Molecular Cloning of a Novel Platelet Protein Showing Homology to the Angiotensin II Receptor C-terminal Domain*

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Oligoscreening of a cDNA library obtained from 4β-phorbol 12-myristate 13-acetate-stimulated human erythroblasts (HEL) resulted in the isolation of a novel clone coding for a protein with a calculated molecular mass of 8110 Da. This protein of 71 amino acids shows significant homology to the carboxy-terminal regulatory domain of angiotensin II type 1 receptors. The homology encompasses four regions of amino acid residues thought to serve as consensus sequences for phosphorylation by serine/threonine kinases such as protein kinase C, which are key mediators of intracellular signaling. Reverse transcription-polymerase chain reaction identified the transcript in human platelets, human megakaryocytic DAMI cells, and HEL cells. High stringency Northern blotting revealed a tissue-specific distribution of three transcript species, with predominant expression in skeletal muscle and pancreas. Rabbit anti-peptide antiserum was used to immunoblot protein lysates from washed resting platelets and from 4β-phorbol 12-myristate 13-acetate-stimulated DAMI and HEL cells. These immunoblots revealed the presence of an intense ~8-kDa protein band in platelets and HEL cells and a faint band of identical size in DAMI cells.

Cell growth and function are regulated through the action of a variety of extracellular ligands binding to specific receptors, initiating intracellular signaling mediated by sequentially activated protein kinases. Serine/threonine kinases such as protein kinase C are critically important enzymes modulating cellular function (1).

Platelets are the progeny of bone marrow megakaryocytes that circulate in blood to effect hemostasis and thrombosis. They are not only critically important for human physiology and pathobiology, but have also been used to study basic principles of cell signaling. Megakaryocytic cell lines such as HEL,1 DAMI, and MEG-01 have been used to study megakaryocyte biology as well as to provide RNA from which cDNA libraries have been constructed and the cDNA of numerous platelet proteins cloned (2).

Angiotensin II (AII) is a potent effector octapeptide that mediates cardiovascular, neuronal, and renal function (3). Biological effects of AII are mediated by two distinct receptors, termed AT1 and AT2. AT1 receptors have been cloned from several mammalian sources (4-9), and the AT2 receptor has been cloned from rats (10, 11). AII signaling in adult tissues is almost exclusively mediated by the AT1 receptor (3). AT1 and AT2 receptors share an amino acid identity of 32% (10, 11). The amphibian AII receptors that have been cloned are functionally similar to AT1 receptors, but do not bind the non-peptide AII-binding antagonist losartan (12, 13). All AT1 receptors and the amphibian AII receptors have several Ser/Thr residues in the carboxy-terminal cytoplasmic tail that may serve as substrates for phosphorylation by protein kinase C (14). This phosphorylation may drive the agonist-induced internalization of AT1 receptors (as well as other G protein-coupled receptors), resulting in cellular desensitization (15).

This report describes the isolation of a novel cDNA clone coding for a protein with a calculated molecular mass of 8110 Da. This protein shows significant homology to the regulatory C-terminal domains of the amphibian AII and mammalian AT1 receptors (59 and 45%, respectively, over a 40-amino acid stretch). This homology includes four consensus protein kinase C phosphorylation sites and a receptor internalization signal (14, 15). Immunoblotting with an anti-peptide antiserum demonstrates that the native protein is expressed in human platelets and cultured cells of human megakaryocytic lineage.

EXPERIMENTAL PROCEDURES

Isolation of the cDNA Clone—A 4β-phorbol 12-myristate 13-acetate-stimulated HEL cell cDNA library in λgt11 (CLONTECH) was oligoscreened simultaneously with two 30-mer oligoprobes (probe 1, 5′-GATGAACCICTICAGTCICCICCIGACCGI-3′; and probe 2, 5′-GAGGCCTICTACGCICCCICIGACAG-3′). The oligoprobes were based on the N-terminal sequence of a protein purified from shear-activated platelets (16). Phages (105) were screened, and one clone showed strong hybridization to the oligoprobes. Hybridization was done overnight at 55 °C in 6 × SSC, 0.5% SDS, 5 × Denhardt’s reagent, and 100 μg/ml sonicated denatured salmon sperm DNA. The final wash was performed in 2 × SSC, 0.5% SDS at 55 °C for 30 min. Plaques were lifted on Colony/PlaqueScreen hybridization transfer membranes (DuPont NEN) and were treated as described by the manufacturer. Methods for bacterial and phage plating, labeling of oligoprobes, and purification were as described by Wallace and Miyada (17). Techniques for prehybridization and hybridization and other routine molecular biology methods were as described by Sambrook et al. (18).

Cloning and DNA Sequencing—One phage that showed the strongest hybridization to the oligoprobes was plaque-purified. Phage DNA was isolated by the method of Elliott and Green (19). This DNA was digested with EcoRI, and a 1.8-kilobase insert was cloned in pCDNAIV (Invitrogen). DNA sequencing with purified recombinant plasmid (RP15a) was done with T7 and SP6 promoter primers and with internal sequence primers. A Sequenase kit (U. S. Biochemical Corp.) was used for plasmid sequencing.

Synthesis of all oligonucleotides was done by Operon Technologies, Inc. (Alameda, CA). Homology searches were done using EUGENE software at the Molecular Biology Computational Resource Center at the National Institutes of Health.

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1The abbreviations used are: HEL, human erythroblasts; AII, angiotensin II; AT1, receptor, angiotensin II type 1 receptor; AT2, receptor, angiotensin II type 2 receptor; RT-PCR, reverse transcription-polymerase chain reaction.
Baylor College of Medicine. The BLAST program of the National Center for Biotechnology Information was used for database comparisons.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from platelets and 100 ng/ml 4β-phorbol 12-myristate 13-acetate-stimulated HEL and DAMI cell cultures using RNA STAT-60 reagent (Tel Test “B” Inc., Houston, TX). Platelets were purified from 250 ml of freshly drawn human blood by the albumin density gradient method as described (20). RT-PCR was carried out with a Gene-Amp RNAPCR kit (Perkin-Elmer). RT reactions were done using random hexamers as primers. Two primers were used for PCR amplification (forward primer, 5'-GTGACTGGTTAAGGTGTGTGCCACTGTACATA-3' (nucleotides 57–86); and reverse primer, 5'-GGCTCTGGGCTGTGCTCAGTGTCTTTGGCC-3' (nucleotides 1761–1790)).

PCRs were carried out at 95°C for 2 min (one cycle), 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min (35 cycles) and with a final extension at 72°C for 7 min. RT-PCR products were electrophoresed in 1% agarose, transferred to GeneScreen Plus (DuPont NEN), and hybridized to a RNA probe generated from pRP15a using T7 RNA polymerase, [32P]CTP (DuPont NEN), and an in vitro transcription kit (Promega).

Northern Blotting—A human multiple tissue Northern blot panel was purchased from CLONTECH (Palo Alto, CA) and probed with labeled antisense RNA generated using SP6 RNA polymerase, [32P]CTP, and an in vitro transcription kit. Prehybridization and hybridization were done in 6 x SSC, 0.5% SDS, 5 x Denhardt’s reagent, and 100 µg/ml sonicated denatured salmon sperm DNA at 65°C. Unincorporated label was removed using a Bio-Spin 30 column (Bio-Rad).

Immunoblotting—A polyclonal antiserum was prepared by immunizing rabbits with a synthetic peptide comprising putative amino acid residues 5–19 (FCSLGDRAACSVITA), a region without homology to any known proteins or cDNAs. The synthetic peptide and antiserum were prepared by Genosys Biotechnologies, Inc. (The Woodlands, TX).

Lysates from albumin gradient-washed platelets and from 100 ng/ml 4β-phorbol 12-myristate 13-acetate-treated HEL and DAMI cells were prepared by adding to cells a solution containing 50% glycerol, 10% dithiothreitol, 8% SDS, and trace bromphenol blue, followed by immediate boiling for 10 min. SDS-polyacrylamide gel electrophoresis separations of 200 µg of lysate protein were performed on 10–20% gradient slab gels, and proteins were subsequently transferred to polyvinylidene difluoride membranes as described previously (20). Protein detection was carried out by established methods (18) using 3% skim milk in Tris-buffered saline as the blocking buffer. Goat anti-rabbit antibody conjugated to alkaline phosphatase (Life Technologies, Inc.) was used as the secondary antibody. In the competition experiments, 200 µg/ml peptide was added to the primary antibody-containing solution.

RESULTS AND DISCUSSION

Analysis of the nucleotide sequence of the cloned HEL cell cDNA (Fig. 1) shows that the putative synthesis of a 71-amino acid protein (pRP15a) begins at an initiation codon following the Kozak rules for eukaryotic protein synthesis initiation (21). The 1815-base pair cDNA has the consensus polyadenylation signal sequence (AATAAA) 19 bases from the 3'-end and a selective mRNA destabilization signal (ATTTA) in the 3'-untranslated region (22). This signal is also present in the AII receptor 3'-untranslated sequences (see below).

Comparison of amino acid residues 21–60 of pRP15a with the C-terminal domain of angiotensin receptors reveals significant homology (Table I). pRP15a shares 34% identity and 59% homology (identical plus chemically similar amino acids) with the amphibian AII receptor. pRP15a shares an average identity of 26% and an average homology of 45% with members of the family of mammalian AT1 receptors. The homology between pRP15a and AII receptors involves four potential consensus sequences for phosphorylation by Ser/Thr kinases such as protein kinase C (23). Hunyady et al. (15) have hypothesized that in the rat AT1 receptor and other G protein-coupled receptors, phosphorylation at these Ser/Thr residues drives ligand-induced receptor internalization. More specifically, they have identified the sequence Ser-Thr-Leu in the C-terminal domain of the rat AT1 receptor as crucial for receptor internalization. pRP15a has the sequence Ser-Phe-Leu (residues 32–34) (Table I).
in vitro for the Src family of protein tyrosine kinases. Its homology to the regulatory carboxyl-terminal region of mammalian AT1 and amphibian AII receptors supports the significance of the homology between pRP15a and the regulatory cytoplasmic C terminus of AT1 receptors remains to be established.

RT-PCR demonstrates that the transcript is present in platelets and in cultured platelet progenitor cell lines (Fig. 2). These results indicate that pRP15a gene expression occurs in cells of hematopoietic lineage (27).

High stringency Northern hybridization of a human multiple tissue blot (Fig. 3) shows that there is a tissue-specific distribution of transcripts of varying size. A transcript of ~1.3 kilobases is predominant in skeletal muscle, and pancreas shows two transcript species of ~1.65 and ~1 kilobases. Brain and liver also have low level expression of these transcripts (data not shown). These three transcript sizes do not correspond to the length of the 1815-basepair cDNA clone and may therefore represent either alternative processing of a common RNA precursor or four different gene products with a high degree of homology.

In vitro translation of pRP15a using rabbit reticulocyte lysate (Life Technologies, Inc.) and [35S]methionine produces a band with a molecular mass of ~8 kDa as well as a series of smaller internal peptides that often mask the 8-kDa protein (data not shown). Immunoblotting of protein lysates shows an intense narrow band of ~8 kDa in platelets and HEL cells and a faint band of identical size in DAMI cells. These bands are not seen on immunoblots performed in the presence of 200 μg/ml immunizing peptide (Fig. 4).

pRP15a is likely to be a membrane-associated protein because this protein could not be expressed in bacteria using a vector under the control of a prokaryotic promoter (pBlue-Script). This necessitated the cloning of the 1.8-kilobase insert into a eukaryotic expression vector like pcDNA1. Hydropathy analysis revealed a single N-terminal hydrophobic domain, thereby providing additional evidence that pRP15a is membrane-associated (data not shown).

In summary, pRP15a appears to represent a novel protein that may serve as a substrate for phosphorylation by protein kinases. Its homology to the regulatory carboxyl-terminal regions of mammalian AT1 and amphibian AII receptors supports the hypothesis that pRP15a may have some signaling function.

### Table I

Comparison of pRP15a (residues 21–60) with the carboxyl-terminal amino acids of AII receptors from Xenopus (xAT1) (12), human (hAT1) (6), bovine (bAT1) (5), rat (rAT1a) (4), rabbit (RbAT1) (8), and pig (PoAT1) (9) sequences have been aligned for maximum homology. Amino acids that are identical or chemically homologous to pRP15a are boxed. The asterisks indicate residues that are consensus sequences for phosphorylation by protein kinase C.

| Sequence | xAT1 | hAT1 | bAT1 | rAT1a | RbAT1 | PoAT1 | pRP15a |
|----------|------|------|------|-------|-------|-------|--------|
| 21–60    |      |      |      |       |       |       |        |

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