

Inhibition of BETA2/NeuroD by Id2

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Abbreviations: bHLH, basic helix-loop-helix; BETA2, β -cell E box trans-activator 2; RIPE3, rat insulin promoter enhancer 3

Abstract

Id (Inhibitor of Differentiation) proteins belong to a family of transcriptional modulators that are characterized by a helix loop helix (HLH) region but lack the basic amino acid domain. Id proteins are known to interact with basic helix-loop-helix (bHLH) transcription factors and function as their negative regulators. The negative role of Id proteins has been well demonstrated in muscle development and some in neuronal cells. In this study, we investigated the effect of Id on the function of BETA2/NeuroD, a bHLH transcription factor responsible for neuron and endocrine cell specific gene expression. cDNAs of several Id isoforms were isolated by yeast two-hybrid system using the bHLH domain of E47, a ubiquitous bHLH partner as a bait. Id proteins expressed in COS M6 cells, were found in both cytosolic and nuclear fractions. Electrophoretic mobility shift assay showed that coexpression of Id2 proteins inhibited BETA2/NeuroD binding to its target sequence, E-box. Id2 inhibited E-box mediated gene expression in a dose dependent manner in BETA2/NeuroD expressing HIT cells. Id coexpressed with BETA2/NeuroD in HeLa cells, inhibited the stimulatory activity of BETA2/NeuroD. These results suggest that Id proteins may negatively regulate tissue specific gene expression induced by BETA2/NeuroD in neuroendocrine cells and the inhibitory role of Id proteins during differentiation may be conserved in various tissues.

Keywords: bHLH, Id, BETA2/NeuroD, neuronal differentiation

Introduction

Basic helix-loop helix (bHLH) transcription factors play a key role in the induction of cell type-specific gene expression (for review, see Weintraub, 1993), especially in the process of myogenesis (Weintraub *et al.*, 1991; Jan and Jan, 1993). A muscle specific class B bHLH protein, such as myoD, myogenin, Myf5, and MRF4 (Olsen, 1990; Wright, 1992; Weintraub, 1993), interacts with a ubiquitous bHLH protein of the class A, E proteins (Lassar *et al.*, 1991). Then the heterodimer binds to the E box (CANNTG) of target genes (Murre *et al.*, 1989) and commits cells to undergo differentiation processes and adopt muscle specific phenotypes.

Id proteins possess helix-loop-helix (HLH) domains but lack the basic domain (Norton *et al.*, 1998). Four isoforms of Id family (Id1-Id4) interact with the Class A bHLH proteins (Jen *et al.*, 1992) and more weakly with some Class B bHLH proteins (Langlands *et al.*, 1997). Due to the lack of the basic DNA-binding domain in Id proteins, heterodimers containing Id proteins cannot bind to the E-box sequences (Benezra *et al.*, 1990). Therefore Id proteins act as dominant-negative regulators of bHLH proteins. During muscle development, Id proteins interact with muscle specific bHLHs including MyoD and myogenin, and block muscle differentiation (Benezra *et al.*, 1990; Jen *et al.*, 1992; Neuhold and Wold, 1993; Melnikova *et al.*, 1999). Id proteins are also known as positive regulators of cell proliferation. Id2 binds to the pocket of Rb tumor suppressor protein and inactivate the anti-proliferative function of Rb (Iavarone *et al.*, 1994; Lasorella *et al.*, 2000). Thus, Id2 may have dual roles; one as a cell cycle promoting factor and the other as a negative regulator of differentiation.

Like muscle development, bHLH transcription factors play key roles in sex determination (Caudy *et al.*, 1988), hematopoiesis (Mellentin *et al.*, 1989) and neurogenesis (Guillemot *et al.*, 1993) as well as development of endocrine cells. Interestingly, a common bHLH factor, BETA2/NeuroD, regulates both differentiation of neurons and endocrine cells. NeuroD was first cloned as a neurogenic bHLH factor which induces terminal differentiation in postmitotic neurons (Lee *et al.*, 1995). The same protein was isolated as a pancreatic β -cell E box trans-activator 2 (BETA2) which regulates β -cell specific expression of the insulin gene (Naya *et al.*, 1995). Targeted mutations of BETA2/NeuroD cause reduced number of pancreatic β -cells as well as defects in development of the central nervous system especially in hippocampus

and cerebellum (Naya *et al.*, 1996; Miyata *et al.*, 1999).

To understand the diverse functions of BETA2/NeuroD in neurons and pancreatic β -cells, we attempted to investigate the interaction of Id proteins and BETA2/NeuroD. First, we isolated three isoforms of Id proteins and examined the interaction of Id2 with BETA2/NeuroD. The results suggest that Id proteins may interfere with the function of BETA2/NeuroD and negatively regulate neuron- and β -cell-specific gene expression.

Materials and Methods

Materials

A yeast strain L40 and pHybTrp/Zeo yeast expression vector were kind gifts from Drs. S. Chae and E. Kim (Bae-Jae Univ., Daejeon, Korea), a mouse embryo 7.5 dpc cDNA library and herring testis DNA were purchased from Clontech (Palo Alto, CA), Luciferase assay system from Promega (Madison, WI), [α - 32 P] dATP from Amersham Pharmacia Biotech (Uppsala, Sweden), TOPO TA cloning kit from Invitrogen (Groningen, CH, Netherlands), yeast selective medium from Bio101 (Vista, CA), Super Signal West Pico Chemiluminescent Substrate from Pierce (Rockford, IL), anti-flag antibody from Sigma-Aldrich (St. Louis, MO), anti-BETA2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and Immobilon-P membrane from Millipore (Bedford, MA). All cell culture media and related reagents from Life Technologies (Rockville, MD). All other chemicals from Sigma-Aldrich (St. Louis, MO).

Plasmids

The bHLH domain between 1297 and 2133 bp of E47 (Genbank accession number M30313) was amplified by PCR from pSVE2-5 using oligonucleotides 5'-AGA ATT CGA GAA GGA GGA CGA GGA GGA CAC G-3' (forward) and 5'-CGA ATT CAA AAG ACC AGA AAA GGA GAC C-3' (reverse) and cloned into the *EcoRI* site of pHybTrp/Zeo to obtain a bait vector, pHybTrp/Zeo-E47. To construct yeast expression vector pACT2-BETA2 as a positive control of yeast two hybrid screening, *NcoI* fragment (1-790 bp; Genbank accession number U24679) was isolated from pGEX-BETA2 and inserted in pACT2, a yeast prey vector. Id2 was amplified by PCR (forward primer: 5'-CAG CAT GAA AGC CTT CAG-3'; reverse primer: 5'-TCA CTT GTC ATC GTC GTC CTT GTA GTC GCC ACA GAG TAC-3') from pACT2-Id2 library vector and then subcloned into pCR-TOPO vector. The reverse PCR primer contained the sequence for a flag epitope. The Id2Flag cDNA was isolated from pCR-TOPO and

inserted into a mammalian expression plasmid pcDNA3 (pcDNA3-Id2Flag). The pRIPE3(3+)-luc reporter construct was previously described (Kim *et al.*, 2002). Expression vectors for the full length BETA2 with an HA tag, pCHA-BETA2, and for a truncated mutant for BETA2, pCHA-BETA2(1-233), were previously described (Cho *et al.*, 2001).

Yeast two-hybrid screening

pHybTrp/Zeo-E47 was used as a bait to screen a mouse 7.5 cDNA library. The plasmid pHybTrp/Zeo-E47 and the mouse 7.5 cDNA library were concomitantly introduced into a yeast strain L40 following a standard yeast transformation protocol. The transformed yeasts were plated on selective medium (SD-Leu/Trp, SD-Leu/Trp+X-gal, SD-His/Leu/Trp, and SD-His/Leu/Trp+3-AT) and allowed to grow for 4-6 days at 30°C. The positive colonies grown on SD-His/Leu/Trp+3-AT plates were transferred to SD-Leu/Trp+X-gal plates. Plasmids were isolated from X-gal positive cells. To select the clones containing the Id cDNAs, PCR was carried out using Id1, 2, and 3 specific primers (Figure 1A). The pACT2-Id2 vector was amplified in bacterial DH5 α cells. Plasmids detected with the Id2 specific primer were subjected to automatic sequence analysis using the BLAST search program. To further confirm positive interactions between Id2 and E47, the pACT2-Id2 was reintroduced back into yeast together with the bait plasmid and then the growth was verified on selective plates.

Western analysis

COS M6, HIT, and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 μ g/ml streptomycin. COS M6 cells (2×10^6 cells/100 mm dish) were transfected with pcDNA3-Id2Flag using DEAE-dextran as a carrier as previously described (Lopata *et al.*, 1984; Wei *et al.*, 1995). Thirty-six hours after transfection, cells were harvested. Cytosolic and nuclear fractions were prepared from transfected COS M6 cells as previously described by Attardi and Tjian (1993) and subject to Western analysis.

Reporter gene assay

HIT and HeLa cells were transfected with pRIPE3(3+)-luc and expression vectors for BETA2 and E47 by calcium phosphate methods as previously described (Graham and Eb, 1973). For each transfection, pCMV- β gal (0.1 μ g/dish) was included as an internal control and the total amount of DNA was kept constant by adding pcDNA3. Forty eight hours after transfection, the cells were harvested and 10-20 μ g

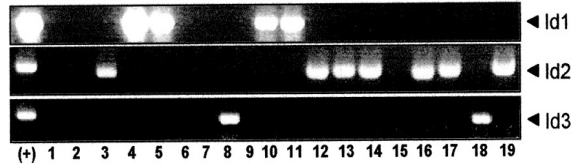
A

Id specific primers

Id1 CCT GCC CGC CTT CGT GGA C
 Id2 CAT CCC ACT CTC GTC AGC CTG C
 Id3 GGG AGT CCC TCT AGG CAC T

Reverse primer

TGA GAT GGT GCA CGA TG



B

Baits	Preys	Growth on selective Media			β-gal	The Number of Clones
		-LT	-HLT	-HLT+3AT		
pHybTrp/Zeo-E47	pACT2	-	-	-	-	NA
	pACT2-Id1	+++	++	+	+	47
	pACT2-Id2	+++	++	+	+	123
	pACT2-Id3	+++	++	+	+	41
	pACT2-BETA2	+++	++	+	+	NA

C

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ATGAAAGCCTTCAGGTC CCGTGAGTCCGTTAGGAAAAACAGCCTGTC GGACCACAGCTTG 60
M K A F R S G E S V R K N S L S D H S L

GGCATCTCCC GGAGCAAACCCCGGTGGACGACCCGATGAGTCTGCTCTACAACATGAAC 120
G I S R S K T P V D D P M S L L Y N M N

GACTGCTACTCCAAGCTCAAGGAAGTGGTCCAGCATCCCCAGAACAAAGGAGTGACC 180
D C Y S K L K E L V P S I P Q N K K V T
      Helix 1                               Loop
AAGATGGAAATCCTGACGACGTCATCGATTACATCTTGGACCTGACAGATCGCCCTGGAC 240
K M E I L Q H V I D Y I L D L Q I A L D
      Helix 2
TCGCATCCCCTATCGTCAGCCTGCATCACCAGAGACCTGGACAGAACCCAGGCGTCCAGG 300
S H P T I V S L H H Q R P G Q N Q A S R

ACGCGCCTGACCCACCTGAACAACCGACATCAGCATCCTGCTTGCAGGCATCTGAATTC 360
T R L T T L N T D I S I L S L Q A S E F

CCTTCTGAGCTTATGTC GAATGATAGCAAAGTACTCTGTGGCGACTACAAGGACGACGAT 420
P S E L M S N D S K V L C G D Y K D D D
                                     Flag
GACAAGTGA 429
D K .
    
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Figure 1. Id proteins are identified as interacting partners of a bHLH protein, E47. Yeast strain L40 was transformed with a bait DNA (pHybTrp-E47) and mouse 7.5 dpc cDNA library and screened by standard methods. (A) The positive clones containing Id cDNAs were identified by PCR using specific primers for isoforms (upper). The most representative PCR result was shown (lower). Most clones contained Id cDNAs except those in lanes of 1, 2, 6, 7, 9, and 15 lanes were visible by agarose gel. (B) Yeast clones containing both E47 and Id proteins were subjected to growth tests on selective medium, LT (SD-Leu/Trp), HLT (SD-His/Leu/Trp), HLT+30 mM 3-AT and β-galactosidase assay on LT plates. The yeast growth rates were presented as fast (+++), slow (+), and no growth (-). NA: not applicable. (C) The full length sequence of Id2 with a flag tag at the C terminus. The open reading frame of Id2 is identical to the sequence in Genbank (accession number: M69293).

proteins were used for luciferase assay as suggested by manufacturer. Luciferase activity was normalized for the transfection efficiency with respect to the β -galactosidase activity.

Electrophoretic mobility shift assay (EMSA)

Binding activity of BETA2/NeuroD was determined as previously described (Cho *et al.*, 2001). Briefly, COS M6 cells were transfected with indicated amounts of pcDNA3-I₂Flag and pCHA-BETA2(1-233), expression vectors for I₂ or BETA2, respectively. Nuclear extracts were prepared as mentioned above. The ³²P-labeled double stranded oligonucleotide containing the RIPE3 sequence, a rat insulin promoter enhancer, and 4 μ g of nuclear extracts were incubated in 20 mM Hepes (pH 7.9), 7% glycerol, 60 mM LiCl, 0.5 mM PMSF, 5 mM MgCl₂, 2 mM DTT and 2 μ g poly dI-dC for 30 min at room temperature. To verify specific binding, competition assays were carried out with unlabeled probe in 20 and 60-fold excess. For supershift assays, 0.4 μ g/lane of anti-BETA2 antibody was added to the binding reaction. Samples were loaded onto 4% polyacrylamide gels in TBE buffer and electrophoresed at 8 V/cm. Gels were dried and autoradiography was performed.

Results and Discussion

I₂ proteins as binding partners of E47

To search for binding ligands for E47 in mouse embryos, we used the bHLH domain of E47 as a bait in yeast two-hybrid screening of a mouse 7.5 dpc cDNA library. A total of 8×10^5 independent colonies were screened and 411 clones were grown in the absence of histidine, leucine and tryptophan, SD-His/Leu/Trp (HLT) plates. The clones containing both E47 and I₂ proteins could survive in HLT plates and express β -galactosidase. These clones could survive in a more stringent condition containing 30 mM 3-amino-1,2,3-triazole (3-AT). 3-AT is a competitive inhibitor of the yeast HIS3 protein (His3p) and used to suppress background growth on SD medium lacking histidine by inhibiting low levels of His3p. The yeast clone expressing both E47 and BETA2 was grown in the same condition and used as a positive control, which is in a good agreement with a previous report that BETA2 forms a heterodimer with E47 and regulates expression of the insulin gene (Naya *et al.*, 1995).

To determine the efficiency of our yeast screening system, we tempted to count the number of I₂-containing clones. We isolated yeast plasmids, and carried out PCR analysis (Figure 1A). The forward primers were designed specific for I₂ isoforms, and

the reverse primer for common sequences in pACT2 vector. Out of 411 clones, 47, 123, and 41 clones were proved to contain the cDNAs encoding I₁, I₂, and I₃, respectively (Figure 1B). These data indicate that I₂ proteins interact with E47 and our system has operated efficiently.

Inhibition of BETA2-mediated gene expression by I₂ protein

Although all isoforms of I₂ proteins are known to negatively regulate differentiation of myogenesis, I₂ has been studied the most with respect to myogenesis. Thus, we focused on I₂ in subsequent studies and generated an expression vector for I₂, pcDNA3-I₂Flag, in the background of pcDNA3. The pcDNA3-I₂Flag was designed to contain the flag epitope at the C terminus of I₂ (Figure 1C and 2A). Figure 2B shows a schematic diagram of expression vectors in this study. To verify proper expression of I₂Flag fusion proteins we transfected pcDNA3-I₂Flag into COS M6 cells. Both cytosolic and nuclear fractions were prepared and subjected to Western analysis using an antibody against the flag epitope (Figure 2B). The I₂Flag fusion protein was detected as a 18 kDa as expected.

To determine whether I₂ blocks BETA2 binding to its target sequence, E-box, we performed EMSA using a double-stranded oligonucleotide containing the E-box sequence of the rat insulin promoter enhancer 3 (RIPE3) (Figure 2C). The source for BETA2 was obtained from the nuclear fraction of COS M6 cells after cells were transiently transfected with pCHA-BETA2(1-233), an expression vector for BETA2 (1-233), and pSVE2-5, an expression vector for E47. We have previously shown that truncation of the activation domain at the C-terminus (amino acids between 234-323) of BETA2 yields stronger binding to the E-box (Cho *et al.*, 2001). Moreover, BETA2(1-233) still retains the bHLH domain which allows us to examine the interfering functions of I₂ proteins. Specific complex derived from dimerization of BETA2(1-233) and E47 was confirmed by competition with an 20 and 60-fold of unlabeled probes (competitor, lane 2 and 3) and supershift assay using anti-BETA2 antibody (lane 4). The supershifted band indicates the specific dimerization of BETA2 and E47.

I₂ proteins were added to DNA binding reaction by cotransfecting COS M6 cells with pcDNA3-I₂Flag in addition to pCHA-BETA2(1-233) and pSVE2-5. Specific complex containing BETA2(1-233) and E47 disappeared in proportion to the expression degree of I₂. Together with yeast two hybrid data, this result indicates that I₂ interferes with heterodimerization of BETA2 and E47, and thereby blocks E-box binding of BETA2. The result also suggests that analogous

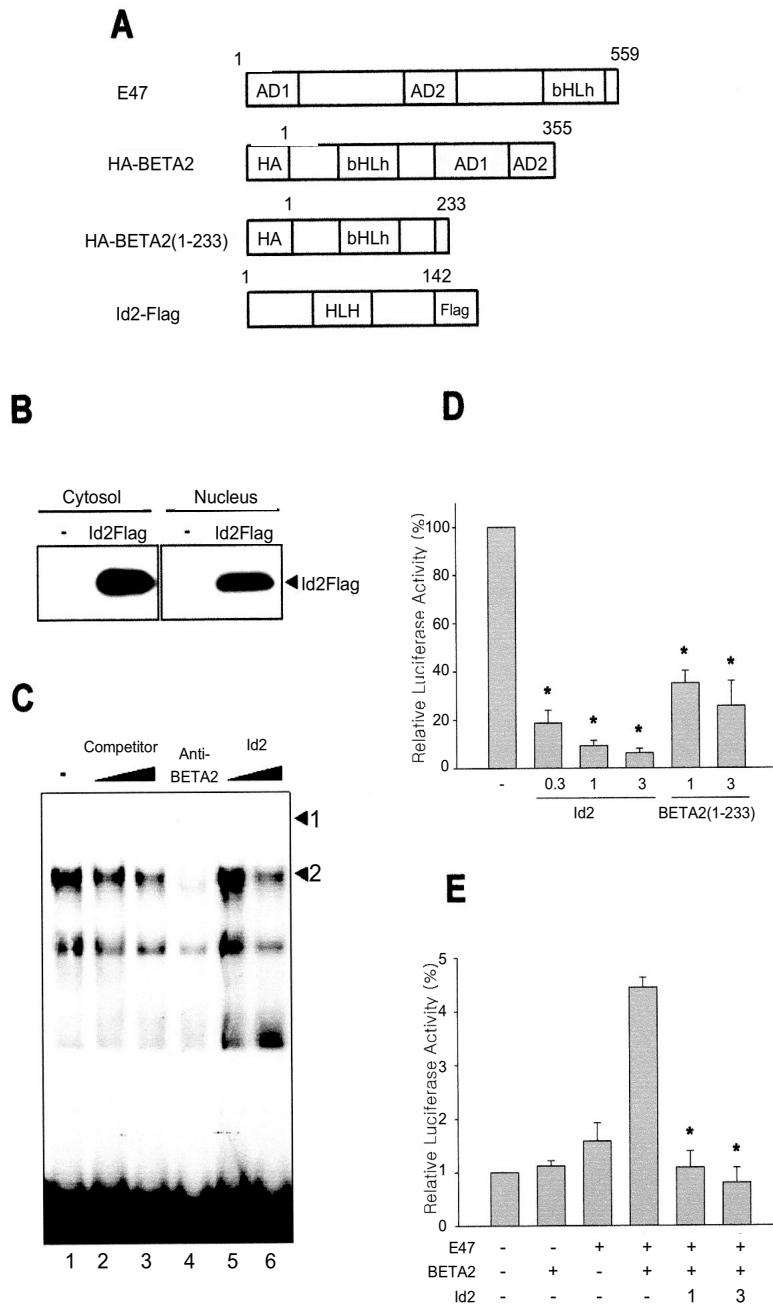


Figure 2. Id2 inhibited E-box mediated gene expression. (A) A schematic diagram of expression vectors. BETA2 and BETA2(1-233) were epitope tagged with hemagglutinin (HA) at the N-termini. Id2 was tagged with flag at the C-terminus. bHLH, basic helix-loop-helix domain; AD, activation domain. (B) After COS M6 cells were transiently transfected with pcDNA3 or pcDNA3-Id2Flag, cytosolic and nuclear fractions were prepared. Western analysis was performed using anti-flag antibody. Id2Flag fusion protein was detected in both cytosol and nuclear fractions. (C) Nuclear extracts were prepared by co-transfection of pHA-BETA2(1-233) and pSVE2-5 into COS M6 cells. Oligonucleotides containing RIPE3 were labeled with ^{32}P and used as a probe. Specific binding of BETA2(1-233)/E47 (complex 2 in lane 1) was confirmed by competition with an excess amount of unlabeled oligonucleotides competitor (complex 2 in lane 2 and 3) and by supershift (complex 1 in lane 4) caused by anti-BETA2 antibody. This specific band was disappeared by co-expression of Id2 (complex 2 in lane 5 and 6). (D) HIT cells were transiently transfected with 0.3 μg pRIPE3(+3)-luc and indicated amounts of pcDNA3-Id2Flag or pcHA-BETA2(1-233). Expression of Id2 proteins inhibited luciferase activity. (E) HeLa cells were transiently transfected with 0.3 μg pRIPE3(+3)-luc, 1 μg or 3 μg of pcDNA3-Id2Flag, 0.3 μg pcHA-BETA2, and 0.3 μg pSVE2-5. Co-expression of pcHA-BETA2 and pSVE2-5 synergistically increased the luciferase activity by 4.5 fold. Co-expression of Id2 reduced luciferase activity in a dose dependent manner. The values were normalized to β -galactosidase activity from three independent experiments and presented as averages \pm standard errors (SE) with respect to the value of reporter gene alone ($*p < 0.001$).

mechanisms may play a role during differentiation of neuronal or endocrine cells. During myogenesis, Id2 competitively interacts with E47 and negatively regulates myoD, a muscle specific bHLH factor (Benezra *et al.*, 1990; Jen *et al.*, 1992).

To investigate the effect of Id2 on BETA2-mediated gene expression, reporter gene assays were carried out in hamster insulin tumor (HIT) cells. HIT cells are known to express endogenous BETA2 and E47 (Kim *et al.*, 2002). We transiently transfected HIT cells with a reporter gene, pRIPE3 (3+)-luc, containing three copies of RIPE3 (Kim *et al.*, 2002), pcDNA3-Id2Flag, and pcHA-BETA2(1-233) in various combination. Earlier study showed that BETA2(1-233) lacks the activation domain and function as a dominant negative regulator of the endogenous BETA2 (Cho *et al.*, 2001). In this experimental condition, transfection of pcHA-BETA2(1-233) decreased the insulin promoter activity. Importantly, expression of Id2Flag inhibited the pRIPE3 (3+)-luc activity in a dose dependent manner as BETA2(1-233) did. Thus, 3 µg of pcDNA3-Id2Flag was sufficient to reduce the luciferase activity by $93 \pm 1.8\%$. Compared to BETA2(1-233), the effect of Id2 was more dramatic, which might be due to that Id2 had a higher affinity for E47 than BETA2(1-233) or more Id proteins were made than BETA2(1-233). Further study is necessary to clarify this.

To confirm that Id2 represses transactivation activity of BETA2, we performed a luciferase assay with the same reporter gene, pRIPE3 (3+)-luc, in HeLa cells. Since HeLa cells are fibroblasts and devoid of endogenous BETA2 (Kim *et al.*, 2002), they are suitable to determine the direct effect of Id2 on BETA2. Expression of BETA2 or E47 alone increased luciferase activity minimally. Co-expression of both BETA2 and E47 increased luciferase activity about 4.5-fold compared to that of the reporter gene alone. Importantly, coexpression of Id2 reduced the luciferase activity derived by BETA2 and E47 in a dose dependent manner. Thus, in the presence of 1 µg of pcDNA3-Id2Flag, the luciferase activity was decreased to the control level. The results both in HIT and HeLa cells indicate that Id2 represses transactivation activity of BETA2.

In this study, Id2 was shown to interact with E47 in the yeast two hybrid system. The results confirm earlier report of Jen *et al.* (1992) demonstrating that Id and E proteins co-fractionate and co-immunoprecipitate in myoblast cell extracts. Furthermore, our study showed that the interaction of Id2 and E47 lowers the binding of BETA2 to the E-box. This may be due to that Id2 competes with BETA2 for the E47 binding and the heterodimers containing Id2 cannot bind to the E-box. The reduced binding to the E-box causes less transactivation by BETA2, since the functional complex of BETA2 requires E47 as a partner.

In BETA2-expressing HIT cells, Id2 reduces the E-box mediated gene expression. Similarly Id2 inhibits the transactivation activity of BETA2 in HeLa cells.

As mentioned earlier, BETA2/NeuroD may play important roles in development of the nervous system and pancreatic islet cells. BETA2 is also known to be essential for other endocrine cells, including secretin producing enteroendocrine cells (Mutoh *et al.*, 2000) and POMC secreting pituitary corticotroph cells (Poulin *et al.*, 2000). The present study indicates that Id proteins may negatively regulate tissue specific gene expression induced by BETA2/NeuroD both in neurons and endocrine cells and the inhibitory role of Id proteins during differentiation may be conserved in various tissues.

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