Modulation of basic helix-loop-helix transcription complex formation
by Id proteins during neuronal differentiation

Annika Jögi #, Paula Persson #, Anna Grynfeld*, Sven Påhlman, and Håkan Axelson

From the Department of Laboratory Medicine, Division of Molecular Medicine, Lund University, University Hospital MAS, S-205 02 Malmö, Sweden

* Present address: Department of Medical Biochemistry and Microbiology, Uppsala University, BMC, P.O. Box 582, S-751 23 Uppsala, Sweden

# These authors contributed equally to this work.

To whom correspondence should be addressed: Håkan Axelson, Department of Laboratory Medicine, Division of Molecular Medicine, Lund University, University Hospital MAS, Entrance 78, S-205 02 Malmö.
Phone: +46-40337621; Fax: +46-40337322;
E-mail: hakan.axelson@molmed.mas.lu.se

Running title: Id-HES-1 interaction in neuronal cells

Keywords: bHLH, Id, HES-1, neuroblastoma, sympathetic nervous system, differentiation, protein-protein interaction.
Summary

It is assumed that the Id helix-loop-helix (HLH) proteins act by associating with ubiquitously expressed basic HLH (bHLH) transcription factors, such as E47 and E2-2, which prevents these factors from forming functional hetero- or homodimeric DNA-binding complexes. Several tissue-specific bHLH proteins, including HASH-1, dHAND, and HES-1, are important for development of the nervous system. Neuroblastoma tumors are derived from the sympathetic nervous system and exhibit neural crest features. In differentiating neuroblastoma cells, HASH-1 is downregulated, and there is coincident upregulation of the transcriptional repressor HES-1, which is known to bind the HASH-1 promoter. We found that the three Id proteins expressed in neuroblastoma cells (Id1, Id2, and Id3) were downregulated during induced differentiation, indicating that Id proteins help keep the tumor cells in an undifferentiated state. Studying interactions, we noted that all four Id proteins could dimerize with E47 or E2-2, but not with HASH-1 or dHAND. However, the Id proteins did complex with HES-1, and increased levels of Id2 reduced the DNA-binding activity of HES-1. Furthermore, HES-1 interfered with Id2/E2-2 complex formation. The ability of Id proteins to affect HES-1 activity is of particular interest in neuronal cells, where regulation of HES-1 is essential for the timing of neuronal differentiation.
Introduction

Neuroblastoma is a childhood tumor derived from sympathetic neuroblasts of the peripheral nervous system (1). This part of the nervous system originates from the neural crest, which, in addition to the cells of the peripheral nervous system, gives rise to cartilage and smooth muscle cells, as well as melanocytes (2). In recent years, several transcription factors that control differentiation of the developing nervous system have been identified, and many of these belong to the basic helix-loop-helix (bHLH) family of proteins (3). This family can be divided into different subgroups on the basis of structure, expression pattern, and DNA-binding activity (4,5). The E proteins (also designated class A), have four mammalian members: E2-2, HEB, and the two E2a gene products E12 and E47. These proteins are ubiquitously expressed, and they can bind DNA as homodimers or as heterodimers with the class B or tissue-specific bHLH transcription factors. The homo- or heterodimeric DNA-binding complexes activate transcription of target genes that contain the E-box motif CANNTG in their promoter (4). There is also a subgroup of tissue-specific bHLH proteins that repress transcription, typified by Hairy/Enhancer of Split homologue-1 (HES-1) (6). The basic DNA-binding domains of these proteins contain a proline that alters the DNA-binding specificity, as compared to other bHLH proteins (6). The HES-related proteins can achieve their repressive functions in two ways: by binding DNA and recruiting the corepressor transducin-like Enhancer of split (TLE); or, in a non-DNA-binding manner, by interfering with complex formation between E and bHLH proteins (6,7). From a mechanistic point of view, the latter function resembles the effect of the Id (inhibitors of differentiation or inhibitors of DNA-binding) proteins, which are a group of regulatory proteins in the helix-loop-helix (HLH)
network. The Id proteins are dominant-negative inhibitors since they lack the basic DNA-binding domain (8). The four mammalian Id proteins (Id1–Id4) form transcriptionally inactive heterodimers, primarily with the E proteins, and thereby prevent the E proteins from forming functional heterodimers with tissue-specific bHLH proteins (9).

*Mammalian achaete-scute homologue-1* (*MASH-1* in the mouse and *HASH-1* in humans) is a vital bHLH protein in the developing sympathetic nervous system (SNS). *MASH-1* is expressed in restricted regions of the embryonic brain and in sympathetic and enteric precursor cells (10). Gene-targeting experiments have shown that *MASH-1* is needed for proper development of autonomic and olfactory neuroblasts, neuroendocrine cells of the lung, and certain regions of the telencephalon (11-13). Another tissue-specific bHLH protein is dHAND, which is expressed in the embryonic sympathetic and enteric nervous systems (14-16).

Neuroblastoma cells show characteristics of developing sympathetic neuroblasts, and we have previously studied the expression of *dHAND* and *HASH-1*, in both primary tumors and neuroblastoma cell lines. At the mRNA level, we detected expression of these two genes in all neuroblastoma cell lines and a majority of the primary tumors we analyzed (16-18). Furthermore, during induced differentiation of neuroblastoma cells, we observed rapid downregulation of *HASH-1* that was accompanied by a transient upregulation of *HES-1* (17). HES-1 is an important protein in the Notch-1 signaling cascade that has been shown to bind the *HASH-1* promoter and thereby inhibit expression of *HASH-1* (19). Accordingly, downregulation of *HASH-1* induced by increased expression of HES-1 may be a prerequisite for neuronal differentiation of neuroblastoma cells (17).
In the present study, we analyzed expression of the Id genes in relation to some tissue-specific bHLH factors that are expressed in differentiating neuroblastoma cells. We found varying levels of Id1, Id2, and Id3, but not Id4, in neuroblastoma cell lines, and the expression of these proteins was downregulated when differentiation was induced. To further elucidate the dynamics of the bHLH network, we also performed experiments to ascertain the capacity of Id proteins to interact with bHLH proteins involved in neurogenesis. The results show that the Id proteins form complexes with HES-1, both \textit{in vitro} and \textit{in vivo}, which demonstrates novel functions of HES-1 and the Id proteins and provides evidence of an additional level of regulation within the bHLH network. This could have implications for understanding of the formation of the neural crest and the lineage determination of neuronal cells, as well as the function of these proteins in general.
Experimental procedures

Cell culture

The neuroblastoma cell lines SH-SY5Y and SK-N-BE(2) and the neuroepithelioma cell line SK-N-MC were grown in Eagle’s Minimum Essential Medium (MEM, Life Technologies) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37 °C in 5% CO2. The SH-SY5Y cells were induced to differentiate by exposure to 16 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and the SK-N-BE(2) cells were differentiated with 10 µM all-trans retinoic acid (RA, Sigma) for 0, 2, 8, 24, and 96 h. The neuroblastoma cell lines (IMR-32, LA-N-1, LA-N-2, and LA-N-5) and the Chinese hamster ovary cell line (CHO) were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). The rat pheochromocytoma cell line PC12 was grown in RPMI 1640 medium supplemented with 10% horse serum, 5% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Western blot analysis

Total cell homogenates were prepared in NP40 lysis buffer (1% NP40, 10% glycerol, 20 mM Tris HCl [pH 8.0], 137 mM NaCl, and 4% complete protease inhibitor cocktail mix [Roche]). Using 40 µg of protein per lane, we performed SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted to PVDF filters (Immobilon). The filters were probed with polyclonal anti-Id1 or anti-Id3 antisera (Santa Cruz) diluted 1:1000 or polyclonal anti-Id2 antiserum (Santa Cruz) diluted 1:200. HES-1 and HASH-1 were detected with polyclonal anti-HES-1 antiserum (kindly provided by Dr. Tetsuo Sudo, (20)) diluted 1:8000 and a
monoclonal anti-MASH-1 antibody (Pharmingen) diluted 1:125. Fusion proteins containing VP16, GAL4, and EGFP domains were respectively detected with a monoclonal anti-VP16 antibody (Santa Cruz), a monoclonal anti-GAL4-DNA-binding domain antibody (Santa Cruz), and a polyclonal anti-GFP antiserum (Clontech). Immunoreactivity was visualized by enhanced chemiluminescence (Pierce).

**Mammalian two-hybrid analysis**

Mammalian two-hybrid analyses were performed using the Checkmate system (Promega). Constructs encoding VP16 transactivating (pACT) and GAL4-DNA-binding (pBIND) proteins were created by cloning PCR-generated fragments into BamHI/SalI digested vectors. The fragments comprised the following amino acids: dHAND, 1–217; E2-2, 1–668; E47, 508–654 (spanning the bHLH region); HASH-1, 1–180; HES-1, 90–281; Id1, 1–154; Id2, 1–134; Id3, 1–119; Id4, 1–162. The pBIND-MyoD vector was provided in the Checkmate kit (Promega). The pG5\(\text{\textit{luc}}\) reporter plasmid contains five GAL4 binding sites upstream of the coding sequence for firefly luciferase. The pBIND vector also encodes *renilla* luciferase downstream of a constitutively active CMV promoter, hence it was possible to use a Dual Luciferase kit (Promega) for both luminometric measurement of the interaction and determination of transfection efficiency. The analyses were done on CHO cells (1.5 \times 10^5 per 35-mm dish), which were transfected with the appropriate vectors and the pG5\(\text{\textit{luc}}\) reporter (0.6 µg of each plasmid per dish) using Lipofectamine (Life Technologies). The experiments were performed in triplicate, and the results were recorded as the relative luciferase activity (i.e., the ratio of firefly luciferase activity to *renilla* luciferase activity). The expression plasmids were sequenced, and expression of all proteins was verified by Western blot analysis. The mammalian two-hybrid
system was also employed to assess the ability of HES-1 and Id2 to interfere with the formation of bHLH dimers in a dominant-negative manner. CHO cells were cotransfected with pACT-E2-2 and pBIND-HASH-1 or pBIND-Id2 (0.2 µg of each plasmid per dish), along with increasing amounts of pMYC-HES-1 or pEGFP-Id2 (0.2–0.8 µg of plasmid per dish). The total amount of DNA was equalized with either pMYC or pEGFP. The pMYC-HES-1 and pEGFP-Id2 were made as described above, but, for HES-1, the full-length sequence (amino acids 1–281) was used. The results are given as percentage of the initial activity.

**Transient transfections and coimmunoprecipitations**

The following expression plasmids were used: pBIND-HASH-1, pBIND-HES-1, pBIND-Id2, pBIND-E2-2, pBIND-E47, pEGFP-HASH-1, and pEGFP-HES-1. To make pEGFP-HASH-1 (amino acids 1–238) and pEGFP-HES-1 (amino acids 1–281), PCR-generated fragments were inserted into a BamHI/XhoI-digested pEGFP-C2 vector (Clontech). All transfections were performed using Lipofectamine according to the recommendations of the manufacturer (Life Technologies). CHO cells were plated (1 × 10^6 cells/100-mm dish) and transfected with 5 µg of total DNA. Sixteen hours after transfections, total cell lysates were prepared in modified RIPA buffer (1% NP40, 50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 4% complete protease inhibitor cocktail mix [Roche]). The lysates were centrifuged at 14,000×g for 15 min to remove debris and then precleared with protein G Sepharose beads (Amersham Pharmacia Biotech) for 10 min at 4 °C. Immunoprecipitations were performed with a monoclonal anti-GAL4-DNA-binding domain antibody. The immune complexes were recovered on protein G Sepharose beads for 1 h at 4 °C and then washed four times with phosphate-buffered saline (PBS). The precipitated
proteins were resuspended in sample buffer, boiled for 5 min, fractionated by SDS-PAGE on a 12.5% gel, and thereafter blotted to a PVDF-filter. The filter was incubated with polyclonal anti-GFP antiserum diluted 1:100. Immunoreactivity was detected by enhanced chemiluminescence (Pierce).

For coimmunoprecipitations of endogenous proteins, PC12 cells were plated (10 × 10^6 cells/100-mm dish), and cell lysates were prepared in modified RIPA buffer (as above). Immunoprecipitations were performed with 1 µg of polyclonal anti-Id1 antiserum (Santa Cruz) or 1 µg of rabbit immunoglobulin (negative control, Dako) for 1.5 h at 4 °C. The immune complexes were precipitated with protein G Sepharose for 1 h at 4 °C and then washed twice with modified RIPA buffer and twice with PBS. The samples were separated by electrophoresis on a 12.5% acrylamide gel and the proteins were transferred to a PVDF filter. HES-1 was detected with polyclonal anti-HES-1 antiserum diluted 1:16,000, and Id1 with polyclonal anti-Id1 antiserum diluted 1:1000. Immunoreactivity was detected as described above.

**Electrophoretic mobility shift assay**

Using Lipofectamine, pACT-HES-1 or pACT-Id2 were transfected into CHO cells seeded at a density of 1 × 10^6 cells/100-mm plate. Sixteen hours after transfection, the cells were harvested in lysis buffer (10 mM Tris HCl, 50 mM NaCl, 30 mM Na₃P₂O₇, 50 mM NaF, and 5 µM ZnCl₂ [pH 7.1], supplemented with 1% Triton X100, 5% protease inhibitor cocktail [Sigma], and 6.7 mM DTT). Transcription and translation of Id2 *in vitro* were carried out using a TNT-coupled reticulocyte lysate kit (Promega) and the pACT-Id2 plasmid. DNA binding reactions were done in a total volume of 35 µl containing 20 mM HEPES (pH 7.3), 50 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 8% glycerol, 5 µg/ml aprotinin, 1 mM β-mercaptoethanol, 10 mM DTT, 0.1 mg/ml
salmon sperm DNA, and 0.2 ng $^{32}$P-labeled wildtype (wt) oligonucleotide (5’-CCGCCAGGCGCACGCACTGCAACAA-3’). Competition experiments were done with 175 ng of unlabeled wt oligonucleotide or 175 ng of N-box-mutated oligonucleotide (5’-CCGCCAGGCGACTAAGCTGCAACAA-3’). For supershift experiments, the cell extracts were pre-incubated with polyclonal anti-HES-1 antiserum (CeMines) for 10 min at room temperature. Thereafter, the labeled probe was added, and the samples were further incubated for 30 min at 30 °C. Id2 translated \textit{in vitro} or an equal amount of mock-translated rabbit reticulocyte lysate was mixed with protein extract from HES-1-transfected CHO cells, and the mixture was pre-incubated for 10 min at room temperature, after which the labeled probe was added. In the Id2 competition experiments, increasing amounts of cell extract from Id2-transfected CHO cells were mixed with constant amounts of cell extract from HES-1-transfected CHO cells and then pre-incubated for 10 min at room temperature. Cell extract from untransfected CHO cells was used as a negative control. The binding reactions were then subjected to electrophoresis on a 4% polyacrylamide gel (30:1 acrylamide:bis-acrylamide).
Results

Expression of Id proteins in neuroblastoma cell lines

The expression patterns of Id1, Id2, Id3, Id4, HASH-1, and HES-1 were analyzed at the protein level in neuroblastoma cell lines and in two other cell lines derived from related tumors, the rat pheochromocytoma cell line PC12 and the neuroepithelioma cell line SK-N-MC (Fig. 1). The PC12 cells and all but one of the neuroblastoma cell lines (i.e., SH-SY5Y, IMR-32, LA-N-2, LA-N-1, and SK-N-BE(2), but not LA-N-5) expressed detectable levels of HASH-1. HES-1 was clearly detected in PC12 and SK-N-MC cells, and low levels of this protein was also found in four neuroblastoma cell lines (SH-SY5Y, LA-N-5, LA-N-1, and SK-N-BE(2)). These results corroborated our previous mRNA data showing that there is an inverse correlation between HASH-1 and HES-1 expression in neuroblastoma cells and that HES-1, but not HASH-1, is expressed in SK-N-MC cells (17). Varying levels of Id1 and Id2 were expressed in all neuroblastoma cell lines tested: high levels of Id1 in SH-SY5Y, IMR-32, LA-N-1, and SK-N-BE(2); and substantial expression of Id2 in LA-N-2 and SH-SY5Y (Fig. 1). All the neuroblastoma lines except IMR-32 and LA-N-1 expressed Id3 (Fig. 1), whereas none of the cell lines investigated expressed Id4 (data not shown).

Expression of Id proteins in differentiating neuroblastoma cells

In many cell systems, Id expression decreases during differentiation (9). To study the expression pattern of HASH-1, HES-1, and the Id proteins in differentiating neuroblastoma cells, we used two different cell systems: SH-SY5Y, which lacks N-
myc amplification; and SK-N-BE(2), in which each cell contains approximately 85 copies of the N-myc gene (21). The SH-SY5Y cells were induced to undergo robust and well-characterized sympathetic neuronal differentiation by exposure to 16 nM TPA (22,23), and the SK-N-BE(2) cells were induced to differentiate with 10 µM RA, which led to a less distinct neuronal differentiation (17,18). Within 2 h of TPA treatment, the level of HES-1 in the SH-SY5Y cells reached a maximum, and there was a concurrent downregulation of HASH-1 expression (Fig. 2); neither of these proteins was detected after 96 h of differentiation. In accordance with these findings, we have previously studied the corresponding genes and observed a similar rapid downregulation of HASH-1 at the mRNA level, with a concomitant transient upregulation of HES-1 (17). In differentiating SH-SY5Y cells, the Id1 level remained constant up to 24 h, but had decreased markedly after 96 h (Fig. 2). By comparison, Id2 and Id3 were downregulated more quickly: both showed a distinct decrease after only 8 h, and, after 96 h, Id3 was undetectable, and the level of Id2 was low (Fig. 2).

After 2 h of differentiation induced by 10 µM RA, the SK-N-BE(2) cells exhibited a decrease in the level of HASH-1, and this was accompanied by an increase in HES-1 (Fig. 3). After 96 h, the former protein was barely detectable, and the level of HES-1 had decreased to baseline, which reflects our previously reported mRNA data (17). The changes in Id levels that occurred in this cell line with progressing differentiation were not as marked as those observed in SH-SY5Y cells undergoing TPA-induced differentiation. Moreover, in the SK-N-BE(2) cells, the level of Id1 was virtually constant, while the level of Id2 decreased to some extent, and there was a slight transient upregulation of Id3 expression after 8 h (Fig. 3). Thus, the SK-N-BE(2) and SH-SY5Y cells were similar in regard to regulation of
expression of HASH-1 and HES-1, but they exhibited slightly different Id expression patterns.

Mammalian two-hybrid analysis of interactions between Id proteins and neuronal bHLH transcription factors

We used the mammalian two-hybrid system to investigate the ability of the Id proteins to form complexes with bHLH transcription factors (24). Expression plasmids encoding GAL4 fusion proteins of Id1 to Id4 and VP16 fusion proteins of a panel of bHLH transcription factors were constructed (Fig. 4A). Western blot analyses revealed that the levels of expression of the different fusion proteins were similar (Fig. 4B and C). The modest variations that were noticeable were not correlated with the degree of reporter gene activation in the mammalian two-hybrid assays, as exemplified by HES-1 and dHAND (Fig. 4B), as well as dHAND and Id1 (Fig. 4C). As expected, there was a clear interaction between Id1 and the ubiquitously expressed E proteins E47 and E2-2 (Fig. 4B). Comparable results were obtained with the Id2, Id3, and Id4 constructs (data not shown), and, in the subsequent mammalian two-hybrid experiments, all four Id proteins displayed similar capacities to interact with bHLH proteins. In agreement with previous reports (25,26), we found that all four Id proteins interacted with MyoD (Fig. 4B and data not shown). Moreover, although the four Id proteins did not form complexes with the tissue-specific proneuronal transcription factors dHAND and HASH-1, they did dimerize with HES-1 (Fig. 4B and data not shown).

The ability of HES-1 to dimerize with both the Id and the E proteins distinguished it from the other tissue-specific bHLH transcription factors we investigated. The difference between dHAND and HES-1 in regard to interaction
capacity was further illustrated by coexpression of VP16-dHAND or VP16-HES-1 with GAL4 fusion proteins of a panel of HLH/bHLH factors. We thereby generated a “fingerprint” of the abilities of dHAND and HES-1 to interact with other proteins of the HLH network (Fig. 4C and D). The background activity of each GAL4 fusion protein was assessed in combination with the empty pACT vector. These analyses highlighted the following characteristic difference in the interaction patterns of these two proteins: HES-1 formed complexes with the E and the Id proteins, whereas dHAND dimerized only with the E proteins.

**Mammalian two-hybrid analysis of dominant-negative effects of HES-1 and Id2**

We extended the mammalian two-hybrid analyses to ascertain whether HES-1 and Id2 interfere with the formation of HLH dimers in a dominant-negative manner. Increasing amounts of HES-1 had no effect on dimerization between HASH-1 and E2-2 (Fig. 5A). In contrast, Id2 decreased the reporter gene activity even at the initial concentration we tested (Fig. 5B). At this level, the EGFP-Id2 fusion protein was hardly detectable by Western blot analysis. This effect was most likely due to the formation of E2-2/Id2 dimers, since the Id proteins do not interact with HASH-1 (Fig. 4B and data not shown). Accordingly, we investigated the effect of HES-1 on formation of the E2-2/Id2 dimer and discovered that reporter gene activation decreased with increasing amounts of HES-1 (Fig. 5C). Theoretically, this may have been due to binding of either Id2 or E2-2 to HES-1. However, HES-1 did not affect the interaction between E2-2 and HASH-1 (Fig. 5A), hence we suggest that this inhibitory effect of HES-1 was mainly the result of complex formation with Id2. Thus, in these experiments *in vivo*, HES-1 sequestered Id2 and in that way prevented it from dimerizing with the E protein E2-2.
Coimmunoprecipitation of Id2 and HES-1

We performed coimmunoprecipitation assays to prove that HES-1 and Id proteins can interact in mammalian cells. CHO cells were transfected with expression plasmids for EGFP-tagged HES-1 or HASH-1 and GAL4-tagged Id2 (Fig. 6A), and Western blotting revealed that EGFP-HES-1 and EGFP-HASH-1 were expressed at similar levels (data not shown). Sixteen hours after transfection, the cells were lysed, and Id2 with associated proteins were immunoprecipitated with an anti-GAL4 antibody conjugated to protein G Sepharose beads. The precipitates were subjected to Western blot analysis using a polyclonal anti-EGFP antiserum, which showed that HES-1, but not HASH-1, was coimmunoprecipitated with Id2 (Fig. 6A). Use of an empty GAL4-expressing vector showed that the Id2 part of the fusion protein was necessary for coimmunoprecipitation to occur. We included the E proteins E2-2 and E47 as positive controls in these experiments. Our findings confirmed the results obtained using the mammalian two-hybrid system, that is, both E2-2 and E47 formed complexes with HES-1, whereas there was no detectable association between HASH-1 and HES-1 or between HASH-1 and Id2 (Fig. 6A). Apparently, the amounts of HES-1 immunoprecipitated by Id2, E47, and E2-2, respectively, were to some extent correlated with the level of reporter gene activation observed in the mammalian two-hybrid system, because we found that more HES-1 was coimmunoprecipitated with Id2 than with the E-proteins. Furthermore, E47 was less efficient in precipitating HES-1 than E2-2 (Fig. 4B and 6A). It will be necessary to use other experimental techniques to determine whether these observations reflect different affinities between the various HLH proteins.
We continued our work by examining the ability of HES-1 to form complexes with the Id proteins in cells derived from the sympathetic nervous system. We chose to analyze Id1 instead of Id2 due to the superior immunoprecipitating capacity of the anti-Id1 compared to the anti-Id2 antiserum. The rat pheochromocytoma cell line PC12 was used, because these cells express high levels of HES-1 and Id1. We found that the anti-Id1 antiserum did immunoprecipitate HES-1 from PC12 extracts (Fig. 6B), which confirmed the results of the coimmunoprecipitations using extracts from transfected cells. Therefore, we conclude that Id1 can complex with HES-1 in sympathetic neuronal cells.

**Id2 inhibits binding of HES-1 to an N-box oligonucleotide**

HES-1 and other hairy-related bHLH proteins bind to a distinct hexameric sequence called the N- or C-box (CACNAG) (6). For electrophoretic mobility shift assays, we transfected CHO cells with a VP16-tagged HES-1 expression construct and then prepared cell lysates. A prominent complex appeared when 32P-labeled N-box-containing oligonucleotide was mixed with cell extract from HES-1-transfected CHO cells, whereas no such complex was detected when this oligonucleotide was mixed with extract from untransfected cells (Fig. 7A, lanes 2 and 4). The complex was supershifted when we mixed extract from HES-1-transfected cells with an anti-HES-1 serum, which confirms that this complex arose due to HES-1 DNA-binding activity (Fig. 7A, lane 3). To verify the specificity of this binding, we performed competition experiments, in which an excess of unlabeled wt or N-box-mutated oligonucleotide was added to the reaction mixtures, and found that only the wt oligonucleotide could diminish the DNA-binding of HES-1 (Fig. 7A, lanes 5 and 6). To study the effect of
Id2 on DNA binding by HES-1, we pre-incubated extract from HES-1-transfected cells with Id2 translated *in vitro* or, as a control, with an equal amount of a lysate of mock-translated rabbit reticulocyte lysate. The results show that binding of HES-1 to the N-box oligonucleotide was decreased by the *in vitro* translated Id2, but not by the control mock-translated lysate (Fig. 7B, lanes 2 and 3). Extending these experiments, we mixed increasing amounts of cell extract from *Id2*-transfected CHO cells with a constant amount of cell extract from *HES-1* transfected CHO cells, and found that binding of HES-1 to the N-box-containing oligonucleotide decreased with increasing Id2 concentration (Fig. 7C). Together, these results show that HES-1 can bind an N-box-containing oligonucleotide, and that addition of Id2 translated *in vitro* or extract from *Id2*-transfected cells decreases this DNA-binding activity of HES-1.
Discussion

To gain a better understanding of the biological activity and function of the different Id proteins, we analyzed these proteins in regard to their expression patterns and their ability to form complexes with bHLH factors expressed in differentiating neuroblastoma cells. All the neuroblastoma cell lines we investigated expressed Id1 and Id2, and most of them also expressed Id3.

It has been reported that, in a number of model systems, Id levels are downregulated during induced differentiation, and there is concomitant cell cycle withdrawal (27). Consistent with those findings, we have previously noted that all Id genes expressed in our experiments (Id1, Id2, and Id3) were downregulated during TPA-induced differentiation and growth inhibition in SH-SY5Y cells (28), but it is not clear whether the downregulation of Id proteins is a prerequisite for the decreased proliferation. By comparison, in the present study, we detected only modest changes in Id levels in SK-N-BE(2) cells that had been induced to differentiate. The differences in Id levels may reflect the varying extent of neuronal differentiation in these two cell lines, with a more robust neuronal phenotype in the SH-SY5Y cells than in the SK-N-BE(2) cells. Considering the decreased proliferation during induced differentiation, it should be mentioned that Id proteins also have several effects on cell cycle regulatory proteins. One such effect is the direct interaction between Id2 and proteins of the retinoblastoma family (pRB, p107, and p130) (29). It is assumed that Id2 interferes with the association between pRB and E2F-DP1 by binding hypophosphorylated pRB, which results in E2F-DP1-driven transcription of genes required for S-phase progression. Thus, a possible explanation for the reduced proliferation seen in neuroblastoma cells undergoing induced differentiation is that a lower level of Id2 increases the pool of free hypophosphorylated pRB. In support of
this concept, ectopic expression of the Id proteins leads to a blocked differentiation and increased proliferation in several model systems (9). However, we could not determine whether this is also the case in neuroblastoma cells, because they died upon overexpression of Id proteins (data not shown). Of specific interest for the genesis of neuroblastoma is a study indicating that Id2 is a transcriptional target of N-myc (30). Since N-myc amplification, and thereby dysregulated expression, is a cardinal feature of high stage neuroblastoma tumors these findings could shed light on some puzzling features of neuroblastomas, such as the lack of mutations in the pRB pathway (31).

Within the bHLH network, the Id proteins exert their effect primarily by binding to E proteins. This was readily revealed by the mammalian two-hybrid analyses, which showed that all four Id proteins interacted with E47 and E2-2 (Fig. 4B and data not shown). We observed that the Id proteins interacted with MyoD, as also reported by other investigators (8,26), but not with the proneuronal bHLH proteins HASH-1 and dHAND. These data corroborate the concept that the Id proteins act by sequestering the E proteins, thereby preventing complex formation between proneuronal bHLH proteins and their obligatory protein dimerization partners (4). Furthermore, we found that HES-1 interacted with all four Id proteins, and the difference in interaction pattern between HES-1 and the proneuronal bHLH protein dHAND was clearly demonstrated by generating mammalian two-hybrid interaction “fingerprints” (Fig. 4C and D). Whereas dHAND interacted solely and specifically with the E proteins, HES-1 formed complexes with both the E and Id proteins. Although the level of reporter gene activation in the mammalian two-hybrid system does not necessarily reflect the exact strength of the interaction, our coimmunoprecipitation data support the suggestion that the association between HES-1 and Id2 is of considerable strength (Fig. 6A). Moreover, the coimmunoprecipitation
experiments of endogenous proteins showed that a considerable fraction of HES-1 in PC12 cells is complexed with Id1 (Fig. 6B), which is the predominant Id protein in this SNS-derived cell line. This clearly suggests that the interaction influences the activity of both proteins *in vivo*.

We extended the mammalian two-hybrid analyses to include a third factor that was expressed together with the GAL4 and VP16 fusion proteins. We reasoned that if this additional protein bound either of the two fusion proteins, we would detect decreased reporter gene activation. Indeed, introduction of increasing amounts of Id2 to cells with a transactivating HASH-1/E2-2 complex showed that Id2 had a dominant-negative effect on the HASH-1/E2-2 reporter gene activation (Fig. 5B). We also found that HES-1 can interfere with the dimerization between Id2 and E2-2, but not that between HASH-1 and E2-2, which suggests that HES-1 *in vivo* can modulate the dominant-negative effect that Id2 has on an E protein without directly interfering with dimerization between HASH-1 and E2-2 (Fig. 5). This reveals a novel level of regulation within the HLH network: in an *in vivo* situation in which the four proteins compared in this experiment are present, Id2 may act as a dominant-negative regulator of the HASH-1/E2-2 complex, and HES-1 will, in turn, act as a negative regulator of Id2, without affecting the association between HASH-1 and E2-2. Our results may also have implications beyond the bHLH network, since HES-1 might interfere with other functions of the Id proteins, such as the interaction between Id and pRB (29), and in that way directly affect cell proliferation. Furthermore, Id2 decreased the binding of HES-1 to an N-box-containing oligonucleotide (Fig. 7C). Thus, Id2 can sequester HES-1 and prevent it from binding DNA in a manner similar to the dominant-negative effect that the Id proteins exert on the E proteins.
Low levels of HES-1 are expressed in many types of cells, and this expression is at least partly controlled by Notch-1 signaling (32). Moreover, there is a negative HES-1 autoregulation component at N-boxes in the HES-1 promoter (33), and Id protein levels may therefore influence this autoregulatory loop. Interestingly, studies of *Drosophila* have provided genetic evidence linking the function of the Notch signaling cascade with extramacrochaetae (emc), the *Drosophila* orthologue of the Id proteins. In *Drosophila*, the function of Notch-1 is important for proper wing formation, and, during that event, there is an association between expression of *Notch* and *emc*. This suggests that, in certain developmental stages, *emc* collaborates with downstream genes that are targets of Notch-1, such as the hairy-related *Enhancer of split-mb* gene (34). However, biochemical studies of the *Drosophila* proteins have excluded a direct interaction between emc and the hairy-related proteins, in contrast to our results which are based on the mammalian homologues and show a relatively strong interaction between HES-1 and the Id proteins (Fig. 4B and C, and Fig. 6A).

Both HES-1 and Id2 play important roles in development of the neural crest. Gene-targeting experiments in mice have shown that HES-1 controls the proper timing of neurogenesis and regulates neural tube morphogenesis by repressing expression of proneuronal genes such as MASH-1 (35-37). In the chicken, it has been demonstrated that overexpression of Id2, which is normally expressed in the cranial neural folds, converts ectodermal precursor cells into a neural instead of an epidermal lineage (38), and the authors suggested that epidermalization may be promoted by a bHLH partner of Id2. This bHLH partner should have properties that are compatible with the known functions of HES-1 as an inhibitor of neuronal differentiation.

Mutual regulation of HES-1 and Id proteins may also be important in other tissues and cell types in which involvement of HES-1 has been implicated, for
instance the developing CNS (39), β cells of the pancreas (40), and tumors such as small cell lung cancer (41). During differentiation in SH-SY5Y neuroblastoma cells, HES-1 is transiently upregulated before the Id proteins are downregulated (Fig. 2). Consequently, it is possible that differentiation is initiated by the short-lived upregulation of HES-1 and that the subsequent downregulation of Id proteins is necessary during later stages of differentiation and for cessation of proliferation. In this scenario, the interaction between HES-1 and the Id proteins could have several effects, one of which might be to balance the level of functional HES-1, and, conversely, HES-1 might affect the activity of the Id proteins. Furthermore, the induction of HES-1 during neuroblastoma cell differentiation may result in decreased levels of free Id2 and an increased pool of hypophosphorylated pRB, which would impede cell cycle progression. Concomitantly, the HES-1/Id2 interaction may relieve the dominant-negative effect of Id2 on the E proteins, allowing them to dimerize with the proneuronal bHLH proteins and permitting the differentiation program to proceed. HES-1 that is not associated with Id proteins may have a negative autoregulatory effect and also repress HASH-1 transcription. Our finding that interaction can occur between HES-1 and Id protein may be of importance not only in further studies of neuroblastoma cell differentiation, but also to elucidate the role of these proteins in general.
**Acknowledgments**

We thank Dr. Karim Dib for helpful advice on the coimmunoprecipitation experiments. This work was supported by grants from the Swedish Cancer Society, the Children’s Cancer Foundation of Sweden, Inga and John Hain’s Foundation, Åke Wiberg’s Foundation, the Crafoord Foundation, HKH Kronprinsessans Lovisas förening för barnsjukvård, Hans von Kantzow’s Foundation, and Malmö University Hospital Research Funds.
References

1. Hoehner, J. C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L., and Påhlman, S. (1996) Lab Invest 75(5), 659-675
2. Groves, A. K., and Bronner-Fraser, M. (1999) Curr Top Dev Biol 43, 221-58
3. Lee, J. E. (1997) Curr Opin Neurobiol 7(1), 13-20
4. Massari, M. E., and Murre, C. (2000) Mol Cell Biol 20(2), 429-440
5. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., and et al. (1989) Cell 58(3), 537-544
6. Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992) Genes Dev 6(12B), 2620-2634
7. Palaparti, A., Baratz, A., and Stifani, S. (1997) J Biol Chem 272(42), 26604-26610
8. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) Cell 61(1), 49-59.
9. Norton, J. D. (2000) J Cell Sci 113(Pt 22), 3897-38905
10. Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T., and Anderson, D. J. (1991) Genes Dev 5(9), 1524-1537.
11. Borges, M., Linnoila, R. I., van de Velde, H. J., Chen, H., Nelkin, B. D., Mabry, M., Baylin, S. B., and Ball, D. W. (1997) Nature 386(6627), 852-855.
12. Casarosa, S., Fode, C., and Guillemot, F. (1999) Development 126(3), 525-534.
13. Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993) Cell 75(3), 463-476.
14. Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D., and Olson, E. N. (1997) Nat Genet 16(2), 154-160.
15. Srivastava, D., Cserjesi, P., and Olson, E. N. (1995) Science 270(5244), 1995-1999.
16. Gestblom, C., Grynfeld, A., Öra, I., Örtoft, E., Larsson, C., Axelson, H., Sandstedt, B., Cserjesi, P., Olson, E. N., and Påhlman, S. (1999) Lab Invest 79(1), 67-79
17. Grynfeld, A., Pahlman, S., and Axelson, H. (2000) Int J Cancer 88(3), 401-410.
18. Söderholm, H., Örtoft, E., Johansson, I., Ljungberg, J., Larsson, C., Axelson, H., and Påhlman, S. (1999) Biochem Biophys Res Commun 256(3), 557-563
19. Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B., and Ball, D. W. (1997) Proc Natl Acad Sci U S A 94(10), 5355-5360.
20. Kaneta, M., Osawa, M., Sudo, K., Nakauchi, H., Farr, A. G., and Takahama, Y. (2000) J Immunol 164(1), 256-264.
21. Squire, J. A., Thorner, P. S., Weitzman, S., Maggi, J. D., Dirks, P., Doyle, J., Hale, M., and Godbout, R. (1995) Oncogene 10(7), 1417-1422.
22. Påhlman, S., Odelstad, L., Larsson, E., Grotte, G., and Nilsson, K. (1981) Int J Cancer 28(5), 583-589
23. Bjelfman, C., Meyerson, G., Cartwright, C. A., Mellstrom, K., Hammerling, U., and Pålman, S. (1990) *Mol Cell Biol* **10**(1), 361-370.
24. Dang, C. V., Barrett, J., Villa-Garcia, M., Resar, L. M., Kato, G. J., and Fearon, E. R. (1991) *Mol Cell Biol* **11**(2), 954-962.
25. Langlands, K., Yin, X., Anand, G., and Prochownik, E. V. (1997) *J Biol Chem* **272**(32), 19785-19793.
26. Finkel, T., Duc, J., Fearon, E. R., Dang, C. V., and Tomaselli, G. F. (1993) *J Biol Chem* **268**(1), 5-8.
27. Norton, J. D., Deed, R. W., Craggs, G., and Sablitzky, F. (1998) *Trends Cell Biol* **8**(2), 58-65.
28. Pålman, S., Ruusala, A. I., Abrahamsson, L., Odelstad, L., and Nilsson, K. (1983) *Cell Differ* **12**(3), 165-170.
29. Lasorella, A., Iavarone, A., and Israel, M. A. (1996) *Mol Cell Biol* **16**(6), 2570-2578.
30. Lasorella, A., Noseda, M., Beyna, M., and Iavarone, A. (2000) *Nature* **407**(6804), 592-598.
31. Brodeur, G. M., Maris, J. M., Yamashiro, D. J., Hogarty, M. D., and White, P. S. (1997) *J Pediatr Hematol Oncol* **19**(2), 93-101.
32. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) *Science* **284**(5415), 770-776.
33. Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., and Kageyama, R. (1994) *J Biol Chem* **269**(7), 5150-5156.
34. Baonza, A., de Celis, J. F., and Garcia-Bellido, A. (2000) *Development* **127**(11), 2383-2393.
35. Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995) *Genes Dev* **9**(24), 3136-3148.
36. Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S., and Kageyama, R. (1994) *Embo J* **13**(8), 1799-17805.
37. Castella, P., Wagner, J. A., and Caudy, M. (1999) *J Neurosci Res* **56**(3), 229-240.
38. Martinsen, B. J., and Bronner-Fraser, M. (1998) *Science* **281**(5379), 988-991.
39. Nakamura, Y., Sakakibara, S., Miyata, T., Ogawa, M., Shimazaki, T., Weiss, S., Kageyama, R., and Okano, H. (2000) *J Neurosci* **20**(1), 283-293.
40. Jensen, J., Pedersen, E. E., Galante, P., Hald, J., Heller, R. S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O. D. (2000) *Nat Genet* **24**(1), 36-44.
41. Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B., and Ball, D. W. (1997) *Proc Natl Acad Sci U S A* **94**(10), 5355-5360.
Figure 1.

Western blot analysis of neuroblastoma and related cell lines, showing the expression patterns of HASH-1, HES-1, Id1, Id2, and Id3. Cell extracts (each containing 40 µg of protein) from the neuroblastoma cell lines SH-SY5Y, IMR-32, LA-N-5, LA-N-2, LA-N-1, and SK-N-BE(2), as well as the rat pheochromocytoma cell line PC12 and the human neuroepithelioma cell line SK-N-MC, were subjected to 12.5% SDS-PAGE. The proteins were transferred to a PVDF filter and were detected using antibodies directed towards HASH-1, HES-1, Id1, Id2, and Id3.

Figure 2.

Expression patterns of HASH-1, HES-1, Id1, Id2, and Id3 in differentiating SH-SY5Y cells. Cell extracts (40 µg) from SH-SY5Y cells treated with 16 nM TPA for 2 to 96 h was subjected to Western blotting. The samples were fractionated by SDS-PAGE in a 12.5% gel and then transferred to a PVDF filter. The proteins were detected using antibodies directed towards HASH-1, HES-1, Id1, Id2, and Id3.

Figure 3.

Western blot analysis of HASH-1, HES-1, Id1, Id2, and Id3 expressed in RA-treated SK-N-BE(2) cells. The cells were treated with 10 µM RA for 2 to 96 h. Cell extracts containing 40 µg of protein were subjected to 12.5% SDS-PAGE and then transferred to a PVDF filter. Proteins were detected using antibodies directed towards HASH-1, HES-1, Id1, Id2, and Id3.
Figure 4.

Mammalian two-hybrid analysis of the ability of Id proteins to dimerize with bHLH transcription factors in vivo. (A) Schematic representation of the GAL4 or VP16 fusion proteins tested in the mammalian two-hybrid system. The proteins were cloned in frame with the GAL4 domain in the pBIND vector or the VP16 domain in the pACT vector. The amino acids of the proteins included in the individual constructs are indicated. The shaded boxes represent the GAL4 or VP16 domains, and the striped boxes indicate the approximate locations of the HLH domains. All constructs were sequenced and found to express proteins of the expected molecular weights. (B) A mammalian two-hybrid system was employed to study the ability of Id1 to form heterodimers with a collection of bHLH transcription factors expressed in human neuroblastoma cells, using MyoD as a positive control. Plasmids encoding the DNA-binding GAL4 domain fused to Id proteins were cotransfected into CHO cells, together with plasmids encoding the VP16 transactivating domain fused to bHLH factors. The degree of interaction is given as the “relative luciferase activity”, which was calculated as the ratio of firefly luciferase activity to renilla luciferase activity. The interaction patterns obtained in experiments with Id2, Id3, and Id4 (data not shown) were identical to the pattern for Id1. The Western blot panel shows the expression levels of the VP16 fusion proteins. The mammalian two-hybrid system was used to analyze VP16 fusion proteins of either HES-1 (C) or dHAND (D) regarding their ability to form complexes with a panel of GAL4-fused HLH proteins. The shaded columns represent the relative luciferase activity recorded when pACT-HES-1 or pACT-dHAND was cotransfected with different pBIND-HLH plasmids. The unshaded columns represent the background relative luciferase activity detected.
when the various pBIND-HLH vectors were cotransfected with empty pACT vector. Expression of GAL4 fusion protein was analyzed by Western blotting (C).

**Figure 5.**

*Analysis of the ability of Id2 and HES-1 to interfere with formation of bHLH dimers in vivo.*

The expression vectors of the mammalian two-hybrid system were used to assess the capacity of HES-1 and Id2 to interfere with the formation of transcriptionally active bHLH-dimers in a dominant-negative manner. (A) Effect of HES-1 on the interaction between HASH-1 and E2-2. CHO cells grown in 35-mm culture dishes were cotransfected with pBIND-HASH-1 and pACT-E2-2 (0.2 µg each) and increasing amounts (0.2–0.8 µg) of pMyc-HES-1. The total amount of DNA was balanced with empty pMyc-vector. (B) Effect of Id2 on the interaction between HASH-1 and E2-2. The cells were cotransfected with HASH-1 and E2-2 as described above, as well as increasing amounts (0.2–0.8 µg) of pEGFP-Id2. (C) Effect of HES-1 on the interaction between Id2 and E2-2 interaction. The CHO cells were cotransfected with constant amounts of pBIND-Id2 and pACT-E2-2, and increasing levels of pMyc-HES-1, as in A. The interaction between the GAL4 and the VP16 fusion proteins is recorded as the relative luciferase activity, representing the ratio of firefly luciferase activity to *renilla* luciferase activity. The value representing the activity of the GAL4 and VP16 fusion proteins alone was considered to be 100%. Western blot analysis using anti-HES-1 (A) or anti-GFP (B) antiserum was performed to confirm that the protein levels did increase with increasing amounts of expression vector.

**Figure 6.**
Coimmunoprecipitation assays confirming interactions between the Id proteins and HES-1. (A) CHO cells were transfected with the indicated plasmids, using pEGFP-HASH-1, pBIND-HASH-1, and pBIND as negative controls. Cell extracts were immunoprecipitated with a monoclonal anti-GAL4 DNA-binding domain antibody, and the immunoprecipitated protein complexes were subjected to 10% SDS-PAGE and blotted to a PVDF filter. Crude cell extract from CHO cells transfected with pEGFP-HES-1 and pBIND-Id2 was run in a parallel lane. Coimmunoprecipitated EGFP-HES-1 fusion protein was detected by Western blotting with a polyclonal anti-GFP antiserum. (B) Coimmunoprecipitation of endogenous HES-1 and Id1 proteins from extracts of PC12 pheochromocytoma cells. The proteins were immunoprecipitated with a polyclonal anti-Id1 antiserum or with normal rabbit immunoglobulin (Ig) as a negative control. The immunoprecipitate was subjected to 12.5% SDS-PAGE, and proteins were transferred to a PVDF filter. Crude cell extract from PC12 cells was run in a parallel lane. Immunodetection was done with polyclonal anti-HES-1 or anti-Id1 antiserum, as indicated.

Figure 7.
Electrophoretic mobility shift assay of N-box binding activity in cell extracts from HES-1-transfected CHO cells.
(A) A \(^{32}\)P-labeled oligonucleotide containing a HASH-1 promoter sequence, including the N-box CACGCA, was incubated with extract from HES-1-transfected CHO cells (lanes 2, 3, 5, and 6). In lane 3, a polyclonal anti-HES-1 antiserum was added to the reaction mixture. Lane 4 shows untransfected CHO cell extract; the complex that appeared in this lane was of unknown origin. As competitors, an excess of the unlabeled wt oligonucleotide probe was added (lane 5), or an unlabeled mutant
version of the oligonucleotide (lane 6). (B) Cell extract from HES-1-transfected CHO cells was mixed with a $^{32}$P-labeled N-box oligonucleotide. Before adding the labeled probe, aliquots of the extract were pre-incubated with rabbit reticulocyte lysate (lane 2) or an equal amount of Id2 translated in vitro (lane 3). (C) Increasing amounts (3, 5, 7, and 9 µg, lanes 3-6) of cell extract from Id2 transfected CHO cells were mixed with a constant amount of cell extract from HES-1-transfected CHO cells, after which the labeled N-box oligonucleotide was added. The same amounts of cell extract from untransfected CHO cells (i.e., 3, 5, 7 and 9 µg, lanes 7-10) were used as a negative control.
Fig. 1

PC12  SH-SY5Y  IMR-32  LA-N-5  LA-N-2  LA-N-1  SK-N-MC  SK-N-BE(2)

HASH-1

HES-1

Id1

Id2

Id3
Fig. 2

[Image: Western blot analysis of HASH-1, HES-1, Id1, Id2, and Id3 in SH-SY5Y cells treated with 16 nM TPA at different time points (0 h, 2 h, 8 h, 24 h, 96 h).]
Fig. 3

| 10 µM RA | 0 h | 2 h | 8 h | 24 h | 96 h |
|----------|-----|-----|-----|------|------|
| HASH-1   |     |     |     |      |      |
| HES-1    |     |     |     |      |      |
| Id1      |     |     |     |      |      |
| Id2      |     |     |     |      |      |
| Id3      |     |     |     |      |      |
Fig. 4

A

GAL4

GAL4

GAL4

GAL4

GAL4/VP16

GAL4/VP16

GAL4/VP16

GAL4/VP16

VP16

VP16

Id1 (1-154)
Id2 (1-134)
Id3 (1-119)
Id4 (1-162)
E2-2 (1-668)
E47 (508-654)
HASH-1 (1-180)
dHAND (1-217)
MyoD (1-318)
HES-1 (90-281)
Fig. 4

B

GAL4-Id1

relative luciferase activity

VP16 fusion proteins:
empty, HES-1, HASH-1, dHAND, E47, E2-2, MyoD

Western: VP16
Fig. 4

C

Western: GAL4

D

Relative luciferase activity

E2-2  E47  HASH-1  id1  id2  id3  id4

VP16  VP16-dHAND

Relative luciferase activity

E2-2  E47  HASH-1  id1  id2  id3  id4

VP16  VP16-HES-1
Fig. 5

A

% relative luciferase activity

pMyc-HES-1(µg) 0 0.2 0.4 0.8

Western: HES-1

B

% relative luciferase activity

pEGFP-Id2(µg) 0 0.2 0.4 0.8

Western: EGFP-Id2

C

% relative luciferase activity

pMyc-HES-1(µg) 0 0.2 0.4 0.8

Western: HES-1
|                | Crude lysate | IP: α- GAL4 |
|----------------|-------------|-------------|
| pEGFP-HES-1    | +           | +           |
| pBIND-Id2      | +           | -           |
| pBIND-HASH-1   | -           | +           |
| pBIND          | -           | -           |
| pEGFP-HASH-1   | -           | +           |
| pBIND-E47      | -           | -           |
| pBIND-E2-2     | -           | -           |

**Fig. 6**

A

EGFP-HES-1
Fig. 6

B

Crude lysate
IP: α-Id1
IP: Rabbit Ig

HES-1

Id1
Fig. 7

A

| Antibody Competition | probe only | HES-1 | HES-1 | control | HES-1 | HES-1 |
|----------------------|------------|-------|-------|---------|-------|-------|
|                      | -          | -     | α-HES-1 | -       | -     | wt    |
|                      |            |       |        |         |       | mut   |

1 2 3 4 5 6
Fig. 7

B
Fig. 7

C
Modulation of basic helix-loop-helix transcription complex formation by Id proteins during neuronal differentiation
Annika Jögi, Paula Persson, Anna Grynfeld, Sven Pålman and Håkan Axelson

J. Biol. Chem. published online December 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107713200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts