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CHARACTERIZATION OF A HUMAN cDNA THAT ENCODES A FUNCTIONAL RECEPTOR FOR PLATELET ACTIVATING FACTOR

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SUMMARY: We have cloned a cDNA for the platelet activating factor (PAF) receptor by screening an HL-60 granulocyte cDNA library with degenerate oligonucleotide probes based on conserved sequences of rhodopsin-type receptors. The 342-amino acid receptor contains 7 putative transmembrane domains, but lacks sites for N-linked glycosylation at the N-terminus. Stably transfected fibroblasts expressing the cloned cDNA responded to sub-nanomolar PAF stimulation with calcium mobilization, which could be inhibited by the PAF antagonist L-659,989. The PAF receptor message was detected as a single species of approximately 4 kb in human placenta, lung, and differentiated HL-60 granulocytes. Expression of the cloned cDNA in undifferentiated HL-60 cells devoid of endogenous PAF receptor resulted in specific and saturable binding of the PAF antagonist WEB 2086 with a dissociation constant of 30.7 nM.

Platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a lipid mediator that possesses diverse and potent effects on a variety of cells and tissues. PAF was originally identified as a soluble substance released from IgE-stimulated basophils that could aggregate platelets (1-3). Its cellular effects have since been found to involve the activation of neutrophils, protein phosphorylation, glycogenolysis, induction of expression of regulatory genes, and activation of arachidonic acid and phosphoinositide metabolites (reviewed in Ref. 4,5). PAF actions are mediated by specific receptors on the plasma membrane of target cells. These receptors are high-affinity binding sites for PAF, with dissociation constants in the nanomolar or sub-nanomolar range (chapters 6 and 8 in Ref. 5). Binding of PAF to the receptor correlates with its action, as both can be inhibited by specific PAF receptor antagonists. PAF was found to stimulate GTPase activity in platelets and neutrophils (6,7), and GTP specifically inhibits PAF binding to platelet and neutrophil membranes (8). These findings suggest that the PAF receptor is coupled to guanine nucleotide regulatory proteins (G proteins).

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Abbreviations: PAF, platelet activating factor; G proteins, guanine nucleotide regulatory proteins.

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The scarcity of PAF receptors on plasma membrane has so far hindered purification and structural studies of the receptor. We report here the isolation of a human PAF receptor cDNA with degenerate oligonucleotide probes based on the conserved sequence of rhodopsin-type receptors. Characterization of the cloned human PAF receptor revealed that it is a new member of the rhodopsin-type receptor family.

MATERIALS AND METHODS

cDNA cloning and sequencing. Poly(A)$^+$ RNA was prepared from HL-60 cells differentiated with dibutyryl cAMP (500 μM) for 46 h. A cDNA library was constructed from 10 μg of the RNA in the lambda gt11 vector, as described (9,10). The two probes used for this experiment were based on the conserved sequences of transmembrane domains II and VII of rhodopsin-type receptors and contained the sequence 5'-CTGAACCTNICCNTGGC(GT)GAC-3', and 5'-AACCC(CT)(AG)T(CT)AT(CT)TACTG(TC)T(GT)-3', where N was an equal molar mixture of all four bases. Approximately 300,000 plaques from the library were screened with the two probes labeled with [^{32}P]phosphate by phosphorylation of the 5'-OH as described (9) and two phage isolates with inserts of 1.5 (KP132) and 1.1 kb (KP101) were obtained. KP132 was subcloned into the PUC19 plasmid vector and sequenced to completion by the dideoxy method. Partial sequencing of the 1.1 kb insert (KP101) showed that it overlaps with KP132 but lacks the 5' portion of the coding sequence.

Expression of the cDNA. A PstI - EcoRV restriction fragment of the KP132 (nucleotide -63 to 1097, Fig. 1) was inserted into the mammalian expression vector SFFV-neo (11) to obtain KP132neo. The linearized plasmid (10 μg) was introduced into mouse L cell fibroblasts (ATCC CCL 1.1) by the calcium phosphate method (9). For transfection of HL-60 cells, 10 μg of the linearized vector was used for electroporation of 5X10^6 cells with a Bio-Rad Gene Pulser electroporator set at 200 V, 960 μF. Stably transfected cells were selected by there resistance to G418 and were collected as a group for functional analysis.

Calcium response. Transfected cells were assayed for ligand-induced calcium mobilization with the use of indo-1, as described before (10). Fluorescent measurements of calcium-bound and free indo-1 were made using an SLM 8000 photon-counting spectrofluorometer (SLM-Aminco), with excitation wavelength of 340 nm and emission wavelengths of 400 nm and 490 nm, respectively. Calcium levels were expressed as the ratio of emission at 400 nm to 490 nm.

Binding assay. Stably transfected HL-60 cells were washed once with PBS, and resuspended to 1X10^7 cells in 1 ml of binding buffer (10 mM HEPES, 145 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 0.5 mM Na_2HPO_4, 6 mM glucose, and 0.1% BSA, pH 7.4). Binding was initiated by the addition of [^{3}H]WEB 2086 (NEN, specific activity 14.1 Ci/mmol). After an incubation at 25°C for 1 h, unbound ligand was separated from bound ligand by centrifugation of the cells through silicon oil (12). The cell pellet and aqueous phase were counted with liquid scintillation spectrometry to determine bound and unbound ligand, respectively. Non-specific binding was measured by including PAF (20 μM) to the binding buffer.

Northern blot analysis. Poly(A)$^+$ RNA from undifferentiated and differentiated HL-60 cells (see above) was separated by formaldehyde agarose gel electrophoresis, and the RNA transferred to nylon membrane. An additional blot containing poly(A)$^+$ RNA from human tissues was obtained from Clontech Laboratories. An 1160-bp cDNA fragment (nucleotides -63 to 1097), labeled with [^{32}P]dCTP (specific activity, 7X10^8 cpm/μg DNA), was added to 2X10^6 cpm/ml in rapid hybridization buffer (Amersham), and the blots were hybridized at 65°C for 3 h. Final wash of the blot was at 65°C in 1XSSC, 0.1% SDS (plus 2% sodium pyrophosphate for the multiple tissue blot) for 15 minutes. The wet blot was exposed to Kodak XAR-5 film at -70°C for 4 days with an intensifying screen.

Sequence analysis. DNA and protein sequence analysis was conducted with the aid of computer softwares from the Genetics Computer Group (13).

Others. PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained from Calbiochem. PAF antagonist L-659,989 was a kind gift from Merck & Co.

RESULTS AND DISCUSSION

G protein-coupled receptors of the rhodopsin family share extensive sequence homology within the transmembrane domains (14). We utilized this feature for cloning the cDNA of human PAF receptor by synthesizing oligonucleotide probes based on these sequences (see "Materials and Methods"). The [^{32}P]-labeled probes were employed for screening a cDNA library made from granulocytic HL-60 cells, which express the PAF receptor (15). The insert from one of the two positive isolates (KP132) was sequenced in its entirety. KP132 contained a 5' non-coding sequence of 266 bp, an open reading frame of 1026 bp encoding a protein of 342 amino acids, a stop codon, and a 3' non-coding sequence of 256 bp (Fig. 1). The 3' non-coding sequence lacks a poly(A) tail and the consensus polyadenylation signal AATAAA, indicating that this is a partial cDNA. A search of the NFPR protein data base (release 27) with the deduced amino acid sequence did not find any identical proteins, but did reveal sequence homology to other G protein-coupled receptors. The deduced protein sequence was most homologous to the N-formyl peptide receptor sequence (16), with 29% identical residues overall. Hydropathy plot analysis (not shown) of the deduced protein sequence revealed 7 stretches of hydrophobic residues found in all rhodopsin-type G protein-coupled receptors. Thus, KP132 is a new member of the rhodopsin gene family.

The protein encoded by KP132 was identified as a functional PAF receptor based on its pharmacological properties in stably transfected cells. We first expressed the KP132 cDNA in mouse L cell fibroblasts, which we used for functional expression of the N-formyl peptide receptor (10). Stably transfected cells were tested for ligand-specific signal transduction by measuring calcium mobilization. Among a panel of chemoattractant ligands screened, the transfected cells responded to PAF (Fig. 2), but not to N-formyl peptide or C5a (not shown). Calcium response in transfected L cells was PAF dose-dependent between 10^{-11} M and 10^{-7} M of PAF, with half-maximal response at approximately $3X10^{-10}$ M (Fig. 2A). We subsequently found that L cells contained endogenous PAF receptors that contributed a significant portion of the calcium response at PAF concentration above 10^{-8} M, when the response nearly reached maximum (Fig. 2A). The PAF-induced calcium response appeared to be the result of an increase in the intracellular level of free calcium, as addition of EGTA (5 mM) did not alter the amplitude of the calcium response (not shown). PAF antagonist L-659,989 (17) inhibited the response in a dose-dependent manner (Fig. 2B). Pertussis toxin, at a concentration (100 ng/ml,

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                                        -266 CTGGTGGCCTTTAATACCTGGCTGTT -241
GCTGAAAGGTCTTTAGAAACGGCGCTAACAGCAGGTTTGTGGAATGCCGGATCGCTCAAC -181
GGCCTGACGTGGGCAAAAACCTCGCCTTCCGCACCCATCATTATATTGATGCTCATTGCC -121
GCCGCCTTACTGGTACGCCGGATGCGCTTGCTGGAAATGGGACACACGGTCACTGCAGCT  -61
GAAGCCGCTGCCCCTGCTACAGGCACCACCAGGACCAGCTGATCATTCCAGCCCACAGCA   -1

ATGGAGCCACATGACTCCTCCCACATGGACTCTGAGTTCCGATACACTCTCTTCCCGATT   60
 M  E  P  H  D  S  S  H  M  D  S  E  F  R  Y  T  L  F  P  I   20

GTTTACAGCATCATCTTTGTGCTCGGGGTCATTGCTAATGGCTACGTGCTGTGGGTCTTT  120
 V  Y  S  I  I  F  V  L  G  V  I  A  N  G  Y  V  L  W  V  F   40
                  I
GCCCGCCTGTACCCTTGCAAGAAATTCAATGAGATAAAGATCTTCATGGTGAACCTCACC  180
 A  R  L  Y  P  C  K  K  F  N  E  I  K  I  F  M  V  N  L  T   60

ATGGCGGACATGCTCTTCTTGATCACCCTGCCACTTTGGATTGTCTACTACCAAAACCAG  240
 M  A  D  M  L  F  L  I  T  L  P  L  W  I  V  Y  Y  Q  N  Q   80
          II

GGCAACTGGATACTCCCCAAATTCCTGTGCAACGTGGCTGGCTGCCTTTTCTTCATCAAC  300
 G  N  W  I  L  P  K  F  L  C  N  V  A  G  C  L  F  F  I  N  100

ACCTACTGCTCTGTGGCCTTCCTGGGCGTCATCACTTATAACCGCTTCCAGGCAGTAACT  360
 T  Y  C  S  V  A  F  L  G  V  I  T  Y  N  R  F  Q  A  V  T  120
       III

CGGCCCATCAAGACTGCTCAGGCCAACACCCGCAAGCGTGGCATCTCTTTGTCCTTGGTC  420
 R  P  I  K  T  A  Q  A  N  T  R  K  R  G  I  S  L  S  L  V  140

ATCTGGGTGGCCATTGTGGGAGCTGCATCCTACTTCCTCATCCTGGACTCCACCAACACA  480
 I  W  V  A  I  V  G  A  A  S  Y  F  L  I  L  D  S  T  N  T  160
          IV

GTGCCCGACAGTGCTGGCTCAGGCAACGTCACTCGCTGCTTTGAGCATTACGAGAAGGGC  540
 V  P  D  S  A  G  S  G  N  V  T  R  C  F  E  H  Y  E  K  G  180

AGCGTGCCAGTCCTCATCATCCACATCTTCATCGTGTTCAGCTTCTTCCTGGTCTTCCTC  600
 S  V  P  V  L  I  I  H  I  F  I  V  F  S  F  F  L  V  F  L  200
                                    V

ATCATCCTCTTCTGCAACCTGGTCATCATCCGTACCTTGCTCATGCAGCCGGTGCAGCAG  660
 I  I  L  F  C  N  L  V  I  I  R  T  L  L  M  Q  P  V  Q  Q  220

CAGCGCAACGCTGAAGTCAAGCGCCGGGCGCTGTGGATGGTGTGCACGGTCTTGGCGGTG  720
 Q  R  N  A  E  V  K  R  R  A  L  W  M  V  C  T  V  L  A  V  240

TTCATCATCTGCTTCGTGCCCCACCACGTGGTGCAGCTGCCCTGGACCCTTGCTGAGCTG  780
 F  I  I  C  F  V  P  H  H  V  V  Q  L  P  W  T  L  A  E  L  260
       VI

GGCTTCCAGGACAGCAAATTCCACCAGGCCATTAATGATGCACATCAGGTCACCCTCTGC  840
 G  F  Q  D  S  K  F  H  Q  A  I  N  D  A  H  Q  V  T  L  C  280

CTCCTTAGCACCAACTGTGTCTTAGACCCTGTTATCTACTGTTTCCTCACCAAGAAGTTC  900
 L  L  S  T  N  C  V  L  D  P  V  I  Y  C  F  L  T  K  K  F  300
                   VII

CGCAAGCACCTCACCGAAAAGTTCTACAGCATGCGCAGTAGCCGGAAATGCTCCCGGGCC  960
 R  K  H  L  T  E  K  F  Y  S  M  R  S  S  R  K  C  S  R  A  320

ACCACGGATACGGTCACTGAAGTGGTTGTGCCATTCAACCAGATCCCTGGCAATTCCCTC 1020
 T  T  D  T  V  T  E  V  V  V  P  F  N  Q  I  P  G  N  S  L  340

AAAAATTAGTCCCTGCTTCCAGGCCTGAAGTCTTCTCCTCCATGAACATCATGGACTGAG 1080
 K  N ---                                                     342

CTGGGGGAAGAAGGGATATCTACTGTGGTCTGGGCACCACCTCTGTGGGCACTGGTGGGC 1140
CATTAGATTTGGAGGCTACCTCACCTGGGCAGGGATGATGGCAGAGCCAGGCTGTTGGAA 1200
AATCCAGAACTCAAATGAGCCCCTTCATCCGCCTGTGGGGCATACTACAGTAACTGTGAC 1260
TTGATGACTTTATCTGAGTCCTTAT 1285
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Figure 1. Nucleotide sequence of the KP132 cDNA insert. Nucleotide residues are numbered beginning with the first initiation codon of the longest open reading frame. The 5' non-coding sequence is indicated by negative numbers and contains two stop codons in the same reading frame (-255 to -253; -21 to -19). The deduced amino acid sequence is shown beneath the nucleotide sequence with putative transmembrane domains underlined.

16 h) that reduced formyl peptide-induced calcium response by 90% in similarly transfected L cells (10), did not inhibit PAF-induced calcium mobilization (not shown).

Studies of the ligand-binding properties of the cloned PAF receptor was complicated by the endogenous PAF receptors in transfected L cells. We therefore expressed the cloned receptor in undifferentiated HL-60 cells devoid of endogenous PAF receptors (15). Stably transfected HL-60 cells were incubated with [^{3}H]WEB 2086, a specific PAF antagonist that

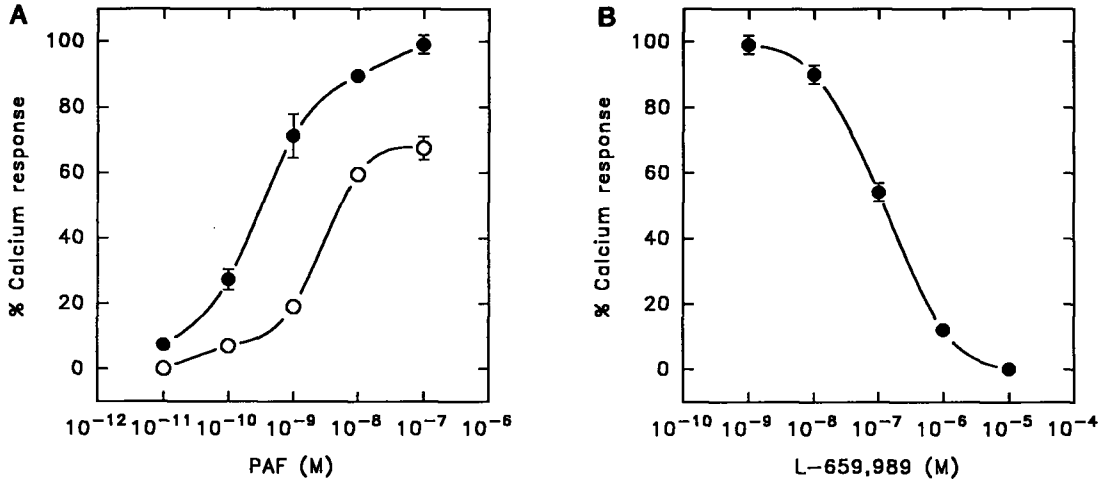

Figure 2. PAF-induced calcium response in transfected L cells. A. Calcium mobilization in L cells transfected with KP132neo (●) or with SFFV-neo vector only (○), in response to PAF of various concentrations. Calcium response was measured in duplicate, and the maximal response (100%) was obtained with 10^{-7} M PAF. Data shown (means and s.d.) are representative of two separate experiments. B. Inhibition of calcium response in KP132neo-transfected L cells by the PAF antagonist L-659,989. PAF was added to 1 nM throughout the experiment, and the maximal response was measured in the absence of L-659,989. Data shown are means and s.d. of duplicate measurements and are representative of two experiments.

recognizes the PAF binding site (18). The cells displayed specific and saturable binding to [^{3}H]WEB 2086, with 15,660 binding sites per cell (Fig. 3A). Untransfected cells or vector-transfected cells did not show significant binding (data not shown). Scatchard analysis, derived

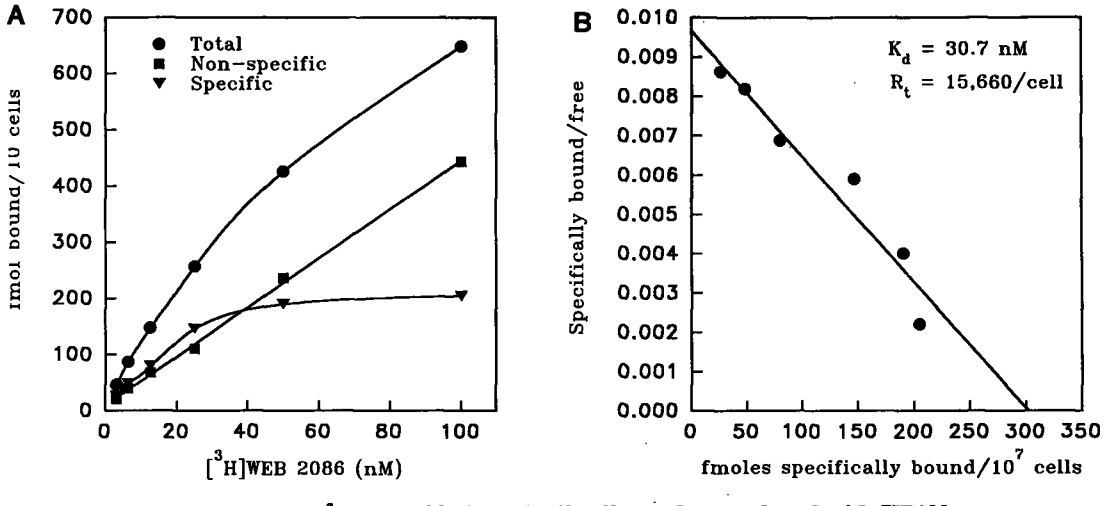

Figure 3. Binding of [^{3}H]WEB 2086 to HL-60 cells stably transfected with KP132neo. A. Equilibrium binding of [^{3}H]WEB 2086 was measured in duplicate in the absence (●) and presence (■) of 20 μM PAF. Each point represents the mean of duplicate determinations. B. Scatchard analysis of [^{3}H]WEB 2086 binding data from A.

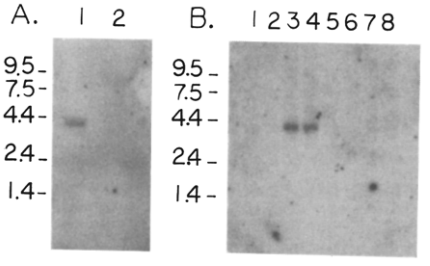

Figure 4. Expression and tissue distribution of the human PAF receptor message. An 1160-bp fragment of the PAF receptor cDNA was used to probe northern blots as described under "Materials and Methods". A. Each lane contains 5 μg of poly(A)$^+$ RNA from differentiated (lane 1) and undifferentiated (lane 2) HL-60 cells. B. Each lane contains 2 μg of poly(A)$^+$ RNA from the indicated human tissues: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. The mobility of RNA size standards is shown at the left of each panel.

from the saturation isotherm of specifically bound [^{3}H]WEB 2086, suggested a single class of binding sites with a dissociation constant of 30.7 nM (Fig. 3B). This value was comparable to the dissociation constants for PAF receptors in intact neutrophils (18.9 nM), guinea pig and human lung membranes (16.8 and 22.6 nM), determined with [^{3}H]WEB 2086 (19,20).

The transcript for the cloned PAF receptor cDNA was detected in differentiated HL-60 cells as a single species of approximately 4.0 kb (Fig. 4A). It was not found in undifferentiated HL-60 cells. The transcript was also detected in human placenta and lung, but was not found in heart, brain, liver, skeletal muscle, kidney, or pancreas (Fig. 4B). These tissues, however, may contain PAF receptor messages that are too low to be detected by the current procedure, since PAF has been shown to have biological effects on at least some of these tissues. The possible existence of an additional PAF receptor (21) in these tissues can not be ruled out.

In summary, the above results indicate that KP132 cDNA encodes a functional human PAF receptor. During the course of this work, Honda et al. (22) reported the expression cloning of a PAF receptor from guinea pig lung. Comparison of these two receptors revealed the following: (1) Both receptors contain 342 amino acids, with 82.75% sequence identity. (2) The human receptor does not have sites for N-linked glycosylation at the N-terminus, whereas the guinea pig receptor has an N-linked glycosylation site at position 4. (3) Northern blot analysis identified two additional species of PAF receptor message in guinea pig tissues that were not found in human tissues. This discrepancy may arise from the use of probes with different length. We used an 1160-bp fragment of the human PAF receptor cDNA that contains mostly coding sequence in order to minimize non-specific cross-hybridization by the 3' non-coding sequence. As discussed above, the possible existence of another PAF receptor remains to be investigated. The cloning of the PAF receptor provides a foundation for future studies of the structural and functional aspects of this interesting and biologically important receptor.

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