The developmental expression of vasoactive intestinal peptide (VIP) in cholinergic sympathetic neurons depends on cytokines signaling through LIFRβ-containing receptors

Chi Vinh Duong, Markus Geissen and Hermann Rohrer*

Max-Planck-Institut für Hirnforschung, Abteilung Neurochemie, Deutschordenstr. 46, 60528 Frankfurt / Main, Germany
*Author for correspondence (e-mail: rohrer@mpih-frankfurt.mpg.de)

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SUMMARY

Sympathetic ganglia are composed of noradrenergic and cholinergic neurons. Cholinergic sympathetic neurons are characterized by the expression of choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VACHT) and the vasoactive intestinal peptide (VIP). To investigate the role of cytokine growth factor family members in the development of cholinergic sympathetic neurons, we interfered in vivo with the function of the subclass of cytokine receptors that contains LIFRβ as essential receptor subunit. Expression of LIFRβ antisense RNA interfered with LIFRβ expression and strongly reduced the developmental induction of VIP expression. By contrast, ganglion size and the number of ChAT-positive cells were not reduced. These results demonstrate a physiological role of cytokines acting through LIFRβ-containing receptors in the control of VIP expression in sympathetic neurons.

Key words: LIF, Cytokine, Transmitter, Plasticity, Chick, Noradrenergic, Cholinergic, VIP

INTRODUCTION

During development, a large variety of different neuronal phenotypes is generated from pluripotent neural precursor cells. The specification of neuronal identity involves series of cellular interactions, in particular extrinsic signals produced in the vicinity of proliferating neural precursor cells (Edlund and Jessell, 1999; Jessell, 2000; Christiansen et al., 2000). At the time of cell cycle withdrawal, major specification steps take place in many neuronal lineages. The final steps of neuronal differentiation are dependent on interactions with other cells in the circuit where a particular neuron becomes integrated. These late differentiation steps include not only the facilitation or weakening of synaptic contacts (Sanes and Lichtman, 1999; Davis, 2000) but also the stabilization or the change of the modality of the neuron, the neurotransmitter phenotype and neuropeptide expression (Francis and Landis, 1999; Lewin and Barde, 1996; Ernsberger and Rohrer, 1999).

Sympathetic ganglion development serves as an excellent model system with which one can study the emergence of distinct cell types, noradrenergic and cholinergic neurons, from a common progenitor pool. Early during development, noradrenergic sympathetic neurons, characterized by the expression of tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and pan-neuronal genes, are specified in sympathetic ganglion primordia by members of the bone morphogenetic protein (BMP) family (Reissmann et al., 1996; Shuh et al., 1996; Schneider et al., 1999; Varley et al., 1995).

In a later differentiation phase, which is controlled by yet unknown signals, a large population of these cells transiently co-expresses the cholinergic marker genes ChAT and VACHT in avian sympathetic ganglia (Ernsberger et al., 1997; Ernsberger and Rohrer, 1999). The subsequent, final differentiation steps result in the selective expression of noradrenergic and cholinergic marker genes in different subpopulations of sympathetic neurons and the co-expression of the neuropeptide VIP in cholinergic neurons (Ernsberger et al., 1997; Ernsberger et al., 2000; Ernsberger and Rohrer, 1999). VIP-positive, non-noradrenergic sympathetic neurons innervate large feed arteries and the vasculature in muscle and skin of several species (Gibbins, 1992; Dehal et al., 1992; Grasby et al., 1997; Guidry and Landis, 2000), including the chick embryo (U. Zechbauer and H. R., unpublished). As VIP is a powerful vasodilator (Fahrenkrug, 1989), it is assumed that the VIP-positive non-noradrenergic sympathetic neurons have a vasodilatory effect on the vessels they innervate (Morris et al., 1995). Cholinergic sympathetic neuron differentiation in mammalian species has been studied in most detail for neurons that innervate the sweat glands in rat and mouse foot pads (Landis and Keefe, 1983; Leblanc and Landis, 1986). These studies demonstrate that the cholinergic neurotransmitter phenotype is induced by factors that are secreted by the innervated sweat gland target tissue (Guidry and Landis, 1995; Schotzinger and Landis, 1988; Schotzinger and Landis, 1990; Habecker et al., 1997).

The cytokine growth factors LIF (Yamamori et al., 1989),
CNTF (Ernsberger et al., 1989; Saadat et al., 1989), GPA (Leung et al., 1992; Heller et al., 1995) CT-1 (Pennica et al., 1995; Geissen et al., 1998), OSM (Rao et al., 1992a) and IL-6 (Mártínez et al., 1998) have been implicated in the induction of cholinergic and peptidergic properties of sympathetic neurons, because they are able to induce ChAT and co-expressed neuropeptides and to reduce noradrenergic gene expression in cultured sympathetic neurons. These cytokine family members and the recently discovered composite cardiotoxin-like cytokine/cytokine-like factor-1 (CLC/CLF) (Senaldi et al., 1999; Shi et al., 1999) act through specific receptor complexes that contain gp130 as common signal transducing receptor subunit (Bravo and Heath, 2000; Taga and Kishimoto, 1997). The gp130 receptor family and their ligands can be subdivided into receptors that contain, as signaling subunits, either homodimers of gp130 (IL-6), heterodimers of gp130 and OSMR (OSM), or gp130/LIFβ heterodimers (CNTF, CT-1, LIF, CLC/CLF) (Taga and Kishimoto, 1997; Elson et al., 2000; Lelièvre et al., 2001). Additional ligand-binding α-receptor subunits can associate with these core signaling receptors to form tripartite or even more complex (CLC) receptor complexes. These α-receptor subunits are responsible for the selective activation of a receptor subclass by different ligands, whereas LIF can bind and activate directly the gp130/LIFβ receptor (Stahl and Yancopoulos, 1994; Bravo and Heath, 2000; Taga and Kishimoto, 1997).

The role of neuropoietic cytokines in the cholinergic differentiation of sweat gland innervation has been studied by various loss-of-function approaches that have revealed that all known neuropoietic cytokines can be excluded as candidate cholinergic sweat gland factors (Habecker et al., 1995; Habecker et al., 1997; Francis et al., 1997). However, a cholinergic differentiation activity that displays the characteristics of a neuropoietic cytokine is present in homogenates of sweat gland tissue and supernatants of cultured sweat glands (Rao and Landis, 1990; Rohrer, 1992; Rao et al., 1992b). The ChAT- and VIP-inducing activity in sweat gland homogenates can be blocked in vitro by neutralizing anti-LIFβ antibodies and activates the cytokine receptor pathway (Habecker et al., 1997). Although these results support the notion that neuropoietic cytokines control the cholinergic differentiation of sweat gland innervation, the physiological importance of neuropoietic cytokines remains to be demonstrated in vivo by loss-of-function experiments. This is important, because the production and response to neuropoietic cytokines is rapidly induced in neural cell types upon in vivo lesioning or in tissue culture (Freidin et al., 1992; Rao et al., 1993; Zigmund, 1996; Yao et al., 1997). In the chick embryo, we have demonstrated that neuropoietic cytokines are essential for sympathetic neuron differentiation; however, only a part of the properties that are induced in cultured sympathetic neurons are controlled by cytokines in vivo. Using antisense RNA to block the function of gp130, we observed that the developmental expression of VIP, but not of ChAT and VACHT, is dependent on gp130-cytokine family members (Geissen et al., 1998).

To further characterize the cytokines involved in the induction of VIP in avian sympathetic neurons, we have investigated which class of cytokine receptors is required for this differentiation step. As gp130 is the only signaling cytokine receptor subunit known in the chicken, the chick homolog of LIFβ was cloned, using a reverse transcriptase approach. Chicken LIFβ is expressed in sympathetic ganglia throughout development, which is in agreement with the notion that LIF-related cytokines are involved in the control of VIP expression. To test this hypothesis, we developed an in vitro antisense RNA strategy to interfere with cytokines acting through LIFβ and subsequently applied this approach in vivo. The expression of LIFβ antisense RNA in sympathetic ganglia strongly reduced the number of VIP-expressing cells, whereas the ganglion size and the number of ChAT-expressing cells were unaffected. Together with the previous results from the gp130 knockdown experiments, the present data strongly implicate cytokines that act through heterodimeric gp130/LIFβ receptors in the physiological control of VIP-expression in avian sympathetic neurons.

**MATERIALS AND METHODS**

**Cloning of chick LIFβ**

To obtain a chick LIFβ-cDNA fragment, degenerate primers (sense primer, 5'-AA(AG)CCCNCT(C/G)CA(AG)TTCT(C/T)CA(A/G)AA-3'; antisense primer, 5'-TC(G/A)AT(G/A)ATNGNGNGG(A/G)CA(G/A)-TA-3') based on human and mouse LIFβ sequences were used for RT-PCR. As the amplified sequence showed high homology to the other known LIFβ sequences, this fragment was used to screen a lambda ZAPII cDNA-library derived from embryonic day 8 (E8) sympathetic ganglia (Heller et al., 1995) and an adult chick brain library (Stratagene). The combined sequences could be obtained by 5'-RACE (Roche Diagnostic) on E9 liver cDNA (sense primer, 5'-CCATATACGATTTCTGGAAACA-3'; antisense primer, 5'-GCGATATCTGACTTCCAGTTC-3'; and nested antisense primer, CATTCCGAGTACCTCTCAATC-3'). The PCR-based sequences were confirmed by sequencing of several independent clones. The chick LIFβ nucleotide sequence will appear in the EMBL/GenBank/DDBJ databases under the accession number AJ416111.

**Transient transfection of sympathetic neurons in culture**

Vectors expressing sense and antisense LIFβ-RNA were created by subcloning a LIFβ-PCR-flank of ClaI-restriction sites into a pbK-CMV-vector (Stratagene). The fragment contains the 5'-untranslated region and the translation startpoint (bases -84 to +893), (sense primer, 5'-CCATCGATGCGCCGTGGCTGCAATAAACGAGCCAG-3'; antisense primer, 5'-CCATCGATTGCCACACTAATTTGTCCTCAGTGC-3'). Cultures of embryonic day 7 (E7) chick sympathetic neurons were prepared and maintained as previously (Ernsberger et al., 1989). DNA (8 µg sense/antisense LIFβ and 2 µg lacZ-expressing plasmid) were used to co-transfect about 1.6×10⁴ neurons in a 35 mm culture dish (Heller et al., 1995), using the calcium phosphate method. Twenty-four hours after co-transfection, the neurons were treated with 0.8 ng/ml CNTF to induce VIP expression. After further 3 days, the neurons were double-stained for VIP and β-galactosidase as described previously (Heller et al., 1995). The proportion of VIP-immunoreactive (VIP-IR) neurons in the β-gal-positive cells was determined. The significance of the difference between sense and antisense transfections was determined by Student’s t-test (pairwise comparison).

**Retroviral constructs and expression of antisense LIFβ RNA in sympathetic ganglia in vivo**

The LIFβ-fragment used in the in vitro transfection study was subcloned in sense and antisense orientation into the RCAS (B) proviral cloning vector (Hughes and Kosik, 1984; Morgan and Fekete, 1996) and verified via sequence analysis. DF-1 chicken embryonic
fibroblasts (American Type Culture Collection, Manassas) were transfected with the retroviral vector and cultured in DMEM containing 10% fetal calf serum and 1% Pen/Strep. Complete infection of the fibroblast culture was verified by staining with the AMV-3C2 anti-gag-antibody. The AMV-3C2 monoclonal antibody developed by D. Boettiger was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Iowa City, USA.

The in ovo chicken embryo manipulations and the in vivo infections were performed as described (Geissen et al., 1998). Fertilized virus-free chicken eggs [SPAFAS, Charles River (Sulzfeld)] were incubated at 38°C for 2 days. The embryos were staged (Hamburger and Hamilton, 1951) and aggregates of virus-producing fibroblasts [antisense RCAS (B) or sense RCAS (B)] were implanted into the embryos at brachial level between the neural tube and the last somite formed (Reissmann et al., 1996). At E14, the embryos were killed by decapitation. After removal of internal organs, the trunk and cervical region of the embryo were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for about 12 hours, kept for 2 days in 15% sucrose (in 0.1 M phosphate buffer, pH 7.3), embedded in Tissue-Tek (Sakura Finetek Europe BV) and sectioned. Consecutive 14 µm cryostat cross-sections were separately collected and analyzed for expression of reverse transcriptase (RT), VIP-, ChAT-, SCG10-, TH- and LIFRβ mRNA by in situ hybridization methods and LIFRβ protein expression by immunohistochemistry.

In situ hybridization

Non-radioactive in situ hybridization on cryosections and preparation of digoxigenin- or fluorescein-labeled probes for chick RT, VIP, ChAT, SCG10 and TH were carried out as described previously (Ernsberger et al., 1997). The probe to detect endogenous LIFRβ mRNA covers bases +2269 to +4114, and thus does not overlap with the sense/antisense LIFRβ fragment expressed via the RCAS (B) virus.

Immunohistochemistry on sections

Sections were equilibrated to room temperature, blocked for 1 hour with PBST (PBS, 0.1% Tween) containing 10% donkey serum, and subsequently incubated overnight at room temperature with an antibody raised against a LIFRβ C-terminal peptide (1:1000, Santa Cruz Biotechnology). After three washing steps of 15 minutes each in PBST, the sections were incubated with secondary antibody (Cy3-coupled anti-rabbit, 1:100, Molecular Probes) for 30 minutes at room temperature. After a further three washing steps, twice in PBST, once in PBS, the sections were mounted in PBS/glycerol (1:1) and finally analyzed by fluorescence microscopy.

Immunohistochemistry on cultured neurons and fibroblasts

Sympathetic neuron cultures were prepared from E7 chick paraver tebral sympathetic ganglia as described previously (Ernsberger et al., 1989). Chick embryo fibroblasts were maintained in culture as described above. Culture dishes were washed once for 15 minutes with KRH (125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 2H₂O, 25 mM Hepes, 1.2 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 5.6 mM glucose) and fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.3) for 15 min. After washing (three times) with PBST (0.1% Triton, 1% BSA in PBS), the neuron cultures were incubated (40 minutes) with rabbit antibody against VIP (1:100, Incstar; Sorin Diagnostics, Düsseldorf) and mouse anti-β-galactosidase antibodies (1:1000, Promega). Fibroblast cultures were incubated with AMV-3C2 anti-gag antibody (1:100) for 40 minutes. The antibody incubations were followed by four washing steps in PBST and PBT2 (0.1% Triton, 0.1% BSA in PBS) twice each. After 30 minutes of incubation with the secondary antibody (1:500, Cy3-coupled anti-rabbit, Cy3-coupled anti-mouse or FITC-coupled anti-mouse), the cultures were washed in PBT2 and PBS twice each. The cultures were mounted in PBS/glycerol and analyzed by fluorescence microscopy.

Morphometric analysis

The number of VIP- and ChAT-positive cells was counted on all sections infected by the virus indicated by the expression of RT mRNA. On alternate sections, the area of SCG10 mRNA expression was quantified morphometrically, using the Metamorph Imaging System (Version 4.0, Universal Imaging Corporation), and the number of VIP- and ChAT-positive cells was referred to the SCG10-positive area and expressed as number of VIP+ or ChAT+ cells/mm².

RT-PCR

Total RNA was isolated from E7, E8, E9, E10, E11 and E14 chick sympathetic ganglia (RNeasy Kit, Qiagen), treated with RNase-free DNaseI prior to reverse transcription (RT-PCR Kit, Stratagene). Semi-quantitative PCR reactions were performed in the linear range, using the following primers (sense primer, 5'-GCAATGCTTCAA-GTGCC-3'; antisense primer, 5'-CTGATCAGCCAGAACTGG-3') to obtain a 300 bp fragment of LIFRβ.

RESULTS

Cloning of chick LIFRβ and expression analysis

A fragment of the chick LIFRβ was obtained by RT-PCR using degenerate primers based on human (Gearing et al., 1991) and mouse (Tomida, 1995) LIFRβ sequences. This fragment was used to screen a lambda-cDNA library from E8 sympathetic ganglia. The screen resulted in the identification of several independent clones that contained the complete 3'-part of LIFRβ, including the transmembrane region. Major parts of the sequence of the extracellular region were obtained by secondary screens of an adult chick brain library. The 5'-terminal 300 bp region was identified by 5'-RACE. The sequences of all PCR based fragments were confirmed by analyzing several independent clones.

The combined sequences resulted in a sequence with an open reading frame encoding a 1083 amino acid protein (Fig. 1). The predicted amino acid sequence shows 51% and 49% identity to the human and mouse LIFRβ sequences, respectively, and identifies the cloned receptor as chick homolog of mammalian LIFRβ. The sequence displays a very low homology to the mammalian OSMR [32% and 31% identity to human (Mosley et al., 1996) and mouse (Tanaka et al., 1999) OSMR, respectively]. The chick LIFRβ displays characteristic traits of the gp130-family of cytokine receptors: the WSXWS motif (Bazan, 1990) is present twice in the extracellular part of the receptor subunit, as observed for mammalian LIFRβ (Fig. 1).

The results obtained for in situ hybridization and RT-PCR (Fig. 2) were confirmed by expression in spinal cord motoneurons, sensory dorsal root ganglia and paravertebral sympathetic ganglia at all stages analyzed (E7-E14). The expression in sympathetic ganglia was significantly higher than in neighboring tissues such as muscle and bone, but lower than in DRG and motoneurons (Fig. 2A).
The expression of LIFRβ in sympathetic ganglia was confirmed by RT-PCR analysis, using ganglia from E7 to E14 embryos (Fig. 2B). The expression of LIFRβ antisense RNA in cultured chick sympathetic neurons interferes with cytokine-induced VIP expression by the expression of antisense RNA from the LIFRβ 5′-terminal region (bases −83 to +893) (Fig. 3). The extent of inhibition was in the range observed after antisense-inhibition of other cytokine receptor subunits (Heller et al., 1995; Geissen et al., 1998).

Expression of LIFRβ antisense RNA interferes with the developmental VIP expression in vivo
To express antisense and sense RNA of LIFRβ in vivo, the avian-specific retroviral RCAS vectors (Hughes and Kosik, 1984; Morgan and Fekete, 1996) were used. Aggregates of virus-producing DF-1 cells were implanted into the neural crest migration pathway. Sympathetic ganglia in the cervical-thoracic region of stage 39/40 embryos (E13/14) were analyzed on serial frozen sections for the extent of virus infection and effects on VIP expression. The use of DF-1 cells resulted in a very low expression of VIP mRNA, which is consistent with the results obtained by in situ hybridization.
efficient infection of the paravertebral sympathetic ganglia on both sides of the embryos. The overall development of the embryos and of the nervous system, as judged by morphological criteria, was apparently not affected by the infection with LIFRβ sense or antisense RCAS virus (not shown). Sympathetic ganglion development was followed by the expression of the adrenergic marker gene TH and the panneuronal marker gene SCG10. All infected embryos showed neither differences in the expression of TH and SCG10 nor in morphological criteria, compared with control embryos (Fig. 4C,G,D,H). However, sympathetic ganglia expressing antisense RNA for LIFRβ displayed a reduced expression of VIP mRNA, when compared with sense RNA-expressing ganglia and uninfected controls (Fig. 4B,F). This effect was quantified by determining the number of VIP mRNA-positive cells per sympathetic ganglion area. The area of pan-neuronal SCG10 expression was analyzed by in situ hybridization, quantified morphometrically and the number of VIP-positive cells referred to the SCG10-expressing area. This analysis revealed a highly significant reduction in the number of VIP-positive neurons in ganglia expressing LIFRβ antisense RNA ($P<0.0001$) (Fig. 5G). As the sympathetic ganglion area, i.e. the area of SCG10-positive neurons is not significantly different between sense and antisense infected embryos (38808±5237 μm² and 35343±7520 μm²; $n=6$), the reduced proportion of VIP-positive cells reflects a reduction in the number of VIP-expressing cells per ganglion.

**LIFRβ antisense RNA specifically affects developmental VIP expression, but not ChAT expression in sympathetic ganglia**

The reduced number of VIP-positive cells could, in principle, either be due to a loss of VIP-positive cells or due to the lack of VIP expression. As the proportion of VIP-positive cells in the ganglia is relatively low, a loss of VIP-positive cells would be difficult to exclude, even if the ganglion size is not significantly different between sense and antisense treated embryos. However, as VIP is expressed by a subpopulation of sympathetic neurons that co-express the cholinergic marker genes VACHT and ChAT (Ernsberger et al., 1997; Ernsberger and Rohrer, 1999; Landis and Keefe, 1983; Elfvin et al., 1993), a loss of VIP-positive cells should also be reflected by a reduction in the number of ChAT/VACHT-positive cells. The quantitative analysis of ChAT expression revealed that the number of ChAT-expressing cells was not reduced in ganglia expressing LIFRβ antisense RNA (Fig. 5B,E,G). This result demonstrates that cytokines act through LIFRβ to control the expression of VIP, but are neither involved in the expression of ChAT, nor in the survival of the ChAT- and VIP-expressing cell population.

The quantification of ChAT- and VIP-positive cells in sections of cervical to thoracic ganglia at E13/14 (Fig. 5G,H) showed that VIP-positive cells are present in lower numbers than ChAT-expressing cells. Double-in situ hybridization for VIP and ChAT in E18 control embryos (Fig. 6A-D) revealed...
complete co-expression of VIP and CHAT at the cervical and thoracic levels analyzed, confirming the results of a previous analysis (Ernsberger and Rohrer, 1999). Thus, the lower numbers of VIP-expressing cells at E13/14 is most likely due to a continued increase of VIP expression levels in cholinergic sympathetic neurons between E14 and E18.

To investigate the specificity of LIFRβ antisense RNA treatment, we analyzed the expression of LIFRβ in embryos infected with RCAS containing sense or antisense LIFRβ sequences. The infection protocol results in the infection of sympathetic ganglia, whereas sensory DRG and spinal cord remain mostly uninfected (Fig. 7A,C) (Geissen et al., 1998). Thus, the endogenous LIFRβ expression in the DRG and spinal cord can be used as reference for LIFRβ levels in sympathetic ganglia. LIFRβ protein expression, analyzed by immunohistological techniques, was specifically reduced in antisense-infected sympathetic ganglia (Fig. 7D) when compared with sense-infected ganglia (Fig. 7B) and uninfected DRG and spinal cord (Fig. 7D). Similar results were obtained using in situ hybridization for endogenous LIFRβ-mRNA (not shown). Taken together with the selective reduction of VIP, but not of CHAT, SCG10 and TH expression, these results illustrate a specific inhibition of cytokine effects in sympathetic ganglia by LIFRβ antisense RNA.

Fig. 4. Sympathetic ganglia infected with LIFRβ antisense-RCAS retrovirus exhibit a decreased number of VIP-expressing neurons. Chick embryos were infected with LIFRβ sense-RCAS (A-D) or LIFRβ antisense-RCAS (E-H) at E2 and analyzed by in situ hybridization for viral infection (RT) (A,E), and the expression of VIP (B,F), TH (C,G) and SCG10 (D,H) on alternate thoracic sections of E13/14 embryos. Sympathetic ganglia expressing antisense LIFRβ mRNA (F) displayed a strong reduction in the number of VIP-positive cells as compared to ganglia infected with sense-RCAS (B). By contrast, the expression of TH mRNA (C,G) and SCG10 mRNA (D,H) is not altered. Scale bar: 100 μm.

Fig. 5. Quantification of VIP and ChAT expression in E13/14 sympathetic ganglia. The number of VIP- (A,D) and ChAT- (B,E) positive neurons was determined in cervical and thoracic ganglia infected by LIFRβ antisense-RCAS and LIFRβ sense-RCAS. The area of neuronal SCG10-positive cells in the sympathetic ganglia section was determined morphometrically, as described in Materials and Methods. The numbers of VIP- (G) and ChAT-positive (H) cells were referred to the SCG10-positive area. Scale bar: 100 μm. 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).
DISCUSSION

The generation of the different neurotransmitter phenotypes in the autonomic nervous system depends on extrinsic signals. In vitro studies suggested a role for cytokine family members in the acquisition of cholinergic and peptidergic (VIP) characteristics. For chick sympathetic ganglia, we have previously demonstrated an essential role for cytokines in the developmental expression of VIP, but not of cholinergic marker genes (Geissen et al., 1998). The present study confirms and extends these results by demonstrating that the cytokine receptor subunit LIFRβ is essential for VIP expression in sympathetic neurons, but is not involved in the control of ChAT expression. Taken together, these results support the notion of separate control mechanisms for the expression of VIP and ChAT genes and implicate cytokines signaling through LIFRβ-containing receptor complexes in the control of VIP expression during sympathetic neuron development.

Cholinergic sympathetic neurons are generated from noradrenergic sympathetic neurons during normal development. For rat and mouse cholinergic sympathetic neurons, there is convincing evidence from the analysis of sweat gland innervation that this switch of neurotransmitter phenotype is due to signals secreted from the innervated target tissue (reviewed by Francis and Landis, 1999). The sweat gland innervating sympathetic fibers display noradrenergic properties before they reach their targets and switch to the expression of cholinergic traits and VIP after target contact (Landis and Keefe, 1983; Leblanc and Landis, 1986; Guidry and Landis, 1998). Members of the cytokine family of growth factors are good candidates for target-derived cholinergic differentiation factors, as they are able to induce ChAT and VIP expression in cultured sympathetic neurons (Yamamori et al., 1989; Ernsberger et al., 1989; Saadat et al., 1989; Heller et al., 1995; Rao et al., 1992b; Habecker et al., 1995; Ernsberger et al., 1997). In addition, crude extracts of sweat gland tissue contain cytokine family members that act through LIFRβ-containing receptors (Habecker et al., 1997). Loss-of-function approaches suggest that none of the known cytokines is involved in the cholinergic differentiation of sweat gland innervation (Masu et al., 1993; Rao et al., 1993; Habecker et al., 1995; Habecker et al., 1997; Francis et al., 1997). This presumably also includes the CNTFRα-interacting CLC/CLF (Elson et al., 2000), as cytokines that act through CNTFRα have been excluded as candidate factors in sweat gland homogenates (Habecker et al., 1997). Thus, the physiological importance of cytokines for cholinergic differentiation in mammals is still unclear.
In the chick, after the initial phase of noradrenergic differentiation at E3, cholinergic genes ChAT and VACHT are widely expressed in sympathetic neurons from E7 onwards (Ernsberger et al., 1997) (M. Stanke and H. R., unpublished). At E10, several days after the onset of ChAT and VACHT expression, the first VIP-positive cells are detectable (New and Mudge, 1986; Ernsberger et al., 1997). With increasing age, the noradrenergic properties and the characteristic cholinergic genes VACHT, ChAT and VIP become restricted to two separate cell populations: TH+/DBH+ neurons that are devoid of cholinergic gene expression and ChAT+/VACHT+/VIP+ neurons that are devoid of noradrenergic marker genes (Ernsberger et al., 1997; Ernsberger and Rohrer, 1999). The timing of VIP expression correlates with the innervation of peripheral targets by sympathetic fibers. The only target of cholinergic sympathetic fibers known so far in the chick are blood vessels in muscle and skin (U. Zechbauer and H. R., unpublished). These results suggest a two-step differentiation process for avian sympathetic neurons that involves an initial induction of ChAT and VACHT at E7, followed by a later, target-dependent induction of VIP. In vitro, chick sympathetic neurons were also shown to express ChAT and VIP in response to cytokines (Ernsberger et al., 1989; Heller et al., 1995; Ernsberger et al., 1997). The first in vivo evidence for a physiological role of neuromodulatory cytokines was obtained by the inhibition of gp130 expression in chick sympathetic ganglia (Geissen et al., 1998), resulting in a strong reduction of VIP, but not of ChAT expression. However, the identity of the cytokine that controls VIP expression in avian sympathetic ganglia is unclear. So far, only one cytokine, GPA, has been cloned from the chick, which is closely related to mammalian CTGF (Leung et al., 1992). GPA is an unlikely candidate for the target-dependent VIP-inducing cytokine, owing to its strong expression in glial cells of peripheral nerves (Leung et al., 1992) and because the expression of CNTFRα antisense RNA does not affect VIP expression in sympathetic ganglia (M. G., unpublished). The latter result also excludes chick homologs of CLC/CLF as potential candidates.

The cytokine family of growth factors can be subdivided into different classes, depending on the receptor subunit composition (reviewed by Taga and Kishimoto, 1997). IL-6-like factors use receptor complexes that contain gp130 homodimers, OSM uses a heterodimer composed of gp130 and OSMR or gp130/LIFRβ, LIF, CNTF, CT-1, CLC/CLF and related factors activate receptor complexes that contain gp130/LIFRβ heterodimers as signaling subunits. The present study aimed to characterize the VIP-inducing cytokine with respect to its receptor subtype by interfering with the expression of LIFRβ in sympathetic ganglia. To this purpose, the chick homolog of mammalian LIFRβ was cloned, LIFRβ expression was analyzed and an antisense RNA approach was developed to knock down LIFRβ expression.

The sequence of the cloned cDNA is most homologous to mammalian LIFRβ (Gearing et al., 1991; Tomida, 1995) and is only distantly related to the mammalian OSMR sequences (Mosley et al., 1996; Tanaka et al., 1999). In addition, the number of WSXWS motifs is characteristic for LIFRβ but not for OSMR or gp130. In the cytoplasmic domain, the amino acid sequence is highly conserved, which is also reflected by the crossreactivity of LIFRβ antisera raised against a C-terminal peptide of human LIFRβ. The analysis of LIFRβ expression in the developing nervous system by in situ hybridization showed high expression levels in neuronal populations known to respond to neurotropic cytokines, i.e. spinal cord motoneurons (Sendtner et al., 1991; Martinou, 1992; Li et al., 1995), sensory DRG neurons (Murphy et al., 1993; Rodig et al., 1998) and sympathetic ganglia (Yamamori et al., 1989). The continuous expression of LIFRβ mRNA in sympathetic ganglia between E7 and E14 was confirmed by RT-PCR.

To interfere with the expression of LIFRβ an antisense RNA approach was developed. The expression of antisense RNA from the 5’ region of the LIFRβ produced an in vitro inhibition of cytokine-mediated VIP-induction, whereas sense RNA was without effect. The inhibition was not complete, but this was also not expected, as only the synthesis of new receptors can be blocked by antisense RNA, whereas receptors present in the plasma membrane and/or in the ER/Golgi on their way to the cell surface are still able to signal. The inhibition achieved is in the range of effects observed previously in this system, using antisense RNA for CNTFRα or gp130 (Heller et al., 1995; Geissen et al., 1998).

Antisense or sense RNA for LIFRβ was expressed in vivo in chick sympathetic ganglia by the use of RCAS retroviral vectors. The infection protocol, involving the implantation of aggregates of virus-producing cells into the area where the sympathetic ganglion primordia form, resulted in strongly infected sympathetic ganglia in virtually all embryos analyzed. By contrast, spinal cord was never infected and DRG only in a few cases. The expression of LIFRβ antisense or sense RNA had no obvious morphological effects on embryo development or the developing nervous system. This was confirmed by the analysis of pan-neuronal (SCG10) gene expression in sympathetic ganglia, DRG and the spinal cord. Quantification of sympathetic ganglion size did not show any difference between antisense and sense controls. This is in agreement with previous studies that also failed to observe an effect on sympathetic ganglion size in response to gp130 knockout in chick embryos (Geissen et al., 1998) or in sympathetic ganglia of LIFRβ knockout mice (Stanke et al., 2000). Further evidence for the specificity of the antisense RNA approach comes from the demonstration that LIFRβ expression is reduced in antisense-expressing sympathetic ganglia. In contrast to the unaffected general development of sympathetic ganglia, the cholinergic subpopulation displayed a strongly reduced VIP expression, although the number of ChAT-positive cells remained constant.

As VIP is exclusively expressed by cholinergic neurons during normal development (Geissen et al., 1998; Ernsberger and Rohrer, 1999), the decreased number of VIP-positive cells can thus not be explained by the death of VIP-positive cells. The unchanged number of ChAT-positive cells in antisense-treated ganglia rather indicates that cholinergic neurons, which would normally express VIP, are still present in the absence of LIFRβ activation, but do not induce VIP expression. These results support a two-step model of cholinergic sympathetic differentiation that has been implicated from the different developmental onset of ChAT/VACHT and VIP expression (Ernsberger et al., 1997; Ernsberger and Rohrer, 1999). The effect of LIFRβ antisense treatment is stronger after in vivo expression as compared with the in vitro experiments. This may be due to the continuous expression of antisense RNA in the infected cells, starting with the infection at E2 and resulting in a strongly decreased LIFRβ expression. The residual presence
of some VIP-positive cells can be explained by assuming that some sympathetic neurons cells are not infected. This is indeed quite likely, as the first sympathetic precursors withdraw from the cell cycle between E2 and E3 (Rothman et al., 1978). An alternative possibility, that is also compatible with the results, would be that a minority of cholinergic sympathetic neurons would express VIP in response to factors that do not act through LIFRb/gp130 cytokine receptors.

The expression of cholinergic marker genes in the absence of gp130 and LIFRβ signaling raises the question of which factor(s) induce the expression of ChAT and VACHT. Recent studies described a selective expression of receptors for GDNF-related factors and the neurotrophin NT-3 in the chick cholinergic sympathetic neuron subpopulation (Ernsberger et al., 2000; Brodski et al., 2000). The signaling receptor subunit Ret, which mediates the action of GDNF, neurturin, artemin and persephin, as well as the NT3 receptor trkC, are expressed by cholinergic sympathetic neurons at late stage of development when noradrenergic and cholinergic genes are expressed by different subpopulations (Ernsberger et al., 2000; Brodski et al., 2000). Although NT3 is able to induce the expression of ChAT in explants of E12 sympathetic ganglia (Brodski et al., 2000) it is not known whether this effect could be also observed at E7, when ChAT and VACHT expression starts during normal development. As the control of ChAT expression changes strongly during sympathetic neuron development (Ernsberger et al., 1997) and as the gene expression pattern induced by NT3 in vitro differs from that of cholinergic neurons in vivo, the physiological relevance of these in vitro observations remains to be confirmed by in vivo loss- or gain-of-function experiments.

In summary, we report several important aspects of the control of chick sympathetic neuron development by neuropoietic cytokines, including the cloning of the chick cytokine receptor subunit LIFRβ, its developmental expression and its essential role for the expression of VIP in sympathetic ganglia. Furthermore, we provide considerable evidence to suggest that cholinergic marker genes are expressed independently of the VIP gene. Although many aspects of the regulatory network of cholinergic neuron differentiation remain unsolved, the present study provides a solid basis for the identification and functional analysis of cytokine family members that control the expression of the important vasodilator VIP during the development of autonomic neurons.

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