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Regulation of Id1 and Its Association with Basic Helix-Loop-Helix Proteins during Nerve Growth Factor-Induced Differentiation of PC12 Cells

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Cell differentiation in the nervous system is dictated by specific patterns of gene expression. We have investigated the role of helix-loop-helix (HLH) proteins during differentiation of PC12 pheochromocytoma cells in response to nerve growth factor. Gel mobility shift assays using PC12 cell nuclear extracts demonstrated that active basic HLH complexes exist throughout differentiation. Addition of exogeneous Id1 protein, a negative regulator of basic HLH proteins, disrupted specific complexes formed by PC12 cell nuclear extracts on a CANNTG consensus oligonucleotide. To identify possible novel basic HLH proteins in these complexes, a glutathione *S*-transferase–Id1 fusion protein was used to screen a PC12 cell cDNA expression library. A single clone representing the rat E2-2 gene was identified. Sequential immunoprecipitations with antibodies to each HLH protein revealed an association between Id1 and E2-2 that could be detected in both untreated and nerve growth factor-treated PC12 cell lysates. These experiments define a new HLH interaction between Id1 and E2-2 in neuronal cells and suggest that neuronal differentiation may be regulated by HLH proteins in a distinctive manner.

Elucidation of the nuclear events which regulate the development and differentiation of neural cells in mammalian systems has been furthered by the discovery of the *Drosophila melanogaster* proneural genes (18). The proneural genes were originally identified genetically as essential for the development of the *Drosophila* nervous system. These genes were found to be members of a large family of structurally related transcriptional regulators, the helix-loop-helix (HLH) proteins. HLH proteins are involved in the regulation of tissue-specific gene expression in a variety of cell lineages.

Mammalian homologs of known *Drosophila* HLH genes have been identified by PCR. *MASH 1* and *2* (20) and *HES 1*, *3*, and *5* (1, 11, 31) are vertebrate counterparts of the genetically defined *Drosophila* loci *achaete-scute*, *hairy*, and *Enhancer of split*. In addition, the widely expressed HLH proteins E12 and E47 (27) and Id1 (6) are homologs of proteins encoded by the *Drosophila daughterless* and *emc* loci. All of these *Drosophila* loci are required for the appropriate pattern of neurogenesis. HLH protein genes which are expressed in neuronal tissues but which are not homologous to specific *Drosophila* genes, such as *NSCL1,2*, have been isolated (4, 5). The large number of different neuronal HLH protein genes implies a complex network of transcriptional regulation for appropriate development and differentiation of the neuronal phenotype.

Differentiation of myoblasts to myotubes in cell culture has provided a molecular mechanism for how HLH proteins regulate differentiation (22, 35). Muscle-specific HLH proteins such as myoD dimerize with the ubiquitous HLH proteins E12 and E47 (E12/E47), the products of the E2A gene, to form a high-affinity DNA-binding complex. These basic HLH (bHLH) proteins contain a basic region adjacent to the HLH domain which confers DNA binding to a consensus sequence, CANNTG. This myoD-E12/E47 complex transactivates muscle-specific genes containing this consensus sequence, such as the muscle creatine kinase enhancer. The formation of this myoD-E12/E47 complex is negatively regulated by Id1, an HLH protein which lacks a basic domain. bHLH proteins, when complexed with Id1 or related Id proteins, no longer bind to DNA. In myoblasts, Id1 levels are high and Id1 is complexed with E12/E47 (19). As differentiation proceeds, Id1 levels become reduced, and Id1 is no longer found complexed with E12/E47. This allows for the formation of a myoD-E12/E47 complex and transcriptional activation. This exchange of partners is crucial, since differentiation is delayed when Id1 is overexpressed (19). Elevated levels of Id1 can also inhibit the differentiation of a number of cell types (19, 24, 32, 36), suggesting that this may be a common mechanism for regulating HLH protein-protein interactions.

Although a large number of HLH protein genes which are putatively involved in mammalian neuronal development and differentiation have been isolated, their exact roles are not yet understood (1, 5, 9, 11, 14, 20, 23, 28). In order to define HLH protein interactions which may regulate neuronal differentiation, we have used Id1 as a probe of HLH protein action in PC12 cells. Examination of Id1 transcription during nerve growth factor (NGF)-induced differentiation indicated that Id1 message was upregulated, in contrast to the decrease in Id1 levels observed in other cell types undergoing differentiation. Gel mobility shift assays of PC12 cell nuclear extracts binding to an oligonucleotide containing the CANNTG consensus sequence revealed active bHLH protein complexes throughout differentiation. Addition of exogenous Id1 to these complexes indicated that they were sensitive to disruption by Id1. To identify potential partners of Id1, we performed interaction cloning using labeled Id1 protein to probe a PC12 expression library. A single clone, identified as a partial cDNA of the rat E2-2 gene, was isolated. Coimmunoprecipitation experiments establish that Id1 and E2-2 can form a complex in PC12 cells. These results indicate that the interactions between HLH pro-

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teins during NGF-induced differentiation of PC12 cells are distinctly different from those observed in other cell types.

MATERIALS AND METHODS

Cell culture. PC12 cells were grown in Dulbecco's modified Eagle's medium–10% fetal bovine serum–5% horse serum (GIBCO)–1 mM glutamine–100 U of penicillin per ml–100 μg of streptomycin (GIBCO) per ml. The cells were treated with 50 ng of NGF or epidermal growth factor (EGF) (Bioproducts for Science, Indianapolis, Ind.) per ml, with medium changes every 48 h.

RNA isolation and Northern (RNA) analysis. RNA was isolated as described by Sambrook et al. (30). Briefly, PC12 cells were lysed by addition of 3 ml of 4 M guanidinium thiocyanate to each 100-mm-diameter dish. This lysate was passed through a 23-gauge needle until it was no longer viscous. The lysate was layered on 2 ml of 5.7 M cesium chloride–0.1 M EDTA and centrifuged overnight at 30,000 × *g* in a Sorvall AH650. The RNA was resuspended from the pellet, subjected to electrophoresis, and transferred to nitrocellulose. DNA fragments representing cDNA clones were labeled to a specific activity of $>1 \times 10^8$ cpm/μg by using the random-primed DNA labeling kit (Boehringer Mannheim) and isolated on a Sephadex G-50 spin column. Nitrocellulose filters were baked at 80°C for 4 h and prehybridized in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–500 μg of salmon sperm DNA per ml at 42°C for 4 h. Hybridizations were performed at 42°C overnight in 50% formamide–5× SSC–1× Denhardt's solution–10% dextran sulfate–100 μg of salmon sperm DNA per ml. After hybridization, filters were washed three or four times with 0.2× SSC–0.1% sodium dodecyl sulfate (SDS) at 55°C and subjected to autoradiography. Filters were reused after being washed twice with 5 mM Tris (pH 8.0)–0.2 mM EDTA–0.05% sodium PP_i–1× Denhardt's solution at 65°C.

Nuclear extract preparation and DNA binding assays. Nuclear extracts were prepared as described by Dignam et al. (8). The CANNTG consensus sequence from the muscle creatine kinase enhancer was used in all DNA binding reactions. The sequence of the oligonucleotide is GATCCCCCCAACACCTGCTGCC TGA. The upper strand was labeled with [γ-^{32}P]ATP (3,000 mCi/mmol) (Dupont-NEN) by using T4 polynucleotide kinase and then annealed to a 10-fold molar excess of the opposite strand in 100 mM NaCl–1 mM Tris (pH 7.6). DNA binding reactions were done with a total volume of 20 μl containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.1 μg of double-stranded poly(dI-dC), and 0.2 ng of double-stranded labeled probe. Binding reaction mixtures were incubated at room temperature for 15 min, subjected to electrophoresis on a 5% polyacrylamide gel (40:1.33, acrylamide-bisacrylamide), and run in 1× TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) at 140 V for 2 h at room temperature. The gels were dried and autoradiographed.

E47 protein was prepared from RNA generated from the E47P plasmid (27). The in vitro translation reactions were performed by using rabbit reticulocyte lysate (Promega) according to the manufacturer's specifications. For the Id1 disruption assay, the nuclear extracts were incubated with the amounts of glutathione *S*-transferase (GST) or GST-Id1 protein (gift of R. Benezra) (19) indicated below for 15 min at 37°C prior to the addition of a reaction cocktail containing the labeled oligonucleotide. Supershift assays were performed as described above, except that the nuclear extract was preincubated with 1 μl of E12/E47 antiserum (gift of C. Murre) for 15 min at room temperature prior to the addition of a reaction cocktail containing the labeled oligonucleotide.

Isolation of rat Id cDNA. A λgt10 cDNA library made from RNA from PC12 cells treated with NGF for 2 weeks (gift of Jim Eberwine, University of Pennsylvania) was screened with a ^{32}P-labeled mouse Id cDNA fragment (6). A cDNA insert was isolated, subcloned into Bluescript KS, and sequenced in its entirety (3a).

GST fusion proteins. GST fusion proteins were constructed in the GSTag vector (a gift of David Ron) by using PCR according to the specifications of the manufacturer (Perkin-Elmer) (29). The GST-rat Id1 fusion protein was cloned into the *Bam*HI and *Eco*RI sites of the vector, incorporating amino acids 1 to 176 and utilizing the Id1 termination codon (6). The GST–RE2-2 fusion protein was cloned into the *Bam*HI and *Eco*RI sites of the vector, incorporating the first amino acid of the isolated cDNA and utilizing the termination codon of the cDNA. GST fusion proteins were labeled to 10^7 cpm/μg with protein kinase A (Sigma) at 1 U/μl and [γ-^{32}P]ATP (6,000 Ci/mmol) in PK buffer (50 mM KPO_4, 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM dithiothreitol) for 30 min at 37°C. The unincorporated ATP was removed by using a Sephadex G-50 column (Boehringer-Mannheim). For cleavage with thrombin, the fusion protein was incubated with 0.01 U of bovine thrombin (Sigma) per μl in 50 mM Tris (pH 7.5)–150 mM NaCl–2.5 mM $CaCl_2$ at room temperature for at least 1 h.

Library screening. Expression library screening was performed in a manner similar to that described by Ayer et al. (2). Approximately 10^6 phage from a PC12 cell λgt11 expression library (Clontech) in *Escherichia coli* Y1090 were plated on dishes (150 by 15 mm) and incubated at 37°C. Seven hours after plating, filters (supported nitrocellulose; Schleicher & Schuell) which had been presoaked in 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were overlaid on the plates, and the proteins were allowed to transfer overnight at 37°C. The proteins on the filters were denatured and renatured in sequential guanidine hydrochloride washes (29). The filters were incubated twice in 6 M guanidine hydrochloride in basic buffer (20 mM HEPES [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Nonidet P-40 [NP-40]) for 10 min at 4°C with gentle agitation. The 6 M guanidine solution was subsequently diluted 1:1 with basic buffer and incubated for 10 min at 4°C with agitation. This was repeated four or five times. Subsequently, the filters were washed twice (10 min each wash) with basic buffer alone. The filters were then incubated in blocking buffer (basic buffer plus 5% nonfat dry milk) at 4°C with agitation for 4 h to overnight. Protein screenings were performed with approximately 3 μg of labeled, thrombin-cleaved fusion protein in screening buffer (basic buffer plus 1% nonfat dry milk plus 5% glycerol) at 4°C for 4 to 5 h with gentle agitation. After this incubation, the filters were washed four times (10 min each wash) with phosphate-buffered saline (PBS)–0.2% Triton X-100 at 4°C. Two or three additional washes with PBS–100 mM KCl–0.2% Triton X-100 were performed as described above. The filters were then exposed to film. The DNA from positive phage was isolated as described previously (30), and the insert was amplified by PCR with λgt11 primers (New England Biolabs) according to the specifications of the manufacturer (Perkin-Elmer). The insert was subcloned into the pCR II cloning vector by using the TA cloning kit (Invitrogen). The insert was sequenced with the Sequenase sequencing kit (U.S. Biochemicals).

Antibody generation and characterization. The GST–RE2-2 fusion protein was used to generate a polyclonal rabbit antibody (Pocono Farm and Laboratory). The serum was affinity purified by protein A chromatography. The resulting serum was used in all experiments. A chemiluminescence detection kit (Amersham) was used for Western blot (immunoblot) analysis.

Immunoprecipitations were performed with cell lysates from ^{35}S-labeled PC12 cells. PC12 cells were labeled with 1.5 mCi of ^{35}S-Express label (Dupont-NEN) in Dulbecco's modified Eagle's medium lacking cysteine and methionine–10% dialyzed fetal bovine serum–5% dialyzed horse serum for 4 h. Cells were harvested in antibody buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 0.5% NP-40), the Id antibody was added at a 1:100 dilution, and the mixture was incubated for 4 h at 4°C with rocking. Protein A-Sepharose beads were added and incubated with the sample for 1 h at 4°C with rocking. The protein A-Sepharose beads were subsequently washed three times with radioimmunoprecipitation assay (RIPA) buffer. After the final wash, the protein A-Sepharose beads were resuspended in sample buffer, boiled for 10 min, analyzed on an SDS-polyacrylamide gel, and enhanced for fluorography.

Coimmunoprecipitation. Coimmunoprecipitations were done essentially as described by Jen et al. (19). PC12 cells plated in three 150-mm-diameter plates at 70% confluence were labeled with 5 mCi of ^{35}S-Express label for 4 h at 37°C for each time point. Cells were harvested and lysed in 400 μl of HB buffer (0.3 M sucrose, 10 mM Tris [pH 8.0], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40). The sample volume was brought up to 1 ml in HB buffer, and anti-RE2-2 antiserum was added to the lysate at a 1:1,000 dilution. After incubation for 4 h, 10 mg of swollen protein A-Sepharose beads was added and the mixture was incubated for an additional hour. The beads were collected by brief centrifugation and washed three times with HB buffer. After the final wash, the beads were divided into four equal aliquots. Sample buffer was added to one aliquot, and then the mixture was stored at −20°C. The other three aliquots were washed with 400 μl of RIPA buffer for 1 h. The beads were pelleted by brief centrifugation, and the RIPA supernatant was transferred to a fresh tube. To the RIPA supernatant, either anti-Id1 antiserum, anti-Id1 antiserum preincubated overnight with GST-Id1 protein, or anti-Id1 antiserum preincubated overnight with GST protein (as described in reference 19) was added. The antisera were added at a 1:100 dilution. The samples were incubated for 4 h. After this incubation, 10 mg of swollen protein A-Sepharose beads was added and the mixture was allowed to incubate for an additional hour. The protein A-Sepharose beads were collected by brief centrifugation and washed three times with RIPA buffer. After the final wash, the beads were resuspended in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and enhanced for fluorography. All incubations were done with gentle rocking at 4°C.

RESULTS

Id1 is expressed during NGF-induced differentiation. In many cell culture differentiation systems, Id1 mRNA down-regulation coincides with the onset of differentiation. During differentiation of $10T_{1/2}$ cells, myeloid precursors, B lymphocytes, erythroleukemic cells, F3 myoblasts, and F9 embryonal carcinoma cells, Id1 expression is decreased in response to differentiative signals (6). Additionally, during development, Id1 mRNA is seen predominantly in neuronal precursors and is reduced upon differentiation (9, 10).

In order to examine Id1 mRNA regulation during neuronal differentiation in vitro, we have used a well-characterized cell culture model of neuronal differentiation, PC12 cells (15). NGF treatment of PC12 cells leads to differentiation to a sympathetic neuronal phenotype. Northern analysis with the

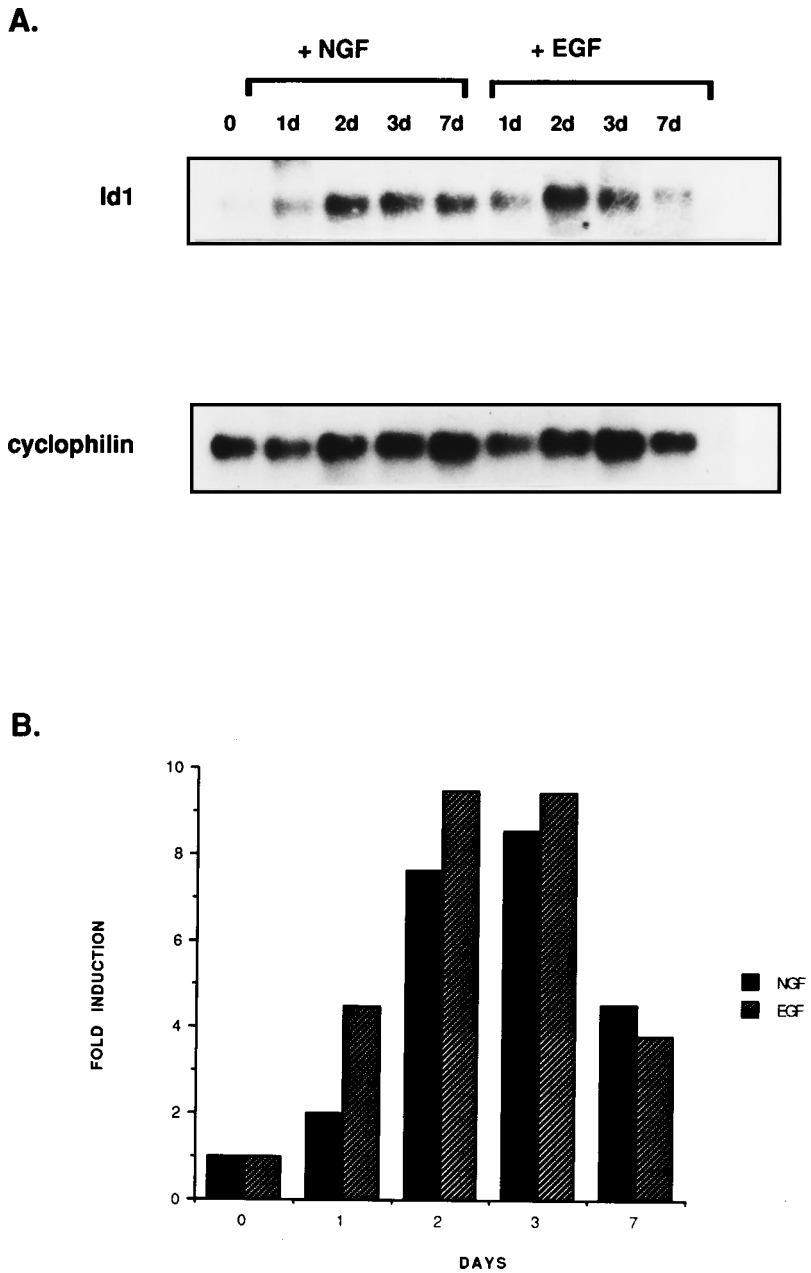

FIG. 1. Id1 mRNA is upregulated in response to growth factor treatment. (A) Northern analysis of 20 μg of total mRNA isolated from PC12 cells which were untreated (lane 0) or treated with NGF (50 ng/ml) or EGF (50 ng/ml) for the numbers of days (d) indicated above the lanes. The gel was probed with the rat Id1 cDNA (upper panel) and then stripped and reprobed with the cyclophilin cDNA (lower panel). (B) Fold induction of Id1 mRNA calculated after normalization to the cyclophilin message. Densitometric analysis was performed on an LKB scanning densitometer.

rat Id1 cDNA was performed on total RNA isolated from NGF-treated PC12 cells. The Id1 message was present in untreated cells and was increased eightfold by NGF treatment (Fig. 1). After 7 days of NGF treatment, when the cells have undergone differentiation, Id1 mRNA levels are decreased to threefold above untreated levels.

PC12 cells express receptors for a number of other growth factors, such as EGF. EGF acts as a mild mitogen on PC12 cells but does not induce neuronal differentiation. The regulation of Id1 mRNA in response to EGF treatment is similar to the response to NGF treatment. This behavior is similar to that of other NGF-induced genes in PC12 cells, in which the majority of early NGF-activated genes are also induced by EGF. The increase in Id1 mRNA during the course of NGF treatment is clearly different from Id1 regulation that has been observed in other cell culture differentiation systems, in which Id1 mRNA substantially decreases. These results indicate that steady-state levels of Id1 mRNA and its role during NGF-mediated differentiation are distinctive.

bHLH DNA binding activity is present in PC12 cells throughout NGF-induced differentiation. In order to determine the activity of bHLH proteins in PC12 cells, nuclear extracts were isolated from PC12 cells and a CANNTG consensus oligonucleotide was used to probe for active bHLH

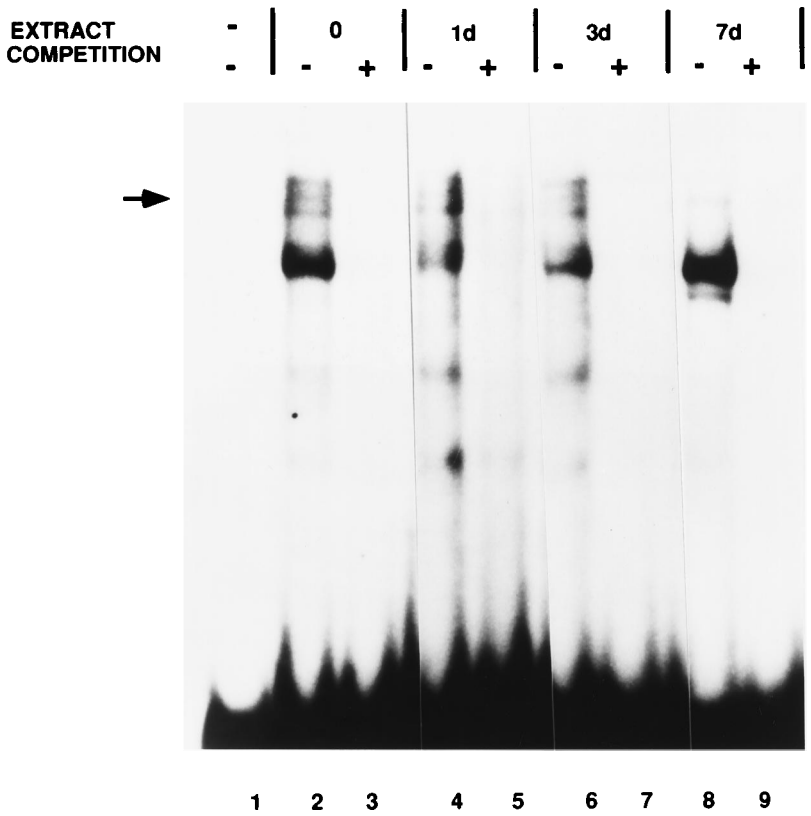

FIG. 2. CANNTG-binding proteins are present in PC12 cell nuclear extracts throughout differentiation. Electrophoretic mobility shift assays of nuclear extracts isolated from PC12 cells which were untreated (lanes 2 and 3) or treated with NGF (50 ng/ml) for 1 (lanes 4 and 5), 3 (lanes 6 and 7), or 7 (lanes 8 and 9) days. Lane 1 contains probe alone. In lanes 3, 5, 7, and 9, 100 ng of unlabeled wild-type oligonucleotide was added. Complexes not inhibited by addition of 100 ng of mutant oligonucleotide (data not shown) (arrow) are indicated.

protein complexes. A consensus oligonucleotide from the muscle creatine kinase enhancer, which has been used extensively to characterize active HLH protein complexes in a variety of cell types (21, 24), was employed to assay bHLH activity from PC12 cells. Complexes are formed on this oligonucleotide when it is incubated with both untreated and NGF-treated nuclear extracts (Fig. 2). A series of complexes (Fig. 2, arrow) is specific to the wild-type consensus site, as an added mutant oligonucleotide did not effectively compete for DNA binding (data not shown). The patterns of DNA-protein complexes did not appear to differ significantly between extracts isolated from untreated cells and extracts from cells treated for 1, 3, or 7 days with NGF (50 ng/ml). The only apparent difference in complex formation was when nuclear extracts from PC12 cells treated with NGF for 7 days were assayed. At day 7, a decrease in the intensity of the uppermost band was observed.

PC12 cell bHLH protein complexes are sensitive to disruption by exogenous Id. bHLH proteins have been classified into two groups on the basis of their susceptibility to Id inhibition (34). The proteins in these two groups bind to the same consensus sequence but display different dimerization properties and different susceptibilities to Id1 inhibition. The first group, represented by the products of the E2A gene (E12 and E47) and myoD, is inhibited by Id1 or Id2. These proteins are members of the previously described class A and class B CANNTG-binding proteins, respectively (22). The second group, represented by c-Myc, TFE3, USF, and AP4, are bHLH zipper proteins, which are not inhibited from binding DNA by Id1 or Id2. These proteins are members of the previously described class C CANNTG-binding proteins (22). Thus, classification of HLH protein-DNA complexes can be determined by their sensitivity to inhibition by Id.

Id1 is capable of inhibiting DNA binding through heterodimerization with class A and class B bHLH proteins (6). These classes of bHLH proteins have been implicated in neuronal differentiation (13, 16, 18). If, upon addition of exogenous Id1, a protein complex is no longer able to bind to DNA, a member of this complex would be either a class A or a class B bHLH protein. In order to determine the sensitivity of the PC12 cell CANNTG-binding complexes to Id1, a GST-Id1 bacterially expressed fusion protein was preincubated with the PC12 cell nuclear extracts at 37°C for 15 min prior to the addition of labeled probe (Fig. 3). As a control, various amounts of GST-Id1 fusion protein were added to E47 protein produced by in vitro translation. Twenty nanograms of added GST-Id1 was sufficient to disrupt DNA binding (Fig. 3A, lanes 5 and 6). Addition of GST protein alone had no effect on DNA binding (Fig. 3A, lane 7). PC12 cell proteins which are specific to the CANNTG consensus sequence were sensitive to the addition of exogenous GST-Id1 protein (Fig. 3B, lanes 3, 6, 9, and 12, arrow). This suggests that the proteins in these complexes are members of the previously described class A or B CANNTG-binding proteins (34).

E12-E47 polyclonal antibodies do not supershift complexes. Id1 has the capability of forming heterodimers with a number of bHLH proteins and displays a high affinity for the E12 and E47 proteins. Since E12 has been shown to be expressed in PC12 cells (26), we investigated whether these proteins are

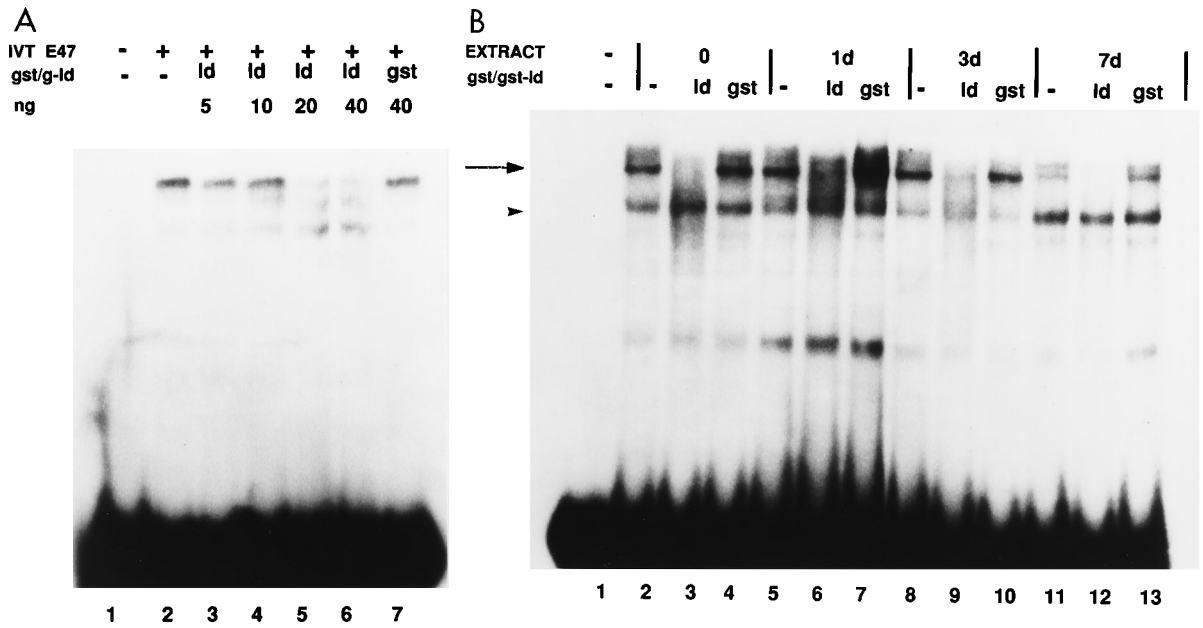

FIG. 3. CANNTG-binding proteins are sensitive to addition of exogenous gst-Id1 protein. (A) Electrophoretic mobility shift assays of in vitro-translated (IVT) E47 protein. Results are shown for probe alone (lane 1), 2 μl of E47P programmed reticulocyte lysate (lane 2), or 2 μl of E47P programmed reticulocyte lysate preincubated at 37°C for 15 min with the indicated amounts of GST-Id1 protein (lanes 3 to 6) or with 40 ng of gst (lane 7). (B) Electrophoretic mobility shift assays of nuclear extracts isolated from PC12 cells which were untreated (lanes 2 to 4) or treated with NGF (50 ng/ml) for the indicated numbers of days (d). Nuclear extracts were preincubated for 15 min at 37°C with 20 ng of GST-Id1, (lanes 3, 6, 9, and 12) or with 20 ng of GST (lanes 4, 7, 10, and 13). Lane 1 contains probe alone. A nonspecific complex (arrowhead) and the Id inhibitable complex (arrow) are indicated.

represented in the complexes observed here. We performed a supershift assay using a polyclonal anti-E12/E47 antiserum (gift of C. Murre). The complexes formed from PC12 cell nuclear extracts were unaffected by the addition of the anti-E12/E47 antiserum (Fig. 4A, lanes 3, 6, 9, and 12). In contrast, in vitro-translated E47 displayed an altered mobility upon the addition of antibody, with the complex shifted to the well of the gel (Fig. 4B, lane 2). This result indicates that although E12 is expressed in PC12 cells, it is not detected in these DNA-binding complexes by using this polyclonal antiserum.

Interaction cloning of Id1 partners from PC12 cells. In order to identify possible targets of Id1 in PC12 cells, we screened a PC12 cell λgt11 cDNA library using ^{32}P-labeled Id1 protein. A GST-Id1 fusion protein containing the entire rat Id1 coding region in the GSTag vector (gift of D. Ron), which contains a recognition site for protein kinase A, was constructed. The protein was labeled to a high specific activity with protein kinase A and used to probe the PC12 cell expression library. Approximately 10^6 phage were screened, from which 24 possible positive phage were isolated. Of these 24, 1 phage rescreened on further probing with the Id1 protein. The positive phage was plaque purified and isolated, and its insert was sequenced (Fig. 5). The insert of 1,087 nucleotides corresponded to a partial cDNA which encodes the rat homolog of ITF2 (E2-2) (17) (designated RE2-2). The rat cDNA sequence shares 89% identity with the published human E2-2 sequence. Alignment of the human and rat sequences revealed the insertion of four amino acids in the rat sequence, RSRS, just N terminal to the basic domain. Comparison of the deduced amino acid sequence of the bHLH regions of rat E2-2 with the human ITF2 (E2-2), ITF1, and HEB genes, revealed 97, 81, and 93% identity, respectively. Human ITF2 (E2-2) was originally cloned as a protein which binds to the μE5 site in the immunoglobulin H enhancer (17).

Generation of a polyclonal anti-RE2-2 antibody and characterization of RE2-2 protein in PC12 cells. A GST–RE2-2 fusion protein was constructed, and bacterially expressed fusion protein was used as an antigen for the generation of a polyclonal antiserum. This antiserum (termed anti-RE2-2) was used to characterize the RE2-2 protein in PC12 cells. Immunoprecipitation of ^{35}S-labeled PC12 cell lysates reveals a single dominant species (approximately 70 kDa) recognized by the antibody (data not shown). The molecular mass predicted from the human cDNA is approximately 65 kDa. Western analysis, using the anti-RE2-2 antiserum, of PC12 cells treated from 1 to 7 days with NGF showed no significant regulation of the RE2-2 protein during differentiation (Fig. 6).

In vivo association of Id1 and RE2-2. In other differentiation systems, the action of Id1 has been shown to be mediated through interactions with E12- and E47-like proteins (19). Because the regulation of Id1 mRNA in PC12 cells in response to NGF is distinctly different from that in the previously characterized systems, the interaction of Id1 with a putative protein partner (RE2-2) might provide some insight into the action of Id1 in PC12 cells. To investigate a possible association of Id1 and RE2-2 in PC12 cells, we used a coimmunoprecipitation strategy previously published by Jen et al. (19).

PC12 cells were labeled with [^{35}S]cysteine and methionine, and cell lysates were immunoprecipitated at low stringency with anti-RE2-2. The immunocomplexes were isolated on protein A-Sepharose beads and were then subjected to a detergent wash in order to release any proteins associated with RE2-2. This detergent wash was then immunoprecipitated with anti-Id1 antibodies (gift of R. Benezra), and the immunocomplexes were subjected to SDS-PAGE and fluorography. This strategy was devised because the anti-Id antiserum disrupts HLH protein interactions in vitro (19). A protein with an apparent molecular mass of 18 kDa was immunoprecipitated from PC12 or P2 myoblasts by using the anti-Id1 antiserum alone (Fig. 7A,

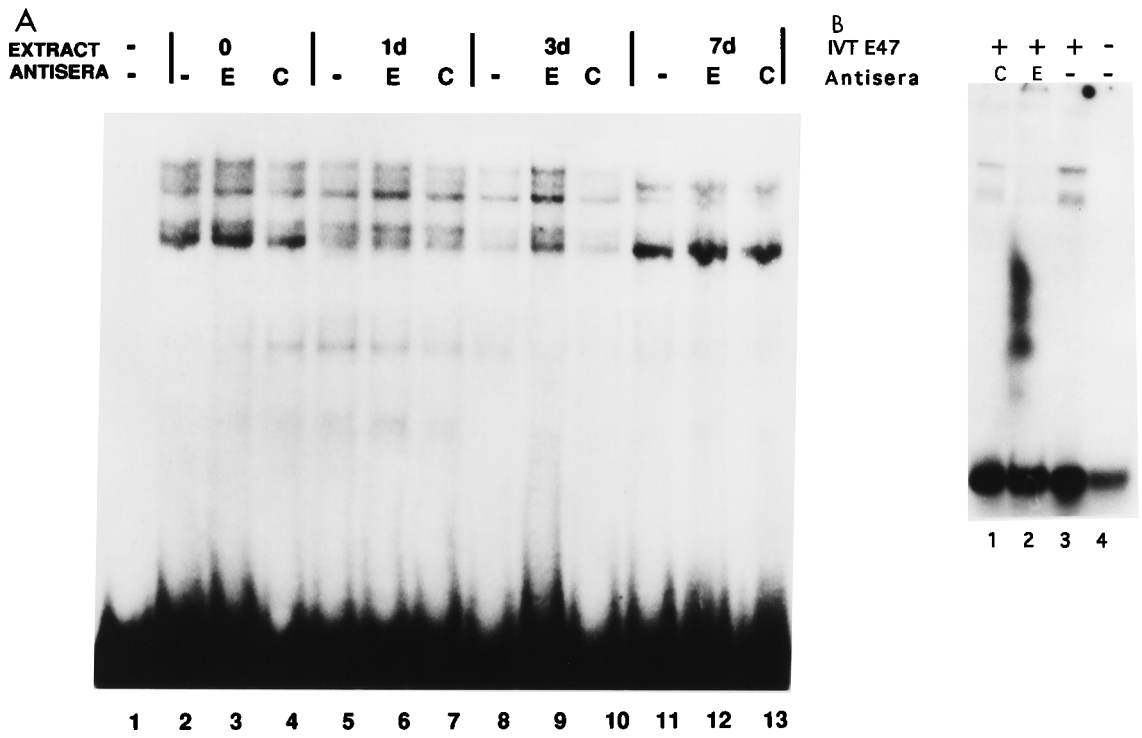

FIG. 4. Id1 inhibitable complexes do not contain E12/E47 proteins. (A) Supershift assays of nuclear extracts isolated from PC12 cells which were untreated (lanes 2 to 4) or treated with NGF (50 ng/ml) for the indicated numbers of days (d). Nuclear extracts were preincubated with 1 μl of anti-E12/E47 antiserum (lanes 3, 6, 9, and 12) or with antiserum to an irrelevant antigen (lanes 4, 7, 10, and 13). Lane 1 contains probe alone. (B) Supershift assay of in vitro-translated (IVT) E47. E47 was translated from reticulocyte lysate (2 μl) preincubated with antiserum to an irrelevant antigen (lane 1), antiserum to E12/E47 (lane 2), no addition (lane 3), or the probe alone (lane 4).

lanes 1 to 4). Sequential immunoprecipitation with anti-RE2-2 followed by anti-Id1 antiserum resulted in immunoprecipitation of Id1 from untreated PC12 cells (Fig. 7B, lanes 5 and 7). In contrast, when the anti-Id1 antiserum was preincubated with GST-Id1, coimmunoprecipitated Id1 was no longer evident (Fig. 7B, lane 6). Id1 was immunoprecipitated when the anti-Id1 antiserum was preincubated with GST protein alone (Fig. 7B, lane 7).

PC12 cells treated with NGF for 24 h were assayed in a similar manner. After 24 h of NGF treatment, PC12 cells exhibit many markers of differentiation, such as neurite outgrowth. Immunoprecipitation of Id1 from these cells was observed (Fig. 7C, lanes 8 and 10). Again, this interaction during NGF-induced differentiation of PC12 cells is in contrast to the muscle differentiation system, in which Id1 was immunoprecipitated from anti-E12 immunocomplexes from undifferentiated myoblasts but not differentiated myotubes (19). This further confirms that the regulation and action of Id1 in PC12 cells are distinct from the functions of Id1 in other previously characterized differentiation systems.

DISCUSSION

Direct protein-protein interaction modulates the activity of many different classes of transcription factors. The activity of HLH proteins can be regulated by protein-protein interactions through two different mechanisms. bHLH proteins can heterodimerize to alter binding site preference (7). Additionally, dominant negative HLH proteins, such as Id1, can inhibit bHLH proteins from binding to DNA, also through heterodimerization (6, 34). The capacity of these proteins to oligomerize allows for complex regulation of their activity. Tissue-specific expression of one class of bHLH protein genes (class A) and ubiquitous expression of another (class B) allow for the coordination of activity which is necessary for appropriate development and differentiation.

Drosophila neurogenesis is controlled in part by the proneural HLH protein genes. Recent studies have indicated that the mammalian homologs of these proneural genes may play a similar function in mammalian development and differentiation (16). However, for the majority of mammalian neuronal HLH protein genes identified to date, the interactions between the proteins which may regulate their activity are not yet known. In order to better understand these regulatory interactions during neuronal differentiation, we have examined Id1 expression and interactions in PC12 cells. Our data indicate that Id1 regulation during NGF-induced differentiation does not mimic the regulatory role proposed for Id1 in other cellular differentiation systems. In contrast to the situation in many cell culture differentiation systems, Id1 message levels are upregulated in response to NGF, during the ongoing program of morphological and biochemical changes associated with NGF treatment. Attempts to overexpress Id1 in PC12 cells were not fruitful, a result which may be related to the inherent difficulty in expressing Id1 at high levels in many cell lines (19). However, our results indicate that Id1 may serve in a novel regulatory role during neuronal differentiation.

Assays of bHLH activity from NGF-treated PC12 cells have not revealed any significant regulation. Although the complexes characterized in this study were specific to the oligonucleotide used, there remains the possibility that the probe may not have contained sufficient sequence specificity to reveal NGF regulation. In this regard, supershifts were not detected

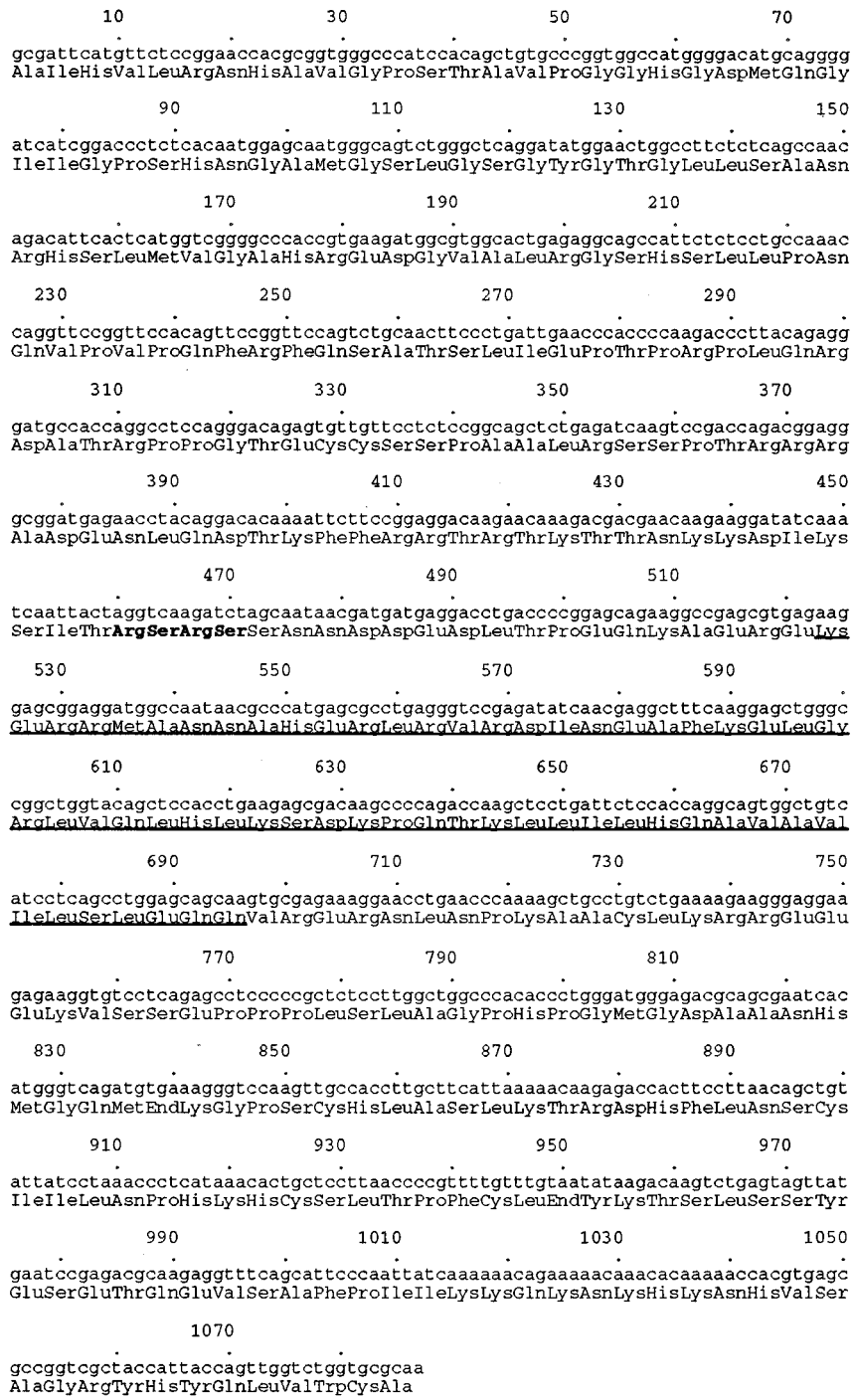

FIG. 5. Nucleotide sequence of partial RE2-2 cDNA, with the predicted amino acid translation below. The location of four amino acids inserted relative to the human sequence (17) (boldface) and the bHLH domain (underlined) are indicated.

with the anti-E2-2 antibody (data not shown), indicating that these complexes may not be NGF specific. Although bHLH proteins bind to a consensus sequence (CANNTG), it has been shown that both the internal bases and the flanking sequence can alter the specificity and affinity of binding (7, 12, 33). Site selection analysis for mammalian neuronal bHLH proteins will permit the use of DNA sequences which contain the sequence specificity necessary to monitor regulation of DNA binding during differentiation.

The identity of Id1's partners during neuronal differentiation is unknown. Interaction cloning was used to isolate potential partners from a PC12 cell expression library. Our screen resulted in the cloning of a partial cDNA, corresponding to the bHLH domain of rat ITF2 (RE2-2). Human ITF2 (E2-2) was originally cloned as a protein which binds to the μE5 region of the immunoglobulin H enhancer (17). E2-2 is a ubiquitous bHLH protein which heterodimerizes with tissue-specific bHLH proteins to regulate tissue-specific expression (22). An-

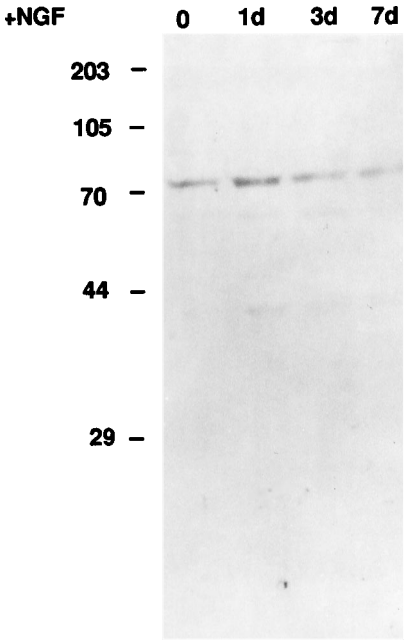

FIG. 6. NGF treatment of PC12 cells does not regulate RE2-2 protein. Results of Western analysis of NGF-treated PC12 cells using polyclonal anti-RE2-2 antiserum are shown. Cells were treated for the indicated numbers of days (d) with NGF (50 ng/ml). A 200-μg sample of cell lysate was subjected to electrophoresis on an SDS–12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Western analysis was performed, and immunoreactive bands were detected by chemiluminescence. Antiserum was added at a 1:5,000 dilution. The positions of molecular size markers (in kilodaltons) are shown on the left.

tibodies generated against the RE2-2 protein show that it is expressed at a uniform level throughout NGF-induced differentiation.

To determine whether Id1 and E2-2 associate in vivo, coimmunoprecipitation experiments were carried out with untreated and NGF-treated PC12 cells. Our results indicate that Id1 and E2-2 associate in vivo. Id1 and E2-2 are also found to interact in NGF-treated PC12 cells, in contrast to the muscle differentiation system, in which subsequent to the onset of differentiation Id1 and E12/E47 are no longer complexed (19). The use of polyclonal anti-E2-2 antibodies does not allow us to rule out the possibility that we may be immunoprecipitating Id1 associated with other E2-2-like proteins expressed in PC12 cells. However, the association experiments indicate that E2-2 is a strong candidate as a putative target of Id1 inhibition in PC12 cells. This raises the possibility that the upregulation of Id1 levels allows for titering the amount of available E2-2. A time course analysis of this association would address this possible function of Id1 in PC12 cell differentiation. In addition, other Id family members (28, 34) may also function in a regulatory capacity during neuronal differentiation. Further information concerning the interactions of tissue-specific bHLH proteins and ubiquitously expressed bHLH proteins will provide more insight into the functional consequence of the Id1–E2-2 interaction.

The knowledge of the function of mammalian neuronal HLH proteins will be expanded when in vivo partners during development and differentiation are further defined. The target genes for the majority of these bHLH proteins are unknown, but their identification will assist in defining how these transcription factors contribute to a neuronal phenotype. The unique regulation of Id1 seen here indicates that study of the HLH proteins expressed in neuronal cells may yield new in-

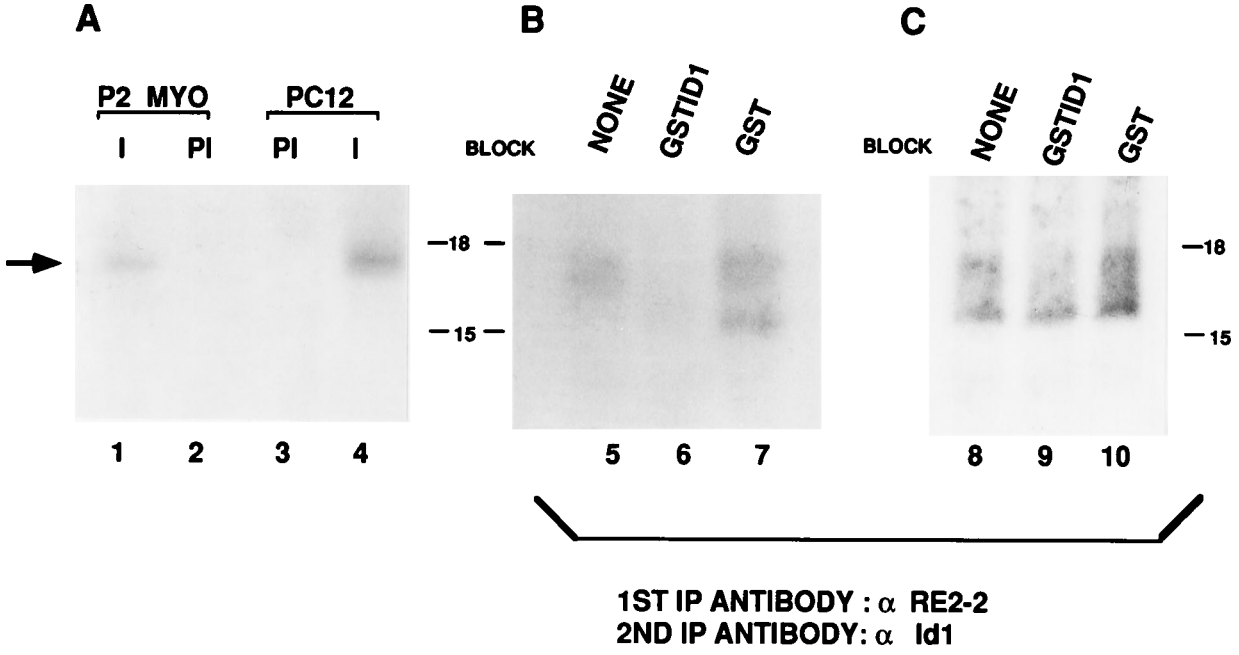

FIG. 7. In vivo association of Id1 and RE2-2 in PC12 cells. (A) ^{35}S-labeled lysate from P2 myoblasts (P2 Myo) (lanes 1 and 2) or PC12 cells (lanes 3 and 4) immunoprecipitated with anti-Id1 antiserum (I) or preimmune antiserum (PI). The position of Id1 is indicated (arrow). (B) ^{35}S-labeled, untreated PC12 cells. Immunoprecipitation at low stringency was performed first with anti-RE2-2 antiserum and then with a RIPA wash of anti-RE2-2 immunocomplexes. The material from the RIPA wash was then immunoprecipitated at high stringency with anti-Id1 antiserum which had not been preincubated (lane 5) or which had been preincubated with GST-Id1 (lane 6) or with GST (lane 7). (C) ^{35}S-labeled PC12 cells treated with NGF (50 ng/ml) for 24 h. Immunoprecipitation was performed at low stringency with anti-RE2-2 antiserum followed by immunoprecipitation of a RIPA wash of anti-RE2-2 immunocomplexes at high stringency with anti-Id1 antiserum which had not been preincubated (lane 8) or which had been preincubated with GST-Id1 (lane 9) or with GST protein (lane 10).

sights into the roles of these transcription factors during neurotrophin action.

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