Identification and Characterization of a Retinoic Acid-regulated Human Homologue of the unc-33-like Phosphoprotein Gene (hUlip) from Neuroblastoma Cells*

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A cDNA, 7G1, was isolated from retinoic acid (RA) differentiated neuroblastoma cells whose expression was high in human fetal brain and spinal cord mRNA but undetectable in adult brain or non-neuronal tissues. Sequence analysis indicates that 7G1 is homologous to the Caenorhabditis elegans gene unc-33. A 5.5-kilobase pair full-length cDNA from a human fetal brain cDNA library contains an 1710-base pair open reading frame. Because the predicted 570 amino acid sequence of 7G1 shares 88% identity with the murine Ulip gene product, an unc-33-like phosphoprotein, we refer to 7G1 as the human Ulip (hUlip). hUlip is also similar to the bacterial enzyme D-hydantoinase and the recently described vertebrate gene products CRMP62, TOAD-64, CRMP1, CRMP2, and mUNC. RA stimulates an increase in hUlip mRNA that is transcriptionally regulated. RA stimulates an increase in polypeptides of 58, 60, 65, and 70 kDa with the 58- and 65-kDa species being dephosphorylated forms of the 60- and 70-kDa species. This study presents a model in which to study the regulation and expression of the hUlip gene, a member of an emerging family of molecules that potentially mediates signals involved in axonal outgrowth.

Human neuroblastomas (NB) are a unique in vitro model in which to explore the cellular and molecular mechanisms that regulate the growth and differentiation of human peripheral nervous system tissue (1, 2). In the presence of biological response modifiers such as retinoic acid (RA) (3), increases in cAMP (4), phorbol esters (5), and interferons (6), the proliferation of these tumor cells is decreased, and there is an increase in neurite extension and neurotransmitter expression, and cells acquire some electrophysiologic properties similar to normal neurons. Several NB cell lines treated in vitro with RA increase Trk gene expression are induced to change the expression of several molecular markers, thus recapitulating steps of the normal embryonic development (7–10). Our studies on the mechanisms of RA-induced differentiation of NB cells show that RA induces an increase in TrkB mRNA transcription as well as protein production. Furthermore, in neuroblastoma cell lines constitutively producing BDNF, RA-induced TrkB expression leads to an activation of the TrkB signal transduction pathway that stimulates neurite extension and differentiation (11). These data suggest that RA may stimulate gene expression and lead to the activation of signal transduction pathways usually suppressed in the transformed NB cell.

To isolate molecular effectors important during activation of the in vitro differentiation of human NB cells, we screened a cDNA library made from NB cells treated for 14 days with RA and identified several genes whose expression changed during RA treatment. One such gene named 7G1 was isolated and found to detect a 5.5-kb mRNA species that was markedly increased after RA treatment of NB cells (12, 13). DNA sequence analysis indicates that 7G1 shares a striking homology to the Caenorhabditis elegans unc-33 gene (14). To isolate the full-length hUlip cDNA, five hundred thousand phage from a human fetal brain cDNA library, hUlip expression in human tissues was detected by Northern blot analysis and in vitro transcription analysis, and cells acquire some electrophysiologic properties similar to normal neurons. Several NB cell lines treated in vitro with RA increase Trk gene expression are induced to change the expression of several molecular markers, thus recapitulating steps of the normal embryonic development (7–10). Our studies on the mechanisms of RA-induced differentiation of NB cells show that RA induces an increase in TrkB mRNA transcription as well as protein production. Furthermore, in neuroblastoma cell lines constitutively producing BDNF, RA-induced TrkB expression leads to an activation of the TrkB signal transduction pathway that stimulates neurite extension and differentiation (11). These data suggest that RA may stimulate gene expression and lead to the activation of signal transduction pathways usually suppressed in the transformed NB cell.

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and a \(^{32}\)P-labeled 5-kb insert of hUlip clone 1 or GAPDH. Washing conditions were as described (24). Membranes were exposed to X-Omat AR film at \(-70^\circ\)C using a intensifying screen. Nuclear RUN-ON assay was performed as described previously (26).

In Vitro Transcription and Translation of the hUlip Gene—A pBluescript SK\(^{+}\) phagemid containing hUlip cDNA clone 2 served as a template for in vitro transcription using the T7 polymerase Ribo MAX kit (Promega), and the resulting mRNA was translated in vitro with rabbit reticulocyte lysate (Promega) in the presence of \([^{35}\text{S}]\)methionine.

For the immunoprecipitation, the translated product was diluted 10-fold with protein extraction buffer (1% Nonidet P-40, 20 mM Tris, pH 8.0, 157 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml of aprotinin, 20 mM leupeptin, 1 mM sodium vanadate, 10 mM NaF) and incubated with anti-peptide A serum and protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) overnight at 4 \(^\circ\)C. After washing with extraction buffer, immunoprecipitates were eluted by boiling 5 min in 1 \(\times\) protein sample buffer (1 \(\times\) = 62.5 mM Tris, pH 6.0, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue) and analyzed by SDS-PAGE.

Protein Analysis—Cells (1 \(\times\) 10\(^6\)) were plated in 100-cm\(^2\) dishes for 24 h and treated with indicated concentrations of RA or control solvent for the indicated times. Cells (1 \(\times\) 10\(^6\)) were lysed in 1 ml of protein extraction buffer at 4 \(^\circ\)C for 30 min, insoluble material was removed by centrifugation at 10,000 \(\times\) g, and protein concentration was determined by protein assay kit (Bio-Rad). 20 \(\mu\)g of proteins were electrophoresed on 10% SDS-polyacrylamide gels (PAGE) and transferred to nitrocellulose. Filters were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20 (TBST) and hULIP proteins were detected using an antiserum (1:1000) raised against Peptide A. Peptide A corresponds to amino acids 499–511 of hULIP and ULIP. The anti-peptide A serum is specifically blocked by Peptide A and not the corresponding peptide in the TOAD-64/rCRMP2 gene.3 The anti-peptide A serum was originally described to detect the TOAD-64 protein, which is now known to be Ulip/rCRMP4 (14, 15). The blots were washed, and bound antibodies were detected with the ECL kit (Amer sham Corp.).

For protein phosphatase treatment, after cells were lysed with lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.5, 157 mM NaCl, 2 mM MgCl\(_2\), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml of aprotinin, 20 mM leupeptin, 0.1 mM dithiothreitol), 20 \(\mu\)g of protein extracts were incubated with 15 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) at 37 \(^\circ\)C for 30 min and analyzed by autoradiography.

**RESULTS**

Expression and Regulation of hUlip—The hUlip gene was originally isolated as a partial cDNA clone (7G1) from a cDNA library generated from NB cells that had been treated for 14 days with RA (12). To study the kinetics of RA inducibility of the hUlip gene, we analyzed RNA isolated from SMS-KCNR NB cells at various times after treatment. An increase in hUlip steady-state mRNA levels was not detected after 15 min of RA treatment (Fig. 1A, lane 2). However, within 1 h a small increase in hUlip mRNA was detected (Fig. 1A, lane 3) that was markedly increased after 2 days of RA treatment (Fig. 1A, lane 4). hUlip expression peaked between 4–8 days (Fig. 1A, lanes 5 and 6) and remained relatively unchanged up to 18 days after RA treatment (Fig. 1A, lanes 7 and 8). At this time, the transcriptional induction of hUlip gene was approximately 20-fold higher in RA-treated than in control cells (Fig. 1B).

This is not due to a generalized increase in transcription stimulated by RA because the specific transcription of the retinoblastoma and CDC2 genes was decreased less than 2-fold after RA treatment, whereas N-myc transcription was reduced approximately 50-fold by RA as has been previously described (24). The transcriptional regulation of hUlip is consistent with a previous observation that RA stimulated a 2-fold increase in 7G1 transcription in nuclei from cells treated for 2 days with RA and a 22-fold induction in nuclei from cells after 14 days of RA treatment (13).

A Northern analysis of normal human fetal and adult tissues indicated that hUlip mRNA is detected in 59-day fetal brain (Fig. 1C, lane 1) and an increased level of hUlip mRNA is observed in a 135-day brain sample (lane 7). However, two different samples of adult brain mRNA (regions undefined) did not express hUlip mRNA (lanes 8 and 9). Detectable levels of hUlip mRNA are also found in fetal spinal cord mRNA but not in adult dorsal root ganglion or peripheral nerve mRNA (Fig. 1C, lanes 10, 11, and 12). Expression of hUlip was not detected in RNA samples from adult muscle, fetal limb, adrenal, or pancreas (Fig. 1C, lanes 1, 2, 3, 13, and 14).

Sequence Analysis and Comparisons—The 7G1 clone contained a 3.7-kb cDNA, and recent sequence analysis comparisons showed that 7G1 shared homology with the C. elegans gene unc-33. The highest homology matched a 3’ region of the unc-33 cDNA. Using a polymerase chain reaction generated 800-bp DNA fragment to the most 5’ region of the 7G1 cDNA as a probe, we isolated a full-length cDNA insert of 5.5 kb from a human fetal brain library. Sequencing of both strands and computer analysis of the 5.5-kb cDNA indicated an open reading frame of 1710 bp predicted a 62-kDa polypeptide with a pl of 6.4 (Fig. 2). Sequencing indicated that 7G1 was identical to this 5.5-kb clone in the 3’ region of the molecule. A more detailed nucleotide data base search revealed several highly homologous sequences. The highest homology, 98% identity at
FIG. 2. Nucleotide sequence and amino acid translation of 7G1 and hUlip. hUlip nucleotide sequence and the putative translation in amino acids according to IUPAC single letter code. The underlined region corresponds to the region of overlap with the 7G1 clone. The boxed area represents the peptide used to generate anti-peptide A serum.
protein level, was observed with the mouse unc-33-like phosphoprotein Ulip (14) (Fig. 3). The human sequences, hCRMP1 and hCRMP2 (17), showed a lower level of homology ranging from 68% (hCRMP2) to 75% (hCRMP1) (data not shown).

Amino acid sequence comparisons of the Ulip gene revealed domains within the Ulip protein that share significant homologies with the \( \beta \)-hydantoinase protein from \( Pseudomonas \) \( putida \) (14). During the process of sequence analysis, we noted that the hUlip gene product shared a significant number of conserved amino acid residues with the protein sequence of the \( B. \) \( stearothermophilus \) \( \beta \)-hydantoinase enzyme (36% homology) and the \( C. \) \( elegans \) unc-33 gene product (30% homology). By alignment of these three proteins we identified four conserved internal domains, A, B, C, and a D region of homology within C in which the level of identity was significantly higher than the average (60–76%) (Fig. 4). Further analysis is required to assess if these regions may be significant in the function of the hUlip protein.

**Analysis of hULIP Protein Expression and Regulation**—To identify the hUlip gene product, we prepared in vitro translated hULIP protein. The 2-kb hUlip mRNA that encompassed the coding region of hUlip was transcribed by T7 polymerase in vitro from the pBluescript II SK(−) hUlip cDNA clone 2 and translated into protein using a rabbit reticulocyte lysate in the presence of \(^{35}\text{S}\)methionine and analyzed by SDS-PAGE (Fig. 5A). \(^{35}\text{S}\)Methionine-labeled proteins ranging from 35 to 60 kDa are detected, and the 60-kDa labeled protein corresponds to the expected size from the deduced amino acid structure of the hUlip gene.

To study the regulation of hULIP proteins, we used a rabbit anti-peptide A serum (18), which is raised against the peptide YDGPVFDLTTTPK (amino acids 499–511; Figs. 3 and 6). To determine whether the anti-peptide serum recognizes the hULIP protein, the in vitro translated hULIP protein was reacted with specific anti-peptide A or a control antiserum in the absence or the presence of the immunizing peptide, and the immunoprecipitates were analyzed by SDS-PAGE (Fig. 5A). These results indicate that the mRNA transcripts synthesized from hUlip cDNA translate a 60-kDa protein that is recognized by the anti-peptide A serum and competed out by co-incubation with the immunizing peptide. Immunoprecipitation with a normal rabbit antiserum did not detect hUlip proteins. The smaller peptide fragments synthesized by the rabbit reticulocyte lysate may represent truncated protein products of the hUlip gene, because immunoprecipitation of these truncated proteins by the anti-peptide A serum was also inhibited by the immunizing peptide (Fig. 5A).

FIG. 3. Sequence comparison between hUlip and Ulip. Comparison of hUlip protein sequence with the highly related mouse Ulip protein showing 98% of identity in amino acid residues. Mismatched amino acid residues were indicated by asterisks. Shaded boxes represent the consensus sites for protein kinases such as protein kinase C (Ser14, Ser147, Thr172, Ser214, Thr229, Ser256, and Ser258), casein kinase II (Thr264, Ser275, Ser281, Ser303, Thr313, Thr315, Ser326, Ser329, Ser332, Thr343, and Thr345), protein kinase A (Ser368, Ser372, and Ser375), and proline-directed kinases (Ser404, Thr414, and Ser416). Potential \( N \)-myristoylation sites were indicated by dots.
of the family because the corresponding peptides (amino acids 499–511) from hUlip-related proteins. The black background indicates identical residues with peptide A. In rUlip/rCRMP4, because only 5’ end of the coding region has been reported, its amino acid sequence of this region is not available (15).

The finding that peptide A (499–511 amino acids) specifically inhibits antibody binding to all hUlip proteins (Fig. 5B) indicates that these four proteins all contain an identical antigenic epitope. The p70, p65, and p60 hUlip proteins are detected within 48 h after RA stimulation, the p58 hUlip protein is detected after 9–14 days of RA treatment. The differential regulation of hUlip proteins suggests that they may be translational modifications of a smaller number of proteins. Studies of ULIP, the murine counterpart, indicate that it is a phosphoprotein (14). To test whether the different proteins detected by the anti-peptide A serum in RA-treated NB cells may be phosphorylated forms of a single protein, protein extracts from 6 day RA-treated KCNR NB were incubated with alkaline phosphatase at 37 °C. After a 30-min incubation, proteins were resolved by SDS-PAGE, blotted, and analyzed with the anti-peptide A serum (Fig. 5D). Western analysis of phosphatase treated extracts resulted in the detection of proteins with apparent molecular masses of 70, 65, 60, and 58 kDa. These studies indicate that the p70 and p60 hUlip proteins may be phosphorylated forms of the p65 and p58 proteins, respectively. The kinetics of detection of the underphosphorylated forms of the proteins suggests that RA may be inducing a specific phosphatase activity.
hULIP protein expression was evaluated in three neuroblastoma cell lines whose differentiation response when treated with RA was variable. In KCNR, RA induced a high level of expression of all three hULIP related proteins in KCNR (Fig. 5E, lane 2). In the NGP NB cell line, the 70- and 60-kDa hULIP proteins are constitutively expressed (Fig. 5E, lane 3), and the level of expression is not significantly altered by RA (Fig. 5E, lane 4). RA is a poor inducer of differentiation in the cell line SY5Y (11), and expression of hULIP proteins in SY5Y (Fig. 5E, lanes 5 and 6) was at a level not detected when compared with the levels of hULIP proteins in KCNR and NGP. Prolonged exposure of blots revealed that RA did induce hULIP in SY5Y (data not shown).

**DISCUSSION**

This paper describes the identification, cloning, and sequence of the human homologue of the mouse Ulip (14) and the rat CRMP4 (15) gene. An analysis of fetal and adult human tissues indicates that hULIP is developmentally regulated like its rodent counterparts. By utilizing RA-induced differentiation of human neuroblastoma cells, we have found that the hULIP gene is transcriptionally regulated during the process of induced differentiation, and there is a dramatic increase in hULIP protein expression during neurogenesis.

hULIP is a member of a family of evolutionarily conserved and structurally related genes. Homologous sequences were isolated from different species: d-hydantoinase from *P. putida*, unc-33 from *C. elegans* (16), CRMP62 from chicken (17), the rat gene TOAD-64 (19), the mouse genes Ulip (14) and mUNC, 4 the human genes CRMP1 and CRPM2 (17) several EST sequences (28), and four rat genes CRMP1-4 (15). The presence of multiple sequences with different levels of homology even within the same specie suggests that multiple unc-33-like genes are present among the vertebrates. hULIP has an overall homology with the bacterial enzyme d-hydantoinase and the *C. elegans* unc-33 of only 36 and 39%, respectively, yet it is possible to detect areas of the proteins that have a higher level of identity in the primary sequence (Fig. 4). A previous study identified at least three areas of homology that may represent functional domains in the protein by comparison of Ulip with the d-hydantoinase of *P. putida* (14). However, by comparing hULIP to the d-hydantoinase of *B. stearothermophylus*, we have detected an additional region D within the C region with a high number of conserved residues present in the human, bacterial, and worm genes that may have functional significance. To date it has not been possible to assign enzymatic functions to these domains in other unc-33-like proteins (CRMP62 and Ulip), and studies are in progress to address this issue in hULIP. It may be possible that those conserved areas underlie functional domains of the protein. Such a hypothesis is supported by the evidence that the function of the chicken unc-33 homologue, CRMP62, as a collapsin response mediator may be suppressed by injecting an antibody raised against a peptide within the first conserved domain A into the cell (17).

Clues to the role these proteins play during differentiation have been inferred from the functional identification of several members of this family; all members share homology with unc-33, a *C. elegans* mutant with uncoordinated movements and defects in axonal outgrowth (16); chicken CRMP1 was identified as a mediator of collapsin-induced growth cone retraction (17); and TOAD-64 is one of the earliest and most abundant proteins expressed in post-mitotic neurons during corticogenesis and migration, yet its expression decreases dramatically in adult neural cells (18, 19); Family members have an intracellular cytoplasmic location and the proteins, including hULIP, contain consensus sites of phosphorylation for protein kinase C, casein kinase II, protein kinase A, and "proline-directed" kinases as well as N-myristoylation sites. This has lead to speculation that these proteins may be intracellular mediators of collapsin signal transduction and play a role in axonal guidance during neurite outgrowth.

The analysis of hULIP distribution in fetal and adult human tissue samples of the central and peripheral nervous system is consistent with the reports for the tissue distribution of the murine homologue of hULIP as well as other members of this gene family in other species (14–19). We found a high level of specific mRNA in fetal brain and spinal cord samples but not in any of the non-neuronal and adult tissues examined. This finding may underlie a tissue-specific regulation of hULIP expression and suggests that, like its rodent counterparts, it has a specific role in developing the human nervous system. Members of this family of genes have been almost exclusively localized in rodent neural tissues by *in situ* analysis (15, 17), although Ulip expression has been found in muscle localized at the neuromuscular junction (14).

The finding of differential expression and regulation of hULIP in neuroblastoma cell lines enables biochemical and functional studies of the hULIP gene and protein to be performed that may be important in determining its functional role during neural development. The expression of hULIP mRNA by Northern blot analysis was increased in RA-treated SMS-KCNR cells after 24–48 h of treatment reaching the peak at 8 days and stabilizing thereafter (Fig. 1A). The increase in hULIP parallels the kinetics of neurite extension that peak 6–8 days after RA treatment (24). This increase in the steady-state levels of hULIP mRNA is due in part to an increase in gene transcription as shown by RUN-ON assays (Fig. 1B), indicating that chromatin changes are required to activate the expression of this gene in differentiating neuroblasts. The kinetics of induction of hULIP transcription increase gradually with time after RA treatment (2-fold at 2 days, 20-fold at 7 days (Fig. 1B), and 22-fold at 14 days (13)), indicating that the effects of RA may be indirect and not mediated by RA receptors. hULIP mRNA expression is also increased in RA-treated NB cells at a time in which cells have arrested in G1, of the cell cycle and neuritogenesis begins. This is similar to the pattern of expression of TOAD-64 that is absent in the mitotic precursors of corical neurons but is highly expressed once cells have stopped dividing (18, 19). However, constitutive hULIP expression can be detected in the proliferating neuroblastoma cell line, NGP, and this suggests that arrest of cell growth may be coincident and not required for expression of hULIP in neuroblastoma. Conversely, it is possible that during normal development hULIP expression is coordinately regulated with arrest of cell growth, yet this process is disrupted as a consequence of genetic changes leading to tumorigenesis in some neuroblastoma cell lines.

During RA-induced growth arrest and neurite extension in KCNR NB cells, four immunologically related hULIP proteins with apparent molecular masses of 58, 60, 65, and 70 kDa were detected. The specificity of the anti-peptide A antiserum to hULIP, contain consensus sites of phosphorylation for protein kinase C, casein kinase II, protein kinase A, and "proline-directed" kinases as well as N-myristoylation sites. This has lead to speculation that these proteins may be intracellular mediators of collapsin signal transduction and play a role in axonal guidance during neurite outgrowth.

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detected later after RA treatment and in lower abundance compared with p60 and p70, respectively. This suggests that during RA-induced differentiation a phosphatase may be induced. The products of the chicken, rat, and mouse genes, CRMP62, TOAD64, and Ulip, have been shown to be phosphoproteins. In particular the phosphorylation of several proteolytic peptides are altered after nerve growth factor treatment of PC12 cells, indicating a possible involvement of the unc-33-like proteins. In particular the phosphorylation of several proteo-

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