Proprotein convertase PACE4 is down-regulated by the basic helix–loop–helix transcription factor hASH-1 and MASH-1

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PACE4 is a mammalian subtilisin-like proprotein convertase that activates transforming growth factor (TGF)- β -related proteins such as bone morphogenetic protein 2 (BMP2), BMP4 and Nodal and exhibits a dynamic expression pattern during embryogenesis. We recently determined that the 1 kb 5'-upstream region of the PACE4 gene contains 12 E-box (E1–E12) elements and that an E-box cluster (E4–E9) acts as a negative regulator [Tsuji, Yoshida, Hasegawa, Bando, Yoshida, Koide, Mori and Matsuda (1999) *J. Biochem. (Tokyo)* **126**, 494–502]. It is known that the mammalian *achaete-scute* homologue 1 (MASH-1) binds specifically to an E-box (CACCTG) sequence in collaboration with E47, a ubiquitously expressed basic helix–loop–helix (bHLH) factor. To identify the roles of the bHLH factor and E-box elements in regulating PACE4 gene expression in neural development, we analysed the effects of human *achaete-scute* homologue 1 (hASH-1) on PACE4 gene expression with various neuroblastoma cell lines. The expressions of PACE4 and hASH-1 are correlated inversely in these cell lines. The overexpression of hASH-1 or MASH-1 causes a marked decrease in endogenous

PACE4 gene expression but has no effect on the expression of other subtilisin-like proprotein convertases such as furin, PC5/6 and PC7/8. In contrast, other neural bHLH factors (MATH-1, MATH-2, neurogenin 1, neurogenin 2, neurogenin 3 and E47) did not affect PACE4 gene expression. Furthermore, an E-box cluster was a negative regulatory element for the promoter activity in NBL-S cells expressing hASH-1 at high level as determined by a luciferase assay. Binding of hASH-1 to the E-box cluster was confirmed by gel mobility-shift assay. In the present study we identified the PACE4 gene as one of the targets of hASH-1, which is a key factor in the initiation of neural differentiation. These results suggest that the alteration of PACE4 gene expression by hASH-1 causes rapid changes in the biological activities of TGF- β -related proteins via post-translational modification of these proteins.

Key words: bHLH factor, E-box, neuroblastoma, neurogenesis, processing protease.

INTRODUCTION

Recently, basic helix–loop–helix (bHLH) transcription factors have come to be recognized as important in neural development. Generally, tissue-specifically expressed bHLH factors (class B type) form functional heterodimers through the HLH domain with E proteins (class A type), expressed ubiquitously, and act as a transcriptional regulator by binding to the E-box (nucleotide sequence CANNTG) present in the promoter of the target genes [1]. These factors precisely regulate the timing of neural differentiation in both *Drosophila* and mammals [2]. The bHLH transcription factor mammalian *achaete-scute* homologue 1 (MASH-1) (in rat), a mammalian homologue of the *Drosophila* *achaete-scute* complex, is especially important to the early stages of neural development [3]. For example, MASH-1 mutant embryos exhibit a loss of neuronal progenitors in the olfactory system; mice homozygous for the mutation die at birth from an absence of olfactory functions required for breathing and feeding [4]. MASH-1 is transiently expressed in the precursor cells of olfactory neurons and is down-regulated after differentiation [4]. Studies *in vitro* have shown that MASH-1 can form a functional heterodimeric complex with E12/E47, which are ubiquitously expressed E2a gene products, and that this complex binds specifically to the E-box (CACCTG) sequence [5]. Although these factors are considered to promote neurogenesis by forming an interacting network that regulates the transcription of several genes, the target genes for MASH-1 and the molecular mech-

anisms underlying this important step in the neural differentiation are poorly understood. Human protein *achaete-scute* homologue 1 (hASH-1) was cloned from a human medullary thyroid cancer cDNA library, and is 95% similar in amino acid sequence to MASH-1 [6]. It has been shown that hASH-1 is highly expressed in medullary thyroid cancer, small-cell lung cancer and olfactory neuroblastoma [6,7].

Various transforming growth factor (TGF)- β -related differentiation factors, such as bone morphogenetic protein (BMP), are also essential for neural differentiation [8]. These TGF- β -related factors are synthesized as inactive precursors and then activated by limited proteolysis at an RXX/RR↓ site [9]. Recent findings suggest that subtilisin-like proprotein convertase (SPC) family proteases regulate these factors by proteolytic activation [10–13]. So far, seven members (furin, PC1/3, PC2, PACE4, PC4, PC5/6 and PC7/8) of the SPC family have been identified in mammals [14]. All SPCs are Ca²⁺-dependent serine proteases and share structural similarities, although they vary in tissue distribution. PACE4 displays dynamic expression patterns in olfactory neuron lineages [15] and the expression of PACE4 mRNA is up-regulated in some tissues that exhibit high expression levels of BMPs [16,17]. Moreover genetic studies clearly indicated an involvement of PACE4 in the activation of differentiation factors, such as BMPs, during embryogenesis. Constam and Robertson [18] showed that embryos lacking PACE4 die prenatally, displaying holoprosencephaly (cyclopia). In contrast, the most interesting feature of the 5'-flanking region of the PACE4 gene is the

Abbreviations used: bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; hASH-1, human *achaete-scute* homologue 1; MASH-1, mammalian *achaete-scute* homologue 1; RT-PCR, reverse-transcriptase-mediated PCR; SPC, subtilisin-like proprotein convertase; TGF, transforming growth factor.

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presence of 12 E-box elements within 1 kb upstream of the transcription initiation site [19]. We showed that the E-box cluster (E4–E9) was a strong negative regulatory element; the promoter activity was greatly increased by the deletion or mutation of this cluster [20]. Six tandem repeats (E4–E9) of a nonadecamer (GGCCTGGGGGTTCACCTGC) containing an E-box (underlined) are located in this region and the CACCTG sequence is a specific binding site for MASH-1 and hASH-1. Human neuroblastoma, which is a common paediatric tumour derived from the neural crest, retains the ability to differentiate into neuronal cell types on treatment with various agents and could be used as a model of neural differentiation [21].

In the present study we examined the effect of hASH-1 on PACE4 gene expression by using human neuroblastoma cell lines to elucidate whether this bHLH factor acts as a regulator of PACE4 gene expression. Our results showed clearly that the expression of the SPC-family protease PACE4 is suppressed by the binding of hASH-1 to the CACCTG sequence in the E-box cluster.

MATERIALS AND METHODS

Cell culture and transfection

Human neuroblastoma cell lines (SH-SY5Y, NBL-S, GANB, SK-N-DZ and NMB) were grown at 37 °C under air/CO₂ (19:1) in RPMI 1640 medium supplemented with 10% (v/v) FBS (fetal bovine serum), 100 µg/ml streptomycin and 100 unit/ml penicillin. Neuroblastoma cells were transfected by the lipofection method with Trans IT[®]-LT1 transfection reagent (Mirus, Madison, WI, U.S.A.) as described previously [20].

Northern blot analysis

Total RNA was isolated from neuroblastoma cell lines as described previously [20]; 10 µg was resolved by electrophoresis on a 1% (w/v) agarose gel containing 6.7% (v/v) formaldehyde, then transferred to a nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.). The human cDNA fragments of PACE4 (nt 1687–2754), furin (nt 1561–2050), PC5/6 (nt 1801–2385), PC7/8 (nt 1315–1992) and hASH-1 (nt 371–1051) were labelled with [³²P]dCTP by using the Bca BEST labelling kit (Takara, Kyoto, Japan) and used as probes. Hybridization and washing were performed as described previously [15]. The density of each band on the X-ray film was quantified with NIH image software.

Construction of expression plasmid

For the transient expression of hASH-1 in neuroblastoma cells, hASH-1 cDNA was cloned into the *Hind*III and *Bam*HI sites of a pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) vector. The expression plasmids for MASH-1, MATH-1, MATH-2 and E47 were kindly provided by Professor Ryoichiro Kageyama (Kyoto University, Kyoto, Japan). The expression plasmids for neurogenin 1, neurogenin 2 and neurogenin 3 were kindly provided by Professor François Guillemot (Louis Pasteur Université, Strasbourg, France).

Reverse-transcriptase-mediated PCR (RT-PCR) analysis

Total RNA (5 µg) from SH-SY5Y cells was reverse-transcribed with SUPER SCRIPT[®] II (Gibco BRL, Gaithersburg, MD, U.S.A.). PCR was performed with Ex Taq polymerase (Takara) in accordance with the manufacturer's protocol. The sense and anti-sense primers for hASH-1 were 5'-CCCAAGCTT(GATTC-CGCGACTCCTTGCC)-3' (nt 403–422) and 5'-CTGGCGG-

CCGC(TCGCACCAGGGCCTGACCAG)-3' (nt 1159–1179) respectively. PCR was performed with denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min and extension for 2 min at 72 °C, for 20 cycles.

Preparation of anti-(hASH-1) antibody

To generate a polyclonal antibody specific for hASH-1, full-length hASH-1 cDNA (nt 433–1149) was cloned into the *Bam*HI and *Hind*III sites of pQE30 vector with an N-terminal hexahistidine tag (Qiagen GmbH, Hilden, Germany). This fusion protein was expressed in *Escherichia coli* strain BL21 cells. Its expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside and the cultures were grown at 37 °C for 4 h. The fusion protein was solubilized with 8 M urea/50 mM imidazole/0.5 M NaCl/20 mM Tris/HCl (pH 7.9) by homogenization and sonication, then specifically bound to a Ni-nitrilotriacetic acid-agarose affinity chromatography column (Qiagen). The protein was purified on the column in accordance with the manufacturer's protocol. hASH-1 protein was eluted with 8 M urea/50 mM imidazole/0.5 M NaCl/20 mM Tris/HCl (pH 7.9). The final preparation yielded a single band of 28 kDa on SDS/PAGE; this protein was extracted and used for the immunization of rabbits. The specificity of the antiserum was confirmed by immunoprecipitation and Western blot analysis of endogenous hASH-1 protein from neuroblastoma cell lines. Anti-(hASH-1) IgG was purified by Protein A-Sepharose affinity chromatography, and used for gel mobility-shift assay and Western blot analysis. Moreover, we confirmed that the anti-(hASH-1) IgG used did not react with other bHLH proteins such as MATH-1, MATH-2, E47, neurogenin 1, neurogenin 2 and neurogenin 3 (results not shown).

Western blot analysis

Nuclear extracts (20 µg of protein) from each neuroblastoma cell line were subjected to SDS/PAGE [12% (w/v) gel] and the electrophoresed proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% (v/v) skimmed milk in 20 mM Tris/HCl buffer, pH 7.6, containing 0.14 M NaCl and 0.1% (v/v) Tween-20 (TBST) for 1 h, then incubated with an anti-(hASH-1) IgG antibody (2 µg/ml) for 2 h, followed by horseradish-peroxidase-conjugated anti-rabbit IgG second antibody (1:5000 final dilution; Bio-Rad) for 1 h. The hASH-1 protein was detected with a chemiluminescence kit (Pierce, Rockford, IL, U.S.A.) and Konica X-ray film in accordance with the manufacturer's protocol.

Luciferase and β-galactosidase assay

The construction of reporter plasmids and the luciferase and β-galactosidase assays were performed as described previously [20]. The luciferase activities were normalized on the basis of β-galactosidase activity. The results are presented as luciferase activities (means ± S.D. for a single experiment performed in triplicate). The experiments were repeated at least three times to ensure reproducibility.

Preparation of nuclear extract and gel mobility-shift assay

Nuclear extracts were prepared from NBL-S cells as described by Dignam et al. [22]. Protein concentration was determined by the method of Bradford, with BSA as the standard [23]. The sequences of the top strands of oligonucleotides used as probes or competitors in the gel mobility-shift assay were as described previously [20]. Nuclear extracts were incubated with anti-(hASH-1) IgG or control IgG antibody immobilized with Protein

A–Sepharose (50 μ g per 10 μ l gel) for 2 h at 4 °C. The removal of hASH-1 protein from the nuclear extract by treatment with Protein A–Sepharose immobilized with anti-(hASH-1) IgG was confirmed by Western blot analysis (results not shown). The complementary strands of each oligonucleotide were annealed and purified by PAGE; the double-stranded probe was labelled with [γ - 32 P]ATP with the use of T4 polynucleotide kinase. The DNA binding reaction was performed in 10 μ l of a reaction mixture containing 1 μ g of poly(dI-dC)·poly(dI-dC), 10 mM Tris/HCl, pH 7.5, 1 mM MgCl₂, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% (v/v) glycerol, 3 μ g of pretreated nuclear extracts and radiolabelled probe (5×10^4 c.p.m.). The mixture was incubated at 4 °C for 30 min and loaded on a 4% (w/v) polyacrylamide gel. The gel was dried and analysed with a BAS1500 bioimaging analyser (Fuji Photo Film, Tokyo, Japan).

RESULTS

PACE4 expression increases in inverse proportion to the hASH-1 levels in various neuroblastoma cell lines

We first analysed mRNA levels of the SPC-family proteases PACE4, furin, PC5/6 and PC7/8 and the bHLH transcription factor hASH-1 in a variety of cultured neuroblastoma cell lines (SH-SY5Y, NBL-S, GANB, SK-N-DZ and NMB) by Northern blot analysis. As shown in Figure 1(A), PACE4 transcript (4.4 kb) was detected in GANB (lane 1), SH-SY5Y (lane 2) and NBL-S (lane 3) cells but not in NMB (lane 4) or SK-N-DZ (lane 5) cells. hASH-1 mRNA (3 kb) was expressed strongly in NMB and SK-N-DZ cells and slightly in NBL-S cells, but little in SH-SY5Y and GANB cells. Expression of hASH-1 protein (28 kDa) was confirmed by Western blot analysis (Figure 1B). Thus PACE4 was highly expressed in GANB and SH-SY5Y cells, which produce hASH-1 at a low level, whereas it was not detected in NMB and SK-N-DZ cells, which express hASH-1 at a high level

as shown in Figure 1(C). These results indicate that the expression level of PACE4 was inversely proportional to that of hASH-1 in neuroblastoma cell lines. The expression of other SPC-family proteases such as furin, PC5/6 and PC7/8 was also examined: all of the cell lines expressed furin (4.4 kb) and PC7/8 (4.4 kb) but their expression levels were not correlated with the expression level of hASH-1 (Figure 1C). In addition, PC5/6, which is the convertase most related in structure to PACE4, was hardly detected in any of these cell lines (Figure 1A). These results indicate that PACE4 had a clear inverse correlation with hASH-1 but furin, PC5/6 and PC7/8 do not.

Effect of hASH-1 on the mRNA level of PACE4 and other SPC-family proteases in SH-SY5Y cells

To see whether hASH-1 overexpression directly affected the level of mRNA of PACE4 and other SPC-family proteases, the hASH-1 cDNA was transiently transfected into SH-SY5Y cells. As shown in Figure 2(A), PACE4 expression was analysed by Northern blotting. The hASH-1 transcript was expressed at a high level 2 h after transfection; its expression was maintained for 12 h as determined by RT-PCR analysis. To identify the expression of hASH-1 at the protein level, we also performed Western blot analysis on nuclear extracts from hASH-1-transfected SH-SY5Y cells. Consistent with the mRNA expression pattern was the observation that the hASH-1 protein was also expressed within 6 h and thereafter (Figure 2B). In contrast, the expression of PACE4 mRNA decreased rapidly after transfection (down to 29% at 2 h; to 18% at 6 h) as shown in Figure 2(A). However, hASH-1 expression had no effect on the expression of furin and PC7/8 (Figure 2C). These results show that the expression of the PACE4 gene was efficiently suppressed by induction of hASH-1 and this event was specific for PACE4 among SPCs.

Figure 1 Expression of hASH-1 and SPC-family proteases in various neuroblastoma cell lines

(A) Total RNA (10 μ g) from GANB (lane 1), SH-SY5Y (lane 2), NBL-S (lane 3), NMB (lane 4) and SK-N-DZ (lane 5) cells was analysed by Northern blotting as described in the Materials and methods section. The cDNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. (B) For the immunodetection of hASH-1 protein, nuclear extract (20 μ g of protein) from these cell lines was analysed by Western blotting. hASH-1 protein was detected as single 28 kDa band in nuclear extract from NBL-S cells. (C) The density of each band shown in A was quantified with NIH image software. Results are expressed as percentages of the SPC mRNA level in each cell.

Figure 2 Effect of hASH-1 overexpression on the level of mRNA for SPC-family proteases in SH-SY5Y cells

The levels of mRNA for SPC-family proteases and hASH-1 were analysed by Northern blotting and RT-PCR as described in the Materials and methods section. (A) Upper panels: total RNA was extracted from SH-SY5Y cells at 0 h (lane 1), 2 h (lane 2), 6 h (lane 3), 8 h (lane 4) and 12 h (lane 5) after hASH-1 transfection. Lower panel: the expression patterns of PACE4 (●) and hASH-1 (○) in (A), plotted using NIH image software. (B) Western blot analysis of hASH-1 with anti-(hASH-1) antiserum. Lane 1 was a control nuclear extract; lanes 2–4 were nuclear extracts at 6, 8 and 12 h respectively after hASH-1 transfection. (C) Total RNA was extracted from SH-SY5Y cells at 0 h (lane 1), 2 h (lane 2) and 4 h (lane 3) after hASH-1 transfection. The experiments were repeated three times to ensure reproducibility.

Table 1 Effect of overexpression of other bHLH factors on the level of PACE4 mRNA in SH-SY5Y cells

The cDNA of each bHLH factor was transfected into SH-SY5Y cells and the level of PACE4 mRNA 6 h after transfection was analysed by Northern blotting. The expression of their mRNA species was confirmed by RT-PCR analysis. The level of PACE4 mRNA is given relative to that of control experiments (100%). The control was transfected with empty expression vector. The experiments were repeated three times; results are means $\pm$ S.D.

bHLH factor	PACE4 mRNA 6 h after transfection (% of control)
Control	100
MASH-1 + E47	21.3 $\pm$ 8
MATH-1 + E47	98.6 $\pm$ 9
MATH-2 + E47	91.5 $\pm$ 4
Neurogenin 1 + E47	85.0 $\pm$ 5
Neurogenin 2 + E47	93.8 $\pm$ 11
Neurogenin 3 + E47	95.5 $\pm$ 4
E47	88.0 $\pm$ 9

Specificity of bHLH transcription factors for PACE4 expression

To define the specificity of suppression of PACE4 expression by hASH-1, we examined the effect of other neural bHLH transcription factors such as MASH-1, MATH-1, MATH-2, E47, neurogenin 1, neurogenin 2 and neurogenin 3 on the expression in SH-SY5Y cells (Table 1). MASH-1 [24] and multiple *atonal* homologues including MATH-1 [25], MATH-2 [26], neurogenin 1 [27], neurogenin 2 [28] and neurogenin 3 [29] are expressed in the developing nervous system and are important in neural differentiation. It is known that these bHLH factors interact with the E-box element (CANNTG) in collaboration with E47, a

mammalian *daughterless* homologue that is expressed ubiquitously [30]. The expression vectors containing cDNA species for the above bHLH factors (MASH-1, MATH-1, MATH-2, neurogenin 1, neurogenin 2 and neurogenin 3) were co-transfected with that of E47 into SH-SY5Y cells. The amounts of PACE4 mRNA decreased specifically (to $21.3 \pm 8\%$) after co-expression with MASH-1 and E47 for 6 h but were not affected by other bHLH factors (MATH-1, MATH-2, neurogenin 1, neurogenin 2, neurogenin 3 and E47) compared with control (transfected with the empty vector) (Table 1). These results indicate that PACE4 expression was specifically suppressed by MASH-1 as well as hASH-1.

Function of the E-box cluster in the promoter activity of the human PACE4 gene in neuroblastoma cells

The 5'-flanking region of the PACE4 gene contains 12 E-boxes (E1–E12) within 1 kb upstream of the transcription initiation site. Previously, we showed that the E4–E9 E-box cluster in the 5'-upstream region of the PACE4 gene acted as a major negative regulatory element in HepG2 and GH4C1 cells [20]. hASH-1 is presumed to be a possible candidate for a molecule that binds to the E-box motif (CACCTG) within the region from –796 to –649 of the 5'-flanking region of the PACE4 gene. To examine which of these E-box elements was functional in the regulation of PACE4 gene expression by hASH-1 in neuroblastoma cells, various deletion mutants of the 5'-flanking region were ligated to the promoterless luciferase reporter gene (pGL3B), as shown in Figure 3(A). Each construct was transfected into NBL-S and SH-SY5Y cells by lipofection and the luciferase activity of the cell lysate was measured. The former cells expressed higher levels of hASH-1, whereas the latter cells expressed a low level of hASH-1, as shown in Figure 1. Deletion of the region from –796 to –649, in which there were six repeats of the GGCCTGGGGTTCACCTGC sequence containing the E-box (E4–E9), caused an 8-fold increase in luciferase activity in NBL-S cells (Figure 3B). To confirm that the E-box cluster functioned as a negative regulatory element, the promoter activity of the internal deletion mutant –982 (Δ –982, deleted from –783 to –649) was also examined in NBL-S cells. Compared with the –982 construct, the internal deletion of this region (–782 to –650) caused a 2-fold increase in luciferase activity (Figure 3B). In contrast, there was a weak positive region between –982 and –796. However, the –982 construct, from which E12 had been deleted, had no significant effect on the promoter activity relative to the full-length construct (WT). These results indicate that the CACCTG E-box sequence located in the E-box cluster (E4–E9) functioned as a silencer element in NBL-S cells. To see whether hASH-1 could suppress PACE4 gene expression in this region, a –796 construct, a mutant of the E4 (–796EM4) or E9 (–796EM9) E-box (CACCTG was mutated to GTCCTG), were transiently co-transfected into SH-SY5Y cells with an expression vector for hASH-1. Whereas the –796 construct on co-expression of hASH-1 showed approx. 50% of the promoter activity of the wild-type, –796EM4 or –796EM9 had no significant effect on the promoter activity of hASH-1 (Figure 3C). These results indicate that the E-boxes between E4 and E9 had a negative effect on the promoter activity of hASH-1 for the PACE4 gene.

To identify the endogenous E-box (CACCTG)-binding protein that interacted with the E4–E9 E-boxes, we performed a gel mobility-shift assay with nuclear extract from NBL-S cells and E9 E-box oligonucleotide (GGCCTGGGGGTTTACCTGCACGGCCTGGGGGTT; –773 to –741 of the PACE4 gene). To clarify the activity of hASH-1 to bind the E9 probe, nuclear extract from NBL-S cells preabsorbed with anti-(hASH-1) IgG

Figure 3 Deletion analysis of the E-box cluster (E4–E9) of the PACE4 gene promoter region in neuroblastoma cells

(A) Diagram of the 5'-upstream region of the PACE4 gene. A series of 5'-end deletion mutants and internal deletion mutants of the promoter fragment were ligated into a luciferase plasmid (pGL3-Basic). Numbers indicate base pairs from the start of translation. E-boxes (filled rectangles numbered 1–12) are also shown at the top of the figure. (B) The luciferase activity (NBL-S cells) of each construct was expressed relative to the baseline luciferase activity of a promoterless construct (pGL3-Basic vector) set at 1. (C) Effects of hASH-1 overexpression and site-directed mutagenesis of the E-box sequence (E4, E9) on the promoter activity. The E-box sequences of E4 and E9 (CACCTG) were mutated to GTCCTG. The 796 construct and E-box mutants (–796EM4 and –796EM9) were co-transfected into SH-SY5Y cells with an expression vector for hASH-1. After 20 h, cell lysates were recovered for luciferase assay. The luciferase activity of each construct is expressed relative to the luciferase activity of the 796 construct set at 1. Results are means $\pm$ S.D. Abbreviation: WT, wild-type.

was also used for the gel mobility-shift assay. Incubation of the E9 probe with the nuclear extract produced a single major band (Figure 4A); however, this retarded band was greatly reduced when the nuclear extract was preabsorbed with anti-(hASH-1) IgG. Most bHLH factors preferentially bind to the CACGTG or CAGCTG E-box element but hASH-1 and MASH-1 are highly specific for the CACCTG sequence, unlike other bHLH proteins such as MyoD [31]. The sequence specificity of the E9-binding protein was further analysed by competition experiments. The competition was complete with a 10-fold molar excess of unlabelled E9 probe (CACCTG) but not with E10 (CATTTG), standard E-box (CAGGTG) or EM9 (GTCCTG) oligonucleotide containing a mutation in the E9 E-box sequence (Figure 4B). Furthermore, this band was not detected when the EM9 probe was incubated with nuclear extracts. These results clearly indicate

Figure 4 Gel mobility-shift assay of nuclear proteins interacting with the E9 E-box sequence

Double-stranded E9 oligonucleotides were used as a probe for binding analysis. (A) E9 probe was incubated with nuclear proteins (3 μ g) prepared from NBL-S cells pretreated with control IgG or hASH-1 IgG as described in the Materials and methods section. (B) E9 probe was incubated with nuclear proteins prepared from NBL-S cells in the presence of unlabelled ('cold') competitors as described in the Materials and methods section. Competitor oligonucleotides (E9, standard E-box, E10 and EM9) were added at a 10-fold molar excess relative to radiolabelled DNA.

that hASH-1 protein is a major CACCTG-binding protein in the nuclear extract. Persson et al. [32] showed that hASH-1 does not form homodimers. It is highly likely that heterodimers of hASH-1 and E-proteins (E47/E12/E2-2) bind to the CACCTG sequence in the E-box cluster of the PACE4 gene.

DISCUSSION

The timing of the progression from the growth phase to the differentiation phase is controlled by positively and negatively acting bHLH transcription factors during vertebrate neural development [33]. The bHLH factor *achaete-scute* complex genes are required for the development of the nervous system in *Drosophila* [34] and are transiently expressed in neuronal precursors before the expression of the mature neuronal phenotype [35]. The proneural genes *achaete* (*ac*) and *scute* (*sc*), two members of the *achaete-scute* complex [36,37], confer on *Drosophila* epidermal cells the ability to become sensory mother cells. In imaginal discs, *achaete* and *scute* are expressed in groups of cells, the proneural clusters, which are thought to delimit the areas in which sensory mother cells arise. Mammalian homologues of *Drosophila* *achaete-scute* genes have been recently characterized and provide useful information for the analysis of the mechanisms of mammalian neurogenesis. The MASH-1 gene was isolated on the basis of sequence homology with the *Drosophila* proneural *achaete-scute* genes from a sympathoadrenal progenitor cell line [24] and is expressed in specific regions of the developing central nervous system or the peripheral nervous system during embryogenesis [4].

Until recently, little was known about the transcriptional regulation of the PACE4 gene. In the present study we have found a novel character of hASH-1 that act as a negative regulator of the PACE4 gene in neuroblastoma cells. This conclusion was supported by the following findings. First, the endogenous expressions of PACE4 and hASH-1 mRNA species were inversely correlated in various neuroblastoma cell lines. Other SPCs such as furin, PC5/6 and PC7/8 did not show such a correlation. Secondly, PACE4 gene expression was markedly decreased in response to ectopic expression of hASH-1 in SH-SY5Y cells. hASH-1 had no effect on the expressions of furin and PC7/8. Thirdly, PACE4 gene expression in SH-SY5Y cells was specifically suppressed by MASH-1 as well as hASH-1. The other bHLH factors such as MATH-1, MATH-2, neurogenin 1, neurogenin 2, neurogenin 3 and E47 had no effect on this expression. Fourthly, the E-box (CACCTG) cluster (−796 to −649) was a negative regulatory element for the promoter activities of the PACE4 gene in NBL-S cells as determined by luciferase assay. Similar results were obtained in mutation and co-expression experiments on hASH-1 in SH-SY5Y cells. Finally, we showed that hASH-1 proteins could bind specifically to the CACCTG sequence of the E-box in the promoter region of the human PACE4 gene by gel mobility-shift assay with anti-(hASH-1) IgG and various competitors.

The bHLH family are important transcription factors for the development of various tissues [38]. Generally, the E-box element in the 5′-upstream region of tissue-specific genes is recognized by various bHLH transcription factors. MATH-1 [25] and MATH-2 [26], which are typical bHLH factors expressed in the nervous system, have been shown to bind to the E-box motif, CAGGTG or CAGCTG. Although these sequences are located within 1 kb 5′-upstream of the PACE4 gene (CAGGTG, E2 and E11; CAGCTG, E12), deletion of the E2, E11 or E12 E-box had no effect on the promoter activity of the PACE4 gene in neuroblastoma cell lines (results not shown; Figure 3B). Neurogenin 2 binds to CATCTG E-box element [28], whereas this sequence is not located within the upstream region of the PACE4 gene. In fact, ectopic expression of MATH-1, MATH-2 and neurogenin had no effect on the expression of the PACE4 gene. Previously, hASH-1 was shown to be able to interact with a specified sequence (CACCTG) of the E-box in collaboration with E2-2 [32]. These reports and the present results strongly suggest that hASH-1 binds to the CACCTG E-box sequence in the promoter region of the PACE4 gene. An inverse correlation in the expression of PACE4 and hASH-1 was also seen in several cancer cell lines. hASH-1 was highly expressed in a human medullary thyroid carcinoma (TT cells) [6] and small-cell lung carcinoma (NCI-H146) [39], whereas PACE4 was hardly detected in either cell line [40]. So far, all *achaete-scute* homologue factors are known to act as a positive regulator in neurogenesis. Negative effects of bHLH factors on the gene expression have been reported by several groups. MyoD, which is a bHLH factor required for myogenesis, acts as a negative transcription regulator of cyclin B1 [41]. PACE4, unlike furin, shows a restricted distribution in both endocrine and non-endocrine cells. It is found at a high level in pituitary, cerebellar Purkinje cells, olfactory bulb, heart, liver and pancreatic islets [15,42]. PACE4 exhibits highly regulated expression patterns during embryogenesis [18]. Previously we showed that the E-box cluster in the PACE4 gene acts as a negative regulatory element in both HepG2 and GH4C1 cells, although neither cell type expresses hASH-1 [20]. MASH-1 is specifically expressed in the nervous system. These results suggested the involvement of cell-specific bHLH transcription factors other than hASH-1 in the negative regulation of PACE4 expression via the CACCTG sequence in

these cells. Although the profiles of promoter activity in HepG2 and GH4C1 cells indicate a strong positive regulatory element, the negative regulatory activity of the E-box cluster in neuroblastoma cells was stronger than the positive activity in both cell lines. Therefore it is highly likely that hASH-1 has a crucial role in controlling PACE4 expression in neuroblastoma cells.

Very little is known about the genes controlling the development of the olfactory receptor neuron lineage. So far, MASH-1 is the only transcription factor shown to be required for this process. A null mutation of MASH-1 in mice has been shown to affect the olfactory neurons [4]. Although PACE4 is expressed at a high level in the mitral cells of the olfactory bulb, it is first expressed in the olfactory epithelium [15]. We therefore suggest the following physiological functions of PACE4 in the olfactory system. MASH-1 is expressed in the early stage of neural differentiation and acts as a neural determination factor. As development proceeds, MASH-1 is down-regulated and PACE4 expression is induced in the olfactory epithelium. These cells might migrate via the activation of cell adhesion molecule by PACE4 and the patterning of the olfactory system is stimulated by signals from these cells.

Recently, Akamatsu et al. [17] showed the temporospatial expression of PACE4 in the rat molar tooth during development. They also reported that other SPCs including furin, PC5/6 and PC7/8 were expressed at constitutive levels throughout the development. Constam and Robertson [18] also demonstrated dynamic expression patterns of PACE4 throughout embryonic development. A balance between the TGF- β superfamily, such as BMP4, and the secreted BMP antagonists, such as noggin, has been implicated in both neural induction and axis determination [43]. At the early embryonic stage, BMP4 acts as a neural inhibitor, whereas BMP antagonists can promote neural differentiation. Moreover, Baker et al. [14] showed that Wnt signalling in the *Xenopus* embryo inhibits BMP4 expression and activates neural development. As reported by Constam and Robertson [18], PACE4 can process proBMP4 to active BMP4, and embryos lacking PACE4 display various degrees of holoprosencephaly. Other SPCs, such as furin, cannot compensate for the loss of PACE4. These findings suggest that PACE4 functions as a proBMP4-activating enzyme during embryonic development. hASH-1 is expressed transiently before the differentiation to neural cells [45]. Therefore PACE4 expression is highly likely to be suppressed by hASH-1 transiently. This suppression of PACE4 gene expression might cause a decrease in biological activities of TGF- β -related proteins, such as BMP, and promote neural development. Taken together, these results and recent findings strongly suggest that PACE4 is one of the target genes of hASH-1 and is involved in the initiation of neural development.

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