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Transmembrane Receptors

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Transformation of Receptor Tyrosine Kinases into Glutamate Receptors and Photoreceptors

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Abstract: *Receptor tyrosine kinases (RTKs) are key regulators of cellular functions in metazoans. In vertebrates, RTKs are mostly activated by polypeptides but are not naturally sensitive to amino acids or light. Taking inspiration from Venus kinase receptors (VKRs), an atypical family of RTKs found in nature, we have transformed the human insulin (hIR) and hepatocyte growth factor receptor (hMET) into glutamate receptors by replacing their extracellular binding domains with the ligand-binding domain of metabotropic glutamate receptor type 2 (mGluR2). We then imparted light sensitivity through covalent attachment of a synthetic glutamate-based photoswitch via a self-labelling SNAP tag. By employing a Xenopus laevis oocyte kinase activity assay, we demonstrate how these chimeric RTKs, termed light-controlled human insulin receptor (LihIR) and light-controlled human MET receptor (LihMET), can be used to exert optical control over the insulin or MET signaling pathways. Our results outline a potentially general strategy to convert RTKs into photoreceptors.*

Signal transduction is achieved by transmembrane proteins that undergo conformational changes upon extracellular ligand binding and activate intracellular signaling pathways or effect changes in transmembrane potential.[1] These transmembrane proteins include ion channels, G-protein-coupled receptors (GPCRs), and receptor-linked enzymes, such as receptor tyrosine kinases (RTKs). In humans, 58 RTKs are encoded, and they comprise an important class of receptor-linked enzymes.[2] They are involved in the control of critical cellular processes such as metabolism, differentiation, and the cell cycle, thus making them important targets in diabetes and cancer therapy.

A small and distinct class of RTKs is the Venus kinase receptors (VKRs; Figure 1).[3] Originally discovered in parasitic worms (e.g., *Schistosoma mansoni* (SmVKR)[4]) and insects [e.g., in the honeybee *Apis mellifera* (AmVKR)[5]], they are widespread in invertebrates but absent among vertebrates. These VKRs are activated by a variety of ligands, such as amino acids and ions,[3] and have recently received increased attention due to their involvement in gametogenesis and egg production in parasitic helminths[6] as well as in pathogen vector mosquitoes.[7] As such, they are novel drug targets for various infectious diseases. VKRs are named after their extracellular Venus-flytrap-like ligand-binding domain (LBD). This domain, also called "clamshell" domain, belongs to a larger family of LBDs that consist of two lobes, which close around their ligand.[8] Derived from prokaryotic periplasmic binding proteins, LBDs of this type have been incorporated in transporters, ion channels, and GPCRs in the course of evolution.[9] Prominent eukaryotic examples include ionotropic receptors, such as AMPA receptors, and the eight metabotropic glutamate receptors found in the human genome.

The diversity of receptors and ion channels can be further increased through engineering of both extracellular sensory domains and intracellular downstream signaling domains.[10] For instance, the extracellular LBD of the human insulin receptor has been fused to kinase domains of other RTKs to turn on different signaling pathways.[11] Chimeric GPCRs have been engineered that combine the photosensitivity of opsins with the signaling pathways of G_s- or G_q-coupled receptors (OptoXR).[12] All of these chimeras have been engineered from domains within a class of receptors, but domain swapping between receptor classes has been reported

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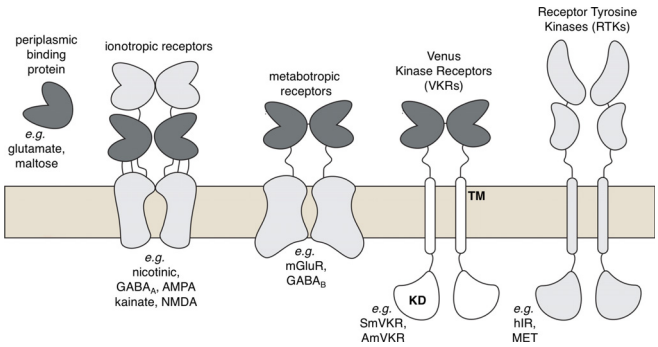


Figure 1. Proteins with Venus-flytrap-like or clamshell ligand-binding domains (LBDs). Presumably originating from periplasmic binding proteins, the Venus-flytrap domain (dark grey) has been found in all classes of transmembrane proteins, such as ion channels, GPCRs, and receptor-linked enzymes like tyrosine kinase, but VKRs are only found in invertebrates. Vertebrate RTKs do not bear clamshell LBDs.

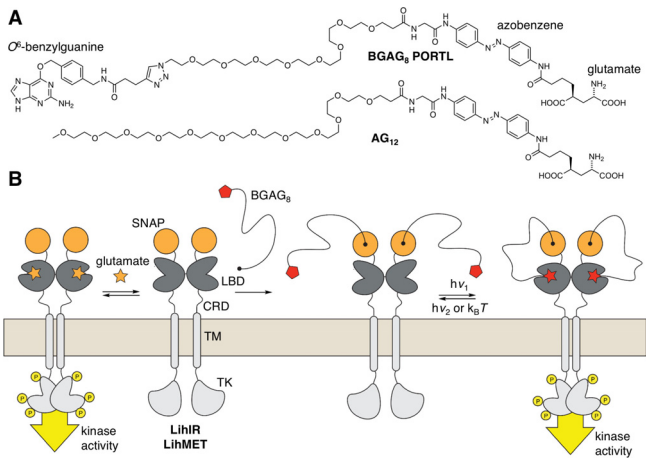


Figure 2. Design of the chimeric light-controllable glutamate receptors LihIR and LihMET. A) Chemical Structure of the PORTL BGAG$_8$ (BG = benzylguanine for bioconjugation, A = azobenzene as a photoswitch with G = glutamate head group) and the freely diffusible analog AG$_{12}$. The index refers to the number of PEG units in the linker or side chain. B) LihIR and LihMET comprise SNAP-mGluR2(LBD + CRD)-RTK-(TM + TK) constructs. Kinase activity can be evoked by addition of glutamate (left) or by bioorthogonal labelling of the SNAP tag with BGAG$_8$ and subsequent illumination.

as well. For example, extracellular antigen recognition domains can be fused to different intracellular signaling domains and expressed in T cells to obtain chimeric antigen receptor T cells (CAR-Ts),[13] which show promise in cancer immunotherapy. Similarly, "Syn-notch" receptors bear an antigen recognition domain and signal through the Notch pathway.[14] Finally, a plethora of chimeric proteins have been engineered that combine fluorescent proteins with sensory domains and have been used to quantify endogenous small molecules.[15] However, to the best of our knowledge, synthetic receptors have never been created by combining protein modules from GPCRs and RTKs.

We now report that it is possible to convert both the human insulin receptor (hIR) and the human hepatocyte growth factor receptor (hMET) into glutamate receptors. This was achieved by exchanging the extracellular LBDs of these RTKs with the Venus-flytrap domain of the GPCR mGluR2. Following this, we equipped the engineered receptors with a glutamate-based photoswitch,[16] which led to two new synthetic photoreceptors termed LihIR and LihMET.

The human insulin receptor (hIR) and the human hepatocyte growth factor receptor (HGFR or hMET) are among the best-studied RTKs due to their prominent role in metabolic and regenerative diseases, as well as in cancer. We therefore chose their transmembrane (TM) and intracellular tyrosine kinase (TK) domains and merged them with the extracellular LBD and the cysteine-rich domain (CRD) of mGluR2. The stiff CRD was retained to serve as a lever and to juxtapose the intracellular TKs following LBD closure. mGluR2 forms constitutive dimers held together by a disulfide bond between the LBDs,[17] similar to hIR. In addition, an N-terminal HA-tag and a self-labelling protein tag (SNAP)[18] was added to enable the eventual attachment of a photoswitch (see below). Both chimeras, termed LihIR and LihMET, were evaluated for their ability to initiate cellular tyrosine kinase signaling upon glutamate and light activation (full sequences, including details on cloning, are provided in the Supporting Information). The topology of our constructs and their activation by glutamate and after BGAG labelling using light, respectively, are shown in Figure 2.

Xenopus laevis frog oocytes are an excellent model system to study kinase function since they express all the required components for deciphering mammalian cell signaling pathways.[19] In stage VI oocytes, activation of mitogen-activated protein kinase (MAPK) pathway by progesterone (PG) or by ectopic expression of RTKs triggers a cellular process called germinal vesicle breakdown (GVBD, described in detail in Figure S1 in the Supporting Information), This GVBD reports kinase activation and can be easily monitored through visual inspection (Figure 3 A). Therefore, we expressed LihIR and LihMET chimeras in oocytes by cRNA microinjection and, after 5 h, confirmed full-length expression of both chimeras by HA immunoprecipitation and subsequent detection at around 160 kDa by western blotting (Figure 3 B). As anticipated, glutamate treatment initiated intracellular phosphorylation of LihIR and LiMET, as revealed by a phosphotyrosine-specific antibody (PY20), and furthermore by downstream phosphorylation of Akt and ERK2, which correlates with positive GVBD. Viability and correct maturation of the oocyte batch were confirmed using progesterone (PG) for GVBD, Akt, and ERK2 activation. To demonstrate that glutamate activation proceeds through the orthosteric site of the mGluR2-LBD, oocytes were pre-incubated with LY341495, a high-affinity mGluR2 antagonist. This inhibitor only blocked glutamate-induced GVBD and had no effect on PG treatment, thus demonstrating orthosteric binding of glutamate to the mGluR2-LBD of LihIR and LihMET. LihIR and LihMET dead kinase (DK) mutants[6] did not respond to glutamate either, thus confirming that the tyrosine kinase activity of the chimeras initiates intracellular signaling (Figure S2).

Insulin receptors are dimers wherein changes of the relative orientation of the TK domain upon TM domain motion regulates catalytic activity and downstream signaling

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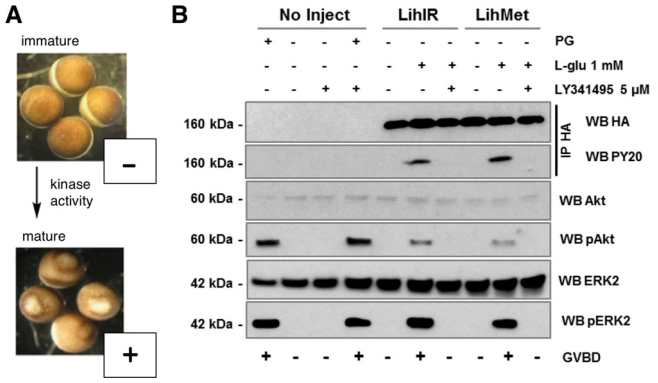


Figure 3. LihIR and LihMET kinase activity assay in *Xenopus* oocytes. A) Oocytes undergo germinal vesicle breakdown (GVBD) in response to outside stimuli that result in an increase of MAPK activity, observable by the formation of a white spot on the brown oocyte pole. B) Immunoprecipitation (IP) and western blotting (WB, 15 oocytes per conditions) show downstream phosphorylation of receptor and effectors only in presence of glutamate and in absence of the orthosteric competitive antagonist LY341495. Each condition was assayed for GVBD in 20 oocytes. >80% of the oocytes undergoing GVBD is considered a positive readout (+). Progesterone (PG) served as positive control.

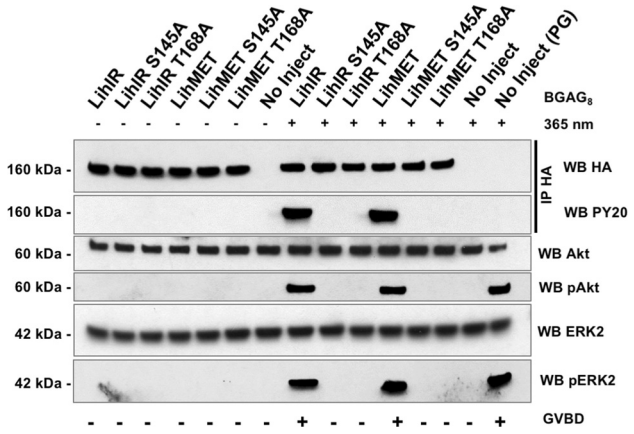


Figure 4. Light activation of $BGAG_8$-labeled LihIR and LihMet. 10 *Xenopus* oocytes were used for each GVBD assay and 25 each for IP and WB (in total 60 oocytes per vertical lane). Point mutations S145A and T168A abolish glutamate binding and serve as negative controls.

pathways.[20] To explore this coupling, we introduced both a single amino acid extension and a truncation of the TM domain. Exposure of the truncated version to glutamate had no effect. By contrast, the extended construct was still able to signal through ERK2 but not through Akt. Moreover, this extended mutant was able to phosphorylate JNK (Figure S3). This modified intracellular signaling pathway confirms that small differences in receptor sequence can result on a pronounced change of signal bias.

Having achieved the conversion hIR and hMET into glutamate receptors, we next imparted light sensitivity to our protein chimeras. To this end, we chose the photoswitchable orthogonal remotely tethered ligand (PORTL) approach, which was developed by our research groups to enable precise photocontrol of mGluRs in vivo.[21] This system relies on robust self-labelling protein tags, such as N-terminally fused SNAP tags, that covalently react with the benzyl guanine $BGAG_8$ (benzylguanine-azobenzene-glutamate).[16] The structure of this molecule is shown in Figure 2A and the assembly and function of the synthetic photoreceptor is schematically shown in Figure 2B. Accordingly, we labelled LihIR and LiMET with $BGAG_8$ and illuminated the oocytes with a 365 nm light source. Indeed, we observed all downstream events that were previously recorded after glutamate exposure (Figure 4).

To confirm the need for covalent receptor labelling, we tested the freely diffusible ligand AG_{12}, which lacks a benzyl guanine for bioconjugation (Figure S4). Exposure to AG_{12} (100 µM) in the dark, followed by irradiation (365 nm), did not result in activation of either chimera. This is in line with our previous findings with mGluRs, where covalent attachment was needed to ensure the high local concentration necessary for successful PORTL photocontrol.[16]

As expected, the LihIR and LihMET dead-kinase mutants did not activate following $BGAG_8$ labeling and 365 nm illumination (Figure S4). To demonstrate that glutamate binding to the mGluR2-LBD is a prerequisite for activation of our chimeras, we generated the point mutations S145A and T168A, which are known to abolish orthosteric glutamate-binding in the mGluR2-LBD.[22] Indeed, these mutants could not be activated with $BGAG_8$ (Figure 4) or with glutamate (Figure S5). This confirms that orthosteric binding is required to trigger tyrosine kinase activity and downstream signaling cascades.

In conclusion, by using a combination of protein engineering and tethered photopharmacology,[23] we have created two RTKs that contain a ligand-binding domain that is sensitive to small molecules. Furthermore, we converted these engineered receptors into light-sensitive RTKs. Initial attempts to convert VKRs themselves into photoreceptors had been unsuccessful. Since our PORTL approach to the optical control of mGluR2 worked well[16,21,24] and since the downstream effects of hIR and hMET were much better understood than those of VKRs, we ultimately settled on a more audacious approach merging two receptors involved in very different signal transduction pathways. Our results confirm that the recombination of modules from vastly different families can lead to functional transmembrane receptors with interesting functional properties.

The optical control of RTK pathways has also been achieved using optogenetic strategies. "Opto-RTKs" have been created by fusing aureochrome LOV domains to various intracellular kinase domains.[25] The resulting constructs lacked extracellular LBDs and were insensitive to ligands but could be activated with blue light. Spatiotemporal control of RTK signals could also be achieved through light-induced cryptochrome oligomerization.[26] Our systems, by contrast, rely on chimeric proteins that retain an extracellular LBD and require the covalent attachment of synthetic photoswitches. LihIR and LiMET are minimally modified on their intracellular domains and presumably retain most of the interactome of their parents (hIR and hMET). They can be conditionally made photosensitive through the addition of the synthetic molecule $BGAG_8$. This allows another level of

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control and prevents undesired light-activation after expression of a photoreceptor.

The ability to control RTKs with small molecules that have well-defined pharmacology, such as glutamate and its analogues, opens new perspectives in chemogenetics.[27] It should be possible, for instance, to engineer RTKs that respond to diffusible ligands that are normally inactive or to tethered ligands that do not effectively activate native receptors before bioconjugation. The tethered photopharmacology described herein should enable the activation of distinct RTK signaling pathways in mammalian cells with higher spatiotemporal precision than conventional pharmacology. Studies in this direction are ongoing in our laboratories.

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Conflict of interest

The authors declare no conflict of interest.

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