FEBS Letters 580 (2006) 1812–1816

Identification of the nuclear export signal in the helix–loop–helix inhibitor Id1

Jun Makita[a,b], Hisanori Kurooka[a], Kentaro Mori[a], Yoshio Akagi[b], Yoshifumi Yokota[a,*]

[a] *Department of Molecular Genetics, School of Medicine, University of Fukui, 23-3, Shimoaizuki, Matsuoka, Fukui 910-1193, Japan*
[b] *Department of Opthalmology, School of Medicine, University of Fukui, 23-3, Shimoaizuki, Matsuoka, Fukui 910-1193, Japan*

Received 19 December 2005; revised 31 January 2006; accepted 16 February 2006

Available online 24 February 2006

Edited by Veli-Pekka Lehto

Abstract Id proteins play important roles in cellular differentiation and proliferation by negatively regulating basic helix–loop–helix transcription factors. Although their intracellular localization may change depending on the biological situation, little is known about the molecular determinants underlying such changes. Here we report the identification of a nuclear export signal (NES) in Id1. The identified NES was different from that of Id2, but had the ability to confine heterologous green fluorescent protein to the cytoplasm. Thus, our results indicate that the intracellular localization of Id1 is regulated differently from that of Id2.

Keywords: Helix–loop–helix; Id1; Id2; Nuclear export signal; Nuclear localization signal

1. Introduction

Id proteins are negative regulators of basic helix–loop–helix transcription factors such as MyoD that participate in the regulation of cell differentiation, proliferation and cellular function [1,2]. Four members of the Id gene family, Id1–Id4, have been identified in mammals and the protein products share a similar overall structure consisting of a highly conserved helix–loop–helix (HLH) domain and less conserved N- and C-terminal regions [1]. Studies using gene-deficient mice have demonstrated that Id proteins are essential for various processes including angiogenesis, tumorigenesis, neurogenesis, and immune cell development [2,3].

The functions of Ids are strictly regulated at the levels of both transcription [4,5] and protein stability [6,7]. In addition, accumulating evidence has suggested that their subcellular localization also seems to be important in exerting their functions [8–11]. For example, Wang et al. [8] demonstrated that the translocation of Id2 from the nucleus to the cytoplasm is observed at the onset of the differentiation of oligodendrocytes from their precursors obtained from the optic nerve. However, the molecular mechanisms underlying the subcellular localization of Id proteins remain to be elucidated.

*Corresponding author. Fax: +81 776 61 8164.
E-mail address: yyokota@fmsrsa.fukui-med.ac.jp (Y. Yokota).

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; GFP, green fluorescent protein; HLH, helix–loop–helix; NES, nuclear export signal; NLS, nuclear localization signal

Since small proteins with molecular weights of less than 40 kDa can freely pass through the nuclear pore [12,13], the molecular weights of Id proteins, ranging from 13 to 18 kDa, suggest that passive diffusion is involved in determining the subcellular distribution of Id proteins. However, it is also possible that their subcellular localization is under the control of intrinsic nuclear localization signals (NLSs) and nuclear export signals (NESs) that are recognized by specific import and export receptors, respectively [13,14]. In fact, we recently reported that Id2 shuttles between the nucleus and cytoplasm via its own NLS and NES [15]. We also showed that Id1 possesses a similar NES-like sequence in the C-terminal region but it is non-functional [15]. This observation raised the interesting possibility that the subcellular distributions of these structurally related proteins are governed by distinct molecular determinants.

We report here the identification of a functional NES in Id1. Our findings establish that distinct molecular determinants govern the intracellular localization of Id1 and Id2. Furthermore, they suggest that Id1 and Id2 play different roles in the regulation of cell differentiation and proliferation under the influence of the cellular environment.

2. Materials and methods

2.1. Plasmids

The N-terminally GFP × 2-tagged Id1 was generated by inserting the full-length mouse Id1 cDNA [16] into the *Hin*dIII–*Bam*HI sites of the tandem-duplicated GFP (GFP × 2) plasmid [15]. For construction of a series of deletion and point mutants of green fluorescent protein (GFP)-tagged Id1, fragments were amplified by PCR with appropriate primer sets using Id1 cDNA as a template and inserted into the *Hin*dIII–*Bam*HI sites of the tandem-duplicated GFP (GFP × 2) plasmid, as described previously [15]. For the NES-WT (wild type) and -Mut (mutant) constructs, annealed oligonucleotides were inserted into the *Hin*dIII–*Bam*HI sites of the tandem-duplicated GFP (GFP × 2) plasmid. All of the constructs were verified by DNA sequencing.

2.2. Cell culture and DNA transfection

Mouse NIH3T3 fibroblasts were transfected with a GFP × 2 construct using the FuGENE 6 reagent (Roche Molecular Biochemicals) as described previously [15].

2.3. Microscopic analysis

Twenty-four hours after transfection in 4-well Lab-Tek chamber slides (Nalge Nunc International), the cells were fixed with 3% formaldehyde in PBS for 15 min at room temperature and subsequently mounted with Vecta Shield reagent containing DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (Molecular Probe) to stain the nuclei. Microscopic analysis was conducted as described previously [15].

0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.038

2.4. Western blotting

Twenty micrograms of the total cell extract prepared as described [15] were separated by 9–12% SDS–PAGE and the resolved proteins were transferred onto a nitrocellulose filter (Amersham Pharmacia Biotech). The filter was blotted with anti-GFP monoclonal antibody (GF200; Nacalai) and immuno-reactive bands were visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech) with a LAS-3000 image analyzer (FUJIFILM).

3. Results

3.1. The region spanning the HLH and C-terminal domains of Id1 is required for cytoplasmic localization

To study the molecular basis of the subcellular localization of Id1, we used Id1 and Id1 mutants that were N-terminally tagged with GFP. The GFP tag was tandemly duplicated (GFP × 2) to produce an appropriate molecular mass that would reduce or prevent the passive diffusion of the proteins to be analyzed. The subcellular localization of the proteins was analyzed in NIH3T3 cells after transient transfection. While GFP × 2 was uniformly distributed throughout the cells, GFP × 2-Id1(WT) was found to be localized in the cytoplasm (see Fig. 1A), as was the case for Id1 with a single GFP tag [15], suggesting the presence of an NES in the Id1 protein. To locate the region required for the cytoplasmic localization of Id1, we constructed a series of deletion mutants of Id1 as shown in Fig. 1A. Among them, the cytoplasmic localization was retained in the GFP × 2-Id1(59–148), GFP × 2-Id1(1–115) and GFP × 2-Id1(1–132) mutant constructs, showing that amino acid residues 1–59 and 115–148 are dispensable for the

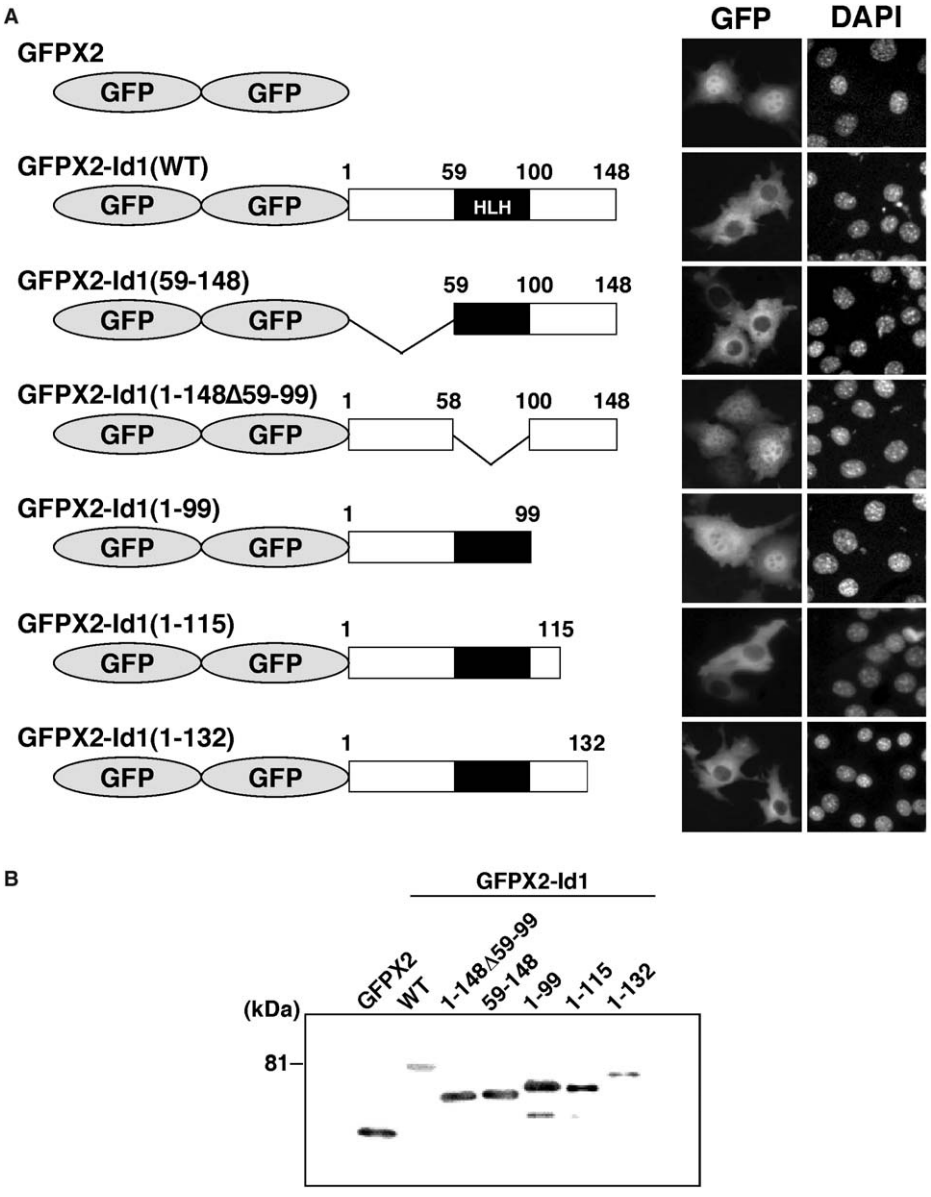

Fig. 1. Subcellular localization of GFP-tagged Id1 and its deletion mutants. (A) A schematic representation of the GFP × 2-Id1 fusion proteins is shown on the left. The helix–loop–helix (HLH) domain is indicated as a black box. The numbers above a box indicate the positions of the amino acid residues of Id1 protein. Representative images of NIH3T3 cells transiently transfected with the fusion constructs are shown in the right panels. Panels labeled by GFP and DAPI demonstrate the localization of the fusion proteins and the nucleus, respectively. (B) Protein expression of the fusion constructs. Protein expression in NIH3T3 cells transiently transfected with each construct was analyzed by Western blotting with anti-GFP antibody.

cytoplasmic localization of Id1. On the other hand, GFP × 2-Id1(1–148Δ59–99) and GFP × 2-Id1(1–99) were distributed throughout the cells, demonstrating that the NES of Id1 is present within the region encompassed by amino acid residues 59–115. As shown in Fig. 1B, Western blotting using an anti-GFP antibody demonstrated that the fusion proteins were produced with the appropriate molecular masses that were expected from the respective constructs.

3.2. Identification of NES of Id1 in the junction of the HLH region and the C-terminal domain

We previously reported that two potential NES-like motifs are present in Id1: one at the junction of the HLH region and the C-terminal domain, and the other in the C-terminal domain [15]. Since mutational analysis indicated that the latter is not active [15], we investigated the activity of the former NES-like sequence present within the region spanning positions 91–102 (Fig. 2A). This NES-like sequence, consisting of six hydrophobic amino acid residues at positions 91, 92, 95, 98, 100 and 102, is well conserved among the four members of the Id protein family, as shown in Fig. 2B. To examine the NES activity of the region, we first constructed six mutants of GFP × 2-Id1 by substituting one of these six hydrophobic amino acid residues with alanine in each mutant. However, alanine substitution at position 95 or 98 resulted in the degradation of the mutant protein for some unknown reason, which hampered the evaluation of the NES activity of the region (data not shown). We then constructed 6 point-mutants of GFP × 2-Id1: V91Q, I92Q, I95Q, L98Q, L100Q and L102Q, by substituting one of these six conserved hydrophobic residues with glutamine in each mutant, and analyzed the effect on the subcellular localization of the GFP × 2-Id1 fusion protein (see Fig. 2C). While GFP × 2-Id1 mutants V91Q, I92Q and L102Q were localized in the cytoplasm like GFP × 2-Id1(WT), the other mutants I95Q, L98Q and L100Q were found in both the cytoplasm and nucleus (see Fig. 2C). Western blot analysis using an anti-GFP antibody demonstrated that all of these substitution mutants produced proteins of the expected molecular mass (see Fig. 2D). These findings suggested that the hydrophobic amino acid residues present at the

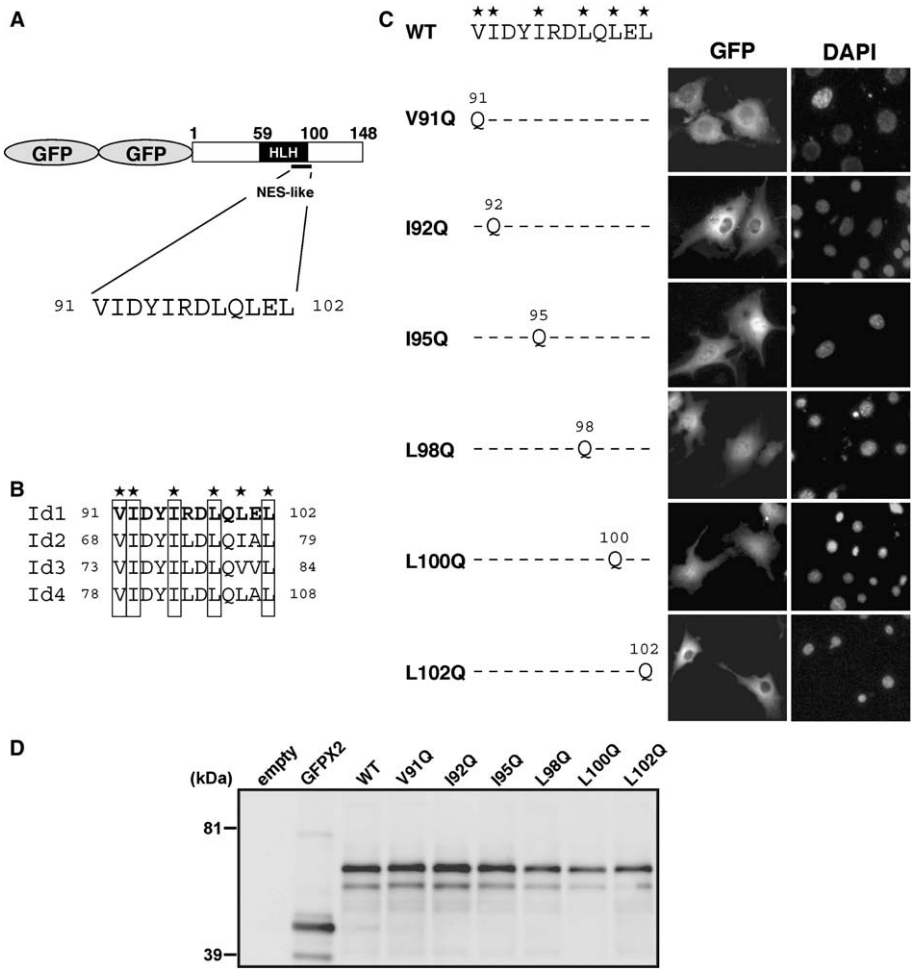

Fig. 2. Effects of point mutations in the NES-like sequence on subcellular localization of Id1. (A) The position of the NES-like sequence of Id1 is shown schematically in the GFP × 2-Id1 fusion protein. The helix–loop–helix (HLH) domain is indicated as a black box. The numbers above a box indicate the positions of the amino acid residues of Id1 protein. (B) Asterisks indicate conserved hydrophobic amino acid residues in the NES-like sequences in the Id protein family. Positions of amino acid residues are indicated. (C) Mutants containing the residues replaced by glutamine are shown at the bottom. Dashes represent unchanged amino acids. Representative images of NIH 3T3 cells transiently transfected with the mutant constructs are shown in the right panels. Panels labeled GFP and DAPI demonstrate the localization of the fusion proteins and the nucleus, respectively. (D) Protein expression of the fusion constructs.

positions from 95 to 100 are important for the cytoplasmic localization of Id1.

3.3. A stretch of 14 amino acid residues is sufficient for active NES

To examine the activity of the NES-like sequence spanning positions 91–104, we generated the GFP × 2-Id1(91–104)WT construct and evaluated the subcellular localization of the fusion protein (see Fig. 3). In contrast to the distribution of the tandem-duplicated GFP protein shown in Fig. 1, the addition of the NES-like sequence of Id1 restricted the subcellular localization of GFP × 2 to the cytoplasm, demonstrating that the identified NES-like sequence of Id1 is active (see Fig. 3A). We next constructed GFP × 2-Id1(91–104)Mut in which glutamine substitution was introduced into three hydrophobic amino acids at positions 95, 98 and 100 (see Fig. 3). As a result of the glutamine substitution, the cytoplasmic localization of the fusion protein was changed to distribution throughout the cells (see Fig. 3). Similar results were obtained by substituting the same hydrophobic amino acid residues with alanine (data not shown). Western blot analysis with an anti-GFP antibody showed the expected size of GFP × 2-Id1(91–104)WT and GFP × 2-Id1(91–104)Mut proteins (see Fig. 3B). These results demonstrated that Id1 has an active NES-like sequence at the junction between the HLH and C-terminal regions.

4. Discussion

Our previous mutational analysis identified a leucine-rich segment in the C-terminal region of Id2, spanning positions 106–115, as an active NES, and also showed that the leucine-rich sequence at the junction of the HLH and the C-terminal domains of Id2 was non-functional with respect to nuclear export [15]. Whereas similar NES-like sequences were found in the corresponding regions of Id1, the one present in the C-terminal region did not contribute to the cytoplasmic localization of Id1, probably due to the presence of alanine in stead of the leucine residue at the most important fourth position of NES [15]. These results raised the possibility that the molecular determinants of the subcellular localization are different for the different members of the Id protein family, even though similar intracellular distribution is observed. This notion is now supported by the findings presented in this report.

A leucine-rich signal that mediates nuclear export of proteins was first identified in the human immunodeficiency virus-encoded Rev protein and the protein kinase A inhibitor [17]. The sequence of L-X(2 or 3)-[LIVFM]-X(2 or 3)-L-X-[LI] has generally been accepted as the consensus NES [18,19], although the comparison of the identified NESs has revealed a variety of deviations from the consensus [19]. We identified the NES sequence of Id1 in the region spanning positions 91–100, VIDYIRDLQL, which agrees with the proposed consensus sequence, except for the first position. Furthermore, it has been noted that an NES sequence has a tendency to contain acidic amino acid residues, glutamate and aspartate, in the spaces between conserved hydrophobic amino acid residues [19] and this feature is also observed in the NES of Id1. The identified NES of Id1 thus shares the structural characteristics of NESs reported so far.

We previously demonstrated that Id1 and Id2 were localized in the cytoplasm, while Id3 and Id4 showed nucleo-cytoplasmic distribution [15]. In addition, the present study shows that

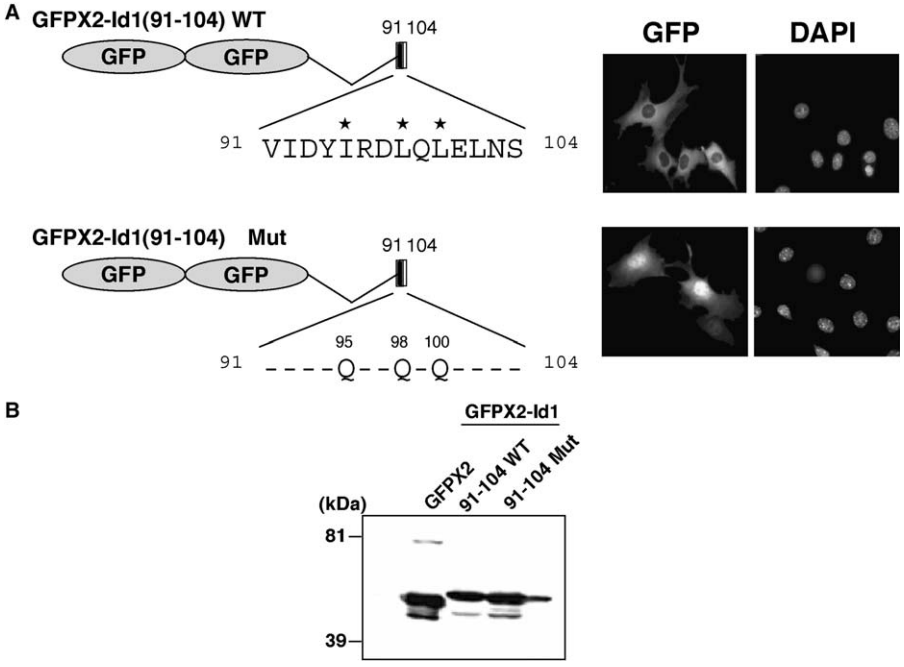

Fig. 3. Activity of NES-like sequence of Id1. (A) Structures of GFP × 2 proteins tagged with the NES-like sequence of Id1 are shown on the left. Positions of amino acid residues indicate the regions of Id1 used for tagging. Asterisks in GFP × 2-Id1(91–104)WT indicate the amino acid residues substituted with glutamine in GFP × 2-Id1(91–104)Mut. Dashes in GFP × 2-Id1(91–104)Mut represent unchanged amino acids. The right panels show representative images of NIH 3T3 cells transiently transfected with GFP × 2-Id1(91–104)WT or GFP × 2-Id1(91–104)Mut. Panels labeled GFP and DAPI demonstrate the localization of the tagged proteins and the nucleus, respectively. (B) Protein expression of the fusion constructs.

the structurally distinct NESs of Id1 and Id2 function to localize the respective proteins to the cytoplasm. Thus the molecular determinant of subcellular localization appears to be specific to each of the Id proteins. Although the phenotypic difference among the Id-deficient mice can be ascribed largely to the similar but distinct expression patterns of the Id gene family members [2,3], it has been demonstrated that the Id proteins display biological activity specific to the respective proteins in some cellular contexts. In human breast cancers, for example, Id1 expression is related to the aggressive and invasive phenotype of tumors, while Id2 is associated with the differentiation state of tumor cells and the expression level correlates with the non-invasive phenotype of tumors [20,21]. The present study reveals that the cytoplasmic localization of Id1 is dependent on the NES sequence, not due to the lack of NLS, and suggests that Id1 may change the subcellular localization in response to the cellular situation. It is tempting to speculate that the differential regulation of subcellular localization underlies the biological functions of the Id proteins.

The region where the NES of Id1 is located is highly conserved among the Id family members, implying that this region may function as an NES in all of the Id proteins. In fact, though mutations in the NES-like sequence of Id2 did not affect its cytosolic distribution [15], the tagging of GFP with a sequence almost equivalent to the NES region of Id2, MEILQHVIDYILDLQIAL, directs the distribution of the protein to the cytoplasm [Kurooka, unpublished observations]. Therefore, we cannot exclude the possibility that, depending on a cellular context, the NES-like sequence in the region play a role as a common regulator of the subcellular localization of Id proteins.

In conclusion, we identified the NES sequence of Id1 and demonstrated that the intracellular localization of Id1 and Id2 is regulated via different molecular elements. Further elucidation of molecular determinants of the intracellular localization of Id proteins would advance our understanding of how Id proteins contribute to the regulation of cellular differentiation and proliferation under the influence of the environment surrounding the cell.

Acknowledgements: This work was supported by Grants-in-Aid (#17390092 and #17790184) and the 21th Century COE program "Biomedical Imaging Technology Integration Program" from Japan Society of the Promotion of Science (JSPS), the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] Norton, J.D., Deed, R.W., Craggs, G. and Sablitzky, F. (1998) Id helix–loop–helix proteins in cell growth and differentiation. Trends Cell Biol. 8, 58–65.
[2] Ruzinova, M.B. and Benezra, R. (2003) Id proteins in development, cell cycle and cancer. Trends Cell Biol. 13, 410–418.
[3] Yokota, Y. (2001) Id and development. Oncogene 20, 8290–8298.
[4] Hara, E., Yamaguchi, T., Nojima, H., Ide, T., Campisi, J., Okayama, H. and Oda, K. (1994) Id-related genes encoding helix–loop–helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. J. Biol. Chem. 269, 2139–2145.
[5] Barone, M.V., Pepperkok, R., Peverali, F.A. and Philipson, L. (1994) Id proteins control growth induction in mammalian cells. Proc. Natl. Acad. Sci. USA 91, 4985–4988.
[6] Bounpheng, M.A., Dimas, J.J., Dodds, S.G. and Christy, B.A. (1999) Degradation of Id proteins by the ubiquitin-proteasome pathway. FASEB J. 13, 2257–2264.
[7] Fajerman, I., Schwartz, A.L. and Ciechanover, A. (2004) Degradation of the Id2 developmental regulator: targeting via N-terminal ubiquitination. Biochem. Biophys. Res. Commun. 314, 505–512.
[8] Wang, S., Sdrulla, A., Johnson, J.E., Yokota, Y. and Barres, B.A. (2001) A role for the helix–loop–helix protein Id2 in the control of oligodendrocyte development. Neuron 29, 603–614.
[9] Matsumura, M.E., Lobe, D.R. and McNamara, C.A. (2002) Contribution of the helix–loop–helix factor id2 to regulation of vascular smooth muscle cell proliferation. J. Biol. Chem. 277, 7293–7297.
[10] Tu, X., Baffa, R., Luke, S., Prisco, M. and Baserga, R. (2003) Intracellular redistribution of nuclear and nucleolar proteins during differentiation of 32D murine hemopoietic cells. Exp. Cell Res. 288, 119–130.
[11] Samanta, J. and Kessler, J.A. (2004) Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development 131, 4131–4142.
[12] Mattaj, I.W. and Englmeier, L. (1998) Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. 97, 265–306.
[13] Gorlich, D. and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. 15, 607–660.
[14] Suntharalingam, M. and Wente, S.R. (2003) Peering through the pore: nuclear pore complex structure, assembly, and function. Dev. Cell 4, 775–789.
[15] Kurooka, H. and Yokota, Y. (2005) Nucleo-cytoplasmic shuttling of Id2, a negative regulator of basic helix–loop–helix transcription factors. J. Biol. Chem. 280, 4313–4320.
[16] Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L. and Weintraub, H. (1990) The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. Cell 61, 49–59.
[17] Fischer, U., Huber, J., Boelens, W.C., Mattaj, I,W. and Luhrmann, R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell 82, 475–483.
[18] Bogerd, H.P., Fridell, R.A., Benson, R.E., Hua, J. and Cullen, B.R. (1996) Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization–selection assay. Mol. Cell. Biol. 16, 4207–4214.
[19] la Cour, T., Gupta, R., Rapacki, K., Skriver, K., Poulsen, F.M. and Brunak, S. (2003) NESbase version 1.0: a database of nuclear export signals. Nucleic Acids Res. 31, 393–396.
[20] Lin, C.Q., Singh, J., Murata, K., Itahana, Y., Parrinello, S., Liang, S.H., Gillett, C.E., Campisi, J. and Desprez, P.Y. (2000) A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res. 60, 1332–1340.
[21] Itahana, Y., Singh, J., Sumida, T., Coppe, J.P., Parrinello, S., Bennington, J.L. and Desprez, P.Y. (2003) Role of id-2 in the maintenance of a differentiated and noninvasive phenotype in breast cancer cells. Cancer Res. 63, 7098–7105.