing.

RESULTS

Cloning of chick gp130

To obtain a chick gp130 cDNA, degenerate oligonucleotides were designed from the highly conserved transmembrane region of mammalian gp130 homologues (Hibi et al., 1990; Wang et al., 1992; Saito et al., 1992) and tested for their ability to amplify a chick homolog by RT-PCR. A fragment of the expected size was obtained, subcloned and sequenced. This clone encoded a sequence showing strong homology to mammalian gp130 and was subsequently used to screen a cDNA library from chick embryonic heart cells (Heller et al., 1995). A cDNA clone was isolated, containing most of the coding sequence for a chick homologue of gp130 but missing 312 bp of the 5′-coding region, including the ATG, when compared to the known mammalian cDNA sequences. To obtain the N-terminal sequence, 5′-RACE with nested primers was performed (Frohmann et al., 1988). The longest fragment (413 bp, covering 341 bp of unknown gp130 sequence) was obtained in two independent RACE amplifications. This N-terminal sequence displayed 74% similarity (61% identity) to human gp130 in the processed region.

A combination of sequences obtained from lambda and RACE clones resulted in a sequence containing an open reading frame that encodes a protein of 918 amino acids (Fig. 1). The predicted primary sequence shows high homology (61% identical amino acids) to human gp130 (Hibi et al., 1990). It includes a Kozak-sequence around the putative start-codon, a potential hydrophobic leader sequence (residues 1-23) and the characteristic cytokine receptor domain including the WS-X-WS motif (residues 314-318) (Bazan, 1990). Comparison with mammalian gp130 sequences

Fig. 1. Sequence comparison of the predicted amino acid sequence of the chick gp130 (c) and its human ortholog (h). Identical amino acids are indicated by asterisks. The transmembrane region (aa 624-645) is boxed, cysteine residues necessary for ligand binding are indicated by closed arrows and the WS-X-WS-domain is marked by a horizontal arrow. Tyrosine residues taking part in signal transduction via STAT molecules are indicated by open arrows. The JAK kinase binding motif is indicated by filled circles.

revealed a conserved location of all 15 cysteine residues and of potential N-glycosylation sites in the extracellular domain of chick gp130. Seven out of the eleven potential N-glycosylation sites are identical between chick and human gp130. In the intracellular region, all five tyrosines are found at conserved locations. In addition, the tyrosine-based motif YXXQ of the last four COOH-terminal tyrosines in gp130, involved in Stat3 tyrosine phosphorylation and activation (Stahl et al., 1995), is also present in chick gp130. The proline-rich box1 motif PXXPXP, implicated in JAK kinase binding to cytokine receptors (Tanner et al., 1995) is also located at a conserved, membrane-proximal region in chick gp130.

gp130 is expressed in sympathetic ganglia between E7 and E14

The expression of gp130 mRNA was investigated by non-radioactive in situ hybridization and RT-PCR. On coronal sections at the thoracic region, gp130 mRNA was detectable in spinal cord motoneurons, sensory dorsal root ganglia (DRG) and sympathetic ganglia at E14 (Fig. 2A) and E12 (not shown). Expression was also detectable in bone marrow cells (Fig. 2A). At these ages other tissues (muscle, bone) showed lower signals that could not be distinguished from background staining. At E7, many tissues showed low levels of gp130 mRNA expression, with elevated levels in spinal cord and DRG (not shown). RT-PCR analysis detected gp130 mRNA in sympathetic ganglia at all stages analysed between E7 and E14 (Fig. 2B); this is in agreement with findings that cytokine-mediated effects on VIP expression can be elicited throughout this developmental period (Ernsberger et al., 1989, 1997).

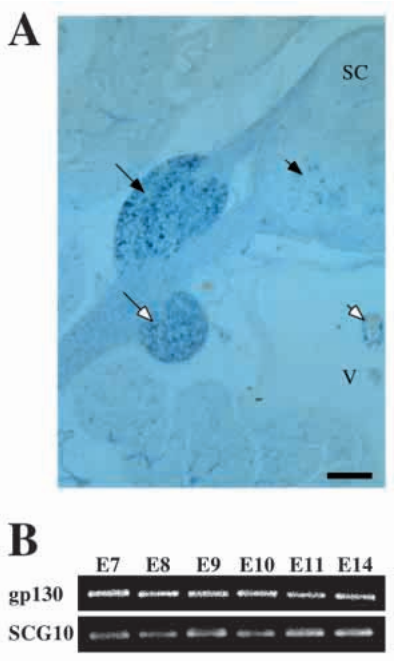

Fig. 2. gp130 mRNA is expressed in sympathetic ganglia, DRG and spinal cord motoneurons. (A) Thoracic sections of chick embryos at E14 were analysed by in situ hybridization. gp130 mRNA is detectable in spinal cord motoneurons (arrowhead), DRG (arrow) and sympathetic ganglia (open arrow). In addition, bone marrow cells (open arrowhead) are positive. SC, spinal cord; V, vertebra. Bar, 200 μm. (B) RT-PCR analysis indicates expression of gp130 mRNA in sympathetic ganglia between embryonic days (E)7 and 14. The expression of the neuronal marker gene SCG10 was analysed in parallel to control for equal amounts of mRNA. No PCR products were obtained using the DNaseI-treated total RNA prior to reverse transcription as a template for PCR under saturating conditions (gp130/SCG10, 35 cycles).

Expression of gp130 antisense RNA in cultured sympathetic neurons interferes with the action of neuropoietic cytokines

To establish conditions that interfere with the expression and function of gp130, cultures of E7 sympathetic neurons were used. We had previously demonstrated that CNTF, GPA and (a) factor(s) present in heart cell conditioned medium (HCM) induce expression of VIP-immunoreactivity (VIP-IR) in these cells (Heller et al., 1995). In the absence of added cytokines, no VIP expression is detecteable in these cultures (Ernsberger et al., 1989; Heller et al., 1995). In agreement with recent findings in cultures of rat sympathetic neurons (Pituello et al., 1997), we observed that the neuropoietic cytokine cardiotrophin-1 (CT-1) (Pennica et al., 1995a) also induced

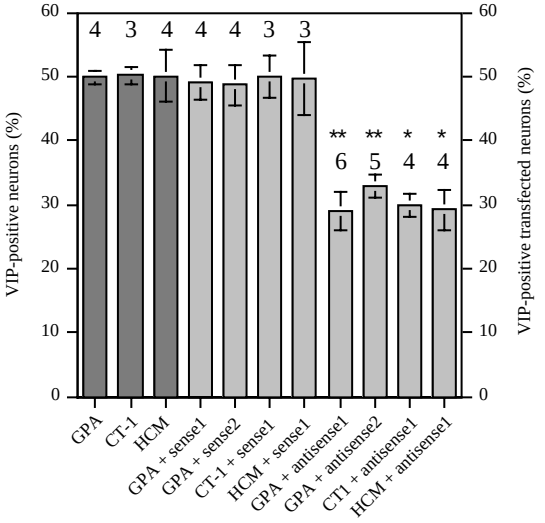

Fig. 3. E7 sympathetic neurons transfected with a gp130 antisense RNA expression vector exhibit decreased responsiveness to GPA, CT-1 and HCM. Primary cultures of sympathetic neurons, cotransfected with pcDNA-LacZ in combination with either sense- or antisense-expression vectors (sense1, sense2, antisense1, antisense2) or nontransfected control cultures were treated with GPA, CT-1 or HCM and analysed for VIP expression. Under control conditions GPA, CT-1 and HCM induced VIP-immunoreactivity in about 50% of the neurons (darkly shaded columns, left *y*-axis). Co-transfected cells were identified by immunostaining for β-galactosidase, and the proportion of transfected cells immunoreactive for VIP was determined (light columns, right *y*-axis). Neurons transfected with gp130 antisense RNA expression vectors exhibited significantly reduced responsiveness to GPA, CT-1 and HCM as compared with either non-transfected cells or with neurons transfected with sense-CMV, respectively. Data represent the mean ± s.d. of several independent experiments as indicated by the numbers. *, $P<0.001$; **, $P<0.0001$ (Student's *t*-test, pairwise comparison between sense and antisense experiments).

VIP. When used at saturating concentrations (300 ng/ml), about 50% of sympathetic neurons expressed VIP-IR after a culture period of 4 days, as observed for the other cytokines tested (Fig. 3).

To interfere with the expression and function of cytokine receptors in sympathetic neurons the cells were transfected with expression vectors containing gp130 cDNA sequences in sense or antisense orientation (sense-CMV; antisense-CMV). Two different, non-overlapping sense and antisense constructs were used that contained fragments from the extracellular domain of gp130 (sense1/antisense1: bp −29 to +341; sense2/antisense2: bp 342-1874). In order to identify transfected cells, expression vectors coding for β-galactosidase were cotransfected.

Cells transfected during the first hours of culturing with either sense1-, sense2-, antisense1-, or antisense2-CMV were treated after 1 day with different cytokines and analysed for the expression of VIP after a total culture period of 4 days. Expression of gp130 antisense RNA interfered with the action of GPA, HCM and CT-1, whereas cells expressing gp130 sense RNA responded to a similar extent as untransfected cells (Fig. 3). CNTFRα-mediated signaling (GPA) and cytokines not acting through CNTFRα (HCM (Heller et al., 1995) and CT-1 (Pennica et al., 1996), data not shown) were affected to a similar extent. The limited extent of the inhibition observed with the expression of both gp130 antisense and GPARα antisense RNA (Heller et al., 1995) may be explained by the presence of residual receptors still present on the cell surface or in intracellular transit towards the cell membrane (see below).

After establishing that expression of antisense gp130 RNA interferes with the action of all cytokines tested, we addressed the question of whether the RNA expression levels induced by RCAS retroviral vectors (Morgan and Fekete, 1996) are sufficiently high to interfere with the function of gp130. In addition, we wanted to determine whether the extent of inhibition would be higher upon delayed application of the cytokine. Retroviral vectors have to integrate into the genome for efficient expression. As immature E7 sympathetic neurons proliferate in vitro, these cells can be infected by these viruses and in vitro cultures can be used to test the efficiency of antisense RNA expression by retroviral vectors. RCAS vectors were constructed that contain gp130 cDNA sequences (bp −10 to 641) in sense or antisense orientation (sense-RCAS or antisense-RCAS) and E7 cells were infected at the onset of culture. Treatment with GPA started after 4 days and cells were analysed for expression of virus and for VIP-IR after 12 days. The proportion of VIP-expressing cells in the population of gp130 antisense RNA expressing cells was 5.5-fold lower than in cells expressing gp130 sense RNA (Fig. 4). No difference was observed between noninfected control cultures and cells infected with sense-RCAS. The lower proportion of VIP-IR cells under control experimental conditions as compared to the experiments shown in Fig. 3 is most likely due to prolonged culture periods that may affect VIP expression or the ability of the neurons to respond to cytokines. The larger effect of antisense RNA expression under these conditions (Fig. 4) supports the notion that delayed application of the cytokines allows more time for receptor turnover, which reduces the amount of receptor and leads to increased inhibition.

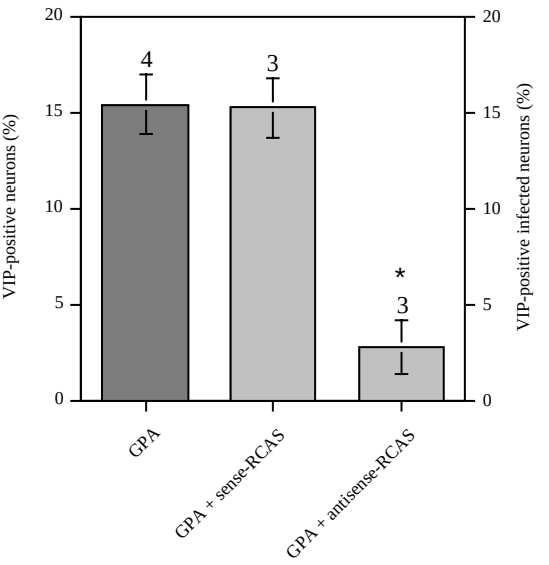

Fig. 4. E7 sympathetic neurons infected with gp130 antisense-RCAS retrovirus exhibit decreased responsiveness to GPA. Primary cultures were infected with antisense-RCAS, sense-RCAS or were not infected. Cultures were treated daily with GPA from day 4 onwards and analysed for VIP expression and expression of the viral gag protein after 12 days. Under control conditions GPA induced VIP-IR in about 15% of the neurons (dark column, left *y*-axis). Neurons infected with antisense-RCAS diplayed a strongly reduced responsiveness to GPA as compared to cells infected with sense-RCAS (light columns, right *y*-axis) or noninfected controls. Data represent the mean ± s.d. of several independent experiments as indicated by the numbers. *, $P<0.001$ (Student's *t*-test, pairwise comparison between sense and antisense experiments).

Expression of gp130 antisense RNA interferes with VIP expression in vivo

To interfere with cytokine receptor function in sympathetic neurons in vivo, sympathetic neuron precursor cells were infected with antisense-RCAS or sense-RCAS in E2 chick embryos (stage 14) and the effect on VIP expression was analysed in E14 sympathetic ganglia. E14 embryos were chosen since a large number of VIP-positive cells are detectable in control sympathetic ganglia and since most cells

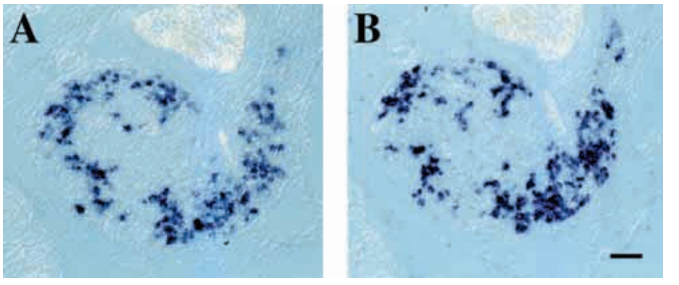

Fig. 5. Expression of VIP and ChAT in E14 paravertebral sympathetic ganglia. Non-radiactive in situ hybridization was performed on alternating tissue sections from the thoracic region of E14 chick embryos to demonstrate mRNA for VIP (A) and ChAT (B). The comparable staining pattern indicates that VIP and ChAT are coexpressed to a large extent. Bar, 50 μm.

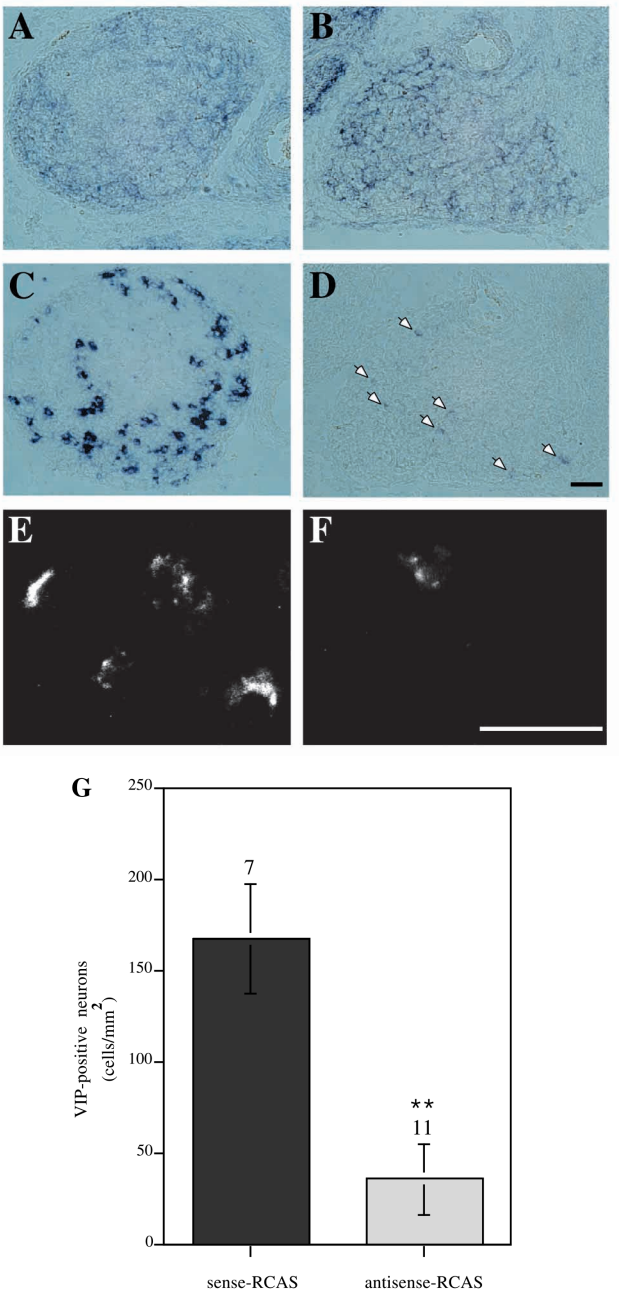

Fig. 6. Sympathetic ganglia infected with gp130 antisense-RCAS retrovirus exhibit a decreased number of VIP-expressing neurons. Ganglia were infected with antisense-RCAS (B,D,F) or sense-RCAS (A,C,E) at E2 and analysed for viral infection (A,B) and VIP expression (C-F) in sections of E14 embryos. Expression of virus-encoded gp130 antisense RNA (B) and sense RNA (A) was detected by in situ hybridization using cRNA probes directed against the inserted gp130 fragment. Infection was confirmed using an antibody directed against the viral GAG-protein (not shown). VIP-IR (E,F) and VIP mRNA (C,D) were analysed by immunofluorescence and in situ hybridization, respectively. Ganglia expressing gp130 antisense RNA displayed a strong reduction in the number of VIP-positive cells as compared to ganglia infected with sense-RCAS. In addition, the signal intensity of VIP-IR and in situ hybridization in remaining VIP-positive cells was lower in gp130 antisense expressing cells as compared to controls. Cells with low VIP mRNA expression are indicated by arrows in D. Bars, 50 μm (A-D), 12.5 μm (E,F). (G) Quantification of VIP expression in E14 sympathetic ganglia. The number of VIP-IR neurons per ganglion section and the area of the ganglion section were determined in areas infected by antisense-RCAS and sense-RCAS. The area of sympathetic ganglion sections was determined by confocal microscopy as described in Materials and methods. Data represent the mean ± s.d. of several independent experiments as indicated by the numbers. **, $P<0.0001$ (Student's *t*-test, pairwise comparison between sense and antisense experiments).

expressing VIP mRNA seem to coexpress ChAT mRNA at this stage (Fig. 5). To initiate infection, aggregates of virus-producing chick embryo fibroblasts were implanted into the migration pathway of neural crest cells in E2 chick embryos. Implants were positioned at the level of somites 18-23, which corresponds to the cervical/thoracic region (cervical/thoracic vertebrae C13-T5). The embryos were sectioned in the cervical/thoracic region and alternate sections were analysed for viral infection using probes for antisense or sense gp130 RNA (Fig. 6A,B) and the viral gag protein (not shown). Although the probe directed against virus-encoded gp130 sense RNA also detects endogenous gp130 mRNA, infected areas were recognized by increased in situ hybridization signal and, in addition, controlled by staining for gag protein (data not shown). Parallel sections were analysed for the expression of VIP mRNA and protein (Fig. 6C-F).

The number of VIP-IR cells and VIP mRNA-positive cells was strongly reduced in sympathetic ganglia that expressed antisense gp130 RNA, as compared to ganglia infected with sense-RCAS (Fig. 6E,F) and noninfected control embryos (data not shown). The effect on VIP expression was quantified by determining the number of VIP-IR cells and referring to areas of the corresponding ganglion sections. It was observed that the density of VIP-IR cells was reduced in gp130 antisense RNA-expressing ganglia to 21% of the density in sense controls (Fig. 6G). In addition to a reduction in the number of VIP-IR cells, the intensity of the remaining VIP-IR and VIP mRNA signals was also considerably diminished in ganglia expressing gp130 antisense RNA (Fig. 6D,F). The density of VIP-IR cells in sympathetic ganglia of noninfected embryos and embryos infected with sense-RCAS were not different (noninfected 176±24 VIP-IR cells/mm^2; mean±s.d.; n=3, sense 166±30 VIP-IR cells/mm^2; mean±s.d.; n=7). As the ganglion section areas (i.e. ganglion volumes) were not significantly different between antisense and controls (antisense 92±14×10^3 μm^2, controls 99±28×10^3 μm^2; mean±s.d.; n=9 for both groups), it is concluded that the reduction in VIP cell density is due to a smaller number of VIP-positive cells per ganglion.

To demonstrate that the expression of gp130 antisense RNA interferes with gp130 expression, endogenous gp130 mRNA levels were analysed using a gp130 cRNA probe that did not overlap with the region used in the retroviral vector. The levels of endogenous gp130 mRNA were reduced to background levels in all ganglia expressing antisense gp130 RNA (Fig. 7; four embryos analysed). In contrast, gp130 mRNA levels were not affected in ganglia infected with sense-RCAS (not shown). The effect of gp130 antisense RNA expression on VIP expression closely correlated with the extent of viral infection of sympathetic ganglia (e.g. along the antero-posterior axis of the sympathetic chain), but not with the infection of non-neural tissues (e.g. muscle, bone). Further evidence for the specificity

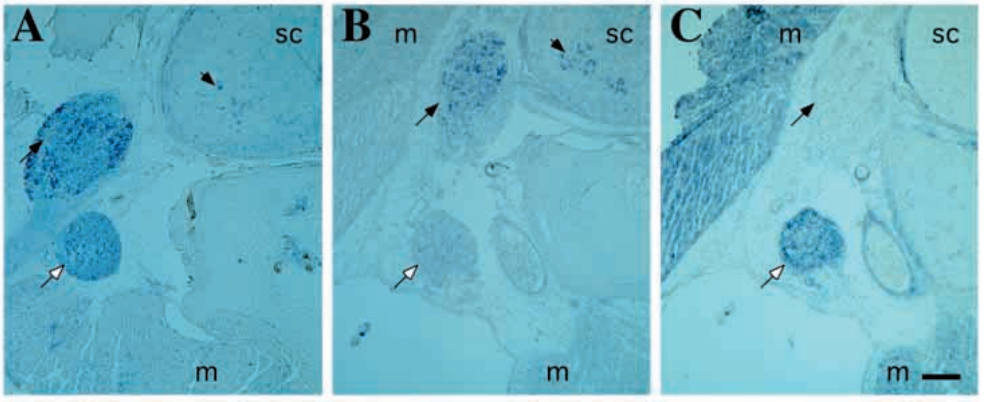

Fig. 7. Expression of gp130 antisense RNA in vivo interferes with the expression of endogenous gp130 mRNA. Alternating sections of an infected embryo (B,C) were stained with in situ RNA probes detecting the virus-encoded gp130 antisense RNA (C) or endogenous gp130 mRNA (B). Expression of endogenous gp130 mRNA in infected embryos (B) should be compared to the normal pattern of gp130 mRNA of an uninfected E14 embryo (A) (or Fig. 2A). Note that expression of gp130 antisense RNA (C) in sympathetic ganglia (open arrow) results in a strong decrease in endogenous gp130 mRNA (B), whereas uninfected DRG neurons (arrow) and motoneurons (arrowhead) display normal expression levels of endogenous gp130. Endogenous gp130 mRNA was detected using an antisense cRNA probe (endgp130, see Materials and methods) that does not overlap with the mRNA expressed by the RCAS. m, muscle; sc, spinal cord; Bar, 200 μm.

of the effect of antisense gp130 expression is the observation that VIP expression in the (noninfected) spinal cord was not impaired (data not shown), providing an internal control for the VIP staining on the same section.

The role of gp130-mediated cytokine signaling in the developmental expression of ChAT, TH and general neuronal markers

Recent findings on cholinergic differentiation of sympathetic neurons in chick paravertebral ganglia revealed an onset of ChAT expression before sympathetic neurons have contacted their target tissues (Ernsberger et al., 1997). This led to the suggestion that the mature cholinergic sympathetic neuron phenotype may be generated by two differentiation stages: an early, target-independent induction of ChAT (and VAChT) and a later step involving signals from the target (Ernsberger et al., 1997). The present finding that cytokine signaling is involved in the control of VIP expression raised the question whether the early onset of ChAT expression also depends on the action of cytokines.

In sections of E14 sympathetic ganglia of normal, noninfected embryos, ChAT mRNA and VIP mRNA positive cells are found in similar numbers (Fig. 5). In contrast to VIP expression, no apparent difference was detected in ChAT mRNA expression between gp130 antisense RNA and sense control embryos (Fig. 8A,B). This suggests that the initial onset of ChAT mRNA expression and the maintenance of ChAT mRNA expression until E14 is not dependent on cytokine-mediated signaling. Thus, this result argues against a role of neuropoietic cytokines in the proliferation, survival or differentiation of ChAT mRNA expressing cells up to this stage of development.

To address the question whether cytokine receptor signaling in vivo affects exclusively the VIP-expressing subpopulation or is (also) involved in the general development of sympathetic neurons, the expression of the adrenergic marker tyrosine hydroxylase (TH) and of the general neuronal marker SCG10 were analysed by in situ hybridization. No apparent difference was observed in the expression of TH and SCG10 between embryos expressing sense or antisense gp130 RNA (Fig. 8C-F). This result, together with the lack of effect on ganglion size,

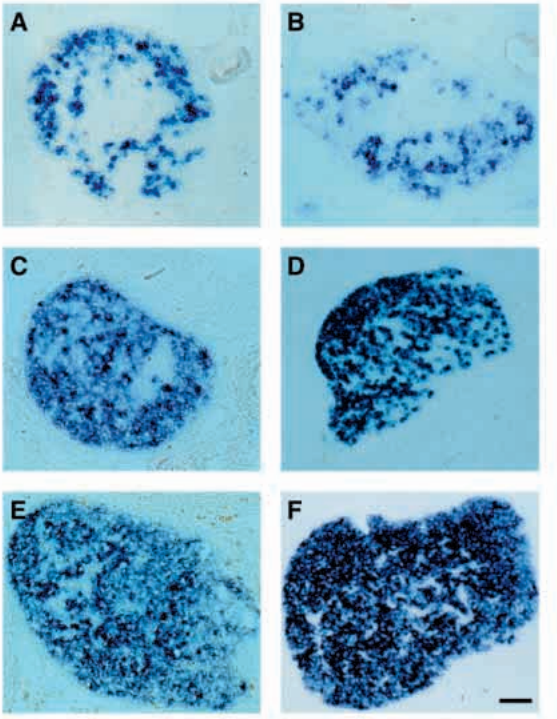

Fig. 8. ChAT, TH and SCG10 expression in E14 sympathetic ganglia is not changed by expression of gp130 antisense RNA. Sections of infected sympathetic ganglia were used for in situ hybridization with specific cRNA probes for ChAT (A,B). No obvious difference in the extent of ChAT expression was observed between antisense RNA expressing ganglia (B) and sense controls (A). Representative micrographs are shown. In alternating sections the number of VIP expressing neurons was strongly reduced by the expression of gp130 antisense RNA (not shown). Alternating sections of infected sympathetic ganglia were used for in situ hybridization with specific cRNA probes for TH (C,D) and SCG10 (E,F). No obvious change in the expression level was observed in infected ganglia expressing gp130 antisense RNA (D,F) as compared to gp130 sense RNA controls (C,E). The more intense staining for SCG10 and TH in ganglia expressing antisense RNA (A,C) is not a consistent finding. Bar, 50 μm.

argues against a general role of cytokines in sympathetic ganglion development.

DISCUSSION

The sympathoadrenergic lineage, giving rise to noradrenergic and cholinergic sympathetic neurons and chromaffin cells, has been intensively used to define signals involved in the specification of transmitter phenotype. The structurally related cytokines LIF, CNTF, GPA, OSM and CT-1 induce the expression of the cholinergic marker ChAT and the expression of the neuropeptide VIP that is co-expressed with ChAT in cultured sympathetic neurons. We now demonstrate that VIP expression in sympathetic ganglia is prevented by interfering with the expression of gp130, the common signal transducing subunit of neuropoietic cytokine receptors. This result provides the first evidence for a physiological role of gp130-mediated signaling in the developmental control of neurotransmitter phenotype in sympathetic neurons.

Cloning and expression of chick gp130

In order to interfere with the function of neuropoietic cytokines, the receptor subunit gp130 was cloned from the chick. The predicted amino acid sequence shows high homology (61% identity) to human gp130. The sequence displays features characteristic of receptors for neuropoietic cytokines, including multiple fibronectin type III repeats and the WS-X-WS sequence. Comparison with human gp130 demonstrated conservation of all 15 cysteine residues, the tyrosine residues and the proline-rich box1 motif in the cytoplasmic domain, essential for signal transduction (Stahl et al., 1995; Tanner et al., 1995). In addition, 7 potential N-glycosylation sites are at the equivalent position as predicted in the human gp130 sequence (total number of potential N-glycosylation sites is 11 in chick and 10 in human gp130).

The analysis of murine gp130 expression by northern blotting led to the conclusion that gp130 is ubiquitously expressed in the adult (Saito et al., 1992). In the embryo, expression is detected throughout development; however, the tissue specificity of murine gp130 expression has not been analysed (Saito et al., 1992). Thus, it is of interest that in the chick embryo a much stronger expression of gp130 mRNA was observed in neural tissues as compared to mesodermal tissues like muscle. Expression in bone marrow cells is in agreement with the known role of cytokines in haematopoiesis. The high level of gp130 mRNA in spinal cord motoneurons, sensory DRG neurons and sympathetic neurons is highly reminiscent of the expression pattern of CNTFRα (Heller et al., 1995; v. Holst et al., 1997a; MacLennan et al., 1996), and is in line with the demonstration of functional CNTF receptors in these neuron populations (Oppenheim, 1996; Sendtner et al., 1991; Ernsberger et al., 1989).

Antisense RNA for gp130 inhibits cytokine-induced VIP expression in vitro

To analyse the biological function of neuropoietic cytokines in sympathetic neurons, it was our aim to block neuropoietic cytokine receptor function by interfering with gp130 expression. To this end, an antisense RNA approach was developed and initially tested in cultures of E7 sympathetic neurons. We had previously demonstrated that expression of antisense RNA for CNTFRα in such cultures successfully blocks the action of CNTF and GPA, but not of (a) factor(s) present in heart cell conditioned medium (HCM) (Heller et al., 1995). Cardiotrophin-1 induces VIP expression in chick sympathetic neurons through a receptor complex that also does not involve CNTFRα (M. Geissen, unpublished observation). Expression of antisense RNA for gp130 is now found to interfere not only with the action of CNTF and GPA, but also of HCM and CT-1. Both gp130 antisense and CNTFRα antisense RNA expression (Heller et al., 1995) resulted in only a partial block of cytokine-mediated VIP-induction. This may be due to the short, 24-hour interval between the onset of antisense RNA expression and the addition of cytokines. This interval may not be sufficient to eliminate all receptors from the cell surface. This issue of timing is even more important when retroviral vectors are used, as the virus must first integrate into the host's DNA before RNA is transcribed. Thus, to test the efficiency of gp130 antisense RNA expression mediated by RCAS vectors, conditions were used that allow long-term survival of sympathetic neurons and to increase the time interval between viral infection and cytokine addition to 4 days. The reduction of cytokine-mediated VIP-induction in cells infected with antisense gp130 retrovirus to 18% of controls suggested that antisense RNA levels in most infected cells are sufficiently high to lead to an impairment of cytokine receptor function, and that this approach may be used to investigate gp130 function in vivo. The observation that some neurons responded to neuropoietic cytokines although they had been infected by antisense-RCAS may be explained by variations in the levels of antisense RNA or a slow turnover of the gp130 protein that would allow for residual functional receptors.

Antisense RNA for gp130 prevents the expression of VIP in vivo

The retrovirus-based antisense RNA approach to interfere with cytokine signaling was applied in vivo by infecting neural crest precursors of sympathetic neurons in E2 chick embryos. This results in a massive infection of primary and secondary paravertebral sympathetic ganglia in the region of implantation (Reissmann et al., 1996). In addition to sympathetic ganglia, sensory DRG and mesodermal tissues like muscle were also infected to a variable extent. It should be noted that the use of RCAS vectors in the chick embryo circumvents the problem of early death of the embryo observed in gp130(–/–) mice (Yoshida et al., 1996) by a locally restricted loss of cytokine signal transduction.

Analysis of VIP expression in antisense gp130 RNA expressing ganglia revealed a reduction in the density of VIP-IR cells to 21% of control infections and ganglia from noninfected embryos. The reduction in VIP mRNA expression suggests that gp130 antisense treatment interferes with the induction of VIP expression. The strong decrease in the levels of endogenous gp130 mRNA in ganglia expressing viral-derived gp130 antisense RNA supports the notion that the reduction in VIP expression is caused by the loss of gp130 mRNA and protein. Further evidence for the specificity of gp130 antisense RNA treatment is the close correlation between antisense RNA expression and low number of VIP-expressing cells along the antero-posterior axis of the

paravertebral sympathetic chain. It should also be noted that VIP expression in spinal cord, which remained uninfected, was not impaired in embryos where sympathetic ganglia displayed a strong reduction in VIP expression.

The expression of the noradrenergic marker TH and the general neuronal marker SCG10 were not altered in ganglia expressing gp130 antisense RNA. Together with the finding that section areas/ganglion volumes were not influenced, these results argue against a role of neuropoietic cytokines for general development of sympathetic ganglia and neuron survival. This conclusion is in agreement with the observation that effects on sympathetic ganglion development were not reported in mice lacking functional genes for CNTF (Masu et al., 1993), CNTFRα (DeChiara et al., 1995) or LIF (Rao et al., 1993). The reduction in the density of VIP-expressing cells may be explained by a selective survival effect for VIP-positive cells or by an effect on their differentiation. Several arguments are in favor of a differentiation effect. (1) The number of VIP-expressing cells is reduced in gp130 antisense-expressing ganglia, whereas ChAT expression is unaffected. As the vast majority of VIP-positive cells co-express ChAT in E14 sympathetic ganglia, a selective survival effect of neuropoietic cytokines on the survival of VIP-expressing cells can be excluded. (2) The in vitro effects of cytokines on cholinergic differentiation of chick and rat sympathetic neurons are due to the increased expression of cholinergic marker genes (Patterson and Chun, 1977; Yamamori et al., 1989; Ernsberger et al., 1989; Saadat et al., 1989).

As VIP and ChAT are coexpressed in a subpopulation of sympathetic neurons at late embryonic stages (Ernsberger et al., 1997 and the present study) and neuropoietic cytokines induce both VIP and ChAT in cultures of chick and rat sympathetic neurons (Yamamori et al., 1989; Fann and Patterson, 1994; Ernsberger et al., 1989; Saadat et al., 1989), it is of considerable interest that ChAT expression is not prevented in ganglia expressing gp130 antisense RNA. This finding may be explained by assuming that the antisense approach results in low, residual gp130 levels that would be sufficient to allow cytokine-mediated ChAT-induction, but not VIP-induction. However, the observation that cytokine-mediated VIP-induction in cultured sympathetic neurons requires lower cytokine concentrations than ChAT-induction (Fann and Patterson, 1993) argues against this explanation. In our retrovirus infection protocol, sympathetic ganglia and their precursors are massively infected as early as E3, which argues against the possibility that the earlier expressed ChAT gene escapes inhibition as compared to the later expressed VIP gene. We favor an alternative interpretation for the maintained ChAT expression, i.e. that ChAT and VIP are differentially regulated during development of chick sympathetic ganglia. This notion is strongly supported by our recent finding that ChAT expression in sympathetic neurons starts in vivo at E6, several days before the onset of VIP expression (Ernsberger et al., 1997). In addition, cultured E7 sympathetic neurons were observed to respond to CNTF by an induction of VIP, whereas ChAT expression was not increased. The effect of cytokines on ChAT activity and mRNA expression in older sympathetic neurons observed in vitro (Ernsberger et al., 1997) may not play a major role in vivo, as there was no apparent change of ChAT mRNA expression after gp130 antisense expression. Taken together, our observations suggest a two-step model of cholinergic differentiation involving an early onset of ChAT expression, followed by a delayed expression of VIP. Neuropoietic cytokines acting through gp130 are of physiological importance for VIP expression in chick sympathetic neurons, but not for the onset and maintenance of ChAT expression. It should be pointed out that the present study analysed a population of cholinergic sympathetic neurons that may include mostly, if not exclusively, vasomotor neurons. Thus, the results and the proposed differentiation pathway may be specific for vasodilatory cholinergic sympathetic neurons as compared to sweat gland innervating neurons (reviewed by U. Ernsberger and H. Rohrer, manuscript submitted). The important issues now are to characterize the unknown factor(s) involved in the early onset of ChAT expression and to identify which member of the neuropoietic cytokine family is involved in the developmental control of VIP expression.

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