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cDNA SEQUENCE ANALYSIS OF THE HUMAN BRAIN INSULIN RECEPTOR

Kathleen A. Kenner*, Jyotirmoy Kusari[£], and Kim A. Heidenreich[ϑ †]

*Department of Medicine, Division of Endocrinology/Metabolism, University of California, San Diego, La Jolla, California 92093, and the Medical Research Service, Veterans Administration Medical Center, San Diego, California 92161

[£]Department of Physiology, School of Medicine, Tulane University Medical Center, New Orleans, Louisiana 70112

[ϑ]University of Colorado Health Sciences Center, Denver, Colorado 80262 and the Veterans Affairs Medical Research Center, Denver, Colorado 80220

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Brain tissue mRNA was amplified using polymerase chain reaction (PCR) with eight overlapping sets of primers that span the cDNA coding sequence for the human placental insulin receptor. Only the A isoform (lacking exon 11) of the receptor was detected. No difference was found in the predicted amino acid sequence of brain derived insulin receptor cDNA compared with the receptor from human placenta. A silent polymorphism was detected at nucleotide position 1698 (amino acid 523), confirming that mRNA corresponding to both alleles of the human brain receptor was sequenced. Our findings indicate that the unique glycosylation properties of brain insulin receptors do not stem from differences in primary structure, but rather are due to tissue-specific differences in post-translational processing.

The insulin receptor is a heterotetrameric glycoprotein located within the external plasma membrane, and is responsible for mediating the cellular responses specific to insulin (for review see 1). Insulin receptors are translated from a single open reading frame and then post-translationally modified by cleavage into separate α and β subunits, and by the addition of N-linked oligosaccharides (2-4). Insulin receptors are comprised of two α subunits and two β subunits, which are joined by disulfide bonds. The α subunits are extracellular and contain the insulin binding region. The β subunits traverse the plasma membrane and contain insulin-dependent tyrosine kinase activity within the cytoplasmic domain. Insulin induced receptor tyrosine kinase activity is believed to play a role in insulin signal transduction, by initiating a cascade of phosphorylation events that leads ultimately to insulin's biologic effects.

†To Whom correspondence should be addressed: Kim A Heidenreich, the Department of Pharmacology C-236, University of Colorado Health Sciences Center, 4200 East 9th Ave., Denver, Colorado 80262. Fax (303) 377-5685.

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The brain responds to insulin in a manner that is unique among insulin target tissues. In contrast to classic peripheral target tissues such as liver, adipose and muscle, in which the major action of insulin involves the regulation of glucose homeostasis, acute glucose metabolism in the brain is virtually unaffected by insulin (5). On the other hand, various aspects of neuronal development and neuromodulation are regulated in an insulin-responsive manner. In neuronal cultures, insulin supports the growth of neurons in the absence of other growth factors (5-7). In addition, insulin increases neuronal protein synthesis (8,9) and stimulates the accumulation of transcripts for tubulin and neurofilament proteins (10). Both of these events coincide with increased neurite outgrowth (6). Insulin also promotes the emergence of neurotransmission at nascent synapses of fetal cholinergic retinal neurons (11), and promotes the differentiation of dorsal root ganglion cells into catecholaminergic neurons (12).

Although brain insulin receptors have properties that are similar to those of peripheral receptors, including catalytic traits and temperature and pH dependency (13), a number of physical and functional properties are unique. The binding of insulin to brain receptors does not display negative cooperativity (13,14) as it does in peripheral receptors. Brain insulin receptors bind certain insulin analogs with higher affinity than peripheral receptors (13) and express different antigenic determinants (15). The most distinguishing structural feature of insulin receptors in brain is the reduced size of the receptor subunits. After denaturing gel electrophoresis the apparent molecular weights of the α and β subunits from brain insulin receptors are 115,000 and 85,000, respectively. These molecular weights are 5,000-10,000 smaller than their counterparts in peripheral tissues (16) and the size discrepancy can be accounted for by differences in N-linked glycosylation. The unique structure of insulin receptors in the brain is a characteristic that has been conserved throughout animal species as diverse as mammals, birds, reptiles and amphibians (17).

Crude analysis of the primary structure of insulin receptors from rat brain and adipose tissues by peptide mapping has suggested that the peptide backbones may be homologous. Northern blot analysis of insulin receptor transcripts from rat brain and adipose tissues has provided similar evidence (18). However, these techniques could have failed to detect small differences, resulting from brain-specific splicing of the insulin receptor message. Alternative splicing of the insulin receptor message is known occur, generating at least two distinct isoforms that differ by the presence of 12 amino acids at the carboxyl end of the α subunit. The two resulting insulin receptor isoforms are expressed in distinct ratios in various tissues (19). This alternative splicing event cannot account for the structural heterogeneity described above, but does not preclude the presence of another similar event in brain. In order to determine if insulin receptors from brain have unique primary structure, we undertook the sequence analysis of the entire coding region of the insulin proreceptor from human brain and compared our results with previously published sequence information derived from human placental insulin receptor cDNA (20, 21).

Materials and Methods

Materials

Post-autopsy adult human brain was obtained from Dr. Henry Powell (Department of Pathology, University of California, San Diego, La Jolla, CA). B2-(2-nitro,4 azidophenyl)-des-Phe^{B1}-insulin (NAPA-DP-insulin) was a gift of Dr. Dietrich Brandenburg (Deutches Wolforschunginstitut, Aachen, Germany). $\gamma[^{32}P]$ATP (6000 μCi/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). *Thermus aquaticus* (*Taq*) polymerase was purchased from Perkin-Elmer Cetus (Norwalk, CT). Moloney murine leukemia virus (MmULV)- reverse transcriptase, AMV reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase, and deoxynucleotides were purchased from Gibco-Bethesda Research Labs (Gaithersburg, MD) or Promega (Madison, WI). Dideoxynucleotides were purchased from Pharmacia Biochemicals Inc. (Milwaukee, WI). Reagents for sequencing with "Sequenase 2.0", including T7 DNA polymerase and deoxy- and dideoxy nucleotides were purchased from United States Biochemical (Cleveland, OH). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Photoaffinity Labeling

Insulin receptors were photo-affinity labeled using the photoreactive analog of insulin B2-(2-nitro, 4-azidophenyl)-des-Phe^{B1}-insulin (NAPA-DP-insulin). This compound is an insulin agonist and has been used previously to specifically label insulin receptors on target tissues (22, 23). The preparation and binding characteristics of this photoprobe have been previously reported (24). An aliquot of brain or placental membranes was incubated with [^{125}I]NAPA-DP-insulin (40 ng) in minimal essential media containing 10 mM Hepes (pH 7.5) and 1% BSA. Incubations were carried out in the dark at 16°C for 30 min.

Nonspecific binding was determined in the presence of 10 μg/ml of unlabeled insulin. At the end of the incubation, samples were placed on ice, 10 cm under a 20 watt long wave UV lamp (366 nm) for 3 min. After photolysis, the membranes were washed 3 times to remove unbound hormone and then solubilized in 2X Laemmli sample buffer (25), containing 2 mM phenylmethylsulfonyl fluoride and 50 mM dithiothreitol.

Preparation of Total Cellular RNA

Total cellular RNA was isolated from post-mortem adult human brain by centrifugation through cesium chloride, according to the method of Chirgwin (26). Briefly, frozen tissue was rapidly homogenized in 50 volumes of DEPC-treated water, containing 4 M guanidium isothiocyanate, 0.05 M tris-Cl, pH 7.5, 0.01 M Na-EDTA, and 5 % (v/v) β-mercaptoethanol. The tissue homogenate was centrifuged at 8,000 x g for 10 min at 12°C, and the subsequent supernatant was retained. Sarkosyl was added to a final concentration of 2%. RNA was then isolated by centrifugation through a CsCl cushion, followed by phenol/chloroform extraction and ethanol precipitation, and stored at -70°C.

DNA Synthesis and Amplification

Eight distinct, overlapping sets of single stranded 5' and 3' oligonucleotide DNA primers complementary to the human placental insulin receptor mRNA sequence (20,21) were used to amplify insulin receptor-specific sequences from human brain RNA using polymerase chain reaction (PCR, 27). Collectively, these primers spanned the region between nucleotides 126 and 4269 (Figure 2), according to the numbering system of Ullrich et. al. (20). This region included the coding sequences of both the α and β subunits of the human placental insulin receptor. To avoid the subcloning step for sequencing PCR-amplified DNA, the 5' oligonucleotide of each set of primers contained a T7 phage promoter sequence (TAATACGACTCACTATAGGGAGA) at its 5' end. Thus the 5' end of the amplified DNA contained this phage promoter and could be used for direct sequence analysis (28). A specific first-strand cDNA copy of the insulin receptor mRNA sequence was made using the 3' primer of each set as a primer for reverse transcriptase. Synthesis and subsequent amplification of the cDNA was carried out according to the methods previously described by Kusari et. al. (29).

Direct Sequencing

After PCR the amplified DNA was directly transcribed and sequenced using the T7 phage promoter and AMV reverse transcriptase, according to the procedure described earlier by

Kusari et. al. (29). The nucleotide sequence of some regions of the amplified DNA was difficult to analyze using reverse transcriptase. In these cases (nucleotides 2454-2466 and 2520-2534) the sequence was determined by Sequenase (United States Biochemical Corp., Cleveland, OH), using single stranded DNA as the template (30).

Results and Discussion

Differences in the size of mature insulin receptor subunits from brain and peripheral tissues have been demonstrated in a number of species (17,31). To confirm that subunit size differences are also found in human tissues, receptors from human brain and placenta were photo-affinity labeled with [^{125}I]NAPA-DP-insulin. After photolysis and washing to remove noncovalently bound hormone, proteins were solubilized in Laemmli sample buffer, containing 50 mM dithiothreitol and analyzed by SDS-PAGE. Labeled proteins were visualized by autoradiography. Only the receptor α subunit was labeled using this technique. As shown in Figure 1, the α subunit from human placenta displayed a relative molecular weight of 125,000, whereas the corresponding subunit from human brain displayed a lower relative molecular weight of 115,000.

To ascertain whether differences exist in the human brain insulin receptor primary sequence, total cellular RNA was isolated from post-mortem human brain, and used as a source of insulin receptor mRNA. The nucleotide sequence of the human brain insulin receptor was determined and compared to the sequence published for the human placental

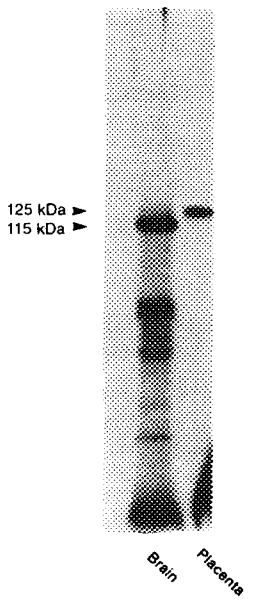

Figure 1. Autoradiogram of proteins from human brain and placenta covalently labeled with ^{125}I-NAPA-DP-insulin. Tissue homogenates were incubated with 40 ng/ml of ^{125}I-NAPA-DP-insulin at 16°C for 30 min. After photolysis and washing to remove noncovalently bound hormone, proteins were solubilized and resolved by sodium dodecyl sulfate gel electrophoresis through a 5-15% acrylamide gel under reducing conditions. The molecular weights of the specifically labeled proteins are indicated.

insulin receptor (20, 21). A schematic representation of the published insulin receptor message is shown in Figure 2. Analysis of the receptor's primary structure reveals that it is comprised of a series of distinct domains subserving specialized functions. A single open reading frame encodes both the α and β subunits. Viewed from left to right, the initial 5' untranslated region is followed by a 21 amino acid signal sequence. Adjoining this sequence is the extracellular α subunit, containing a cysteine-rich ligand binding domain. A short midspan sequence encodes the peptides at which the nascent protein will divide into the α and β subunits of the mature protein. Distal to the cleavage site is the β subunit, including a small extracellular region, a single transmembrane segment, and a large intracellular region containing the catalytic tyrosine kinase domain.

Eight overlapping sets of single stranded oligonucleotide primers were used to amplify regions of brain mRNA (A-H) by polymerase chain reaction (PCR). The locations of the amplified fragments are indicated along the schematic representation of the insulin receptor message. Arrows indicate the primer binding sites that served to delimit each of the amplified segments in the complimentary sense and antisense directions. The PCR primers were specifically synthesized so that the amplified cDNA products contained the T7

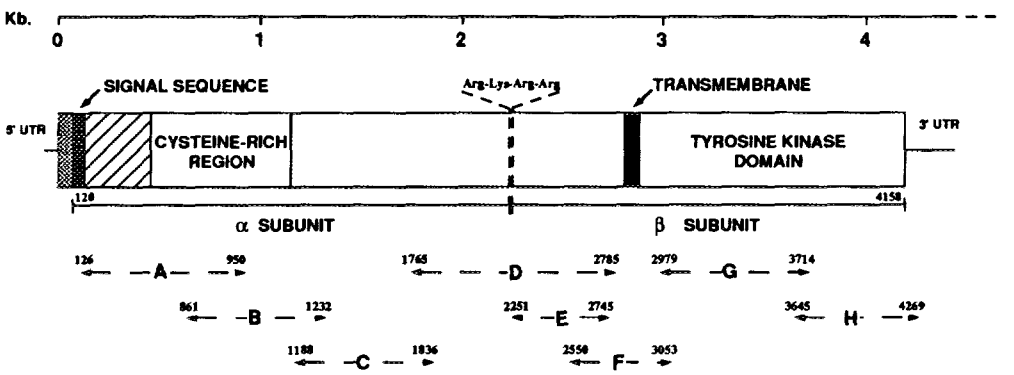

Figure 2. Structure of human insulin receptor (IR) message. The single open reading frame encodes both α and β subunits. The schematic contains a 5' untranslated region, followed by a 21 amino acid signal sequence. The α subunit is extracellular and contains a cysteine-rich putative ligand binding domain. The β subunit contains extracellular, transmembrane and intracellular regions, and has an intracellular tyrosine kinase domain. Eight overlapping sets of single-stranded oligonucleotide primers were used to amplify regions of mRNA (A-H) by polymerase chain reaction (PCR). The sequences of the upstream and downstream primers used for PCR amplification are as follows: A) **VA66**-GCATGGATATCCGGAACA, **956**-AGAACTCGCGGAGGCAAGGGCTGC; B) **VA79**-GTACTACCACTTCCAGGAC, **1238**-GAAGAAATTTCAGGGTATCT; C) **VA67**-AGCTAGAAGCCAACCTC, **1841**-GCCATCTTTGTGGAAGACCCTG; D) **VA87**-AGAACCACCCAGGGTGGCTGA, **J044**-GGAACTACAGCGTGCGAAT; E) **708**-TACCTGCACAACGTGGTT, **VA55**-GACACCCGCAAGCACTTC; F) **VA80**-GAAGCCAAGGCTGATGACA, **VA37**-TGCTCTGTGTACGTGCCGGA; G) **VA95**-ACCGCTTTACGCTTCTTCA, **VA91**-ACCAGCTTGGCAGAAC; H) **VA96**-GTCTTCACCACTTCTTCTGAC, **VA27**-ACTCTACCATGTCCAATGGA. The combined regions include the coding sequence for both the α and β subunits. Arrows indicate primer binding sites.

promoter at the 5' end. This modification provided several key advantages. The amplified cDNA was competent for use directly in sequence analysis, simplifying the sequencing procedure. In addition, transcription of PCR amplified cDNA into RNA during the sequencing procedure reamplified message specific fragments, greatly reducing the likelihood of producing inaccurate sequence results. Most importantly, this method enabled the simultaneous sequencing of a population of mRNA segments representing both gene alleles (27). PCR amplified fragments ranged in size from 366 to 1040 nucleotides in length. The sizes of the PCR products were confirmed by gel electrophoresis through 1% agarose, followed by ethidium bromide luminescence of the PCR product bands adjacent to DNA size standards (data not shown). The sizes of the PCR products determined after gel electrophoresis were comparable to the sizes calculated theoretically from the published human placental sequence (20). The coding sequences for both the α and β subunits were represented in the combined set of eight PCR fragments that were utilized in sequence analysis.

Since the primers used for PCR amplification were derived from the placental insulin receptor sequence, there is a small possibility that existing differences in brain primary structure were not detected. However, in order for this scenario to occur, the brain insulin receptor sequence would need to differ at two adjacent overlapping primer sites and within a region that was not amplified by other neighboring primer sets. Earlier studies have indicated that brain insulin receptors might contain subtle differences, but do not contain any extensive differences in primary structure (18). Therefore the use of overlapping PCR products virtually ensured the amplification and detection of brain receptor-specific sequences, even with the possible presence of background levels of peripheral receptor message.

Amplified insulin receptor PCR fragments were transcribed into RNA and sequenced using a modification of the dideoxy sequencing method (29). Each fragment was sequenced using 2 to 5 nested 19-oligonucleotide primers. Analysis of the brain receptor message enabled us to deduce its primary structure, and to compare its nucleotide sequence with that from the human placenta. By this method only the A isoform of the insulin receptor was detected (data not shown). Our data confirms a similar finding by Seino and Bell (19), derived using differential PCR techniques. A single sequence polymorphism was detected at nucleotide position 1698, using the numbering system of Ullrich et. al. (20). Figure 3 shows a partial nucleotide sequence from nucleotide 1695 to nucleotide 1701. Position 1698 is uniquely characterized by the concurrent appearance of two separate nucleotides. The two resulting codons, GCA and GCG both encode the amino acid alanine. Both sequence isoforms have been reported separately in previous reports, as indicated in Table 1 (20, 21, 32). Their subsequent coexpression here suggests that they are probably a naturally occurring silent polymorphism of the human insulin receptor gene.

A variety of tissue sources have been utilized for the analysis of human insulin receptor nucleotide sequences, and for the subsequent deduction of likely amino acid sequences.

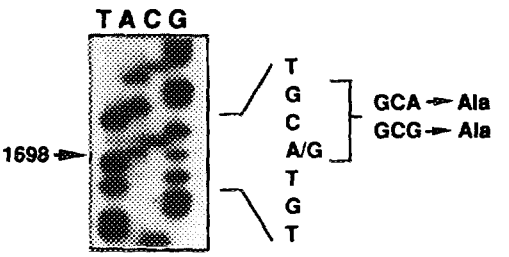

Figure 3. Partial nucleotide sequence of insulin receptor cDNA from human brain, containing a silent nucleotide polymorphism. At nucleotide position 1698, two separate nucleotide bands, (A) and (G), are detected (arrow), instead of a single band, (A) or (G). The two resulting codons, GCA and GCG, both encode the amino acid alanine.

Table 1 outlines differences that have arisen so far in the various reports. Confirmed sequence polymorphisms at nucleotide positions 957, 1686, and 1698 encode silent changes in the insulin receptor message. Two alternative nucleotides have also been reported at positions 559, 1391, 1522, 2711, 2713, and 3846. These could be either unconfirmed sequence polymorphisms or the results of sequencing errors. Sequence references are in order from left to right: 1) Ullrich et. al.(20); 2) Ebina et. al.(21); 3) Seino et. al. (32); 4) present data.

These data indicate that brain and peripheral insulin receptors have homologous primary structures. A report by Goldstein and Dudley (33) reviewed the occurrence of receptor isoforms resulting both from differences in receptor primary structure and from differences in processing of nascent receptors. Heteroduplex mapping was the method used to identify differences within insulin receptor transcripts from various rat tissues, including brain. With this method Goldstein and Dudley detected only the receptor heterogeneity resulting

Table 1: Comparison of insulin receptor nucleotide and amino acid sequences from various human tissues. Amino acids and nucleotides are numbered according to the numbering system of Ullrich *et al.* (1985). (*)Confirmed sequence polymorphisms; (underlined) unconfirmed sequence polymorphisms or sequencing errors. Sequence references are in order from left to right: 1. Ullrich *et al.* (1985), 2. Ebina *et al.* (1985), 3. Seino *et al.* (1989), 4. present data.

		Tissue Source							
Nucleotide	Amino Acid codon	Term Placenta cDNA 1		Term Placenta cDNA 2		Fetal Liver genomic DNA 3		Adult Brain cDNA 4	
559	144	TAC	Tyr	CAC	His	TAC	Tyr	TAC	Tyr
*957	276	CAG	Gln	CAA	Gln	CAA	Gln	CAG	Gln
1391	421	ATC	Ile	ACC	Thr	ATC	Ile	ATC	Ile
1522	465	CAG	Gln	AAG	Lys	CAG	Gln	CAG	Gln
*1686	519	GAC	Asp	GAT	Asp	GAT	Asp	GAC	Asp
*1698	523	GCA	Ala	GCG	Ala	GCG	Ala	GCA/G	Ala/Ala
2711	861	GAC	Asp	GTC	Val	GTC	Val	GTC	Val
2713	862	ACC	Thr	TCC	Ser	TCC	Ser	TCC	Ser
3846	1239	AAC	Asn	AAG	Lys	AAG	Lys	AAG	Lys

from the alternative splicing of exon 11, substantiating the data presented here. Our data indicate that differences in the relative molecular weight and functional characterisitics of brain insulin receptors result from different patterns of glycosylation and/or from other unknown effects of the unique cellular environment of the brain.

Acknowledgments

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