Review

Protein translocation as a tool: The current rapamycin story

Mateusz Putyrski, Carsten Schultz*

Cell Biology and Biophysics Unit, EMBL Heidelberg, 69117 Heidelberg, Germany

Article info

Article history:
Received 20 February 2012
Revised 27 April 2012
Accepted 29 April 2012
Available online 11 May 2012

Edited by Thomas Reiss and Wilhelm Just

Keywords:
Rapamycin
Chemical dimerizer
Protein–protein interaction
Protein translocation
Signal transduction

Abstract

In cell biology and pharmacology, small chemicals are mostly used as agonists and antagonists against receptors and enzymes. The immunosuppressant rapamycin can serve an entirely different purpose: if employed sensibly, it might function as an inducer of dimerization that is able to rapidly activate enzyme activity inside the intact cell. A number of very recent developments such as photoactivatable derivatives make rapamycin an even more attractive tool for basic science.

1. Introduction

Protein translocation is one of the key events that help regulate cell functions. Among the most prominent examples are proteins that enter or leave the nucleus or another organelle, often in a regulated fashion. By this means, different cellular compartments are able to communicate and exchange material. In cytosolic signal transduction networks, protein translocation from the cytosol to internal membranes or the inner leaflet of the plasma membrane represents a principal way of how signals may be dispersed without the need to cross from one cellular compartment to another. In fact, the shear concentration of signaling partners at a 2-dimensional surface seems to be sufficient to increase the local concentration, resulting in efficient interaction and signal transmission. An example is the interaction of Akt and PDK1 (3-phosphoinositide dependent protein kinase-1) which usually requires the translocation of both proteins to the plasma membrane [1]. As both Akt and PDK1 bear PH domains that recognize the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate \( \text{PtdIns}(3,4,5)P_3 \), they accumulate at the same membrane region, phosphorylate each other and thereby fulfill the next signaling step within the network. Interestingly, both molecules also phosphorylate and interact with each other when artificially brought in close proximity within the cytosol [1]. This means that the intrinsic activity of the complex is at least partially independent from the membrane but requires the small molecule switch, in this case the phosphoinositide \( \text{PtdIns}(3,4,5)P_3 \), to induce proximity. The change from the 3-dimensional space of the cytosol to the membrane surface results in a concentration effect of at least one order of magnitude, although a true concentration is tough to determine at a membrane. The residence of lipids in certain membrane compartments leads to an even higher protein density, potentially up to a factor of 100. It remains the question, why the coincidental interaction of two signaling molecules does not produce a significant signal. In most cases, a kiss-and-run interaction is simply not producing enough molecular events (such as phosphorylation) to give rise to a sustainable signal, which would be resistant to the opposing action of the counteracting enzymes (such as ubiquitously distributed phosphatases). As a result, the inner leaflet of the plasma membrane and also organelle membranes may be considered distinguished cellular compartments, even if physical shielding is not provided. When observed by realtime imaging, translocation of a protein from the cytosol to a membrane seems to be very fast on the timescale relevant for non-excitable mammalian cells. Usually, translocation events are complete within a few seconds, which corresponds to one or two frames when observed in a standard live cell imaging setup. From this, it becomes apparent, that translocation must be considered a very fast vehicle to alter intracellular events. Most other techniques for manipulation are based on changing protein numbers rather than localization. This includes transfection/overexpression and RNAi methods that require 10–72 h for showing an often incomplete effect, or various techniques that specifically activate selective degradation of the protein of interest [2,3]. The later shows significant effects within tens of minutes to hours [4]. Therefore, intracellular translocation is

* Corresponding author.
E-mail address: schultz@embl.de (C. Schultz).

0014-5793 © 2012 Federation of European Biochemical Societies. Published by Elsevier B.V. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.febslet.2012.04.061

FEBS Letters 586 (2012) 2097–2105
significantly faster in comparison and the time required for artificially induced translocation by small molecules such as rapamycin is most probably determined by the pace of cell entry of the chemical dimerizer rather than the speed of protein translocation.

2. The principle of induced translocation

As just mentioned, some small molecules are able to induce protein–protein interaction. Collectively they are called ‘chemical inducers of dimerization’ or CIDs. The most prominent feature of CIDs is the ability to bind two proteins/protein domains simultaneously. If these proteins are of the same kind, we speak of homodimerizers. If two different proteins are bound, the compounds are named heterodimerizers. Surprisingly and unfortunately, the number of known chemical dimerizers is quite limited. Historically, studies on the mechanism of action of immunosuppressants FK506 and cyclosporin A (CsA) paved the path to the development of the CID technology [5]. As a result, FK1012, a synthetic dimer of FK506, gave rise to an entire family of chemical homodimerizers used to connect two copies of FK506 binding protein with a mass of 12 kDa (FKBP12), a ubiquitous prolyl cis–trans isomerase [6]. Importantly, the development of chemical heterodimerizers has been heavily influenced by the discovery of the mechanism of action of rapamycin, yet another immunosuppressive drug, which potently inhibits the protein kinase TOR (target of rapamycin), more exactly the TORC1 multiprotein complex (TOR complex 1), of which TOR is one of the constituents [7]. The mechanism of TORC1 inhibition by rapamycin is of particular importance. Within a cell, the rapamycin molecule first binds to FKBP12 and only then the FKBP12–rapamycin complex binds to TOR (precisely to the FRB (FKBP and rapamycin binding) domain of the kinase), rendering TORC1 enzymatically inactive (Fig. 1) [8,9]. Therefore, two proteins genetically fused to FKBP12 and FRB, respectively, are brought into close proximity in the presence of rapamycin. Since the first report describing the application of such a strategy in cells for inducing gene expression [10], rapamycin and its derivatives have clearly dominated the field of small molecule heterodimerizers. Applications range from using rapamycin-inducible FKBP/FRB interactions in proof-of-concept experiments when designing new methods for monitoring protein–protein interactions to inducible regulation of gene expression in living organisms [11]. It is also important to underline that inducible homodimerization, mostly by employing derivatives of FK1012, is an important and widely used tool in chemical biology. For a broader overview on CID applications, please consult recent reviews on the topic [6,12,13] and the comprehensive list of publications available on the website of the commercial supplier for dimerizer technology [11]. The main focus of this review is the use of inducible heterodimerization to modulate cell signaling in an acute and highly precise manner.

3. Dimerizer-induced modulation of cellular signal transduction

The field of signal transduction is particularly attractive for applying tools that specifically manipulate single enzyme activities in living cells. Typically enzyme inhibitors or, more rarely, activators are used. However, CIDs provide a good alternative to rapidly modulate enzyme activity. This offers the possibility to trigger signaling events downstream of the plasma membrane receptor level and permits to dissect signaling networks, because only one specific branch of the network is activated. By using several orthogonally acting chemical dimerizers simultaneously, it might become possible in the future to reconstitute even fairly complex signaling patterns.

Fig. 1 depicts a typical approach that involves the translocation of a constitutively active form of a protein of interest (POI) to the plasma membrane. If the protein is an enzyme and finds its substrate exclusively at the plasma membrane, no activity will be observed as long as enzyme and substrate are physically separated. After translocating the enzyme to the plasma membrane, the enzyme–substrate complex is formed successfully and the signal is triggered. One of the possible protein families especially amenable to such manipulations are G proteins. The availability of both constitutively active (GTPase deficient) and dominant negative (incapable of productive interaction with GTPase activating proteins (GEPs)) makes them convenient targets for the dimerizer-inducible approach [14–17]. Another group of important proteins which was shown to be successfully manipulated in this way are enzymes involved in the metabolism of membrane lipids, especially phosphoinositides [18–22]. The physical separation of (mislocalized) cytoplasmic enzymes and its substrate at the membrane makes it theoretically easy to construct an inducible tool.

Indeed, the list of currently available rapamycin-inducible signal molecules contains numerous examples of proteins belonging to these groups (Table 1). Established CID-dependent tools cover a large fraction of the cellular signaling modules, ranging from small G-proteins, heterotrimeric G-protein subunits, phosphoinositide metabolizing enzymes, receptor- and non-receptor tyrosine kinases, serine/threonine protein kinases to transmembrane receptors without enzymatic function and signaling adapter proteins.

Without doubt, the most commonly used CID-dependent molecular tool is the rapamycin-inducible phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] 5-phosphatase. It was indepen-
monomeric G-proteins relies on the translocation of their cognate guanine nucleotide exchange factors (GEFs) [16,20,40,41]. The latter mode of stimulation assures physiologically relevant activation levels (only endogenously available amounts of the G-protein can be activated), as it does not require over-expression of the mutated G-protein isoforms. Currently, various small GTPases of the Rho, Ras and Arf families have been successfully activated by chemical dimers [14–17,20,40,41] and it is plausible that inducible activation of other monomeric G-proteins could be achieved in a similar manner. Surprisingly, the group of heterotrimeric G-proteins, an important family of intracellular signal transducers, has joined the set of the rapamycin-inducible signaling molecules only recently [39]. \(G_{\alpha q}\) and \(G_{\alpha s}\), crucial mediators of cellular calcium and cAMP signaling, respectively, have been successfully activated by induced plasma membrane recruitment. Also \(G_{\beta y}\) heterodimers have been shown to possess independent signaling functions by affecting intracellular calcium transients [39]. Interestingly, rapamycin-dependent \(G_{\beta y}\) dimers have been previously reported in the literature. Inducible recruitment of the heterodimer to the Golgi membranes was used to study \(G_{\beta y}\) effects on the integrity of this organelle [61], but not to assess \(G_{\beta y}\) signaling competence at the plasma membrane, the major site of action of heterotrimeric G-proteins.

Engineered kinase alleles which can be allosterically activated by rapamycin and its analogues were introduced by the group of Klaus Hahn and constitute another recent addition to the palette of CID-dependent tools for manipulating cellular signal transduction [29]. Inducible activation of different serine/threonine and tyrosine protein kinases (FAK (focal adhesion kinase), Src and p38) was achieved by inserting an engineered FKBP variant into the kinase catalytic domain. Upon addition of rapamycin, binding of inserted FKBP and co-expressed FRB molecule resulted in selective activation of the engineered kinase constructs. This approach to inducible activation does not require translocation of the initially mislocalized protein constructs, thus the modified kinase is activated at its endogenous intracellular location. The generic nature of this method holds the promise that other kinases are also amenable to such a form of activation. Importantly, the exploratory value of these novel tools is even more enhanced by further developments of Hahn and co-workers, namely caged rapamycin derivatives and light-induced activation of kinase constructs [62] (see below).

4. Further applications of inducible heterodimerization

Among the plethora of applications for inducible heterodimerization and related techniques, there are many that do not rely on the acute and selective activation of signal transduction pathways. However, many of these applications provide very important tools for studying/manipulating various aspects of cell biology and have direct repercussions on the intracellular signal transduction. Below selected examples of such applications are described. A more complete overview on CID-related techniques is available elsewhere [11–13].

Conditional protein splicing developed in the group of Tom Muir is one of the prominent methods to achieve post-translational activation of protein constructs [63–66]. The protein of interest can be divided into two separate polypeptides, each expressed as a separate fusion with one of the rapamycin binding domains (FKBP or FRB). These fusions are additionally equipped with one of the two fragments of a split intein. In the presence of the heterodimerizer, binding of FKBP and FRB induces proximity of the two intein fragments resulting in the formation of a functional intein and leading to protein splicing. The latter allows covalent linkage of the two parts of the protein of interest and reconstitutes the
Table 1
Overview of the CID-dependent signaling modules.

| Protein target | Site of action | POI construct | Membrane anchor | Monitored event | References |
|----------------|---------------|---------------|-----------------|-----------------|------------|
| Akt            | PM            | FRB-Akt2      | PM               | 2xFKBP-Akt1 APH | [23,25]    |
| c-Abl          | PM            | PM-Abl-2xFKBP | –               | Akt/p70S6K     | [26]       |
| Fyn            | PM            | FKBP-Fyn      | PM              | Akt/PKB activation | [23–25] |
| Lck            | PM            | FKBP-Lck      | PM              | Akt/PKB activation | [23–25] |
| Lyn            | PM            | FKBP-Lyn      | PM              | Akt/PKB activation | [23–25] |
| β-Arrestin 2   | GPCR and PM   | FRB-β-arrestin | V1aR/2V2R-FKB   | GPCR internalization, ERK activation | [28] |
| FAK, Src, p38α | Endogenous location | Variant of FKBP inserted into catalytic domain of respective kinase | – | Kinase activation | [29] |
| Fas            | PM            | PM-Abl-2xFKBP | PM              | Fas activation, induction of apoptosis | [30–32] |
| FGFR           | PM            | FKBP-FGFR1    | PM              | RTK activation by homo- and heterodimerization | [33] |
| ErBb           | PM            | p75-527-533   | PM              | – | [34] |
| c-Kit          | PM            | PM-c-Kit-2xFKBP | PM              | NRTK activation | [26] |
| PDGFR          | PM            | PM-FDGFR     | PM              | NRTK activation | [26] |
| IR             | PM            | PM-Inp54p     | PM              | NRTK activation | [26] |
| Gxq, Gαs, Gβγ | PM            | FRB-Gxq-C9S, C10S, Q209L | PM | Intraacellular calcium and cAMP signaling | [39] |
| Myotubularin 1 | Endosome      | FRB-myotubularin | 2xFKBP-Rab5a | Depletion of endosomal PtdIns3P pool | [18,22] |
| PH domain of PLCδ | PM/mt       | FRB-PH PLCδ  | PM              | Unmasking of PtdIns4P at the PM | [20] |
| PI3K           | PM            | FKBP-p85P15K(e1420-615) | PM | Synthesis of PtdIns(4,5)P2 from PtdIns4P | [19,20] |
| PI4P5K         | PM            | FKBP-PI4P5K(e1a79-635) | PM | Synthesis of PtdIns(3,4,5)P3 from PtdIns4P | [19,20] |
| PtdIns(4,5)P2 5PTase | PM           | FKBP-InP54p(aa121-331) | PM | Depletion of the PM PtdIns(4,5)P2 pool | [19,21] |
| Rac1           | PM            | Rac1 G12 V-FRB | CD25516-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Rac2           | PM            | FKBP-Rac1(2/V1aR)/ACAX | CD25516-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Tiam1          | PM            | FKBP-Tiam1(aa1012-1591) | PM | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Cdc42          | CD25512-2xFKBP | CD25512-2xFKBP | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| RhoA           | PM            | FKBP-RhoA(C10A5) | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Ras            | PM            | FKBP-RasGRF(aa1003-1273) | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Sos            | PM            | 3xFKBP-Sos1 | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Rem            | PM            | FKBP-Rem(aa2a2-265) | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| Sec7 (ArfGGEF) | PM            | FKBP-Sec7(arfgef) | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| STIM1          | ER            | FRB/2xFKBP replacing EF-hand and SAM domains of STIM1 | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| TCR, TGF-βRI, TGF-βRII | PM          | Inter alia: | CD25512-2xFKBP | Small G-protein activation/inactivation, PM protrusions | [14,16] |
| VAMP2/Synaptobrevin | Presynaptic vesicles | VAMP2-2xFKBP | CD25512-2xFKBP | Inhibition of synaptic transmission | [49] |
| WASP           | PM            | FRB-WASP-APH  | CD25512-2xFKBP | PM protrusions | [15] |

Abbreviations used: cAMP – cyclic adenosine monophosphate, CD – cytoplasmic domain, CyP – cyclophilin, ED – extracellular domain, ER – endoplasmic reticulum, ERK – extracellular signal-regulated kinase, FAK – focal adhesion kinase, FGFR – fibroblast growth factor receptor, GEF – guanine nucleotide exchange factor, GPCR – G-protein-coupled receptor, IR – insulin receptor, IRβ – insulin receptor β chain, MoA – monoamine oxidase, mt – outer mitochondrial membrane, NRTK – non-receptor tyrosine kinase, PDGFR – platelet-derived growth factor receptor, PH – pleckstrin homology, PI3K – phosphoinositide 3-kinase, PI4P – phosphatidylinositol 4-phosphate, PI4P5K – phosphatidylinositol 4-phosphate 5-kinase, PKB – protein kinase B, PLC – phospholipase C, PM – plasma membrane, plasma membrane targeting peptide, PtdIns(4,5)P2 – phosphatidylinositol 4,5-bisphosphate, PtdIns(4,5)P2 5PTase – phosphatidylinositol 4,5-bisphosphate 5-phosphatase, PtdIns3P – phosphatidylinositol 3-phosphate, RTK – receptor tyrosine kinase, SAM – sterile alpha motif, SOCE – store-operated calcium entry, STIM1 – stromal interaction molecule 1, TCR – T cell receptor, TGF-βRII – transforming growth factor β receptor I/II, TM – transmembrane domain, V1aR – vasopressin receptor type 1a, V2R – vasopressin receptor type 2, VAMP2 – vesicle-associated membrane protein 2, WASP – Wiskott–Aldrich syndrome protein.

*Detailed design of the construct not described.*
functional enzyme/protein molecule in a rapamycin-dependent fashion.

Another approach to regulate protein function by the Muir group addresses the regulation of protein stability by reconstitution of a split ubiquitin (Ub) molecule [67]. In this method, called SURF (split ubiquitin for the rescue of function), the protein of interest is fused to a split Ub fragment tagged with FRB and a degradation signal which leads to constitutive proteasomal degradation of the fusion construct. In the presence of the dimerizer and the FKBP fusion of the second Ub fragment, the functional Ub molecule forms. Upon cleavage by endogenous deubiquitinating enzymes, the protein of interest becomes released from the degradation signal and stays intact. There are numerous other methods to regulate protein stability/degradation in a chemically-controlled manner [2–4]. For example, inducible protein degradation was achieved by rapamycin-dependent targeting of proteins to the subunits of the yeast proteasome [68]. In another method, the protein of interest was fused to a destabilizing variant of the FRB domain, which promoted constitutive degradation of the entire fusion construct. In this system, the protein of interest is inducibly stabilized upon dimerizer/FKBP binding [69,70]. Yet another approach, developed by Tom Wandless and collaborators, relies on mutated variants of FKBP, which, upon fusion to the protein of interest, influence the stability of the construct. Depending on the particular variant of the method, addition of the small molecule ligand of FKBP leads either to construct stabilization [71,72] or degradation [73].

A novel method for inducible inactivation of proteins on a time scale of seconds to minutes is provided by the so-called anchor-away approach. When applied in yeast, this technology led to rapamycin-induced trapping of nuclear proteins (fused to FRB) in the cytoplasm, by complexing it with the FKBP-tagged large ribosomal subunit [74]. Another variant of the anchor-away approach for rapid inducible protein inactivation was applied in HeLa cells [75]: the endogenous pool of the protein of interest, in this case adaptor protein-1 and -2 (AP-1/2), was depleted by siRNA and, simultaneously, the knock-down resistant copies of the protein were expressed as FKBP fusions. When FRB domains were expressed on the surface of the outer mitochondrial membrane, addition of rapamycin caused fast inactivation of the protein of interest by its rerouting to the mitochondrial membrane, resulting in impaired intracellular vesicle trafficking. Due to its speed of action, which minimizes the risk of compensatory mechanisms, the anchor-away technique, although relatively cumbersome, may present a valuable alternative to standard protein knock-down by siRNA. It is worth noting that the recently described method of inducible liberation of phosphatidylinositol 4,5-bisphosphate at the plasma membrane principally relies on the anchor-away approach [20].

Inducible regulation of nuclear trafficking is closely related to CID-dependent re-routing of intracellular proteins within the anchor-away method. Until now, rapamycin-induced nuclear import/export has been most meticulously studied in yeast [76–78], although reports on its application in mammalian cultured cells are also available [79–81]. In both cases, induced shuttling of proteins from the nucleus to the cytoplasm (or vice versa) is a relatively slow process, requiring tens of minutes until the steady state is reached [76,79,80]. This most probably relates to the endogenous kinetics of nuclear trafficking. Nevertheless, inducible nuclear translocation of proteins constitutes a valuable tool, which could be used for example to study DNA replication, transcription, or DNA repair.

The reversible aggregation approach takes advantage of the FKBP-F36M mutant, which has the tendency to spontaneously dimerize in the absence of its cognate ligand (AP21998, a synthetic rapamycin mimic) [82]. Thus, protein fusions equipped with several copies of FKBP-F36M tend to create macromolecular clusters, which can be reversibly solubilized by addition of the drug. Notably, this approach has been adapted to trigger protein secretion, which was induced by drug-evoked disruption of molecule clusters within the endoplasmic reticulum [11,83,84].

5. Effects of rapamycin on cell function and the use of rapalogs

As already mentioned, rapamycin is an immunosuppressant in that it potently inhibits TORC1, an important protein kinase involved in numerous aspects of cell biology such as cell growth, proliferation and regulation of autophagy [7,85]. Therefore, application of rapamycin in cellulo or in vivo may have pleiotropic detrimental effects on the living system under investigation [7]. In some applications in cultured cells, the short experiment time may justify application of rapamycin, as it takes usually longer periods of time to unravel its potential toxic effects. Otherwise, application of non-toxic synthetic rapamycin analogues (so-called rapalogs) may be highly advisable. Rapalogs (Fig. 3) have been created by using the ‘bump-and-hole’ approach [6,86]. The part of rapamycin skeleton which is responsible for binding of the endogenous FRB domain of TOR becomes equipped with a bulky substituent (the ‘bump’), which abolishes any binding to wild type FRB. In order to restore the dimerizing potency of such a modified molecule, a heterologously expressed FRB domain contains mutations which enlarge its rapamycin-binding pocket (the ‘hole’) to accommodate the bump. As a result, the rapalog molecule is devoid of inhibitory effects towards TOR kinase, but still readily induces dimerization of FKBP and the mutated FRB domain. There are several examples of different rapamycins in the literature (Fig. 3) [6,16,78,81], with the most popular compound AP21967 being commercially available. It is important to note that particular care must be taken while synthesizing and purifying rapalogs in house, since even minute contamination with rapamycin causes extensive inhibition of TOR kinase [87]. Interestingly, the toxicity of rapamycin is not a big obstacle for applications in yeast, since there are rapamycin-insensitive strains with TOR mutations and deleted endogenous FKBP12 [74,78].

6. Reversibility and caged rapamycin

Because of the high affinity of rapamycin towards its protein binding partners ($K_{d} = 0.2 \text{nM}$ for rapamycin–FKBP binding and $K_{d} = 12 \text{nM}$ for FKBP-rapamycin–FRB [88]), rapamycin-inducible tools for manipulating signal transduction can be considered irreversible (in cellulo), even though in some cases out-competing FKBP binding to rapamycin by high concentrations of FK506 is attempted, usually with relatively moderate success (e.g. [89]).

Recent advances in the rapamycin-inducible protein dimerization technique allow both high temporal and increased spatial control of the activated event. This can be achieved by applying caged rapamycin, i.e. rapamycin molecules equipped with a photo-labile substituent. Ideally, after applying caged rapamycin onto cells, the presence of the substituent should prevent effective dimerization between FRB and FKBP domains. Local and brief illumination with light would release the functional dimerizer, thus inducing the FRB–FKBP interactions only in the illuminated region. Currently available caged dimerizers do not fulfill these ideal expectations yet, but nevertheless constitute a big step in this direction. cRb, developed in the lab of Takanari Inoue [90], features a nitrobenzyl cage linked to HE-Rapa, a rapamycin analogue which, just as its parent molecule, is a potent dimerizer of FRB and FKBP proteins (Fig. 4A). The cage connects the dimerizer with a biotin molecule. Unfortunately, cRb was still able to induce interactions between FRB and FKBP, indicating that the steric hindrance of biotin and the nitrobenzyl cage was insufficient to prevent FRB–FKBP heterodimerization. However, incubation of cRb with avidin prior to its application onto cells resulted in trapping of the dimerizer in the
extracellular medium, until the photolinker was destroyed by irradiation with light. After local illumination of the cell boundary with UV light, dimerizer-induced plasma membrane ruffle formation (by activation of CID-dependent Rac1 GEF) was observed, mostly restricted to the vicinity of the irradiated area of the cell. While an elegant solution to the problem of ‘leaky’ rapamycin derivatives, this approach to spatial control of CID-regulated events has a few limitations. Most of all, its application may be restricted to events happening at the plasma membrane. In addition, diffusion of the light-liberated dimerizer may hinder attempts to induce dimerization in a highly spatially restricted manner.

In an alternative approach, Hahn and co-workers expanded the concept from CID-regulated protein kinases [29] to light-inducible ones [62]. pRap consists of a rapamycin molecule, linked at its C40 position to a nitrobenzyl photo-cage (Fig. 4B). Interestingly, in this case the steric obstacle was sufficient to inhibit binding of FRB and engineered FKBP (iFKBP, inserted into the catalytic domain of FAK). Upon illumination with UV light, pRap released rapamycin which effectively activated the inducible kinase construct. However, until now pRap has not been demonstrated to be activatable with sub-cellular spatial resolution. Moreover, for both novel caged rapamycin derivatives (i.e. cRb and pRap) no thorough analyses were performed of how modifications of the rapamycin skeleton influence the membrane permeability of the respective compounds. It is conceivable that the caged species exhibit much reduced potential of diffusing through the lipid bilayer. Then, at least to some extent, uncaging would simply serve to release molecules which cross the plasma membrane more readily, rather than to remove the steric hindrance of FRB–FKBP binding. Certainly, more work is needed to fully establish reliable caged dimers. Their potential use in vivo by applying two-photon uncaging should provide a sufficient compensation for the significant synthetic effort.

7. Alternative chemical dimerizers

While rapalog-based dimerization approaches hold great promise for manipulating cell function, the methods still have limitations. For instance, despite the proven selectivity of certain rapalogs towards particular mutants of the FRB domain [81], to our knowledge there are no communications on successful application of two different rapamycin derivatives simultaneously in the same cell for regulating two processes in an orthogonal fashion. Such an approach would require using FKBP as a universal anchor for protein translocation, and two different FRB domain mutants within the independently translocatable fusion constructs. A truly orthogonal solution could probably be best provided by using CIDs that employ totally different molecular components such as SNAP tag [91] or beta-lactamase fusions [92,93]. A review of cross-linking alternatives has recently been compiled [94].

An appealing approach to orthogonal chemical dimerizers relies on engineered natural compounds (and their receptor proteins) from biological pathways which are distinct from the ones present in animal cells. Recent reports describe application of two different plant hormones which successfully act as inducers of dimerization. In one promising example, engineering of the abscisic acid (ABA) signaling pathway resulted in the development of a novel heterodimerizer, with a structure completely unrelated to rapamycin (Fig. 4C) [95]. As a matter of fact, ABA was used concomitantly with rapamycin in a truly orthogonal fashion. However, in the current design of this novel heterodimerization system, the ABA-induction kinetics seems to be severely inferior to the one of rapamycin and its analogues. Another new example of a similar strategy comes from Miyamoto and colleagues [96], who adopted the plant hormone gibberellin (GA3) as an inducer of dimerization. To circumvent the problems of membrane permeability of GA3, its negatively charged carboxyl group was masked by an acetoxy-methyl (AM) group, resulting in GA3-AM (Fig. 4D). Once in the cytoplasm, the protecting AM group is readily hydrolyzed by endogenous esterases, thus liberating a functional dimerizer molecule. Application of GA3-AM onto cells resulted in rapid (comparable to the speed of action of rapamycin) dimerization of engineered gibberellin binding proteins. Moreover, rapamycin and GA3-AM were shown to act independently from each other, enabling precise and timely control of two different intracellular events within a single living cell. With this quite spectacular achievement, chemical biology is approaching a fascinating possibility of constructing complex artificial signaling networks in cellulo and in vivo. Analysis of the biological outcome will certainly shed new light on endogenous signal transduction mechanisms.

8. Limitations and future developments

During the past decade, the chemical dimerizer concept has emerged as a truly orthogonal technique for manipulating enzyme activity in living cells. The truth is, however, that it is only used by a handful of labs because the cloning effort is fairly high and the constructs are not readily available. For this reason, it would be beneficial to have generally available stably transfected cell lines featuring, for instance, rapamycin-binding domains as membrane-targeted constructs. So far, one of the limitations when using rapamycin, rapalogs or even caged rapamycin derivatives is the lack of switching off the signal, as rapamycin is practically not released from its binding proteins. It would therefore be fascinating to have a photo-destructable
version that would permit the generation of transient signaling patterns mimicking those observed in intact cells. The largest impact, however, would have the generation of new orthogonal chemical dimerizers and their use in a physiologically relevant model system. The fully independent activation of several branches of a signaling network in a time-dependent fashion would open new doors for studying the crosstalk of signaling elements.

9. Conclusion

Chemical dimerizers such as rapamycin are some of the few fast acting molecules that can be combined with genetically encoded signaling components, meaning that enzymes may be activated independently of a ligand designed to bind the enzyme. This complements the artificial activation of signaling molecules by membrane-permeant allosteric enzyme activators such as second messenger mimics [97]. The combinatorial use of these fast acting tools will be essential to unravel complex intracellular networks and for validating models produced by systems biology efforts in the future.

Acknowledgements

We apologize for not being able to mention all relevant work in the field due to space restrictions. Work of the Schultz lab is funded by the EMBL, the ESF (EuroMembrane TraPPs, Schu 943/7-1), the DFG (Transregio 83), the Helmholtz Association (SBCancer and LungSysII), the German Lung Research Center and the EU (Integrated project LIVIMODE). The authors declare no competing financial interests.
References

[1] Ding, Z. et al. (2011) Physical association of PKD1 with AKT1 is sufficient for pathway activation independent of membrane localization and AKT1 carboxy terminus. PLoS ONE 6, e18096.

[2] Ouyang, X. and Chen, J.K. (2010) Synthetic strategies for embryonic development. Chem. Biol. 17, 590–606.

[3] Raina, K. and Creswe, C.M. (2010) Chemical inducers of targeted protein degradation. J. Biol. Chem. 285, 11057–11060.

[4] Stankunas, K. and Crabtree, G.R. (2007) Exploiting protein destruction for constructive use. Proc. Natl. Acad. Sci. USA 104, 11111–11112.

[5] Hinterding, K., Alonso-Diaz, A. and Waldmann, H. (1998) Organic Synthesis and Bifunctional Signal Transducer. Angew. Chem., Int. Ed. 37, 688–749.

[6] Clackson, T. (2007) Controlling Protein–Protein Interactions Using Chemical Inducers and Disrupters of Dimerization: in Chemical Biology. From Small Molecules to Systems Biology and Drug Design (Schreiber, S.L., Kapoor, T.M. and Biological Signal Transduction. Angew. Chem., Int. Ed. 37, 688–749.

[7] Zoncu, R., Efeyan, A. and Sabatini, D.M. (2011) MTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell. Biol. 12, 21–35.

[8] Liang, J., Choi, J. and Clardy, J. (1999) Refined structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Crystallogr. 55, 736–744.

[9] Choi, J., Chen, J., Schreiber, S.L. and Clardy, J. (1998) Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science 273, 239–247.

[10] Rivera, V.M. et al. (1996) A humanized system for pharmacologic control of Freiberg, R.A., Spencer, D.M., Choate, K.A., Peng, P.D., Schreiber, S.L., Crabtree, G.R. and Khavari, P.A. (1996) Specific triggering of the Fas signal transduction pathway in normal human keratinocytes. J. Biol. Chem. 271, 31666–31669.

[11] Burgoyne, R.D. (2010) Role of phosphoinositides in STIM1 dynamics and store-operated calcium entry. Biochem. J. 434, 533–542.

[12] Zhan, L., Xiang, B. and Muthuswamy, S.K. (2006) Organelle-specific, rapid induction of molecular activities and protein expression. Proc. Natl. Acad. Sci. USA 104, 11491–11496.

[13] Muthuswamy, S.K., Gilman, M. and Brugge, J.S. (1999) Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol. Cell. Biol. 19, 6845–6857.

[14] Pruschy, M.N., Spencer, D.M., Kapoor, T.M., Miyake, H., Crabtree, G.R. and Schreiber, S.L. (1994) Mechanistic studies of a signaling pathway activated by the organic dimerizer FK1012. Chem. Biol. 1, 163–172.

[15] Spencer, D.M., Wandless, T.J., Schreiber, S.L. and Crabtree, G.R. (1993) SIG1-Orai1 involves an intramolecular switching mechanism. Sci. Signal. 3, 115–136.

[16] Prakriya, M., Wu, M.M. and Lewis, R.S. (2008) Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. Nature 454, 533–542.

[17] Zoncu, R. et al. (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. USA 104, 3793–3798.

[18] Ružička, T. (2010) Organelle-specific, rapid induction of molecular activities and protein expression. Proc. Natl. Acad. Sci. USA 104, 11511–11512.

[19] Suh, B.C., Inoue, T., Meyer, T. and Hille, B. (2006) Rapid chemically induced pathway activation independent of membrane localization and gene expression. Nat. Med. 2, 1028–1032.

[20] Korzeniowski, M.K., Manjarres, I.M., Varnai, P. and Balla, T. (2009) Activation of ONE 3, e3068.

[21] Luik, R.M., Wang, B., Prakriya, M., Wu, M.M. and Lewis, R.S. (2008) Rapid and reversible chemical inactivation of synaptic transmission in genetically targeted neurons. Neuron 48, 727–735.

[22] Zoncu, R. et al. (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. USA 104, 3793–3798.

[23] Li, B., Desu, S.A., MacCorlkie-Chosnek, R.A., Fan, L. and Spencer, D.M. (2002) A novel conditional cell survival switch reversibly protects cells from apoptosis. Gene Ther. 9, 233–244.

[24] Li, B., Sun, A., Youn, H., Hong, Y., Terranova, P.F., Thrasher, J.B., Xu, P. and Spencer, D.M. (2007) Controlling signal transduction with synthetic ligands. Science 262, 1019–1024.

[25] Holsinger, L.J., Spencer, D.M., Austin, D.J., Schreiber, S.L. and Crabtree, G.R. (1996) Specific triggering of the Fas signal transduction pathway in normal human keratinocytes. J. Biol. Chem. 271, 31666–31669.

[26] Belshaw, P.J., Chen, L., Ho, S.N., Randazzo, F., Crabtree, G.R. and Schreiber, S.L. (1996) Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. Cell Biol. 6, 837–847.

[27] Belshaw, P.J., Spencer, D.M., Crabtree, G.R. and Schreiber, S.L. (1996) Controlling programmed cell death with a cyclophilin-cyclosporin-based chemical inducer of dimerization. Chem. Biol. 3, 731–738.

[28] Zoncu, R. et al. (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. USA 104, 3793–3798.

[29] Zoncu, R. et al. (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. USA 104, 3793–3798.

[30] Zoncu, R. et al. (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc. Natl. Acad. Sci. USA 104, 3793–3798.

[31] Nakatsu, F., Perera, R.M., Lucast, L., Zoncu, R., Domin, J., Gertler, F.M., Toomre, D. and De Camilli, P. (2010) The inositol 5-phosphatase SHIP2 regulates endocytic clathrin-coated pit dynamics. J. Biol. Chem. 295, 2037–2045.

[32] Belshaw, P.J., Chen, L., Ho, S.N., Randazzo, F., Crabtree, G.R. and Schreiber, S.L. (1996) Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. Cell Biol. 6, 837–847.

[33] Belshaw, P.J., Spencer, D.M., Crabtree, G.R. and Schreiber, S.L. (1996) Controlling programmed cell death with a cyclophilin-cyclosporin-based chemical inducer of dimerization. Chem. Biol. 3, 731–738.

[34] Freeman, K.W. et al. (2003) Conditional activation of fibroblast growth factor receptors (FGFR) I, but not FGFR2, in prostate cancer cells leads to increased osteopontin induction, extracellular signal-regulated kinase activation, and in vivo proliferation. Cancer Res. 63, 6237–6243.

[35] Suh, B.C., Inoue, T., Blau, C.A., Rotheke, H. and Murry, C.E. (2001) Control of myoblast proliferation with a synthetic ligand. J. Biol. Chem. 276, 4119–41196.

[36] Rivera, V.M. et al. (1996) A humanized system for pharmacologic control of Freiberg, R.A., Spencer, D.M., Choate, K.A., Peng, P.D., Schreiber, S.L., Crabtree, G.R. and Khavari, P.A. (1996) Specific triggering of the Fas signal transduction pathway in normal human keratinocytes. J. Biol. Chem. 271, 31666–31669.

[37] Belshaw, P.J., Chen, L., Ho, S.N., Randazzo, F., Crabtree, G.R. and Schreiber, S.L. (1996) Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. Cell Biol. 6, 837–847.
