(Fig. 4*b*). The predicted intracytoplasmic loop between M5 and M6 is much smaller in the peptidergic receptors compared with the α- and β-adrenergic receptors, the dopamine and the serotonin (5-hydroxytryptamine) receptors. In neurotransmitter receptors, this segment is apparently regulatory along with the C terminus and confers specificity on the interaction with a particular class of GTP-binding protein[20]. The C5a receptor and the formyl peptide receptor in leukocytes perform pharmacologically similar if not identical functions, and the predicted cytoplasmic C region for these two receptors has ~50% sequence identity over 45 amino acids, with multiple serine and threonine residues as potential phosphorylation sites. The second predicted cytoplasmic loop, between M3 and M4, is identical for eight out of sixteen residues. Five of the eleven amino acids between the M2 and M3 membrane-spanning regions are also identical in the C5a and formyl peptide receptors; this segment is predicted to loop out on the extracellular side of the membrane and may thus be part of a binding site. Unique to the C5a receptor is the presence of seven aspartic acid residues in the first extracellular region. This confers acidic properties on this putative binding site which complement the basic nature of the C5a molecule (pI ~8.6).

Southern analysis of human genomic DNA reveals a simple restriction pattern for the C5a receptor, consistent with a single gene (data not shown). Isolation and sequence analysis of genomic clones for the C5a receptor show no evidence for introns within the coding sequence, a feature shared by many other receptors for GTP-binding proteins. This is in contrast to the receptors for the tachykinins (substance P and substance K) where five introns are present in each gene[18]. Northern blot analysis of other human myeloid cells reveals the distribution expected for the C5a receptor, as shown in Fig. 2*b*. Notably, the receptor is present in the myeloid series and absent in the lymphoid series.

The human C5a receptor has the highest abundance as both message and protein product of any of the rhodopsin superfamily of receptors yet cloned. In contrast to the other receptors in this family (adrenergic, serotoninergic, dopaminergic, FSH/LH, substance P and substance K), the C5a receptor and the formyl peptide receptor function in a concentration gradient of ligand, and internalize bound receptor while cells are crawling to the source of the stimulus. Thus the unique biology of chemotaxis may require a recycling pool of receptor and high levels of expression. Control of expression of the C5a receptor gene, clearly available in both the U937 and HL-60 cell systems, will be facilitated by the present study, as will the structure-activity relationships which characterize the GTP-binding proteins and receptors relevant to the inflammatory response. The strategy used here should be useful for molecular cloning of other leukocyte receptors such as those for leukotrienes, platelet-activating factor, interleukin 8, and adenosine, which are present on U937 cells. □

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Cloning and expression of cDNA for a human thromboxane A_2 receptor

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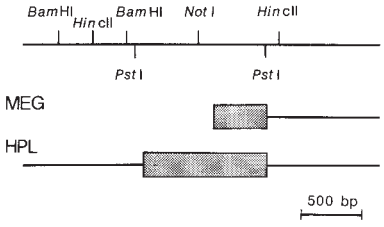
THROMBOXANE A_2 is a very unstable arachidonate metabolite, yet a potent stimulator of platelet aggregation and a constrictor of vascular and respiratory smooth muscles[1]. It has been implicated as a mediator in diseases such as myocardial infarction, stroke and bronchial asthma[2]. Using a stable analogue of this compound we recently purified the human platelet thromboxane A_2 receptor to apparent homogeneity[3]. Using an oligonucleotide probe corresponding to its partial amino-acid sequence, we have obtained a complementary DNA clone encoding this receptor from human placenta and a partial clone from cultured human megakaryocytic leukaemia cells. The placenta cDNA encodes a protein of 343 amino acids with seven putative transmembrane domains. The protein expressed in COS-7 cells binds drugs with affinities identical to those of the platelet receptor, and that in *Xenopus* oocytes opens Ca^{2+}-activated Cl^- channel on agonist stimulation. Northern blot analysis and nucleotide sequences of the two clones suggest that an identical species of the thromboxane A_2 receptor is present in platelets and vascular tissues. This first report on the molecular structure of an eicosanoid receptor will promote the molecular pharmacology and pathophysiology of these bioactive compounds.

Human platelet receptor for thromboxane A_2(TXA_2), purified as described[3], was subjected to proteolysis, and four partial amino-acid sequences were determined. Part of the sequence was used to design a 41-mer oligonucleotide probe, with which we screened a cDNA library of MEG-01 cultured human megakaryocytic leukaemia cells[4,5], and isolated one positive clone (MEG) with a 1.4-kilobase (kb) insert. Nucleotide sequencing revealed that MEG was a partial clone. We therefore used a 586-base pair (bp) *Hin*cII MEG fragment as a probe to screen a human placenta cDNA library. Placenta was chosen as a cDNA source because it showed most intense signals on northern blot analysis (see below). One positive clone, HPL, was isolated that had a 2.9-kb insert containing an entire coding region.

Figure 1*a* and *b* show the restriction map and nucleotide (2,932 bp) and deduced amino-acid sequences derived from HPL. The open reading frame (1,029 bp) codes for a protein of 343 amino acids (relative molecular mass (M_r) 37,429). MEG covered the nucleotide sequence from 581 to 1,941 of HPL with two substitutions, and the deduced amino-acid sequence in the overlapping region was identical. The N-terminal sequence of HPL is identical to that of one of the fragment peptides and there are two putative N-glycosylation sites where no signal was found on peptide analysis. Hydrophobicity analysis[6] of the deduced amino-acid sequence revealed seven hydrophobic stretches that could represent transmembrane domains (data not shown). Comparison of the sequence with those of the rhodopsin-type receptors is shown in Fig. 1*c*. There is a significant

a



b

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                                                                                                                    G  -991
TAATGCAGAGATAATAAAACTTCTTAGGTCCATAGGTCTTATAATAATTTAATAACCTAAACATGGTATACAAATTCCTCCAAACCCAATAACATAATT  -892
ATAGTTTCAAAAAGTTCCCCAAACTTTCAAGTTAGATTTATTGCTTTGATGAGTGGCTTTAAATATGAAAAGTCTTGCCTGTGAAGGGCAATCCTTTT  -793
CCCGTGGACTGGGATCTATAGAAATACAGAAATGTGCCCAGGGGTTCATCTCCCTAATAACCATCATTCACATTTCTCAACCTCCCTAATAACCAGCCA  -694
CCATGTGAGAAGGATCCACAGTTACTGTTTATGACTATAATTAACTAGTACCTGGGACTGGTCAGTGGAGTTGGTTGCAACCTGATGCTAAGGATGTCA  -595
AAGTTGTCTCGGCCTCTGTTCCCAGCCAGTAAGTAATTCCCTGGCCTCGGGCCATACCCCCTAATCTTGGTCAGCTGATTATGACAGGCAGACAGCACA  -496
GTAAATAACACTATATATTAAGAAAACCCAAAGCATATGTATCAATGGTATATACCCAACAGCATCCTAGGAATGGAGAGTCTGTAGCAAGGGCCTCCA  -397
ATGTGAAGGTCAACACAGTCACTGTGATGCGTGTATTTCCATTTTGTAAAGCATGATCTCTGGTGGTCATTTTTATCTTCCTAACTTATTGGAAAAGTC  -298
TCCTGTTTTGGGGGCCCGCCCCTGGTCACAGCCAGACTGACTCAGTTTCCCTGGGAGGTCCCGCTCGAGCCCGTCCTTCCCCTCCCTCTGCCCGCCCCC  -199
AGCCCTCGCCCCACCCTCGGCGCCCGCACATCTGCCTGCTCAGCTCCAGACGGCGCCCGGACCCCCGGGCGCGGGATCCAGCCAGGTGGGAGCCCCGCA  -100
GATGAGGTCTCTGAAGGTGTGCCTGAACCAGTGCCAGCCTGCCCTGTCTGCAGCATCGGCCTGATGGGGTGGTGACTGATCCCTCAGGGCTCCGGAGCC    -1

ATG TGG CCC AAC GGC AGT TCC CTG GGG CCC TGT TTC CGG CCC ACA AAC ATT ACC CTG GAG GAG AGA CGG CTG ATC     75
Met Trp Pro Asn Gly Ser Ser Leu Gly Pro Cys Phe Arg Pro Thr Asn Ile Thr Leu Glu Glu Arg Arg Leu Ile     25

GCC TCG CCC TGG TTC GCC GCC TCC TTC TGC GTG GTG GGC CTG GCC TCC AAC CTG CTG GCC CTG AGC GTG CTG GCG    150
Ala Ser Pro Trp Phe Ala Ala Ser Phe Cys Val Val Gly Leu Ala Ser Asn Leu Leu Ala Leu Ser Val Leu Ala     50

GGC GCG CGG CAG GGG GGT TCG CAC ACG CGC TCC TCC TTC CTC ACC TTC CTC TGC GGC CTC GTC CTC ACC GAC TTC    225
Gly Ala Arg Gln Gly Gly Ser His Thr Arg Ser Ser Phe Leu Thr Phe Leu Cys Gly Leu Val Leu Thr Asp Phe     75

CTG GGG CTG CTG GTG ACC GGT ACC ATC GTG GTG TCC CAG CAC GCC GCG CTC TTC GAG TGG CAC GCC GTG GAC CCT    300
Leu Gly Leu Leu Val Thr Gly Thr Ile Val Val Ser Gln His Ala Ala Leu Phe Glu Trp His Ala Val Asp Pro    100

GGC TGC CGT CTC TGT CGC TTC ATG GGC GTC GTC ATG ATC TTC TTC GGC CTG TCC CCG CTG CTG CTG GGG GCC GCC    375
Gly Cys Arg Leu Cys Arg Phe Met Gly Val Val Met Ile Phe Phe Gly Leu Ser Pro Leu Leu Leu Gly Ala Ala    125

ATG GCC TCA GAG CGC TAC CTG GGT ATC ACC CGG CCC TTC TCG CGC CCG GCG GTC GCC TCG CAG CGC CGC GCC TGG    450
Met Ala Ser Glu Arg Tyr Leu Gly Ile Thr Arg Pro Phe Ser Arg Pro Ala Val Ala Ser Gln Arg Arg Ala Trp    150

GCC ACC GTG GGG CTG GTG TGG GCG GCC GCG CTG GCG CTG GGC CTG CTG CCC CTG CTG GGC GTG GGT CGC TAC ACC    525
Ala Thr Val Gly Leu Val Trp Ala Ala Ala Leu Ala Leu Gly Leu Leu Pro Leu Leu Gly Val Gly Arg Tyr Thr    175

GTG CAA TAC CCG GGG TCC TGG TGC TTC CTG ACG CTG GGC GCC GAG TCC GGG GAC GTG GCC TTC GGG CTG CTC TTC    600
Val Gln Tyr Pro Gly Ser Trp Cys Phe Leu Thr Leu Gly Ala Glu Ser Gly Asp Val Ala Phe Gly Leu Leu Phe    200

TCC ATG CTG GGC GGC CTC TCG GTC GGG CTG TCC TTC CTG CTG AAC ACG GTC AGC GTG GCC ACC CTG TGC CAC GTC    675
Ser Met Leu Gly Gly Leu Ser Val Gly Leu Ser Phe Leu Leu Asn Thr Val Ser Val Ala Thr Leu Cys His Val    225

TAC CAC GGG CAG GAG GCG GCC CAG CAG CGT CCC CGG GAC TCC GAG GTG GAG ATG ATG GCT CAG CTC CTG GGG ATC    750
Tyr His Gly Gln Glu Ala Ala Gln Gln Arg Pro Arg Asp Ser Glu Val Glu Met Met Ala Gln Leu Leu Gly Ile    250

ATG GTG GTG GCC AGC GTG TGT TGG CTG CCC CTT CTG GTC TTC ATT GCC CAG ACA GTG CTG CGA AAC CCG CCT GCC    825
Met Val Val Ala Ser Val Cys Trp Leu Pro Leu Leu Val Phe Ile Ala Gln Thr Val Leu Arg Asn Pro Pro Ala    275

ATG AGC CCC GCC GGG CAG CTG TCC CGC ACC ACG GAG AAG GAG CTG CTC ATC TAC TTG CGC GTG GCC ACC TGG AAC    900
Met Ser Pro Ala Gly Gln Leu Ser Arg Thr Thr Glu Lys Glu Leu Leu Ile Tyr Leu Arg Val Ala Thr Trp Asn    300

CAG ATC CTG GAC CCC TGG GTG TAT ATC CTG TTC CGC CGC GCC GTG CTC CGG CGT CTC CAG CCT CGC CTC AGC ACC    975
Gln Ile Leu Asp Pro Trp Val Tyr Ile Leu Phe Arg Arg Ala Val Leu Arg Arg Leu Gln Pro Arg Leu Ser Thr    325

CGG CCC AGG TCG CTG TCC CTC CAG CCC CAG CTC ACG CAG CGC TCC GGG CTG CAG TAG GAAGTGGACAGAGCGCCCCTCCC  1055
Arg Pro Arg Ser Leu Ser Leu Gln Pro Gln Leu Thr Gln Arg Ser Gly Leu Gln                                 343

GCGCCTTTCCGCGGAGCCCTTGGCCCCTCGGACAGCCCATCTGCCTGTTCTGAGGATTCAGGGGCTGGGGGTGCTGGATGGACAGTGGGCATCAGCAGC  1154
AGGGTTTTGGGTTGACCCCAATCCAACCCGGGGACCCCCAACTCCTCCCTGATCCTTTTACCAAGCACTCTCCCTTCCTCGGCCCCTTTTCCCATCCA  1253
GAGCTCCCACCCCTTCTCTGCGTCCCTCCCAACCCCAGGAAGGGCATGCAGACATTGGAAGAGGGTCTTGCATTGCTATTTTTTTTTTAGACGGAGTC  1352
TTGCTCTGTCCCCCAGGCTGGAGTGCAGTGGCGCAATCTCAGCTCACTGCAACCTCCACCTCCCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCTGAG  1451
TAGCTGGGACTATAGGCGCGCGCCACCACGCCCGGCTAATTTTTGTATTTTTAGTAGAGACGGGGTTTCACCGTGTTGGCCAGGCTGGTCTTGAACTCC  1550
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CTCACTCTGTGGCCCAGCCTGGAGTACAGTGGCACGATCTCGGCTCACTGCAACCTCCGCCTCCCGGGTTCAAGCGATTCTCGTGCCTCAGCCTCCCGA  1748
GCAGCTGGGATTACAGGCGTAAGCCACTGCGCCCGGCCTTGCATGCTCTTTGACCCTGAATTTGACCTACTTGCTGGGGTACAGTTGCTTCCTTTTGAA  1847
CCTCCAACAGGGAAGGCTCTGTCCAGAAAGGATTGAATGTGAAACGGGGGCACCCCCTTTTCTTGCCAAAATATATCTCTGCCTTTGGTTTTAT        1941
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c

◀ FIG. 1 *a*, Restriction map of TXA_2 receptor clones from human placenta (HPL) and cultured human megakaryocytic leukaemia cells (MEG). Stippled boxes indicate coding region. *b*, Nucleotide and deduced amino-acid sequence of HPL. The nucleotide sequence was numbered in the 5′ to 3′ direction beginning with the first ATG of the open reading frame. The 5′-most ATG found in frame with all the partial amino-acid sequences was assigned as the site of translation initiation. There are 18 other ATGs in the 5′-noncoding region, all of which terminate after a short stretch of reading frame. The putative polyadenylation signal is shown by a double underline. The deduced amino-acid sequence is numbered at the right-hand end of each line, beginning at the initiator methionine (Met). Sequences obtained from proteolytic peptides are underlined, and possible N-glycosylation sites are shown by asterisks. MEG covers the nucleotide sequence from 581 to 1941 of HPL with two substiutions; both T→C at the positions of 795 and 924 of HPL. These substitutions do not alter the amino acids encoded by these codons. *c*, Alignment of the amino-acid sequences of receptors. Deduced amino-acid sequences of the human TXA_2 receptor (TXR), the bovine rhodopsin[8] (Opsin), the human β_1-adrenergic receptor[25] (β_1), the human α_2-adrenergic receptor-C4[26] (α_2), the porcine muscarinic M1 receptor[27] (M1) and the rat substance P receptor[28] (SPR) were aligned to optimize homology. The putative transmembrane domains are bracketed and the conserved amino acids are boxed.

METHODS. Human platelet TXA_2 receptor was purified as described[3] and proteolysed with cyanogen bromide, lysyl endopeptidase and trypsin. Fragment peptides were isolated by reversed phase HPLC and sequenced by automated pulse-liquid phase protein sequencer. Four partial amino-acid sequences were obtained. Part of these sequences (VATWNQILDPIVYI (single-letter notation)) was used to design a 41-mer oligonucleotide probe (GTIGCIACITGGAACCAGATCCTIGACCCIATCGTITACAT). MEG-01 cDNA library was screened with this probe, radiolabelled at the 5′-end. One positive clone, MEG, was isolated. The sequence of a 1.4-kb insert in this clone revealed one open reading frame which contained two of the partial amino-acid sequences of the purified protein. A 586-bp fragment of MEG was then used to screen a placental cDNA library. One positive clone, HPL, was obtained and analysed. DNA sequencing was carried out on double-stranded templates using dideoxy chain termination method.

homology between this and other receptors, particularly in the putative transmembrane regions, suggesting that it belongs to this receptor family. As in the rhodopsin and substance P receptor, there is no Asp in the third transmembrane domain of the TXA_2 receptor, a residue which is presumed to bind the amino group of the ligand in adrenergic receptors[7]. On the other hand, the TXA_2 receptor has Arg 295 which is located at the position analogous to Lys 296 of bovine rhodopsin in the seventh transmembrane domain. The latter amino-acid residue was assigned for retinal attachment in the rhodopsin molecule[8]. These structural features may reflect the acidic nature of the ligand for the TXA_2 receptor. The third cytoplasmic loop consists of less than 30 residues, and its N-terminal end has significant homology with rhodopsin. But the C-terminal end of the loop lacks the basic amino-acid cluster present in the other receptors. These regions are involved in binding to and determining the specificity of a coupling G protein[9,10]. The G-protein coupling to the TXA_2 receptor is insensitive to pertussis and cholera toxins and remains to be identified[11-13]. The short C-terminal tail contains six serine and threonine residues and shows some homology to the adrenergic receptors, especially the β_1 receptor. This structure may be involved in agonist-induced desensitization of the signalling pathway, as like the β receptors, the TXA_2 receptor is desensitized on agonist stimulation[14].

To establish that HPL encodes the TXA_2 receptor, we subcloned a 2.0-kb fragment of HPL into a eukaryotic expression vector, CDM8[15], and transfected the plasmid into COS-7 cells. Ligand binding was examined with the selective TXA_2 receptor antagonist, [^{3}H]S-145[16]. The membranes of transfected COS cells bound [^{3}H]S-145 in a saturable manner with the dissociation constant, K_d, of 1.2 nM (Fig. 2*a*), which is comparable to that observed with membranes from fresh platelets. Binding specificity was analysed with several TXA_2 analogues as well as various prostanoids (Fig. 2*b*). The TXA_2 agonist STA_2 and antagonists S-145 and ONO-3708 competed for the [^{3}H]S-145 binding with an order of potency identical to that observed with the TXA_2 receptor in other cells[3,4,16,17]. Other prostaglandins and an inactive TXA_2 metabolite, TXB_2, were at least two orders of magnitude less active in the competition.

The TXA_2 receptor stimulation results in phosphatidylinositol turnover and Ca^{2+} mobilization[18]. We therefore expressed the clone in *Xenopus* oocyte where agonist stimulation linked to phosphatidylinositol turnover opens endogenous Ca^{2+}-dependent Cl^- channels[19]. Application of 1 μM STA_2 to the oocyte injected with the *in vitro*-transcribed messenger RNA of the clone evoked a fast inward current of a few hundred nanoamperes followed by a slow phase of current of low amplitude (Fig. 3*a*). This response was seen at concentrations of STA_2 as low as 1 nM (data not shown). PGD_2 and $PGF_{2\alpha}$ did not evoke a response at 1 μM (Fig. 3*b*, *c*). Other prostaglandins such as PGE_1, PGE_2 and PGI_2 and TXB_2 did not evoke response, either, under these experimental conditions (data not shown). Thus,

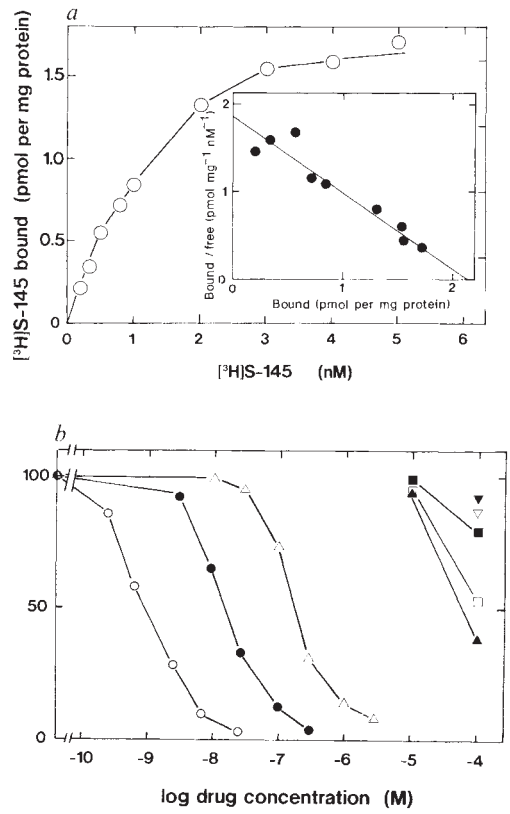

FIG. 2 Binding of [^{3}H]S-145 to transformed COS-7 cell membranes. *a*, Saturation isotherms of the specific binding of [^{3}H]S-145 to membranes from COS-7 cells expressing the human TXA_2 receptor. The results shown are representative of three experiments each conducted in duplicate. Inset, Scatchard plot of the same data. The estimated maximal binding, B_{max}, and K_d values were 2.2 pmol per mg protein and 1.2 nM, respectively. *b*, Specificity of [^{3}H]S-145 binding to COS-7 cells. Competition of specific [^{3}H]S-145 binding was carried out with S-145 (○), ONO-3708 (●), STA_2 (△), PGD_2 (▲), $PGF_{2\alpha}$ [□], PGE_2 (■), PGI_2 (▼) and TXB_2 (▽). Representative curves of two to three experiments each conducted in duplicate are shown.

METHODS. HPL was digested with *Bam*HI to remove most of the 5′-untranslated region, and its 2.0-kb fragment was inserted between the *Hin*dIII–*Xba*I site of eukaryotic expression vector CDM8[15]. A modified DEAE-dextran method[29] was used for the transfection of COS-7 cells. At 60 h after transfection, cells were collected and membranes were prepared by homogenizing the cells as described[29] in the presence of 1 mM benzamidine and 0.3 mM PMSF. The membranes were finally suspended in 20 mM Na-MES, pH 6.4, and 5 mM EGTA, and binding experiments with [^{3}H]S-145 (24 Ci mmol^{-1}) were carried out as described[16]. Competition experiments used 1.25 nM [^{3}H]S-145 and various concentrations of ligands as indicated.

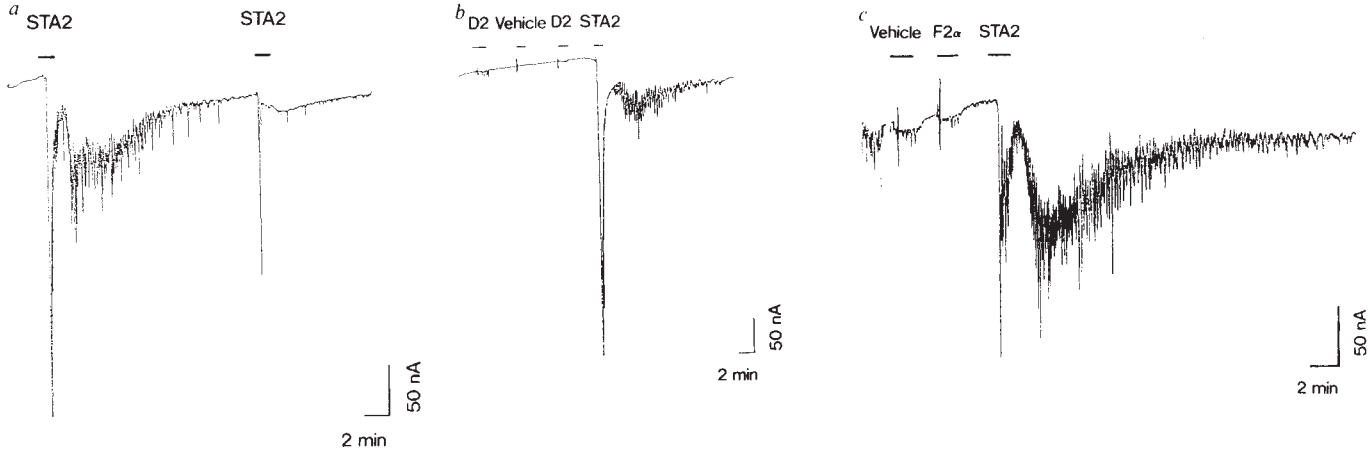

FIG. 3 Responses to a TXA_2 agonist (*a*) and other prostaglandins (*b*, *c*) recorded from *Xenopus* oocytes injected with the *in vitro* transcribed mRNA of the cloned human TXA_2 receptor cDNA. The mRNA synthesized *in vitro* from the 2.0-kb *Bam*HI subclone of HPL was injected into oocytes and electrophysiological responses were measured after 48 h. A TXA_2 agonist, STA_2, and other prostaglandins dissolved in ethanol at 100 μM were added to the bath at 1 μM concentration, and responses of oocytes were recorded by the two-micropipette voltage clamp method as described[19]. D2, PGD_2; $F2_\alpha$, $PGF_{2\alpha}$.

the protein encoded by HPL possesses the ligand-binding properties characteristic of the TXA_2 receptor and evokes a response expected for this receptor.

Northern blot analysis of the TXA_2 receptor mRNA expression in various tissues is of interest because of a controversy over the presence of receptor subtypes[20–22]. As extensive analysis using poly(A)$^+$RNA from various rat tissues yielded few signals, probably owing to the species difference of the receptor[23], we analysed only poly(A)$^+$RNA from human placenta and lung and from cultured MEG-01 cells (Fig. 4). Placenta and lung are representative tissues rich in the TXA_2 receptor. A main hybridization band was observed at 2.8 kb in all three lanes under stringent condition, which was most intense with placenta RNA. In this lane, a minor band was present at 3.5 kb which also became apparent on extended exposure in the MEG-01 poly(A)$^+$RNA lane. These results, together with the above findings that MEG and HPL encode the identical amino-acid sequence, suggest that the same species of mRNA are expressed in platelet precursor cells and vascular-rich tissues.

Bioactive arachidonate metabolites, collectively called eicosanoids, comprise a large family of substances consisting of more than 30 members, and each member of the family acts on a specific receptor to stimulate a variety of activities[24]. This cloning of an eicosanoid receptor will facilitate elucidation of receptor structures for other members of this family and make way for the development of more selective drugs to control these pathophysiologically important mediators. □

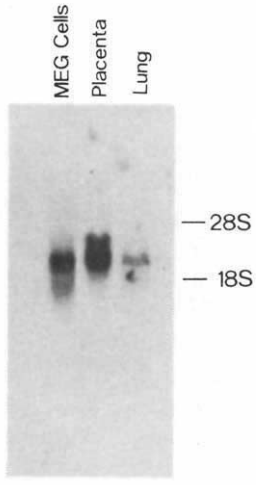

FIG. 4 Northern blot analysis of TXA_2 receptor transcription in MEG-01 cells, human placenta and lung. Each lane contained 20 μg of poly(A)$^+$ RNA. Positions of 28S and 18S rRNA are indicated.

METHODS. Total RNA was prepared from cultured cells and tissues using the guanidinium thiocyanate–CsCl method, and poly(A)$^+$ RNA was isolated using an oligo-(dT) cellulose column. Twenty micrograms of each poly(A)$^+$ RNA was subjected to formaldehyde agarose gel electrophoresis, transferred to Biodyne membrane and UV-cross-linked. The filter was prehybridized in 5 × SSC buffer, 5 × Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 200 μg ml^{-1} heat denatured salmon testis DNA, 50% formamide and 0.1% SDS at 42 °C for 4 h, and hybridized in the same solution at 42 °C overnight with a 586-bp *Hin*cII fragment of MEG clone labelled with [^{32}P]dCTP by random-priming (4×10^6 c.p.m. ml^{-1}). The filter was washed twice in 0.1 × SSC, 0.1% SDS at 65 °C for 1 h and exposed to an X-ray film for 12 days in the presence of an intensifying screen at −80 °C.

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