The sympathetic innervation of sweat glands undergoes a target-induced noradrenergic to cholinergic/peptidergic switch during development. Similar changes are induced in cultured sympathetic neurons by sweat gland cells or by one of the following cytokines: leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), or cardiotrophin-1 (CT-1). None of these is the sweat gland-derived differentiation activity. LIF, CNTF, and CT-1 act through the known receptors LIF receptor β (LIFRβ) and gp130 and well defined signaling pathways including receptor phosphorylation and STAT3 activation. Therefore, to determine whether the gland-derived differentiation activity was a member of the LIF/CNTF cytokine family, we tested whether it acted via these same receptors and signal cascades. Blockade of LIFRβ inhibited the sweat gland differentiation activity in neuron/gland co-cultures, and extracts of gland-containing footpads stimulated tyrosine phosphorylation of LIFRβ and gp130. An inhibitor (CGX) of molecules that bind the CNTFRα, which is required for CNTF signaling, did not affect the gland-derived differentiation activity. Soluble footpad extracts induced the same changes in NBFL neuroblastoma cells as LIF and CNTF, including increased vasoactive intestinal peptide mRNA, STAT3 dimerization, and DNA binding, and stimulation of transcription from the vasoactive intestinal peptide cytokine-responsive element. Thus, the sweat gland-derived differentiation activity uses the same signaling pathway as the neuroepoietic cytokines, and is likely to be a family member.

The sympathetic innervation of rodent sweat glands undergoes a switch in neurotransmitter properties during development. The sympathetic axons innervating rat sweat glands are initially noradrenergic, but as the innervation matures, noradrenergic markers decrease and cholinergic and peptidergic properties are acquired, so that the mature sweat gland innervation co-expresses acetylcholine and vasoactive intestinal peptide (VIP) (1–3). The alteration in the neurotransmitter phenotype is induced by interactions with the sweat gland target tissue (4–7) and can be elicited in cultured sympathetic neurons by the addition of sweat gland cells (8, 9) or extracts of sweat gland-containing footpads (8–12).

Similar neurotransmitter changes can be stimulated in cultured sympathetic neurons by the cytokines ciliary neurotrophic factor (CNTF; Refs. 13–15), leukemia inhibitory factor (LIF; Refs. 12 and 16–18), and cardiotrophin-1 (CT-1; Refs. 19 and 20), which induce cholinergic function and VIP production while decreasing catecholamine content. A closely related cytokine, oncostatin M (OSM), has a much smaller effect on sympathetic neuron phenotype (21). Many other growth and differentiation factors have been tested for their effects on sympathetic neuron phenotype, and these do not promote the acquisition of both cholinergic and peptidergic function in sympathetic neurons (22–24). Thus, CNTF, LIF, and CT-1, members of a larger family of molecules known as the neuroepoietic cytokines, are currently the only proteins known to produce the changes in sympathetic neuron phenotype in vitro that are observed during the development of the sweat gland innervation in vivo. Analysis of mice lacking CNTF and LIF reveals, however, that in the absence of both cytokines the sweat gland sympathetic innervation still acquires cholinergic properties and VIP immunoreactivity (25). In addition, an antisense that blocks CT-1 induction of cholinergic differentiation in cultured sympathetic neurons does not inhibit the sweat gland-induced increase in cholinergic function (19). Therefore, although LIF, CNTF, and CT-1 are the only currently identified proteins that produce all of the changes in sympathetic neuron phenotype induced by sweat glands, it is unlikely that any of them is responsible for the differentiation activity present in sweat glands.

The LIF/CNTF/CT-1 family of cytokines elicits similar effects in a variety of cell types (20, 26, 27) but share little amino acid homology. Despite the lack of sequence identity, these molecules are thought to share common three-dimensional structure (28–30) and are known to share receptor subunits and signal transduction pathways (for a review, see Refs. 31–35). These molecules use two common receptor subunits, LIF receptor β (LIFRβ) and gp130 (20, 36–41), while CNTF requires an additional α subunit (CNTFRα) for receptor binding and activation.

The abbreviations used are: CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; CT-1, cardiotrophin-1; OSM, oncostatin M; LIFRβ, LIF receptor β; CNTFRα, CNTF receptor α; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; VIP, vasoactive intestinal peptide; ChAT, choline acetyltransferase; STAT, signal transducers and activators of transcription; CyRE, cytokine-responsive element.
viation (42, 43). The transmembrane receptor subunits LIFRβ and gp130 do not have intrinsic kinase activity, but their intracellular domains are constitutively associated with Jak tyrosine kinases (Jak1, Jak2, or Tyk2) (44, 45). Cytokine binding induces association of the receptor subunits, resulting in the activation and phosphorylation of the Jak kinases and subsequent phosphorylation of both the receptor subunits and downstream signaling proteins (40, 44, 46, 47). One of the best characterized effector molecules that is stimulated in response to LIF and CNTF is STAT3 (47–50), a member of the STAT family of DNA-binding proteins (51, 52). Tyrosine phosphorylation of STAT3 in the cytoplasm leads to its dimerization and translocation to the nucleus (51, 53), where it binds to DNA and stimulates the transcription of a number of genes, including the VIP gene (52).

The cytokines LIF and CNTF alter sympathetic neuron phenotype and stimulate production of VIP in NBFL neuroblastoma cells through activation of LIFRβ/gp130 and the Jak-STAT pathway (52, 54). We have therefore used primary sympathetic neurons and NBFL neuroblastoma cells to investigate whether the sweat gland–derived differentiation activity shares these receptors and downstream signaling pathways. We present evidence that the gland-derived activity requires LIFRβ, results in the activation of both LIFRβ and gp130, and does not require the CNTFRα. In addition, it causes the activation of STAT3 and stimulates transcription of the VIP gene via a cytokine-responsive element in the VIP promoter. Therefore, the sweat gland–derived differentiation activity shares signaling mechanisms with these cytokines and appears to be a novel member of the LIF/CNTF cytokine family.

**MATERIALS AND METHODS**

**Primary Cell Culture**—Cultures of sympathetic neurons were prepared from the superior cervical ganglia of newborn rats as described by Hawrot and Patterson (55) and modified by Rao and Landsis (56). To reduce the number of nonneuronal cells, neurons were preplated for at least 2 h, and then for 2 days after plating they were grown in the presence of the antimitotic agent cytosine arabinoside (araC; 10 μM). Cells were treated with cytokines or footpad extracts for 15 min before harvesting in ice-cold PBS. Nuclear extracts were isolated, and binding reactions were performed as described previously (52). The G3 synthetic complementary oligonucleotides 5′-GGGATTTCCGTAATACGACTCACTATAGG-3′, or 5′-TCATGAGTGCACGATGTACG-3′, were annealed and labeled with [32P]dCTP using Superscript reverse transcriptase (Life Technologies, Inc.). Nuclear extracts (approximately 15 μg of protein) were incubated with 0.5 ng of labeled probe (approximately 250,000 cpm) for 20 min at room temperature before electrophoretic separation on a 5% nondenaturing polyacrylamide gel (37.5:1) in 0.5 × TBE at 200 V. Antibodies, when used, were added 10 min prior to the addition of the probe. STAT1 and STAT3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**CNTF Immunoblotting**—Footpad and sciotic nerve extracts were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose for identification of CNTF. Blots were blocked 1 h with 5% low fat dry milk in TBS-T and incubated overnight with anti-CNTF (the kind gift of Peter Richardson, Montreal General Hospital) diluted 1:2000 in blocking solution. Bound antibodies were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000) and visualized by chemiluminescence (NEN Life Science Products).

**Receptor Phosphorylation**—NBFL cells were grown to confluence on 10-cm plates. Cells were treated with cytokines or footpad extracts for 15 min before washing in ice-cold PBS. Nuclear extracts were isolated, and binding reactions were performed as described previously (52). The G3 synthetic complementary oligonucleotides 5′-GGGATTTCCGTAATACGACTCACTATAGG-3′, or 5′-TCATGAGTGCACGATGTACG-3′, were annealed and labeled with [32P]dCTP using Superscript reverse transcriptase (Life Technologies, Inc.). Nuclear extracts (approximately 15 μg of protein) were incubated with 0.5 ng of labeled probe (approximately 250,000 cpm) for 20 min at room temperature before electrophoretic separation on a 5% nondenaturing polyacrylamide gel (37.5:1) in 0.5 × TBE at 200 V. Antibodies, when used, were added 10 min prior to the addition of the probe. STAT1 and STAT3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**RESULTS**

**The Sweat Gland-derived Differentiation Factor Requires the LIFRβ**—The neuroepithelial cytokines share two receptor subunits, gp130 and LIFRβ (36, 38, 39, 41). To test whether the sweat gland–derived differentiation factor uses LIFRβ, we asked whether blocking LIFRβ prevented sweat gland–induced changes in the sympathetic neuron phenotype. Sympathetic neurons were co-cultured with sweat gland cells or treated with cellular debris was removed by centrifugation, and supernatants were assayed for dopamine content by high pressure liquid chromatography with electrochemical detection as described (63).

Statistical significance of results was analyzed by ANOVA using Statview 4.0. Asterisks indicate values that differ significantly from control values.

**RNA Isolation and Analysis**—Cytoplasmic RNA was isolated from NBFL cells treated with cytokines or footpad extracts by Nonidet P-40 lysis (64). RNA was separated by electrophoresis on formaldehyde, 1.3% agarose gels and electrotansferred onto GeneScreen membranes. Northern blots were hybridized as described with a 580-base pair fragment of human VIP (65) and rehybridized with a probe for the unregulated internal reference gene cyclophilin (66) to correct for loading differences. Total cytoplasmic RNA was isolated from sweat gland cells, neuron gland co-cultures, and the sciatic nerves, sweat glands, and rear footpads of adult rats using RNAzol B (67). To prepare RNA for PCR analysis, 0.5 μg of total RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamer primers. PCR reactions were carried out with CNTF: forward, 5′-ACAGTGTGATTGGGATGG-3′; reverse, 5′-AGGGAGAAGAGGTATGAGC-3′, oncostatin M: forward, 5′-ACCTACAGCGAAGACAGC-3′; reverse, 5′-TGAAGACCTCCTCCACATG-3′; or β-actin: forward, 5′-TCATGAGTGTCAGGTCCATCGT-3′; reverse, 5′-CCTAGAAGCTTCTGCGTGCAGT-3′, specific oligonucleotide primers. Reactions with cytokine-treated cells were subjected to gel electrophoresis, and some samples were denatured and transferred to Nytran membranes. Amplification reactions were carried out through 30 cycles, and the products were detected with a biotinylated CNTF oligonucleotide probe (5′-TCTGGGAGGTGAGATGACTGAGGCG) using the Flash rad-free chemiluminescence system (Stratagene).

**Nuclear Extract Preparation and Gel Retardation Assays**—NBFL cells were grown to confluence on 10-cm plates. Cells were treated with cytokines or footpad extracts for 15 min before harvesting in ice-cold PBS. Nuclear extracts were isolated, and binding reactions were performed as described previously (52). The G3 synthetic complementary oligonucleotides 5′-GGGATTTCCGTAATACGACTCACTATAGG-3′, or 5′-TCATGAGTGCACGATGTACG-3′, were annealed and labeled with [32P]dCTP using Superscript reverse transcriptase (Life Technologies, Inc.). Nuclear extracts (approximately 15 μg of protein) were incubated with 0.5 ng of labeled probe (approximately 250,000 cpm) for 20 min at room temperature before electrophoretic separation on a 5% nondenaturing polyacrylamide gel (37.5:1) in 0.5 × TBE at 200 V. Antibodies, when used, were added 10 min prior to the addition of the probe. STAT1 and STAT3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Primary Cell Culture**—Cultures of sympathetic neurons were prepared from the superior cervical ganglia of newborn rats as described by Hawrot and Patterson (55) and modified by Rao and Landsis (56). To reduce the number of nonneuronal cells, neurons were preplated for at least 2 h, and then for 2 days after plating they were grown in the antimitotic agent cytosine arabinoside (araC; 10 μM). Cultures of sympathetic neurons, sweat gland cells and sweat gland/neuron co-cultures were established as described previously (9). Co-cultures were harvested to assay neuronal choline acetyltransferase activity 5–7 days after the addition of sweat gland cells. Cytokines and antibodies were diluted in L15-CO2 and filter-sterilized before the addition onto cells.

**Transfections**—NBFL neuroblastoma cells were maintained as described previously (56). Cells were transfected overnight by calcium phosphate precipitation, with 12 μg of luciferase reporter plasmid and 4 μg of Rous sarcoma virus-chloramphenicol acetyltransferase plasmid/35-mm plate. Cells were treated with cytokines or footpad extracts beginning 6 h after transfection; 36 h later, cells were harvested and assayed for luciferase and cholinergic acetyltransferase activity. Luciferase was normalized to chloramphenicol acetyltransferase activity to control for transfection efficiency.

**Animals**—Mice with targeted disruption of the genes for CNTF or LIF have been described elsewhere (57, 58). Founder breeding pairs for protein concentrations were determined using the Pierce protein assay concentrated by centrifugation through Centricon 10-kDa filters, and activity was assayed for luciferase and chloramphenicol acetyltransferase activity. Activity was normalized to chloramphenicol acetyltransferase activity.

**Extraction Preparation**—Footpad extracts were prepared from the rear footpads of mice and rats as described previously (9, 12). Extracts were concentrated by centrifugation through Centricon 10-kDa filters, and protein concentrations were determined using the Pierce protein assay kit.

**Biochemical Assays**—Cholinergic function was determined by measuring the activity of choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine, using the method of Fonnum (61) as modified by Rao (11). Dopamine was quantified as a measure of catecholamine production (62). Co-cultures were homogenized in ice-cold 0.2% perchloric acid,
LIF or retinoic acid in the presence of antisera that block LIFRβ function. Five to seven days after treatment and/or co-culture, cells were assayed for adrenergic and cholinergic function. The addition of two polyclonal antisera raised against the mouse LIFRβ (a kind gift of Immunex Corp., Seattle, WA) (68), each at a dilution of 1:100, prevented the LIF-induced increase in ChAT activity and suppression of catecholamine content in sympathetic neurons. Similarly, the addition of anti-LIFRβ to sympathetic neurons that were co-cultured with sweat gland cells inhibited both the induction of cholinergic and the loss of adrenergic properties in these neurons (Fig. 1, A and B). The retention of normal catecholamine content indicated that the lack of ChAT induction was not the result of compromised neuron health due to antibody treatment. Anti-LIFRβ did not inhibit the retinoic acid-induced increase in ChAT activity and decrease in catecholamine content, which is mediated through the retinoic acid receptor α (69), suggesting that the blockade of ChAT induction and catecholamine suppression was specific to inhibition of LIFRβ. Similar results were observed in sympathetic neurons treated with footpad extracts from wild type and CNTF −/− mice rather than co-cultured with sweat gland cells (data not shown), indicating that inhibition of LIFRβ on neurons rather than gland cells was responsible for the lack of neuronal ChAT induction. These results suggest that the sweat gland-derived differentiation factor acts through the LIFRβ to induce changes in sympathetic neuron phenotype.

Since the differentiation factor produced by sweat gland cells required activation of LIFRβ, we determined whether LIF itself was contributing to the differentiation activity in co-cultures. LIF does not appear to be produced by sweat glands in vivo (12, 25, 60) or by gland cells cultured alone, which do not produce any cholinergic differentiation activity (9). Cyclic AMP can, however, induce the production and secretion of LIF in primary cultures of certain cell types that do not otherwise produce LIF (70, 71). This raises the possibility that co-culture of sweat gland cells with sympathetic neurons, which release norepinephrine that can increase cAMP levels in sweat glands (72), could result in the production of LIF by the gland cells. Therefore, sweat gland cells derived from animals that lack the gene encoding LIF were co-cultured with sympathetic neurons to test whether LIF contributed to the cholinergic differentiation activity in co-cultures. Gland cells that lacked the ability to make LIF retained the ability to induce cholinergic function in sympathetic neurons, indicating that the gland cell-derived differentiation activity in co-cultures is not LIF (Fig. 1C). Similarly, the addition of anti-LIF antibodies (the kind gift of Dr. Keiko Fukada, SUNY Stony Brook) (73) to neuron/sweat gland co-cultures did not inhibit the induction of cholinergic function in sympathetic neurons (data not shown).

The Sweat Gland-derived Differentiation Factor Stimulates Phosphorylation of gp130 and LIFRβ—The receptor subunits gp130 and LIFRβ do not have intrinsic kinase activity, but when bound by a ligand such as LIF or CNTF they are phosphorylated on tyrosine residues (38). NBFL neuroblastoma cells, which respond to LIF and CNTF (52, 56), express gp130 and LIFRβ and provide a well characterized system with which to pursue biochemical experiments that are difficult to carry out with the limited number of cells available in primary neuron cultures. We therefore investigated whether treatment of NBFL neuroblastoma cells with footpad extracts resulted in tyrosine phosphorylation of gp130 and LIFRβ (Fig. 2). NBFL cells were treated with CNTF (10 ng/ml) or with 300 μg/ml of extracts from sweat gland-containing footpads. Extracts from both wild type (wt-FP) and CNTF −/− (ko-FP) mice were used in these experiments, since footpad extracts, as described below, contain not only the sweat gland-derived factor but also CNTF derived from Schwann cells associated with the footpad sensory innervation (10, 12). The receptor subunits gp130 and LIFRβ were immunoprecipitated from previously frozen cell lysates and immunoblotted with an anti-phosphotyrosine antibody. Treatment of NBFL cells with CNTF or with footpad extracts from either wild type or CNTF −/− mice resulted in phosphorylation of gp130 and LIFRβ as determined by immunoblotting with an anti-phosphotyrosine antibody (Fig. 2, C). To determine whether LIF contributed to this phosphorylation, we cultured NBFL cells with footpad extracts from wild type and LIF−/− mice (Fig. 2, D). Since footpad extracts from LIF−/− mice do not inhibit the induction of cholinergic activity in co-cultures (compare Figs. 1 and 2, A and D), we predicted that LIF−/− footpad extracts would not induce the phosphorylation of gp130 and LIFRβ. As predicted, extracts from LIF−/− mice did not induce phosphorylation of these receptors. These results suggest that LIF contributed to the phosphorylation of these receptors and was required for the differentiation activity in co-cultures.
increased phosphorylation of both the gp130 and LIFRβ receptor subunits, suggesting that a gland-derived differentiation factor activated a receptor complex including these subunits. Footpads from wild type mice stimulated a greater increase in receptor phosphorylation of the two subunits than did footpads from CNTF knockouts, consistent with the presence of both a gland-derived activity and CNTF in extracts from wild type footpads. Co-immunoprecipitation with one receptor antibody usually brings down a complex containing the other receptor as well. We observed co-immunoprecipitation in prefrozen lysates from cells treated with CNTF and wild type extracts but not in lysates from cells treated with CNTF −/− extracts. Given the relative amounts of phosphorylation induced by these treatments, it seems likely that co-immunoprecipitation from cells treated with CNTF −/− extract is simply below the level of detection.

The Sweat Gland-derived Differentiation Factor Does Not Require CNTFRα—Because the sweat gland-derived differentiation factor activated LIFRβ and gp130, both of which are used by CNTF, we determined whether the gland-derived activity utilized the CNTFRα. This membrane-tethered receptor forms a complex with gp130 and LIFRβ and is required for CNTF signaling but is not used by other known cytokines (40, 42, 43). To test whether the gland-derived factor bound to CNTFRα, we assayed the ability of a covalent dimer composed of the human CNTFRα and the extracellular domain of human gp130 to inhibit the cholinergergic differentiation activity in sweat glands. This soluble dimer, CGX, has a higher affinity for CNTF than the heterotrimeric CNTFRα complex and can inhibit signaling through the cell surface receptor by binding molecules that interact with the CNTFRα.2 The addition of 500 ng/ml CGX did not inhibit the ability of murine CT-1 (150 ng/ml; kindly provided by Diane Pennica, Genentech), which does not bind CNTFRα (37), to induce cholinergergic function in sympathetic neuron cultures, but it completely blocked induction of cholinergergic function in response to murine CNTF (10 ng/ml), indicating that the human dimer bound mouse CNTF. The same concentration of CGX had no effect on the ability of sweat gland cells, derived from either rats or CNTF −/− mice, to induce cholinergergic function in sympathtetic neurons (Fig. 3). This suggests that although the gland-derived factor activates LIFRβ and gp130, two components of the CNTF receptor complex, it does not require the CNTFRα subunit.

Based on the failure of the differentiation activity present in neuron/sweat gland co-cultures to interact with the CNTFRα, it seems unlikely that the factor released by sweat glands shares extensive homology with CNTF. Previous immunoprecipitation experiments, however, had suggested that while LIF was absent from footpad extracts, CNTF and/or a CNTF-like molecule accounted for a portion of the ChAT- and VIP-inducing activity present in rat footpad extracts (10, 12). When we analyzed the expression of CNTF, we found that CNTF mRNA was detectable in footpad total RNA by RT-PCR (Fig. 4A) but not by Northern blot (12), and CNTF protein was identified by immunoblot using chemiluminescence but not by alkaline phosphatase (Fig. 4B) (12). Previous immunocytochemical analysis indicated that CNTF immunoreactivity is not detectable in sweat glands but is instead present in myelinating Schwann cells associated with the footpad sensory innervation (10, 12). Consistent with this observation, CNTF mRNA is not detectable by RT-PCR in neuron/sweat gland co-cultures, which contain few Schwann cells (Fig. 4A). Although the CNTF detected in footpad extracts and RNA does not appear to be produced by sweat glands, it contributes to the cholinergergic inducing activity present in these extracts. Therefore, mice lacking CNTF were used as a source for footpad extracts to ensure that the effects on NBFL cells were due to the sweat gland-derived differentiation activity.

Footpad Extracts Stimulate Transcription VIP mRNA in NBFL Cells—Treatment of cultured sympathetic neurons with CNTF, LIF, or footpad extracts causes an increase in VIP mRNA and peptide content (10, 12–14, 17, 18, 21, 23, 24, 54). The same change occurs in the NBFL cell line in response to CNTF and LIF (56), making this a useful model system to identify the molecules involved in mediating cytokine responses like increased VIP expression (52, 74). To determine whether the differentiation activity in footpad extracts could induce VIP mRNA in NBFL cells as it does in sympathetic neurons, NBFL neuroblastoma cells were treated with recombinant CNTF (10 ng/ml) or with 300 μg/ml footpad extracts

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from CNTF −/− mice. Four hours after treatment, RNA was isolated and analyzed by Northern blot. The addition of footpad extracts from CNTF −/− mice increased VIP mRNA in NBFL cells, while the level of RNA encoding the housekeeping gene cyclophilin remained constant, indicating that similar levels of RNA were present in each sample. Therefore, footpad extracts contain a differentiation activity distinct from CNTF that can stimulate expression of a cytokine-responsive gene (Fig. 5).

One way in which cytokines regulate expression of inducible genes is through activation of members of the STAT (51) family of transcription factors. The neuropoietic cytokines preferentially activate STAT3 (47–50), and in NBFL cells activation of this transcription factor in response to LIF or CNTF contributes to increased transcription of the VIP gene (52). Because the activity in footpad extracts induced expression of VIP mRNA in NBFL cells, we asked whether it also activated STAT3, using DNA mobility shift assays with a probe containing the STAT site from the VIP cytokine-responsive element (CyRE). Incubation of nuclear extracts prepared from NBFL cells treated for 15 min with either CNTF (10 ng/ml) or footpad extracts from CNTF −/− or wild type mice (300 µg/ml), with a STAT probe led to the formation of protein-DNA complexes that were absent from untreated NBFL cells (Fig. 6, A and C). The mobility of these DNA-protein complexes suggested preferential formation of a STAT3 homodimer, with formation of STAT1-containing homo- and heterodimers at high cytokine concentrations. Preincubation of these nuclear extracts with STAT1 and STAT3 antibodies showed that treatment of NBFL cells with footpad extracts from CNTF −/− mice led to induction of STAT3 specifically.

In contrast, treatment of NBFL cells with the cytokine CT-1 (100 ng/ml) did not increase VIP mRNA (data not shown) or activate STAT3 (Fig. 6C), although CT-1 increases VIP expression in sympathetic neurons (19). To ensure that the CT-1 used in these experiments was active, sympathetic neurons were treated with the CT-1 (100 ng/ml) and assayed for ChAT activity, which was increased almost 5-fold compared with control neurons (4.7 ± 0.68; n = 4 ± S.E.). These data suggest that NBFL cells, which respond to the other known neuropoietic cytokines as well as the gland-derived differentiation activity, are unresponsive to CT-1.

Because STAT activation contributes to transcriptional activation of the VIP gene by neuropoietic cytokines, we assessed whether the activity in footpad extracts could drive transcription of the VIP gene through activation of the CyRE. NBFL neuroblastoma cells transfected with a VIP CyRE-luciferase reporter plasmid were treated with 10 ng/ml recombinant CNTF or 300 µg/ml of footpad extracts from CNTF −/− mice for 36 h. Analysis of the resulting luciferase activity showed that an activity in the footpad extracts stimulated transcription from the VIP CyRE (Fig. 7). Thus, a differentiation activity present in footpads induced VIP transcription through the same cytokine response element by which LIF and CNTF induce VIP. This provides further evidence that the gland-derived activity is a member of the neuropoietic cytokine family.

**Fig. 4. CNTF mRNA and protein are present in rat footpad.** A, RT-PCR analysis of CNTF mRNA expression in rat footpad and sciatic nerve. RNA from sympathetic neuron/sweat gland co-cultures (co-cult), sciatic nerve, rat footpad (FP), and a water negative control were reverse transcribed and amplified with CNTF-specific primers, and products were identified by Southern blot. Amplification products were detected in footpad and sciatic nerve CDNA samples, but not in the co-culture cDNA or reverse transcription negative control. B, anticiNTF Western blot of footpad and sciatic nerve extracts. Recombinant CNTF (rCNTF), and 200-µg samples of soluble protein from rat footpad or sciatic nerve were blotted, incubated with anti-CNTF diluted 1:2000, and visualized with chemiluminescence.

**Fig. 5. Footpad extracts induce VIP mRNA in NBFL cells.** NBFL neuroblastoma cells were grown in control medium (control) or treated with 10 ng/ml recombinant CNTF (rCNTF) or with 300 µg/ml soluble protein extracted from the footpads of CNTF −/− mice (ho-FP). Four hours later, total RNA was isolated, and VIP and cyclophilin mRNA were detected by Northern blot.

**OSM Gene:** The sweat gland-derived cholinergic differentiation activity acts through the same receptors and signaling pathways used by CNTF, LIF, and CT-1. Although none of these cytokines can account for the gland-derived differentiation activity, OSM is also a member of this cytokine family (29, 30). Like the other neuropoietic cytokines, OSM can signal through a LIFRβ-gp130 heterodimer (39, 75, 76), but it can also use an OSMRβ-gp130 heterodimer (77). Recombinant human OSM stimulates only small increases in cholinergic and peptidergic function in rat sympathetic neurons (21), suggesting it is an unlikely candidate for the gland-derived differentiation activity. Murine OSM was recently cloned, however, and shown to share only 48% homology with the human protein (78), raising the possibility that murine OSM could induce phenotypic changes in rat neurons more effectively than does human OSM. Consistent with this possibility, human OSM induces a large increase in VIP mRNA in human NBFL cells (21). Therefore, we investigated whether OSM was expressed in sweat glands. Preliminary analysis of OSM mRNA expression by RT-PCR indicated that OSM mRNA was detected in footpads from wild type mice and from Tabby mutant mice, which lack sweat glands (25). Therefore, to determine whether OSM was present in sweat glands or in other footpad tissues, the sweat gland-containing central region was dissected away from the dermis and epidermis of adult rat footpads (72), and OSM mRNA expression was analyzed using RT-PCR. Oncostatin M mRNA was detected in RNA from thymus (data not shown) and from...
whole footpad as previously reported but was not detected in sweat gland RNA (Fig. 8), indicating that OSM is not expressed in gland cells. Therefore, OSM is unlikely to be the gland-derived differentiation factor.

**DISCUSSION**

Our results indicate that the target-derived differentiation factor that induces cholinergic and peptidergic properties in the developing sweat gland sympathetic innervation exerts its effects on neurotransmitter and neuropeptide expression via the same mechanisms used by the neuropoietic cytokines. We focused on the signaling pathways used by this family of molecules because these cytokines, acting alone, induce the appropriate combination of changes in sympathetic neuron phenotype (12, 14–20), while other types of growth and differentiation factors do not (21–23). Although previous studies indicate that the gland-derived differentiation activity is not LIF, CNTF, or CT-1 (19, 25, 60), several lines of evidence suggest that the gland-derived activity is a member of this family. The differentiation activity produced by cultured sweat gland cells requires the LIFR

FIG. 6. Footpad extracts induce formation of STAT3 dimers that bind to the VIP CyRE. A, DNA mobility shift assay using a probe (G3; GATTTCCTGGAAATTAG) that contains the STAT3 binding domain of the human VIP CyRE. The G3 probe was combined with nuclear extracts from NBFL cells that had been treated for 15 min with 10 ng/ml CNTF or 300 µg/ml of footpad (FP) or sciatic nerve (scn) extracts, isolated from CNTF −/− (ko) mice. STAT-DNA complexes are identified by arrows. B, supershift assay using the G3 probe and antibodies against the STAT1 and STAT3 DNA-binding proteins. Only anti-STAT3 interacts with the protein-DNA complex. C, the G3 probe was combined with nuclear extracts from NBFL cells that had been treated for 15 min with 100 ng/ml CT-1 or 300 µg/ml footpad extract from wild type mice (wt-FP). Data shown are representative of results obtained with three independently isolated sets of extracts.

FIG. 7. Footpad extracts stimulate transcription from the VIP CyRE. A, NBFL cells were transfected with a VIP CyRE-luciferase control plasmid and a Rous sarcoma virus-chloramphenicol acetyltransferase control plasmid. B, 6 h after transfection, cells were treated with 300 µg/ml footpad (FP) or sciatic nerve (scn) extracts isolated from CNTF −/− mice, and 36 h later they were harvested and assayed for luciferase and chloramphenicol acetyltransferase activity. Luciferase activity was normalized to chloramphenicol acetyltransferase activity to control for transfection efficiency. Data shown are representative results obtained in three independent experiments.

FIG. 8. OSM mRNA is not detected in sweat glands. RT-PCR analysis of β-actin and OSM expression in RNA isolated from the sweat glands (SG) or whole footpads (FP) of adult rats. Each two-lane pair represents RNA from a separate animal, reverse transcribed in a single reaction and amplified in separate reactions with the two primer pairs. β-Actin was amplified in samples from all animals, while OSM mRNA was detected in footpad but not in sweat glands.
LIFRβ and gp130, suggesting that both of these molecules are a part of its signal transduction complex. Cytokines that act through gp130, including CNTF and LIF, stimulate the tyrosine phosphorylation of STAT3, allowing it to form stable dimers and bind to DNA. The specificities of these interactions are determined by the particular cytokine receptor and STAT protein, since different cytokine receptors interact with distinct STAT DNA-binding proteins (49, 83). STAT3-specific docking protein, since different cytokine receptors interact with distinct dimers and bind to DNA. The specificities of these interactions through gp130, including CNTF and LIF, stimulate the tyrosine phosphorylation of LIFRβ and gp130 and activates the STAT3 DNA-binding protein is consistent with the activity in footpad extracts acting through a LIFRβ-gp130 heterodimer.

While LIFRβ and gp130 are the signal-transducing components of the receptor used by the sweat gland-derived activity, we cannot exclude the possibility that another subunit is required for binding and formation of the receptor complex. Our data indicate that the CNTFRA is not required, but it is possible that the activity in sweat glands requires a non-signal-transducing receptor subunit analogous to the CNTFRA. These nonsignaling receptor components have been proposed to be a general mechanism by which cytokine actions are restricted to specific tissues, in light of the widespread distribution of the LIFRβ and gp130, which mediate the actions of multiple ligands. Consistent with this suggestion, recent studies revealed a membrane-tethered component of the CT-1 receptor complex (37, 84) that increases the affinity of CT-1 binding. It is unlikely, however, that the gland-derived activity requires the novel CT-1 receptor component, since NBFL neuroblastoma cells are not responsive to recombinant murine CT-1 but are responsive to the murine sweat gland differentiation activity. The fact that sympathetic neurons but not NBFL cells respond to CT-1 indicates that there are differences in the receptors expressed by these two cells, and such differences complicate comparisons between the two cell types. While part of its receptor complex may remain unidentified, all of the receptor components required by the gland-derived differentiation activity are present in NBFL neuroblastoma cells and sympathetic neurons.

Sympathetic neurons respond to CNTF, LIF, and CT-1 just as they respond to the gland-derived activity, with the induction of VIP and ChAT expression and reduction of catecholamine content. The sweat gland-derived activity resembles these molecules functionally, and LIF, CNTF, and CT-1 transcripts can be detected in developing rat footpads using RT-PCR, but several lines of evidence indicate that these family members do not account for the differentiation activity present in the glands. The most direct evidence comes from the analysis of mice lacking CNTF, LIF, or both CNTF and LIF. If either or both of these molecules were required for the induction of cholinergic and peptidergic function in the sweat gland sympathetic innervation, then the loss of these proteins would result in mice lacking cholinergic, VIP immunoreactive sympathetic innervation in the footpads. In each line of transgenic mice, however, the sweat gland sympathetic innervation acquires cholinergic properties and VIP immunoreactivity during development (25, 60). Furthermore, neither CNTF nor LIF can account for the cholinergic differentiation activity in neuron/sweat gland co-cultures, since sweat glands that lack the genes encoding CNTF or LIF retain the ability to induce cholinergic function in sympathetic neurons. Similarly, sweat gland cells co-cultured with sympathetic neurons in the presence of antibodies that inhibit CT-1 function induce cholinergic properties in those sympathetic neurons (19). Finally, mRNA encoding the other closely related neuroepoietic cytokine, OSM, was not detected in sweat gland tissue by RT-PCR analysis. Thus, these cytokines do not appear to account for the differentiation activity produced by sweat glands in vivo or by gland cells cultured with sympathetic neurons.

While CNTF is not the sweat gland-derived differentiation factor in vivo, or in neuron/sweat gland co-cultures, previous immunoprecipitation data suggested that multiple factors with differentiation activity are present in footpad extracts, including CNTF or a CNTF-like molecule (10, 12). CNTF is present in myelinating Schwann cells associated with the footpad sensory innervation rather than in sweat gland cells (10, 12), and while we detected CNTF mRNA and CNTF protein in samples prepared from whole footpad, CNTF mRNA was not detected in neuron/sweat gland co-cultures. These cultures contain a few nonmyelinating Schwann cells, which do not express detectable CNTF, and no myelinating Schwann cells. Although CNTF is not in sweat glands, the presence of CNTF in footpad extracts complicates the use of these extracts as a source of the gland-derived differentiation activity and led us to use tissue from CNTF −/− mice. In the absence of CNTF, footpad extracts contain a STAT3-inducing activity that stimulates phosphorylation of the LIFRβ and gp130 portion of the CNTF receptor complex, but this activity is reduced compared with that present in wild type footpads that contain both a gland-derived activity and CNTF. The cholinergic inducing activity in extracts that is distinct from CNTF is produced by the sweat glands, since extracts of footpads from tabby mutant mice, which lack sweat glands (85), contain very little differentiation activity (9).

Our data indicate that the sweat gland-derived differentiation activity is a ligand for the LIFRβ-gp130 receptor complex. Further, the previous studies summarized above provide compelling evidence that the sweat gland differentiation factor is not one of the known ligands LIF, CNTF, CT-1, or OSM. Comparison of mice lacking either LIFRβ or gp130 with mice lacking LIF, CNTF, or both, indicates that there are additional ligands that act through these receptors. Mice lacking LIFRβ show significant motor neuron loss, appear to have a decreased number of astrocytes, and do not survive past the first postnatal day (86, 87), while mice lacking gp130 die between embryonic day 12.5 and birth (88). In contrast, the cytokine knockout mice have no developmental deficits but exhibit motor neuron degeneration as they age, which is more severe in mice lacking both CNTF and LIF (57, 89). These differences in phenotype suggest that there are unknown ligands for the LIFRβ and gp130 that are particularly important during neural development. Our data suggest that one such ligand is the sweat gland-derived differentiation activity.

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