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ORIGINAL PAPER

Structural and functional evolution of the $P2Y_{12}$-like receptor group

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Abstract Metabotropic pyrimidine and purine nucleotide receptors (P2Y receptors) belong to the superfamily of G protein-coupled receptors (GPCR). They are distinguishable from adenosine receptors (P1) as they bind adenine and/or uracil nucleotide triphosphates or diphosphates depending on the subtype. Over the past decade, P2Y receptors have been cloned from a variety of tissues and species, and as many as eight functional subtypes have been characterized. Most recently, several members of the $P2Y_{12}$-like receptor group, which includes the clopidogrel-sensitive ADP receptor $P2Y_{12}$, have been deorphanized. The $P2Y_{12}$-like receptor group comprises several structurally related GPCR which, however, display heterogeneous agonist specificity including nucleotides, their derivatives, and lipids. Besides the established function of $P2Y_{12}$ in platelet activation, expression in macrophages, neuronal and glial cells as well as recent results from functional studies implicate that several members of this group may have specific functions in neurotransmission, inflammation, chemotaxis, and response to tissue injury. This review focuses specifically on the structure-function relation and shortly summarizes some aspects of the physiological relevance of $P2Y_{12}$-like receptor members.

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Introduction

Most cellular effects of nucleotides are mediated through two main families of specific cell surface receptors, the ionotropic P2X and metabotropic P2Y nucleotide receptors. The metabotropic receptors belong to the superfamily of G protein-coupled receptors (GPCR). The GPCR superfamily comprises at least five structurally distinct families/subfamilies (GRAFS classification) which share little sequence homology among each other [1]. The rhodopsin-like receptors (also called family A or 1) form the largest family in vertebrates. Eight different mammalian P2Y ($P2Y_{1,\ 2,\ 4,\ 6,\ 11–14}$) receptor subtypes, all belonging to the rhodopsin-like receptors (family A), have been identified and proven to function as receptors for extracellular nucleotides so far [2]. Based on their preferential agonists, P2Y receptors have been subclassified into adenine nucleotide-activated ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$), pyrimidine nucleotide-activated ($P2Y_4$, $P2Y_6$), ATP/UTP-activated ($P2Y_2$), and UDP-sugar-activated ($P2Y_{14}$) receptors. Most structural and functional studies were performed with the classic P2Y receptors $P2Y_1$ and $P2Y_2$ [3]. However, in comparison to other GPCR, large-scale structure/function data are rare, mainly due to difficulties in finding suitable P2Y-free heterologous expression systems [4]. Pharmacological characterization on both native and recombinant P2Y receptors is further hampered by the release of endogenous nucleotides and the hydrolysis or conversion of exogenous and endogenous nucleotides by ectoenzymes (for review see [5]). ATP and AMP are metabolized by cell surface enzymes, the ecto-NTPDase 1 (nucleoside triphos-

phate diphosphohydrolase 1, ectoapyrase, CD39, metabolizes ATP to AMP) and the 5′-ectonucleotidase (CD73, metabolizes AMP to adenosine). In addition, a nucleoside diphosphokinase catalyzes the transfer of the gamma phosphate of nucleoside triphosphates to nucleoside diphosphates. For example, in the presence of ATP, nucleoside diphosphokinase catalyzes the conversion of UDP to UTP. These specific problems may have contributed to many false and controversial declarations of P2Y receptors being activated by nucleotides [6–9]. Recent success in expressing GPCR in yeast, which is discussed below, may circumvent these problems and may engage structure-function-relation studies.

Although in-depth phylogenetic analysis of GPCR groups indicates clustering of P2Y receptors, when compared with other GPCR of family A [1, 10], the adenine nucleotide-activated $P2Y_1$ and $P2Y_{12}$, for example, display only minor structural relation at the protein level. Phylogenetic analysis even revealed that nucleotide specificity e.g. for ADP evolved independently in $P2Y_1$ and $P2Y_{12}$. So we suggest that grouping of P2Y receptors should be performed on phylogenetic aspects rather than on ligand specificity.

Based on their amino acid sequences P2Y receptors can be subdivided into at least two groups (Fig. 1). One comprises $P2Y_{1, 2, 4, 6, 11}$, and the second group contains $P2Y_{12–14}$. Additionally, several other structurally related GPCR, such as CysLT1R, GPR91, GPR99, and GPR34, cluster into both groups (Fig. 1). These receptors are activated by structurally different ligands such as leukotrienes, organic acids, and phospholipids. Probably because of their structural relation several P2Y receptors display agonist promiscuity. It was shown that not only ADP but also leukotriene E4 (LTE_4) activates $P2Y_{12}$ at nanomolar concentrations [11]. Cysteinyl leukotriene receptors (CysLT1R and CysLT2R) have been described to be activated by UDP in addition to activation by cysteinyl leukotrienes [12, 13]. Further, there is evidence that CysLT1R antagonists, such as pranlukast and montelukast, inhibit activation of $P2Y_1$ and $P2Y_6$ by 2MeS-ADP (2-methylthio-ADP) and UDP, respectively [14]. Although $P2Y_{12}$ and cysteinyl leukotriene receptors are clinically used targets for prevention of thrombocyte aggregation (clopidogrel) and treatment of asthma (montelukast), respectively, the structural basis and (patho)physiological relevance of dual/multiple agonist specificity is obscure. Close structural relations between P2Y and other non-nucleotide receptors as well as the mentioned examples of ligand promiscuity indicate that nucleotides are probably only one of many natural agonists on the genuine P2Y receptors.

Structural evolution of $P2Y_{12}$-like GPCR

Discovery and deorphanization of group members

The ADP-($P2Y_{12}$)-like receptor group is the most recently identified P2Y receptor group and includes $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (see Fig. 1, Table 1). Based on high structural

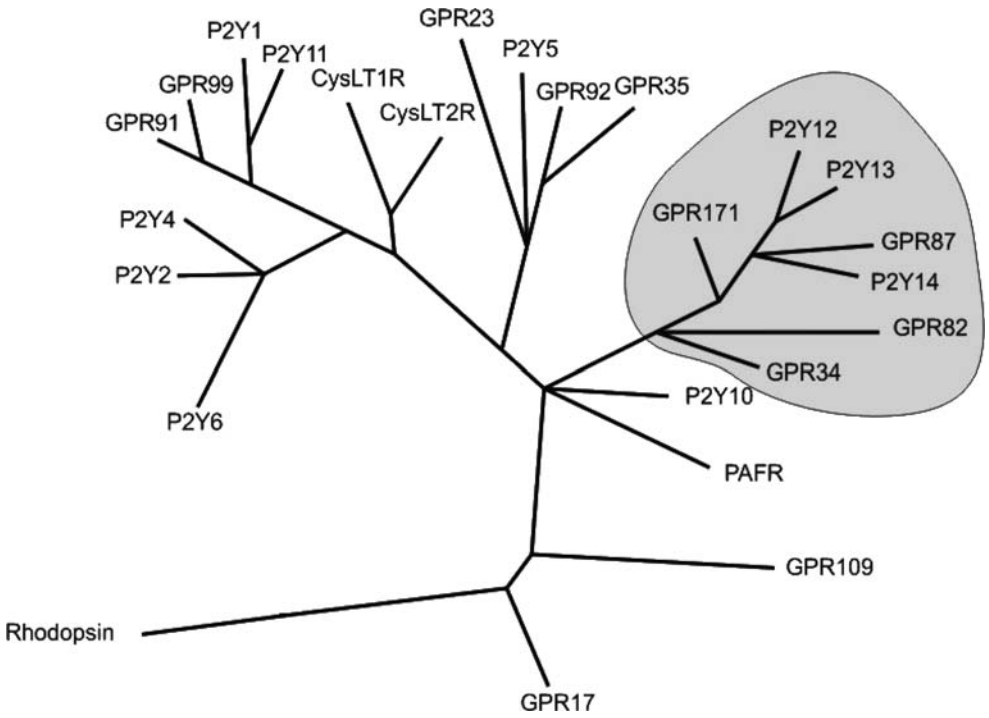

Fig. 1 Phylogenetic relation of human P2Y receptors. To evaluate the structural relation of P2Y receptors and related GPCR, amino acid sequences of human orthologs were aligned and phylogenetic relations were estimated using CLUSTAL W. The derived tree was essentially identical to former analysis [2, 10]. The $P2Y_{12}$-like receptors cluster into a group (*framed*) that is distinct from other nucleotide receptors such as $P2Y_1$ and $P2Y_2$

Table 1 Members of the $P2Y_{12}$-like receptor family

Receptor	G protein coupling	Natural agonist	Tissue expression
$P2Y_{12}$	G_i	ADP, CysLT-E4, phosphoribosyl pyrophosphate	Platelets, brain (glia) [19]
$P2Y_{13}$	G_i	ADP, diadenosine triphosphate	Spleen and adult brain, lower expression in placenta, lung, liver, spinal cord, thymus, spleen, small intestine, uterus, stomach, testis, fetal brain, adrenal gland, lymph node, bone marrow, peripheral blood mononuclear cells, leukocytes [22, 45]
$P2Y_{14}$	G_i	UDP-glucose, UDP-galactose, UDP-glucuronic acid, UDP-*N*-acetylglucosamine	Brain (astrocytes, glia), placenta, adipose tissue, stomach, intestine, spleen, lung (epithelium), heart [15, 16, 71]
GPR87	G_i	n.k	Placenta, thymus, small intestine, colon, prostate, uterus, testis, peripheral blood leukocytes [28, 29]
GPR171	n.k	n.k	n.k
GPR34	G_i	*lyso*-phosphatidylserine	Brain, glia cells, mast cells, spleen, heart, kidney, liver [27, 31, 34, 72]
GPR82	n.k	n.k	Testes, epididymis (unpublished own results)

Based on phylogenetic analyses and structural similarities the seven GPCR clusters in a group which is distinct from other P2Y receptors (see Fig. 1). Although they share structural features it appears from current studies that not all members are receptors for nucleotides. The table lists the currently known receptor agonists, the receptor's G protein specificity and expression pattern
n.k not known

similarity, the *lyso*-phosphatidylserine receptor (GPR34) and several orphan GPCR (GPR34-like, GPR82, GPR87, GPR171) are considered members of the structural $P2Y_{12}$-like receptor group (3,11). Most members of this group were deorphanized by the so-called reverse pharmacology approach. The orphan receptors predicted from sequence data are now often used to ascertain the ligands by testing them on tissue extracts and subsequent fractionation or on huge libraries of bioactive compounds, whereas, traditionally, the bioactive ligand was used to identify the receptor (classic approach).

As the first member of the $P2Y_{12}$-like receptor group $P2Y_{14}$ (synonyms KIAA0001, GPR105, VTR 15–20) was identified [15, 16] and then deorphanized in the year 2000 [17]. UDP-glucose was found to activate $P2Y_{14}$ by screening multiple receptors, each expressed in individual yeast strains, against a large library of over 700 known and putative natural GPCR agonists.

In 2001, $P2Y_{12}$ (initially named SP1999) was discovered and identified as the platelet clopidogrel-sensitive ADP receptor by a number of groups [18–21]. The $P2Y_{12}$ was deorphanized with both the classic and the reverse pharmacology approaches. Hollopeter et al. used subfractionated transcripts from platelets and isolated the cRNA (complementary RNA, encoding e.g. the $P2Y_{12}$ receptor) which mediated ADP-induced increases in K^+ current after injection into *Xenopus* oocytes [19]. In contrast, fractionated rat spinal cord extracts were assayed for Ca^{2+} mobilization in cells transiently transfected with $P2Y_{12}$ and chimeric Gα subunit [21]. Almost in parallel, ADP was shown to be an agonist for $P2Y_{13}$ (former GPR86), a very close structural neighbor of $P2Y_{12}$ [22–24]. 2MeS-ADP is the most potent agonist at $P2Y_{12}$ followed by ADP, adenosine 5′-O-2-thiodiphosphate, and ATP. (*N*)-methanocarba-2-methylthio-ADP, a highly potent agonist at $P2Y_1$, exhibited no and only minor agonist activity at $P2Y_{12}$ and $P2Y_{13}$, respectively [25].

Based on structural relations GPR34 [26, 27], GPR82 [28], GPR87 [29], and GPR171 (synonym H963) [30] also belong to the $P2Y_{12}$-like group (see Fig. 1). Except for GPR34, which was recently identified as a receptor for *lyso*-phosphatidylserine [31] by a reverse pharmacology approach, all the other $P2Y_{12}$-like receptors are still orphan GPCR.

The evolutionary origins

Based on structural similarities $P2Y_{12}$-like receptors cluster into a group distinct from other P2Y receptors (see Figs. 1 and 2). This $P2Y_{12}$-like receptor group can be subdivided into two subgroups. One subgroup encompasses $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, GPR87, and GPR171, the other subgroup GPR34, GPR34-like, and GPR82 (Fig. 2). This may suggest that both subgroups evolved from gene duplications starting from two related members. The grouping is further supported by shared genomic localization. Chromosomal clustering in the human genome is found for $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, GPR87, and GPR171 at 3q24-3q25 as well as for GPR34 and GPR82 at Xp11.4. This clustering is evolutionary well preserved. In the zebrafish genome $P2Y_{12}$,

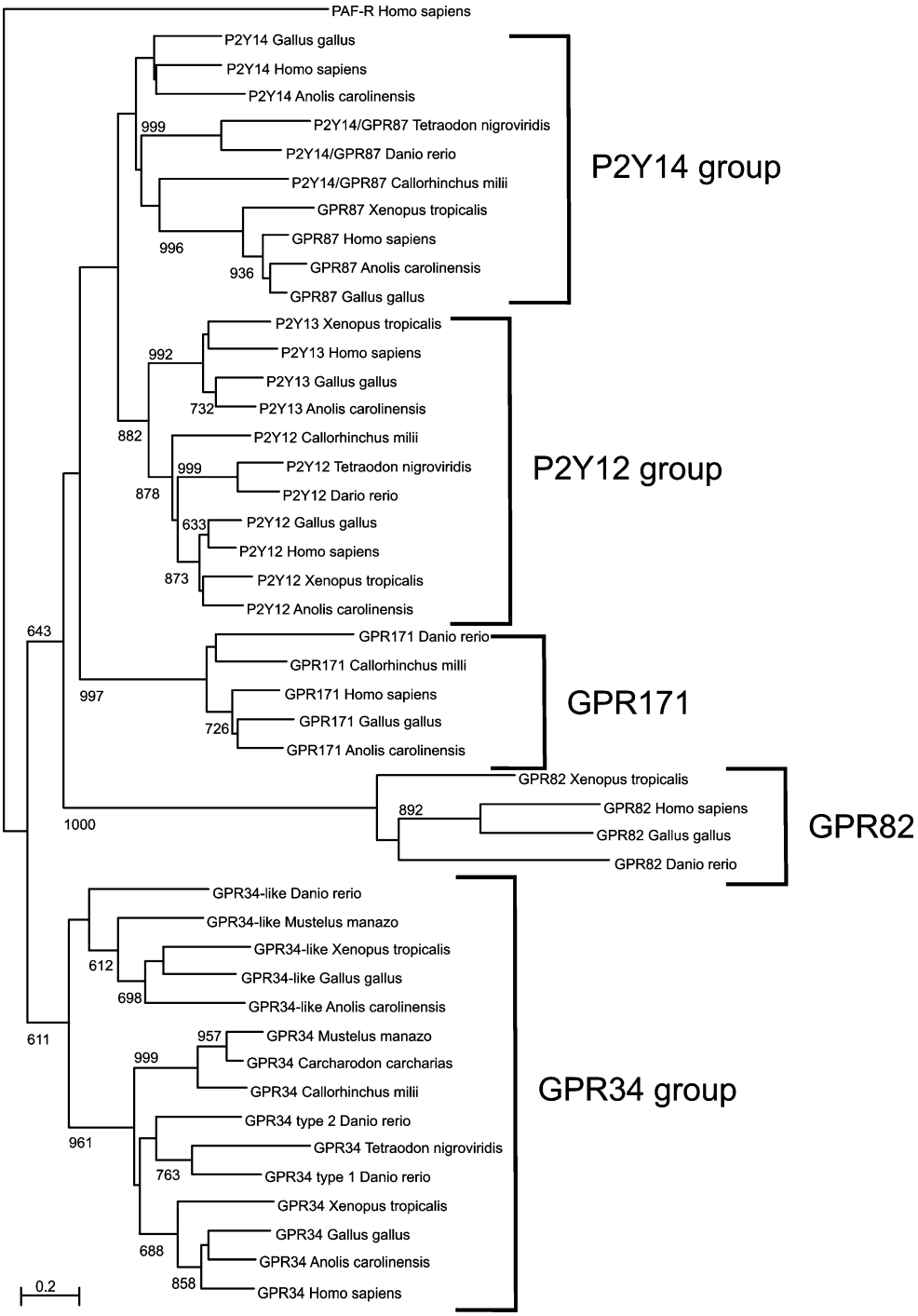

Fig. 2 Evolutionary origin of $P2Y_{12}$-like receptors. To subclassify and evaluate the phylogenetic relations between the $P2Y_{12}$-like receptors, the amino acid sequences of orthologs from one species of each vertebrate class (if available) were aligned using CLUSTAL W (identity matrices) and a phylogenetic tree was constructed (1,000 iterations). Bootstrap values >600 were considered as significant to support a new branch

$P2Y_{14}$/GPR87, and GPR171 cluster at chromosome 15 and GPR34 type 2 and GPR82 are tandemly arranged at chromosome 9. Gene clustering is not only found for vertebrate $P2Y_{12}$-like receptors but also for several other GPCR genes. Multiple copies of related GPCR genes, such as the human protease-activated receptors on 5q13, trace amine-associated receptors (TAAR, human 6q23.2), and CC-chemokine receptors (human 3p21.3), are the result of intrachromosomal gene duplications. The most impressive example is found in odorant receptors, where in humans, chromosome 11 contains nearly half of the odorant receptor repertoire, including a single cluster of more than 100 odorant receptors [32]. Gene amplification can be viewed as a dynamic and reversible regulatory mechanism that facilitates adaptation to variable environments. Clustered genes may confer selective benefits via their ability to be co-regulated and co-amplified. Indeed, even transcripts encoding a fusion protein of GPR34 and GPR82 have been

observed (own unpublished results). However, the biological relevance of $P2Y_{12}$-like receptor clustering in respect to transcriptional activity at their respective genomic loci, co-regulation, and transcript diversity need to be determined in the future.

Since $P2Y_{12}$, $P2Y_{14}$/GPR87, GPR34, and GPR34-like are all found in sharks (*Mustelus manazo*, *Carcharodon carcharias*) and chimeras (*Callorhinchus milii*) one can assume that $P2Y_{12}$-like receptors arose more than 450 million years (Myr) ago, before cartilaginous and bony fish split. Our own polymerase chain reaction (PCR) amplification attempts as well as the ongoing lamprey (*Petromyzon marina*) and sea urchin (*Strongylocentrotus purpuratus*) genome projects revealed no $P2Y_{12}$-like sequences yet, suggesting their origin in the very early *Gnathostomata* or, less likely, a loss in agnate and all non-vertebrates.

Gene duplication can be a primary source of the genetic material from which genes with new functions evolve. One copy of a duplicated gene may become mutated and acquire unique functionality without risking the fitness of the organism ensured by the homolog. On the other hand, if not advantageous, continuous accumulation of mutations (neutral drift) will eliminate one of the genes, a process named pseudogenization. In fish genomes, there is only one respective ortholog with relation to $P2Y_{12}$/$P2Y_{13}$ and to GPR87/$P2Y_{14}$. In the case of $P2Y_{12}$/$P2Y_{13}$ the fish ortholog is more closely related to $P2Y_{12}$ implicating that $P2Y_{13}$ evolved later in tetrapod evolution probably by $P2Y_{12}$ duplication. However, one cannot rule out that the gene was eliminated in the common ancestor of sharks and bony fishes. The evolutionary mechanism of $P2Y_{14}$ and GPR87 evolvement remains unsolved since their structural relation to the fish orthologs is similar. The absence of $P2Y_{14}$ in the African craw frog (*Xenopus*) genome but the presence of ortholog sequences in all other sequenced tetrapod genomes does not necessarily implicate that $P2Y_{14}$ derived from GPR87 in early tetrapod evolution. Again, $P2Y_{14}$ gene loss in amphibians must be considered.

Orthologs of GPR34, GPR87/$P2Y_{14}$, and $P2Y_{12}$ are found in all tetrapods and bony and cartilaginous fishes investigated so far. However, not all members of the $P2Y_{12}$-like group appear to be present in all vertebrate classes. GPR82 has an ortholog in zebrafish but is not yet found in the genomes of pufferfishes and the lizard (*Anolis carolinensis*) genome. Although GPR171 is found in reptiles, birds, mammals, zebrafish, and chimera it appears to be absent in African craw frog, stickleback, and pufferfishes. This may implicate specific functions of some $P2Y_{12}$-like receptors which are less important in some species or are compensated by other receptors and mechanisms.

Signatures of gene inactivation (pseudogenization) are rarely found for members of the $P2Y_{12}$-like group. Following gene duplication in the common ancestor of evolutionary basic fishes, like eels and carps [33], pseudogenization of one GPR34 subtype was identified in a salmon species (Keta salmon) (own unpublished results). The GPR34-like receptor is an evolutionary old $P2Y_{12}$-like receptor being present in cartilaginous and bony fishes, amphibians, and birds but not in mammals (Fig. 2). Sequences with residual relation to the GPR34-like receptor are found in the platypus genome but it is most likely a pseudogene.

Genomic organization of $P2Y_{12}$-like receptors

Comparison of transcript and genomic sequences provides information on intron/exon structure of a GPCR gene. Since the gain or loss of spliceosomal introns are unique events in evolution, they can serve as markers for phylogenetic analysis. Further, such analyses may reveal splice variants and may be informative about the promoter structure and gene regulation. Introns are the basis of alternative splicing, exon skipping, and RNA editing events and, therefore, can contribute to receptor diversity at a supragenomic level.

Most coding regions of human $P2Y_{12}$-like receptors do not contain introns (Table 2). One exception is GPR87 where the genomic sequence encoding the receptor's N terminus is interrupted by an intron in mammalian and avian orthologs. Another intron which is rarely removed (cryptic intron) has been identified in the N terminus-encoding sequence of GPR34 [34].

The genomic organization of the individual $P2Y_{12}$-like receptor genes is not well conserved during vertebrate evolution (see Table 2). For example, the $P2Y_{12}$ gene gained an intron in bony fish evolution disrupting the open reading frame of the transmembrane domain 2 (TMD2)-encoding part. Similarly, one of the two GPR34 paralog genes acquired an intron in the more recent bony fish evolution [33]. The two long-standing alternative explanations for the origin of introns, the intron-early theory and intron-late theory, remain a matter of continuous debate not only for GPCR [35]. The intron-early theory suggests that introns are extremely ancient characteristics of genes and that early genes were created through the intron-mediated shuffling of exons. However, numerous gene and genome comparison studies provided evidence that at least some introns are more recently acquired (intron-late theory). The $P2Y_{12}$-like receptor group is, therefore, a nice example where introns were acquired in the coding region in some species during more recent evolution.

In contrast to the coding region, the 5′ region of most members of the $P2Y_{12}$-like receptor group displays a distinct intron/exon organization with sometimes multiple transcription starts. Also, the gene sizes strongly differ between the individual and even closely related receptors.

Table 2 Genomic organization of $P2Y_{12}$-like GPCR. *n.a.* not analyzed because of lack of mRNA information

Member	Number of introns in the 5′ non-coding region	Number of introns in the coding region	Approx. size of genomic region[a]
$P2Y_{12}$			
Human	1–2	–	47 kbp (2 different transcript starts)
Mouse	4	–	46.5 kbp
Chicken	n.a.	–[b]	n.a. (>1 kbp)
African clawed frog	–	1 (N terminus)	4 kbp
Zebrafish	n.a.	–[b]	n.a. (>1 kbp)
Stickleback	1	1 (TMD2)	2 kbp
Pufferfish	n.a.	1 (TMD2)	>2 kbp
$P2Y_{13}$			
Human	–	1 (rare transcript, N terminus, NM_176894)	3.2 kp (2 different transcript starts)
Mouse	1	–	3 kbp
Chicken	n.a.	–[b]	n.a. (>1 kbp)
African clawed frog	–	1 (N terminus)	14–19 kbp
$P2Y_{14}$			
Human	2	–	66 kbp (2 different transcript starts)
Mouse	1–2	–	16 kbp
Chicken	n.a.	–[b]	n.a. (>1 kbp)
GPR87			
Human	1	1 (N terminus)	22.7 kbp
Mouse	1	1 (N terminus)	16 kbp
Chicken	2	1 (N terminus)	9.5 kbp
African clawed frog	n.a.	–[b]	n.a. (>1 kbp)
$P2Y_{14}$/GPR87			
Zebrafish	n.a.	–[b]	n.a. (>1 kbp)
Pufferfish	n.a.	–[b]	n.a. (>1 kbp)
GPR171			
Human	2	–	5 kbp
Mouse	1	–	4.5 kbp
Chicken	1	–	2.3 kbp
Zebrafish	n.a.	–[b]	n.a. (>1 kbp)
GPR82			
Human	2	–	4 kbp
Mouse	2–3	–	6 kbp
Chicken	n.a.	–[b]	n.a. (>1 kbp)
African clawed frog	n.a.	–[b]	n.a. (>1 kbp)
Zebrafish	n.a.	–[b]	n.a. (>1 kbp)
GPR34			
Human	3–4	1 cryptic (N terminus)	8.2 kbp
Mouse	3–4	–	9.1 kbp
Chicken	1	–	2.5 kbp
African clawed frog	2	–	5.2 kbp
Zebrafish			
Type 1	1	–	7.3 kbp
Type 2	–	–	1.2 kbp
Pufferfish	n.a.	–[b]	n.a. (>1 kbp)

Except for GPR87, human $P2Y_{12}$-like receptor members contain no intron in the coding region. However, this intronless gene structure is not well preserved in all vertebrates. The table summarizes the number of introns within the 5′ and coding region of selected vertebrate genes and estimates the size of the gene

[a] Based on the 5′ longest transcript

[b] Complete open reading frame within the genomic sequence but intron within the very N terminus cannot be ruled out because of lack of mRNA information

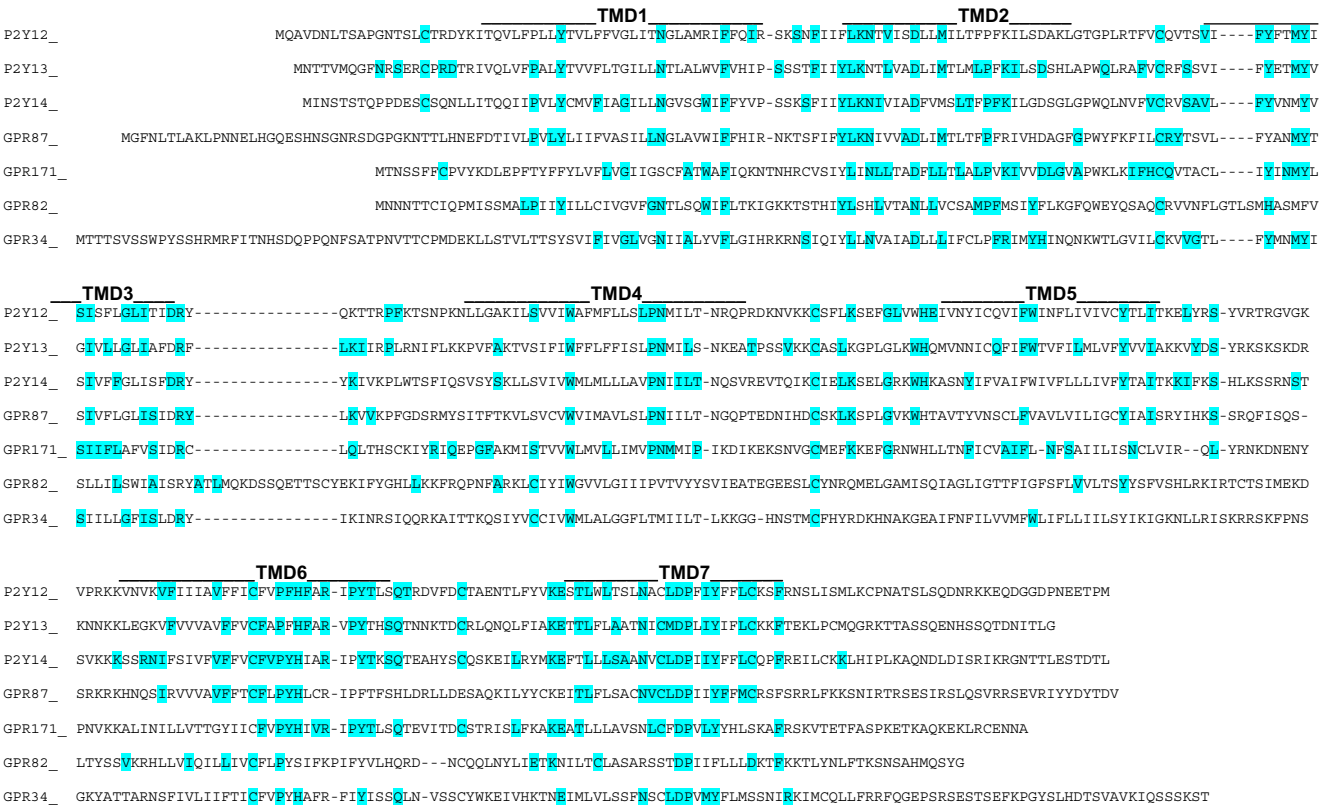

Fig. 3 Conserved residues in $P2Y_{12}$-like receptors. To identify conserved group and member-specific positions the amino acid sequences of $P2Y_{12}$ (74 orthologs), $P2Y_{13}$ (31 orthologs), $P2Y_{14}$ (38 orthologs), GPR87 (51 orthologs), GPR171 (41 orthologs), GPR82 (34 orthologs), and GPR34 (133 orthologs) were aligned using CLUSTAL W. Residues that are 100% conserved among the respective orthologs were boxed. Only a few positions are almost fully conserved among all members of the $P2Y_{12}$-like group (position number refers to the relative numbering system by Ballesteros and Weinstein [73]): TMD1: Phe/Tyr$^{1.39}$, Phe$^{1.57}$; TMD2: Leu$^{2.43}$, Asn/Asp$^{2.50}$, Pro/Ala$^{2.58}$; TMD3: Tyr/His$^{3.33}$, Arg/Gln$^{3.50}$; TMD4: Trp$^{4.50}$; TMD6: Cys/Ser$^{6.47}$; Pro$^{6.50}$; TMD7: Asp$^{7.49}$, Pro$^{7.50}$, and the two Cys residues bridging extracellular loops 1 and 2. The approximate positions of the seven transmembrane domains (*TMD*) are given above the sequences

Introns in the 3′-untranslated region (UTR) of $P2Y_{12}$-like receptors have not been found yet. Large and complex organized 5′ non-coding regions of a gene may provide the basis for multiple promoter regions, cis-acting elements, and a variable 5′ UTR of the mRNA. Alternative structures of the 5′ UTR can contribute to expression regulation and alternative translation start points. For example, 5′ UTRs often contain small open reading frames (ORF) which can be translated via leaky scanning at the ribosome. Such leaky scanning can reduce translation of the downstream main ORF as shown for several genes including GPCR [36, 37].

It is of interest to note that both genes, GPR34 and GPR82, are located in antisense orientation within a large intron of the CASK gene. This position is conserved during vertebrate evolution. The CASK gene encodes a calcium/calmodulin-dependent serine protein kinase that is a member of the membrane-associated guanylate kinase (MAGUK) protein family. Since GPR34 and GPR82 transcripts are antisense orientated to CASK one can speculate that transcripts may regulate expression of CASK or vice versa. Such hypotheses will be addressed in receptor-deficient mouse models (see below) in the future.

Key residues defining the individual member

Many attempts have been made to identify structural signatures which are helpful in annotation and grouping of P2Y receptors. On the basis of available sequence data for validated P2Y receptors, key residues were extracted to define $P2Y_{12}$-like and $P2Y_1$-like receptor groups [11, 38, 39]. In recent studies, we have shown for several GPCR that a significant number of orthologs is required for identification of functional motifs and key residues [33, 40–42]. By mining public databases and by amplifying and sequencing $P2Y_{12}$-like orthologs we have acquired large sets of sequences of $P2Y_{12}$ (74 orthologs), $P2Y_{13}$ (31 orthologs), $P2Y_{14}$ (38 orthologs), GPR87 (51 orthologs), GPR171 (41 orthologs), GPR82 (34 orthologs), and GPR34 (133 orthologs) to determine structural conservation of the

Table 3 Sequence conservation of $P2Y_{12}$-like GPCR

Receptor	K_a/K_s (mean ± SD)	Pi (mean ± SD)
$P2Y_{12}$	0.049±0.018	0.145±0.022
$P2Y_{13}$	0.141±0.070	0.187±0.023
$P2Y_{14}$	0.103±0.036	0.190±0.024
GPR87	0.046±0.027	0.134±0.018
GPR171	0.078±0.012	0.158±0.021
GPR34	0.082±0.037	0.137±0.026
GPR82	0.202±0.049	0.167±0.034

To compare the sequence conservation of $P2Y_{12}$-like receptors, ortholog sequences [relative positions 1.48 (in TMD1) to 7.68 (in C-term)] from 18 species were aligned and DNA polymorphism analyses were performed using DnaSP (version 4.1). The K_a/K_s ratio is calculated from the number of nonsynonymous substitutions per nonsynonymous site (K_a) and the number of synonymous substitutions per synonymous site (K_s) for any pair of sequences. The nucleotide diversity (Pi) is the average number of nucleotide differences per site between two sequences. The orthologs of the following species were analyzed because their genome contained all members of the $P2Y_{12}$-like group: *Bos taurus, Equus caballus, Canis familiaris, Pteropus vampyrus, Ornithorhynchus anatinus, Monodelphis domestica, Callithrix jacchus, Pan troglodytes, Homo sapiens, Macaca mulatta, Microcebus murinus, Pongo pygmaeus, Tarsius syrichta, Cavia porcellus, Mus musculus, Rattus norvegicus, Tursiops truncates, Gallus gallus*

members and to identify amino acid sequence motifs and key residues that are unique for the $P2Y_{12}$-like receptor group and the individual members (Fig. 3). These data sets were obtained from vertebrate species ranging from evolutionary old bony and cartilaginous fishes to the more modern mammals representing 450 Myr of evolution. The overall identity between $P2Y_{12}$-like members is rather low ranging from 19% amino acid identity (human GPR82 vs human GPR87) to 47% (human $P2Y_{12}$ and human $P2Y_{13}$). Between the respective fish and mammal receptor orthologs $P2Y_{12}$ (~49%) shows the highest identity followed by GPR171 (~48%), GPR34 (~40%), and GPR82 (~35%). Since not all $P2Y_{12}$-like GPCR have orthologs in all vertebrate classes, we analyzed the structural conservation within this receptor group by comparing K_a/K_s values (ratio of the number of nonsynonymous substitutions per nonsynonymous site and the number of synonymous—or silent—substitutions per synonymous—or silent—site) of the $P2Y_{12}$-like receptor ortholog set from species containing all group members (Table 3). Although all $P2Y_{12}$-like receptors display a purifying selection mode of evolution (K_a/K_s <<1 indicates high conservation and elimination of deleterious mutations), there are significant differences between the members of this receptor group. $P2Y_{12}$ and GPR87 were kept most conserved during evolution in birds and mammals. By contrast, GPR82 appears to be less constrained as already indicated by a relatively low conservation at the amino acid level (see above).

As shown in Fig. 3, members of the $P2Y_{12}$-like group share only eight fully conserved residues ($Phe^{1.57}$, $Leu^{2.43}$, $Trp^{4.50}$, $Pro^{6.50}$, $Asp^{7.49}$, $Pro^{7.50}$, and both Cys residues bridging extracellular loops 1 and 2) when sequences of more than 400 receptors of this group are compared. Although the ADP receptors $P2Y_{12}$ and $P2Y_{13}$ share more than 50 fully conserved residues none of these conserved residues is exclusively found in $P2Y_{12}$ and $P2Y_{13}$ but rather present also in other $P2Y_{12}$-like receptors (see Fig. 3). Further, only a few residues are member specifically conserved (GPR171: $Gln^{3.53}$ and $Asn^{5.59}$; $P2Y_{13}$: $Met^{7.48}$; GPR82: $Leu^{6.44}$ and $Asp^{7.57}$; GPR34: $Met^{7.52}$) and have not been found in other $P2Y_{12}$-like receptors so far. These facts suggest that ligand and signaling specificity is determined by a combination of many, more or less conserved determinants. It has been proposed that $His^{6.52}$/$Arg^{6.55}$ and $Lys^{7.35}$/$Glu^{7.36}$/$Leu^{7.39}$ within TMD6 and the ECL3, respectively, may present such motifs required for nucleotide binding [38]. However, such residue combination is also present in some GPR87, GPR171, and GPR34 orthologs which are not activated by ADP. This does not rule out that these residues are involved in nucleotide binding of e.g. $P2Y_{12}$ but it implicates additional positions which determine ligand specificity. In-depth structure-function analysis e.g. by mutagenesis studies are required to identify key positions and their structural properties.

Physiological relevance of $P2Y_{12}$-like receptors

Functional characterization of $P2Y_{12}$-like receptors in heterologous expression systems

Signal transduction of the ADP receptor $P2Y_{12}$ via pertussis toxin-sensitive G_i proteins and adenylyl cyclase inhibition is well established [22]. Similar G_i protein-coupling specificity was found for $P2Y_{13}$ [22, 24], $P2Y_{14}$ [43], and *lyso*-PS receptor GPR34 [31, 33]. Because adenylyl cyclase inhibition assays are usually less sensitive and robust several other experimental setups were established to measure function of $P2Y_{12}$-like GPCR. It has been shown that $G\alpha_{15}$ and $G\alpha_{16}$ can be activated by a wide variety of GPCR [44]. The ability of $G\alpha_{16}$ to bypass the selectivity of receptor/G protein interaction was also useful to measure activation of $P2Y_{13}$ [45]. It has been demonstrated that replacement of the four or five C-terminal amino acids of $G\alpha_q$ with the corresponding $G\alpha_i$ residues (referred to as $G_{\Delta 6qi4}$ [46]) confers the ability to stimulate the PLC-β pathway onto G_i-coupled receptors [47]. Successful heterologous expression and activation by apply-

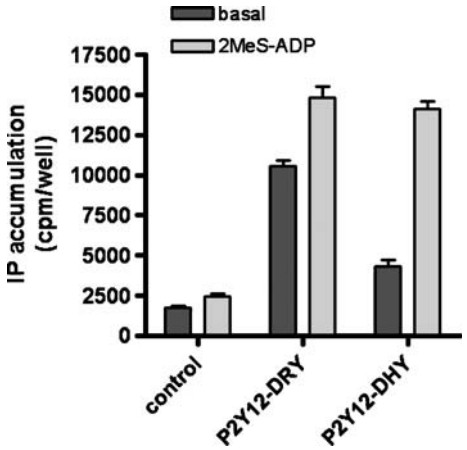

Fig. 4 High basal activity is a genuine property of $P2Y_{12}$. Basal activity of the wild-type $P2Y_{12}$ (DRY motif) and a mutant $P2Y_{12}$ (DHR motif) was determined in transiently transfected COS-7 cells. G_i coupling of $P2Y_{12}$ was rerouted to IP production by co-transfection of a chimeric G protein $G_{\Delta 6qi4}$ [46]

ing chimeric $G\alpha_{qi4}$ has been demonstrated for $P2Y_{12–14}$ and *lyso*-PS receptor GPR34 [25, 33, 48].

We have previously shown that $P2Y_{12}$ as well as the *lyso*-PS receptor GPR34 display increased basal activity in functional assays when compared with other GPCR [33]. This high basal activity can be either discussed as the natural ground state of the receptor activity equilibrium or as the effect of receptor agonists that are present in the cell culture medium or that are released from cells. Analyzing mutations in the highly conserved DRY motif of $P2Y_{12}$ we recently showed that basal activity is abolished in the DHY mutant but agonist-induced activation remains intact (Fig. 4). We conclude that the basal activity of the ADP receptor is rather a genuine property of this receptor and not due to continuous stimulation by agonists. High basal activity appears to be a general feature of all members of the $P2Y_{12}$-like receptor group. In our initial ortholog screen we identified two GPR87/$P2Y_{14}$ receptors, carp GPR87/$P2Y_{14}$ types 1a and 1b (AY241103, AY241102), which differ in only ten amino acid positions. Interestingly, carp GPR87/$P2Y_{14}$ type 1b displays higher basal activity and studies are ongoing to identify residues that promote constitutive activity.

Because many standard mammalian expression systems endogenously express P2Y receptors clear-cut functional studies are difficult to perform. One exception is the 1321N1 human astrocytoma cell line which does not express P2Y receptors. However, transient expression of GPCR in 1321N1 cells is limited by low transfection efficiency and stable transfection of these cells is usually required. Heterologous expression of GPCR in yeast was initially established for large-scale purification of receptor proteins. These advances in the expression of heterologous GPCR in the yeast *Saccharomyces cerevisiae* have led to the development of sensitive and selective assays of ligand-induced GPCR activation (reviewed in [49]). To facilitate a more systematic genetic analysis of GPCR function, e.g., by saturating random mutagenesis, we took advantage of a yeast expression system in which parts of the mammalian GPCR signaling system (GPCR and chimeric G protein) are linked to a modified yeast pheromone pathway (Fig. 5) [50]. The coupling of receptor activity to the genetically engineered yeast pathway allows for rapid and economical screening of substance libraries and randomly modified receptor libraries. Functional studies in yeast may have an advantage especially for P2Y receptors because one can (at least partially) circumvent specific problems in working with nucleotide receptors (see above). Only two NTPDases (GDA1 and YND1/APY1) have been found within the entire yeast genome which are mainly expressed in the Golgi apparatus [51]. Although conversion of nucleotides by nucleotidases and ectoapyrases occur also in yeast and one cannot exclude conversion of extracellular nucleotides by yeast enzymes, there is no endogenous plasma membrane P2Y receptor in yeast which can mediate and, therefore, interfere with transmembrane signaling of heterologously expressed

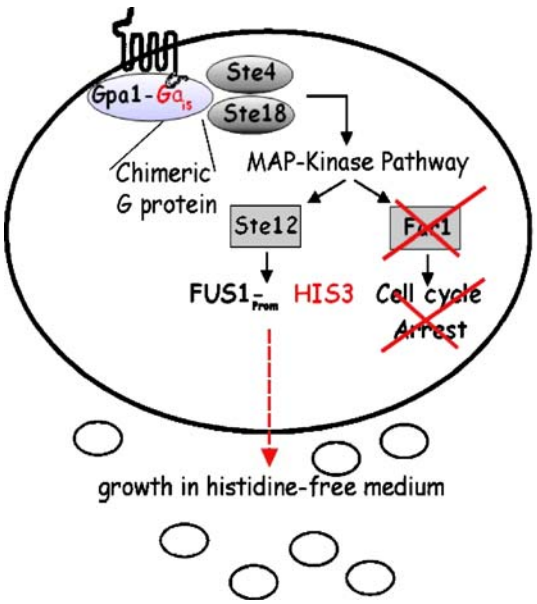

Fig. 5 Functional expression and in vitro evolution of GPCR in yeast. Genetically modified yeast cells are transformed with a mammalian GPCR. Following agonist activation and constitutive receptor activity, the receptor couples to a chimeric G protein [backbone yeast G protein Gpa1 in which the C-terminal five amino acids were replaced by the respective mammalian G protein sequence (e.g., from the G_i protein)]. Activation of the chimeric G protein enables yeast cells to grow on histidine-free medium by utilizing parts of the yeast mating pathway [49]. *Gpa* yeast ortholog of the mammalian G protein alpha subunit, *Ste4* yeast ortholog of the mammalian G protein beta subunit, *Ste18* yeast ortholog of the mammalian G protein gamma subunit, *Ste12* transcription factor that is activated by a MAP kinase signaling cascade, *Far1* cell cycle regulator that directly inhibits the yeast cyclin-dependent kinase Cdc28-Cln

P2Y receptors in yeast. We and others have successfully used this system for expression and functional study of $P2Y_{12}$ [52], $P2Y_{14}$ [53], and *lyso*-PS receptor GPR34 (unpublished data). As shown in Fig. 6a, $P2Y_{12}$-expressing yeast cells grow only in the presence of the agonist 2-methylthioadenosine 5′-diphosphate (2MeS-ADP).

Constitutively active GPCR are useful tools in studying the action of inverse agonists and activation mechanisms in GPCR. A constitutively active $P2Y_{12}$ was generated by replacing the endogenous C terminus with the corresponding part of the human $P2Y_1$ receptor [54]. Pharmacological evaluation of several $P2Y_{12}$ antagonists revealed AR-C78511 (an adenosine derivative) as a potent $P2Y_{12}$ inverse agonist. Traditional mutagenesis approaches are limited to screen for activating mutations because the number of mutant proteins that can be investigated is usually relatively small, primarily because of technical reasons and the time and costs needed to generate and analyze large numbers of mutant receptors. To circumvent these limitations, yeast has emerged as a highly useful host for the in vivo reconstitution of mammalian GPCR. Therefore, we applied a random mutagenesis and screening approach in yeast to identify key residues in maintaining ground stage of the human $P2Y_{12}$. PCR-based random mutagenesis was optimized to induce approximately four mutations in 1 kbp. Several dozen clones were selected and are now under in-depth investigation. For example, we identified a triple mutant $P2Y_{12}$ ($Leu^{115}Gln/Phe^{177}Ser/Arg^{224}Gly$), and individual characterization revealed $Leu^{115}Gln$ (TMD3) to be mainly responsible for constitutive activity in the yeast expression system (Fig. 6b). These promising results in applying the yeast expression system await further efforts to identify functionally relevant determinants in $P2Y_{12}$-like receptors.

Establishing in vivo function of $P2Y_{12}$-like receptors using mouse models

Selective receptor ligand and receptor-deficient animal models are suitable tools to evaluate the physiological relevance of distinct GPCR. Enormous efforts have been undertaken in the development of selective and clinically useful ADP and other nucleotide receptor ligands. But except for $P2Y_{12}$, dissection of the physiological relevance of all other $P2Y_{12}$-like GPCR is at the very early stage. Since the pharmacological properties of ligands have been reviewed in detail elsewhere [2] we only shortly summarize available data on receptor-deficient animal models.

Based on the clinical success of irreversibly bound $P2Y_{12}$ antagonists, such as clopidogrel, the pivotal role of ADP in arterial thrombogenesis is well established. Although the combined action of $P2Y_1$ and $P2Y_{12}$ is necessary for the full platelet aggregation response to ADP, mice deficient for $P2Y_{12}$ already display reduced platelet adhesion/activation, thrombus growth, and stability [18, 55]. Except for the altered platelet functionality $P2Y_{12}$-deficient mice appear normal under standard laboratory conditions. In consent with this finding, we occasionally identified a frameshift (T insertion at base pair position 667) in the $P2Y_{12}$ coding sequence of several individuals of the Asian house mouse (inbred laboratory strain of *Mus musculus castaneus* from of the Laboratoire Génome Populations Interactions Adaptation at the Universite Montpellier, France) during ortholog screening which showed no obvious phenotype in standard laboratory captivity. The platelet function was, however, not tested. The identified $P2Y_{12}$ inactivation in some Asian house mice may reflect either natural polymorphisms present in the wild population or a new polymorphism that has been

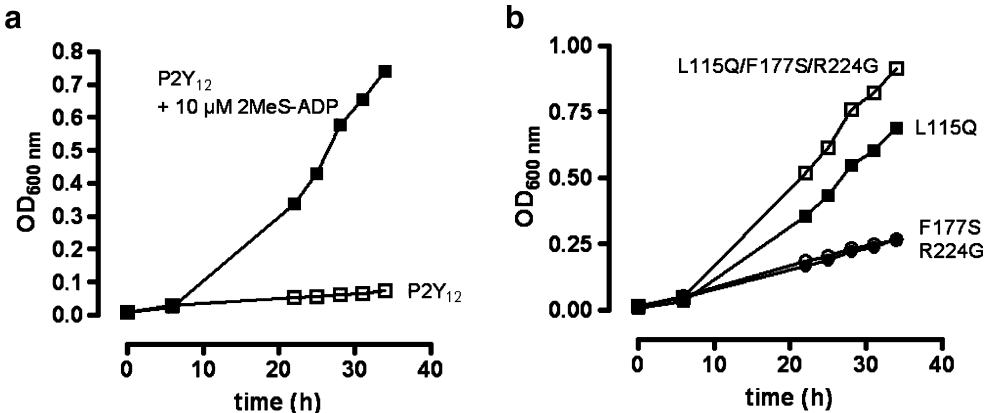

Fig. 6 Functional expression and random mutagenesis of $P2Y_{12}$ in yeast. **a** The human $P2Y_{12}$ receptor was transformed into modified yeast (see Fig. 4). The agonist 2-methylthioadenosine 5′-diphosphate (2MeS-ADP) induces a robust yeast cell growth. **b** The entire coding region of the human $P2Y_{12}$ was subjected to random mutagenesis and transformed yeast cells were selected for growth in agonist-free U^-/H^-medium. Colonies that grow under this condition contain a constitutively active $P2Y_{12}$. Exemplarily, mutations of a triple mutant ($L^{115}Q/F^{177}S/R^{224}G$) were individualized and tested separately for constitutive activity. The data indicate that $L^{115}Q$ mainly contributes to the constitutive activity of the triple mutant

introduced during captivity. To distinguish between these possibilities, we analyzed the position 667 in $P2Y_{12}$ from 88 wild *M. m. castaneus* trapped in Taiwan [41]. All contained the intact $P2Y_{12}$ allele indicating that the inactive $P2Y_{12}$ allele is very rare or absent in this wild *M. m. castaneus* population and favors the hypothesis of its acquisition in captivity (unpublished observations).

The fact that receptor deficiency is per se compatible with viability and fertility appears to be true also for other $P2Y_{12}$-like receptor-deficient species and mouse models. For example, mice individually lacking GPR82 and GPR34 are viable, fertile, and produced viable offspring (own unpublished observation). Further, there are several vertebrate classes and species naturally deficient in distinct $P2Y_{12}$-like receptors. For example, $P2Y_{13}$ is absent in fish genomes sequenced so far, GPR82 is not present in pufferfish genomes, and GPR171 is absent in pufferfish and *Xenopus* genomes.

It appears that many functions of $P2Y_{12}$-like receptors are more distinct and their disclosure requires specific challenging conditions. Indeed, $P2Y_{12}$-deficient mice revealed an unexpected phenotype when specifically challenged. CNS injury is accompanied by release of nucleotides, serving as signals for microglial activation or chemotaxis. Microglia cells express several purinoceptors, including $P2Y_{12}$. Microglia in $P2Y_{12}$-deficient mice showed significantly diminished directional branch extension toward sites of cortical damage in the living mouse. These results imply that $P2Y_{12}$ is a primary site at which nucleotides act to induce microglial chemotaxis at early stages of the response to local CNS injury [56].

Variants of $P2Y_{12}$-like receptor genes within human populations

Activating and inactivating mutations in GPCR have been made responsible for more than 30 different human diseases [57]. As expected from its pivotal role in platelet activation inactivating mutations in $P2Y_{12}$ can cause a congenital bleeding disorder. Only a few missense ($Met^{1}Arg$, $Pro^{258}Thr$, $Arg^{256}Gln$, $Arg^{265}Trp$) and frame-shifting (frameshift at amino acid position 240) mutations have been reported [19, 58–60]. Unexceptionally, the missense mutations found are at highly conserved positions and cause an impairment of receptor function. There are no other human diseases identified yet which are associated with dysfunction of $P2Y_{12}$-like receptors.

The antithrombotic effect of clopidogrel is considerably variable and the $P2Y_{12}$ gene was screened for possible sequence variants. Five nucleotide variations were found in the human $P2Y_{12}$ gene, two of them silent substitutions in the coding region [61]. Several studies were initiated to investigate the impact of $P2Y_{12}$ polymorphisms on atherosclerosis [62, 63] and clopidogrel efficiency in preventing neurological events [64] showing no or some association to one haplotype. In single nucleotide polymorphism (SNP) projects, such as HapMap [65], SeattleSNP etc., silent variations have been identified at amino acid position Val^{4}, Asn^{6}, Gly^{12}, and Phe^{182} of $P2Y_{12}$. For example the C/T variation at amino acid position Phe^{182} is only found in the African population whereas the G/T variation at position Gly^{12} is present only in European and Asian populations. One missense mutation ($Glu^{330}Gly$; receptor C terminus) has been detected in SNP projects. This variant is absent in European and Asian populations but displays an allelic frequency of about 15% in the African population. The functional relevance of this missense mutation has not been studied yet but a Gly at position 330 is naturally found in many other $P2Y_{12}$ orthologs including primate $P2Y_{12}$ almost ruling out a specific input in receptor function.

One silent variant (C/T at Ile^{59}) and one substituting variant (T/C at $Met^{158}Thr$) were identified in the coding region of $P2Y_{13}$. The Thr^{158} variant is less frequent in African populations (~4%) when compared to European (~21%) and Asian (~18%) populations. Met^{158} is not conserved and a Thr at this position is found in the dolphin (*Tursiops truncates*).

Only two silent substitutions (A/G at Ala^{35} and T/C at Phe^{240}) have been identified in $P2Y_{14}$ so far. The GPR171 gene contains two more frequent silent substitutions (T/C at Tyr^{19}, A/C Thr^{58}) and one missense variant (A/G at $Ile^{283}Val$, frequency about 6%) which is located within the DPXXY motif of TMD7. Ile^{283} is quite conserved among vertebrate GPR171 orthologs. The functional relevance of Val^{283} has not been studied yet but it occurs naturally in the zebrafish ortholog.

In the human GPR87 gene three silent polymorphisms (G/A at Pro^{46}, G/A at Leu^{179}, C/T at Tyr^{355}) and one nonsynonymous polymorphism (C/T at $Thr^{205}Met$) are present. The position 205 is not conserved and Met^{205} is found in several rodents. Only rare silent substitutions in the coding regions have been found in GPR82 (C/T at Asp^{313}) and in GPR34 (A/G at Val^{296}) [34].

SNP stochastically occur in individual genomes and can amount to a reasonable frequency in populations by drift but also by selection. There are several approaches and methods which are suitable to distinguish between drift and selection. Population genetic models predict that selection can leave “footprints” in closely linked genomic regions. Several methods were developed to detect signatures of selective sweeps in genomic sequences [66]. All methods require data on allele variation (mainly SNP) within populations. Large genome-wide analyses have scanned the human genome for signatures of positive selection on the basis of nonsynonymous and synonymous substitution ratios or single nucleotide polymorphism (SNP) data. Several loci which contain GPCR genes have been

identified using such methods, but the $P2Y_{12}$-like receptor-containing loci showed no strong signatures of recent positive selection in these studies [67–70].

Conclusion

$P2Y_{12}$-like receptors which are grouped mainly by phylogenetic relations have been an inherent part of the vertebrate GPCR repertoire since more than 450 Myr. Although sharing features in respect to structural determinants and signal transduction the activating ligands are heterogeneous such as nucleotides, nucleotide derivatives, leukotrienes, and phospholipids. We are at the very beginning of understanding the physiological importance of the individual members. The nature of the ligands, first functional data, and expression of several members in migrating cells point at functions in immunologic response and tissue damage response. Doubtless, upcoming receptor-deficient mouse models and selective receptor ligands will help to unveil the functions of $P2Y_{12}$-like receptor members. Further, one should consider combined receptor-deficient mouse models within this group but also with other P2Y receptors to uncover phenotypes which are hidden by receptor redundancy, e.g., in the case of ADP receptors.

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