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Review

G protein-coupled receptor overexpression with the baculovirus–insect cell system: a tool for structural and functional studies

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Abstract

G protein-coupled receptors, whose topology shows seven transmembrane domains, form the largest known family of receptors involved in higher organism signal transduction. These receptors are generally of low natural abundance and overexpression is usually a prerequisite to their structural or functional characterisation. The baculovirus–insect cell system constitutes a versatile tool for the maximal production of receptors. This heterologous expression system also provides interesting alternatives for receptor functional studies in a well-controlled cellular context.

Keywords: Baculovirus; G protein-coupled receptor; Insect cell; G protein; Stable expression; Overexpression

1. Introduction

G protein-coupled receptors (GPCRs) form the fourth largest superfamily in the human genome with more than 600 genes identified to date [1]. They have in common a topology based on seven-transmembrane α-helical domains and coupling to heterotrimeric G proteins (Gαβγ). GPCRs mediate the effect of numerous ligands including neurotransmitters, chemoattractants, hormones, cytokines and sensory stimuli such as photons and odorants. Binding of extracellular ligands initiates the signal transduction cascade by triggering conformational changes in the receptor that promote Gα subunit activation [2,3]. Following nucleotide exchange (GDP replacement by GTP), the tightly associated Gα and Gβγ subunits separate from each other and from the receptor. Both components are then free to interact and modulate the activity of downstream elements of the signalling cascades such as adenylyl cyclase or ion channels. Signal transduction is tightly regulated by receptor phosphorylation and subsequent interaction with arrestins as well as by the interplay of G-protein subunits with ''regulators of G-protein signalling'' (RGS). Tremendous progress has been accomplished within the last few years in dissecting GPCR-mediated signal transduction pathways, but the molecular mechanisms underlying ligand recognition and signal transduction through the membrane are restrained by the lack of detailed receptor structures. To date, only the 3D structure of rhodopsin has been solved at high resolution [4].

Structural characterisation of a protein may be achieved using different biophysical and biochemical approaches that often require several milligrams of protein per assay. The low natural abundance of most GPCRs precludes the use of such methods of investigation. For more than 10 years, efforts have been made to overcome this problem, leading to the use of heterologous organisms for protein production [5–7].

Escherichia coli is an attractive expression system because it is easy to scale-up and cheap to grow, but there are some drawbacks associated with the prokaryotic nature of the organism. For example, the low percentage of GC nucleotides in the genome when compared to mammalian genes or the existence of rare codons often result at least for soluble proteins in low expression levels or truncated forms [8,9]. Heterologous expression in bacteria also suffers from the inability of prokaryotes to perform any post-translational modifications (e.g. glycosylation, fatty acid acylation, phosphorylation) some of which are known to be of critical

Abbreviations: ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; Sf, *Spodoptera frugiperda*

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relevance for protein function. Also, the lack of G proteins in bacteria affects the properties of the expressed GPCRs though successful interaction with a Gα subunit in fusion [10] or added to the membrane-bound receptor has been demonstrated [11]. Therefore, *E. coli* is most often chosen to provide material suitable for structural studies. In spite of many efforts invested in the design of fusion proteins and/or selection or engineering of bacterial strains, GPCR expression levels at the inner membrane are still often poor (most often less than 200 receptors/cell) and receptor production is often perceived as toxic by the cell. However, in the best cases, up to 3500 copies per cell of functional muscarinic m_1 receptor (0.66 mg/l culture) have been produced in *E. coli*, allowing the purification of milligrams of receptor [12]. Folding of some heterologous membrane proteins may be hindered by the lack of appropriate chaperones leading to accumulation of misfolded proteins in the cytoplasm and formation of the so-called inclusion bodies which requires subsequent refolding of the protein [13,14].

Yeast is another small organism for which large-scale culture is easily achieved. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* as well as the methylotrophic *Pichia pastoris* have been used successfully for GPCR expression [15]. Expression levels reported for GPCRs were generally about 1–2 pmol/mg membrane proteins but serotonin $5\text{-}HT_{5A}$, β_2- and α_2-adrenergic as well as dopamine D_2 receptors were produced at levels well exceeding 10 pmol/mg membrane proteins [15]. Yeast cells offer a cellular context able to perform post-translational modifications similar to other more complex eukaryotic cells. However, N-glycosylation of mammalian membrane proteins in yeast seems to be very inefficient [5,6]. It is also worth noting that the yeast cell is surrounded by a cell wall that may hinder recovery of nonsecreted proteins.

GPCR expression in mammalian cells represents the closest alternative to their native environment. Heterologous protein production may be achieved through transient or stable expression and various cell lines are available (e.g. Cos, CHO, BHK 21, HEK 293, GH3). This system is widely used for functional studies especially when post-translational modifications such as N-glycosylation or proteolytic processing are a prerequisite to function. Usually, GPCR expression levels do not exceed 5–10 pmol/mg membrane proteins but some host cell lines, for example, HEK 293, are able to grow in suspension and are suitable for large-scale biofermentors. However, very high expression levels of the β_2-adrenergic or opioid receptors could not be maintained in suspension culture [7,16]. Unfortunately, scaling up of adherent cell cultures is a rather arduous and costly process, so mammalian cells are not altogether preferred for GPCR overproduction.

In this context, the baculovirus system offers a very attractive alternative that has been widely taken advantage of. It is, however, important to bear in mind that the right combination between receptor and heterologous host has to be found and that no universal system is likely applicable to all GPCRs. When dealing with structural aspects, amounts of protein produced are the limiting step while post-translational modifications as well as a cellular environment close to the native one are essential when studying functional aspects. Both criteria can rarely be fulfilled by one single heterologous expression system. Depending on the final goal, one system may prove more adapted than another and expression may be performed in several heterologous systems simultaneously to address different questions.

2. What is the baculovirus system?

In the eukaryotic expression system based on the use of baculovirus, protein production results from infection of insect cells by recombinant viruses encoding the gene(s) to be expressed. The cDNA of interest is inserted into a plasmid transfer vector that is introduced by homologous recombination into the genome of a baculovirus, usually *Autographa californica* multiply embedded nuclear polyhedrosis virus (AcMNPV). In most cases, the very strong polyhedrin promoter drives expression. Polyhedrin is naturally produced at very high levels (up to 20% of the total protein synthesised) and is nonessential for viral propagation in cell culture, and it can therefore be replaced by any gene of interest. Other promoters can also be used such as the p10 promoter. Both the polyhedrin and p10 promoters are ‘very late’ promoters because they are only switched on about 24 h post viral infection (Fig. 1). Both promoters drive the expression of two major components of occlusion bodies that are located within the nucleus and can be described to form a proteinaceous matrix in which viral particles are embedded and protected from the external environment. Several copies of the polyhedrin and p10 promoters can also be used simultaneously when two or more proteins are to be expressed [17]. The system is therefore suitable for production of multimeric proteins [18,19] or for the reconstitution of partnerships such as those taking place in the early steps of the signalling cascade (see below). Expression of heterologous proteins under the control of the very late promoters peaks usually 48–72 h post infection. The viral cycle is lytic and causes cell death about 4–5 days post infection; there is, therefore, considerable interest in promoters that would start protein production earlier, allowing more time for post-translational modifications to take place. The basic protein promoter drives the production of a small protein involved in DNA compaction as early as 6–8 h post viral infection. It has been successfully used for heterologous protein production including GPCRs, but maximal expression levels were markedly lower than those reached under the control of the polyhedrin promoter (Ref. [20], Massotte unpublished data). Therefore, this promoter is not much favoured for overexpression.

Since its first description for heterologous protein expression almost 20 years ago [21], the baculovirus system has inherited many technical improvements along the years to

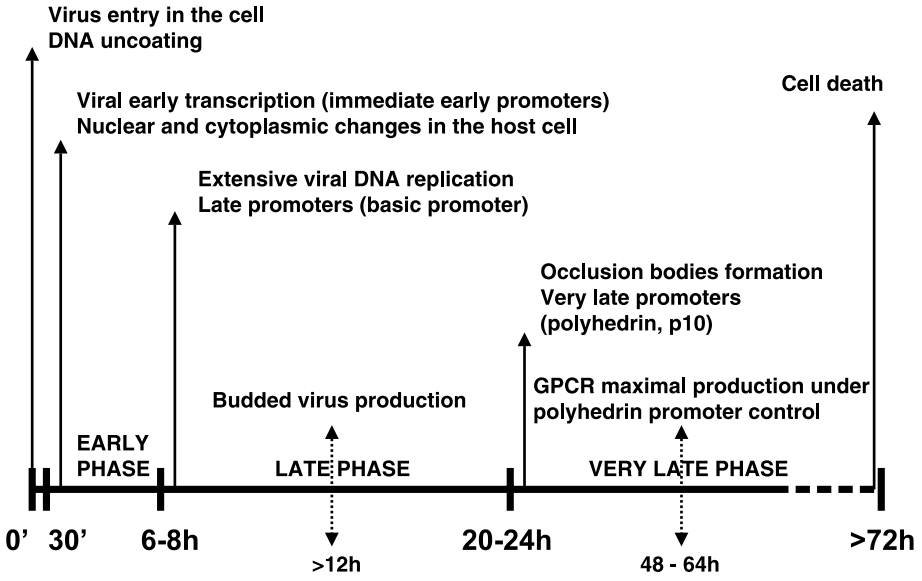

Fig. 1. Time-scale of baculovirus infection showing the three successive phases of infection. Vertical arrows point out the main events taking place respectively in the early, late and very late phases. Promoters used for GPCR expression are indicated within parentheses. An approximate time-scale for the main events is also mentioned.

become a relatively simple technique that can be readily introduced into most laboratories [22,23]. Protocols are well established and most of the tools for recombinant virus generation as well as cell culture and protein production are commercially available. The narrow host cell range restricted mainly to insects makes baculoviruses attractive because they do not require extensive biosafety measures. Indeed, baculoviruses can infect various mammalian cells including hepatocytes, but they show no evidence of replication in such cells [24,25].

Insect cells are semi-adherent, they grow easily in suspension and they can be adapted to serum-free media allowing easy scale-up in large biofermentors. Insect cells are also able to perform post-translational modifications identical to those of mammalian cells (e.g. phosphorylation, fatty acid acylation, glycosylation) and recombinant proteins exhibit characteristics very similar if not identical to their native counterparts. It is worth mentioning that N-glycosylation is often more simple than in mammalian cells and remains of the high-mannose type [26]. Cells derived from *Spodoptera frugiperda* ovarian tissue (Sf9 and Sf21) are the most widely used but attempts have been made to broaden the number of available cellular lines to improve protein production [*Trichoplusia ni* (High Five™)] [27–29] or to produce glycosylation patterns of the complex and hybrid types in cell lines derived from *T. ni* and *Estigmena acrea*, respectively [30,31].

3. GPCR overexpression for structural studies

Structural studies require fairly large amounts of protein, often exceeding milligram quantities, of a receptor in a fully functional state. Heterologous expression with the baculovirus–insect cell system ensures production of good protein quantities in cells performing post-translational modifications essentially identical to those occurring in naturally producing tissues. This guarantees high-level expression of recombinant GPCRs with characteristics almost identical to their native counterparts, making them ideal for subsequent structural studies.

3.1. GPCR maximal levels of expression

Baculovirus-infected insect cells have proven very efficient for membrane protein production and have achieved successful overexpression of numerous GPCRs such as several dopamine, serotonin, muscarinic, opioid or adrenergic subtypes. As reviewed by Grisshammer and Tate [5] in 1995, amounts of receptors produced were generally in the range of 5–30 pmol/mg protein and $1–2 \times 10^6$ receptor sites were generally present per cell. These amounts are 25–600 times higher than those obtained in the naturally producing mammalian cells. Since Grisshammer and Tate's comprehensive review, some yields of expression have been improved and other receptor types successfully expressed. In several cases, receptor production exceeded well 30 pmol/mg protein (Table 1).

3.2. Development of tools to improve GPCR expression and purification

Several attempts have been made to improve GPCR expression with the baculovirus system. Membrane proteins, like secreted soluble polypeptides, travel through the cell secretory pathway to reach the cell outer membrane.

Table 1
GPCRs expressed for the first time or for which expression was improved since 1995

Receptor	Type	Origin	Signal sequence	Maximal expression level	Reference
Adenosine	A_{2A}	Human		19 ± 4 pmol/mg	[50]
				26 ± 4 pmol/mg	[135]
Adenosine	A_{2A}	Human	*N*-(his)$_6$-flag	48 ± 5 pmol/mg	[50]
Bradykinin	B_2	Human		2 pmol/mg	[146]
				2.57 pmol/mg	[77]
Cannabinoid	CB_1	Human		15 pmol/mg	[144]
Cannabinoid	CB_2	Human		33 pmol/mg	[144]
Dopamine	D_{2S}	Human	mellitin	6–10 pmol/mg	[27]
Dopamine	D_{2S}	Rat		5–8 pmol/mg	[109]
Dopamine	D_{2L}	Rat		5–8 pmol/mg	[109]
Dopamine	D_3	Human		10–12 pmol/mg	[153]
Endothelin	B	Human		100 pmol/mg	[49]
Gonadotropin-releasing hormone		Rat		7800 ± 650 receptors/cell = 10 fmol/mg	[154]
Histamine	H_2	Rat		6.6 ± 0.6 pmol/mg	[102]
Hydroxytryptamine	5-HT_{1A}	Human		5–22 pmol/mg	[60]
Hydroxytryptamine	5-HT_{1B}	Human	gp64	22 ± 1.5[a] pmol/mg	[37]
Hydroxytryptamine	5-HT_{1D}	Human	gp64	31.6 ± 1.8 pmol/mg or 21.8 ± 6.1[a] pmol/mg	[37]
Hydroxytryptamine	5-HT_{2C}	Rat		50–70 pmol/mg	[145]
Hydroxytryptamine	5-HT_{5A}	Human	none	23 ± 2 pmol/mg	[38]
Hydroxytryptamine			gp64	63 ± 11 pmol/mg	[38]
Kinin	B_1	Human		150 fmol/mg	[146]
Luteinizing hormone		Mouse		0.8 pmol/mg	[20]
Opioid	delta	Human		85000–110000 receptors/cell[b]	[48]
Opioid	kappa	Human		30000–50000 receptors/cell[b]	[48]
Opioid	mu	Human	none	400000–500000 receptors/cell[b]	[48]
Opioid			gp64	2.7×10^6–5.4×10^6 receptors/cell = 12–25 pmol/mg	[29]
PACAP		Human		82.6 ± 3.8 pmol/mg	[40]
Substance P		Human		65 pmol/mg	[155]
Thrombin		Human		0.78 mg/l = 16 nmol/l	[156]

For older references, see Ref. [5].
[a] Measured with an agonist.
[b] As a rough estimate for conversion of expression levels, 10^6 receptors/cell is about 10 pmol/mg membrane proteins (for a 45-kDa protein) [5].

Hence, solutions brought to address secretion or folding problems of secreted proteins are often of use for membrane polypeptides. Fusions to a variety of signal sequences from baculoviral, prokaryotic or eukaryotic origin successfully increased the amount of soluble secreted proteins [32–35]; the N terminus of all GPCRs is predicted to be on the extracellular surface of the plasma membrane, so attempts were therefore made to use a similar strategy to increase GPCR production. In contrast to other protein families, most GPCRs are not synthesised as a pro-receptor with an N-terminal signal sequence preceding the mature protein coding sequence. In fact, addition of a cleavable signal sequence ahead of the GPCR coding sequence improved expression levels (Table 1) whether derived from the *Apis melifera* melittin in the case of the human dopamine D_{2S} receptor [27] or the influenza hemagglutinin in the case of the human β_2-adrenergic receptor [36]. The signal sequence of the baculoviral gp64, a glycoprotein involved in membrane fusion, also led to high expression levels of the human mu opioid [29], the 5-HT_{1B} and 5-HT_{1D} [37] or 5-HT_{5D} [38] receptors.

Crude cell membrane extracts from baculovirus-infected cells contain immature GPCRs probably representing a fraction of proteins trapped in the membranes of endoplasmic reticulum (ER) and Golgi apparatus as a result of the biosynthetic pathway saturation. A step based on the biological activity of the receptor such as a ligand affinity column was often included in purification schemes to eliminate nonfunctional misfolded receptors [39–41]. Engineering of the insect cells could also be undertaken to try and prevent the formation of misfolded receptor and therefore optimise GPCR expression. As already mentioned, secreted and membrane proteins share in common the requirement of moving along the secretory pathway to be respectively released in the extracellular environment or routed to the plasma membrane, explaining why addressing secretion or folding problems of secreted proteins may result in improvements in membrane protein production. Co-expression of chaperones such as the ER-resident BiP (immunoglobin heavy chain binding protein), calreticulin or the ER membrane-bound calnexin appeared an attractive strategy (reviewed in Ref. [42]) that could be applied to the GPCR family. Calnexin associates with a number of mem-

brane or secreted glycoprotein intermediates and prevents them from entering unproductive folding pathways [43]. It has successfully increased expression of the serotonin transporter by almost 200% [44] and expression of the human taurine transporter fused to glutathione *S*-transferase by 54% [45]. Catalytic enzymes, on the other hand, accelerate folding along the proper pathway. Their use for membrane proteins was shown in the case of the human dopamine transporter, the expression of which was increased in an insect cell line stably transformed with the peptidyl-prolyl *cis–trans* isomerase that catalyses the *cis–trans* isomerisation of an X-Pro bond [46].

Influence of the culture medium on maximal receptor expression has also been reported. In culture conditions in which Sf9 cells had been adapted to serum-free medium, the human mu opioid receptor fused to the gp64 signal sequence was expressed about twice as much as in Sf9 cells grown in the presence of serum and at levels comparable to those obtained in *T. ni* cells adapted to serum-free medium [29]. This latter observation suggests that higher expression levels observed in *T. ni* cells compared to Sf9 are due to serum-free culture conditions rather than to a cell line effect [29]. High levels of expression (20 nmol/l) were also obtained for the human β_2-adrenergic receptor fused to the hemagglutinin signal sequence and a C-terminal $(His)_6$ tag in Sf9 cells adapted to serum-free medium [39] and production of bovine rhodopsin was increased up to double in Sf9 cells adapted to serum-free medium [47]. Interestingly, opioid receptor expression peaked earlier after viral infection in cells adapted in serum-free medium (Sf9 or *T. ni*) when compared to expression in the presence of serum, suggesting that serum depletion exerts a direct influence on the cell metabolism and hence protein production [29].

Efforts have also been made to facilitate downstream purification of the receptors. A hexahistidine $(His)_6$ tag has often been added to the protein which allows the use of an affinity step on an ion chelating resin for an efficient purification step. A systematic study using human opioid receptors examined the influence of this tag on the receptor production. The authors showed that addition of a $(His)_6$ tag did not interfere with ligand binding whether located at the N or C terminus of the receptor [29,48]. However, $(His)_6$ addition at the N terminus reduced drastically the expression levels of delta, kappa and mu opioid receptors, whereas $(His)_6$ addition at the C terminus proved to be less deleterious. In fact, $(His)_6$ addition at the C terminus reduced expression only 2-fold in Sf9 and *T. ni* cells, but $(His)_6$ addition at the N terminus decreased the expression level at least 6- to 8-fold in Sf9 cells and 20-fold in *T. ni* cells [29,48]. Addition of a $(His)_6$ tag at the C terminus of the human dopamine D_{2s} receptor also had a negative influence on the receptor processing as revealed by a reduced amount of glycosylated receptor [27]. On the other hand, no decrease in the level of expression was reported for the endothelin B receptor following addition of a $(His)_6$ tag at its C terminus [49] and addition of a $(His)_6$-Flag tag at the N terminus of the human adenosine A_{2a} receptor increased two to three times expression levels when compared to the native sequence [50]. As outlined by those examples, prediction of expression levels after the addition of a tag is very difficult because the way a sequence alters the expression level appears to be rather receptor specific.

Noteworthy novel strategies are still explored with the hope to improve the current state of art. In such an attempt, the β_2-adrenergic [51] as well as the muscarinic m_1 and dopamine D_1 [52] receptors could be recovered from extracellular baculovirus particles. This is a consequence of the budding process during which the viral particles released from the cell acquire an envelope or ''loosely fitting'' lipid bilayer derived from the cell plasma membrane. Only mature fully processed receptors were identified in extracellular baculovirus particles. Also, those receptors were coupled to the adenylyl cyclase as demonstrated by its stimulation following agonist treatment. This approach may therefore offer an easy alternative for producing homogenous populations of functional GPCRs although the amounts produced per litre of culture are relatively low.

3.3. Large-scale production in biofermentors

The amounts of GPCR produced per cell and litre of culture with the baculovirus system represent a great improvement compared to natural expression yields (up to several hundred-fold increase), but structural studies are very demanding and large-scale cultures have to be envisaged. Insect cells are semi-adherent, allowing both growth under attached conditions (rollers, micro-carriers) or in suspension (shakers, airlift, or stirred tank). However, the cells are also very sensitive to shear damage resulting from gas bubbling. This can be reduced by different additives such as Pluronic F-68, a tensioactive compound that prevents the cells from attaching to the gas–medium interface [53]. Biofermentors allow not only large culture volumes but also control of optimised conditions for protein production. Oxygen proves to be a crucial rate-limiting component because its consumption increases drastically following viral infection. Volumes of 60 l or more are used in industry but, at the laboratory scale, biofermentors are rarely larger than 5–10 l. Over the years, numerous protocols for culture and infection in spinner flasks or biofermentors have been established [54–58].

One drawback of the baculovirus system is the requirement for high-titre viral stocks to infect cultures. The systematic study performed on opioid receptor production showed that the optimal multiplicity of infection for most constructs tested was lower in Sf9 cells compared to *T. ni* cells [29]. This is of importance if one considers the buildup of large viral stocks. Because the virus is lytic, attention has also to be paid when deciding for optimal multiplicity of infection and very often the use of a semicontinuous system or repeated batches was preferred over continuous production. Also, culture in a continuous reactor is limited to a

maximum of 30 days. For longer times, an inevitable loss of production happens, probably due to a high proportion of defective viral particles [59].

Despite the variety of biofermentor designs that have been studied and used in academic work and industry, no single system has proven to be universally superior in productivity and easiness of scale-up. Both attached and suspension growth systems present advantages though the latter is often favoured because of its simplicity and the long-standing experience from microbial fermentation.

3.4. Post-translational modifications

Among post-translational modifications, palmitoylation plays a major role in the regulation of GPCR function. This reversible fatty acid acylation corresponds to the covalent attachment of palmitic acid to cysteine residues via a hydroxylamine-labile thioester bond and it is often very difficult to study due to the low natural abundance of the receptors. Heterologous expression using mammalian cell lines such as CHO or HEK 293 partially alleviated this problem. Nevertheless, some GPCRs are still too poorly expressed in those cells. Indeed, the low specific activity of [^{3}H] or [^{14}C] palmitate used in metabolic experiments makes it extremely difficult to detect palmitoylation of GPCRs expressed in mammalian cells. Therefore, overexpression in insect cells with the baculovirus system proved to be very helpful because insect cells perform post-translational modifications identical to mammalian ones with the exception of glycosylation (see below). Palmitoylation of the human dopamine D_{2s} [27], human 5-HT_{1A} [60], human 5-HT_{1B} [61], GluR6 kainate [62], murine 5-$HT_{4(a)}$ [63] and human mu opioid (Massotte, unpublished data) receptors have been established using the baculovirus expression system. Palmitoylation is a dynamic process [64] and the high level of receptor expression also allowed the determination of its kinetics as well as increased turnover upon agonist stimulation [65,66]. Recent studies in mammalian cells have confirmed the biological role of the dynamic palmitoylation process which validates the use of insect cells for those studies [67–71].

GPCR agonist-induced phosphorylation has been similarly demonstrated in baculovirus-infected insect cells for the human m_2 [72] and m_3 muscarinic [73], human 5-HT_{1B} [61] and 5-HT_{1A} [74], dopamine D_1 [75] and D_2 [27], substance P [76] and bradykinin B_2 [77] receptors. Phosphorylation is critically involved in receptor desensitisation and advantage has also been taken of its co-expression with a given G protein-coupled receptor kinase (GRK) to get better insights in this central regulatory mechanism (see GPCR Overexpression for Functional Studies).

Most proteins present at the extracellular surface of mammalian cells are glycosylated. Two main roles have been postulated for the need of N-glycosylation. Inside the cell, it helps proteins to fold and assemble correctly in the ER where proteins like calnexin and calreticulin act as chaperones that recognise and bind to the carbohydrate portion of newly synthesised proteins. Misfolded glycoproteins are then selectively eliminated from the ER by a stringent process of conformation-based quality control [43]. Outside the cell, carbohydrates provide specific recognition structures for interaction with a variety of external effectors intervening in processes such as the modulation of neural plasticity [78]. Also, sialic acids are known to play an important role in the in vivo clearance rate of proteins [31]. The functional role(s) of glycosylation in the case of most GPCRs is still unclear, but it has been shown that many GPCRs do not depend on the presence of *N*-glycans for ligand recognition [79–81]. One interesting case is the human mu opioid receptor where a single nucleotide polymorphism has been identified resulting in the replacement of asparagine 40, a putative site for N-glycosylation, by an aspartate residue [82]. This mutation appeared rather frequently in different clinical studies aiming at identifying polymorphisms associated with addictive disorders and it has been suggested that the absence of glycosylation may influence the physiological responses to drug intake.

Glycosylation in insect cells remains mostly very simple of the high mannose type. The inability of insect cells to perform complex glycosylation is apparently due to the absence or insufficient amounts of the terminal glycosyltransferases that are required to convert N-linked side chains to complex forms [83]. Metabolic engineering can be used to extend the glycoprotein processing capability of insect cells and to express heterologous glycoproteins with glycans that more closely resemble those produced by higher eukaryotes. The paradigm is based on the incorporation of mammalian glycosyltransferases into the insect cell by stable transformation of the cells that now express the enzymes mainly under the control of baculoviral immediate-early promoters (Refs. [83,84] and references therein). Another approach focused on the search for insect cell lines that contain endogenous enzymes able to perform a more complete sugar triggering such as *E. acrea* [30]. Strict control of growth and infection parameters, especially dissolved oxygen, was also used to improve glycosylation by endogenous enzymes [85].

Upon GPCR overexpression with the baculovirus system, a large portion of the recombinant protein is often not glycosylated at all as a likely consequence of the saturation of the cell machinery. Overall glycosylation is thought to influence protein stability, folding and solubility and, hence, receptor expression levels (Ref. [86] and references therein), but ligand binding and signalling properties were, in most cases, identical to those of the naturally produced receptors [27,40,48,86,87]. Heterogeneity in glycosylation may affect 3D crystallisation attempts, but glycosylated proteins can be easily separated from unglycosylated ones on lectin affinity columns [88]. Though it is generally admitted that glycosylation introduces perturbations and flexibility that are undesirable during crystallogenesis [89], several soluble glycoproteins have been crystallised (e.g. Refs. [90–92])

as well as the GPCR rhodopsin [4] and, in two cases at least, sugars were crucial for crystal lattice formation by forming bridges between adjacent proteins [93,94].

3.5. Influence of membrane lipid composition

Compared to mammalian cells, the Sf9 cell plasma membrane contains higher amounts of unsaturated lipids and lower amounts of cholesterol to ensure membrane fluidity at the organism growth temperature (27–28 °C) [95]. Moreover, baculovirus infection changes the lipid composition of the membrane through enrichment in phosphatidylcholine [95]. Several reports have pointed out the influence of membrane lipid composition on GPCR activity, showing that acyl chain saturation [96], negatively charged lipids [97] and/or cholesterol [98–100] constitute parameters that sometimes influence receptor functionality. For example, the human oxytocin [101] and the histamine H_2 [102] receptors were expressed in a low-affinity state in Sf9 cells; addition of cholesterol by incubation of the cells with cholesterol-β-cyclodextrin complexes led to an increased amount of high-affinity binding sites. Thus, in some cases, overexpression increases the amount of receptors produced in a nonactive conformation by reducing the overall availability of less abundant membrane components.

4. GPCR overexpression for functional studies

Functional as well as structural studies require characteristics of the recombinant protein indistinguishable from the native one. It should also be kept in mind that the functional activity of a GPCR is strongly dependent upon cell type and receptor density: for instance, the presence and relative concentrations of signal transduction elements may drastically affect ligand and G-protein binding properties [103,104]. All these parameters ought to be taken into account when addressing pharmacological properties such as ligand affinities or when depicting the nature of, and interrelations between, signal transduction components. The baculovirus expression system has proven to be very successful in studying the intrinsic properties of receptors and their G protein-coupling specificity.

4.1. Endogenous Gα subunit composition

The G proteins $G_s\alpha$ and $G_q\alpha$ have been unambiguously identified in Sf9 cells [20,105,106], but some debate remains concerning the presence of $G_{i/o}\alpha$ subunits. Several laboratories were unable to detect $G_{i/o}\alpha$ subunits with antibodies raised against their mammalian counterparts [20,105–107]. On the other hand, a partial cDNA encoding a $G_{o\text{-like}}\alpha$ was isolated from insect cells [61] and $G_o\alpha$, but not $G_i\alpha$, could be detected by immunoblotting [108]. The presence of a $G_{o\text{-like}}\alpha$ was further supported by the recovery of high-affinity agonist binding sites for the 5-HT_{1A} receptor when it was expressed at a level comparable to naturally occurring ones (150 fmol/mg membrane protein) [108]. Similarly, dopamine D_2 coupling to endogenous $G_{i/o}\alpha$ proteins could be detected if the receptor was expressed at levels below 1 pmol/mg membrane protein with no more than 40 fmol/mg membrane protein high-affinity binding sites [109]. Therefore, the absence of coupling to endogenous G proteins reported by other laboratories may result from the high expression levels of receptor that were achieved (>27 pmol/mg proteins) [106,107]. Interestingly, Leopoldt et al. [110] detected $G_{i/o}\alpha$ subunits in uninfected Sf9 cells, but reported endogenous Gα protein down-regulation on prolonged baculovirus infection as shown by (1) decreased [^{35}S]-GTPγS incorporation, (2) pertussis toxin (PTX)-mediated [^{32}P]-ADP ribosylation, (3) G protein immunoreactivity, (4) [α^{32}P]-GTP-azidoanilide photolabelling of Gα and (5) [^{32}P]-GTP labelling of Gβ. As an example, PTX-mediated [^{32}P]-ADP ribosylation was weak 62 h post infection and virtually undetectable 75 h post infection. In addition, coupling to endogenous G proteins by the histamine H_1 and H_2 receptors was detected 28 h post infection, but no coupling was found at 48 h post infection [110]. Down-regulation of endogenous G proteins following baculovirus infection, as well as different selectivity and/or sensitivity of the antibodies used for endogenous G protein detection and identification, are two factors that might reconcile the apparent contradictions in $G_{i/o}\alpha$ content and receptor coupling states reported by the various authors.

Post-translational modifications of heterotrimeric G proteins have been extensively studied in Sf9 cells with no differences being observed when compared to mammalian cells. For example, Gα subunits are palmitoylated, which is essential for interaction with the receptor [111–113], and $G_{i/o}\alpha$ subtypes are myristoylated [114]. Similarly, post-translational prenylation and carboxylmethylation of the Gγ subunit take place following βγ dimer formation. These latter modifications are essential for Gα – Gβγ, Gβγ-receptor and Gβγ-effector interactions [115].

4.2. Overexpression as a tool for ligand screening

The high expression levels achieved in baculovirus-infected insect cells result in amounts of receptors that allow easy characterisation of their pharmacological profiles, which in turn identifies those receptors as a good support for drug screening and development. It is now well established that antagonist binding properties of the receptors expressed in insect cells are identical to those observed for receptors obtained from naturally producing sources. In many examples, however, only the guanidine nucleotide insensitive low-affinity state for agonist could be detected [40,48,107,116]. This most likely reflects the fact that the number of receptors expressed with the baculovirus system exceeds enormously the number of endogenous G proteins with which they can interact. It

follows that only a tiny amount of the receptors are in the high-affinity binding state for agonist that corresponds to receptors interacting with heterotrimeric G proteins. In such a situation, antagonists as well as inverse agonists will bind to those uncoupled receptors, but agonist binding will be limited. In many cases, the receptor has been expressed together with its cognate Gα partners to ensure that the G proteins are no longer limiting; this allows complete characterisation of all types of ligands, including agonists (see below).

4.3. Identification of interacting G protein partners

The baculovirus–insect cell system represents an excellent choice for studying interactions of a given receptor with its cognate G protein partners. Because well-defined receptor–heterotrimeric G protein combinations can be produced, unambiguous identification and characterisation of specific interactions can be performed. By providing low endogenous background and controlled expression of the effectors, the system allows differentiation between the effects of agonists, receptor subtypes, Gα subunits and/or Gβγ combinations. The fact that specific receptor subtypes can be expressed in a defined cell type allows the unambiguous assignment of ligand selectivity which would be impossible from naturally expressing tissues in which several receptor subtypes co-localise, for example, the muscarinic subtypes m_1–m_5 [117–119]. This may be of special interest if a given receptor subtype is involved in a pathology, such as those belonging to the 5-HT receptor family, and one wishes to modulate its activity without affecting the other subtypes [120].

Examples of identification and characterisation of GPCR–heterotrimeric G protein partnerships are given in Table 2. Four different experimental paradigms were used to collect information. Each approach offers advantages and drawbacks regarding its ability to mimic the receptor–G protein ratio present in the native cellular environment.

4.3.1. Co-expression of the GPCR and G proteins

In this approach, no direct control can be exerted on the ratio between the different effectors. The amount of the different partners present within a given cell is variable and depends on the number of different recombinant baculoviruses that have co-infected the cell. Gβγ subunits are sometimes encoded on a single recombinant baculovirus in an attempt to enhance co-infection efficiencies [17]. Whether expression levels of receptor and G protein influence each other reciprocally [121,122] or not [20] is still an open question.

Studies of the interactions between the co-expressed partners can be performed using membrane- or whole-cell-based experiments. When using membranes, differences in coupling between closely related receptors such as dopamine D_{2L} and D_{2S} receptors were revealed as attested by the increased affinity of D_{2S} for $G_{i1}\alpha\beta_1\gamma_2$ compared to D_{2L} and differential cAMP inhibition [109]. Examples of functional studies performed using intact cells include the demonstration of the preferred coupling of the human dopamine D_{2s} receptor for $G_{i1}\alpha$ over $G_{i2}\alpha$ as assessed by GTPγS binding, GTPase activity and the measurement of intracellular cAMP on intact cells [121]. Electrophysiology has also been a valuable tool to monitor agonist-evoked intracellular responses. For example, the activation of potassium channels upon addition of acetylcholine in Sf9 cells expressing the muscarinic m_3 receptor was shown by whole-cell current recordings and patch-clamp techniques [123]. In another study, 5-HT_{1A} or 5-$HT_{4(a)}$ receptors were co-expressed in baculovirus-infected Sf9 cells together with a cyclic nucleotide-sensitive and voltage-activated cation channel from the moth *Heliothis virescens* and either $G_i\alpha\beta_1\gamma_2$ or $G_s\alpha\beta_1\gamma_2$ proteins, respectively. Hyperpolarization-activated inward currents were measured in the whole-cell patch-clamp configuration, which allowed the variation in the intracellular cAMP concentration in response to receptor activation by serotonin to be determined [71,124]. Fluorescent Ca^{2+}-sensitive compounds have also been used to probe small variations in intracellular Ca^{2+} concentration caused by agonist-induced stimulation of GPCRs. For example, the regulation of the human bradykinin B_2 receptor in Sf21 cells has been observed in cells loaded with Fura-2-AM to monitor intracellular Ca^{2+} mobilisation [77] while loading of Sf9 cells with Fura-2 allowed detection of the increase in free Ca^{2+} induced by endothelin receptors [125]. Recently, however, limitations in the usefulness of Sf9 cells for the analysis of receptor/$G_q\alpha$ coupling and $G_q\alpha$-mediated activation of effectors have been raised [126].

4.3.2. Expression of receptor–Gα fusion proteins

Receptor–Gα fusions allow a 1:1 ratio between the partners as a result of their forced interaction. Because each GPCR molecule is expressed together with the Gα subunit of interest, it alleviates problems related to Gα availability. This approach was successfully used for ligand screening [127]. The imposed 1:1 coupling stoichiometry also shed light on the mechanistic insights of G protein activation and has been mainly used to study the β_2-adrenergic receptor signalling properties [128–132].

4.3.3. Addition of purified G proteins to membrane-bound receptors

The main advantage of this method over the co-expression protocol resides in better control of the amounts of the different partners interacting, because receptor and purified G protein subunits can be separately quantified and mixed at defined ratios. This paradigm was often chosen to examine the role of Gβγ in cell signalling [133]. It revealed the impact of Gβγ composition on receptor–Gα coupling and, hence, on the choice of a signalling pathway. Examples include the demonstration that the nature of Gβ influences directly receptor/$G_s\alpha$ coupling [134,135] and that $G\gamma_1$ only is able to promote rhodopsin/$G_t\alpha$ interaction [136].

Table 2
Determination of coupling specificity in baculovirus-infected Sf9 cells between defined GPCR subtypes, Gα and Gβγ subunits

Receptor	Type	Origin	Identified interacting Gα proteins	Identified non-interacting Gα proteins	Gβγ subunits	Reference
Adenosine[a]	A_{2a}	Human	G_s, $\beta_4\gamma_2>\beta_2\gamma_2=\beta_3\gamma_2>\beta_1\gamma_2$	$\beta_5\gamma_2$		[134]
Adenosine[b]	A_{2a}	Human	$G_s\beta_4\gamma_2$	$G_s\beta_1\gamma_2$		[135]
Adrenergic[c]	β	Turkey	G_s		Bovine brain	[137]
Adrenergic[b]	β_1	Rat	G_s	G_z, G_q, G_{12}, G_{13}	$\beta_1\gamma_2$	[157]
Adrenergic[a]	β_1	Rat	G_s, $\beta_1\gamma_2=\beta_3\gamma_2=\beta_4\gamma_2>\beta_2\gamma_2$	$\beta_5\gamma_2$		[134]
Adrenergic[b]	α_{2B}	Human	G_s, G_i			[158]
Cannabinoid[a]	CB_1	Human	$G_i>G_o$		Bovine brain	[144]
Cannabinoid[a]	CB_2	Human	$G_i\ggg G_o$		Bovine brain	[144]
Dopamine[b]	D_{2L}	Rat	$G_o>G_{i2}$ (agonist dependent)		$\beta_1\gamma_2$	[159]
Dopamine[b]	D_{2S}	Human	$G_{i1}>G_{i2}$		$\beta_1\gamma_2$	[121]
Endothelin[a]	B	Human	$G_q\beta_1\gamma_2$, $G_q\beta_5\gamma_2$, $G_{i1}\beta_1\gamma_2$	$G_{i1}\beta_5\gamma_2$		[133]
Formyl peptide[d]		Human	$G_{i1}=G_{i2}=G_{i3}$		$\beta_1\gamma_2$	[160]
Histamine[e]	H_1	Guinea pig	$G_{q/11}$		Endogenous Gβγ	[20]
Histamine[e]	H_1	Guinea pig	$G_{q/11}$	G_i, G_{12}	Endogenous Gβγ	[110]
Histamine[e]	H_2	Rat	$G_{q/11}$, G_s	G_i, G_{12}	Endogenous Gβγ	[110]
Histamine[e]	H_2	Human	$G_s\ggg G_q$			[126]
Histamine[b,d]	H_2	Human	G_s mammalian	G_q mammalian		[126]
Hydroxytryptamine[e]	5-HT_{1A}	Human	G_o		Endogenous Gβγ	[108]
Hydroxytryptamine[b]	5-HT_{1A}	Human	$G_{i1}=G_{i2}=G_{i3}=G_o=G_z$	G_s, G_q	$\beta_1\gamma_2=\beta_1\gamma_3=\beta_1\gamma_5<\beta_1\gamma_1$	[60]
Hydroxytryptamine[b]	5-HT_{1A}	Human	G_z	G_s, G_{12}, G_{13}, G_q	$\beta_1\gamma_2$	[157]
Hydroxytryptamine[a]	5-$HT_{1A,\ 1B}$	Human	$G_{i3}>G_{i1}$, G_{i2}, G_o	G_t	Bovine brain purified or Gγ retina	[161]
Hydroxytryptamine[a]	5-HT_{1D}	Human	$G_{i3}>G_{i1}$, G_{i2}, G_o	G_t		
Hydroxytryptamine[a]	5-HT_{1E}	Human		G_t, G_{i1}, G_{i2}, G_{i3}, G_o		
Hydroxytryptamine[b]	5-HT_{1D}	Human	G_{i1}, G_{i2}, G_{i3}, G_o	G_q	$\beta_1\gamma_2$	[37]
Hydroxytryptamine[b]	5-$HT_{4(a)}$	Murine	G_{13}, G_s	G_{i2}, G_{12}, G_q	$\beta_1\gamma_2$	[162]
Hydroxytryptamine[b]	5-HT_{5A}	Human	G_{i1}, G_{i2}, G_{i3}, G_o	G_z, G_s, G_q, G_{11}, G_{12}, G_{13}, G_{16}	$\beta_1\gamma_2$	[38]
Luteinizing hormone[e]		Mouse	G_s (cAMP)		Endogenous Gβγ	[20]
Luteinizing hormone[b]		Mouse	$G_{i2}>G_{i3}$, G_s (PLC)	G_{i1}, G_q, G_{11}	$\beta_1\gamma_1$, $\beta_2\gamma_2$	[20]
Lysophosphatidic acid[b]	Edg2-Vzg1	Mouse	G_{i1}, G_{oA}, G_{11}	G_s	$\beta_1\gamma_2$	[122]
Muscarinic[a]	m_1	Human	$G_q\beta_1\gamma_2$, $G_q\beta_5\gamma_2$			[133]
Muscarinic[c]	m_2	Human	G_o, $G_z>G_{i1}$, G_{i3}	G_s	Bovine brain	[137]
Opioid[b]	mu	Human	G_{i1}	G_{i2}	$\beta_1\gamma_2$	[163]
Opioid[b]	kappa	Rat	G_{i1}, G_{oA}		$\beta_1\gamma_2$	[17]
PACAP[c]		Human	G_s		Bovine brain	[40]
Sphingosine1-phosphate[b]	Edg-1	Human	G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_z	G_s, G_q, G_{12}, G_{13}	$\beta_1\gamma_2$	[164]
Sphingosine1-phosphate[b]	Edg-3	Human	G_{i2}, G_q, G_{13}		$\beta_1\gamma_2$	[164]
Sphingosine1-phosphate[b]	Edg-5	Rat	G_{i2}, G_q, G_{13}		$\beta_1\gamma_2$	[164]
Substance P[e]		Human	G_o, $G_{q/11}$, G_s		Endogenous Gβγ	[155]
Substance P[b]	NK_1	Human	G_q, G_{12}, $G_{13}>G_z$	G_s	$\beta_1\gamma_2$	[157]
Thrombin[b]		Human	G_q, G_{12}, $G_{13}>G_z$	G_s	$\beta_1\gamma_2$	[157,162]

[a] Addition of purified Gα to membranes expressing the receptor.
[b] Co-expression.
[c] Reconstitution of purified partners in lipid vesicles.
[d] GPCR–Gα fusion.
[e] Coupling to endogenous Gα.

4.3.4. Reconstitution in lipid vesicles of partners expressed with the baculovirus system

This methodology takes advantage of the ability to express and purify large amounts of GPCRs and heterotrimeric G proteins and to introduce them into a fully artificial environment where all parameters can be controlled. Coupling of the β-adrenergic receptor to $G_s\alpha$ but not $G_{i/o}\alpha$ was observed using this system, while the muscarinic m_2 receptor did not couple to $G_s\alpha$ but mainly to $G_o\alpha$ and $G_z\alpha$ and to a much lesser extent to $G_{i1}\alpha$ or $G_{i3}\alpha$ [137]. Similarly, reconstitution of the purified human PACAP receptor into lipid vesicles with $G_s\alpha$ and Gβγ led to GTPγS incorporation into $G_s\alpha$ and the appearance of high-affinity binding sites [40].

4.4. Constitutive activity and inverse agonism

Constitutive activity reflects the spontaneous activity of a receptor in the absence of agonist. This concept has become widely accepted and verified for numerous GPCRs

[37,138–140], and this ligand-independent activity is also acknowledged to play a role in some pathologies [141]. Such constitutive activity is usually very difficult to detect in naturally producing cells because of the very low level of expression (fmol/mg membrane proteins) and heterologous expression in mammalian cells is not completely satisfactory to solve the problem because expression levels are often too low for optimal detection (generally 1–2 pmol/mg membrane protein). In contrast, the high expression levels of uncoupled receptors produced in insect cells with the baculovirus system results in a good basal signal [142–144]. Moreover, overexpression of the receptor together with its cognate Gα partner resulted in amplification of the signal arising from their spontaneous interaction enabling easy demonstration of the receptor constitutive activity (see below). Hence, this system also facilitated drug screening for identification and characterisation of compounds that reduce this basal activity, that is, inverse agonists.

Spontaneous activation of the Gα subunit by the receptor has been shown by co-expression of the GPCR and G protein; for example, the 5-HT_{1A} receptor and $G_z\alpha$ [142], the 5-HT_{1D} receptor and $G_{i1}\alpha$ [37], the 5-HT_{2C} receptor and $G_q\alpha$ [145], the cannabinoid CB_1 receptor and $G_i\alpha$ [144], the lysophosphatidic acid Edg2-Vzg1 receptor and $G_{i1}\alpha$ [122] and the β_2-adrenergic receptor and $G_s\alpha$ [143].There are also examples where basal activity was shown by the expression of a fusion protein between the receptor and the Gα subunit; for example, the histamine H_2 receptor fused to the long or the short variants of $G_s\alpha$ [132] or the β_2-adrenergic receptor fused to the long splice variant of $G_s\alpha$ [128].

4.5. Regulation of GPCR activity

Because of the high level of receptor expressed with the baculovirus system and the possibility to isolate one particular receptor subtype, studies have been performed on aspects of receptor regulation that were impossible in mammalian cells. These studies showed the importance of post-translational modifications and also led to the identification of cellular determinants responsible for those modifications and their modulation. For example, differential agonist-induced phosphorylation of human kinin receptors B_1 and B_2 [146] and human muscarinic m_1 and m_2 receptors [72] were observed. Similarly, identification of the GRKs responsible for agonist-induced phosphorylation of the muscarinic m_3 receptor was achieved by addition of purified GRKs to the membrane-bound receptor [73]. Also, substance P receptor phosphorylation was shown to be mediated by GRK2 but distinctly regulated by Gβγ compared to rhodopsin or the β_2-adrenergic receptor [76]. Another example demonstrated that $G\gamma_{12}$ phosphorylation increased A_1 adenosine receptor coupling to $G_{i1}\alpha$, markedly inhibited type II adenylyl cyclase activation, but did not affect its ability to activate phospholipase C [147].

5. GPCR overexpression in stable Sf cell lines

Stable expression in Sf9 insect cells can be achieved using the baculovirus 'immediate-early' promoters that are recognised and used by insect cell RNA polymerases without requiring any additional viral encoded proteins. During the normal viral replication cycle, these promoters are the first to be switched on following viral entry in the cell (see Fig. 1). Immediate-early promoters have been widely used to engineer cell lines with improved post-translational processing (see above), but very few attempts have been made to express membrane proteins under their control.

Only expression of the β_2-adrenergic receptor as well as the nicotinic acetylcholine receptor α-subunit have been reported in Sf9 cells under the control of the immediate-early promoter, IE1, isolated from the baculovirus *A. californica* (AcIE1) [105,148]. β_2-Adrenergic receptor expression ranged from 24 000 to 350 000 receptors/cell [105] which is well below the 12–17 pmol/mg corresponding to at least 10^6 receptors/cell reported for its expression with the baculovirus system under the control of the polyhedrin promoter [149]. Recently, a nonlytic vector system was described that employs the *Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early 2 (OpIE2) promoter for constitutive expression of proteins in insect cells [150,151]. In an isogenic plasmid background, the OpIE2 promoter is 5- to 100-fold more active than the AcIE1 promoter in transiently transformed Sf9 cells [150]. Recently, this promoter has been successfully used for human mu opioid receptor production [152]. The receptor was fused to the gp64 signal sequence and was detected at the cell surface by confocal imaging. Expression levels were about 11 000–15 000 receptors/cell, which can be estimated at 250 pmol/l culture. This is about 15 times less than the $2–3 \times 10^6$ receptors/cell, corresponding to 3200–4700 pmol/l culture, obtained when the same construct was expressed with the baculovirus system under the control of the polyhedrin promoter [29].

Though significantly lower levels of expression were obtained under the control of 'immediate-early' promoters when compared to the very late polyhedrin promoter, stably transfected insect Sf9 cells may represent a valuable alternative for GPCR large-scale expression. Indeed, stably transformed insect cells not only grow to high density in suspension and adapt easily to serum-free medium but they do not require the production of large-scale high-titre viral stocks and allow continuous culture in a biofermentor. Moreover, compared to mammalian cells, insect cells are considerably less expensive and time-consuming to grow in large volumes and reach higher cell densities.

6. Concluding remarks

Over the years, the baculovirus–insect cell system has proven extremely effective for the production of large amounts of many GPCRs with properties comparable to

those of their native counterparts. Insect cells are indeed able to perform post-translational modifications crucial for GPCR function such as palmitoylation and phosphorylation. Receptors produced in insect cells constitute, therefore, an excellent source of material for structural studies. The expression yields obtained for some GPCRs are high enough to produce the milligram quantities required for solid-state NMR or 3D-crystallisation. However, for many GPCRs, expression levels are still too low for such ambitious goals or would at least require very large culture volumes. GPCR expression and purification, on the other hand, have been improved, but some parameters designed to optimise expression remain elusive and do not always act in a predictable manner. Nevertheless, a lot of information has been gathered on the system that will definitely help future studies and efforts are still made to develop new approaches and optimise current procedures.

The other striking breakthrough emerging from the use of the baculovirus–insect cell system concerns the functional study of GPCRs. Because of the high expression levels that can be reached, many characteristics of GPCRs including palmitoylation and phosphorylation were uncovered that were not observed in mammalian cells because heterologous expression did not produce sufficient amounts of receptors. In addition, the ability to select GPCR and G protein combinations, whether by co-expression or by reconstitution with purified elements, has allowed the detailed characterisation of preferred interactions. Moreover, the baculovirus–insect cell system has become a tool of choice for pharmacologists because it allows the study of ligand specificities on isolated receptor subtypes. The system also provides material suitable for drug screening. This includes the search for inverse agonists by allowing the easy detection of receptor constitutive activity as a result of the high expression levels achieved both for the receptor and its cognate G protein partners.

Advantages of the baculovirus–insect cell system include its simplicity to set up in any laboratory, the fact that it does not require extensive biosafety measures, and that it benefits from over 15 years of experience and of well-established protocols. As a consequence, it represents a valuable tool for GPCR studies whether the ultimate goal is to gain structural information or to better understand the regulation of the first steps of the signalling cascade.

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