rp23 appeared as the expected 23-kD bands, whereas the glycine eluate did not have biologic activity or a protein band (Fig. 3B). With the use of p23-based primers, analyses by Northern (RNA) blot and by reverse transcription polymerase chain reaction (RT-PCR) indicated that mRNA for the IgE-dependent HRF was ubiquitous: It was found in B cells, T cells, mononuclear cells, and fibroblasts. The transcript could not be detected, however, in isolated human skin mast cells or in the rat basophilic leukemic cell line.

Efforts to identify an IgE-dependent HRF have occupied several laboratories for the past decade. Now that it is characterized, we expect that this molecule, which is linked to the intensity of the LPR (*17*) and to the severity of atopic dermatitis in children (*10*), will allow us to better understand the pathogenesis of human allergic disease and to appreciate the nature and significance of the heterogeneity of IgE, which may be a genetically determined polymorphism, may be due to differential glycosylation of the IgE molecule, or may be based on interactions with the alternatively spliced forms of human IgE reported to be present in human atopic serum by Zhang *et al.* (*18*). Efforts are ongoing to decipher whether this molecule interacts solely with IgE or, additionally, binds to its own receptor.

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20. Native material from the U937 macrophage cell line, human recombinant HRF (rp23), mouse recombinant HRF (rp21), and SEEBLUE prestained molecular weight markers (Novex, San Diego, CA), were run on a 4 to 20% tris glycine gradient gel (Novex) by an established method [U. K. Laemmli, *Nature* **227**, 680 (1970)]. The gel was blotted onto nitrocellulose. A rabbit polyclonal antibody generated against rp21 was used as a primary antibody, and the proteins were visualized with goat IgG to rabbit IgG, coupled to alkaline phosphatase. The additional bands that appeared in the lanes containing rp21 and rp23 were caused by nonspecific binding of the antiserum to *E. coli* proteins. The antibody recognizes a 23-kD band in each preparation, corresponding to the native HRF from the U937 line, to 0.04 μg of rp21, and to 0.14 μg of rp23.
21. The following primers were used to amplify the p21 cDNA from the Okayama-Berg vector: 5′ primer, 5′-AAAAGGATCCATGATCATCTACCGGGACC-3′; 3′ primer, 5′-AAAGAATTCTTAACATTTCTCCATCTCTAAGCC-3′. The resultant PCR product was restricted with Bam HI and Eco RI, ligated in frame with GST into the pGEX-2T plasmid, and transfected into JM109 competent cells (Promega). Production and purification of the recombinant GST fusion protein were done by means of affinity chromatography on immobilized glutathione (*16*). To separate p21 from GST, the GST p21 fusion protein was cleaved with thrombin with the use of the Pharmacia bulk GST purification module.
22. Partial purification of NP HRF was as previously described (*5*). After informed consent, peripheral blood was obtained from participants by venipuncture. Mixed leukocytes containing basophils were prepared by dextran sedimentation as previously described [R. P. Schleimer *et al.*, *J. Immunol.* **128**, 1632 (1982)]. Histamine release was done by a standard method (*5*), and histamine was measured by means of an automated fluorometric method [R. P. Siraganian, *J. Immunol. Meth.* **7**, 283 (1975)].
23. A lactic acid method [J. J. Pruzansky *et al.*, *J. Immunol.* **131**, 1949 (1983)] was used to dissociate IgE from basophils. Passive sensitization was accomplished as described [D. A. Levy and A. G. Osler, *ibid.* **97**, 203 (1966)]. After addition of the stimuli, the released histamine was measured as described above.
24. PBMC were isolated with the use of lymphocyte separation media (Ficoll-Paque, Pharmacia), and 5×10^6 cells per milliliter in 80 ml were stimulated with concanavalin A (5 μg/ml) (Sigma) for 24 hours at 37°C in 5% CO_2. Supernatants were harvested, centrifuged, dialyzed against a Pipes buffer, concentrated 26-fold, and placed on 5 ml of cyanogen-activated Sepharose CL-4B (*19*) beads covalently coupled to IgG anti-human p23 (1 mg/ml). The affinity column was washed with four column volumes of phosphate-buffered saline starting buffer, followed by two column volumes of 0.1 M glycine (pH 3) and then two column volumes of 5 M guanidine. The eluates were concentrated 100-fold and tested for basophil histamine-releasing activity as described above. Starting material from the concentrated PBMC supernatants, glycine eluate, guanidine eluate, human recombinant HRF (rp23), and SEEBLUE molecular weight markers were run on a 4 to 20% tris-glycine gradient gel as described above (*20*). The gel was blotted onto nitrocellulose (Schleicher and Schull, Keene, NH). A rabbit IgG polyclonal antibody generated against rp23 was the primary antibody, and a goat IgG to rabbit coupled to alkaline phosphatase was the secondary antibody. The protein immunoblot was developed with the use of enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

25. We thank M. Koots and G. Brawerman (Tufts University, Boston, MA) for supplying p21 cDNA that was isolated from a mouse L cell library, ImmuLogic Corporation for NH_2-terminal sequencing of the four polyvinylidene difluoride transferred bands, and D. Redburn for scientific advice. Supported by NIH grants AI 32651 and AI 07290. For early studies, ImmuLogic Corporation supplied funds for obtaining 50 liters of U937 supernatants.

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Cloning and Characterization of a G Protein–Activated Human Phosphoinositide-3 Kinase

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Phosphoinositide-3 kinase activity is implicated in diverse cellular responses triggered by mammalian cell surface receptors and in the regulation of protein sorting in yeast. Receptors with intrinsic and associated tyrosine kinase activity recruit heterodimeric phosphoinositide-3 kinases that consist of p110 catalytic subunits and p85 adaptor molecules containing Src homology 2 (SH2) domains. A phosphoinositide-3 kinase isotype, p110γ, was cloned and characterized. The p110γ enzyme was activated in vitro by both the α and βγ subunits of heterotrimeric guanosine triphosphate (GTP)–binding proteins (G proteins) and did not interact with p85. A potential pleckstrin homology domain is located near its amino terminus. The p110γ isotype may link signaling through G protein–coupled receptors to the generation of phosphoinositide second messengers phosphorylated in the D-3 position.

Phosphoinositide-3 kinase (PI-3K) activity increases in response to numerous ligands, including those that signal through receptor tyrosine kinases, cytokine receptors, and G protein–coupled receptors (*1–3*). The activation of PI-3K by membrane-bound receptor tyrosine kinases results from recruitment of p110-p85 heterodimers to membrane-bound signaling complexes; this process is mediated by the SH2 domains of p85 that

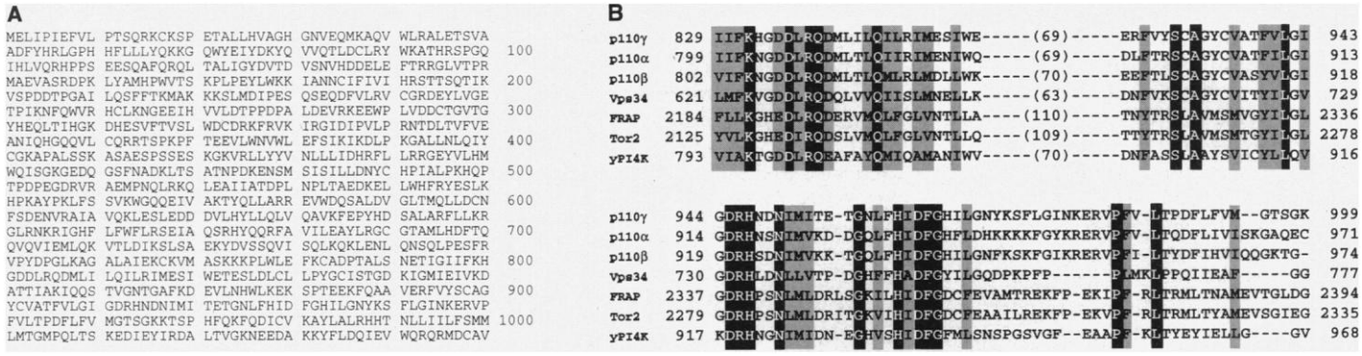

Fig. 1. Sequence analysis of human p110γ cDNA. (**A**) Predicted translation product of the human p110γ cDNA clone. The nucleotide sequence of p110γ has been deposited in the European Molecular Biology Laboratory database (accession number X83368). (**B**) Sequence comparison of part of the kinase domain of p110γ, human p110α (*12*), human p110β (*7*), yeast Vps34 (*8*), human FRAP (*27*), yeast Tor2 (*28*), and yeast PI-4 kinase (yPI4K) (*9*). Amino acid residues identical among all proteins are shown with a black background, and physiochemically conserved amino acids have a shaded background. The GCG program package was used for sequence alignment.

bind to specific phosphotyrosine residues on the receptors (*1*). In contrast, the mechanism by which G protein–coupled receptors induce PI-3K activation is currently unclear. Activation of partially purified neutrophil (*4*) and platelet (*5*) PI-3K activities upon addition of G protein βγ subunits might result from an interaction with a p110-p85 heterodimer (*5*) or might involve a distinct G protein–specific PI-3K isotype (*4*). Here, we report the cloning, expression, and purification of a PI-3K that does not bind p85 and is activated upon addition of either the Gα or Gβγ subunits.

The diversity of the signaling events that regulate PI-3K activity and the evidence that at least two PI-3K isotypes exist (*6*, *7*) warranted a search for new members of the PI-3K family. To isolate complementary DNAs (cDNAs) encoding PI-3Ks, we screened a human bone marrow cDNA library with probes generated from the polymerase chain reaction (PCR) with degenerate oligonucleotide primers that were based on conserved regions of bovine PI-3K (p110α) (*6*), yeast phosphatidylinositol (PtdIns)-3K (Vps34) (*8*), and a yeast PI-4 kinase (*9*). Primers corresponding to the highly conserved amino acid sequences KNGDDLR and HIDFG [residues 803 to 809 and 932 to 936 in p110α (*6*, *10*)] were used to amplify a 402-bp product that was 57% identical to the corresponding region of the gene encoding bovine p110α. We used this PCR fragment as a probe and isolated a set of overlapping clones from a human U937 cDNA library. The largest clone contained an open reading frame with the potential to encode 1050 amino acids (Fig. 1A). The cDNA contained three potential initiation codons preceded by an in-frame stop codon. The most COOH-terminal of these methionine residues conformed best with criteria for the site of initiation of translation (*11*). The protein encoded by this cDNA, termed p110γ, has a predicted molecular mass of 120 kD, which is similar to that of the bovine (*6*) and human (*7*, *12*) p110α and p110β catalytic subunits.

The predicted amino acid sequence of p110γ exhibits 28 to 36% overall similarity to the sequences of other PI-3Ks, including human p110α (36% identity) (*12*), human p110β (33.5% identity) (*7*), and yeast PtdIns-3K (27.7% identity) (*3*, *8*). The highest degree of conservation among these proteins is at the COOH-terminus within the putative kinase domain (*1*) (Fig. 1B). There is no similarity between p110γ and either p110α or p110β in the NH_2-terminal region through which p110α and p110β bind p85α and p85β (*13*). However, the sequence of p110γ close to the NH_2-terminus is similar to the pleckstrin homology (PH) domain (*14*) of Ras–guanosine triphosphatase activating protein (Ras-GAP) (21.7% identity) and dynamin (15.5% identity).

Immunoprecipitation and protein immunoblot analysis with an antiserum to a peptide derived from p110γ revealed a 110-kD protein in lysates of the human leukemic cell lines U937 and K562 (Fig. 2A). The 5.3-kb mRNA encoding the protein was detected in the pancreas, skeletal muscle, liver, and heart (Fig. 2B). To characterize the substrate specificity of p110γ, we used baculoviruses to express the recombinant protein as a glutathione-S-transferase (GST) fusion protein in Sf9 insect cells (*15*). The p110γ-GST fusion protein was affinity-purified on glutathione-Sepharose beads and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 3A); it was found to phosphorylate PtdIns, PtdIns-4 phosphate (PI-4P), and PtdIns-

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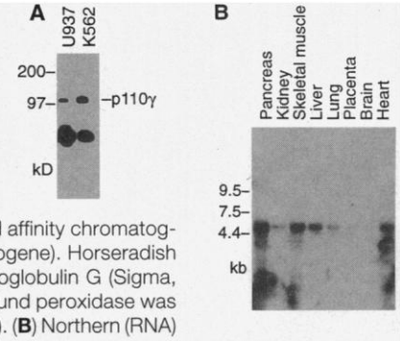

Fig. 2. Distribution of p110γ in mammalian tissue. (**A**) Detection of p110γ in the human leukemic cell lines U937 and K562 by immunoprecipitation with antibodies to p110γ and subsequent protein immunoblotting (*6*) with the same antibodies. A rabbit polyclonal antiserum to p110γ was raised against a 15–amino acid peptide corresponding to a sequence of p110γ (residues 742 to 756, NSQLPESFRVPYDPG) (*10*). The antibodies were purified by protein A chromatography and affinity chromatography with the peptide antigen coupled to Actigel (Sterogene). Horseradish peroxidase–conjugated goat antibody to rabbit immunoglobulin G (Sigma, 1:2000 dilution) was used as a secondary antibody. Bound peroxidase was visualized by enhanced chemiluminescence (Amersham). (**B**) Northern (RNA) blot (Clontech) probed with a random primer–labeled PCR fragment encompassing the p110γ sequence between amino acids 1 and 233. The blot was hybridized and washed according to the manufacturer's specifications.

4,5-bisphosphate [PI(4,5)P_2] in the D-3 position of the inositol ring (Fig. 3B). The activity of p110γ was inhibited by nanomolar concentrations of wortmannin, as are the bovine PI-3Kα and human PtdIns-3K enzymes (*4*, *16*).

To evaluate any interaction between p110γ and the p85 adaptor molecule, we expressed p110γ and p110α as GST fusion proteins either alone or together with p85α or p85β in Sf9 cells. Under these conditions, p110α copurified with p85α and p85β, whereas p110γ did not associate with either the p85α or p85β subunits, consistent with the absence of a p85-binding region at the NH_2-terminus of p110γ.

The failure of p110γ to bind p85 subunits prompted us to search for alternative regulatory mechanisms. The presence of a putative PH domain in p110γ suggested possible regulation by G proteins, because this region mediates the interaction of the β-adrenergic receptor kinase with Gβγ subunits (*17*). Furthermore, G protein–linked receptors induce a rapid accumulation of 3-phosphorylated phosphoinositides in neutrophils (*18*, *19*) and platelets (*5*, *20*), and the partial purification of a Gβγ-stimulated PI-3K enzyme has been described (*4*, *5*). To determine whether p110γ might be activated by G proteins, we added the transducin G protein subunits α_t or $\beta\gamma_t$ to purified GST-p110γ. Both $G\alpha_t$ and $G\beta\gamma_t$ strongly activated p110γ (Fig. 4A). The stimulation of p110γ by $G\alpha_t$ loaded with guanosine diphosphate (GDP) was dependent on the presence of AlF_4^- (*21*), and the stimulation of p110γ by $G\beta\gamma_t$ was completely suppressed by $G\alpha_t$-GDP. In contrast, p85-dependent p110α was not activated by $G\alpha_t$-GDP–AlF_4^- or by $G\beta\gamma_t$. In addition to the stimulation of p110γ by $G\alpha_t$-GDP–AlF_4^-, we observed that $G\alpha_t$ could also activate when it was loaded with the nonhydrolyzable GTP analog guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) (Fig. 4B). Activation was concentration-dependent and showed half-maximal stimulation at about 200 nM $G\alpha_t$–GTP-γ-S. Moreover, p110γ activity was considerably more sensitive to $G\alpha_{i1}$, another subspecies of the $G\alpha_i$ group of Gα subunits ($K_{0.5}$ = 0.5 nM). These data are consistent with the involvement of the G_i subfamily in the stimulation of neutrophil PI-3K activity by agonists of G protein–coupled receptors, such as the formylated Met-Leu-Phe receptor (*19*).

This study defines p110γ as a member of the PI-3K family of enzymes. Our results suggest that induction of cellular PI-3K activity by heterotrimeric G protein–linked receptors (*18*) may occur by a mechanism in which the Gα or Gβγ subunits, or both, directly modulate the activity of p110γ. In this respect, p110γ is similar to the β isoforms of phospholipase C, which are also activated by both the α and βγ G protein subunits (*22*). Thus, it seems that there are two distinct mechanisms by which receptors can trigger increases in PI-3K activity and the generation of 3-OH′ phosphoinositides. The first involves the interaction of p110-p85 heterodimeric PI-3Ks with tyrosine-phosphorylated proteins, such as autophosphorylated receptors or their substrates, through the SH2 domains of the p85 adaptor subunits. The second may involve direct activation and recruitment of the p110γ isotype through an interaction with Gα or Gβγ subunits, which are released upon agonist activation of receptors linked to G proteins. This diversity in mechanisms of activation closely resembles that of the phospholipase C enzymes, various isotypes of which are regulated either by tyrosine phosphorylation or by heterotrimeric G proteins (*23*).

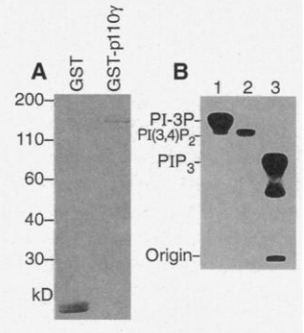

Fig. 3. Expression of p110γ. (**A**) Recombinant GST-p110γ fusion protein or GST alone were expressed in Sf9 insect cells infected with appropriate baculoviruses (*15*), purified, fractionated by SDS-PAGE, and visualized by staining with Coomassie blue. The cDNA for p110γ was cloned from codon 4 into the vector pAcG2T (*15*) to generate the vector pAcG2T-p110γ as a COOH-terminal in-frame fusion with the GST gene. Sf9 cells were transfected with pAcG2T-p110γ and linearized baculovirus DNA (BaculoGold, Pharmingen). A single recombinant baculovirus expressing GST-p110γ was plaque-purified and amplified; expression and purification of the recombinant protein was done according to standard protocols (*13*). (**B**) Lipid kinase assay of purified recombinant p110γ with PtdIns (lane 1), PI-4P (lane 2), and PI(4,5)P_2 (lane 3) as substrates. The kinase assay was done essentially as described (*4*) but in the absence of cholate. Sonicated lipid vesicles (30 μl) containing 320 μM phosphatidylethanolamine, 140 μM phosphatidylcholine, 300 μM phosphatidylserine, 30 μM sphingomyelin, and 320 μM PtdIns, PI-4P, or PI(4,5)P_2 were added to 10 μl of enzyme (0.1 ng) and incubated for 8 min on ice. The assay was started by addition of 20 μM adenosine triphosphate (ATP, 10 μl) containing 10 μCi [γ-^{32}P]ATP and was incubated for 15 min at room temperature. Extracted lipids were separated and visualized as described (*4*). The identity of the 3-phosphorylated phosphoinositides PtdIns-3 phosphate (PI-3P), PtdIns-3, 4-bisphosphate [PI(3,4)P_2], and PtdIns-3,4,5-trisphosphate (PIP_3) was confirmed by anion exchange high-performance liquid chromatography after deacylation of the lipids (*24*).

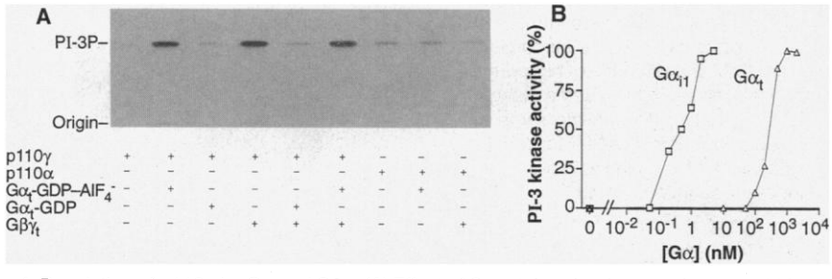

Fig. 4. Regulation of p110γ by Gα and Gβγ. (**A**) Effect of G protein subunits α_t and $\beta\gamma_t$ on p110γ activity. Purified recombinant p110γ or bovine p110α was incubated with G protein subunits, and activity was assayed thereafter with PtdIns as a substrate (*29*). (**B**) Concentration dependence of $G\alpha_i$ and $G\alpha_t$ for p110γ activity. Recombinant p110γ was incubated with different concentrations of $G\alpha_{i1}$–GTP-γ-S (□) purified from bovine brain (*25*) or $G\alpha_t$–GTP-γ-S (△) purified from bovine retina (*26*, *30*), and PI-3K activity was assayed (*31*).

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10. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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29. G_t proteins purified from bovine retina (*24*) were mixed with the lipid vesicles for 10 min on ice before addition of the enzyme. The following protein concentrations were used: p110γ, 10 pM; p110α, 10 pM; $G\alpha_t$-GDP, 2 μM; and $G\beta\gamma_t$, 1 μM. The activated $G\alpha_t$-GDP–AlF_4^- was produced by incubation of Gα-GDP with 20 μM $AlCl_3$ and 10 mM NaF in assay buffer for 1 hour at room temperature (*25*). AlF_4^- alone did not activate the enzyme. The content of Gβγ in the $G\alpha_t$ preparations was estimated to be approximately 2% (mol/mol) by immunoblotting with Gβ antibodies and different amounts of $G\beta\gamma_t$ for calibration. An involvement of βγ impurities in the stimulatory effect of $G\alpha_t$ can be excluded because half-maximal activation by $G\alpha_t$-GDP–AlF_4^- and $G\beta_t$ was observed at concentrations of about 2 μM and 200 nM, respectively.
30. $G\alpha_t$–GTP-γ-S was produced by means of Blue Sepharose (Pharmacia) chromatography (*26*) in the presence of 100 μM GTP-γ-S.
31. Before addition of the enzyme, both Gα proteins were incubated for 1 hour on ice in the presence of 100 μM GTP-γ-S and 5 mM $MgCl_2$, mixed with the PtdIns-containing lipid vesicles, and incubated again for 10 min on ice. Activity was assayed as described (Fig. 3). After separation of extracted lipids by thin-layer chromatography, [^{32}P]PI-3P spots were located with the use of a phosphorimager (Bio-Rad) and were quantitated according to the manufacturer's instructions. In three independent experiments, maximal stimulatory concentrations of $G\alpha_{i1}$–GTP-γ-S (2 nM) and $G\alpha_t$–GTP-γ-S (1 μM) induced 1.5 to 3 times the base-line rate of p110γ activity. Heat denaturation of the Gα proteins (10 min at 100°C) completely abolished this stimulation. GTP-γ-S alone (100 μM) and a separate preparation of $G\alpha_i$-GDP did not stimulate enzyme activity.
32. We thank J. Thorner for unpublished sequence information and A. Ullrich in whose laboratory a partial clone of p110γ was isolated by B.S. This work was supported by grants from Deutsche Forschungsgemeinschaft (SFB 197), the British Council, and Deutscher Akademischer Austauschdienst. B.V. was supported in part by the Belgian NFWO.

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A Gene Outside the Human MHC Related to Classical HLA Class I Genes

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By presenting antigenic peptides to T lymphocytes, major histocompatibility complex (MHC) class I molecules play important roles in the human immune system. Knowledge is limited on the evolutionary history of human MHC class I–related molecules. An expressed class I gene, *MR1*, has now been identified on human chromosome 1q25, outside the MHC. In contrast to other known human divergent class I genes, *MR1* encodes peptide-binding domains similar to those encoded by human leukocyte antigen (HLA) class I genes on chromosome 6 and by nonmammalian classical MHC class I genes. This gene may thus contribute to understanding the evolution of the MHC.

MHC class I molecules present antigenic peptides to $CD8^+$ T cells (*1*). In humans, the HLA class I gene family consists of six members: polymorphic *HLA-A*, *-B*, and *-C*, and oligomorphic *HLA-E*, *-F*, and *-G* (*2*). The functions of HLA-E, -F, and -G molecules are unknown, although HLA-G is thought to play a role in the maternal-fetal interaction (*3*). Several groups of human class I–related molecules—including the CD1 family (*4*), Zn-α2-glycoprotein (Znα2gp) (*5*), MICA (MHC class I chain-related molecule A) (*6*), and a human homolog of the rat neonatal Fc receptor (FcRn) (*7*)—have also been identified. Studies on nonmammalian MHC-related systems suggest a tendency to expand the number of genes in various animals (*8–10*). Even in humans, it is possible that only a fraction of MHC class I–related molecules have been identified to date. To examine this possibility, we have attempted to isolate unknown human class I genes. We now describe a close relative of classical HLA class I genes in the human genome.

The *MR1* gene was discovered by an approach based on our previous polymerase chain reaction (PCR)–based strategy (*11–13*). A candidate human class I gene fragment was amplified from human genomic DNA with the use of two primers that correspond to the two conserved regions in the α3 domain of class I molecules (*14*). The DNA sequence of this PCR product did not correspond to that of any previously described human class I sequence. Because expression of this DNA sequence was detected by Northern (RNA) analysis (Fig. 1) (*15*), a human thymus complementary DNA (cDNA) library was screened with the PCR fragment, and the nucleotide sequence and predicted amino acid sequence (Fig. 2) of the new gene, *MR1*, were determined. Similar to a typical class I molecule, the MR1 protein comprises a signal sequence, three extracellular domains (α1, α2, and α3), a transmembrane domain, and a cytoplasmic domain. The chromosomal location of *MR1* was determined by fluorescence in situ hybridization (FISH) analysis (*16*, *17*) as the distal portion of 1q25 (1q25.3) (Fig. 3A), outside the human MHC (6p21.3). The location of *MR1* on chromosome 1 was also supported by the results of PCR amplification of the *MR1* fragment from somatic cell hybrid DNA (*18*) (Fig. 3B).

The predicted amino acid sequence of MR1 was compared with the sequences of various class I molecules (Fig. 4). The three extracellular domains (α1, α2, and α3) of a class I molecule can be divided into two structurally distinct components: the peptide-binding symmetrical α1 and α2 domains, which contain characteristic α helices, and the α3 domain, which adopts the structure of the immunoglobulin fold. In the α3 domain, the percentage amino acid identity between MR1 and the HLA class I molecules is similar to that between the HLA class I molecules and either other human divergent class I molecules or nonmammalian class I molecules (Fig. 4).

However, comparison of the functionally important peptide-binding domains (α1 and α2) revealed that MR1 exhibits ~40 to 50% amino acid identity with the classical MHC class I molecules of human, mouse, and nonmammalian (for example, chicken) species in both these domains (Fig. 4). Polymorphic

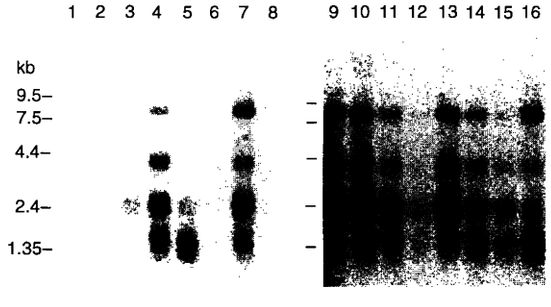

Fig. 1. Northern analysis of *MR1* expression. A 1.15-kb fragment prepared from the *MR1* cDNA clone C7 was hybridized to membranes containing polyadenylated RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes (lanes 1 to 16, respectively) (*15*). Size markers (in kilobases) are indicated on the left.

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