



Interuniversity Attraction Poles (IAP) Phase VI

2007 – 2011

**ANNEX I
TO CONTRACT P6/14**

TECHNICAL SPECIFICATIONS : SECTION I

Information on the network

Title of the project : **G PROTEIN-COUPLED RECEPTORS, FROM STRUCTURE TO DISEASES**

Name of the coordinator : **Marc PARMENTIER**

Institution : **Université Libre de Bruxelles**

I. 1. NETWORK COMPOSITION

BELGIAN PARTNERS *

<u>Coordinator</u> : <u>Partner 1</u> (P1) Name : Marc PARMENTIER Institution : Université Libre de Bruxelles Institution's abbreviation : ULB
<u>Partner 2</u> (P2) Name : Jozef VANDEN BROECK Institution : Katholieke Universiteit Leuven Institution's abbreviation : KUL
<u>Partner 3</u> (P3) Name : Johan THEVELEIN Institution : Katholieke Universiteit Leuven Institution's abbreviation : KUL
<u>Partner 4</u> (P4) Name : Jozef VAN DAMME Institution : Katholieke Universiteit Leuven Institution's abbreviation : KUL
<u>Partner 5</u> (P5) Name : Daniel DESMECHT Institution : Université de Liège Institution's abbreviation : ULG

* Mention only one name per partner. The person listed here should be the one in charge of the operational aspects of the project. Indicate the full name (family name + first name) of the partner.

EUROPEAN PARTNERS * (if applicable)

<p><u>EU-Partner 1</u> (EU1) Name : Leonardo PARDO Institution: Universidad Autonoma de Barcelona Institution's abbreviation : UAB Country : Spain</p>
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* Mention only one name per partner. The person listed here should be the one in charge of the operational aspects of the project. Indicate the full name (family name + first name) of the partner.

I. 2. TITLE AND SUMMARY OF THE PROJECT

Indicate clearly and briefly the project's major objectives and provide a concise description of the project.

A. Title and summary in English (2 pages maximum)

Introduction

G protein-coupled receptors (GPCR) represent the largest family among membrane receptors (Bockaert and Pin 1999). They play a major role in a variety of physiological and pathophysiological processes, and constitute the targets for about half the active compounds presently used as therapeutic agents. Working together in the frame of a previous IAP network, the partners of the present program have played a major role in the characterization of many G protein-coupled receptors in yeast, insects and mammalian species. Based on this previous experience, they now set up a partnership that will further study both general and specific aspects of this important gene family, with the ultimate goal of improving human health. The partnership will use specific receptors and receptor subfamilies as models, in order to approach the field of GPCRs as a whole, as many of the studied aspects can apply to the entire family. The key models that will be studied, on which the partners have built their present expertise, include the glycoprotein hormone and chemoattractant receptors in human and mouse, insect neuropeptide receptors and yeast sugar-sensing receptors. The main focuses of the program will be as follows:

Structure of GPCRs, activation mechanisms and ligand-receptor interactions

We will construct three dimensional models of our receptors of interest, on the basis of the single crystal structure presently available (bovine rhodopsin) in order to raise hypotheses regarding ligand-receptor interactions, activation mechanisms and oligomeric organization. We will systematically test these models by mutagenesis studies, and the results of these experiments will be used to improve the models. This approach will be applied to most receptor classes studied, and the modeling aspects will be supported by our foreign partner EU1 (L. Pardo, Barcelona). For selected receptors, the models will also be used as basis for the virtual screening of chemical libraries, in order to develop small molecules with agonist, antagonist or allosteric effector properties.

Oligomerization of GPCRs

GPCRs were initially considered to act as monomers. More recent data have shown that most receptors form dimers if not higher order oligomers. The ability of GPCRs to homo- and heterodimerize will likely change many aspects of pharmacology in general, and the partnership has recently demonstrated allosteric interactions between receptor protomers. We will investigate further, for different classes of receptors, the functional consequences of dimerization, in terms of pharmacology, receptor activation, signaling properties and regulatory pathways, and will explore whether these observations apply to receptors expressed at physiological levels in native cell populations. Chemokine, glycoprotein hormone and yeast sugar-sensing receptors will be the first families studied in this frame.

Signaling cascades of G protein-coupled receptors

Besides the classical pathways activated by GPCRs through heterotrimeric G proteins, a number of additional pathways, some of which are G protein-independent have been described. In addition, the range of signaling cascades activated by a given receptor can vary according to the agonist. Signaling cascades will be studied for yeast sugar-sensing, insect neuropeptide and mammalian chemoattractant receptors, focusing on new pathways and the protein complexes involved in signal transduction.

Functional characterization of specific receptors in physiological processes

A number of specific receptors, among which several receptors identified by the partners over the previous IAP programs, will be studied in details in order to determine their role in physiological processes. This will involve in vitro analysis of receptor pharmacology and ligand processing, distribution studies, as well as in vivo studies and the design of knock-out and transgenic models. We will study, among others, chemoattractant receptors (ChemR23, FPRL2) and a set of mammalian neuromodulatory receptors, neuropeptide receptors in insects, and the glucose/sucrose sensing GPCR system in yeast and *Candida albicans*.

GPCRs in human diseases and animal disease models

For mammalian receptors, we will further determine their potential involvement in human diseases, with a special focus onto inflammation, cancer, and the neuroendocrine axis. These studies will be conducted both by studying human pathological samples, and by submitting the genetically modified mice to a number of in vivo disease models. These models will be run with the help of a group of pathologists (partner P5). The receptors studied in this frame include glycoprotein hormone, chemoattractant and neuromodulatory receptors. Finally, the influence of allelic variation in GPCR genes or gene clusters in mouse lung disease models will be studied by genetic linkage analysis. If candidate GPCR genes result from this approach, they will be studied more specifically both in human and in mouse models.

Characterization of novel receptors and their ligands

Many orphan receptors for which the ligands and function are still unknown are encoded by the mammalian, insect and yeast genomes (Civelli et al. 1999, Wise et al. 2004). Several partners will focus on the characterization of these receptors, through the identification of their ligand, and the subsequent delineation of their function. In particular, human receptors for new leukocyte chemoattractants, neuropeptides, glycoprotein hormone-like proteins and glucose, insect receptors for neuropeptides, and nutrient-sensing receptors in yeast, will be investigated using evolutionary clues.

Olfactory receptors and evolutionary aspects of the GPCR family

The partnership involves groups specialized in yeast, insect and mammalian receptors. This will bring an evolutionary dimension to the program, with parallel studies of receptor classes in different systems. We intend to interact with another IAP network dedicated to Bioinformatics in order to allow in depth studies of receptor gene families in the growing number of full genomes available in the databases. Correlation between structural and functional evolution of receptor and ligand gene families will be studied in this context. We will also reinstate an avenue of research dedicated to olfactory receptors. Following years of unsuccessful attempts, the reliable functional expression of olfactory receptors became achievable over the recent years. We will build on this recent evolution, and will start a proteomic program in order to identify new proteins involved in the organization of the signaling complex in olfactory neurons, both in mouse and in insects. The evolutionary aspects of olfactory receptors, associated proteins, and ligand specificity will be considered as well.

Integration in international networks

This program will be linked through several of its members to FP6 European consortia dedicated to the same or related topics. This includes a STREP program "GPCRs" involving partner P1 (as coordinator) and EU1, an integrated project "INNOCHEM", which includes partners P1 and P4, a network of excellence "EADGENE" incorporating partner P5 and a Marie-Curie Training Network "CANTRAIN" involving partner P3.

B. Title and summary in Dutch (2 pages maximum)

Inleiding

G-proteïne gekoppelde receptoren (GPCRs) vertegenwoordigen de grootste familie van membraanreceptoren. Ze vervullen een belangrijke rol in diverse fysiologische en pathofysiologische processen, en zijn het doelwit voor zowat de helft van de actieve producten die momenteel aangewend worden als therapeutische agentia. Samenwerkend in het kader van een vorig IUAP-netwerk hebben de partners van het huidig onderzoeksprogramma een vooraanstaande rol gespeeld bij de karakterisering van tal van GPCRs afkomstig van gist, insecten en zoogdieren. Op basis van deze voorafgaande ervaring, hebben ze een consortium opgericht dat zowel algemene als specifieke aspecten van deze belangrijke genfamilie verder zal bestuderen, met als uiteindelijk doel om de gezondheid van de mens te verbeteren. De partners van het netwerk zullen gebruik maken van specifieke receptoren en receptorsubfamilies als model, met als doel om het domein van GPCRs als geheel te benaderen, aangezien vele van de te onderzoeken aspecten van toepassing kunnen zijn voor de volledige familie. De voornaamste modellen die zullen bestudeerd worden, en waarop de aanwezige onderzoeksexpertise van de partners werd uitgebouwd, omvatten de receptoren voor glycoproteïnehormonen en voor chemoattractantia bij de mens en de muis, de receptoren voor neuropeptiden bij insecten en de receptoren voor suikers bij gist. De voornaamste onderzoekslijnen van dit programma zijn de volgende:

De structuur van GPCRs, activeringsmechanismen en ligand-receptor interacties

Op basis van de enige kristalstructuur die momenteel gekend is (runder rhodopsine) zullen we driedimensionale modellen construeren van de receptoren die hiervoor in onze belangstelling staan. Hieruit kunnen dan hypothesen geformuleerd worden betreffende ligand-receptor interacties, activeringsmechanismen en oligomeervorming. We zullen systematisch deze hypothetische modellen testen aan de hand van mutagenesestudies, en de resultaten van deze experimenten zullen dan verder aangewend worden om de geconstrueerde modellen te verfijnen. Deze benadering zal toegepast worden op de meeste receptorklassen onder studie, en de modelleringsaspecten zullen ondersteund worden door onze buitenlandse partner EU1 (L. Pardo, Barcelona). Voor een selectie van receptoren, zullen de geproduceerde modellen aangewend worden als basis voor de virtuele screening van chemische libraries, met als doel om kleine moleculen te ontwikkelen die als agonist of antagonist kunnen werken.

Oligomerisatie van GPCRs

GPCRs werden initieel beschouwd functioneel te zijn als monomeer. Meer recente gegevens hebben echter aangetoond dat de meeste receptoren dimeren, of zelfs meer complexe oligomeren, kunnen vormen. Het vermogen van GPCRs tot het vormen van homo- en heterodimeren zal wellicht een invloed hebben op meerdere aspecten van de farmacologie in het algemeen, en het partnerschap heeft recent het bestaan van allosterische interacties tussen receptorprotomeren aangetoond. Voor verschillende klassen van receptoren zullen we verder de functionele gevolgen van deze dimerisatie bestuderen, op gebied van farmacologie, receptoractivering, signaaltransductie en regulatie, en verder onderzoeken of deze observaties van toepassing kunnen zijn op receptoren die aan fysiologische expressieniveaus aanwezig zijn in natieve celpopulaties. Receptoren voor chemokines, glycoproteïnehormonen en glucose zullen als eerste families in deze context worden onderzocht.

Signaalcascades van G-proteïne gekoppelde receptoren

Naast de klassieke signaalwegen die via heterotrimere G-proteïnen door GPCRs geactiveerd worden, zijn er een aantal additionele pathways beschreven waarvan sommige G-proteïne-onafhankelijk verlopen. Bovendien kan het gamma aan signaalcascades dat geactiveerd wordt door een gegeven receptor variëren naargelang de aard van de agonist. De cascades die instaan voor waarneming van suikers bij gist, van neuropeptiden bij insecten en van chemoattractantia bij zoogdieren zullen nader worden bestudeerd, met focus op nieuwe signaalwegen en op de eiwitcomplexen betrokken bij signaaltransductie.

Functionele karakterisering van specifieke receptoren in fysiologische processen

Een aantal specifieke receptoren, waarvan er verscheidene door de partners zelf geïdentificeerd werden gedurende de vorige IUAP-programma's, zullen meer in detail bestudeerd worden met als

doel om hun rol in fysiologische processen te achterhalen. Dit onderzoek omvat *in vitro* analyses van de receptorfarmacologie en van ligand-processing, distributiestudies, evenals *in vivo* studies en de ontwikkeling van knock-out en transgene modellen. We zullen onder meer enkele receptoren voor chemoattractantia (ChemR23, FPRL2) en een set van neuromodulatorische receptoren bij zoogdieren, neuropeptidereceptoren bij insecten, en het glucose/sucrose-gevoelige GPCR-systeem bij gist en *Candida albicans* bestuderen.

GPCRs bij menselijke ziekten en in diermodellen voor ziekten

Verder zullen we ook de mogelijke betrokkenheid onderzoeken van receptoren bij menselijke aandoeningen, met een speciale focus op inflammatie, kanker, en de neuroendocriene assen. Deze studies zullen zowel worden uitgevoerd door onderzoek op menselijke pathologische stalen, als door het onderwerpen van genetisch gemodificeerde muizen aan een aantal *in vivo* ziektebeelden. Deze modellen zullen getoetst worden dankzij de medewerking van een groep van pathologen (partner P5). De receptoren die in deze context zullen bestudeerd worden, omvatten receptoren voor glycoproteïnehormonen, chemoattractantia en neuromodulators van zoogdieren. Tenslotte zal de invloed worden bestudeerd van allelische variatie in GPCR-genen of -genclusters in muismodellen voor longziekten aan de hand van genetische linkage analyse. Indien deze benadering zou leiden tot kandidaat GPCR-genen, dan zullen deze meer specifiek worden bestudeerd zowel bij de mens als in muismodellen.

Karakterisering van nieuwe receptoren en hun liganden

Vele weesreceptoren, waarvoor nog geen liganden of functies bekend zijn, worden gecodeerd door de genomen van (onder andere) zoogdieren, insecten en gisten. Verscheidene partners zullen zich toeleveren op de karakterisering van dergelijke receptoren, door de identificatie van hun liganden, en de verdere opheldering van hun functies. We zullen hierbij de nadruk leggen op humane receptoren voor nieuwe chemoattractantia van leukocyten, neuropeptiden, glycoproteïnehormoon-achtige eiwitten en glucose, op receptoren voor neuropeptiden bij insecten, en op receptoren betrokken bij nutriëntwaarneming bij gisten, en zullen bij deze benadering tevens gebruik maken van evolutionaire aanwijzingen.

Olfactorische receptoren en evolutionaire aspecten van de GPCR-familie

Het partnerschap omvat groepen gespecialiseerd in receptoren afkomstig van gist, insecten en zoogdieren. Dit voegt een evolutionaire dimensie toe aan het onderzoeksprogramma, met parallelle studies van receptorklassen in verschillende systemen. We hebben dan ook de intentie om te interageren met een ander IUAP-netwerk dat zich toespitst op Bioinformatica om aldus een diepgaande studie te gaan voeren van de verschillende receptorgenfamilies die aanwezig zijn in het gestadig toenemend aantal volledige genomen die beschikbaar zijn in sequentiedatabanken. In deze context, zal de correlatie tussen structurele en functionele evolutie van receptor- en ligandgenfamilies bestudeerd worden. Tevens zullen we een onderzoekslijn herintroduceren die gericht is op de studie van olfactorische receptoren. Een doorbraak in het realiseren van een betrouwbare, functionele expressie van olfactorische receptoren werd immers tijdens de recente jaren verwezenlijkt, en dit na enkele jaren met niet zo succesvolle pogingen. We zullen voortbouwen op deze recente ontwikkeling, en zullen starten met een proteomische analyse met het oog op het identificeren van nieuwe proteïnen betrokken bij de organisatie van het signalerend complex in olfactorische neuronen, zowel bij muizen als bij insecten. De evolutionaire aspecten van olfactorische receptoren, geassocieerde proteïnen, en van ligandspecificiteit zullen eveneens in overweging genomen worden.

Integratie in internationale netwerken

Dit programma zal via verschillende van zijn leden onder meer verbonden zijn met Europese consortia (FP6) gewijd aan dezelfde of gelijkaardige topics. Deze omvatten o.a. een STREP programma "GPCRs" met partners P1 (als coördinator) en EU1, een geïntegreerd project "INNOCHEM" met partners P1 en P4, een excellentienetwerk "EADGENE" met partner P5, en een *Marie-Curie Training Network* "CANTRAIN" met partner P3.

C. Title and summary in French (2 pages maximum)

Introduction

Les récepteurs couplés aux protéines G (RCPGs) représentent la plus grande famille de récepteurs membranaires. Ils jouent un rôle majeur dans de nombreux processus physiologiques et physiopathologiques, et constituent les cibles de près de la moitié des molécules actives utilisées en thérapeutique. En travaillant ensemble dans le cadre d'un réseau PAI précédent, les partenaires du présent programme ont joué un rôle majeur dans la caractérisation de nombreux RCPGs de mammifères, insectes et levures. Sur la base de cette expérience, ils ont mis en place un réseau qui se propose d'étudier plus avant des aspects généraux et spécifiques de cette importante famille de gènes, avec le but ultime d'améliorer la santé humaine. Le partenariat utilisera des récepteurs et sous-familles de récepteurs spécifiques comme modèles pour approcher le domaine des RCPGs dans son ensemble, car de nombreux aspects étudiés pourront être appliqués à la famille entière. Les principaux modèles étudiés, sur lesquels les partenaires ont construit leur expertise actuelle, incluent les récepteurs d'hormones glycoprotéiques et d'agents chimioattractants chez l'homme et la souris, les récepteurs neuropeptidergiques d'insectes, et les récepteurs aux sucres des levures. Les principaux axes seront les suivants :

Structure des RCPGs, mécanismes d'activation et interactions ligand-récepteur

Nous construirons des modèles tridimensionnels de nos récepteurs d'intérêt, sur la base de la seule structure cristallographique disponible actuellement (rhodopsine bovine), de manière à générer des hypothèses concernant les interactions ligand-récepteur, les mécanismes d'activation et l'organisation oligomérique. Nous testerons systématiquement ces modèles par mutagenèse, et les résultats de ces expériences seront utilisés pour améliorer les modèles. Cette approche sera appliquée à la plupart des récepteurs étudiés, et la modélisation sera supportée par notre partenaire étranger EU1 (L. Pardo, Barcelone). Dans quelques cas, les modèles seront aussi utilisés comme base pour le criblage virtuel de librairies chimiques et le développement de petites molécules agonistes, antagonistes ou modulateurs allostériques.

Oligomérisation des RCPGs

Les RCPGs étaient initialement considérés comme des monomères. Des données plus récentes ont montré que la plupart de ces récepteurs forment des dimères ou oligomères d'ordre supérieur. L'aptitude des RCPGs à former des homo- ou des hétéro-dimères va certainement bouleverser de nombreux aspects de la pharmacologie, et le partenariat a récemment démontré l'existence d'interactions allostériques entre protomères de récepteurs. Nous étudierons plus avant, pour différentes classes de récepteurs, les conséquences fonctionnelles de la dimérisation, en termes de pharmacologie, activation, signalisation et régulation, et explorerons si ces observations s'appliquent aux récepteurs exprimés à des niveaux physiologiques dans des populations cellulaires natives. Les récepteurs de chimiokines, d'hormones glycoprotéiques et du glucose seront les premiers récepteurs étudiés dans ce cadre.

Cascades de signalisation des récepteurs couplés aux protéines G

En plus des cascades classiques activées par les RCPGs via les protéines G hétérotrimériques, une série d'autres cascades, parfois indépendantes des protéines G, ont été décrites. De plus, l'éventail de cascades activées par un récepteur donné peut varier en fonction de l'agoniste utilisé. Nous étudierons les cascades de signalisation des récepteurs aux sucres des levures, des récepteurs peptidergiques d'insectes, et des récepteurs d'agents chimioattractants humains, en nous focalisant sur les cascades originales et les complexes protéiques impliqués dans la transduction des signaux.

Caractérisation fonctionnelle de récepteurs spécifiques en physiologie

Une série de récepteurs spécifiques, dont certains identifiés par les partenaires au cours des programmes PAI précédents, seront étudiés en détail afin de déterminer leur rôle dans les

processus physiologiques. Ceci impliquera l'analyse *in vitro* de leur pharmacologie et du processing de leur ligand, des études de distribution, ainsi que des études *in vivo* et la génération de modèles transgéniques et knock-out. Nous étudierons, entre autres, les récepteurs d'agents chimioattractants (ChemR23, FPRL2), de certains agents neuromodulateurs, des récepteurs neuropeptidergiques d'insectes, et les récepteurs du glucose/sucrose de la levure et *Candida albicans*.

Les RCPGs dans les maladies humaines et les modèles animaux de pathologies

Pour les récepteurs de mammifères, nous déterminerons leur implication potentielle dans les maladies humaines, particulièrement l'inflammation, le cancer et les désordres de l'axe neuroendocrinien. Ces études seront conduites en étudiant des échantillons pathologiques humains, et en soumettant les souris modifiées génétiquement à divers modèles de pathologies. Ces modèles seront réalisés avec l'aide d'un groupe de pathologistes (partenaire P5). Les récepteurs étudiés dans ce cadre incluent des récepteurs d'hormones glycoprotéiques, d'agents chimioattractants et d'agents neuromodulateurs. Finalement, l'influence de variations alléliques des gènes ou clusters de gènes de RCPGs dans des modèles murins de maladies pulmonaires seront étudiés par analyse de liaison génétique. Si des RCPGs candidats résultent de cette approche, ils seront étudiés plus spécifiquement chez l'homme et sur modèles murins.

Caractérisation de nouveaux récepteurs et leurs ligands

De nombreux récepteurs orphelins dont les ligands et fonctions sont encore inconnus sont codés par les génomes des mammifères, insectes et levures. Plusieurs partenaires vont s'attacher à la caractérisation de ces récepteurs, par l'identification de leur ligand naturel et de leur fonction biologique. En particulier, les récepteurs humains de nouveaux agents chimioattractants pour les leucocytes, de neuropeptides, de protéines apparentées aux hormones glycoprotéiques et du glucose, les récepteurs peptidergiques d'insectes, et les récepteurs de nutriments de la levure, seront étudiés en utilisant des arguments évolutifs.

Récepteurs olfactifs et aspects évolutifs de la famille des RCPGs

Le réseau inclut des groupes spécialisés dans les récepteurs de levure, d'insectes et de mammifères, apportant une dimension évolutive au programme. Nous interagissons avec un autre réseau PAI dédié à la bioinformatique, de manière à mener des études en profondeur concernant les familles de RCPGs dans le nombre croissant de génomes complets disponibles dans les bases de données. Les corrélations entre l'évolution structurale et fonctionnelle des familles de récepteurs et ligands sera étudiée dans ce contexte. Nous réinitialiserons également une étude des récepteurs olfactifs. Après des années d'essais infructueux, l'expression fonctionnelle de récepteurs olfactifs est en effet devenue possible. Nous construirons sur cette base, et démarrerons un programme de protéomique visant à identifier de nouvelles protéines impliquées dans les complexes de signalisation des neurones olfactifs, chez la souris et les insectes. Les aspects évolutifs des récepteurs olfactifs, des protéines associées, et de leur spécificité de ligands sera considérée également.

Intégration dans les réseaux internationaux

Ce programme sera connecté par certains de ses membres à plusieurs réseaux européens (FP6) dédiés aux mêmes thèmes. Ceci inclut un programme STREP "GPCRs" impliquant le partenaire P1 (comme coordinateur) et EU1, un projet intégré "INNOCHEM", incluant les partenaires P1 et P4, un réseau d'excellence "EADGENE" incorporant le partenaire P5 et un réseau Marie-Curie "CANTRAIN" impliquant le partenaire P3.

I. 3. OBJECTIVES, MOTIVATION AND STATE OF THE ART (5 pages maximum)

Describe the project's objectives and research goals.

Define the problems being addressed by positioning them in relation to the current state of knowledge.

1. PROJECT OBJECTIVES

The first objective of the present program is to improve the understanding of structure-function relationships of G protein-coupled receptors. We aim at understanding what are the structural determinants and changes associated with ligand binding, receptor activation and G protein coupling, and how the knowledge of these structural elements can be translated into predictive tools for the selection and design of molecules acting on receptors with a therapeutic potential. Structural models will be built on the crystal structure of inactive bovine rhodopsin (and other rhodopsin structures that will soon become available) for the class A GPCRs of interest. Hypotheses will be raised regarding i) structural elements involved in the binding of natural ligands, antagonists or allosteric modulators, ii) the structural rearrangements associated with receptor activation, and iii) the coupling with G proteins, β -arrestins or other interacting proteins. These hypotheses will be tested by mutagenesis and a set of functional assays. Experimental results derived from different receptor classes will progressively improve the robustness of the structural models, and their predictive character. A topic of special interest will be GPCR dimerization, and how (hetero)dimerization influences the physiological role of receptors, and their use as therapeutic targets.

The second aim of the program will be to characterize the biological role of specific receptors in the physiology of human, mouse, insects and yeasts, and the involvement of human receptors in disease states. The partnership will focus on a set of receptors, many of which have been discovered in the network over the previous IAP phases. They include glycoprotein hormone receptors, chemokine receptors, receptors for neuromodulatory molecules in human and mouse, insect neuropeptidergic receptors and sugar sensing-receptors. They will be studied in terms of signaling pathways, involvement in immune, neural and endocrine functions, and contribution to the development of human diseases. *In vitro* and *in vivo* models, and genetically modified organisms will be used for this purpose. Orphan receptors will be studied as a source of future programs in the same area, starting by the identification of their natural ligands, one of the main expertises of the partnership. Through this work, we expect to designate receptors as targets for therapeutic agents fulfilling presently unmet medical needs.

2. RESEARCH GOALS

2.1. Design of three dimensional models of G protein-coupled receptors

We will design GPCR models that can be used as reliable tools to understand the basic properties of this class of receptors, including agonist and antagonist binding, dimerization, allosteric interaction between binding sites, structural changes associated with the mechanism of activation, the interaction with the different effector pathways, and the relation between the various intracellular signaling pathways and the existence of multiple active states. The same models will also allow supporting the design of chemical ligands for some of the receptors, which have an obvious interest as drug targets. Modeling will be applied to most of the receptors of interest, and will be performed in close relation with the experimental approaches, both supporting the design of experiments and being fed by its results.

2.2. Understanding the functional significance of GPCR dimers

Dimerization of GPCRs became over the recent years a well established phenomenon. However, apart from a few examples for which heterodimerization of receptor subtypes is necessary to achieve a functional pharmacological entity, it is presently unclear for the majority of receptors what is the functional significance of dimerization. Using a few receptor classes as models (glycoprotein hormone, chemokine, glucose receptors) we will study further the requirement for dimeric or oligomeric assemblies, and the functional consequences of heterodimerization on the pharmacology of the various subtypes *in vitro* and *in vivo*, and on the use of these receptors as drug targets.

2.3. Delineation of new signaling pathways for GPCRs

Besides the classical pathways activated by GPCRs, an increasing number of intracellular cascades have been shown to be modified following GPCR activation. Some of these pathways are regulated independently from G protein activation through the scaffolding of receptors with a range of cytosolic and membrane proteins. Signalling pathways activated by G proteins will be studied by DNA chips and proteomic approaches, using as models, chemoattractant receptors in mammalian cells and the glucose receptor in yeast.

2.4. Understanding the role of receptors in physiological processes and human diseases

We expect to associate a number of receptors from mammalian species (human and mouse), insect species, and yeasts to precise regulatory networks in these organisms. For mammalian receptors, we will go one step further, and will determine their role in human diseases, focusing on immune and inflammatory disorders, cancer, and endocrine diseases. Validated drug targets should therefore result from this program.

2.5. Characterizing new receptors in mammals, insects and yeasts

Orphan receptors will be expressed in recombinant systems, and we will search for their natural agonists within collections of known molecules, and in fractions of tissues and biological fluids. We expect to identify the natural ligands for at least four orphan receptors within the time frame of this program. The new ligand-receptor systems identified will be studied further in vitro, in vivo and in transgenic or knock out organisms, in order to delineate their physiological relevance and, for mammalian receptors, their potential relevance in the frame of specific human diseases, as stated above.

2.5. Linking evolution, structure and function of GPCR families

We will establish connections with partners of another IAP network, in order to study the evolutionary aspects of selected GPCR families (chemokine and olfactory receptors), and integrate this analysis with structural and functional data, with the aim of improving the power of the modeling tools and their predictive character.

3. STATE OF THE ART

3.1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) represent the largest family among membrane receptors. They play a major role in a variety of physiological and pathophysiological processes, e.g. carbohydrate metabolism, regulation of the cardiovascular system, nociception, feeding behaviour, immune response, etc. All GPCRs share a common structural organization with seven transmembrane segments, and a common way of modulating cell function by regulating effector systems through a family of heterotrimeric G proteins (although G protein-independent signalling has been reported as well). Not considering the olfactory and gustatory receptors, more than 350 G protein-coupled receptor types and subtypes have been cloned to date in mammalian species. Among these, about 250 have been characterized functionally, and our groups have largely contributed over the years to this characterization. Due to their accessibility from the extracellular space and their key roles in modulating cell functions, G protein-coupled receptors constitute the targets for the majority of active compounds presently used as therapeutic agents. In this context, newly characterized receptors raise a strong interest in the pharmaceutical industry, and will certainly lead to the development of a significant fraction of novel drugs in the future (Wise et al; 2004, Ribeiro and Horuk 2005).

3.2. Three-dimensional structure of GPCRs and modeling approaches

In contrast to the wealth of available pharmacological data, structural information on GPCRs is still scarce. To date, the only crystal structure available is that of the inactive state of bovine rhodopsin (Li et al 2004; Palczewski et al 2000). Five structural models of rhodopsin are available in the Protein Data Bank, at resolutions from 2.8 Å (PDB identifiers 1F88 and 1HZX) to 2.2 Å (1U19). Rhodopsin-like GPCRs share a large number of conserved sequence patterns in their TM domains (Mirzadegan et al 2003). These patterns allow the use of homology modeling techniques for building three-dimensional models of class A GPCRs using rhodopsin crystal structures as a template. These models are further adapted by state of the art molecular dynamics simulations in an explicit environment.

Drug discovery has traditionally made progress by a combination of random screening and rational design. In practice, the latter approach has often been frustrated by the lack of experimental data that define the structure and properties of the biological target. Today, this situation is starting to change because the structure of bovine rhodopsin has clearly opened a new era. However, the success of the rational design approach is intimately linked with the “quality” of the structure of the biological target. Therefore, GPCR modeling strategies require further improvements in order to deliver reliable models, and such improvements is one of the aim of this partnership.

3.3. GPCR dimerization

Until the past few years, GPCRs were essentially considered as monomeric functional structures. A growing body of evidence has however accumulated recently, which support that GPCRs exist and function essentially as dimers or higher order oligomers. A number of recent reviews have addressed this question (Milligan et al. 2003, Terrillon and Bouvier 2004, Kroeger and Eidne 2004, Bulenger et al. 2005). Different approaches have been used over the years to support the concept of GPCR oligomerization, including co-immunoprecipitation of tagged receptors. New biophysical techniques, such as bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET) and homogenous time resolved FRET (HTRF) are now available to monitor protein interactions in living cells (Van Roessel and Brand 2002, Angers et al. 2002). The reported functional consequences of dimerization vary greatly according to the specific GPCR dimer considered. In most cases, no clear functional consequences have been associated to dimerization. Some heterodimers were reported to display original pharmacological profiles or coupling properties (Jordan and Devi 1999). In a few cases, heterodimerization is an absolute requirement to form a functional receptor. The GABA_{B1} and GABA_{B2} receptor isoforms become functional only when co-expressed (Jones et al. 1998, Kaupmann et al. 1998, White et al. 1998, Pin et al. 2003). Heterodimerization is also required for the transport and function of taste receptors (Nelson et al. 2001, 2002). Altogether, the prominent current hypothesis is that GPCRs assemble as dimers shortly after synthesis in the endoplasmic reticulum, and traffic as such throughout their life in the cell.

3.4. Glycoprotein hormone receptors (GPHRs)

The glycoprotein hormones and their receptors constitute an interesting example of co-evolution. The hormones, follitropin (FSH), lutropin (LH), chorionic gonadotropin (hCG) and thyrotropin (TSH) are dimeric proteins of about 30 kDa made of a common alpha subunit and specific beta subunits. They play key roles in the control of reproduction (FSH, LH, hCG) and metabolism (TSH). The beta subunits are encoded by paralogous genes displaying substantial sequence similarity. The corresponding receptors, FSHr, LH/CGr and TSHr, are members of the rhodopsin-like G protein-coupled receptor family. As such, they contain a “serpentine” portion containing seven transmembrane helices with many (but not all) of the sequence signatures typical of this receptor family. In addition, and a hallmark of the subfamily, they contain a large (350-400 residues) aminoterminal ectodomain responsible for the high affinity and selective binding of the corresponding hormones (Dias and Van Roey 2001, Szkudlinski et al. 2002, Ascoli et al. 2002). The higher sequence identity of the serpentine portions (about 70%) when compared with the ectodomains (about 40%) suggested that the former are interchangeable modules capable of activating the G proteins (mainly G_{αs}) after specific binding of the individual hormones to the latter (Vassart and Dumont 1992). Contrary to other rhodopsin-like GPCRs, binding of the hormones to their ectodomains can be observed with high affinity in the absence of the serpentine (Schmidt et al. 2001, Remy et al. 2001, Cornelis et al. 2001). The intramolecular transduction of the signal between these two portions of the receptors raises interesting mechanistic issues which are still not understood.

Another issue, of evolutionary nature, relates to the shaping of hormone-receptor couples in order to cope with the emergence of chorionic gonadotropin in primates (Szkudlinski et al. 2002). Whereas in all mammals the circulating concentrations of TSH, LH and FSH are in proportion with their K_ds for the corresponding receptors (in the low nanomolar range), in primates and in particular in man, chorionic gonadotropin (hCG), which shares its receptor with LH, may approach micromolar concentrations during the first trimester of pregnancy. This constitutes a challenge to the specificity of recognition regarding the TSH and FSH receptors and is known to be responsible for some spill-over phenomena in trophoblastic diseases, and in rare cases where mutant TSH or FSH receptors become abnormally sensitive to hCG (Rodien et al. 1998, Smits et al. 2003).

3.5. Chemokine receptors

A large number of G protein-coupled receptors contribute to the mounting of immune responses by regulating the trafficking of leukocyte populations. Chemokines constitute one of the major classes of such signaling proteins (Rossi and Zlotnik 2000, Sallusto et al. 2000), with over 40 chemokines and 19 chemokine receptors described so far (Murphy et al. 2000, 2002). Chemokines recruit white blood cells to the sites of inflammation and to the various compartments of lymphoid organs. They play fundamental roles in the host defence against infection by viruses, bacteria and parasites. The protective effects of chemokines can however turn harmful to healthy tissues when their expression is inadequate, and chemokines contribute therefore to the damage in a wide range of acute and chronic inflammatory diseases. They were shown to play a deleterious role in septic shock, rheumatoid arthritis, asthma, acute glomerulonephritis, reperfusion injury, psoriasis, atherosclerosis and many other diseases (Gerard and Rollins 2001, Johnson et al. 2004). The chemokine receptors CCR5 and CXCR4 have also been characterized as the co-receptors that are necessary, together with CD4, to the entry of HIV in its host cells (Moore et al. 2004). Chemokines are also involved in the complex relationships between tumoral and host cells, regulating angiogenesis, cell recruitment and sometimes cell proliferation (Wang et al. 1998, Vicari and Caux 2002, Balkwill 2003, Mantovani et al. 2004). CCR5 is a chemokine receptor that was characterized by partner P1 (Samson et al. 1996a, 1996b, Doranz et al., 1996). This group has since studied various aspects of CCR5 function, including the genetics of receptor mutants, and the structure-function relationships of the receptor and its ligands (Samson et al. 1997, Rucker et al. 1996, 1997, Libert et al. 1998, Blanpain et al. 1999a-c, 2000, 2001, 2002, 2003, Govaerts et al. 2001b), and will continue to use CCR5 as a general model for chemokine receptors.

3.6. Other chemoattractant receptors

Other chemoattractant molecules include the formyl peptides, complement fragments (C3a, C5a) and leukotrienes, among others. Partner P1 has characterized recently two new chemoattractant factors (chemerin and F2L) and their cognate receptors (ChemR23 and FPRL2, respectively) (Wittamer et al. 2003, 2004, 2005, Migeotte et al. 2005). A number of orphan human receptors, structurally related to chemoattractant receptors, are still available in the literature and databases.

ChemR23. ChemR23 is a receptor expressed in dendritic cells (DCs) and macrophages (Samson et al. 1998). Its natural ligand, chemerin, was purified from a human ascitic fluid (Wittamer et al. 2003). Chemerin is structurally related to the cathelicidin precursors (anti-bacterial peptides), cystatins (cysteine protease inhibitors) and kininogens. It is a potent chemoattractant for antigen-presenting cells (both myeloid and plasmacytoid dendritic cells, Vermi et al. 2005) and is abundant in human inflammatory fluids. Chemerin is secreted as an inactive precursor (prochemerin), and requires proteolytic processing for activity. Two neutrophil proteases, cathepsin G and elastase, are able to mediate this activation (Wittamer et al. 2005). Preliminary experiments have shown that prochemerin expression in the mouse B16 melanoma model results in a strong anti-tumor effect. Resolvin E1, an omega-3 lipid mediator with demonstrated anti-inflammatory properties was also proposed recently as a ligand for ChemR23 (Arita et al. 2005) but this activity was not confirmed locally. Given its anti-tumour properties, its involvement in DC recruitment, and the strong production of chemerin in human inflammatory diseases, this new chemoattractant system will be studied further in this program.

FPRL2. Partner P1 has recently characterized the peptide F2L as a high affinity natural ligand of the FPRL2 receptor (Migeotte et al. 2005). FPRL2 belongs to a small family of receptors related to FPR, the receptor for fMLP and other formylated bacterial peptides. This subfamily contains three receptors in human. FPR and FPRL1 are expressed by neutrophils, monocytes, and, for FPR, dendritic cells, while FPRL2 is not expressed in neutrophils, and found exclusively in monocytes and (immature and mature) DCs. FPRL1 is an exceptionally promiscuous receptor, responding to numerous ligands of different origins and of high structural diversity (Le et al, 2001, 2002). F2L is the N-terminal 21 amino acid peptide of the intracellular Heme-binding protein (HBP). It chemoattracts monocytes and immature dendritic cells through the G_i family of G proteins, and is strongly conserved in other vertebrate species. F2L is inactive on FPR, and poorly active on FPRL1. HBP has no well defined biological activity so far (Taketani et al. 1998, Jacob Blackmon et al. 2002). Another human endogenous peptide, humanin, was described in parallel as another high affinity ligand of FPRL1 and FPRL2 (Harada et al. 2004).

3.7. Insect receptors

Several signal transducing receptor families had an ancient origin that was situated before the divergence between protostomian and deuterostomian animals (Vanden Broeck 1996). For the analysis of insect G protein-coupled receptors, the *Drosophila* genome project (Adams et al. 2000) represented a major breakthrough. Since then, the genomes of several other insect species, such as the malaria mosquito, *Anopheles gambiae*, the honeybee, *Apis mellifera*, the silkworm, *Bombyx mori*, and the red flour beetle, *Tribolium castaneum*, most of which are of economic (positive or negative) or medical value, have been (or are being) sequenced. Some insect receptors are believed to be potential targets for pest control agents (Vanden Broeck et al. 1997). In silico analyses revealed the presence of at least 200 genes coding for putative heptahelical transmembrane (7TM) proteins in *Drosophila* as well as in other insect genomes (Brody and Cravchik 2000, Vanden Broeck 2001b, Hill et al. 2002, Fredriksson and Schioth 2005). These can be classified into at least four evolutionary conserved subgroups: 1) the large rhodopsin-like receptor family (A), 2) the secretin receptor family (B), 3) the metabotropic glutamate receptor family (C) and 4) the Frizzled/Smoothed family. In addition, a large group of odorant (OR) and gustatory (GR) receptors was discovered in insects by means of an e-genetics approach using a new algorithm for 7TM protein discovery (Kim and Carlson 2002, Robertson et al. 2003, de Bruyne and Warr 2006). Insect genomes also encode a multitude of putative neuropeptide precursor genes (Vanden Broeck 2001a, Hewes and Taghert 2001, Riehle et al. 2002). In addition, an important fraction of the insect GPCRs shows sequence similarities with mammalian peptide receptors (Hewes and Taghert 2001, Hill et al. 2002). Several of these receptors have been deorphanized in recent years, while others still remain orphans (Claeys et al. 2005). *In vitro* (e.g. cell-based assays, reverse pharmacology) as well as *in vivo* (e.g. analysis of fruit fly mutants) approaches will further contribute to the discovery of specific receptor ligands and to the elucidation of insect GPCR function(s), respectively.

3.8. Yeast GPCRs

As part of the previous consortium, a sugar sensing GPCR system in the yeast *Saccharomyces cerevisiae* has been characterised extensively. Previously, yeast was only known to contain a single GPCR system involved in the mating pheromone pathway. This GPCR system has played an important model role for the elucidation of GPCR functioning and downstream signaling (Dohlman et al. 1991). The discovery of the sugar-sensing GPCR system could play a similar role since here too several novel aspects of GPCR functioning have already been discovered which might be present in other organisms as well. The sugar-sensing GPCR system regulates the cAMP-PKA pathway, which controls a variety of processes in yeast, in particular those associated with growth, stationary phase entry, reserve carbohydrates, stress response, morphological development (pseudohyphal growth) as well as many aspects of metabolism (Thevelein and de Winde 1999).

3.9. Olfactory receptors

Olfaction is responsible for detecting an immense repertoire of structurally diverse molecules. The precise recognition of thousands of odorants requires a massive receptor repertoire (Buck and Axel 2001). Olfactory receptors (ORs) are therefore the largest gene family in the genome of human and other mammalian species, as well as in other vertebrates and invertebrates. In most mammalian species analyzed to date, about a thousand presumably functional ORs have been identified in the genome. In human, about 380 genes are expected to encode functional ORs, while about an equal number of OR pseudogenes are present. ORs are present in the cilia of olfactory neurons. They couple to an olfactory-specific G-protein (G_{olf}), which activates adenylyl cyclase III, and increased cAMP levels activate cyclic nucleotide-gated channels, causing cell membrane depolarization. Specific databases are dedicated to ORs from various species, such as the Yale (<http://senselab.med.yale.edu/senselab/ORDB/>), and HORDE databases (<http://bioportal.weizmann.ac.il/HORDE/>) (Olender et al. 2004).

Most studies conducted over the past decade have led to the hypothesis that individual olfactory neurons express a single OR, giving rise to the "one receptor-one neuron" hypothesis. This hypothesis has served as the basis for models of olfactory coding. However, recent reports regarding *Drosophila* have shown that in insects, a non-canonical receptor Or83b, which is highly conserved across insect orders, dimerizes with odorant and pheromone receptors and is required for efficient localization of these proteins to dendrites of sensory neurons (Dahanukar et al. 2005, Nakagawa et al. 2005). Co-expression of two functional receptors in one class of olfactory receptor neurons was also reported in *Drosophila* (Goldman et al. 2005). It is not known presently if these new concepts might apply to mammalian ORs as well.

3.10. Orphan receptors

Orphan receptors, for which the ligands and function are still unknown, represent an attractive set of future drug targets. Molecular cloning relying on structural similarities has been pioneered by members of the network (Libert et al. 1989, Parmentier et al. 1989), and resulted in the discovery of many orphan receptors. Screening strategies have been developed over the years in order to identify the natural ligands of orphan receptors. Most orphan receptors characterized over the last ten years have been associated to previously characterized molecules. However, new and unpredicted biological mediators have also been purified from complex biological sources. The identification of the ligands of orphan receptors, starting from purely genetic data, is referred to as “reverse pharmacology”. The first success was the identification of a novel neuropeptide, nociceptin, as the natural agonist of an orphan receptor related to the opioid receptor (Meunier et al. 1995, Reinsheid et al. 2005). Subsequently, other peptides and proteins, such as orexins (Sakurai et al. 1998), prolactin-releasing peptide (Hinuma et al. 1998), apelin (Tatemoto et al. 1998), ghrelin (Kojima et al. 1999), prokineticins (Lin et al. 2002), kisspeptin/metastin (Kotani et al. 2001, Ohtaki et al. 2001), and chemerin (Wittamer et al. 2003) have been purified and identified as the natural ligands of previously orphan receptors. Nevertheless, “deorphanising” a receptor remains a tedious venture. Partners of the present program have played a major role in the characterisation of many G protein-coupled receptors, including the purification of natural ligands.

References are included in the main reference list in **Form D**

I. 4. DETAILED DESCRIPTION OF THE PROJECT (15 pages minimum, 25 pages maximum)

- Submit a general description of the project as well as a description detailing each workpackage and indicate the partners involved in each workpackage.
 - Illustrate by means of a table or scheme the interaction between the partners within a workpackage and the interaction between the workpackages.
 - Describe and justify the methods and proposed approaches in relation to the state of the art.
 - Describe and justify how the contribution of the different partners will be integrated.
-

I. General description

The program will articulate along two main lines. Along one line (**WP1** and **WP2**), the structural organization of class A GPCRs will be studied by modeling and mutagenesis. Along the other line (**WP3**, **WP4**, **WP5** and **WP6**), the functional role of specific receptors and receptor classes will be studied *in vitro*, *in vivo* and in animal models, with the aim of identifying their involvement in key physiological or pathological processes. These two main aspects of the program will be supported and linked by the bioinformatics analysis of the GPCR family and its evolution in relation to species evolution and differentiation (**WP7**), an aspect that will be developed as the number of full genomes available in the databases increases at a rapid pace. This program is based on the expertise the network partners have acquired over the previous years, and their demonstrated willingness of working together as a continuation of a previous IAP program. This partnership will study further both general and specific aspects of this important gene family, with the ultimate aim of improving human health.

In **WP1**, models of the GPCRs of interest will be constructed by partner EU1 on the basis of the crystal structure of the inactive state of bovine rhodopsin, the only three-dimensional model of a member of the GPCR family available to date. It is however likely that the crystal structure of an intermediate activation state of rhodopsin will appear in the literature in the near future, potentially allowing to construct more adequate models of receptor activation. These structural models will be used to understand how receptors are maintained in their inactive state, and how the interaction with their ligands can result in the modification of the receptor's structure, allowing its interaction with G proteins and downstream signaling events. Ligand-receptor interactions and activation models will be tested for glycoprotein hormone receptors, CCR5, ChemR23 and insect tachykinin receptors. Ultimately, these models should allow understanding how different ligands can stabilize different conformations of a given receptor, and activate a specific set of downstream events. Such models will also be used to help designing chemical agonists and antagonists of specific receptors. The last aspect of this structural analysis will consider the oligomerization of GPCRs (**WP2**). Homodimerization of GPCRs is now a well established phenomenon. The network will study homo- and heterodimerization of glycoprotein hormone, chemokine and sugar-sensing receptors, focusing on the allosteric interactions between the receptor protomers, the functional consequences in terms of ligand binding, signaling and regulation, and their relevance in terms of therapeutic targeting.

WP3 will focus on the signaling of GPCRs. Chemokine ligand processing, affecting receptor specificity and agonist potency will be considered, as well as synergistic activation through different receptors co-expressed in the same cells. Signaling cascades activated by chemoattractants in dendritic cells will be studied by genomic and proteomic approaches. In parallel, the signaling pathways activated by insect neuropeptide receptors and yeast sugar-sensing receptors will be studied as well, and these pathways will be compared to what is known for mammalian cells. **WP4** will be devoted to the understanding of the role of specific receptors and receptor classes in physiological processes. The mammalian ChemR23, FPRL2, GIR and GPR3, the insect neuropeptide receptors and the yeast sugar-sensing receptors will be studied *in vitro*, *in vivo* and in genetically modified organisms. As a follow-up, **WP5** will focus on the involvement of mammalian receptors in human diseases and mouse models of these diseases, with a special emphasis on inflammation, cancer and endocrine control. Glycoprotein hormone receptors, chemokine receptors, ChemR23 and GPR10 will be studied in this frame. **WP6** will focus on the remaining orphan receptors in mammals, insects and yeasts, with the aim of characterizing some of them functionally. Newly characterised receptors will be studied in details.

WP7 will constitute new aspects studied in the network. We will on one side identify by a proteomic approach new proteins involved in the signalling complexes of olfactory neurons, and will use this knowledge to improve the functional expression of olfactory receptors. Besides, we will implement, in collaboration with members of another IAP program (Bioinformatics and modeling: from genomes to networks), the analysis of

the evolution of GPCR families across species, and will integrate these data with structural and functional aspects. The main families of interest will be chemokine and olfactory receptors.

II. Detailed description of workpackages

WP1. STRUCTURAL ORGANIZATION OF GPCRS

1.1. Structural models of GPCRs (EU1, P1)

Partner EU1 applies bioinformatics tools, ranging from multiple sequence analysis, statistical methods, protein database search, molecular graphics, and molecular dynamics simulations, to study the structure and activity of several types of GPCRs. This group develops *in silico* models of both the amino-terminal extracellular domain of the leucine-rich repeats G protein-coupled receptors subfamily and the membrane embedded heptahelical bundle of Class-I or rhodopsin-like GPCRs. These computer models are used to predict the mechanisms of ligand recognition and receptor activation, which are experimentally validated by side-directed mutagenesis and functional assays. This partner also develops pharmacophore models using structural information derived from i) a series of ligands that are thought to interact with the same binding site and/or ii) the putative residues in the receptor model which might be involved in the binding of the ligand. Chemical 3D-databases are searched using the pharmacophore model as a 3D query for virtual screening and lead optimization. These various approaches will be applied to various receptors used as models, and more details are provided in the next sections.

Besides modeling, this partnership will also contribute, through existing FP6 networks, to international efforts for purifying and crystallizing selected receptors (ChemR23, CCR5), in order to obtain actual structures of receptors. When available, these purified functional receptors will also allow to design biophysics analysis of these receptors in collaboration with groups specialized in membrane proteins (E. Goormaghtigh and V. Raussens team in ULB).

1.2. Activation mechanisms

1.2.1. Glycoprotein-hormone receptors (P1, EU1)

The glycoprotein hormone receptors (GPHRs: thyrotropin receptor, TSHR; follitropin receptor, FSHR; lutropin receptor, LH/CGR) are peculiar as they are composed of a large ectodomain, responsible for hormone recognition and binding, and a canonical heptahelical (or serpentine) domain, typical of rhodopsin-like GPCRs, involved in transduction of the activation signal to the G protein(s). Whereas in other rhodopsin-like GPCRs, binding of the agonists, within the interhelical trough (for small molecules like biogenic amines), or to the short aminoterminal extension and the extracellular loops (for peptides or chemokines), is intimately linked with the activation mechanism, in glycoprotein hormone receptors, these two steps can be separated. The work performed during the previous IAP program lead us to propose a model in which, upon binding of the hormones, the ectodomain would transform into a direct agonist of the serpentine portion of the receptors (Vassart et al. 2004). According to this model, the "immediate" agonist of the serpentine would be the activated ectodomain. The possibility to fully activate GPHRs by introducing a single point mutation in their ectodomain is a strong argument in favor of the model. Besides, in a tight collaboration between partners EU1 and P1, several structural locks have been identified in the serpentine portions of GPHRs, the release of which is implicated in the activation mechanism (Govaerts et al. 2001a, Vassart et al. 2004, Urizar et al. 2005a).

We plan to further explore the mechanism of activation of GPHRs by a combination of two experimental approaches.

Identification of additional key residues involved in activation by random mutagenesis. Up to now, more than 30 residues have been identified, mainly in the TSHR, which upon mutation, cause constitutive activation of the receptor (Van Durme et al. 2006). The TSHR has been chosen to pursue this mapping endeavor, because of its propensity to be activated by mutations (Parma et al. 1997). We plan to saturate the serpentine portion of the receptor with activating mutations by generating a large library of random mutants by a low fidelity PCR approach (GeneMorph II, Stratagene). Individual mutants will be tested for their aptitude to activate G_s, by transfection in HEK293T cells followed by measure of intracellular cAMP. It is expected that about 2500 mutants will be required to probe efficiently the 350 residues of the serpentine domain. The technology to perform these relatively high throughput transfection experiments has been explored, and is now available. The atomic interactions of the novel residues identified will be studied by modelization by partner EU1, in the light of the 3D structure of inactive rhodopsin.

Identification of residues implicated in interactions between the ectodomain and the serpentine domain. This will be attempted by intramolecular cross-linking of the receptor with selected chemicals and identification of cross-linked residues from the proteolytically digested protein by mass spectrometry. Intra-molecular cross-linking has recently been developed as a means to probe the 3D structure of proteins for which crystallographic or NMR structural data are not readily available (Back et al. 2003). Recently, a new type of

cross-linker was designed, which includes two labile bonds. This allows specific release of reporter ions by low energy MS/MS while also allowing specific cleavage of the spacer arm to release cross-linked peptide chains without disrupting their peptide backbones (Tang et al. 2005). The individual peptide chains can then be sequenced separately with additional stages of MS/MS (Trester-Zedlitz et al. 2003).

Our goal is to use this new generation of cross-linkers to identify the contacts residues between the ectodomain (and/or the hormone) and the serpentine region of TSHr. Various ratios between receptor and cross-linker will be used, and the quality of the cross linking reaction will be monitored by SDS-PAGE and western blotting. The complex will be digested by a panel of proteases (trypsin, chymotrypsin...) and analyzed by mass spectrometry. The same approach will be applied to a TSH receptor construct harboring a mutation in its ectodomain, rendering it constitutively active. Comparison of the contact points identified in the wild type and mutant receptors is expected to yield the first structural information on transduction of the activation from the ectodomain to the serpentine domain. Interestingly, these two methodological approaches will also be exploited in the frame of our study of GPHR dimerization (see 2.1).

Identification of mechanisms relating basal activity and specificity in GPHRs. Studies performed in the frame of the previous IAP program have demonstrated the existence of an unexpected relation between activation of GPHRs and specificity of activation by their respective hormones (Smits et al. 2003, Vassart et al. 2004, De Leener et al. 2006). In particular, it was shown that specific mutations of the human FSHR, in the serpentine domain, made the mutant abnormally sensitive to both TSH and hCG. Interestingly, at the same time, these mutations caused slight or definite constitutive activation of the receptor. As, contrary to other species, the wild type human FSHR is totally silent, we hypothesized that silencing has been selected by evolution to avoid promiscuous activation of the FSHR by the extremely high hCG concentrations prevailing during pregnancy (chorionic gonadotropin is only present in higher primates). This hypothesis will be tested experimentally by measuring the sensitivity to hCG of chimeric constructs made of the human FSHR ectodomain (the "recognition" unit) and serpentine portions (the effector) from a wide variety of primate- and non-primate vertebrates. Identification, within the serpentine domains of the various species, of the residues responsible for both silencing and establishment of an effective specificity barrier, will contribute to our understanding of the functioning and evolution of the GPHR family and their corresponding hormones. In addition, it may cast light on the pathophysiology of ovarian hyperstimulation syndrome, a disease caused by promiscuous activation of the FSHR by hCG (see 5.1).

1.2.2. CCR5 (P1, EU1)

CCR5 belongs to the rhodopsin family of GPCRs, thus containing the known restraining intramolecular interactions that maintain the receptor in the inactive state. However, chemokine receptors contain particular signatures, the role of which in receptor activation will be explored by partners EU1 and P1 using molecular modeling, site-directed mutagenesis and functional assays.

Using bovine rhodopsin as template, partners EU1 and P1 have constructed a model of the transmembrane helix bundle of CCR5, and have tested the model experimentally. This has led to the identification of a TxP motif in helix 2, which is shared by all chemokine receptors, and plays an important role in receptor activation (Govaerts et al. 2001). An aromatic cluster at the top of helices 2 and 3 has also been characterized functionally (Govaerts et al. 2003).

Identification of the role of His7.45 in the conserved hydrogen bond network linking Asp2.50 and Trp6.48. Asp2.50 is involved in maintaining Trp6.48 pointing towards TM7 in the inactive state of the receptor through a conserved hydrogen bond network (Li et al. 2004). This conserved network varies among GPCR subfamilies. Rhodopsin forms this network through water molecules #12 and #10 (Li et al. 2004). Asn7.45-containing receptors are able to form the Asp2.50••#12••Asn7.45••Trp6.48 network (Jongejan et al. 2005), whereas Asn3.35/Asn7.45-containing receptors form the Asn2.50••Asn3.35••Asn7.45••Trp6.48 network of interactions (Xu et al. 2005). Mutation of Asn at either positions 3.35 or 7.45 induces constitutive activity. Thus, it is suggested that this diverse but conserved hydrogen bond network impedes the reported conformational transition of Trp6.48, from pointing towards TM7, in the inactive state, to pointing towards TM5, in the active state (Ruprecht et al. 2004). Chemokine receptors replace Asn at position 7.45 by His. We plan to explore the role of His7.45 and its environment in the mechanism of activation of chemokine receptors.

Identification of the role of Arg6.30 in the ionic lock between TMs 3 and 6. The interaction between Arg at position 3.50 of the highly conserved (D/E)R(Y/W) motif in TM3 with its adjacent Asp/Glu residue at position 3.49 and an additional Asp/Glu at position 6.30 near the cytoplasmic end of TM6 is known as the ionic lock (Ballesteros et al. 2001). Charge-neutralizing mutation of Asp/Glu3.49 in TM3 and Asp/Glu6.30 in TM 6 results in increased constitutive activity in a number of structurally-related class A GPCRs (Ballesteros et al. 2001b; Montanelli et al. 2004b). The acidic residue at position 6.30 is only conserved in 32% of the rhodopsin-like sequences. Many GPCRs (34% of the sequences), including CCR5, contain a basic residue at this 6.30 position preventing a direct interaction with R3.50. These receptors will probably possess a totally different

network of interhelical interactions at the intracellular side. We would like to explore this network in the chemokine CCR5 receptor. These residues involved in interhelical interactions will be mutated, and the mutants tested for their constitutive activity and their ability to respond functionally to agonists. These studies should provide new insights into the mechanisms of GPCR activation in general.

1.2.3. ChemR23 (P1, EU1)

In order to extend the observations made on glycoprotein hormone and chemokine receptors to structurally different classes, we will initiate mutagenesis studies on other receptors, including ChemR23, and likely other receptors selected in the course of the program. As an example, from the ChemR23 model (see 1.3.1), a number of mutations has been proposed by partner EU1 (N3.35A, T6.43A and H3.36A), which should affect inter-helical interactions maintaining the inactive state of the receptor, and result in constitutive activity of these mutants. These hypotheses, testing the general activation model of GPCRs, but also the specific ChemR23 structural model, will be challenged by generating these mutants and testing them functionally.

1.3. Ligand-receptor interactions

1.3.1. ChemR23, characterization of two binding sites for chemerin domains (P1, EU1)

The ChemR23 model constructed by partners EU1 and P1 will be used to determine how chemerin or its C-terminal peptides bind and activate the receptor. Partner P1 has identified that, in the chemerin-9 nonapeptide (YFpGqFaFs-COOH), the four aromatic residues, the glycine and the terminal carboxyl group are essential for binding and activation of ChemR23. From the model, Arg^{5.42} was identified as a candidate partner for the carboxyl group. Indeed, position 5.42, in transmembrane helix 5, is involved in ligand binding in many G protein-coupled receptors. Preliminary experiments performed with a R5.42A mutant has shown a strong decrease in the efficacy of the chemerin-9 nonapeptide, while recombinant full-size protein is only weakly affected. These results suggest the existence of an independent binding site for the cystatin-like domain of chemerin. The existence of a second binding site is also supported by the fact that some anti-ChemR23 mAbs block the activity of full-size chemerin, but not of the nonapeptide. The existence of two binding sites is also proposed for other receptors, such as C5aR. This two sites hypothesis will be tested by different approaches, and the two binding sites characterized by mutagenesis.

A binding assay using chemerin-9 is available. Partner P1 will establish a binding assay for the cystatin-like domain of chemerin, using first iodinated full-size chemerin, then the cystatin domain of chemerin alone, which will be expressed and purified by affinity chromatography using available monoclonals. These tracers will be used in saturation and competition binding assays to determine the binding characteristics of the cystatin-like domain, analyse the two binding sites independently, and the potential cooperativity interactions between them.

The binding site of the chemerin-9 nonapeptide will be determined by constructing a set of mutants affecting the potential binding partners in the receptor. These include residues N3.29, L3.32, I3.33, and M3.36 in TM3, R5.42 and F5.43 in TM5, Y6.51, H6.52 and N6.55 in TM6. Alanine mutants will be tested in binding and functional assays, using chemerin, chemerin-derived peptides and mutants thereof. The results will be integrated into the 3D model of ChemR23, and several rounds of mutagenesis will follow, in a permanent interplay between modeling and mutagenesis. Putative side chain interactions between the peptide and the receptor will be tested by concerted mutations on both partners. Also, the chemerin-9 peptide will be studied by infrared spectroscopy and nuclear magnetic resonance, in order to confirm the presence of a putative beta-hairpin structure, suggested by the presence of a Pro-Gly motif. These data will be integrated in the structural model as well.

In parallel, the binding site of the cystatin-like domain of chemerin will be studied, focusing first onto the N-terminal domain of ChemR23. The role of this domain is supported by the presence of a large number of negatively charged residues (Asp, Glu, potentially sulfated Tyr), while chemerin is positively-charged. The N-terminal domain of GPCRs is also frequently involved in the binding of peptide/protein ligands. The contribution of the various ChemR23 domains in chemerin binding will be tested on epitope-tagged chimeras between ChemR23 and GPR1, the most closely related GPCR. In the N-terminal domain, the clusters of negatively charged residues will be neutralized first in groups (²EDEDY⁶ in ²QNQNF⁶, ¹²YGDEY¹⁶ in ¹²FGNQF¹⁶, ...), then individually. The effects of these mutations on the binding and functional properties of various ligands of ChemR23 will be tested, and the results will direct subsequent rounds of mutagenesis. Mutations of the cystatin-like domain of chemerin will then be considered. The binding sites of blocking antibodies will also be mapped using the same tools, as well as chimeras between human and mouse ChemR23.

1.3.2. Molecular pharmacology of insect tachykinin-like peptide receptors (P2, EU1)

The molecular properties determining the interactions between (co)evolutionary conserved neuropeptide/receptor partners will be investigated by means of a combination of molecular modeling, site-

directed mutagenesis, receptor assays and peptide chemistry. The work will mainly focus on tachykinin- or neurokinin-like ligands and their receptors, since these have been identified in both vertebrate and invertebrate species (Vanden Broeck et al., 1999; Kawada et al., 2002). Moreover, the corresponding mammalian versions are among the best studied examples of G protein-coupled peptide receptors belonging to the rhodopsin superfamily (Bhogal et al., 1994; Elling et al., 1995). Previous studies by P2 indeed revealed the existence of insect G protein-coupled receptors that are activated by insect tachykinin-related peptides, or their analogues, causing distinct calcium and cyclic AMP responses in transfected (i.e. receptor-expressing) cells (Torfs et al., 2000, 2001, 2002abc; Poels et al., 2004). In addition, these responses are antagonized by spantides, potent substance P receptor antagonists (Torfs et al., 2002a; Janecka et al., 2005). Multiple sequence comparisons showed that the amino acid sequences of the insect receptors display several features that are typical for the subgroup of neurokinin (NK) receptors (Torfs et al., 2001). Therefore, based on in silico models for both mammalian and insect neurokinin-like peptide GPCRs (partner EU1), and on the available information derived from mutagenesis studies with mammalian NK receptors, receptor mutants will be designed, expression constructs will be prepared and cells will be transfected. Model building and site-directed mutagenesis experiments have been initiated during the past year in the context of the ongoing project (IAP P5/30). The expressed mutated receptors will be further analysed by means of binding and functional assays (available from P1) and their pharmacological as well as signalling properties will be compared with the wild type.

1.4. Design of agonists and antagonists (EU1, P1)

The processes initiated by the recognition of the extracellular ligand by the receptor will extensively depend on the type of receptor since wide ranges of extracellular ligands, from small neurotransmitters to large hormones, are recognized by GPCRs. Each subfamily has probably developed specific structural motifs that allow the receptor to accommodate and respond to its cognate ligand. However, it seems reasonable to propose that in Trp6.48-containing GPCRs (71% of the rhodopsin-like sequences), agonists binding modify the conformation of Trp6.48 (Ruprecht et al. 2004; Jongejan et al. 2005). We have proposed that agonists trigger, by means of an explicit hydrogen bond or an aromatic-aromatic interaction, or both, the conformational transition of Trp6.48 towards TM5 (Lopez-Rodriguez et al. 2005). Thus, GPCRs possess a small cavity between TMs 5 and 6 to accommodate the side chain of Trp6.48 in the active conformation. This small cavity is formed by the side chains at positions 3.40 (L:9%; V:25%; I:42%; M:5%), 5.47 (F:70%; Y:11%), and 6.52 (H:29%; F:20%; N:19%). The role of the Phe/Tyr5.47 and His/Phe6.52 aromatic side chains is to further stabilize the active conformation of Trp6.48 by aromatic-aromatic interactions in the face-to-edge orientation. We propose that inverse agonists occupy this small cavity to impede the transition of Trp6.48 towards TM5, thus decreasing the constitutive activity of the receptor. The fact that this cavity is formed by mostly aromatic amino acids led us to suggest an aromatic ring as an important pharmacophoric element of inverse agonists. This definition of agonism and inverse agonism at the molecular level provides the tool for the rational design of new ligands with a predetermined pharmacological profile at a given receptor.

Glycoprotein hormone receptors. From their mode of activation, with a clear dichotomy between recognition of a large agonist by their ectodomain and transduction of the activation signal via their serpentine portion, glycoprotein hormone receptors have until recently been considered “non-drugable”. However, the existence of auto-antibodies with agonistic properties on the TSHR, in Graves’ disease, has prompted extensive research, culminating in the generation of murine and human monoclonal antibodies (Costagliola et al. 2002, 2004; Ando et al. 2002; Sanders et al. 2002, 2003). Also, there have been recent reports indicating that small molecules, binding to the serpentine domain of GPHRs, can act as effective allosteric modulators, or as true agonists or antagonists (van Straten et al. 2002, Jaschke et al. 2006, Stephen et al. 2006).

Screening for monoclonal antibodies with agonist or antagonist activities. P1 will use, on a large scale, the genetic immunization protocol that they pioneered for GPCRs (Costagliola et al. 1998) to generate monoclonal antibodies directed against the TSH, LH and FSHR. The biological activity of the mAbs will be screened for using stimulation of cAMP generation in available CHO cell lines expressing each receptor. Apart from their potential clinical interest [humanized mAbs recognizing GPHRs might find applications in diagnosis of thyroid cancer metastasis (TSHR), treatment of Graves’ disease (TSHR), stimulation of follicle maturation and ovulation in IVF procedures (FSHR, LHR), hypogonadotropic hypogonadism (FSHR, LHR)...], precise mapping of the epitopes of these mAbs, using mutant receptors will help understanding the mechanism of GPHR activation.

Molecular design of agonists and inverse agonists of GPHRs. GPHRs lack Trp6.48 in TM6 but contain the family-specific Thr6.43Asp6.44 motif (Vassart et al. 2004). Partners EU1 and P1 have proposed that GPHRs form the highly conserved hydrogen bond network linking Asp2.50 and TM6 through Asp2.50••Asn7.45••Asp6.44 (Urizar et al. 2005a). Accordingly, this family of receptors also modifies the side chains at positions 5.47 and 6.52, forming the small cavity between TMs 5 and 6, from aromatic in Trp6.48-containing receptors (Phe/Tyr5.47 and His/Phe6.52) to polar in GPHRs (Asn5.47 and S6.52). Thus, we propose that small molecules would bind in this area of the 7TM bundle and would modulate the

conformation of Asp6.44 in a similar manner to ligands that bind Trp6.48-containing receptors. This working hypothesis will be used to design new agonists and inverse agonists for GPHRs.

Virtual screening of small molecule libraries for (ant)agonists of GPHRs followed by experimental validation. A pharmacophore definition is clearly established as one of the successful computational tools in rational drug design (Guner et al. 2004). The approach that will be followed in this project is the generation of a pharmacophore model from the TM domain of GPHRs, constructing a complementary image of the putative binding site. Various databases will be searched using the pharmacophore model as a 3D query, with the final goal of identifying compounds previously synthesized or commercially available that will fit within the structure of the receptor and therefore might be used as a lead compound. The TSH receptor is chosen (1) because it is the one for which we have the more detailed knowledge of structure-function relationships and a large collection of mutants; (2) because of the potential pharmacological interest of developing an antagonist for a better treatment of Graves' disease. The candidate molecules coming out of the virtual screening, will be tested experimentally for agonistic or antagonistic activities, on CHO cells stably expressing the TSH receptor. Again, apart from their pharmacological interest, understanding how these molecules interact with the receptor (via use of site-directed mutagenized constructs) is expected to further our understanding of the mechanisms of TSHR activation.

Other receptors will be selected in the course of this program for similar virtual screening approaches.

WP2. DIMERIZATION OF GPCRS

2.1. Glycoprotein hormone receptors (P1, EU1)

Partner 1 has recently identified negative cooperativity as an important correlate of homodimerization of glycoprotein hormone receptors (Urizar et al. 2005b). The group will pursue this topic (1) by investigating the possible relation between GPHR dimerization and activation, and (2) by attempting determination of the residues implicated in the dimerization interface.

Relation between GPHR dimerization and activation. Taking advantage of the wide panel of available mutants of the TSHR displaying constitutive activity, partner P1 has started exploring the relation between constitutive activity and negative cooperativity, in a series of such mutants. Preliminary results indicate an inverse relation between constitutive activity of mutants and acceleration of tracer dissociation by excess agonist, taken as an index of negative cooperativity. These results open the following possibilities: (1) do the constitutive mutants still dimerize efficiently?; (2) if no, what is the relation between dimerization and activation by natural agonists?; (3) if yes, is the loss of acceleration of tracer dissociation observed in the most constitutive mutants due to loss of negative cooperativity (both protomers keeping identical affinities, whether one member of the pair is liganded or not), or to absolute cooperativity ("complete" loss of affinity of one member of the pair upon binding of agonist to the other)? We will address the first question by measuring the ability of the mutants to dimerize, using BRET and HTRF technologies (Urizar et al. 2005b). Depending on the results, we will reinvestigate the effect of agonists on the formation of dimers by the same two methods [previous results suggested no effect of activation on dimerization, but this is still controversial (Latif et al. 2002)]. If mutants do still dimerize, we will explore in detail the kinetics of dissociation of tracer at various concentrations from wild-type and mutant receptors, to answer question 3.

Determination of the residues implicated in the dimerization interface. This question has been explored for some rhodopsin-like GPCRs (e.g. Guo et al. 2005, Fotiadis 2006), but not for GPHRs. Considering the possibility for a role of the ectodomain in the dimerization of the GPHRs (as suggested in Fan and Hendrickson 2005), we plan to investigate this point by the two methods detailed under 1.2.1. (1) Use of a library of randomly mutated constructs: Briefly, the idea is to transfect individual mutants in HEK293T cells and to measure independently expression at the cell surface (by flow immunocytometry, FACS) and dimerization (by HTRF). Mutants positive in FACS but negative in HTRF will be sequenced and the residue responsible for the phenotype identified. If no such mutant can be identified, it would strongly suggest that dimerization is an absolute prerequisite to expression of GPHRs at the plasma membrane. (2) Identification of contact residues between protomers by crosslinking. Crosslinking conditions favoring detection of dimers will be selected by western blotting, by adjusting the concentration of linking agents. Crosslinked peptides will be purified and sequenced by MS/MS.

2.2. Chemokine receptors (P1, EU2)

Homodimerization has been demonstrated for CCR2, CCR5, CXCR2 and CXCR4, and is likely applicable to all members of the family (Springael et al. 2005). Heterodimerization has been demonstrated between the closely related CCR2 and CCR5, but also between CCR2 and CXCR4 (Mellado et al. 2001, Blanpain et al. 2002, Issafras et al. 2002). During the previous IAP program, partner P1 has analysed by BRET the interaction between CCR5 and CCR2, and functional analyses have demonstrated negative cooperativity in terms of binding between the two dimer subunits (El Asmar et al. 2004). In cells coexpressing CCR5 and CCR2, a

CCR5-specific ligand (CCL4/MIP-1 β) was able to compete for the binding of a CCR2-specific tracer (CCL2/MCP-1). Thus, only one chemokine can bind with high affinity onto a receptor dimer, and this implies that activation of a protomer by an agonist induces correlated structural changes in the other protomer, either through the dimer interface, or indirectly through docking of the G protein. Recently, partner P1 has demonstrated such allosteric interaction between receptors, using ligand dissociation experiments (Springael et al. 2006). This can have significant consequences *in vivo*, as leukocyte populations frequently express numerous receptors on their surface, and the existence of heterodimers on native leukocyte populations has been demonstrated (Springael et al. 2006). Partner 1 will therefore investigate further the dimerization process of chemokine receptors.

Heterodimerization between various combinations of receptors (CCR5 and CCR1, CCR3, CCR8, CXCR4 or opiate receptors) will be tested by BRET, as well as its consequences on the pharmacology in functional and binding assays. Equilibrium binding assays and dissociation kinetics will be performed, in order to detect non-competitive allosteric interactions between binding sites. Observations made in cell lines overexpressing the receptors will be tested in native cell populations co-expressing the receptors. Heterodimerization and functional cross-talk between protomers might indeed have considerable consequences in the design and testing of chemical receptor agonists and antagonists. These experimental approaches will be combined to the modeling of these allosteric interactions in an adaptation of the so-called extended ternary complex model (Samama et al. 1993, Kenakin 2004). Such model will help setting the best experimental conditions to test hypotheses, and will allow determining the parameters governing the allosteric interactions for various receptor combinations.

The functional consequences of heterodimerization will be investigated. This includes the trafficking properties of receptors in basal conditions, and following interaction with agonists and/or antagonists of each of the protomers. GFP-tagged receptors and confocal microscopy will be used in this frame. The consequences of dimerization on the efficiency of HIV entry will be used for heterodimers involving CCR5 and CXCR4, as this may influence greatly the antiviral therapeutic approaches targeting these receptors.

Partner 1 will use its large collection of CCR5 mutants and chimeras, constructed over the last ten years, and will design additional and specific mutants in order to determine the dimer interface, as well as the potential role of G protein-coupling in the allosteric interaction between protomers. Indeed, a number of mutants with altered functional properties have been identified, and will be tested in BRET, binding and functional assays.

Finally, we will explore complementary avenues of testing GPCR dimerization. One is to restrict the analysis of dimers to receptors that have reached the cell surface, by labeling them with monoclonals and measure their interaction by FRET. This method has been set up for glycoprotein hormone receptors, and will be applied to chemokine receptors as well. A second avenue will be to design an expression system in which only heterodimers are transported to the cell surface. This would allow investigating the binding and functional properties of heterodimers in the absence of homodimers. Such obligate heterodimers exist naturally for GABA_B receptors (Pin et al. 2004). In this case, an ER retention signal of one subunit is masked by the C-terminal domain of the other, allowing the heterodimer to traffic through the Golgi. Attempts to apply these elements to class A GPCRs have failed so far, and this failure is attributed to the relative positioning of the two interacting domains. Other constructs will be designed using the GABA_B structural elements, but we will also consider ER-retention signals and masking domains from other proteins, such as cytokine receptor subunits (Michelsen et al. 2005).

2.3. ChemR23 and other receptors (P1)

The same strategies will be applied to other receptors of interest, selected in the course of the program. ChemR23 will be studied in this frame, as it is expressed together with a number of chemokine receptors in dendritic cells and macrophages, many of which are involved in similar mechanisms (chemotaxis, activation). We will first test the ability of ChemR23 to homodimerize, and will then investigate whether it can heterodimerize with co-expressed chemokine receptors such as CCR5 or CXCR4. The functional consequences of heterodimerization will then be studied if appropriate.

2.4. Sugar sensing receptor in yeast (P3, P1)

Preliminary results have been obtained indicating that the yeast sucrose/glucose-sensing receptor Gpr1 also dimerizes. This has been accomplished using BRET with Gpr1-luciferase and Gpr1-YFP fusion proteins. Partner P3 will investigate whether dimerization of Gpr1 is induced by the agonists sucrose and glucose and the antagonist mannose. Several mutant alleles of Gpr1 are available that are either fully functional, deficient in glucose- and sucrose-induced signaling or specifically in glucose-induced signaling (Lemaire et al. 2004). Partner 3 will check whether these mutant alleles are deficient in receptor dimerization and whether mutant alleles can be found that are deficient in dimerization but not in signaling or vice-versa.

WP3. SIGNALING CASCADES ACTIVATED BY GPCRS

Besides the classical cascades activated by GPCRs, a number of G protein-independent signaling pathways have been delineated over the recent years. In addition, it became increasingly evident that the same receptor can stimulate different intracellular cascades according to the agonist which is used for stimulation. These properties are linked to the hypothesis following which different active states of a receptor may exist. An additional dimension to this complexity is due to the processing of ligands by proteases, particularly for chemokines, which can affect their receptor specificity, or the functional consequences of the interaction with their receptors. Some of these aspects will be studied for yeast, insect and mammalian receptors.

3.1. Spectrum of GPCRs recognized by post-translationally-modified chemokines (P4)

To alter the inflammatory response, chemokines are enzymatically modified (e.g. by NH₂-terminal truncation) yielding increased or decreased biological activity. This phenomenon is explained by changes in receptor recognition by the modified chemokine. Previously unrecognized posttranslational modifications on human chemokines have been partially identified and are under investigation. The impact of the modifications on receptor binding and signaling will be studied in detail by partner P4. Chemokine isoforms with retained binding capacity but impaired signaling potential may function as chemokine antagonist (Mc Quibban et al. 2000).

In particular, we will focus on enzymes (e.g. aminopeptidases) that minimally modify chemokines, but cause drastic effects on their biological activities. For example, a number of naturally occurring NH₂-terminally processed forms (missing 2, 3 or 4 residues) of IP-10/CXCL10 have previously been identified for which nor the protease(s) involved, nor the effect(s) on chemokine activities have been investigated (Proost et al. 1993). Previously, the effect of the serine protease dipeptidylpeptidase IV (DPPIV)/CD26 on IP-10/CXCL10 (cleavage of the 2 NH₂-terminal residues) has been studied in detail, on both leukocyte migration and angiogenesis (Proost et al., 2001). Indeed, NH₂-terminal processing of chemokines does not necessarily imply similar consequences for both activities, since the effect on leukocyte migration is directly linked to GPCR signaling (Murphy, 2002), whereas other mechanisms (e.g. glycosaminoglycan binding) are probably also crucial in angiogenesis (Johnson et al. 2005; Strieter et al. 2005). More specifically, we will isolate novel chemokine isoforms from in vitro cultured fibroblasts, endothelial cells and/or leukocytes stimulated with inflammatory mediators, such as cytokines and cytokine inducers (e.g. Toll like receptor ligands) (Van Damme et al. 1994; Proost et al. 1998a). After purification, chemokine isoforms will be structurally identified by Edman degradation and mass spectrometry.

In order to obtain pure protein of each isoform, synthesis of chemokine isoforms (starting from the COOH terminus) should provide sufficient protein of each form to perform a detailed analysis both in vitro and in vivo. This includes receptor binding (using chemokine receptor transfected cell lines provided by partner P1), receptor mediated signaling (e.g. intracellular calcium mobilization) and migration of various cell types, including freshly isolated leukocytes (e.g. monocytes, lymphocytes, granulocytes and dendritic cells) and cultured endothelial cells. Furthermore, it will be verified whether truncated chemokine isoforms show an altered behaviour in vivo, for instance in the recruitment of leukocytes (e.g. after intraperitoneal injection), and in the stimulation or inhibition of angiogenesis (e.g. cornea assay). Indeed, as shown for some proteolytically truncated chemokines (Proost et al., 1998a and 1998b; Struyf et al., 1999), it is predictable that also other processed chemokines may function as natural chemokine antagonists, both in vitro and in vivo.

Finally, it will be evidenced using a proteomic approach that these chemokine isoforms exist under pathological conditions (vide infra) and ultimately it will be verified whether their appearance may serve as a clinical parameter for inflammatory diseases (e.g. arthritis) or cancer (e.g. ovarian carcinoma) (Schutyser et al., 2002; Proost et al., 2003 and 2004).

3.2. Synergy between chemokines and enhancement of the inflammatory response (P4)

Regakine-1 was first found to synergize with other GPCR ligands including complement factors (C5a), bacterial peptides (fMLP) and other chemokines (IL-8/CXCL8) in the chemoattraction of neutrophilic granulocytes (Struyf et al., 2001; Gouwy et al., 2004). Although this phenomenon could be extended to other chemokines, the cellular mechanisms implicated are not understood. Although ligand or receptor dimerization could be implicated, it is rather expected that individual chemokines bind to their proper receptor and subsequently cause synergy at the level of signal transduction. Chemokine synergy will therefore be investigated in depth using various target cells, GPCR ligands and signal transduction pathways.

With the use of different in vitro test systems (e.g. chemotaxis, shape change, degranulation) on various leukocyte subtypes (including monocytes, lymphocytes, granulocytes and dendritic cells) to analyze synergy between GPCR ligands, multiple possible combinations between constitutive and inflammatory chemokines of different subgroups (CXC and CC) or between chemokines and other GPCR ligands will be investigated.

Relevant GPCR ligand combinations will subsequently be tested *in vivo* (mice or rabbits) by local intradermal or intraperitoneal co-injection (Struyf et al., 2005) or by a combination of local and systemic injections. In the latter case, chemokines which are constitutively present in the circulation and are recruiting leukocytes from the bone marrow to blood could cooperate with locally induced inflammatory chemokines, which subsequently attract these leukocytes to the inflammatory site.

The involvement of different GPCRs in chemokine synergy will be investigated in binding and signaling assays using cell lines that are singly or doubly transfected (Partner P1) with the specific receptors of the synergizing chemokines. Alternatively, selective blockage of one receptor with a specific antagonist should also provide evidence of individual GPCR involvement in the synergy phenomenon. If such cooperation between GPCR ligands is mediated through separate receptors, it will be investigated which signal transduction pathways lead to synergy. Since intracellular calcium concentrations do not seem to be affected during synergistic chemokine interactions, we plan to study other signal transduction pathways, such as the activation of p44/p42 MAP-kinase and the phosphatidylinositol 3-kinase pathway. In addition, it will be investigated whether chemokine binding to glycosaminoglycans is implicated in the observed synergistic interactions. Furthermore, the new confocal microscope of Partner 4 will allow to study signaling at the single cell level.

Finally, it will be questioned whether synergy or antagonism between chemokines can occur on cells other than leukocytes, such as endothelial cells. Therefore angiostatic chemokines (PF-4/CXCL4, IP-10/CXCL10) will be tested in combination, to possibly cooperatively block angiogenic chemokines (GCP-2/CXCL6, IL-8/CXCL8, SDF-1 α /CXCL12).

3.3. Signaling pathways regulated by ChemR23 in dendritic cells (P1)

The signaling pathways leading to leukocyte chemotaxis are incompletely understood, and it is well established that different receptors regulate differentially intracellular cascades and gene expression profiles. We will investigate the signaling cascades activated by ChemR23 in dendritic cells, and will compare this profile to that activated by chemokine receptors such as CCR5 or CXCR4. ChemR23 is coupled to G_{ai}, inhibition of adenylyl cyclase and stimulation of phospholipase C β . We will investigate the transcriptional program stimulated by the activation of ChemR23 in dendritic cells, using DNA microarrays. The genes found to be regulated following ChemR23 activation will be placed within known transduction cascades, by using specific inhibitors of intracellular pathways, and we will test how this profile differs from that regulated by RANTES or SDF-1. As a complement to DNA microarrays, we will analyze signal transduction cascades by two complementary strategies, 2D-gel electrophoresis (2D-DIGE) and multi-dimensional diagonal HPLC, followed by mass spectrometric identification. In both cases, the proteins are separated on the basis of their physico-chemical properties: molecular weight, charge, hydrophobicity (in some cases after specific protein modification by using chemical agents). 2D-DIGE analysis of the lysed cells stimulated by the ligand at various times will give an overview of the major changes occurring in the proteome, including the post-translational modifications such as phosphorylation (Celis et al. 2000, Gromov et al. 2002), while multi-dimensional HPLC will permit to analyse the proteic patterns in a more quantitative way, taking into account low-abundant proteins (Gevaert et al. 2002, 2003).

3.4. Downstream signalling effects of insect GPCRs (P2)

In addition to cellular (cf. 6.4) and *in vivo* (cf. 4.6) studies of the initial steps in insect GPCR-mediated processes by analysing second messenger cascades, we will examine downstream signalling effects at the gene expression level in response to agonist stimulation. *Drosophila* genomic sequence information resulted in the availability of cDNA and oligonucleotide microarrays. These microarrays will be used to carry out an exhaustive analysis of gene expression in the fly mutants/transgenes that are produced under 4.6. Particular emphasis will be on gene expression analysis in freshly eclosed flies, in order to analyse genes that are regulated in response to the release of the neurohormone bursicon. More than 40 years after its initial discovery, this hormone, essential for the sclerotisation of the cuticle after each moult and for inducing wing spreading behaviour in the eclosed adults, was molecularly identified via a collaboration between P1 and P2 (Mendive/Van Loy et al., 2005) as a heterodimeric cystine knot protein that activates the *Drosophila* 'Leucine-rich repeats containing G protein-coupled receptor', dLGR2. This receptor belongs to the highly conserved subfamily of LGRs that also contains human receptors for glycoprotein hormones and relaxin-like peptides. To gain insight in the mode of action and in the regulation of this important hormone, P2 is currently analysing bursicon deficient *Drosophila* mutants and also has different mutants that are devoid of functional dLGR2 (rickets) (Baker and Truman, 2002). This will allow for comparisons of gene expression profiles of mutant (homo- and heterozygotes), bursicon-induced and wildtype flies. To confirm the microarray results, real time RT-PCR will be carried out to quantitatively analyse the transcript expression levels of selected genes.

3.5. Glucose-sensing receptor (P3)

The sucrose/glucose sensing receptor Gpr1 triggers activation of cAMP synthesis in yeast through the G_{α} protein Gpa2 (Colombo et al. 1998). This protein is unusual since it does not associate with classical $G_{\beta,\gamma}$ subunits. Two kelch repeat proteins, Krh1 and Krh2, have been suggested to function as alternative G_{β} subunits (Harashima and Heitman 2002), but more recent work has revealed that they act as effectors of Gpa2 in a bypass pathway of adenylate cyclase, directly linking the G_{α} protein to the catalytic subunits of PKA (Lu and Hirsch 2005; Peeters et al. submitted). The Krh proteins stimulate the association between the catalytic and regulatory subunits. We have also shown that the kelch repeat proteins bind and downregulate mammalian PKA catalytic subunits expressed in yeast. We have established a two-hybrid screening system with the regulatory and catalytic subunits of mammalian PKA expressed in a Krh-deficient yeast strain to isolate mammalian cDNA's encoding kelch repeat proteins with a similar regulatory function on mammalian PKA as the yeast Krh proteins.

We have produced the G_{α} protein Gpa2 in pure form and in large quantity in *E. coli*. This material will now be used for crystallization of the protein and 3D-structure determination in collaboration with Remi Lories at the VUB. Gpa2 can also be produced easily in a pure 1:1 complex with the kelch repeat part of the kelch repeat proteins. We will also try to crystallize this complex and determine the 3D-structure.

Gpa2 is controlled by the RGS protein, Rgs2 (Versele et al. 1999). Recent work has revealed that Rgs2 also acts as an effector for sucrose-controlled gene expression, in particular sucrose-induction of invertase. In the present project we will search by two-hybrid screening and yeast protein micro-arrays for downstream signaling components linking Rgs2 to transcription factor(s) responsible for induction of the invertase encoding gene *SUC2*. We have previously shown that specific mammalian RGS proteins can replace yeast Rgs2 for control of the GTPase activity of Gpa2 (Versele et al. 1999). We will investigate whether these and/or other mammalian RGS proteins can also act as effectors of Gpa2 for sucrose induction of invertase.

Gpa2 also associates itself with a putative cysteine zinc finger transcription factor. We have recently shown that deletion of this gene severely affects sucrose induction of pseudohyphal growth revealing yet another output pathway of the GPCR system. We will investigate whether this Gpa2 interacting protein is a genuine transcription factor using band shift experiments. Micro-array analysis will be used to identify target genes and epistasis analysis to determine the precise interaction point of the protein in vivo with the cAMP-PKA and other signaling pathways that control pseudohyphal differentiation.

Activation of the yeast PKA pathway by nitrogen sources and phosphate in nitrogen- and phosphate-starved cells, respectively, is largely dependent on the presence of glucose. In this case, glucose-sensing is carried out by the Gpr1-Gpa2 GPCR system that activates cAMP synthesis (Giots et al. 2003; Van Nuland et al. 2006). We have previously hypothesized that glucose enhances the availability of free catalytic subunits of PKA and that the nitrogen- and phosphate-induced signaling pathways specifically act on the catalytic subunits to enhance their activity further (Thevelein and de Winde 1999). This model would fit with the action of the Krh proteins which only act on PKA in a limited range of cAMP concentrations. In the absence of cAMP, their deletion has no effect and in the presence of very high cAMP, they do not further enhance PKA catalytic activity. Hence, we will explore whether the Krh proteins might represent a crucial node in the integration of glucose-induced cAMP signaling with nitrogen and phosphate signaling, respectively. Since recent results have shown that inactivation of yeast PDK1 (using the temperature-sensitive *Pkh1^{ts}*, *pkh2 Δ* , *pkh3 Δ* strain) also reduces glucose-induced activation of the PKA target trehalase, PDK1 might also serve as an integrator of the signaling by different nutrients. Hence, we will investigate whether inactivation of yeast PDK1 reduces glucose-induced cAMP signaling and whether overactive alleles of PDK1 can suppress the glucose requirement of nitrogen and phosphate signaling.

In a next step, we will replace yeast PDK1 and PKB separately and together by their mammalian homologues and investigate to what extent they can take over distinct nutrient-induced signaling processes. Mammalian PDK1 and PKB will also be expressed in yeast strains expressing candidate mammalian amino acid, ammonium and phosphate transceptors if the latter are only able to rescue the transport capacity and not the signaling capacity of the corresponding yeast transceptors.

WP4. CHARACTERIZATION OF RECEPTORS IN PHYSIOLOGICAL PROCESSES

A number of specific receptors, among which several were identified by the partners over the previous years, will be studied in details in order to determine their role in physiological processes. This will involve both in vitro studies, as well as in vivo models of genetically modified organisms. We will study among others, chemoattractant receptors and a set of neuromodulatory receptors in human and mouse, neuropeptide receptors in insects, and the glucose/sucrose sensing GPCR system in yeast and *Candida albicans*.

4.1. Human and mouse ChemR23 (P1, P4, P5)

Chemerin was identified by partner P1 as the natural ligand of ChemR23 over the previous IAP program. Partner P1 will, in collaboration with partners P4 and P5, pursue the characterization of this new

chemoattractant system acting on myeloid and plasmacytoid dendritic cells. The following aspects will be considered:

Design of synthetic peptide ligands. Partner P1 has determined that a synthetic nonapeptide corresponding to the C-terminal end of mature chemerin is able to activate the receptor with limited loss of potency as compared to the full-size protein (Wittamer et al. 2004). The synthetic peptides display however a very short half-life in biological media, attributed to proteolytic degradation, limiting their usefulness in long-term *in vitro* assays, as well as *in vivo*. We will therefore pursue our structure-function analysis, with the aim of designing peptide antagonists, as well as agonists and tracers with improved stability. Stable peptides will be a considerable asset in order to investigate the activities of the system in *in vivo* models.

Distribution studies. Monoclonal antibodies directed against human ChemR23 and human chemerin have been developed (Wittamer et al. 2003, Vermi et al. 2005). Rat monoclonals against mouse ChemR23 and anti-mouse chemerin polyclonal antibodies were also generated. A broader range of antibodies will however be necessary to discriminate the various forms of chemerin (prochemerin, the active chemerin forms, and the inactive shorter forms resulting from proteolytic inactivation). The distribution of chemerin and its receptor, as well as the regulation of expression will be studied by different approaches, using RT-PCR, *in situ* hybridization, FACS, Elisa and immunohistochemistry. This study has been initiated for human lymphoid organs, but will be extended to a broader range of human and mouse tissues, in order to understand better in which physiological and pathological situations this system is involved. Primary and secondary lymphoid organs will be analyzed in human and mice (thymus, bone marrow, lymph nodes, amygdala, Peyer's patches and spleen) as well as selected tissues, such as skin, gut and lung, constituting the main entry ports of pathogenic agents.

(Pro)chemerin processing. Partner P1 has identified neutrophil cathepsin G and elastase as two proteases able to activate prochemerin, generating two chemerin forms differing by a single amino acid at the C-terminus (Wittamer et al. 2005). However, we have shown that cell types (including tumor cell lines) that do not express cathepsin G and elastase are also able to generate active chemerin, while other proteolytic activities can inactivate the ChemR23 ligands. We will therefore pursue the analysis of proteases responsible for the processing of pro(chemerin). As for the identification of the neutrophil proteases, we will identify proteolytic activities on the basis of a bioassay (activation of prochemerin or inactivation of chemerin), will determine the nature of the processing by mass spectrometry analysis, and will characterize the protease by using broad range or specific inhibitors, blocking antibodies and recombinant proteases. These studies will allow to determine in which physiological and pathological processes chemerin is generated from its precursor, and will complement therefore the analysis of the expression pattern and regulation, and the immunohistochemical studies.

Biological actions in vitro and in vivo. Chemokines and other immune mediators do interact strongly with extracellular matrix components, such as glycosaminoglycans (GAG), and these interactions play a major role in their bioactivity *in vivo*. For chemokines, GAG binding is indeed necessary for the formation of stable gradients across tissues, and it has been demonstrated that chemokine variants unable to bind GAGs do not recruit leukocytes *in vivo* (while their chemotactic properties are conserved in *in vitro* settings) (Proudfoot et al. 2003). We will therefore evaluate whether chemerin interacts with different classes of GAGs (heparan sulfate, chondroitine sulfate, keratan sulfate) using methods used routinely for chemokines (chromatography using NaCl gradients, kinetics of interaction using the Biacore technology).

We will also investigate, as a complement to our *in vivo* assays described below, the other potential biological roles of the cystatin-like domain of chemerin. We will test whether this domain can inhibit cysteine-proteases (cathepsin B, cathepsin L, autophagins, caspases), or is able to recruit and/or activate by itself leukocyte populations, which would suggest the existence of another receptor for this domain. In addition, we will test this truncated protein on our collection of cell lines expressing orphan receptors.

Knock out models. The technology for generating and analyzing knock out mice is well established in the P1 group (Ledent et al. 1997, 1999, 2005, among others). We have recently obtained mice invalidated for ChemR23. These mice have been bred on a C57Bl6 background. As it is not clear yet whether the activation of ChemR23 is the only function of chemerin, we have also initiated the knock out of the ligand gene. The targeting vector for the prochemerin has been constructed, and this construct will be used to generate knock out animals if considered useful. This would allow to compare the phenotypes obtained for the receptor and ligand knockouts. The phenotype resulting from the functional loss of ChemR23 will be analyzed by different approaches. No obvious defect has been detected so far in pathogen-free situations. More subtle morphological abnormalities will be searched for, particularly in the lymphoid organs, by testing the distribution and relative abundance of the various classes of leukocytes. These animals will also be tested in disease models as described in section 5.3.

4.2 Human and mouse FPRL2 (P1, P4)

Structure-function of F2L. The F2L peptide is highly hydrophobic and extremely tedious to synthesize, purify and use in binding and functional assays. We will therefore aim at the identification of a F2L variant that would keep its biological activity, while displaying more favourable physico-chemical properties. For this purpose, we will first test N- and C-terminal deletions in order to determine the shortest peptide with conserved high affinity for FPRL2. Second, an ala-scan will be run to identify the key residues involved in FPRL2 recognition and activation. Finally, other modifications, such as internal deletions, substitutions of aromatic residues by polar or charged amino acids, will be introduced in an iterative way, up to the characterization of a peptide easier to synthesize and usable as a pharmacological tool.

Characterization of the mouse functional ortholog of FPRL2. The mouse genome encodes 8 receptors within the FPR family (Gao et al. 1998), while only 3 receptors are present in human. It is therefore not obvious to determine the functional ortholog(s) of FPRL2, as this gene family has been subjected to recent amplification and rapid evolution after the mammalian radiation. We have cloned the various mouse genes, and these are being expressed in CHO-K1 cell lines in order to test their functional response to F2L, but also to other peptides described as high affinity agonists of FPRL1 or FPRL2 (humanin, SHAAG peptide, synthetic W hexapeptides, ...), using the aequorin-based assay. Binding assays will then be performed on the receptors of interest, using the appropriate tracers, as well as complementary functional assays allowing to characterize the natural transduction cascades of the receptor. This analysis will allow to determine whether one or several functional orthologs of FPRL2 exist in the mouse, opening the way to the development of mouse models.

Distribution of cells expressing FPRL2 and HBP. Monoclonal antibodies against human FPRL2 have been generated, and used in FACS analysis. These antibodies need to be validated for immunohistochemistry. We will also generate monoclonal or polyclonal antibodies against human F2L and its precursor HBP. These tools will be used to determine the expression of the receptor and ligands in human normal tissues and cell types, as well as in pathological situations, in order to determine in which situations the F2L-FPRL2 system is recruited. Particularly, we will investigate by Western blotting, whether HBP is proteolyzed into the F2L peptide or other intermediates, in normal or pathological situations.

Generation of F2L from HBP. We will investigate how F2L is generated from intracellular HBP, starting from the hypothesis that the peptide might be released following the processes of necrosis or apoptosis. This hypothesis will be tested by using tumoral cell lines in culture, expressing HBP naturally, or overexpressing recombinant HBP, and subjected to different protocols stimulating necrosis or apoptosis (staurosporine, FAS-L, hypoxia, hyperthermia). A biological activity on FPRL2 will be searched for, using our aequorin-based assay, following partial purification by HPLC, and the HBP processing will also be analyzed by Western blotting. Once HBP processing has been detected in a specific system, we will analyze the mechanisms involved, by inhibiting protease classes, interfering with intracellular cascades, or expressing modified HBP genes (truncations and point mutations in putative cleavage sites or motifs possibly involved in non classical secretory pathways).

Animal models. If a functional receptor for F2L can be identified in mouse, we will consider the construction of animal models in this animal, as described in details for ChemR23. Knock-out models for some of the candidate mouse receptors are already available in labs with which we collaborate, and we might therefore have rapid access to these if necessary. Such models will also require the generation of antibodies directed against the mouse receptor and mouse HBP. It is likely that antibodies against human F2L will recognize the mouse peptide, given the strong cross-species conservation. Considering that the FPR-related receptor system is very different in human and mouse (3 versus 8 genes), it is however likely that mouse models will not constitute the ideal system to investigate the contribution of FPRL2 in human diseases.

4.3. Glucocorticoid-induced receptor (P1, P5)

The glucocorticoid-induced receptor (GIR/GPR83) was described as a receptor overexpressed in T lymphocytes following glucocorticoid and/or forskolin treatment (Harrigan et al. 2001). Partner 1 has studied the distribution of GIR transcripts in human and mouse brain. Its relatively broad expression, particularly in striatum, nucleus accumbens and the olfactory tubercle, similar in both species, suggested its involvement in the control of emotions and of neuroendocrine, cognitive and motor functions (Pesini et al. 1998, Brezillon et al. 2001). A knock out model for the GIR/GPR83 receptor was generated and tested in a number of experimental settings. The animals are viable and breed normally. They display a moderate obesity and are hypotensive. In an open field test, they display an hyperactive and anxious phenotype. This hyperactive phenotype might be related with decreased striatal levels of proenkephalin transcripts in the knock out mice. This hyperactivity is reduced by muscarinic agonists and dopaminergic antagonists, suggesting an imbalance between these two systems in the motor control of knock out mice (Laurent et al. unpublished observation). We will explore the dopaminergic and cholinergic systems in the striatum and complement the behavioural characterisation of this knock out model. A modification of leukocyte populations was also observed in blood and lymphoid organs, in terms of relative abundance of lymphocyte populations in a conventional animal

facility, but not under SPF conditions. The animals also displayed a much stronger response in terms of secreted cytokines (TNF α), following an inflammatory challenge (LPS injection). This latter phenotype is similar to that observed for the GPR10 knock out model described below (section 5.4). We are presently investigating whether the hypothalamus-pituitary-adrenal axis is also involved in this process, and the phenotype of this model will be studied further in different directions. In parallel, the natural ligand will be searched for in extracts in the frame of **WP6**. These mice will also be studied further in animal inflammatory and infectious disease models as described for GPR10 in **WP5**.

4.4. GPR3 and other knock out models (P1)

A knock out model for the orphan receptor GPR3 was established by partner P1. Besides other aspects that are presently being studied, an important role of this receptor was identified in oocyte maturation. After becoming competent for resuming meiosis, fully developed mammalian oocytes are maintained arrested in prophase I until ovulation is triggered by the luteotropin surge. Meiotic pause has been shown to depend critically on maintenance of cAMP level in the oocyte and was recently attributed to the constitutive G_s signaling activity of GPR3 (Mehlmann et al. 2004). Partner 1 has now shown that mice deficient for GPR3 are unexpectedly fertile but display progressive reduction in litter size despite stable age-independent alteration of meiotic pause. Detailed analysis of the phenotype confirms premature resumption of meiosis, in vivo, in about one-third of antral follicles from GPR3^{-/-} females, independently of their age. In contrast, in aging mice, absence of GPR3 leads to severe reduction of fertility, which manifests by production of an increasing number of nondeveloping early embryos upon spontaneous ovulation and massive amounts of fragmented oocytes after superovulation. Severe worsening of the phenotype in older animals points to an additional role of GPR3 related to protection (or rescue) of oocytes from aging. GPR3-defective mice may therefore constitute a relevant model of premature ovarian failure due to early oocyte aging (Ledent et al. 2005). We will study further this model, trying to understand how GPR3 acts in this system, and whether its action is mediated exclusively by its constitutive activity or by the action of an endogenous agonist or inverse agonist. In parallel, as this receptor is also widely expressed in the central nervous system, with a distribution suggesting a role in the control of behavioral responses, this model will be studied in a large range of behavioral settings.

We will also pursue the analysis of knock out models for the adenosine A2a receptor (Ledent et al. 1997) and cannabinoid CB1 receptor (Ledent et al. 1999), essentially through collaborations with groups specialized with specific aspects of these receptors' physiology.

4.5. Purinergic receptors and ATP target genes in human dendritic cells (P1)

Partner 1 and others have demonstrated that extracellular ATP affects the maturation of human monocyte-derived dendritic cells (MoDCs), mainly by inhibiting Th1 cytokines, promoting Th2 cytokines, and modulating the expression of costimulatory molecules (Wilkin et al. 2001, La Sala et al. 2001). ATP expression profile in human MoDCs revealed an extensive number of target genes involved in immunosuppression, maturation and chemotaxis (Marteau et al. 2005). More particularly, ATP elicited a drastic up-regulation of two mediators involved in immunosuppression: thrombospondin-1 and indoleamine 2,3-dioxygenase (Marteau et al. 2005). Extracellular ATP released from damaged cells and previously considered as danger signal is thus a potent regulator of mediators playing key roles in immune tolerance (Marteau et al. 2005, Duhant et al. 2002). Partner P1 will pursue this work, and analyse the features of DCs isolated from thrombospondin-1 knock-out mice (maturation state, capacity to activate T lymphocytes) using FACS analysis and mixed lymphocyte reactions. Following the observation that ATP and other adenine nucleotides are able to down-regulate several chemokines and chemokine receptor genes, particularly major monocyte and lymphocyte recruiters (Horckmans et al. 2006), we will also investigate how nucleotides affect the capacity of MoDCs to recruit leukocytes using chemotaxis assays. Pharmacological data support the involvement of the P2Y11 receptor (Communi et al. 1999) in the regulation of these ATP target genes in human MoDCs. Consequently nucleotides derivatives may be considered as useful tools for DC-based immunotherapies. Several other promising genes regulated by ATP in human dendritic cells have been identified on our arrays and the role of these genes in DC physiology will be studied.

4.6. Functional and molecular genetic analysis of insect G protein-coupled receptors (P2)

While the number of orphanized insect GPCRs has rapidly increased in recent years (Claeys et al., 2005), detailed information concerning their in vivo expression and their physiological role(s) is still lacking for most of them. Therefore, the first objective in this workpackage will be to analyse receptor (gene) expression in insect tissues at different developmental stages. This will be done by real-time quantitative RT-PCR. To obtain more detailed information about the exact localisation and cell type specific expression, in situ hybridization experiments will be performed with these tissues or developmental stages in which expression was detected. For small tissues, or even complete *Drosophila* larvae, this can be done on whole mount preparations. The in vivo expression data may result in hypotheses about (a) possible function(s). The

second objective will therefore be to explore the *in vivo* role of the receptor-mediated pathway(s) by means of physiological assays and/or genetic experiments.

The fruit fly, *Drosophila melanogaster*, is particularly well suited for the latter type of experiments because of the availability of a plethora of (molecular) genetic tools and a continuously growing number of mutant fly strains. As a result, some receptor (or ligand) defective mutants are available from mutant fly repositories, and transgenic flies, in which a given GPCR or its signalling pathway is affected, can be generated. For instance, the UAS/GAL4 system can be employed to induce an *in vivo* knock down (via RNA interference), and miss- or over-expression of GPCRs. GAL4 is a yeast transcriptional activator that initiates gene expression via interaction with UAS ('upstream activating sequence'). Stable fly strains (GAL4 drivers) that have GAL4 expression under the control of inducible promoters (e.g. heat shock) are available in the lab of P2. In addition, as a result of enhancer trapping, a lot of transgenic flies exist in which GAL4 expression is driven by tissue- or stage-specific enhancers/promoters (available from the Bloomington *Drosophila* Stock Center). Crosses between specific GAL4 driver strains and UAS target strains can induce expression in well-defined developmental stages or tissues. RNA interference (RNAi) is an elegant method to induce an *in vivo* down regulation of specific transcripts. *Drosophila* embryo's are transformed with a P-element based RNAi vector (pWIZ) in which receptor gene specific inverted repeats, separated by a small intron, can be placed under control of UAS (Lee and Carthew, 2003). When crossed with GAL4 driver fly strains, a double-stranded RNA, causing a specific post-transcriptional gene silencing (via a specific degradation of the corresponding mRNA), is expressed. The methodology for miss- and over-expression of GPCRs is quite similar. However, in this case GPCR-encoding sequences will first be cloned in pUAST (Brand and Perrimon, 1993). Afterwards, crossing with specific GAL4 drivers can lead to miss- and/or over-expression of the receptors. Moreover, the GAL4/UAS system constitutes a very versatile toolbox for integrative physiology in *Drosophila*. For instance, it can also be employed for cell-specific *in vivo* manipulations of second messenger systems and GPCR signalling pathways (Kerr et al., 2004).

After having controlled receptor gene (product) invalidation (mutants) and/or expression (transgenes), the resulting phenotype(s) will be studied in detail. Several recent studies have shown that the analysis of fruit fly mutants can indeed provide important indications about the *in vivo* role of GPCRs (Ishimoto et al., 2000; Kutsukake et al., 2000; Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Bainton et al., 2005; Schwabe et al., 2005). P2 applies this technology to a selection of recently characterized receptors in the fruit fly.

In addition to genetic studies, P2 also employs a wide series of *in vivo* and *in vitro* assays mainly based on larger insect species, such as flesh flies, cockroaches and locusts, which are well suited as physiological research models. These insects can be more easily treated with receptor ligands to study their biological activity. In this context, P2 investigates the role of receptors and their endogenous agonists in the regulation of a variety of important processes, such as cuticle tanning, vitellogenesis and reproduction, pupariation, visceral muscle motility, circadian rhythm, cellular immunity, enzyme activities, phase polyphenism (cf. 6.4.2), etc ... (Mendive et al., 2005; Simonet et al., 2004; Verleyen et al., 2004; Schoofs et al., 1993; Mertens et al., 2005; Franssens et al., 2005; Macours et al., 2004; Borovsky et al., 1996; Hoste et al., 2002; Claeys et al., 2006). Expression analysis, genetic studies and physiology are complementary approaches that may all contribute to the quest for the *in vivo* role of GPCRs and their corresponding ligands.

4.7. Glucose-sensing receptor in yeast and *Candida albicans* (P3)

The sugar sensing GPCR, Gpr1, is a high-affinity sucrose, low-affinity glucose sensor (Lemaire et al. 2004). In the present project, partner P3 will further explore the physiological role of high-affinity sucrose sensing by Gpr1. This group has discovered that low sucrose triggers pseudohyphal growth even in rich media, suggesting that morphogenesis is a positive response to an elicitor rather than the generally held belief that it is a negative response to starvation. Partner P3 will determine sucrose induction of transcriptional targets of the pseudohyphal growth response, such as *FLO11*, by Real-Time PCR. We will also investigate to what extent previously discovered components required for filamentous growth, such as the Mep2 protein suggested as nitrogen starvation sensor (Lorenz and Heitman 1998), are required for low sucrose induction of pseudohyphal growth.

Rapid activation of the yeast PKA pathway can be triggered in respiring cells by addition of the rapidly-fermented sugars glucose and sucrose (Lemaire et al. 2004). In nitrogen-starved cells the same effects on the PKA pathway can be triggered by nitrogen sources like amino acids and ammonium, and in phosphate-starved cells by addition of phosphate (Hirimburegama et al. 1992). In both cases the activation is largely dependent on the presence of glucose and this group shown that the glucose is detected by the same Gpr1-Gpa2 GPCR system that controls cAMP synthesis (Giots et al. 2001; Van Nuland et al. 2006). Activation by nitrogen sources and phosphate is not mediated by cAMP but it requires PKA. Partner P3 has shown that these nutrients are detected by transporters which apparently also act as nutrient receptors ('transceptors') for

activation of the PKA pathway (Holsbeeks et al. 2004). Amino acid activation is mediated by the amino acid transporter Gap1 (Donaton et al. 2003), ammonium activation by Mep2 (Van Nuland et al. 2006) and phosphate activation by Pho84 (Giots et al. 2003).

In the present project partner P3 will explore how these transceptor systems trigger activation of the PKA pathway in concert with the sugar-sensing GPCR system. The group has already shown that activation by nitrogen sources (both amino acids and ammonium), but not by phosphate requires Sch9, the yeast PKB homologue (Thevelein and de Winde 1999; Van Nuland et al. 2006). Recent work in the group has shown that activation by nitrogen sources also requires the yeast PDK1 homologues, Pkh1, Pkh2 and Pkh3. In addition, we have recently shown that inactivation (conversion into alanine) or constitutive activation (conversion into aspartate) of the PDK2 site in yeast PKB abolishes or constitutively activates signaling, respectively. Alteration of the PDK1 site always abolishes signaling. Hence, using custom-ordered phospho-specific antibodies we will test whether nitrogen sources trigger phosphorylation of the PDK1 and/or PDK2 site in yeast PKB. If this is the case, we will check whether it is dependent on yeast PDK1 activity (using the temperature-sensitive Pkh1ts, pkh2 Δ , pkh3 Δ strain) and we will routinely use it as a new read-out for activation of the pathway. Site-directed mutagenesis of the phosphorylated threonine in the activation loop of PKA has revealed that its inactivation partially reduces nitrogen-induced activation of the PKA pathway. Hence, we will test using phosphospecific antibodies whether nitrogen sources trigger phosphorylation of this site and if so, whether it is dependent on yeast PDK1 and PKB.

In recent work we have identified amino acid analogues which are most likely not transported by Gap1 but act as inhibitors of Gap1 transport. Some of these compounds trigger activation of the PKA signaling pathway and remarkably, a specific group of compounds triggers constitutive signaling. We will investigate whether activation by these compounds is dependent on yeast PDK1 and PKB activity and whether they possibly trigger phosphorylation of PKB. Since Gap1 is ubiquitinated, internalized and degraded after addition of amino acids, we will investigate whether nontransported and especially the constitutively-activating agonists trigger ubiquitination and degradation of Gap1. We will screen for similar compounds acting on the ammonium transceptor Mep2. In Gap1, we have identified two residues exposed in the amino acid binding site using SCAM analysis (Substituted Cysteine Accessibility Method). We will investigate whether nontransported and constitutively-activating agonists act through the same amino acid binding site. SCAM analysis will also be applied to the Mep2 transceptor and similar studies conducted as for Gap1. We have identified several competitive inhibitors of transport by Gap1 which do not act as agonists of the signaling pathway. This demonstrates that mere binding of the nutrient ligand to the transceptor is not sufficient for signaling and that apparently the ligand has to trigger a specific conformational change. We will use radioactively labeled forms of such compounds to definitely prove that they are not transported and we will search similar compounds for the Mep2 ammonium transceptor.

For phosphate signaling we have identified glycerol-3-phosphate as a nontransported agonist of the signaling function of the Pho84 transceptor. We will use SCAM analysis to identify amino acid residues in the phosphate-binding site and test whether glycerol-3-phosphate acts through the same site. Pho84 is phosphorylated, ubiquitinated and internalized after addition of phosphate (collaborative work with B. Persson, Kalmar, Sweden) and we will test whether glycerol-3-phosphate also triggers these processes. We will evaluate whether this downregulation of Pho84 is part of a feedback-inhibition system triggered by PKA, similar to the well-known feedback-inhibition mechanisms acting on many GPCR's.

When the promising results recently obtained for dimerization of the Gpr1 receptor with BRET can be confirmed, we will extend the BRET analyses to other aspects of sugar signaling by the GPCR system, such as real-time analysis of Gpr1-Gpa2 interaction, Gpa2-Krh interaction and the interactions between the nutrient transceptors and the components situated immediately downstream in the signaling pathway, such as is possibly true for the yeast PDK1 homologues.

In the last part of the project we will express mammalian amino acid, ammonium and phosphate transporters in yeast mutants deficient in Gap1, Mep2 (Mep1 and Mep3) and Pho84 (as well as the other phosphate transporters), respectively. We will test whether they can restore transport and/or signaling. If transport is restored but not signaling, we will express mammalian PDK1 (and mammalian PKB) in the corresponding mutants and check whether this allows recovery of signaling. Both mammalian PDK1 and PKB have been shown to be functional in yeast (Casamayor et al. 1999; Geyskens et al. 2000).

WP5. ROLE OF GPCRS IN HUMAN DISEASES AND ANIMAL MODELS

The functional characterization of receptors initiated in WP4 will be pursued for human receptors of potential clinical relevance in human diseases and animal (essentially mouse) models of human diseases. Experiments dealing with glycoprotein hormone receptors, chemokine receptors, ChemR23, the prolactin-releasing peptide receptor GPR10 are presented below. However, it is expected that other receptors studied in **WP4**, or deriving from the study of orphan receptors (**WP6**), will be selected during the course of this program

for being studied in this frame as well. A genetic approach will be used to identify putative GPCRs involved in specific inflammatory and infectious diseases in mice (section 5.5). A number of animal models that will be applied to the receptors of interest are presented briefly (section 5.6).

5.1. Glycoprotein hormone receptors (P1)

The ovarian hyperstimulation syndrome (OHSS) is a potentially life-threatening complication of pharmacological ovarian stimulation (Aboulghar et al. 2003). Severe forms complicate around 1% of in vitro fertilization (IVF) cycles and are characterised by a massive ovarian enlargement together with a fluid shift into extravascular compartments. Spontaneous forms of OHSS are very rare and always reported during pregnancy (Olatunbosun et al. 1996). During the previous IAP program, partner P1 and another group have identified mutations in the FSH-receptor gene of patients having presented recurrent spontaneous OHSS (Smits et al. 2003, Vasseur et al. 2003, Montanelli et al. 2004). When tested in vitro, the mutant receptors displayed an abnormally high sensitivity to hCG, providing an explanation for their implication in the OHSS development in vivo. Activating mutations of the FSH receptor gene have not been identified in the much more frequent iatrogenic forms of OHSS. However, recent reports suggested that some common single nucleotide polymorphisms (SNPs) of the FSH receptor gene could play a role in ovarian responses to FSH stimulation (Gromoll et al. 2005). Partner P1 performed a retrospective association study of the Asn⁶⁸⁰Ser polymorphism of the FSH receptor gene in a limited cohort of patients who developed iatrogenic OHSS and suggested that the Asn⁶⁸⁰ allele might be a predictor of symptom severity among OHSS patients (Daelemans et al. 2004). We will progressively extend our patient cohort to 500 cases through collaborations with other IVF centres. In parallel, we will extend the study of the SNPs of the FSH receptor gene to its promoter region and untranslated regions (mainly the 3'UTR) of its transcripts. Indeed, it has been recently suggested that SNPs in the FSH receptor promoter region could modulate the expression of the FSHR via changes in transcription factor binding sites and, similarly, polymorphism of target sites for microRNAs must now be considered as realistic parameters in all gene expression studies (Clop et al. 2006). The level of expression of the FSH receptor will be evaluated on granulosa cells collected at the time of the oocyte retrieval during the IVF treatment and associations will be searched with the SNPs of the FSH receptor gene promoter of the patients.

5.2. Chemokine receptors and chemokine variants in cancer and inflammatory diseases (P4, P1)

During the metastatic process, cancer cells interact with endothelial cells and tissues in a way that is similar to the recruitment of white blood cells to inflammatory areas or lymphoid organs (Wang et al. 1998, Coussens and Werb 2002, Payne and Cornelius 2002, Balkwill 2003). It has been demonstrated that chemokines play a role in this process by determining the preferential sites of metastasis according to the specific receptors expressed in tumor cells (Muller et al. 2001, Mashino et al. 2002). Partner P1 has established tools for studying the role of receptors and their ligands in the oncogenesis process. This has been applied so far to selected members of the chemokine receptor family. We have analyzed the expression of receptors in a set of human tumors, and have selected on this basis a few receptors for testing their role in animal models. The two cellular models that have been selected are the B16 melanoma and the Lewis lung carcinoma (LLC) cell lines. In these models, the consequences of the expression of specific receptors or ligands on the tumor phenotype are analyzed. We have expressed the CCR6 receptor in the LLC line, which resulted in a decrease in the frequency of metastases. We will finalize this model and will modulate the expression of the cognate chemokine (LARC) in the tumour.

Using a proteomic approach, partner P4 will continue to study the presence of chemokine variants and posttranslationally modified chemokines in relation to human diseases, in particular infection, autoimmunity and cancer. In the past, we have studied chemokine expression in gastrointestinal disorders (e.g. inflammatory bowel disease, colon cancer), in septic or autoimmune arthritis and leukemia (Gijssbers et al., 2005; Schutyser et al., 2001; Proost et al., 2004; Struyf et al., 2003) with the help of immunotests (e.g. ELISA, immunohistochemistry). Since these assays do not discriminate between intact and processed chemokine forms, the quantitative abundance of a chemokine not necessarily reflects qualitative biological activity. In a first attempt to measure chemokine isoforms and variants in vivo, substantial volumes of body fluids (e.g. ascites) from cancer patients were purified and screened by ELISA to obtain homogenous chemokine preparations to be identified by NH₂-terminal sequence analysis and mass spectrometry (Schutyser et al., 2002). We aim to fine tune this approach by reducing the starting volume and being still able to identify small quantities of chemokine isoforms by proteomics. Although the equipment is available, success depends on technical limits, i.e. the isolation of chemokines at low concentrations without aspecific loss during processing. This could be achieved by miniaturization of the isolation procedure with a multidimensional capillary on-line chromatography system coupled to mass spectrometry. By focusing on chemokines and not the whole proteome we hope to be able to identify new or existing chemokine forms in body fluids and to quantify these in larger series of patients with autoimmune diseases (synovial fluids) or cancer (ascitic fluids). This could provide new insights on the role of chemokines and the enzymes involved in their processing during pathological conditions and will hopefully lead to the use of chemokine isoform profiles as molecular markers

in the diagnosis or treatment of specific diseases. At a later stage, cellular expression of chemokine receptors in tumor biopsies or purified body fluids from patients will be evaluated by immunoassays as proof of evidence that the presence of (a) particular chemokine(s) can be linked with selective leukocyte infiltration, pathological events or clinical parameters.

5.3. Chemerin in inflammation and cancer (P1, P5)

ChemR23 and chemerin In addition to the distribution studies in normal tissues performed in section 4.1, distribution of chemerin and ChemR23 will be studied in a set of human diseases and animal disease models, using the same tools. In addition to Partners P1 and P5), this activity will involve collaborations with groups specialized in specific human pathologies or animal models, including presently Silvano Sozzani and Alberto Mantovani (Milano and Brescia, Italy), Serge Steinfeld and Jacques Devière (respectively Departments of Rheumatology and Gastroenterology, Erasme Hospital, ULB). The pathological situations considered will include inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases (Crohn and ulcerative colitis), hepatitis and pancreatitis, skin diseases (atopic dermatitis and psoriasis), and samples from clinical or experimental graft rejection or reperfusion injury situations. Chemerin Elisa will allow assay the ligand in blood or other biological fluids. Immunohistochemistry and RT-PCR will detect the expression and distribution of ChemR23 and chemerin in these diseased tissues. The mouse models described in section 5.6 will provide the samples for similar analyses in mice. A collection of samples corresponding to major types of human cancers (lung, breast, colon and ovary carcinomas, melanoma) has also been collected through a network of collaborations with clinical groups. The expression of prochemerin, ChemR23 and the proteases known to process prochemerin will be identified in these various tumors, as well as the leukocyte populations expressing ChemR23 present in the tumors.

Animal disease models. The mouse knock out model for ChemR23 (and possibly prochemerin) will be tested in a range of disease models. These models include skin diseases (particularly psoriasis), arthritis, colitis, hepatitis, pancreatitis, and a number of lung inflammatory and infectious diseases. These models are listed in section 5.6. Other models will be considered according to the first observations.

We will also construct a model in which active chemerin (without the need for processing) will be overexpressed in keratinocytes under control of a keratin-14 promoter. This choice is guided by the fact that prochemerin expression has been reported first in the unaffected skin of patients with psoriasis, and that a retinoic acid analog (tazarotene) was able to promote expression of this gene (Nagpal et al. 1997). Plasmacytoid dendritic cells, attracted by chemerin, are also known to play a key role in psoriasis. We will investigate in these mice the histology of the skin, the presence of plasmacytoid and myeloid DC populations expressing ChemR23, as well as other leukocyte populations. Thereafter, we will test these mice in the skin inflammatory paradigms described in section 5.6, in order to determine whether chemerin expression enhances or decreases the inflammatory reaction. This will complement the analyses of the ChemR23 knockout mice using the same models. If appropriate, we will generate additional transgenic models, expressing either chemerin variants (forms inactive on ChemR23), or expressing chemerin in other cell populations.

Analysis of the anti-tumoral properties of the chemerin-ChemR23 system. We have demonstrated that expression of prochemerin in B16 cells results in a strong anti-tumour effect in vivo. Indeed, following the grafting of the cell line to C57Black6 mice, a significant fraction of the mice reject the tumor, while the growth of the tumor in the remaining mice is much slower. This is unusual for the melanoma B16 cell line, which is very aggressive and known to be resistant to anti-tumor immune defence mechanisms. We have also observed that chemerin-expressing tumors display more efficient angiogenesis and a much milder necrotic pattern. We will extend these observations to other tumoral cell lines, determine how chemerin represses tumor growth, and what are the host cells involved in this process.

We will repeat the experiments using a second tumor cell line, the Lewis Lung Carcinoma (LLC) model, which is also compatible with C57Bl6 mice. LLC cell lines expressing mouse prochemerin (or GFP as control) will be generated. Their production of bioactive chemerin will be tested by Elisa, western blotting and bioassay, and their proliferation rate tested (MTT assay). The cell lines will be grafted into mice, and the tumor parameters (rejection, growth, angiogenesis, leukocyte infiltration, metastases) will be monitored. Thereafter, we will test whether the antitumour effect is mediated by ChemR23 or through another mechanism. Indeed, the N-terminal cystatin-like domain of chemerin might have other functions. We will graft the LLC and B16 cell lines expressing or not prochemerin, into wild-type or ChemR23 KO mice, allowing to determine whether the receptor is involved in the anti-tumor properties of chemerin. In addition, we will generate B16 and LLC cell lines expressing C-terminally truncated forms of prochemerin, namely, bioactive chemerin (without the requirement for proteolytic processing) and inactive chemerin (further deletion of two amino-acids). This will allow testing independently the contribution of the cystatin-like domain and the ChemR23-binding peptide onto the anti-tumor properties, the leukocyte infiltrate and angiogenesis. According to the outcome of these

experiments, we will determine whether additional chemerin variants might be generated in order to delineate more precisely the domains responsible for the modification of tumor phenotype.

We will in parallel investigate the mechanisms by which tumor growth is prevented or delayed. It is indeed paradoxical to observe at the same time increased tumor angiogenesis and cell survival, but an overall delay in tumor growth. In order to understand better these mechanisms, we will analyze more precisely the leukocyte infiltrate of the tumors. Preliminary experiments have identified an increase in dendritic cell infiltrates in the tumors expressing prochemerin, in agreement with the established role of chemerin. This infiltrate will be characterized by immunohistochemistry, from early to late stages following tumor graft, using markers of dendritic cells (CD11c, GR-1/Ly-6G), macrophages (F4/80, CD11b), T (CD3, CD4, CD8) and B (B220, CD19) lymphocytes, as well as NK cells (CD161c). The angiogenic process will be monitored over time by using endothelial cell markers (CD31/PECAM-1, Factor VIII). This analysis will allow analyzing whether different classes of leukocytes are recruited successively during the evolution of the tumors.

We will also test the role of chemerin in a model of skin tumors in mice, as an extension of our psoriasis-like skin-inflammatory model. Repeated skin painting with phorbol esters, following an initial skin contact with the mutagen DMBA, results in a chronic inflammatory syndrome that ultimately leads to the development of skin tumors (section 5.6). This model will be applied to wild-type, chemerin-expressing and ChemR23 KO mice, allowing determining the influence of the chemerin-ChemR23 system in chronic inflammation and inflammation-associated tumorigenesis.

5.4. Prolactin-releasing peptide receptor (P1, P5)

Prolactin-releasing peptide (PrRP) was identified as the ligand of the GPR10 receptor (Hinuma et al. 1998), but the biological actions of this system were soon discovered to go well beyond the control of prolactin release (Roland et al. 1999, Sun et al. 2005). Partner P1 has generated GPR10-knockout mice in order to investigate the role of this new neuropeptidergic system *in vivo*. These mice present no major deficit and breed normally. They were tested in a number of basic behavioural tests. Knock out mice displayed higher nociceptive thresholds and stronger stress-induced analgesia than wild-type mice, differences that were suppressed by naloxone treatment. In addition, potentiation of morphine-induced antinociception and reduction of morphine tolerance were observed in mutants. Intracerebroventricular administration of PrRP in wild-type mice promoted hyperalgesia and reversed morphine-induced antinociception. Finally, GPR10 deficiency enhanced the acquisition of morphine-induced conditioned place preference and decreased the severity of naloxone-precipitated morphine withdrawal syndrome. Altogether, our data identified the PrRP-GPR10 system as a new and potent negative modulator of the opioid system (Laurent et al. 2005). The PrRP-GPR10 system has also been identified as a major regulator of stress responses (Mera et al. 2006), what we have also observed in our knock out model (Laurent et al. 2005 and unpublished data). In addition, GPR10 knockout mice were found to be extremely sensitive to an acute hepatitis model (unpublished data). This might in part be related to inadequate responses of the hypothalamus-pituitary-adrenal axis, but the phenotype is not reversed by administration of physiological glucocorticoid doses. We hypothesize therefore that GPR10 is involved in the regulation of inflammatory responses, at least in our hepatitis model.

We will therefore analyse this further by submitting our mouse knock out model to a set of inflammatory and/or infection models of the liver, but also of other organs, particularly the pancreas, the gut and the lung. These models will be run in collaboration with groups that have the expertise of these models (P5 for lung models, J. Devière, ULB for gastrointestinal models). A short description of the proposed models and the parameters analyzed is provided in section 5.6 below. Following this first step, we will select one or a few models in which the phenotype of the GPR10 KO mice is clearly different from wild-type animals, and will determine what pathways are associated to the excessive inflammatory responses.

A number of variants of the GPR10 gene have been described, some alleles being associated with blood pressure (Bhattacharyya et al. 2003). Following further analysis of the phenotype of the mouse knock out models, we will investigate whether GPR10 variants are associated with the development of the corresponding inflammatory diseases in human.

5.5. QTL analysis in animal models (P5)

As the available collection of mouse lines knock-out for specific GPCRs is still far from being complete, the partners intend to make an inventory of the spontaneous allelic variation at GPCRs loci among the inbred mouse strains available. This inventory will permit the identification of inbred lines that differs in the amino acid sequence or level of expression of a specific GPCR. Comparison of the resistance/susceptibility patterns displayed to an experimental disease by cohorts homozygous for different alleles will then allow to determine whether or not the said GPCR behaves like a major gene or a QTL in the context studied. The diseases to be studied will be those mentioned in the paragraph 4.13.1., i.e. asthma, COPD/emphysema, Sendai-virus pneumonia, respiratory syncytial virus pneumonia and influenza A virus pneumonia. In case a significant

effect is indeed detected, the segregation of the GPCR alleles throughout the progeny will be assessed for confirmation. This will be done by generating and phenotyping the disease resistance/susceptibility pattern of ~50 F1s and ~300 F2s. Along with the verification that the said GPCR indeed controls a part of the severity of the disease, the genomic DNA banks will simultaneously be used to map possible coacting QTLs. Whenever an allelic variation at a GPCR locus is found to control the course of a disease in the mouse, the partners would start a screening of the natural allelic polymorphism occurring at the human orthologous locus. In case a significant polymorphism is found in humans, association and transmission disequilibrium studies would be started to ascertain the role of the said GPCR in humans using cohorts of asthmatics, children hospitalized for severe respiratory syncytial bronchitis, etc.

5.6. Description of mouse models (P5, P1)

A short description of the mouse models of human diseases that are presently available is presented below. These models will be tested on GPCR knock-out mouse lines that are presently available or will be generated in the network, in order to detect potential roles of these receptors in the pathophysiology of these diseases.

5.6.1. Respiratory system

Murine models of allergic bronchopulmonary inflammation have proved to be extremely useful for examination of the basic mechanisms of allergic inflammation and the underlying immunologic response (Wills-Karp 2000). Partner P5 will use an established mouse **model of allergic asthma** (ovalbumin-induced) to evaluate the possibility that some of the GPCRs studied in the network play a role in this setting. This model has been well documented, and the key contribution of CD4⁺ T-lymphocytes and Th2 cytokines has been highlighted (Gavett et al., 1994 ; Garlisi et al., 1995 ; Kaminuma et al., 1997 ; Hogan et al., 1998). GPCR knock-out mouse lines will be challenged repeatedly with ovalbumin and the airway behaviour assessed by use of double chamber plethysmography (Flandre et al., 2003).

Chronic obstructive pulmonary disease (COPD) is one of the commonest reasons for ill health worldwide, and the single most important factor in the development of emphysema is cigarette smoke (Calverlet and Bellamy, 2000 ; Shapiro, 2001). Mice are able to tolerate at least two cigarettes per day for a year with non-toxic carboxyhaemoglobin levels and minimal effects on body weight (Shapiro, 2000). Neutrophil recruitment occurs following the first cigarette and is followed by a more gradual accumulation of macrophages. Early neutrophil influx in the lungs is accompanied by a measurable increase in collagen and elastin degradation products. Epithelial changes including loss of cilia are observed after a couple of months of cigarette exposure and Clara cell hyperplasia after about 6 months (Mahadeva and Shapiro, 2002). The alveolar duct area and enlarged alveolar spaces are clearly seen after 3–6 months of exposure to cigarette smoke in susceptible strains and macrophage mediated destruction appears to be prominent at these time points (Shapiro, 2000).

A model of **Paramyxovirus-associated diseases** of the respiratory tract will involve inoculation of Sendai virus, the murine counterpart of human type-1 parainfluenza virus, which, historically, has been used extensively in studies that have defined the basic biologic properties of paramyxoviruses in general (Chanock et al., 2001). The follow-up of the severity of the experimental disease will include daily monitoring of body weight, double-chamber plethysmographic values and carbon monoxide uptake capacity over 7 days after inoculation as previously described (Faisca et al., 2005). In parallel, histological examinations and lung viral titration will be carried out from day 5 to day 7 after inoculation. The resistance/susceptibility pattern of knockout animals will be tested in this model.

A model of **respiratory syncytial virus (RSV) infection** will be tested. The model will use the natural rodent pneumovirus pathogen, which is also the closest phylogenetic relative of human RSV (Compans et al., 1967), pneumonia virus of mice (PVM). The crucial advantages of this PVM-associated model include the following: (i) clinical picture – morbidity – consistently mimicking that observed in infants with RSV-associated bronchiolitis ; (ii) dramatic granulocytic and eosinophilic infiltrations that parallel the pathological changes observed in humans ; (iii) clear evidence of widespread viral replication in lung tissue; and (iv) clear progression to ARDS as reported for ~3% infants with RSV bronchiolitis (Chambers et al., 1990 ; Hall, 2001 ; Ling and Pringle, 1989). The aforementioned array of clinical, functional, virological and histological parameters will be followed here too (Bui Tran Anh et al., 2006).

The strategy proposed for paramyxoviroses and pneumoviroses will be similarly applied to mouse models of **influenza infection** (genus Influenza A, family *Orthomyxoviridae*), using “murinized” viruses, including H5N1 itself.

5.6.2. Gastrointestinal system

Hepatitis models. Three mouse models of hepatitis will be studied according to the receptors studied. In the **concanavalin A (Con A)-induced hepatitis**, Con A administration in mice induces severe hepatitis, which

is considered to be a model mimicking many aspects of human T cell-mediated liver diseases and fulminant hepatic failure (Tiegs et al, 1992). CD4+ T cells, and especially natural killer T cells, play a key role in triggering liver injury; other cells like macrophages, neutrophils and eosinophils are also activated and participate in hepatitis. In the **galactosamine/lipopolysaccharide hepatitis** model, low sublethal dose of lipopolysaccharide given in association with galactosamine (an amino sugar which induces an inhibition of macromolecules synthesis in the liver) leads to fulminant hepatitis and shock. In this experimental model, TNF- α is the major mediator leading to liver injury (Louis et al., 1997). In the **alcoholic liver disease model**, mice are fed a liquid diet adapted from the classical Lieber-DeCarli ethanol diet (Lieber et al., 1975). After 10 days, this feeding leads to a severe steatosis associated with a hepatocellular injury. The administration of bacterial products to ethanol-fed mice induces a liver inflammatory infiltration with the similar histological features of human alcoholic hepatitis (Gustot et al. 2006). In each of these models, the severity of the liver disease is tested by assaying the time-course of blood liver enzymes (lipase, transaminases), cytokines and chemokines, and by histological analysis (necrosis, infiltration, fibrosis).

Experimental model of **acute pancreatitis**. Administration of the cholecystokinin analog caerulein (50 $\mu\text{g}/\text{kg}$, ip) at a supramaximally stimulating dose to mice, results in mild to severe pancreatitis which develops over hours (Demols et al. 2000). The readout includes the intrapancreatic and systemic measurements of chemokines, cytokines and other mediators by ELISA or PCR, and the measurements of markers of pancreatitis severity (serum hydrolases, histological changes).

TNBS-induced **colitis** model. Intrarectal application of trinitrobenzene sulfonic acid (TNBS) in ethanol leads to an inflammatory response in the colon and cecum of susceptible mice, which resembles the histological and immunological abnormalities observed in human Crohn's disease (Franchimont et al., 2000).

5.6.3. Arthritis model

Collagen-induced arthritis (Holmdahl et al. 1986, Kuhn et al. 2006) is promoted by intradermal immunization of DBA/1J male mice with calf collagen type II emulsified with complete Freund's adjuvant on days 0 and 21. From days 25 through 35 after the initial immunization, mice are scored daily for signs of arthritis in the paws, and animals are further tested for histological alteration and infiltration of the joints. This model will require to transfer the invalidated alleles (ChemR23 and possibly other models) on the DBA background, as all our models are presently kept on a C57Bl6 strain.

5.6.4. Skin inflammatory models

A model of **skin inflammation** mimicking some aspects of **psoriasis** will be used. This model consists in skin painting with a phorbol ester (usually 12-O-tetradecanoyl phorbol-13-acetate [TPA], 0.2 mM in 100 μl of acetone), which results over the next few days in leukocyte infiltration, proliferation of epidermis and hyperkeratosis. Repeated treatment of the mice for 20 weeks, associated with a single initial application of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA, 25 μg) results in the development of **spontaneous skin tumors** (Yuspa et al. 1994).

WP6. IDENTIFICATION OF NOVEL RECEPTORS AND THEIR LIGANDS

Many orphan receptors for which the ligands and function are still unknown are encoded by the mammalian, insect and yeast genomes. Several partners will focus on the characterization of these receptors, through the identification of their ligand, and the subsequent delineation of their function. We will focus on human receptors for leukocyte chemoattractants, neuropeptides, glycoprotein hormone-like proteins and glucose, insect receptors for neuropeptides, and nutrient-sensing receptors in yeast, using evolutionary clues in this approach.

6.1. Mammalian chemoattractant and neuropeptide receptors (P1)

6.1.1. Functional screening assays.

The identification of ligands for orphan receptors is based on the use of specific and sensitive functional assays. As no information is usually available on the signalling cascade activated by orphan receptors, a generic functional assay, independent from the activation of a specific cascade, is generally used. Several of these assays have been proposed and used in the past. We have developed a high throughput functional assay based on the luminescence emitted by recombinant aequorin following intracellular calcium release. In this system, a recombinant cell line is developed, that coexpresses an orphan receptor, apoaequorin and a generic coupling protein. Following preincubation of cells with coelenterazine in order to reconstitute active aequorin, luminescence is recorded in a luminometer following addition of potential agonists. This assay has been validated with a number of characterised GPCRs and is now used routinely for orphan receptor screening and functional testing of receptor mutants. Partner P1 has characterized over the years a large number of new receptor's ligands (Libert et al. 1989, 1991, Parmentier et al. 1989, 1992, Maenhaut et al. 1990, 1991, Meunier et al. 1995, Communi et al. 1996, 1997, 1999, 2001, Detheux et al. 2000, Vakili et al.

2001, Brezillon et al. 2001, 2003, Kotani et al. 2001a, b, Le Poul et al. 2003, among others), including the recent characterization of chemerin as the ligand of ChemR23 (Wittamer et al. 2003) and of the F2L peptide as the ligand of FPRL2 (Migeotte et al. 2005), among others. A collection of about 80 recombinant cell lines expressing orphan G protein-coupled receptors has been built on this scheme.

Partner 1 has however identified a number of orphan receptors that express poorly in this system, as illustrated by the low efficiency of clone generation, the frequent rearrangement of the coding sequence generating the synthesis of non functional receptors, or the low FACS signal obtained with monoclonal antibodies. This problem is frequently correlated with the constitutive activity of the receptor, detected following their transient expression, and the measurement of cAMP (G_s -coupled receptors), inositol phosphates (G_q -coupled receptors), or GTP γ S binding (G_i -coupled). The constitutive activity of the receptor appears therefore as a factor that counter-select the cell lines expressing it at high levels. We have also determined that some receptors naturally coupled to G_s do not couple efficiently to $G_{\alpha 16}$. We will therefore develop specific strategies to detect these problems, and design expression systems that allow functional expression of these receptors. This will imply the following approaches:

- All orphan receptors will be tested for constitutive activity following transient expression in Cos-7 cells and assay of each of the three main cascades.
- Receptors suspected to express poorly will be fused to a tag, for which an efficient monoclonal antibody is available, which will allow to monitor surface expression. As the tag itself may modify the expression or the binding of the receptor ligand, an untagged receptor will always be expressed in parallel.
- An inducible expression vector, based on the tet-on technique has been constructed. CHO-K1 cell lines expressing apoaequorin, $G_{\alpha 16}$ and the tet repressor have been established and validated with a number of model receptors. The selected cell line will be used for the expression of the orphan receptors for which constitutive activity and/or expression problems have been encountered. For each receptor, the doxycyclin concentration will be adapted by measuring the constitutive activation of intracellular cascades.
- Receptors demonstrated to couple through the cAMP pathway (constitutive activity), or belonging to structural classes coupled to this pathway will be either screened using another assay (cAMP accumulation), or expressed in systems based on other coupling proteins, such as chimaeric G proteins.

6.1.2. Characterization of natural or surrogate ligands.

The cell lines expressing orphan receptors and adapted to functional assays will be used for the screening of biological activities in a library of fractions prepared from natural sources. Biological extracts are indeed expected to contain the natural ligands of orphan receptors, more particularly the naturally processed forms of peptides and proteins, containing necessary tertiary structures and post-translational modifications. A large number of extracts, fractionated through a first step of HPLC, are presently available. These extracts originate mainly from pig tissues, although a limited number of extracts has been prepared from human tissues, such as placenta, spleen, leukocyte populations or amygdala. We have also prepared extracts from human clinical samples, such as inflammatory and neoplastic ascites and inflammatory exudates from rheumatoid arthritis. Additional libraries will be generated as needed, according to specific expression or regulation patterns of specific orphan receptors.

The extract preparation and purification schemes have primarily been selected to retain peptides and small proteins. However, other extraction and separation procedures have been adapted to focus on other classes of potential ligands, such as bioamines and other small molecules, medium to large size proteins, or lipids.

The cell lines expressing orphan receptors will be tested initially, with a library of potential ligands that includes biologically active peptides, proteins, lipids and other classes of compounds for which receptors have not been identified yet. There are presently over 600 molecules in this library, and this number is expanded progressively. Thereafter, fractions of hemofiltrate, tissue extracts, conditioned media or clinical samples will be tested in a microplate format.

Specific biological activities will be purified using various multidimensional chromatographic procedures (C18 HPLC, ion exchange, isoelectric focusing, gel filtration) in order to obtain a purity level suitable for mass spectrometry and peptide sequencing (MALDI Q-TOF fragmentation analysis) and identify the primary structure of the peptide or protein. Mass spectrometry analysis will also allow to investigate the natural post-translational modifications necessary for high-affinity binding and signal transduction (proteolysis, glycosylation, acetylation, ...). We will clone the cDNAs and genes for the identified peptide ligands and peptide precursors from human and mouse species. Comparison of the precursor sequence from two species is expected to bring important information concerning the conservation of the active peptide in evolution, and the potential functional role of other peptides derived from the precursor. The peptides or proteins will be

chemically synthesised or expressed in bacteria or eukaryotic cells in order to confirm its specific activity. Non peptidic molecules will be analysed by MS and NMR and synthesized for confirming their activity.

6.1.3. Further characterization of deorphanized receptors.

Newly characterized receptors will be further studied by the same approaches described above for ChemR23 and FPRL2, in **WP5** and **WP6**. Among other aspects, the detailed distribution of deorphanized receptors will be studied by in situ hybridisation and immunohistochemistry, with the aim to contribute to the understanding of the receptor functions. Monoclonal antibodies against receptors will be developed, using DNA immunisation. Antibodies will be raised against peptidic ligands. A number of studies will be carried out to characterise the pharmacology and cellular signaling of these systems, as a step toward the function in vivo. Iodinated, tritiated or fluorescent ligands will be synthesised, and the binding parameters of the receptor, ligand, and ligand analogs will be determined. The coupling of the receptors to various transduction cascades will be determined. Finally, the role of the receptors in vivo will be analyzed by generating or obtaining knock out models, and testing them in a number of assays adapted to their distribution and potential functions.

6.2. Chemokine receptors (P4 and P1)

Particular attention will be given to a set of orphan receptors belonging to the chemokine receptor family. Despite the fact that most putative chemokine receptors are structurally identified, a number of these remain orphan (e.g. HCR). Alternatively, for some chemokines (e.g. PARC/CCL18, regakine-1) with biological activity on specific cell types no receptor has been identified. We are currently investigating a new non-allelic chemokine variant (PF-4var/CXCL4L1) of PF-4/CXCL4 (Struyf et al., 2004), for which also no functional receptor has been found yet. We will first identify all possible target cells of PF-4var/CXCL4L1 and investigate which known chemokine receptors are expressed on responsive cells. For the CC chemokine regakine-1 and its variants, the target cells will be better defined and all possible GPCR, stably expressed by Partner P1, will therefore be screened for functional responses.

In order to perform in vivo experiments, the CXC chemokine PF-4var/CXCL4L1 will be cloned and expressed in E.coli. Purified PF-4var/CXCL4L1 protein will be compared with authentic PF-4/CXCL4 for their capacity to attract leukocytes upon intraperitoneal injection into mice and cell influxes will be evaluated by cell counting and by FACS analysis. In addition, the effect of these different PF-4 molecules on tumorigenesis and angiogenesis will be evaluated in mouse tumor models (B16 melanoma, 3F2T hemangio-endothelioma). The angiostatic effect of these PF-4 forms will be analyzed by immunohistochemical staining and microscopic counting of blood vessels in tumor sections and by FACS analysis of cells isolated from tumoral tissue. Further, the metastatic behaviour of the tumors will be investigated after treatment with PF-4/CXCL4 or PF-4var/CXCL4L1.

Although it has been reported that PF-4/CXCL4 can exert its angiostatic effect through binding to CXCR3B (Lasagni et al., 2003), as well as by its affinity for glycosaminoglycans (Luster et al., 1995), the implication of CXCR3B in the antitumoral effect of PF-4/CXCL4 remains controversial. As a consequence, we will chemically synthesize COOH-terminal peptides (expected to bind glycosaminoglycans only, and not to activate a GPCR) of both PF-4 molecules to compare their angiostatic effect in vitro and their antitumoral activity in vivo (e.g. mouse B16 melanoma). Intact PF-4/CXCL4 and PF-4var/CXCL4L1 will be analyzed with regard to glycosaminoglycan and GPCR binding and signaling in parallel with their corresponding COOH-terminal fragments. Both chemokine receptor transfected cell lines (from Partner P1) and naturally responsive cell types (e.g. endothelial cells or specific leukocyte subtypes) will be analyzed as possible targets.

Similarly, the CC chemokine regakine-1 (Struyf et al., 2001) and its variants will be studied at the receptor level to find out which GPCR and hence possible target cell types are responsive. Indeed, it remains intriguing that for the constitutive plasma chemokines PARC/CCL18 and regakine-1, as well as for PF-4/CXCL4, no functional receptors have been identified yet. It is the aim of this project to define the precise action mechanisms of these chemokines. This finding could help at further understanding the role of these chemokines in normal and pathological processes.

6.3. Glucose-sensing receptors (P3)

Following the discovery of the glucose-sensing GPCR in yeast, we have explored whether a similar glucose-sensing GPCR system might be present in specific mammalian cell types. Intestinal epithelial cells are known to induce expression of SGLT1, the sodium-linked glucose transporter, upon glucose arrival in the gut. Investigation of the possible involvement of a glucose-sensing GPCR system has led to the discovery that the taste receptors, including the sweet taste receptor, are also expressed in the intestinal epithelial cells albeit very weakly. The sweet taste receptor might act as glucose sensor for glucose induction of SGLT1 (Dyer et al. 2005). We have identified by DOP-PCR and micro-array analysis the GPCRs that are expressed in isolated intestinal epithelial cells. Subsequently we have performed gut tissue staining with antibodies against a number of GPCRs to identify those that are expressed in the apical part of the plasma membrane of the

intestinal epithelial cells. We have found only one such GPCR but, interestingly, it displays very strong expression and only in the apical part of the plasma membrane. We have expressed this GPCR in *Xenopus* oocytes and preliminary results indicate that it might be a sugar sensing receptor. If this can be confirmed, the work will be extended with a detailed analysis of the sugar specificity of the receptor and the evaluation of candidate antagonistic compounds.

The opportunistic pathogenic yeast *Candida albicans* contains a clear homologue of the *Saccharomyces cerevisiae* sucrose/glucose sensing GPCR Gpr1. However, it remains unclear whether it also responds to sugars (Maidan et al. 2005). Gpr1 is not required for glucose-induced cAMP signaling in *Candida albicans* but it is required for induction of morphogenesis in glucose-containing media. However, induction by specific amino acids, such as methionine, also depends on Gpr1. In the presence of glucose, methionine triggers Gpr1 internalization, suggesting that specific amino acids might act as ligands of Gpr1. We will delete the *MUP* methionine transporter genes in order to investigate whether methionine uptake by these carriers is required for methionine-induced morphogenesis. We will also use strains with a combination of Gpr1 deletion and an overactive allele of the G_{α} protein Gpa2 to determine whether absence of the receptor abolishes glucose and/or methionine-induced morphogenesis.

6.4. Studies on insect orphan GPCRs (P2)

6.4.1. Reverse pharmacology of orphan GPCRs derived from insect genome data

Sequence information on insect GPCRs is rapidly accumulating as a result of recent genome and 'expressed sequence tags' (EST) sequencing programs. P2 will continue studying orphan GPCRs identified from such programs. Reverse pharmacological approaches, based on the use of functional assays (P1; cf. 6.1.1) (e.g. Ca²⁺ detection by aequorin luminescence, CRE-dependent reporter gene expression, arrestin-GFP translocation) on transfected cells expressing the receptor of interest, will be employed to identify the most potent agonist(s) in natural (tissue) extracts and synthetic compound libraries [collections of possible ligands, such as neuronal/endocrine peptides predicted from the genome data (Vanden Broeck, 2001)]. Specific biological activities from natural extracts will be further purified using various chromatographic procedures in order to obtain a purity level suitable for mass spectrometry and/or peptide sequencing. When the biologically active product is a peptide or protein, of which the sequence can be determined either directly or from a comparison to databases, the corresponding cDNA will be analysed. In addition, the tissue distribution of the identified ligands will be explored by quantitative RT-PCR, immunohistochemistry and in situ hybridization (cf. 4.6). For more detailed analyses of biological activities, both in vitro (on receptor-expressing cells) and in vivo (cf. 4.6), we will prepare or purchase substantial amounts of the newly discovered receptor agonists. Long peptides/proteins will be obtained biosynthetically by expressing the corresponding cDNA in a suitable expression system. Shorter peptide ligands will be synthesized by P4 or purchased from companies specialised in chemical synthesis.

6.4.2. Exploring a novel EST database from desert locust CNS

Most of the insect species that recently entered the 'genomics era', such as fruit flies and mosquitoes (Diptera), honeybees (Hymenoptera), silkworms (Lepidoptera) and beetles (Coleoptera), belong to higher insect orders that are phylogenetically very distant from the heterometabolous insects, such as locusts (Orthoptera), which are important research models since decades, in particular for endocrinological and neurobiological research. A disadvantage of many heterometabolous species appears to be their very large genome size. In order to compensate for the absence of locust genome data, we recently initiated an EST sequencing project of cDNAs representing transcripts expressed in the CNS of the desert locust, *Schistocerca gregaria*, which is considered as (one of) the most feared, swarming locust species (cf. FAO: <http://www.fao.org/ag/locusts/en/info/info/faq/index.html>) and for which P2 has established cultures of both solitary and gregarious animals (cf. locust polyphenism). This work is done in collaboration with a high-throughput sequencing and bioinformatics center (the 'W. M. Keck Center for Comparative and Functional Genomics', Urbana, Illinois, USA), as well as with several colleagues, who are acting at the international forefront of insect science. The major objective is to investigate the molecular basis of population density dependent polyphenism, an extreme form of phenotypic plasticity that is observed in a number of locust species and that may lead to the formation of huge, devastating swarms. Locusts can develop into two distinct 'phases', a (relatively harmless) solitary and a gregarious one, which display very prominent differences in behaviour, development, coloration, morphology and physiology. The complete switch from one phase to the other (phase transition) usually requires several generations and is reversible. The underlying mechanisms that control this fascinating, but enigmatic, multifaceted, natural phenomenon are only poorly understood. The early behavioural changes can be mimicked by frequent physical contacts (Simpson et al., 2001), and appear to be closely correlated with substantial changes in the levels of a variety of neurotransmitters and neuromodulators in the locust central nervous system (Rogers et al., 2004). P2 previously characterized a tyramine receptor from locust CNS (Vanden Broeck et al., 1995; Poels et al., 2001). In the context of the

current proposal, P2 will study novel GPCRs that can be identified in this novel EST database, as well as a variety of cDNAs encoding evolutionary conserved proteins that may play a role in the GPCR signalling pathways (cf. WP7). These GPCRs will be further investigated in more detail by means of transcript profiling studies and functional *in vitro* and *in vivo* assays, in a similar way as described in 4.6 and 6.4.1). Detailed analysis of locust receptors, and the components of their signalling pathways, is likely to generate interesting information regarding the identity of their natural agonist(s), their signalling properties and their *in vivo* role(s).

6.5. Orphan LGRs (P1 and P2)

Leucine-rich G-protein-coupled Receptors (LGRs) constitute a subfamily of receptors related to glycoprotein hormone receptors, with a large extracellular aminoterminal domain containing leucine-rich repeats. Amongst them, LGR4, LGR5 and LGR6 form a cluster for which natural agonists are still unknown. During the previous IAP program, we have established a detailed atlas of murine LGR4 expression, and studied the phenotype of a mouse line invalidated for the LGR4 gene (Van Schoor et al. 2005, Mendive et al. 2006). We plan to pursue the quest for the natural agonist of the LGR4,5,6 cluster by a combination of approaches. (1) bioinformatics: drosophila LGR2 is a convincing orthologous receptor of the LGR4,5,6 vertebrate receptors. We will build on our identification of bursicon as the heterodimeric natural agonist of the drosophila receptor, during the previous IAP phase (Mendive et al. 2005), to define candidate orthologous agonists of the vertebrate receptors, *in silico*. The candidates will be cloned, expressed, and tested functionally on available CHO cell lines expressing LGR4 and LGR5. (2) microarray: profiling of total transcripts will be performed on whole wild type 9 days old fetuses (the time when LGR4 is widely expressed) and compared with data from LGR4^{-/-} knockout fetuses of the same age. Although a large amount of “noise” is expected in such type of experiments, the hope is that the unknown agonist might be over-expressed in the KO animals. (3) localization of agonistic activity: cells expressing both LGR4 (or LGR5) and a luciferase reporter gene under the control of a cAMP-responsive promoter will be built and used in tissue overlay experiments, in an attempt to localize the site of production of LGR agonists. (4) search for agonistic activity in tissue extracts: native protein extracts will be prepared from a panel of tissues [if possible, guided by the experiments under (3), above] and tested on cell lines expressing the LGRs for their capacity to stimulate cAMP production. If (when) an activity is detected, it will be followed through a series of chromatographic purification steps until pure enough to warrant analysis by mass spectrometry.

WP7. OLFACTORY RECEPTORS AND EVOLUTION OF GPCR FAMILIES

This workpackage represents new activities in the network that will be implemented within the partners' teams, in close collaboration with external groups belonging to parallel IAP network applications.

7.1. Olfactory receptors (P1, P2)

The structure of olfactory receptors has been known for 15 years (Buck and Axel 1991), and Partner P1 contributed to the cloning of human ORs, and the demonstration that their repertoire is also expressed in other sites, such as male germ cells (Parmentier et al. 1992). The functional expression of ORs has however represented a major problem, as mammalian ORs are poorly targeted to the cell surface in heterologous systems. Due to these limitations, very few mammalian ORs have been characterized functionally to date. Significant improvements have however been reported recently, such as expression in insect cells (Matarazzo et al. 2005). One of the most convincing progresses is however the demonstration that the transmembrane proteins RTP1, RTP2 (both expressed specifically in olfactory neurons) and REEP1 contribute to the translocation of ORs to the plasma membrane, and promote their functional expression in mammalian cell lines (Saito et al. 2004). Over the recent years, it became therefore possible to design a reliable functional assay, comparable in robustness to those developed for other GPCR families. The partnership will not invest in the large scale deorphanization of ORs, but will have access to large sets of data regarding the recognition of sets of odorants by human ORs, through a biotech company (Chemcom) dedicated to this activity. In this frame, we will invest in two directions:

First, it is our hypothesis that additional membrane or soluble proteins are required for the reconstitution of an efficient signaling complex in the knobs of olfactory neuron cilia, complexes that may resemble those found at neuronal synapses, in which receptors, transduction proteins and channels are organized by a number of chaperones and scaffolding proteins. Some proteins that might be part of such signaling complex include Homer (Gasperini and Foa 2004) and Ric-8B, a putative GTP exchange factor (Von Dannecker et al. 2005). We will explore further this hypothesis by using a proteomic approach. Olfactory mucosa from mouse will be homogenized and the homogenate separated by step ultracentrifugation and sucrose gradients, in order to enrich a fraction in ciliary knobs. Antibodies directed to components of the signaling machinery will be used in the monitoring of this purification, but we might use as well transgenic mice expressing a tagged OR under control of the olfactory marker protein (Danciger et al. 1989), in order to focus specifically on the OR-enriched fraction. Once purified, we will screen by 2D-DIGE and 2D-LC the proteins over-represented in this fraction, as compared to a total membrane fraction of the same initial homogenate. Proteins identified by mass

spectrometry will be considered as part of signaling complexes and will be tested functionally in reconstituted systems in mammalian cell lines co-expressing ORs, G_{olf}, and the cyclic-nucleotide-gated channel. Alternatively, insect cells will be employed as a heterologous system for reconstitution purposes. In case proteins identified in mammalian olfactory signaling complexes would have orthologues encoded in insect genomes, their (transcriptional) expression will be verified in insect sensory and other tissues. For a more direct analysis of proteins present in the receptosomes of insect chemosensory neurons, olfactory tissues will be collected from adult *Bombyx mori* (since silkworms have a well developed and very efficient sense of smell and relatively large antennae) and treated following a similar proteomic approach as outlined above for mouse olfactory mucosa. These comparative analyses will likely contribute to our understanding of the fundamental strategies that are employed by representatives of different metazoan Phyla in chemosensory signaling processes, as well as of their evolutionary diversification.

Second, we will use the functional information regarding the repertoire of odorants by specific receptors in order to integrate this with their structure, in an evolutionary context, as detailed in the next section.

7.2. Bioinformatics and evolution of the GPCR gene family (P1, P2, P4, EU1)

An increasing number of full genomes become available at an increasing pace. Bioinformatics and evolutionary clues have been used by the partnership over the previous IAP phases in order to raise hypotheses regarding the function of orphan receptors, and this approach has in some cases been key to the success of the program (Mendive et al. 2005). We would like now to move into a more systematic study of GPCR evolution, as we believe that the combination of structural, functional and evolutionary aspects in an integrated approach should provide major hints for each of these domains. This partnership will collaborate for this part of the work with groups specialized in bioinformatics, particularly that of Yves van de Peer (University of Gent) who is specialized in the evolution of genomes through the study of gene families, and the correlation between structural evolution and function of gene family members.

It is well established that evolution of genomes and species of increasing complexity is driven for a large part by duplication events, either at the gene level, or at larger scales (segmental or whole genome duplications). The influence of these duplication events in species evolution has been modeled (Maere et al. 2005). The analysis of the human genome has revealed the existence of a large number of ancient or recent duplication events, and much of the differences between the genomes of human and chimpanzee has been attributed to large segmental duplications (Cheng et al. 2005). Some duplication events in human are known to involve olfactory receptor clusters (Bailey et al. 2002).

Extracting functional predictions from sequence comparison among proteins encoded by duplicated genes is not a trivial task. One of the problems relates to the difficulty in making a distinction between conservation driven by purely structural (backbone) and functional (e.g. ligand recognition) criteria. By combining large scale sequence alignments of orthologous and paralogous GPCRs with structural knowledge built on modeling (Partner EU1), and functional knowledge (partners P1, P2 and P4), we will explore whether it is possible to understand better the specificity of receptors, and make predictions regarding agonists of orphan GPCRs. The chemokine and olfactory receptors will mainly be studied as a medium-size and large family of paralogs, respectively, for which we have the structural and functional counterparts in the network.

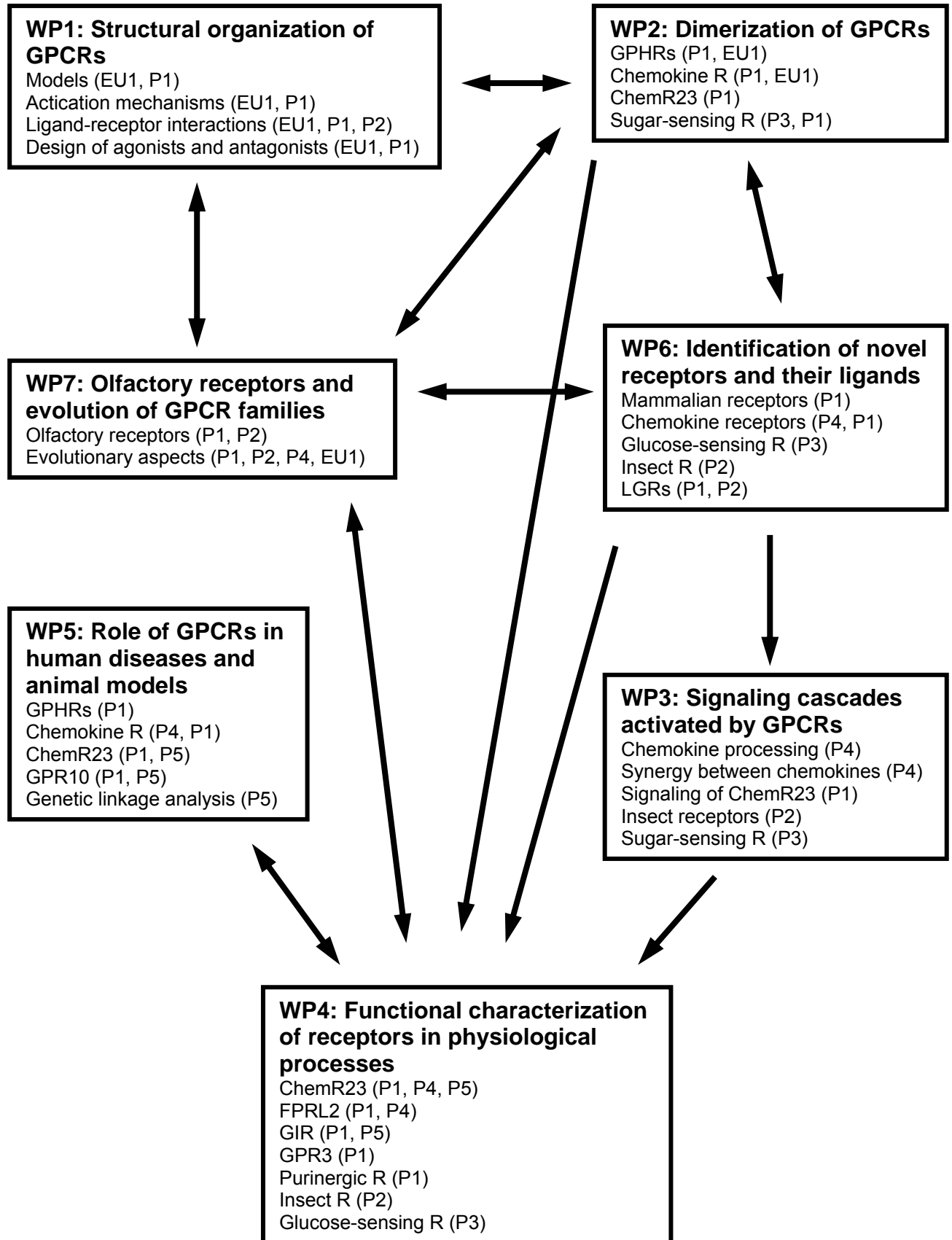
As different receptor classes are known to evolve at different speed, the range of species studied will be adapted to each specific family. The families of chemokine receptors and their chemokine ligands will be studied first in this context. As these families evolve rapidly, and have been shaped by duplication and deletion events in the recent evolution of mammalian species, we will focus on human, chimpanzee, mouse, rat and dog, as well as zebrafish as a distantly related species in which ancestral chemokine receptors and chemokines can be found. Chemokine and chemokine receptor sequences will be aligned, and the Treecon and Treepuzzle softwares (Schmidt et al. 2002) will be used to reconstruct genetic distance-based trees and maximum likelihood trees from the alignments. Using these trees and the structure of the gene clusters in each species, we will infer events of local or segmental duplication, deletion or mutation into pseudogene, and will correlate this information with the known biological function of the proteins and their specific or redundant character. We will also search for evidence of positive Darwinian selection (SCR3 software, Hughes 1990). We will in a second step use monoamine and neuropeptide receptor families, which are more conserved in evolution, allowing similar studies to be extended to all vertebrates and invertebrates (insect species and *C. elegans*). Finally, when sufficient functional data will become available, we will apply the same strategy to mammalian olfactory receptors. As the main mammalian olfactory receptor family is a very large class of molecules sharing a common transduction pathway, and most likely a strongly similar structural organization, we expect that the confrontation of evolutionary data with structural models and even partial functional characterization will help the understanding of this complex system, and will allow to derive accurate predictive tools for further functional characterization.

III. Integration of the partners

The tasks devoted to each partner are clearly stated in the description of the workpackages, as each task of a workpackage is attributed to one or two partners (indicated under brackets after each subtitle). Many of the aspects require the contribution of at least two partners. These shared tasks include the modeling of receptors and the testing of these models by mutagenesis, which involve EU1 and one of the other partners studying the considered receptor experimentally (P1, P2 and P3). They also involve many aspects of the chemoattractant receptor studies, for which partners P1 and P4 have complementary expertises. The phenotypic analysis of knock out animals will involve P1 and the new partner specialized in animal models of lung inflammatory and infectious diseases (P5). Dimerization studies will involve P1, who has developed the tools for studying this phenomenon, and the other groups willing to apply these tools to their receptors of interest (P3, and possibly P2). Finally, the bioinformatics and evolutionary aspects will involve all partners with their respective expertise in specific species (yeast, insects, mammals), receptor families (chemokines, glycoprotein hormone, peptidergic, olfactory receptors) or experimental approaches (genetic studies).

In addition, the network will be integrated at the European and international levels through several of its members, particularly through a number of FP6 European consortia dedicated to topics similar to that of the present program. This includes a STREP program "GPCRs" in which partners P1 (coordinator) and EU1 are involved, an integrated project "INNOCHEM", (partners P1 and P4), a network of excellence "EADGENE" (partner P5) and a Marie-Curie Training Network "CANTRAIN" (partner P3).

A graph illustrating the interactions between the different workpackages and the contribution of each partner to these workpackages is provided hereunder.



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I. 5. PARTICIPATION OF THE PARTNERS IN THE DIFFERENT WORKPACKAGES

Tick off in the table the participation of the different partners in the different workpackages (delete not used rows and columns in the table). Mention for each partner his/her name and the institution's abbreviation.

	PARTNER	WP1	WP2	WP3	WP4	WP5	WP6	WP7
P1	Name : M. Parmentier Institution : ULB	X	X	X	X	X	X	X
P2	Name : J. Vanden Broeck Institution : KUL	X		X	X		X	X
P3	Name : J. Thevelein Institution : KUL		X	X	X		X	
P4	Name : J. Van Damme Institution : KUL			X	X	X	X	X
P5	Name : D. Desmecht Institution : ULG				X	X	X	
EU1	Name : L. Pardo Institution : UAB	X	X					X

I. 6. MAIN SKILLS OF THE PARTNERS

Describe the main skills of each of the partners in relation to the project (15 lines maximum per partner).

Delete not used lines.

P1 - Name : Marc PARMENTIER

Institution : **ULB**

Main Skills : The Institute of Interdisciplinary Research (IRIBHM) is part of the ULB Medical School. About 140 researchers and technicians are working in the Institute over a range of subjects essentially focused on cell signalling, using cell and molecular biology approaches. About one third of the Institute is working on GPCRs and is concerned by the present network proposal. This group has pioneered homology cloning of G protein-coupled receptors, and a large number of original receptors have been isolated and characterised functionally using this approach (including thyrotropin, adenosine A1 and A2a, serotonin 5HT1D α , purinergic P2Y4, P2Y11 and P2Y13, chemokine CCR5, NPFFR2, GPR43, among others). The group also purified a number of natural agonists of orphan receptors, including a truncated HCC-1 variant for CCR5, kisspeptins for GPR54, chemerin for ChemR23 and the F2L peptide for FPRL2. The group identified genetic alterations of GPCRs causing human diseases (thyrotropin receptor in hyperfunctional adenoma, FSH receptor in ovarian hyperstimulation syndrome), or protecting individuals against HIV infection (CCR5), and developed about 20 transgenic (addition or knock out) mouse models so far. In the frame of this past activity, the group has gained a wide experience in all aspects of GPCR characterisation. The heavy equipment available in the Institute includes a Micromass MALDI Q-TOF mass spectrometer, a MassPrep digestion robot, several Waters and Applied Biosystems HPLC systems, Typhoon 2D-DIGE electrophoresis and analysis system, FACS, fluorescence microscopes, confocal microscopes, DNA sequencers, beta and gamma counters, a range of plate luminometers and fluorimeters, Biomek robotic workstations, colony picker and DNA arrayer, microarray scanner, videotracking and other equipment for behavioural analysis, microinjection equipment for cell cultures and mouse embryos, and a SPF animal facility.

P2 - Name : Jozef VANDEN BROECK

Institution : **KUL**

Main Skills : The "Laboratory for Developmental Physiology, Genomics and Proteomics" is studying neuronal and endocrine regulation mechanisms in different ecdysozoan (predominantly insect) species and focuses on molecular, cellular, physiological and evolutionary aspects of the neuro-endocrinology field. Relevant technical expertise: molecular biology, cellular expression technology, receptor and signal transduction studies, reporter gene assays, fluorometry, luminometry, chromatography (HPLC) and sequencing of neuropeptides, proteomics and mass spectrometry (Maldi-TOF, Q-TOF), electrophoretic methods, microfluidics, DNA sequencing, nucleic acid hybridization studies, differential expression analysis, in situ hybridization, immunological localization and detection methods, microarrays, quantitative realtime PCR, insect cultures, physiological assays, molecular genetics.

P3 - Name : Johan Thevelein

Institution : **KUL**

Main Skills : Twenty years of experience in the elucidation of the molecular genetics and cellular physiology of nutrient sensing and signaling in yeast. Expression and characterisation of mammalian genes in yeast and development of specific screens with yeast mutants to isolate functional mammalian substitutes of yeast genes. SCAM (Substituted Cysteine Accessibility Method) analysis of GPCRs and transporter-receptors. Determination of cAMP, metabolites, enzyme activities, cellular transport measurements and various parameters of yeast physiology. Purification of proteins. All modern methods of recombinant DNA technology, gene expression analysis, immunological methods, immunofluorescence, two-hybrid and dual membrane protein interaction

and screening analysis, methods of yeast transformation, mutant and suppressor selection and identification, gene deletion and overexpression in yeast, site-directed mutagenesis and genetic analysis.

P4 - Name : Jozef VAN DAMME

Institution : **KUL**

Main Skills : The Laboratory of Molecular Immunology belongs to the Department of Microbiology and Immunology of the Faculty of Medicine. Our group has a long tradition in cytokine research in relation to inflammation, infection and cancer and is involved in G protein-coupled receptors through research on inflammatory mediators, such as chemokines, complement factors, bacterial peptides and on HIV-1. The laboratory has the skills to produce, isolate and identify novel mediators from in vitro stimulated cell cultures or body fluids from patients with autoimmune diseases or cancer. These include large scale isolation and/or culture of various cell types in vitro and chromatographical purification of secreted proteins thereof to homogeneity and their identification through sequence analysis and mass spectrometry. This can also be implemented on smaller scale, for instance on patient' samples. Specific immunotests are developed to measure cytokines and chemokines in patients and to study their gene regulation in vitro. New chemokine isoforms are chemically synthesized on large scale to perform in vivo experiments using models of inflammation or cancer. Chemokines are evaluated in vitro for their capacity to chemoattract various leukocyte subtypes (freshly isolated from blood) and to activate GPCR in different signal transduction assays, with the help of various technologies including confocal microscopy.

P5 - Name : Daniel DESMECHT

Institution : **ULG**

Main Skills : Follow-up of respiratory diseases in awake and non instrumented mice. Establishment of strain-specific resistance/ susceptibility patterns to respiratory viruses. Tradition and expertise in the study of antiviral function of mammalian Mx GTPases

EU1 - Name : Leonardo PARDO

Institution : **UAB**

Main Skills : This group applies bioinformatic tools, ranging from multiple sequence analysis, statistical methods, protein database search, molecular graphics, and molecular dynamics simulations, to study the structure and activity of several types of GPCRs. We develop in silico models of both the amino-terminal extracellular domain of the leucine-rich repeats G protein-coupled receptors subfamily and the membrane embedded heptahelical bundle of Class-I or rhodopsin-like GPCRs. These computer models are used to predict the mechanisms of ligand recognition and receptor activation, which are experimentally validated by site-directed mutagenesis and functional assays. This partner also develops pharmacophore models using structural information derived from i) a series of ligands that are thought to interact with the same binding site and/or ii) the putative residues in the receptor model which might be involved in the binding of the ligand. Chemical 3D-databases are searched using the pharmacophore model as a 3D query for virtual screening and lead optimization.

I. 7. NETWORK ORGANISATION AND MANAGEMENT (4 pages maximum)

Describe the network's organisation and the practical terms governing collaboration and interaction between the partners (meetings, newsletters, doctoral school, ...).

Network's organization

The partnership is composed of five Belgian academic laboratories, belonging to three Universities, ULB (P1), KUL (P2, P3 and P4) and ULG (P5), and a Spanish group from University of Barcelona as foreign partner (EU1). These groups have complementary expertise in various aspects of GPCR research. The contribution of each partner to the various workpackages is described in detail in Form D, and as a summary table in Form E. From this workprogram, it can be seen that all partners are involved in many aspects of several workpackages, and that a number of aspects do not stand without the involvement of at least two partners. This is a measure of the common interests shared by the network and the necessity of strong and regular interactions between the groups. Most of the partners of the network have a long standing experience of working together. They interacted for five years during the previous IAP program. For these common tasks, the interactions will include exchanges of researchers and regular meetings between the partners concerned by each task, and even more regular contacts by electronic mail. However, formal exchange of information across the entire network will be made through regular meetings on one side, and through the website and newsletter on the other side. The network will be managed by a steering committee.

Steering committee and management

The management of the program will be performed by a steering committee made of the promoters and principal group leaders of the network partners. The steering committee will meet quarterly to evaluate the progress of the network activities, and to determine all necessary actions for the next period. These meetings will be used to organize the annual plenary meetings, plan additional plenary meetings if appropriate, organize the international symposium, redefine the respective responsibilities in the network organization and management that will be determined during the kick-off meeting. It will also be the occasion to discuss potential co-experiments not initially planned in the workprogram, and determine who will, in each laboratory, be in charge of the practical organization of these tasks. In addition, the steering committee will handle all other matters arising from the functioning of the network, including the management of intellectual property, and the potential problems arising from the interactions of the IAP network or specific partners with international (i.e. EU) networks or industrial partners.

Network kick-off meeting

A first meeting of the network will be organized in early 2007. This meeting will be built on the same template as the subsequent plenary meetings (see below). As most partners were also part of the previous IAP program, a scientific session will be the occasion to establish a link between the previous program and the new one. Also a first steering committee will be held, which will define further the responsibilities of each partner in the management of the program, and will determine some of the options regarding practical aspects such as the content of the web site and of the newsletter, and the opportunity of creating a specific Doctoral School. This first meeting will also establish the planning for the next steering committee meetings and the first plenary meeting. We will discuss the format and approximate timing for the International GPCR Meeting (see below), and will formalize the organization of the common research tasks.

Plenary meetings of the network

A plenary meeting will take place at least once a year, likely in November or December, as this will also be the occasion of summarizing the network's achievements in prevision of the annual report. These meetings will necessarily gather all groups, including most senior and junior staff involved in the program. These meetings will occupy a whole day. They will include a scientific session where

partners will describe their overall contribution to the program over the last period, and will focus on some of their main achievements in more details. This will be the occasion for the younger investigators (PhD students and post-docs), to train in presenting their data to a specialized and critical audience. These presentations will of course include the topics of common interest, but also topics that are not planned to constitute co-experiments. These meetings will therefore constitute a forum in which all groups will learn what is going on in the network. Indeed, it is our experience from previous IAP phases, that such meetings are key to the establishment of personal relationships between the PhD students and Post-docs of the various groups, leading to exchange of information, ideas and reagents, and leading ultimately to common and concerted research projects. Besides this scientific session, plenary meetings will include a coordination session of the steering committee, where the partners will evaluate the overall progress of the program, will consider the potential changes in the orientation of the work or in the structure of the partnership, and will redefine the priorities and objectives for the next period. Considering the structure of the network, we propose that the meeting would take place in alternance at ULB and KUL.

International GPCR meeting

We plan to organize over the duration of this IAP program, an international meeting dedicated to GPCRs. This meeting could complement one of our Plenary meetings, or be organized independently. The precise format of this meeting will have to be discussed, but we presently consider a symposium of three days, with an attendance limited to about 150 to 200 participants. This meeting would cover the various interests of the network. A number of European and extra-European speakers will be invited, but spots will be allocated to partners of the IAP network, as well as to participants selected on the basis of abstract submission. This meeting might be combined as well with the regular meeting of one of the EU networks in which IAP partners are involved, in order to combine resources and attractiveness, and allowing assembling an international program of high interest.

Planned interactions between partners

A number of research programs are being run as collaborations between several partners of the network. These represent the follow-up of the interactions that were initiated during the previous IAP phase, but also new developments or planned interactions with the new partner in the program (P5). The activities involving at least two partners include (although this is not exhaustive):

- Modeling of glycoprotein hormone, CCR5, ChemR23 and insect tachykinin receptors (P1, P2 and EU1)
- Activation mechanisms of glycoprotein hormone and CCR5 receptors (P1 and EU1)
- Ligand-receptor interactions for ChemR23 and insect tachykinin receptors (P1, P2 and EU1)
- Design of chemical agonists/antagonists for glycoprotein hormone and ChemR23 (P1 and EU1)
- Dimerization of glycoprotein hormone and chemokine receptors (P1, P4 and EU1)
- Dimerization of sugar-sensing receptors (P1, P3 and EU1)
- Signaling cascades of chemokine receptors (P1 and P4)
- Functional characterization of ChemR23, GPR10 and GIR in physiology (P1 and P5)
- Role of ChemR23, GPR10 and GIR in mouse disease models (P1 and P5)
- Characterization of novel chemokine receptors (P1 and P4)
- Characterization of novel neuropeptide receptors in mammals and insects (P1 and P2)
- Characterization of glucose receptors in mammals (P1 and P3)
- Evolutionary analysis of the GPCR family (P1, P2, P3, EU1)
- Functional characterization of signaling complexes in olfactory neurons (P1 and P2).

Website

A specific web site will be set up for the IAP network. This web site will be split into two parts. One part will be open to public access, and will serve as the promotion tool of the network and partners. A second part will be of restricted access to members of the network, and will serve as a major site of information exchange and as a resource for solving daily research issues. This site should become a daily tool for all participants of the network. Although we will have to build this web site over the first

6 months of the program, and improve it permanently afterwards, we now plan to include the following topics:

Public site

- Description of IAP programs
- Overview of the research program
- Structure of the partnership and links to partners' own web sites
- Expertise of each partner,
- Major achievements of the partnership
- List of publications resulting from the program
- Prizes and honors received by members of the network
- Interuniversity program of lectures devoted to GPCRs (i.e. for Doctoral School PhD students)
- Announcement of the International GPCR Meeting and other events of interest
- Announcement of post-doc or PhD student positions offered by the network, or by other laboratories
- Resources of public interest, such as the glycoprotein hormone receptor mutant database recently set up by partner P1

Restricted access site

- Directory of all scientists and technicians involved in the program in the various partners' laboratories, with contact details
- Expertise of each group, allowing students and post-docs to identify the best contact in the network to obtain information and solve their problems
- Schedule of steering committee meetings
- Program of plenary meetings
- List of resources available in the laboratories of the partnerships, particularly in terms of heavy equipment and facilities (mass spectrometry, 2D-DIGE, confocal microscopy, DNA microarrays,...), but also specific techniques, reagents of broad interest,
- In press (and possibly submitted) manuscripts of the network
- Results of ongoing experiments that may stimulate new collaborations across the network
- Posting of new information, meeting reports, meeting announcements, or external publications of special interest to the whole network
- Forum for questions and suggestions

Newsletter

In order to stimulate exchange of information across the network, and the use of the website, we will establish a regular newsletter, most likely on a quarterly basis, which will incorporate all new information available on the website (newcomers, breakthroughs, publications, meeting announcements, ..). This newsletter will be sent by E-mail to all network participants, and will be posted on the website as well.

Exchange of scientists and students

The mobility of PhD students and post-doctoral researchers between the labs of the partners will be strongly encouraged. This will involve short training for technology transfer, or exchange for longer time periods for performing a whole aspect of a specific program. It could also take the form of young PhDs, who do not elect to go abroad for personal reasons, to spend a postdoctoral training period in another lab of the network. In addition, each partner will use its international connections, including EU networks, to broaden opportunities for the exchange of students and scientists.

Particular attention will be placed on exchanges of researches with our European partner (EU1). As obvious from the workprogram, the European partner will be involved in a number of tasks devoted to modeling, and structure-activity studies of various GPCR classes (WP1 and WP2). This will imply almost daily online interactions between Barcelona and the Belgian labs, but also regular exchange of scientists in order to train them to the modeling approaches. Partners P1 and EU1 have experienced a very fruitful interaction of that kind during the previous IAP program.

Doctoral school

The legal requirement for interuniversity Doctoral Schools is very recent in Belgium. The various partners are associated to one or often several of these recently created Doctoral Schools, which have replaced the pre-existing local Doctoral Schools, and which usually regroup large domains of biomedical sciences (Molecular/cellular biology and biochemistry, Experimental oncology, Neurosciences, Immunology,...). These Doctoral Schools cover the various aspects of the present network activity. At the same time, the scientific management of the PhD students is at present still the responsibility of each host institution, and it is still unclear how this local management will integrate with the responsibilities of the regional Doctoral Schools. We discussed, within the partnership, the possibility of creating a specific Doctoral School dedicated to the Network, or more broadly to the GPCR field in general. No definitive decision has been taken yet, and this will be discussed further at the kick-off meeting in early 2007. We will have by then a clearer vision of how these new Doctoral Schools will function, and what will be their precise prerogatives. In any case, we will contribute to the specific training of our students in GPCR science, by assembling an interuniversity program of lectures devoted to the various aspects of the network activity, and that will be posted on the IAP network website. Such lectures are presently part of the local training programs in each of the partners' Universities. These lectures will be eligible in the number of attended seminars each Ph.D. student has to justify annually, and, if appropriate, will be integrated in the program of the specific Doctoral School we might create.

I. 8. RE-ORGANISATION OF THE PROJECT (maximum 3 pages)

To be completed only if the initial proposal has to be adapted as a result of the selection outcome. If this implies changes in the composition of the network and/or the budget, it may be that it is not longer possible to pursue (achieve) the originally proposed objectives.

In this case, describe and clarify the re-organisation of the project compared to the initial proposal.

Not applicable

I. 9. BUDGET (global distribution per partner for the 5 years)

(in EURO, without decimals)

The detailed distribution per partner is given in Section II

	Name Partner	Institution	Budget
P1	Marc PARMENTIER	ULB	1,400,000
P2	Jozef VANDEN BROECK	KUL	450,000
P3	Johan THEVELEIN	KUL	450,000
P4	Jozef VAN DAMME	KUL	450,000
P5	Daniel DESMECHT	ULG	400,050
EU1*	Leonardo PARDO	UAB	97,250
TOTAL BUDGET			3,247,300

* The budget for the EU-partner is the budget attributed by the IAP-programme only (without the 50% contribution of the EU-partner)

I. 10. PREVIOUS IAP-PHASES

To be completed only if the present network was funded during earlier phases of the IAP programme.

Mention the earlier phases of the IAP programme (I, II, III, IV, or V) and the titles of projects in which the partners of the present network has participated.

Phase II, IAP 2/15, 1990-1996. Molecular genetics of regulation networks. Involved partner **P1** (Gilbert Vassart).

Phase IV, IAP 4/30, 1997-2001. Molecular genetics and pathology of signal transduction. Involved partners **P1** (Gilbert Vassart, as coordinator), **P2** (Arnold De Loof), **P3** (Johan Thevelein) and **P4** (Jozef Van Damme).

Phase V, IAP 5/30, 2002-2006. G protein-coupled receptors: functional genomics, molecular pharmacology, structure-function studies. Involved partners **P1** (Gilbert Vassart, as coordinator), **P2** (Arnold De Loof), **P3** (Johan Thevelein), **P4** (Jozef Van Damme) and **EU1** (Leonardo Pardo).