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E-Cadherin Expression in Postnatal Schwann Cells Is Regulated by the cAMP-Dependent Protein Kinase A Pathway

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KEY WORDS
autotypic junction; cadherin switch; N-cadherin

ABSTRACT
Expression of E-cadherin in the peripheral nervous system is a highly regulated process that appears postnatally in concert with the development of myelinating Schwann cell lineage. As a major component of autotypic junctions, E-cadherin plays an important role in maintaining the structural integrity of noncompact myelin regions. *In vivo*, the appearance of E-cadherin in postnatal Schwann cell is accompanied by the disappearance of N-cadherin, suggesting reciprocal regulation of the two cadherins during Schwann cell development. The molecular signal that regulates the cadherin switch in Schwann cell is unclear. Using a neuron-Schwann cell co-culture system, here we show that E-cadherin expression is induced by components on the axonal membrane. We also show that the axonal effect is mediated through cAMP-dependent protein kinase A (cAMP-PKA) activation in the Schwann cell: (1) inhibition of cAMP-PKA blocks axon-induced E-cadherin expression and (2) cAMP elevation in the Schwann cell is sufficient to induce E-cadherin expression. In addition, cAMP-dependent E-cadherin expression is promoted by contact between adjacent Schwann cell membranes, suggesting its role in autotypic junction formation during myelination. Furthermore, cAMP-induced E-cadherin expression is accompanied by suppression of N-cadherin expression. Therefore, we propose that axon-dependent activation of cAMP-PKA serves as a signal that promotes cadherin switch during postnatal development of Schwann cells. © 2008 Wiley-Liss, Inc.

INTRODUCTION

The formation of myelin sheath is an essential developmental process that enables rapid propagation of action potentials in the nervous system. During myelination in the peripheral nervous system (PNS), individual Schwann cells repeatedly enwrap single axons, forming multiple concentric membrane layers. The subsequent membrane compaction (exclusion of Schwann cell cytoplasm to the cell periphery) and myelin–protein expression complete the myelination process.

The cytoplasmic region forms a continuous network of noncompact myelin that consists of Schmidt-Lanterman incisures, paranodal loops, and inner- and outer-internodal loops. They express a number of adhesion- and adhesion-like proteins that are involved in axon-Schwann cell interaction as well as proteins involved in establishing adherens junctions between each layer of concentric Schwann cell membrane (Arroyo and Scherer, 2000; Menichella et al., 2001). These junctions are termed "autotypic junctions," because unlike typical adherens junctions that are formed between two epithelial cells, they are formed within a single Schwann cell. One of the major components of the autotypic junction is E-cadherin, a type-I transmembrane protein of the classic cadherin family (Fannon et al., 1995). In epithelial cells, cadherins mediate calcium-dependent adhesion between two cells as a component of adherens junction. The extracellular domain of cadherins interact homophilically with cadherins of opposing cells, whereas the cytoplasmic domain connects with the cytoskeleton through a core complex formation with members of the catenin family including β-catenin and p120 catenin (Wheelock and Johnson, 2003; Yap et al., 1997). In myelinating Schwann cells, E-cadherin containing autotypic junctions play an essential role in stabilizing Schmidt-Lanterman incisures through recruitment of p120 catenin to E-cadherin (Tricaud et al., 2005). E-cadherin is also required for the proper establishment and maintenance of the outer abaxonal membrane in myelinated fibers (Young et al., 2002). Furthermore, a recent study has shown that E-cadherin-associated p120 catenin is important for normal myelination (Perrin-Tricaud et al., 2007).

E-cadherin expression in Schwann cell lineage is under strong developmental control. During development, E-cadherin expression is absent in Schwann cell precursors. Instead, the precursors express high levels of N-cadherin, another member of the classical cadherin family (Wanner et al., 2006). N-cadherin function during this stage is thought to involve facilitating axonal

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growth and compaction by mediating contacts among Schwann cell precursors. Later, N-cadherin starts to disappear as Schwann cell precursors convert to Schwann cells. Disappearance of N-cadherin is followed by the appearance of E-cadherin in neonatal Schwann cells. Later, the E-cadherin expression gradually increases and become restricted to the myelinating Schwann cell lineage (Fannon et al., 1995; Menichella et al., 2001).

The molecular cue that triggers E-cadherin expression, and perhaps at the same time suppresses N-cadherin expression, is unknown. A line of evidences suggests that the cadherin switch in Schwann cells might be triggered by components on the axonal membrane. For example, it has been shown that when axon-Schwann cell contact is disrupted in adult PNS following axonal degeneration, E-cadherin expression disappears from the Schwann cells while N-cadherin expression reappears (Fannon et al., 1995; Thornton et al., 2005). When the contact is reestablished following nerve regeneration, E-cadherin reappears and N-cadherin disappears, recapitulating the developmental regulation of cadherin expression. A recent study has shown that treatment of postnatal Schwann cells with neuregulin-1, an axonal protein, restores N-cadherin expression, suggesting that induction of N-cadherin during early Schwann cell development might be regulated by neuregulin-1 on developing axons (Wanner et al., 2006). However, nature of the signal that later suppresses N-cadherin and induces E-cadherin expression is unknown.

Aim of the present study is to define the molecular cue that triggers E-cadherin expression in postnatal Schwann cells. Here we present data showing that the cAMP-dependent protein kinase A (cAMP-PKA) pathway plays an essential role in promoting E-cadherin expression. We also show that cAMP-dependent E-cadherin expression is accompanied by downregulation of N-cadherin, suggesting that cAMP-PKA activation might play a role as a developmental signal that triggers cadherin switch in Schwann cells.

MATERIALS AND METHODS

Antibodies and Growth Factors

For immunofluorescence staining, monoclonal antibody to E-cadherin (clone 36, BD Transduction Laboratories, Franklin Lakes, NJ) was used at a dilution of 1:200 and chicken polyclonal antibody neurofilament (Covance, Berkey, CA) was used at 1:2,000. For Western blot analysis, E-cadherin antibody was used at 1:3,000 and monoclonal antibody to N-cadherin (clone 32, BD Transduction Laboratories) was used at 1:2,500. Monoclonal antibody to β-actin (AC-15, Sigma, St. Louis, MO) and glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MN) was used at 1:5,000 and 1:1,000, respectively. Recombinant sensory and motor neuron derived factor (SMDF; R&D systems, Minneapolis, MN) and glial growth factor (GGF; Cambridge Neuroscience, Norwood, MA) were used at 10 ng/mL. Platelet-derived growth factor -BB(PDGF-BB) (Millipore, Billerica, MA) was also used at 10 ng/mL.

Culture Media

Primary rat Schwann cells were grown in growth medium composed of Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 0.1 mg/mL penicillin–streptomycin, neuregulin-1 EGF-domain (10 ng/mL; R&D systems) and forskolin (5 μM, Sigma, St. Louis MN). A serum-free defined N2 medium was composed of DMEM and F-12 at a 1:1 ratio, supplemented with 5 ng/mL Na selenite, 16 μg/mL putrescine, 125 ng/mL progesterone, 0.2 mg/mL apotransferrin, 0.8 μg/mL insulin, and 1% penicillin–streptomycin. Dorsal root ganglion neurons were maintained in neurobasal medium (Invitrogen, Carlsbad, CA), supplemented with 2% B27 supplement (Invitrogen), 1% glutamine, 1% penicillin–streptomycin, 0.4% glucose, and 25 ng/mL NGF (Millipore).

Preparation of Schwann Cells

Schwann cells were prepared as described previously (Brockes et al., 1979). Briefly, sciatic nerves were harvested from postnatal day 1 or 2 rats and enzymatically dissociated by incubation with 1% collagenase prepared in DMEM for 30 min at 37°C followed by incubation in 0.25% trypsin and 1% collagenase solution for 30 min at 37°C. Cells were spun down and pellets were resuspended in 10% FBS in DMEM, and then plated on two 60-mm culture plates in the same medium. The following day the media was changed to 10% FBS medium containing 10 μM cytosine arabinoside to kill off proliferating fibroblasts. Three days later, in order to remove residual fibroblasts, cells are gently trypsinized, pelleted, and incubated with anti-Thy1 antibody (AbD Serotec, Raleigh, NC) prepared in DMEM at a dilution of 1:1,000. This was followed by incubation in rabbit complement (Jackson Immuno, West Grove, PA) for the same length of time. Schwann cells were then plated on poly-L-lysine-coated 100-mm culture plates and maintained in Schwann cell growth medium.

Preparation of Purified Rat Dorsal Root Ganglion Neuron Cultures

Dorsal root ganglion were removed from rat embryos between E14.5 and E16.5 and dissociated in 0.25% trypsin for 30 min at 37°C. The dissociated dorsal root ganglion (DRG) were plated on 12-mm collagen-coated glass coverslips (1 DRG/coverslip) in DMEM and 10% FBS supplemented with 25 ng/mL of NGF in a 120 μL droplet. To remove proliferating nonneuronal cells, the following day, cultures were flooded in neurobasal medium and B27 supplement mixture containing 15 μM fluorodeoxyuridine (FUdR). Two days later, FUdR was removed from the medium and cultures were maintained in neurobasal medium with the supplements for 2 days before starting a new cycle of FUdR treatment. Cycling in FUdR was continued for 10 days until all nonneuronal cells were removed.

Schwann Cell-DRG Co-Cultures

Prior to seeding onto DRG neurons, Schwann cells were maintained in serum-free N2 medium for 3 days. The growth factor-deprived Schwann cells were trypsinized and plated onto DRG neurons at a density of 200,000 cells/coverslip in DMEM supplemented with 10% FBS and NGF (25 ng/mL). In control cultures, Schwann cells were plated onto coverslips in the absence of neurons (Schwann cell-only), or neurons were maintained in the absence of Schwann cells (neuron-only). E-cadherin expression was assessed on Days 1, 3, 7, and 9 following plating of the Schwann cells. For cAMP-PKA inhibition in co-cultures, Schwann cells were plated onto neurons as mentioned earlier, and 24 h later, 10 μM H-89 (Calbiochem, San Diego, CA) was added to the culture medium. Three and 6 days later, cultures were harvested, and E-cadherin expression was assessed by Western blot analysis. Alternatively, trypsinized Schwann cells (200,000 cells) were incubated in 1 mL of DMEM +10% FBS supplemented with cell permeable myristoylated PKI 14–21 amide (100 μM) (Calbiochem) for 40 min then washed twice in DMEM +10% FBS. The cells were then plated onto DRG neurons as mentioned earlier. Three days later, E-cadherin expression was assessed by Western blot analysis.

Schwann Cell Proliferation Assay

Schwann cells were plated onto poly-L-lysine coated glass coverslips (40,000 cells/coverslip) in DMEM +10% FBS. Sixteen to 20 h later, cells were treated with GGF (10 ng/mL), forskolin (5 μM), or in combination in the presence or absence of H-89 (5 μM) or myristoylated PKA (20 μM). Sixteen hours later, bromo-deoxy-uridine (BrdU; 1 μM) was added to each coverslips. Twenty-four hours later, cells were fixed in cold methanol for 10 min, rehydrated in PBS for 3 min, and then treated with 2 N HCl for 15 min at 37°C. Cells were washed three times in 0.1 M borate buffer (pH 8.5) over a 10-min period and then washed three times in PBS in the same manner. Cells were then incubated in blocking solution (5% normal goat serum supplemented with 0.2% Triton X-100) for 30 min and incubated with monoclonal BrdU antibody (Sigma, St. Louis, MN) prepared in blocking solution for 1 h at room temperature. Alexa-conjugated-goat anti mouse secondary antibody was added for 1 h and before mounting, and cells were incubated with 4′6′-diaminidino-2-pheylindole (DAPI) for 1 min to visualize nuclei.

SDS PAGE and Western Blotting

Cells were lysed in lysis buffer (20 mM Tris pH 7.4, 1% NP-40, 10% glycerol, 2.5 mM ethylene glycol tetraacetic acid (EGTA), 2.5 mM ethylene diamine tetraacetic acid (EDTA), 1 mM sodium ortho-vanadate, 1 mM sodium fluoride, 1 mM phenylmethanesulphonylfluoride (PMSF), 10 μg/mL aprotinin, and 20 μM leupeptin), and the cell lysates were centrifuged at 14,000 rpm at 4°C for 15 min and the supernatants were collected. The protein concentrations were measured using bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) according to manufacturers' instructions. Samples (20–100 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto PDVF membrane. For Western blot analysis, the membranes were incubated in blocking solution (5% nonfat dried milk in TBS) for 1 h followed by incubation with appropriate primary antibodies prepared in TBST (TBS +0.5% Tween +5% BSA) overnight at 4°C. The next day, the membranes were washed three times for 10 min each in TBST then incubated in secondary antibody prepared in blocking solution with 0.5% Tween for 1 h. The blots were then incubated with enhanced chemiluminescence (ECL) Western blotting substrate for 1 min and exposed on X-ray films.

Immunofluorescence Staining

Co-cultures were fixed in 4% paraformaldehyde prepared in PBS for 25 min and permeabilized in methanol for 10 min at −20°C. After rehydrating in PBS, the cultures were incubated in blocking solution (10% normal goat serum +0.3% Triton X-100) for 1 h followed by incubation with primary antibodies overnight at 4°C. Next day, after washing in PBS, the cultures were incubated with the appropriate secondary antibodies for 45 min at room temperature. This was followed by incubation with DAPI for 1 min to visualize nuclei. Coverslips were then mounted onto glass slides using Fluoromount G. Images were acquired using the LSM 510 confocal microscope and analyzed using LSM software (Zeiss, Thornwood, NY).

Quantitative Real-Time PCR

The real-time PCR technique was used to detect the expression of E-cadherin and the N-cadherin. Total RNA was extracted from cultured Schwann cells or Schwann cell-DRG co-cultures. cDNA was amplified from 1 μg of RNA in the presence of 200 U of Moloney murine leukemia virus-RT, 40 U of Rnasin, 1 μg of random primers, 0.5 mM dNTPs, 3 μg of bovine serum albumin, and 1× Moloney murine leukemia virus reaction buffer (Promega, Madison, WI) in a total volume of 30 μL at 42°C for 1 h. cDNA was diluted 10 times for real-time PCR. The PCR mixture consists of 2 μL diluted cDNA, 5 μL of SYBR green-containing PCR master mixture (2×), and 150 nM of each primer in a total volume of 10 μL. The specific primers for real-time PCR were designed by using the primer express™ software from Applied Biosystems (Foster city, CA) and the primer sequences were as follows: E-cadherin sense 5′-TGAAGCCCAG GAAATACACCC-3′ and anti-sense 5′-GGATTAAAGGCG TGCACCAAC-3′; N-cadherin sense 5′-AGCCGATGAAG GAACCACATG-3′ and anti-sense 5′-TTCTGGCAAGTT GATCGGAGG-3′; GAPDH sense 5′-TTCTTGTGCAGT

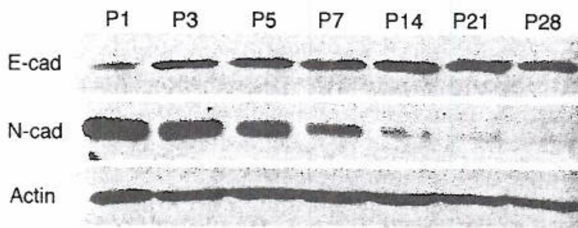

Fig. 1. Cadherin switch during postnatal development of Schwann cells. Sciatic nerve lysates were prepared at different postnatal days ranging from P1 to P28. Expression of E-cadherin (E-cad) and N-cadherin (N-cad) was determined by Western blot analysis. In postnatal sciatic nerve, an increase in E-cadherin expression is accompanied by a decrease in N-cadherin expression. A similar amount of actin across the samples indicates an even protein loading.

GCCAGCC-3′ and anti-sense 5′-CACCGACCTTCATCT TGT-3′. Real-time PCR was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The parameters for the PCR were as follows: 95°C for 15 s and 60°C for 1 min for 40 cycles followed by a melting point determination. If the amplification of the cDNA is specific, it results in a single peak. GAPDH was used as a reference gene and was run in parallel with the samples. A standard curve was generated for each pair of the primers. The expression level for each gene is shown as cycle numbers, which were converted into arbitrary units of DNA using the standard curve. The results were normalized to the mRNA levels of the housekeeping gene GAPDH in the same sample.

RESULTS

Cadherin Switch in Postnatal Peripheral Nerves

Developmental regulation of cadherin expression in peripheral nerve is shown in Figure 1. Sciatic nerve lysates were prepared from postnatal rats at different ages, and both E- and N-cadherin expression was assessed by immunoblotting. E-cadherin protein was expressed at a low level at postnatal day 1 (P1), which then gradually increased in later ages. This is in agreement with a previous study of E-cadherin expression in mouse sciatic nerves (Menichella et al., 2001). In peripheral nerves, E-cadherin expression is restricted to Schwann cells and absent from the axons (Fannon et al., 1995; Menichella et al., 2001); thus, the increase in E-cadherin level shown in Fig. 1 is likely to reflect the change in Schwann cells. Unlike E-cadherin, N-cadherin was expressed at a high level in P1 sciatic nerves followed by a gradual decrease in later ages and was undetectable in P21 nerves. Therefore, expression of E-cadherin and N-cadherin is reciprocally regulated during postnatal development of Schwann cell.

E-Cadherin Expression in Schwann Cells Is Induced by Axonal Components

The signal that induces E-cadherin expression in postnatal Schwann cell is unknown. *In vivo*, it has been shown that E-cadherin expression in Schwann cells is lost when the associated axons degenerate following nerve injury. The expression is restored when axons regenerate and reestablish contact with the Schwann cells, suggesting that E-cadherin expression is regulated by components on the axonal membrane (Menichella et al., 2001). To investigate whether a contact with axonal membrane is sufficient to induce E-cadherin expression in Schwann cells, we assessed E-cadherin induction in Schwann cells associated with axons in a Schwann cell-neuron co-culture system. Schwann cells prepared from neonatal rat sciatic nerves were maintained in serum-free medium for 3 days prior to plating onto DRG neurons. At this time, Schwann cells were devoid of E-cadherin expression (SC Lane, Fig. 2A). E-cadherin was also absent from DRG neurons (DRG lane). When Schwann cells were seeded onto DRG neurons, there was a gradual increase in E-cadherin expression, indicating that axonal components triggered E-cadherin expression in the Schwann cells. To determine whether the expression was also regulated at the mRNA level, we conducted real-time PCR analysis on total RNA collected from the co-cultures (Fig. 2B). As seen with the protein, E-cadherin mRNA was mostly absent from Schwann cell-only and neuron-only cultures and was induced only when the Schwann cells were plated onto neurons.

Peripheral neurons do not express E-cadherin, and hence the expression seen in the co-culture is likely to reflect induction in the Schwann cells (Fannon et al., 1995; Menichella et al., 2001). To confirm this, we performed double-immunofluorescence analysis on Schwann cell-only or 3-day and 7-day co-cultures using antibodies to E-cadherin and neurofilament, a neuronal marker (Fig. 2C). E-cadherin immunoreactivity was not detectable in Schwann cells-only cultures, in the absence of DRG neurons. When plated onto neurons, there was a gradual increase in the protein expression specific to the Schwann cells. Axons visualized by neurofilament expression were devoid of E-cadherin expression.

Cadherins are initially synthesized as inactive propeptide precursors, which are processed posttranslationally by removal of the propeptide fragment and inserted into the membrane as biologically active mature protein (Grunwald, 1993). To determine whether E-cadherin in the axon-associated Schwann cells is expressed as the membrane-bound mature form, we conducted confocal immunocytochemical analysis on 6-day co-cultures to determine the intracellular localization of E-cadherin in Schwann cells (see Fig. 3). Schwann cell-DRG co-cultures were fixed and immunostained for E-cadherin, neurofilament, and GFAP, a Schwann cell specific cytoskeletal protein. Most of the E-cadherin expression was seen at the cell periphery and did not co-localize with the cytoplasmic GFAP, indicating the cell surface expression of the mature protein. A small amount of E-cadherin expression was detected within the cytoplasm, which is likely to represent the precursor form of protein being processed within the cell. The presence of the precursor protein was also detected by Western blot analy-

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Prior to seeding onto DRG neurons, Schwann cells were maintained in serum-free N2 medium for 3 days. The growth factor-deprived Schwann cells were trypsinized and plated onto DRG neurons at a density of 200,000 cells/coverslip in DMEM supplemented with 10% FBS and NGF (25 ng/mL). In control cultures, Schwann cells were plated onto coverslips in the absence of neurons (Schwann cell-only), or neurons were maintained in the absence of Schwann cells (neuron-only). E-cadherin expression was assessed on Days 1, 3, 7, and 9 following plating of the Schwann cells. For cAMP-PKA inhibition in co-cultures, Schwann cells were plated onto neurons as mentioned earlier, and 24 h later, 10 μM H-89 (Calbiochem, San Diego, CA) was added to the culture medium. Three and 6 days later, cultures were harvested, and E-cadherin expression was assessed by Western blot analysis. Alternatively, trypsinized Schwann cells (200,000 cells) were incubated in 1 mL of DMEM +10% FBS supplemented with cell permeable myristoylated PKI 14–21 amide (100 μM) (Calbiochem) for 40 min then washed twice in DMEM +10% FBS. The cells were then plated onto DRG neurons as mentioned earlier. Three days later, E-cadherin expression was assessed by Western blot analysis.

Schwann Cell Proliferation Assay

Schwann cells were plated onto poly-L-lysine coated glass coverslips (40,000 cells/coverslip) in DMEM +10% FBS. Sixteen to 20 h later, cells were treated with GGF (10 ng/mL), forskolin (5 μM), or in combination in the presence or absence of H-89 (5 μM) or myristoylated PKA (20 μM). Sixteen hours later, bromo-deoxy-uridine (BrdU; 1 μM) was added to each coverslips. Twenty-four hours later, cells were fixed in cold methanol for 10 min, rehydrated in PBS for 3 min, and then treated with 2 N HCl for 15 min at 37°C. Cells were washed three times in 0.1 M borate buffer (pH 8.5) over a 10-min period and then washed three times in PBS in the same manner. Cells were then incubated in blocking solution (5% normal goat serum supplemented with 0.2% Triton X-100) for 30 min and incubated with monoclonal BrdU antibody (Sigma, St. Louis, MN) prepared in blocking solution for 1 h at room temperature. Alexa-conjugated-goat anti mouse secondary antibody was added for 1 h and before mounting, and cells were incubated with 4′6′-diaminidino-2-pheylindole (DAPI) for 1 min to visualize nuclei.

SDS PAGE and Western Blotting

Cells were lysed in lysis buffer (20 mM Tris pH 7.4, 1% NP-40, 10% glycerol, 2.5 mM ethylene glycol tetraacetic acid (EGTA), 2.5 mM ethylene diamine tetraacetic acid (EDTA), 1 mM sodium ortho-vanadate, 1 mM sodium fluoride, 1 mM phenylmethanesulphonylfluoride (PMSF), 10 μg/mL aprotinin, and 20 μM leupeptin), and the cell lysates were centrifuged at 14,000 rpm at 4°C for 15 min and the supernatants were collected. The protein concentrations were measured using bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) according to manufacturers' instructions. Samples (20–100 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto PDVF membrane. For Western blot analysis, the membranes were incubated in blocking solution (5% nonfat dried milk in TBS) for 1 h followed by incubation with appropriate primary antibodies prepared in TBST (TBS +0.5% Tween +5% BSA) overnight at 4°C. The next day, the membranes were washed three times for 10 min each in TBST then incubated in secondary antibody prepared in blocking solution with 0.5% Tween for 1 h. The blots were then incubated with enhanced chemiluminescence (ECL) Western blotting substrate for 1 min and exposed on X-ray films.

Immunofluorescence Staining

Co-cultures were fixed in 4% paraformaldehyde prepared in PBS for 25 min and permeabilized in methanol for 10 min at −20°C. After rehydrating in PBS, the cultures were incubated in blocking solution (10% normal goat serum +0.3% Triton X-100) for 1 h followed by incubation with primary antibodies overnight at 4°C. Next day, after washing in PBS, the cultures were incubated with the appropriate secondary antibodies for 45 min at room temperature. This was followed by incubation with DAPI for 1 min to visualize nuclei. Coverslips were then mounted onto glass slides using Fluoromount G. Images were acquired using the LSM 510 confocal microscope and analyzed using LSM software (Zeiss, Thornwood, NY).

Quantitative Real-Time PCR

The real-time PCR technique was used to detect the expression of E-cadherin and the N-cadherin. Total RNA was extracted from cultured Schwann cells or Schwann cell-DRG co-cultures. cDNA was amplified from 1 μg of RNA in the presence of 200 U of Moloney murine leukemia virus-RT, 40 U of Rnasin, 1 μg of random primers, 0.5 mM dNTPs, 3 μg of bovine serum albumin, and 1× Moloney murine leukemia virus reaction buffer (Promega, Madison, WI) in a total volume of 30 μL at 42°C for 1 h. cDNA was diluted 10 times for real-time PCR. The PCR mixture consists of 2 μL diluted cDNA, 5 μL of SYBR green-containing PCR master mixture (2×), and 150 nM of each primer in a total volume of 10 μL. The specific primers for real-time PCR were designed by using the primer express™ software from Applied Biosystems (Foster city, CA) and the primer sequences were as follows: E-cadherin sense 5′-TGAAGCCCAG GAAATACACCC-3′ and anti-sense 5′-GGATTAAAGGCG TGCACCAAC-3′; N-cadherin sense 5′-AGCCGATGAAG GAACCACATG-3′ and anti-sense 5′-TTCTGGCAAGTT GATCGGAGG-3′; GAPDH sense 5′-TTCTTGTGCAGT

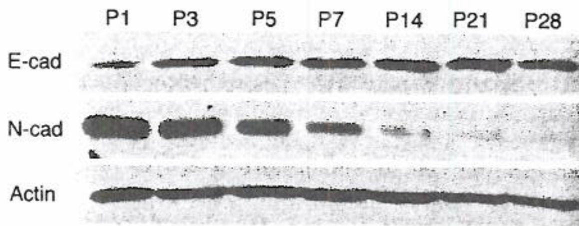

Fig. 1. Cadherin switch during postnatal development of Schwann cells. Sciatic nerve lysates were prepared at different postnatal days ranging from P1 to P28. Expression of E-cadherin (E-cad) and N-cadherin (N-cad) was determined by Western blot analysis. In postnatal sciatic nerve, an increase in E-cadherin expression is accompanied by a decrease in N-cadherin expression. A similar amount of actin across the samples indicates an even protein loading.

GCCAGCC-3′ and anti-sense 5′-CACCGACCTTCATCT TGT-3′. Real-time PCR was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The parameters for the PCR were as follows: 95°C for 15 s and 60°C for 1 min for 40 cycles followed by a melting point determination. If the amplification of the cDNA is specific, it results in a single peak. GAPDH was used as a reference gene and was run in parallel with the samples. A standard curve was generated for each pair of the primers. The expression level for each gene is shown as cycle numbers, which were converted into arbitrary units of DNA using the standard curve. The results were normalized to the mRNA levels of the housekeeping gene GAPDH in the same sample.

RESULTS

Cadherin Switch in Postnatal Peripheral Nerves

Developmental regulation of cadherin expression in peripheral nerve is shown in Figure 1. Sciatic nerve lysates were prepared from postnatal rats at different ages, and both E- and N-cadherin expression was assessed by immunoblotting. E-cadherin protein was expressed at a low level at postnatal day 1 (P1), which then gradually increased in later ages. This is in agreement with a previous study of E-cadherin expression in mouse sciatic nerves (Menichella et al., 2001). In peripheral nerves, E-cadherin expression is restricted to Schwann cells and absent from the axons (Fannon et al., 1995; Menichella et al., 2001); thus, the increase in E-cadherin level shown in Fig. 1 is likely to reflect the change in Schwann cells. Unlike E-cadherin, N-cadherin was expressed at a high level in P1 sciatic nerves followed by a gradual decrease in later ages and was undetectable in P21 nerves. Therefore, expression of E-cadherin and N-cadherin is reciprocally regulated during postnatal development of Schwann cell.

E-Cadherin Expression in Schwann Cells Is Induced by Axonal Components

The signal that induces E-cadherin expression in postnatal Schwann cell is unknown. *In vivo*, it has been shown that E-cadherin expression in Schwann cells is lost when the associated axons degenerate following nerve injury. The expression is restored when axons regenerate and reestablish contact with the Schwann cells, suggesting that E-cadherin expression is regulated by components on the axonal membrane (Menichella et al., 2001). To investigate whether a contact with axonal membrane is sufficient to induce E-cadherin expression in Schwann cells, we assessed E-cadherin induction in Schwann cells associated with axons in a Schwann cell-neuron co-culture system. Schwann cells prepared from neonatal rat sciatic nerves were maintained in serum-free medium for 3 days prior to plating onto DRG neurons. At this time, Schwann cells were devoid of E-cadherin expression (SC Lane, Fig. 2A). E-cadherin was also absent from DRG neurons (DRG lane). When Schwann cells were seeded onto DRG neurons, there was a gradual increase in E-cadherin expression, indicating that axonal components triggered E-cadherin expression in the Schwann cells. To determine whether the expression was also regulated at the mRNA level, we conducted real-time PCR analysis on total RNA collected from the co-cultures (Fig. 2B). As seen with the protein, E-cadherin mRNA was mostly absent from Schwann cell-only and neuron-only cultures and was induced only when the Schwann cells were plated onto neurons.

Peripheral neurons do not express E-cadherin, and hence the expression seen in the co-culture is likely to reflect induction in the Schwann cells (Fannon et al., 1995; Menichella et al., 2001). To confirm this, we performed double-immunofluorescence analysis on Schwann cell-only or 3-day and 7-day co-cultures using antibodies to E-cadherin and neurofilament, a neuronal marker (Fig. 2C). E-cadherin immunoreactivity was not detectable in Schwann cells-only cultures, in the absence of DRG neurons. When plated onto neurons, there was a gradual increase in the protein expression specific to the Schwann cells. Axons visualized by neurofilament expression were devoid of E-cadherin expression.

Cadherins are initially synthesized as inactive propeptide precursors, which are processed posttranslationally by removal of the propeptide fragment and inserted into the membrane as biologically active mature protein (Grunwald, 1993). To determine whether E-cadherin in the axon-associated Schwann cells is expressed as the membrane-bound mature form, we conducted confocal immunocytochemical analysis on 6-day co-cultures to determine the intracellular localization of E-cadherin in Schwann cells (see Fig. 3). Schwann cell-DRG co-cultures were fixed and immunostained for E-cadherin, neurofilament, and GFAP, a Schwann cell specific cytoskeletal protein. Most of the E-cadherin expression was seen at the cell periphery and did not co-localize with the cytoplasmic GFAP, indicating the cell surface expression of the mature protein. A small amount of E-cadherin expression was detected within the cytoplasm, which is likely to represent the precursor form of protein being processed within the cell. The presence of the precursor protein was also detected by Western blot analy-

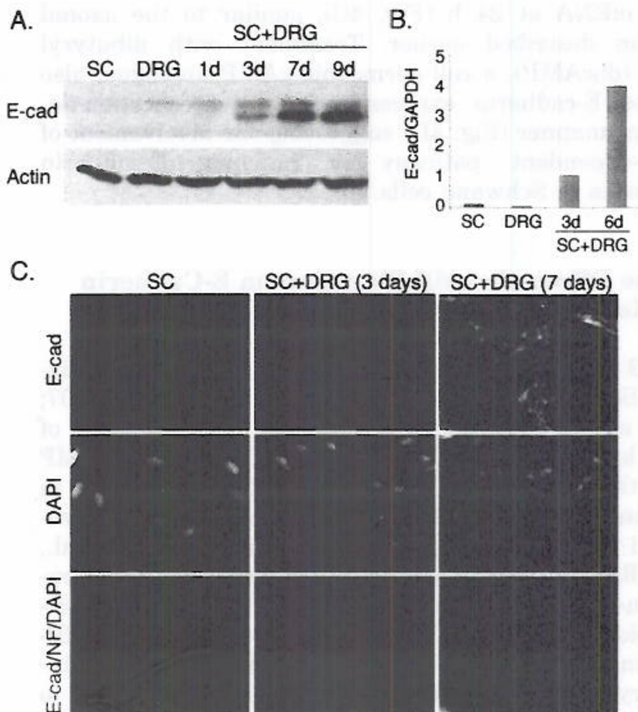

Fig. 2. Axonal components induce E-cadherin expression in postnatal Schwann cells. (A) Growth factor deprived Schwann cells were plated onto DRG neurons (SC+DRG) and E-cadherin expression was determined at 3 and 7 days following seeding of the Schwann cells by immunoblotting. Schwann cell only (SC) and neuron-only (DRG) cultures were used as controls. (B) E-cadherin mRNA level. Complementary DNA were prepared from total RNA collected from the co-cultures - and 6-days after seeding Schwann cells and amplified by real-time PCR with primers specific for E-cadherin. The expression levels of E-cadherin mRNA were normalized to internal house keeping GAPDH mRNA. One representative experiment out of four is shown. (C) Schwann cell specific E-cadherin expression is seen in co-cultures (3 and 7 days) immunostained with antibodies to E-cadherin (red) and neurofilament (NF, green). No expression is detected in NF-positive neurons or in Schwann cell-only cultures. Individual Schwann cells are visualized by the nuclear DAPI staining.

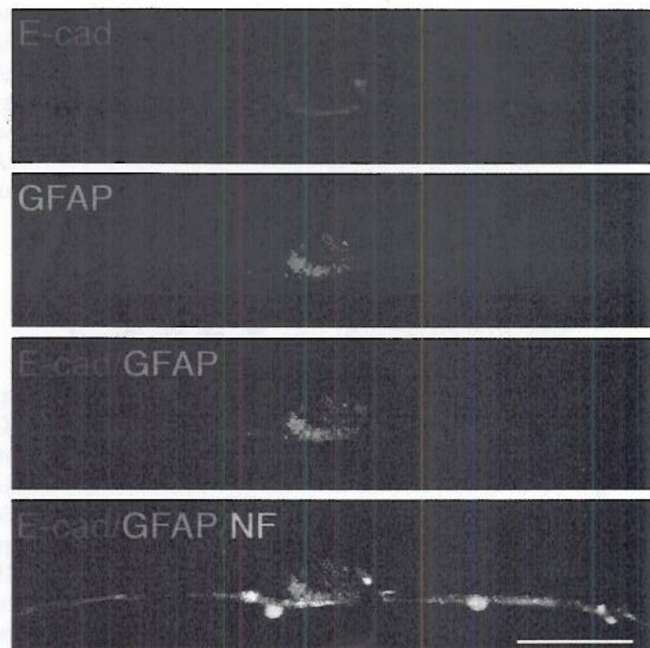

Fig. 3. E-cadherin is expressed on the surface of axon-associated Schwann cells. Confocal images of an axon-associated Schwann cell immunostained for E-cadherin (red), GFAP (green), and neurofilament (blue). The merged image is shown below. Most of the E-cadherin expression is seen on the Schwann cell surface, which is devoid of cytoplasmic GFAP expression. A small amount of the E-cadherin is seen within the cytoplasm, likely representing the unprocessed population of the protein. The Schwann cell is seen in close contact with the axon visualized by the neurofilament (NF) staining. Scale bar: 2 μm.

sis as the slower migrating bands shown in Fig. 2A: in 7- and 9-day co-cultures, most of the E-cadherin was present as the mature form. The confocal images also showed that the E-cadherin$^+$ Schwann cell processes were aligned along the length of the axon (neurofilament$^+$) indicating a direct contact formation between the two cell types.

Taken together, these results suggest that axonal components trigger E-cadherin expression in Schwann cells, possibly by a direct contact between the two cell types, and the expression is regulated both at the mRNA and the protein levels.

Elevation of cAMP in Schwann Cells Mimics Axonal Effect on E-Cadherin Expression

What is the axonal component that triggers E-cadherin expression in Schwann cells? Various growth factors are expressed on the axonal membrane of peripheral neurons with the corresponding receptors expressed on Schwann cell surface. Among these, members of the neuregulin-1 growth factor family have been shown to mediate signaling between peripheral axons and Schwann cells (Garratt et al., 2000; Nave and Salzer, 2006). Elevation of cAMP has also been shown to mediate axonal signaling in Schwann cells (Mirsky and Jessen, 2001). Therefore, we investigated whether neuregulins or cAMP might play a role in regulating E-cadherin induction in Schwann cells. We also tested the effect of PDGF since its function had been implicated in the development and in maintenance of peripheral nerves (Eccleston et al., 1993; Hardy et al., 1992). To assess the role of neureuglin-1, we used two of the isoforms, GGF, a Type-II neuregulin-1, and SMDF, a Type-III neuregulin-1, as these isoforms have been shown to play a key role in regulating Schwann cell development (Garratt et al., 2000; Nave and Salzer, 2006). Forskolin, an adenylyl cyclase activator, was used to assess a role of cAMP-dependent pathway. Growth factor-deprived neonatal rat Schwann cells were treated with forskolin, GGF, SMDF, or PDGF individually or in combination with forskolin and the growth factors. Forty-eight hours later, E-cadherin amount was determined from the cell lysates by immunoblotting (Fig. 4A). E-cadherin expression was absent from control cells without any treatment (NT lane). Addition of GGF, SMDF, or PDGF had minimal or no effect on E-cadherin expression. When cells were treated with forskolin, however, there was a robust increase in E-cadherin expression. A combined treatment of forskolin with GGF, SMDF, or PDGF did not

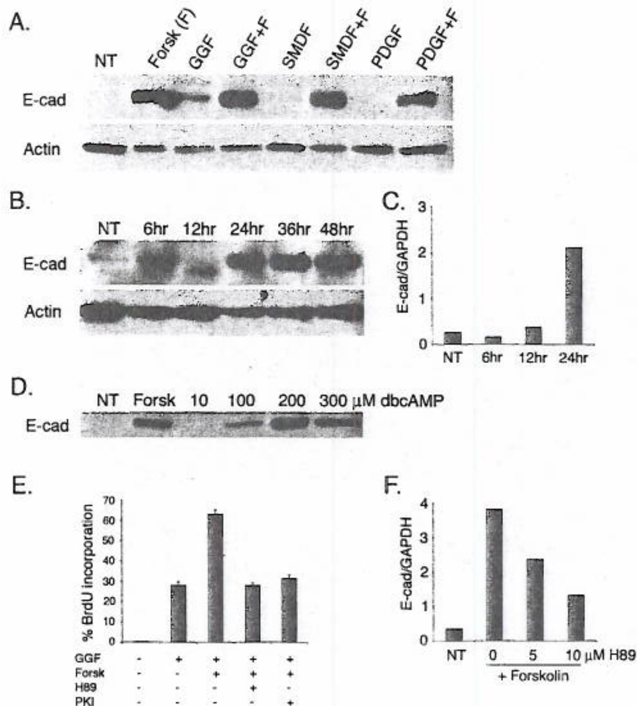

Fig. 4. Cyclic AMP elevation in Schwann cell mimics axon-induced E-cadherin expression. (**A**) Growth factor-deprived Schwann cells were treated with forskolin (F) (5 µM), GGF (10 ng/mL), SMDF (10 ng/mL), PDGF (10 ng/mL), or in combination of forskolin with the growth factors for 48 h and E-cadherin expression was determined from the cell lysates by immunoblotting. Actin expression was used as a loading control. (**B**) and (**C**) Kinetics of E-cadherin expression in Schwann cells following forskolin treatment. Schwann cells were treated with forskolin (5 µM) and E-cadherin protein and mRNA levels were determined at indicated times by immunoblotting (B) or by real-time PCR (C), respectively. (**D**) Induction of E-cadherin expression by dbcAMP. Schwann cells were treated with increasing concentrations of dbcAMP as indicated and E-cadherin amount was determined 48 h later by immunoblotting. As seen with forskolin treatment, dbcAMP also induces E-cadherin expression in Schwann cells. (**E**) H-89 inhibits cAMP-PKA activity in Schwann cells. Schwann cells were treated with GGF or GGF+forskolin in the presence or absence of H-89 (10 µM) or PKI (20 µM). Forty-hours later, the effect of H-89 and PKI on GGF and forskolin induced Schwann cell proliferation was assessed by determining the percentage of BrdU incorporated Schwann cells. The data represents the mean percentage of $BrdU^+$ Schwann cell nuclei (± SEM) from 30 fields pooled from two experiments. (**F**) Forskolin-induced E-cadherin expression is cAMP-PKA dependent. Schwann cells were pretreated with H-89 (5 and 10 µM) for 30 min then treated with forskolin in the presence of H-89. Forty-eight hours later, levels of E-cadherin mRNA were determined. The expression levels of E-cadherin mRNA were normalized to internal house keeping GAPDH mRNA. One representative experiment out of three is shown.

result in a significant change in forskolin-induced E-cadherin expression. Therefore, forskolin treatment alone is sufficient to mimic the axonal effect by inducing E-cadherin expression in Schwann cells.

We further analyzed the mechanism of forskolin-induced E-cadherin expression. To determine the expression kinetics, Schwann cells were treated with forskolin, and E-cadherin expression was determined at various hours following the treatment (Fig. 4B). An increase in E-cadherin protein was detected as early as 24 h following forskolin treatment and the level remained high at later time points. The forskolin effect was also seen at the mRNA level with a significant induction of E-cadherin mRNA at 24 h (Fig. 4C), similar to the axonal function described earlier. Treatment with dibutyryl cAMP (dbcAMP), a cell-permeable cAMP analogue, also induced E-cadherin expression in a concentration-dependent manner (Fig. 4D) suggesting the involvement of cAMP-dependent pathway in inducing E-cadherin expression in Schwann cells.

The Effect of cAMP Elevation on E-Cadherin Induction Is Mediated by the cAMP-PKA

H-89 has been widely used to block cAMP-PKA activity in Schwann cells (Kim et al., 1997; Muja et al.. 2007; Xu et al., 2002); however, it can also block activities of other kinases (Davies et al., 2000). Elevation of cAMP and the subsequent activation of cAMP-PKA in Schwann cells have been shown to synergize with neuregulin-1 to induce Schwann cell proliferation (Kim et al., 1997; Rahmatullah et al., 1998). Therefore, we used neuregulin-induced Schwann cell proliferation as an indirect bioassay to test the specificity of H-89 on cAMP-PKA inhibition. We also used a cAMP-PKA-specific inhibitory peptide PKI (myristoylated-PKI) as a control to assess the specificity of H-89. As seen in Fig. 4E, forskolin treatment greatly enhanced the mitogenic activity of the neuregulin. As expected, treatment with PKI abrogated the forskolin effect suggesting the involvement of cAMP PKA. A similar inhibitory effect was seen in cultures treated with H-89, indicating that the H-89 effect was mediated by its inhibition of the cAMP-PKA.

Next, we used H-89 to determine whether forskolin-induced E-cadherin induction was mediated by the cAMP-PKA pathway. Schwann cells were preincubated with H-89 for 30 min then treated with forskolin. Forty-eight hours later, the E-cadherin mRNA levels were assessed by real-time PCR (Fig. 4F). There was a dose-dependent inhibition of E-cadherin mRNA induction by H-89, suggesting that the forskolin function was mediated through the activation of cAMP-PKA.

Removal of cAMP Mimics the Effect of Axon-Loss on Schwann Cells by Downregulating E-Cadherin Expression

In vivo, injury-induced axonal degeneration results in loss of E-cadherin expression in distal Schwann cells (Menichella et al., 2001), indicating that continuous axonal contact is required to maintain E-cadherin expression. Since cAMP was able to mimic the axonal effect that induces E-cadherin expression, we asked whether a continuous presence of cAMP is needed to maintain the protein level. In other words, whether removal of cAMP elevation would result in downregulation of E-cadherin in Schwann cells. To test this, Schwann cells were treated with forskolin for 48 h to induce E-cadherin expression. Once induced, forskolin was withdrawn from the culture medium and Schwann cells were maintained in the absence of forskolin for various hours. E-cadherin

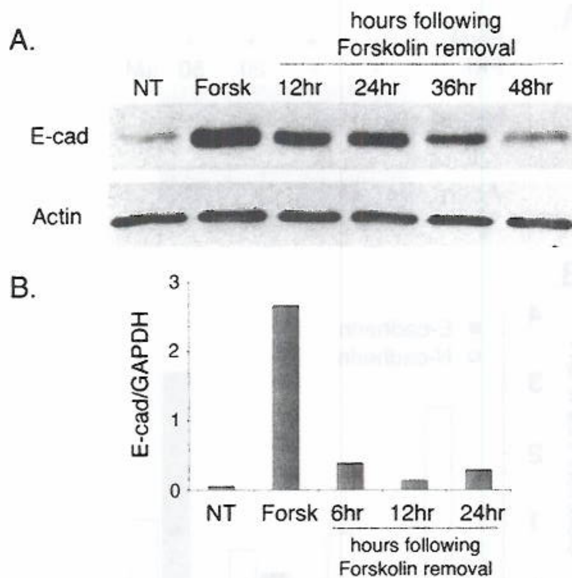

Fig. 5. Forskolin removal mimics the effect of axonal-loss by downregulating E-cadherin. Schwann cells were treated with forskolin (5 μM) for 48 h and subsequently forskolin was removed from the media. (**A**) Changes in the protein level determined by immunoblotting. (**B**) Changes in the mRNA level determined by real-time PCR. One representative experiment out of four is presented.

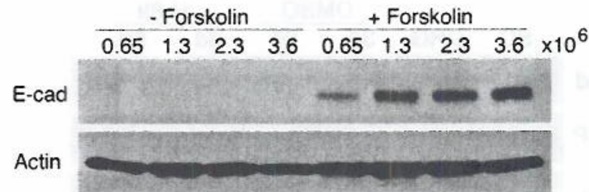

Fig. 6. Contact between Schwann cell membranes promotes E-cadherin expression in a cAMP-dependent manner. Schwann cells were plated at different densities in 60-mm culture dishes (28.26 cm^2), in the absence or in the presence of forskolin (5 μM). E-cadherin amount was determined 48 h later by immunoblotting.

protein levels were determined from the cell lysates prepared at 12, 24, 36, and 48 h following forskolin removal (Fig. 5A). Initial forskolin treatment resulted in a robust E-cadherin induction in Schwann cells (Forsk lane). Following forskolin removal, there was a gradual decrease in the amount of E-cadherin protein level suggesting degradation of the protein. We also determined the effect of forskolin removal on E-cadherin mRNA levels by real-time PCR (Fig. 5B). The effect on mRNA was more dramatic as compared to the protein; within 6 h of removing forskolin, E-cadherin mRNA level was decreased close to the basal level. This result suggests that removal of forskolin results in E-cadherin downregulation by decreasing the amount of protein as well as the mRNA. As a result, continuous cAMP elevation is required to maintain E-cadherin expression level in Schwann cells.

Contact Between Schwann Cell Membranes Promotes E-Cadherin Expression in a cAMP-Dependent Manner

During myelination, E-cadherin levels increase and the expression becomes localized at the autotypic junctions (Fannon et al., 1995; Menichella et al., 2001). Since autotypic junctions are formed when a concentric layer of Schwann cell membrane comes into contact with the previous layer, it is predicted that contact between Schwann cell membranes should regulate E-cadherin expression. To investigate this, Schwann cells were plated in 60-mm culture plates (28.26 cm^2) at densities ranging from 0.65×10^6 cells/28.26 cm^2 (low density, minimal membrane contact) to 3.6×10^6 cells/28.26 cm^2 (high density, maximal membrane contact), in the presence or absence of Forskolin. Two days later, E-cadherin expression was assessed by immunoblotting (see Fig. 6). In cultures without forskolin treatment (−forskolin), there was no detectable level of E-cadherin regardless of the cell density. In the presence of forskolin (+forskolin), however, there was a density-dependent increase in E-cadherin amount. This result suggests that contact between Schwann cell membranes promotes E-cadherin expression in a manner that is dependent on cAMP elevation.

Axon-Induced E-Cadherin Expression Is Blocked by Inhibition of cAMP-PKA Pathway

So far, we have observed that elevation of cAMP in Schwann cells mimics the role of axons that induces E-cadherin expression. In addition, removal of cAMP mimics the effect of axonal-loss by downregulating E-cadherin expression. These results lead to a prediction that axon-induced E-cadherin regulation is mediated by activation of the cAMP-dependent pathway in the associated Schwann cells. To address this, we investigated whether inhibition of the cAMP-PKA pathway in Schwann cell-neuron co-cultures could block axon-induced E-cadherin expression in the Schwann cells. Growth factor-deprived Schwann cells were seeded onto DRG neurons and allowed to establish their contacts with the axons. Twenty-four hours later, co-cultures were treated with H-89 at a final concentration of 10 μM, a concentration that was sufficient to block forskolin-mediated E-cadherin mRNA induction in Schwann cells (Fig. 4F). Control cultures were treated with solvent dimethyl sulfoxide (DMSO). Three and 6 days later, E-cadherin protein levels were determined from the co-culture cell lysates (Fig. 7A). No detectable amount of E-cadherin was seen in control Schwann cell-only (SC) or neuron-only (DRG) cultures. In co-cultures treated with DMSO, there was an increase in E-cadherin expression. In cultures treated with H-89, the presence of axons failed to induce E-cadherin expression. It has been shown previously that inhibition of cAMP-PKA by H-89 treatment blocks Schwann cell proliferation in the co-culture system (Kim et al., 1997). Since it was possible that the reduced E-cadherin levels could have been

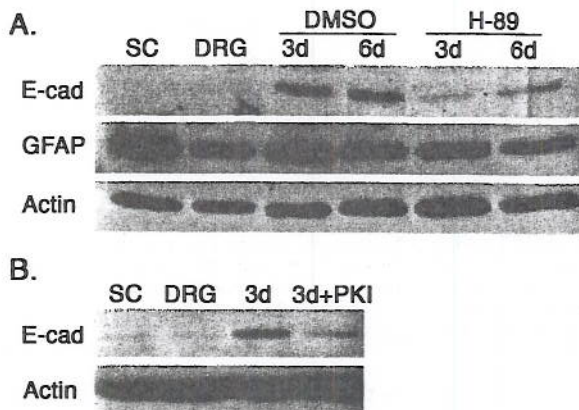

Fig. 7. Axon-induced E-cadherin expression in Schwann cell requires activation of the cAMP-PKA pathway. (**A**) Schwann cells were seeded onto DRG neurons and the next day, cultures were treated with DMSO or H-89 (10 μM). Cell lysates were prepared at 3- and 6-days following seeding of the Schwann cells. Schwann cell only (SC) and neuron only (DRG) cultures were used as controls. Levels of E-cadherin (E-cad), GFAP, and actin were determined by immunoblotting. (**B**) Schwann cells were preincubated with myristoylated PKI (100 μM) for 40 min then washed twice and plated onto DRG neurons. Three days later, E-cadherin expression was assessed by Western blot analysis.

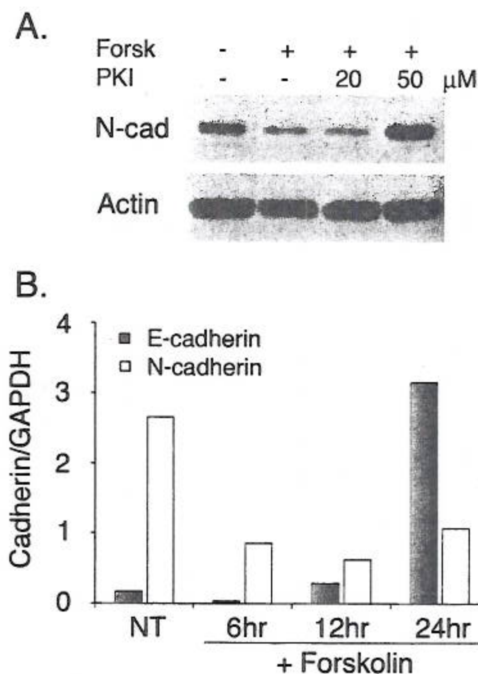

Fig. 8. Forskolin promotes cadherin switch in Schwann cell by suppressing N-cadherin and promoting E-cadherin expression. (**A**) Schwann cells were treated with forskolin (5 μM) in the presence or absence of PKI (20 and 50 μM). Forty-eight hours later, N-cadherin (N-cad) expression was determined by immunoblotting. Actin bands show even protein loading. (**B**) Cyclic AMP induced cadherin switch. Schwann cells were treated with forskolin for indicated times and E-cadherin (gray bars) and N-cadherin (white bars) mRNA level were determined within the same samples by real-time PCR. The mRNA levels were normalized to internal house keeping GAPDH mRNA. One representative experiment out of four is shown.

due to a decrease in Schwann cell number by H-89 treatment, we determined the levels of Schwann cell-specific protein GFAP in the lysates. The GFAP level remained constant across the samples, indicating that there was no significant decrease in the Schwann cell number in H-89 treated culture during the experimental periods.

Cyclic AMP-PKA has been shown to play an important role in supporting neuronal survival. To rule out the possible effect of cAMP-PKA inhibition on the DRG neurons, we used an alternative approach to inhibit cAMP-PKA in a Schwann cell-specific manner. Prior to seeding on the neurons, Schwann cells were preincubated with cell permeable, myristoylated PKI for 40 min in order to introduce the peptide into the cells. The Schwann cells were then washed thoroughly and plated onto DRG neurons and maintained in culture medium in the absence of PKI. Three days later, E-cadherin expression was assessed by immunoblotting. As seen in Fig. 7B, the axon-induced E-cadherin expression was greatly reduced in Schwann cells preincubated with PKI. Since DRG neurons were never exposed to the peptide, this result suggests that axon-induced E-cadherin expression is due to cAMP-PKA activity in Schwann cells, but not in the neurons. Altogether, these results suggest that activation of cAMP-PKA is required for axonal function that induces E-cadherin expression in Schwann cells.

Cyclic AMP-PKA Suppresses N-Cadherin Expression While Promoting E-Cadherin Expression in Schwann Cells: A Possible Role in Mediating Cadherin Switch?

In postnatal peripheral nerves, an increase in E-cadherin expression in the Schwann cell is accompanied by a decrease in N-cadherin expression (see Fig. 1). Our results on cAMP-induced E-cadherin expression, therefore, raise the possibility that the cAMP-dependent pathway plays a role in mediating the cadherin switch by suppressing N-cadherin expression in Schwann cells. It has been shown previously that cultured Schwann cells grown in the absence of axonal contact express a high level of N-cadherin but not E-cadherin (Wanner and Wood, 2002). Therefore, we investigated whether forskolin treatment on cultured Schwann cells could downregulate the N-cadherin expression (Fig. 8A). In control cultures without forskolin treatment, there was a significant level of N-cadherin, in agreement with the previous study. When Schwann cells were treated with forskolin, there was a drastic decrease in N-cadherin levels 48 h later. Pretreatment of cultures with PKI reversed the cAMP effect, suggesting that the inhibitory effect on N-cadherin expression was mediated by the cAMP-PKA.

The opposing effects of cAMP-PKA on E-cadherin and N-cadherin expression suggest that cAMP-PKA might function as the signal that initiates the cadherin switch in postnatal Schwann cells. To address this, Schwann cells were treated with forskolin for various hours and the levels of E-cadherin and N-cadherin mRNA were determined simultaneously within the same cell population by real-time PCR (Fig. 8B). In the absence of for-

skolin treatment, N-cadherin mRNA appeared to be the predominant cadherin mRNA expressed in the cells while E-cadherin mRNA was almost absent. Following forskolin treatment, there was a gradual increase in the E-cadherin mRNA while N-cadherin mRNA began to decrease, indicating the cadherin switch. By 24 h, E-cadherin became predominant over N-cadherin in the Schwann cells. Taken together, our results suggest that elevation of cAMP reciprocally regulates cadherin expression in Schwann cells: it suppresses N-cadherin while promoting E-cadherin expression. Therefore, cAMP elevation might function as a signal that mediates cadherin switch during the postnatal development of Schwann cells.

DISCUSSION

In this study, we have sought to identify molecular cues that trigger E-cadherin expression in postnatal Schwann cells. We report that the expression is mediated by an axonal component that activates the cAMP-PKA pathway in Schwann cells. This is evident by the findings that inhibition of cAMP-PKA blocks axon-mediated E-cadherin expression in Schwann cells and elevation of cAMP is sufficient to trigger E-cadherin induction in the absence of the axons. Furthermore, we show that E-cadherin promoting function of cAMP is accompanied by a function that suppresses N-cadherin expression, suggesting that cAMP-PKA might play a role in mediating cadherin switch in Schwann cells.

Cadherin switching is an integral part of embryonic development (Gumbiner, 2005; Wheelock and Johnson, 2003). In a situation that requires dynamic cellular rearrangement, tissue integrity must be disrupted so that cells can migrate from their original position. For example, when epithelial cells change their relative position within a tissue, they convert to migratory fibroblastic cells, a phenomenon referred to as the epithelium-to-mesenchyme transition (EMT). The EMT is often mediated by downregulation of E-cadherin that disrupts epithelial polarity, which is followed by upregulation of N-cadherin that increases cell motility (Affolter et al., 2003; Gould and Mattingly, 1990). In the PNS, loss of N-cadherin expression and gain of E-cadherin expression during Schwann cell development is reminiscent of the cadherin switch during EMT, only in reverse: N-cadherin expression is associated with migratory Schwann cell precursors (Wanner et al., 2006), whereas E-cadherin expression is enriched in differentiated myelinating Schwann cells (Fannon et al., 1995). Therefore, the cadherin switch in the PNS might play a crucial role by facilitating conversion of migratory embryonic Schwann cells (N-cadherin positive, E-cadherin negative) into non-migratory, polarized myelinating Schwann cells (N-cadherin negative, E-cadherin positive).

Molecular mechanisms that regulate cadherin switch in Schwann cells have not been extensively investigated. A recent study has shown that downregulation of N-cadherin in postnatal Schwann cells can be reversed by treatment with neuregulin-1, which results in re-expression of the protein on the cell surface (Wanner et al., 2006). Neuregulin-1 is expressed on axonal membrane of the PNS neurons and has been shown to play multiple roles in regulating Schwann cell development through activation of the receptor, erbB2 and erbB3, expressed on the Schwann cell surface (Garratt et al., 2000). This raises a possibility that neuregulin-erbB signaling might be involved in the initial induction of N-cadherin expression in Schwann cell precursors. However, it is unlikely that neuregulin-1 is also responsible for the subsequent N-cadherin suppression and E-cadherin induction, since neuregulin and erbB receptor expression persists throughout embryonic and postnatal PNS. Furthermore, we have shown in the present study that neuregulin-1 fails to induce E-cadherin expression in Schwann cells.

Here we show that cAMP-PKA activation fulfills the role as a signal that mediates cadherin switch in Schwann cells by simultaneously suppressing N-cadherin expression and inducing E-cadherin expression. Cyclic AMP has long been considered as an important developmental signal that mediates axon-to-Schwann cell communication. For example, inhibition of cAMP-dependent PKA blocks axon-induced Schwann cell proliferation and myelination (Howe et al., 2001; Kim et al., 1997). Elevation of intracellular cAMP in Schwann cells mimics axon-contact by promoting Schwann cell proliferation, differentiation, and myelin-gene expression (Eccleston, 1992; Lemke et al., 1988; Morgan et al., 1991). Therefore, the cadherin switch in Schwann cells might be another example of the cAMP-dependent function of the axons that regulates Schwann cell development.

Although, the axonal signal that regulates cAMP levels in the associated Schwann cell is unknown, it has been suggested that endogenous cAMP levels of peripheral nerves is under developmental regulation. This is evident by previous studies, which have shown that cAMP in rat sciatic nerve increases during nerve regeneration and decreases following nerve injury (Poduslo et al., 1995; Walikonis et al., 1998). Furthermore, it has been shown that there is a high endogenous cAMP level in myelinated nerve, but not in nonmyelinated nerve, hence associating cAMP elevation with building of the myelin sheath (Poduslo et al., 1995). Therefore, developmental regulation of endogenous cAMP might be associated with the cAMP-dependent axonal function that regulates postnatal development of myelinating Schwann cell lineage in the PNS.

Our data show that cAMP regulates E-cadherin expression both at the protein and the mRNA levels. The mechanism by which cAMP stimulates E-cadherin transcription in Schwann cell is unclear. In thyroid cells, it has been shown that activation of cAMP-PKA induces E-cadherin protein and mRNA by a mechanism that requires protein synthesis, suggesting that the expression is regulated indirectly through induction of a protein or a transcription factor (Brabant et al., 1995). A recent study has identified a cAMP-responsive element (CRE) sequence in the promoter region of E-cadherin

suggesting a cAMP-dependent induction of E-cadherin expression (Mauro et al., 2007). Alternatively, cAMP might function to suppress transcription factors that are known to repress E-cadherin expression (Batlle et al., 2000; Cano et al., 2000). Another mechanism by which cAMP maintains E-cadherin expression on the Schwann cell surface might involve stabilizing the protein by inhibiting E-cadherin degradation This is supported by our data showing that a continuous increase in cAMP within the Schwann cell is required for maintaining E-cadherin expression and removal of forskolin results in rapid degradation of E-cadherin product. Previous work with thymocytes has demonstrated that activation of the cAMP-PKA prevents E-cadherin turnover by inhibiting cell surface degradation and internalization (Larsson et al., 2004).

We further show that cAMP-mediated upregulation of E-cadherin is accompanied by downreguation of N-cadherin protein as well as the mRNA, indicating transcriptional regulation of N-cadherin expression. In ovarian epithelial cells, activation of cAMP-PKA results in downregulation of N-cadherin expression though a mechanism that involves phosphorylation of CREB (Pon et al., 2005). N-cadherin promoter also contains a probable CRE, making it possible that N-cadherin transcriptional suppression might be mediated by cAMP-PKA-dependent phosphorylation of CREB. An alternative way for facilitating N-cadherin decrease is by accelerating its turnover by stimulating the protein degradation. A similar mechanism has been demonstrated in an epithelial cell line in which cAMP induces degradation of N-cadherin via an ubiquitin-proteasome pathway (Pon et al., 2005).

What role does cadherin switch play in regulating postnatal Schwann cell development? As Schwann cells cease their proliferation in neonatal nerves, they begin the differentiation process by building intimate relationships with the associated axons. Nonmyelinating Schwann cells begin to segregate and ensheath a group of small diameter axons. Large diameter axons, destined to become myelinated, become individually wrapped by single myelinating Schwann cells, establishing a unique one-to-one Schwann cell-to-axon relationship. Because N-cadherin has been shown to play a role in enforcing glial-to-glial interaction promoting extensive glial cell contacts, downregulation of the protein in postnatal Schwann cells prior to differentiation might be an essential step to loosen up the glial cell contacts for formation of proper axon-Schwann cell units. Subsequent E-cadherin expression might facilitate Schwann cell differentiation (myelination) by organizing and polarizing membrane proteins and junctional components as seen in other cell types. E-cadherin also participates in forming autotypic junctions that provide stability to structural components of myelinating Schwann cells.

In conclusion, activation of the cAMP-dependent pathway in postnatal Schwann cell serves as a developmental signal that mediates cadherin switch. The factor that triggers cAMP elevation in Schwann cells is likely to be axonal as axon-contact mediated E-cadherin expression is blocked by inhibiting cAMP-PKA activation. Cyclic-AMP-mediated suppression of N-cadherin and induction of E-cadherin therefore is likely to play an important role in regulating postnatal development of Schwann cell.

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