Alternative Splicing of the High Affinity cAMP-Specific Phosphodiesterase (PDE7A) mRNA in Human Skeletal Muscle and Heart*

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To further our understanding of the structure and function of phosphodiesterases of the newly identified family of high affinity cAMP-specific phosphodiesterases (PDE7), we identified and characterized the isoform expressed in human skeletal muscle and the protein product of the previously isolated isozyme HCP1 (designated HSPDE7A1). We report the isolation of a cDNA encoding the full-length skeletal muscle isoform of human PDE7A (HSPDE7A2). The DNA sequence of this skeletal muscle cDNA indicates that PDE7A2 is a novel 5′ splice variant of PDE7A encoding an isoform with a novel, hydrophobic N terminus. The 456-amino acid PDE7A2 protein is detected on Western blots as a band with an apparent mobility of 50 kDa. PDE7A2 is a high affinity cAMP-specific PDE (K_m = 0.1 μM), which is localized to particulate cellular fractions. The PDE7A1 (HCP1) isoform is detected on Western blots as a band with an apparent mobility of 57 kDa, demonstrating that the previously isolated HCP1 cDNA encodes the full-length PDE7A1 protein. The even distribution of PDE7A mRNA among fetal tissues and the relative abundance of its two mRNAs strongly suggest that the expression of PDE7A is regulated throughout development.

Cyclic nucleotides are second messengers involved in a variety of cellular responses to hormones, light, and neurotransmitters. Cyclic nucleotide phosphodiesterases (PDEs)

In addition to the diverse properties of the seven known families of cyclic nucleotide PDEs, PDEs of various families exhibit extensive isozyme diversity, which is derived from the existence of several genes within a family and is compounded by extensive alternative mRNA splicing most prevalent and well documented in the cases of the cAMP-specific PDEs (PDE4) and the calcium, calmodulin-regulated PDEs (PDE1; Refs. 1 and 14–20). While the functional consequences of this diversity are not fully understood, differences in tissue-specific expression and alternative splicing patterns have profound effects not only on tissue distribution of PDEs, but also on their subcellular localization, their kinetic properties, and on their sensitivity to cofactors (1, 6, 8, 10, 19).

Six of the PDE families were identified biochemically as activities fractionated from various tissues and cultured cell lines (1). The seventh family of PDEs consists of a single gene encoding a high affinity cAMP-specific PDE, named HCP1 (designated PDE7A). PDE7A was isolated in a genetic screen for complementation of defects of yeast cells deficient in cAMP PDEs (5). Biochemical characterization demonstrated the high affinity cAMP-specific PDE activity of PDE7A and its insensitivity to potent selective inhibitors of known PDE families. The presence of PDE7A-like activities in cell lines derived from T cells, and their absence from cell lines derived from B cells, was subsequently noted (21). Coupled with its distinct amino acid sequence, these biochemical and pharmacologic properties established PDE7A as a member of a novel, uncharacterized, PDE family.

Among adult human tissues PDE7A is most abundantly expressed in skeletal muscle, and its mRNA is detectable in most tissues examined thus far (5). A 3.8-kb PDE7A mRNA is expressed in skeletal muscle and heart, and a 4.2-kb mRNA is found in the brain, lung, kidney, and pancreas. The HCP1 cDNA isolated in the yeast screen is a partial clone derived from the 4.2-kb mRNA species found in human U118 glioblastoma cells.

We report the isolation of a human PDE7A cDNA derived from the skeletal muscle mRNA and the identification of PDE7A-encoded proteins. This skeletal muscle mRNA encodes an N-terminal alternatively spliced PDE7A isoform. We demonstrate that alternative splicing regulates the subcellular localization of PDE7A-encoded proteins.

EXPERIMENTAL PROCEDURES

Library Screening, Plasmids, DNA Manipulations, and Sequencing—A human skeletal muscle cDNA library (Clontech) was screened using established procedures (22) with nick-translated [α-32P]dCTP-labeled probe derived from the 5′ end of HCP1 (0.4-kb NotI-BglII fragment) and mixed with a 1/50 trace of the 0.3-kb BglII-EcoRI fragment of HCP1. Three classes of overlapping clones, which together span the entire PDE7A2 ORF were cloned and the polyadenylated 3′-untranslated region, were isolated (represented by HSM4; -7, and -14). The inserts from these phage isolates were subcloned into pKS (Strat-
agene) and were subjected to bidirectional fluorescent dyeoxy chain termination nucleotide sequencing using the ABI373A automated sequencer. A 1.5-kb DNA fragment of the HSM7 cDNA was cloned into the yeast expression vector pDa44, containing the ADH1 promoter, a 2 μ replication origin, and the LEU2 selectable marker (pADHS7M). The nucleotide sequence of HSM7 (HSPDE7A2) was submitted to GenBank™.

Fetal Tissues—Fetal tissues were obtained from the late first and second trimester pregnancies and were frozen immediately in liquid nitrogen at the time of harvesting. This study was approved by the Committee on Clinical Investigations of the Albert Einstein College of Medicine, and written consent was obtained from all mothers for the donation of fetal tissues.

Northern Blot Analysis—Total tissue RNA was isolated using TRIzol reagent (Life Technologies, Inc.) and established methods (23). Ten μg of RNA was fractionated on a 1% formaldehyde-agarose gel and transferred onto a nylon membrane (GeneScreen Plus, NEN Life Science Products). Hybridization was performed at 42 °C using nick-translated [α-32P]dCTP-labeled probes under hybridization conditions, stringent washing procedures, and probe stripping protocols described by Michaeli et al. (5). Blots were first hybridized to a 1.2-kb NotI-EcoRI HCP1 fragment probe, and after probe stripping to a 0.75-kb PstI-XbaI human GADPH fragment probe. Blots were subjected to autoradiography onto X-Omat film and onto a Molecular Dynamics PhosphorImager and were quantitated using the NIH Image program.

Reverse Transcriptase Polymerase Chain Reaction—Reverse transcriptase polymerase chain reaction (PCR) was performed on 5 μg of RNA prepared from fetal human tissues according to the manufacturer’s instructions (Life Technologies, Inc.). Controls lacking reverse transcriptase were included in the reactions. To verify the structure of the 5′ end of the PDE7A2 mRNA the following oligonucleotides were used: for reverse transcription, oligo(dT); 724 (GGCCCTGAGTAACATCCGC) (nt 752); and 22-1-9 (AGAGACAGACACTTCAAT) (nt 390). Oligonucleotides used for PCR amplification were: the 5′ oligonucleotide 7+2 (GGGATACCTGTTGGAAAGG) (nt 2–19) was used in all reactions, and it was paired with the following 3′ oligonucleotides: oligonucleotide 7-1 (AGGACGACAGATCTTCAAT) (nt 140) to give a 139-bp PCR product and with oligonucleotide 250 (GGCCCTGAGTAACATCCGC) (nt 278) to give a 277-bp PCR product. As a positive control the 5′ oligonucleotide 256 (nt 284) was paired with oligonucleotide 724 (nt 752) to give a 469-bp PCR product.

To determine the presence of the PDE7A1 and -2 mRNAs in RNA prepared from fetal tissues by reverse transcriptase PCR the following oligonucleotides were used: the 3′ oligonucleotide 22-1-9 was used in all reactions, and it was paired with the following 5′ oligonucleotides: oligonucleotide 22Met1 (5) to give a 311-bp PCR product specific to PDE7A1 and with the 5′ PDE7A1 oligonucleotide 22Met1 (5) to give a 311-bp PCR product specific to PDE7A1.

Antiserum Production and Purification—A rabbit polyclonal antiserum was generated against a 15-amino acid-long synthetic peptide derived from the C terminus of PDE7A (CELSNQLTQTENRLS, Cys added for coupling) which was coupled to maleimide-activated keyhole limpet hemocyanin. This antiserum detects both HSM7 and HCP1 of the HSM7 sequence is identical to that of HSM7, containing novel 5′ sequences that provide an initiator methionine and an N terminus, which is distinct from the one previously reported for HCP1 (Fig. 1). The initiator methionine of HSM7 is preceded by an in-frame termination codon at position –123. The sequence of the HSM7 5′ end was confirmed by reverse transcriptase PCR analysis of fetal skeletal muscle RNA, which was followed by determination of the DNA sequence of the amplified product (see “Experimental Procedures”). While the 5′ end and the N terminus of HSM7 are unique, the remainder of the HSM7 sequence is identical to that of HCP1 throughout the coding region, the 3′-untranslated region, and the polyadenylation site. Thus, human skeletal muscle PDE7A mRNA, represented by the 3.1-kb HSM7 cDNA, and the glioblastoma cell-derived transcript represented by HCP1 are alternatively spliced transcripts of PDE7A. The published HCP1 transcript is therefore designated HSPDE7A1, and the skeletal muscle splice variant HSM7 is designated HSPDE7A2.

HSM7 (PDE7A2) contains an open reading frame encoding a protein of 456 residues with a predicted molecular mass of 52.7 kDa. The 20 N-terminal residues of PDE7A2 are unique to the skeletal muscle isozyme, are hydrophobic, and contain potential sites of myristoylation (glycine residue two) and of palmitoylation (cysteine residue eight). Both PDE7A1-encoded proteins contain a potential cAMP-dependent protein kinase phosphorylation site (serine 58 of PDE7A2). PDE7A1 (HCP1) has two N-terminal repeats each containing a cAMP-dependent protein kinase pseudosubstrate site, but the PDE7A2 alternative splice preserves only one of these sites (Fig. 1B). Thus, while alternative splicing may contribute to post-translational modifications unique to PDE7A2, both PDE7A1-encoded proteins contain motifs that may involve complex interactions with the cAMP-dependent protein kinase.

Expression of PDE7A mRNAs in Fetal Tissues—The expression of PDE7A during early human development was examined by analyzing RNA obtained from fetal tissues. In contrast to the strong predominance of a 3.8-kb mRNA in the adult skeletal muscle, fetal tissues contained comparable amounts of PDE7A RNA, which was composed of both its 4.2- and 3.8-kb mRNAs (Fig. 2). Reverse transcriptase PCR analysis, which distinguished between the PDE7A1 and -2 transcripts, demonstrated the presence of the two independent, alternatively spliced PDE7A mRNAs in the examined fetal tissues (Fig. 3). Thus, it is unlikely that the 3.8-kb PDE7A RNA found in these tissues is a degradation product of the 4.2-kb PDE7A RNA. Normalization of the combined amounts of the two PDE7A mRNAs to those of the housekeeping gene GAPDH mRNA, indicated that PDE7A mRNAs were 2–3-fold more abundant in...
the brain than in the heart, skeletal muscle, kidney, and lung (Fig. 2). Heart and skeletal muscle contained significant amounts of both PDE7A mRNAs, with a relative predominance of the 3.8-kb mRNA, while brain, kidney, and lung contained primarily the 4.2-kb PDE7A mRNA. Thus, both the tissue distribution and the relative abundance of the two PDE7A mRNAs differ considerably among adult and fetal human tissues.

Expression of PDE7A Proteins in Yeast and in Fetal Tissues—To identify the proteins encoded by the two PDE7A mRNAs, we analyzed the expression of PDE7A proteins in yeast and in human fetal tissues on Western blots (Figs. 4 and 5). PDE7A proteins were immunodetected with an anti-PDE7A antiserum raised against a C-terminal peptide common to both PDE7A-encoded proteins. To assess the specificity of the antibodies and the molecular weights of PDE7A-encoded proteins, we examined the expression of PDE7A1 and -2 in yeast and in fetal kidney (Fig. 4). Specific PDE7A-encoded proteins were immunodetected in yeast cells bearing PDE7A expression plasmids and in fetal kidney (Fig. 4A). The detection of PDE7A proteins by anti-PDE7A antibodies was blocked by preincubation of the antibodies with a C-terminal peptide of PDE7A, thereby demonstrating the specificity of the protein bands detected by the anti-PDE7A antibodies (Fig. 4B). The PDE7A1 protein expressed in yeast contained an N-terminal tag derived from influenza hemagglutinin (HA) and migrated as a 59 kDa protein. A smaller PDE7A protein, which migrated as a 57-kDa protein, was found in the soluble fraction of human fetal kidney, as expected for the native, untagged, PDE7A1 protein (Fig. 4A). The PDE7A2 product expressed in yeast migrated as a 50-kDa protein, indicating that, as predicted by its sequence, the PDE7A2 product is smaller than that of PDE7A1. An N-terminally tagged HA-PDE7A2 protein was immunodetected with anti-PDE7A and with anti-HA antibodies as a 52-kDa protein, demonstrating that the 50-kDa PDE7A protein is the full-length product of the PDE7A2 cDNA expressed in yeast (not shown). These observations demonstrate that PDE7A1 encodes a protein with an apparent mobility of 57 kDa and that the PDE7A2 cDNA we isolated encodes a protein with an
apparent mobility of 50 kDa.

To identify PDE7A-encoded proteins and their subcellular localization in fetal tissues, we analyzed the soluble and the particulate fractions of human brain, heart, kidney, lung, and skeletal muscle. A representative subcellular distribution of PDE7A proteins of fetal tissues is depicted in Fig. 5. Immunodetection of PDE7A proteins on Western blots demonstrated that both the soluble and particulate fractions of human brain, heart, kidney, lung, and skeletal muscle contained a 57-kDa PDE7A protein, while a 50-kDa PDE7A protein was present only in the particulate fractions of the heart and skeletal muscle. The fraction of particulate 57-kDa PDE7A protein was significantly higher than the fraction of lactate dehydrogenase activity detected in particulate fractions, suggesting that the localization of the 57-kDa PDE7A to soluble and particulate fractions is not due to cross-contamination (see "Experimental Procedures"). In agreement with the distribution of PDE7A mRNA in fetal tissues, and on the basis of the apparent molecular weights of PDE7A1 and -2 proteins expressed in yeast, it appears that the 57-kDa protein found in the soluble and particulate fractions of all five fetal tissues is the PDE7A1 product, and the particulate 50-kDa protein of heart and skeletal muscle is the PDE7A2 product. The localization of PDE7A2 to particulate fractions is consistent with the hydrophobicity of its N terminus. Taken together, these observations strongly suggest that the PDE7A1 gene product has an apparent mobility of 57 kDa and is located mostly to soluble cellular fractions, and that the PDE7A2 gene product has an apparent mobility of 50 kDa and is located to particulate cellular fractions.

Biochemical Analysis of PDE7A2—To analyze the biochemical properties of PDE7A2 we prepared crude cell extracts from transformants of the PDE-deficient yeast strain 10DAB bearing a PDE7A2 expression plasmid. PDE assays were performed as detailed under "Experimental Procedures" using cAMP as a substrate. Measurable cAMP PDE activity was detected in extracts of 10DAB cells expressing PDE7A2, but not in extracts of 10DAB cells bearing an expression vector that lacked an insert (not shown). The effect of substrate concentration on the velocity of cAMP hydrolysis was determined. The $K_m$ deduced from the linear and the double-reciprocal Lineweaver-Burke plots was 0.1 ± 0.01 $\mu$M cAMP, and the calculated $V_{max}$ was 0.0135 ± 0.001 nmol/min/mg (± standard deviation, Fig. 6). PDE7A2 did not hydrolyze cGMP, and its cAMP PDE activity was not significantly affected by the presence of a wide range of concentrations of cGMP or of the PDE4-specific inhibitor rolipram (see "Experimental Procedures"). These observations demonstrate that PDE7A2 is a high affinity cAMP-specific PDE with kinetic and pharmacologic properties characteristic of PDE7 and distinct from those of PDE3 and 4. Comparable amounts of PDE7A1 and -2 were present in yeast extracts (Fig. 4A), and it therefore appears that the $K_m$ and $V_{max}$ values of PDE7A2 were roughly 2-fold lower than those of PDE7A1, the high affinity cAMP-specific PDE we characterized previously (5). Thus, alternative splicing of PDE7A7 predominant in human skeletal muscle and heart tissues produced small effects on the kinetic properties of PDE7 and drastic effects on its subcellular localization.

**DISCUSSION**

Molecular cloning of cDNAs encoding cyclic nucleotide PDEs demonstrated their extensive isozyme diversity, derived from the existence of multiple gene families and from complex splicing patterns within some PDE families (1, 14–20). Isozyme diversity within the newly isolated high affinity cAMP-specific PDE family, PDE7, is due to the existence of two PDE7A mRNAs. Of these two mRNAs, PDE7A2 is the most prevalent PDE7A mRNA in adult human tissues with abundant expression in skeletal muscle and detectable expression in the heart (5). To further the understanding of PDE7, we isolated a cDNA encoding the human PDE7A skeletal muscle isoform (designat-
ed HSPD7A2). The structure of the PDE7A2 cDNA indicates that the skeletal muscle isoform is a 5′ splice variant of PDE7A with a novel N terminus. The alternative splice of PDE7A2 directs its localization to a particulate cellular fraction.

The PDE7A2 mRNA encodes a variant PDE7 protein with a novel, hydrophobic, 20-residue-long N terminus. We examined the PDE7A2 product in human fetal tissues and as a recombinant protein in yeast and identified the PDE7A2 product as a protein with an apparent mobility of 50 kDa. In agreement with the expression of PDE7A2 mRNA in fetal skeletal muscle and heart, and consistent with the hydrophobicity of its N terminus, the PDE7A2 protein is found in the particulate fractions of fetal skeletal muscle and heart. PDE7A2 is a high affinity cAMP-specific PDE possessing a $K_m$ of 0.1 μM toward its substrate. Thus, in comparison with the properties of the high affinity cAMP-specific PDE, PDE7A1 (HCP1), alternative splicing yields only small effects on the kinetic properties of PDE7A isoforms, but has a dramatic effect on their subcellular localization.

The analysis of PDE7A2-encoded proteins expressed in yeast and in fetal tissues demonstrated that although the HSM7 cDNA isolated in this study is incomplete, it contains the entire coding region of PDE7A2. A similar analysis of the expression of PDE7A1 proteins demonstrated that PDE7A1 encodes a protein with an apparent mobility of 57 kDa. These observations strongly suggest that the incomplete PDE7A1 cDNA we previously isolated contains the complete coding region of PDE7A1 (5). Thus, this study identifies the complete coding regions, and the protein products, of PDE7A1 and PDE7A2. The mRNAs of both PDE7A1 and -2 contain long 5′-untranslated regions of up to 1.1 and 0.7 kb, respectively, that may regulate their expression. In light of the persistent difficulties in cloning in the 5′-untranslated region of the PDE7A1 mRNA, the identification of the PDE7A gene products provides a strong basis for studies of PDE7A function.

Analyses of PDE activities of skeletal muscle do not detail the presence of PDE7-like, high affinity cAMP-specific PDE activities, although the expression of cAMP-specific PDEs (PDE4) has been demonstrated (26–31). Our analysis of PDE7A2 expression demonstrated a strong and direct correlation between the amounts of PDE7A RNA and proteins in several fetal tissues, including the skeletal muscle. Thus, at least in fetal tissues, translational regulation of PDE7A2 is not a plausible cause for the inability to detect PDE7 like activities in skeletal muscle. Rather, it is possible that PDE7-like activities could not be detected in skeletal muscle, and perhaps also in B cells, because only soluble fractions were analyzed, thereby precluding the detection of PDE7A2 activity. Future studies of PDE activities of particulate fractions of skeletal muscle are required to address these issues.

Compartmentalization of cellular proteins is integral to signal transduction. In cAMP signaling, compartmentalization of the cAMP-dependent protein kinase to particulate cellular fractions via the interactions of its regulatory subunit with anchor proteins has been demonstrated in many cell types (reviewed in Refs. 32–34). Compartmentalization of the cAMP-dependent protein kinase is required for cAMP-mediated regulation of glutamate receptor channels (35). In the case of PDE7A, alternative splicing determines the subcellular localization of its protein products without drastically affecting their kinetic properties. While the association of PDE7A2 with particulate fractions can be mediated via hydrophobicity and modifications of its N terminus, the mechanism of partitioning of PDE7A1 between particulate and soluble fractions, most pronounced in the brain, is not clear. As PDE7A1 may be tethered to particulate fractions via protein-protein interactions, its partitioning may be due to the existence of different cell types within a tissue, which either lack, or express different, anchor proteins. Although the functional consequences of the compartmentalization of PDE7A activities are not known, similar effects of N-terminal splicing variability has been demonstrated for PDE4A (10). Three alternatively spliced PDE4A products exhibit 2–5-fold differences in their $V_{max}$ values and small differences in their $K_m$ values and in their sensitivity to the PDE4-specific inhibitor, rolipram. However, these three alternatively spliced PDE4A products vary considerably in their subcellular distribution by being completely, mostly, and weakly particulate (PDE4A1 -8, and -5, respectively). Thus, as is the case for the cAMP-dependent protein kinase, compartmentalization of PDEs via structural changes that are the product of alternative splicing or via dynamic modifications and protein-protein interactions may be fundamental to the performance of isozyme-specific roles in cellular signaling.

The relatively even distribution of PDE7A RNA among human fetal tissues is in sharp contrast to the predominance and overabundance of the PDE7A mRNA in the adult skeletal muscle (5). Unlike adult tissues, several fetal tissues contain both PDE7A1 and -2 mRNAs, perhaps as a result of their expression in different cell types within the tissue. The dramatic reduction of PDE7A expression throughout development observed in most tissues and its persistence and predominance in adult skeletal muscle strongly suggest that the expression of PDE7A is regulated throughout development. Additional studies are required to determine whether the fluctuations in PDE7A mRNA levels are reflected in its activities and to evaluate their functional significance in development. However, a specialized role for PDE7A in muscle signal transduction is suggested by the restricted expression of its splice variant PDE7A2 to adult skeletal muscle and heart and its predominance in fetal skeletal muscle and heart, tissues in which cAMP plays modulatory physiological and developmental roles.

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Note Added in Proof—During the review of this manuscript, similar observations on PDE7A1 of T cells were published by Bloom and Beavo (Bloom, T. J., and Beavo, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14188–14192.)
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