Chemical biology beyond binary codes

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The cellular chemical machinery controlling all living processes is mediated by macroassemblies of enzymes and nucleic acids. These complexes form, break up in different orders and reorganize in ways that result in the chain of events that we call a biochemical 'pathway'. However, all of the methods available to study protein interactions in living cells allow only the observation or recreation of binary complexes. Two recent reports and earlier studies describe the use of chemical 'dimerizers' that can dynamically induce assembly or disassemby of protein complexes. The dimerizer tools create the opportunity to understand the 'real' biology of macromolecular assembly.

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Keywords: Controlled therapeutic delivery; FKBP12; Immunophilin protein; Macromolecular assembler

Received: **27 June 2000**
Revisions requested: **3 august 2000**
Revisions received: **23 August 2000**
Accepted: **26 September 2000**

Published: **10 October 2000**

Chemistry & Biology 2000, **7**:R217–R221

1074-5521/00/$ – see front matter

PII: S1074-5521(00)00040-5

Those of us who, at one time or another in their careers, have studied the immunophilin proteins have learned two things: never to be surprized by new discoveries about the biological functions of these proteins nor by their ingenious application to probing or even recreating biochemical processes. In two recent reports, Rollins et al. and Rivera et al. demonstrate yet another significant example of how these proteins can be put to work to study cellular biochemistry [1,2]. Based on recent results from Tim Clackson's group at Ariad Pharmaceuticals and in collaboration with Jim Rothman at the Sloan-Kettering Cancer Institute and Lelio Orci at the University of Geneva, these reports describe an elegant strategy to assemble and disassemble complexes of the immunophilin FKBP12 and apply this approach to create cells that can secrete biologically active hormones under the control of designed FK506 analogs. These results suggest a novel strategy for controlled therapeutic hormone delivery in humans. Furthermore, they suggest the possibility of a strategy by which we may be able to go beyond simple binary approaches to studying activated responses to protein assembly in living cells: a way to probe or recreate the macroassemblies of proteins and nucleic acids that make up the chemical machinery of the cell.

What makes FKBP12 interesting as a tool in chemical biology, indeed both physical realization and metaphor for the whole field, is its relationship to the natural products FK506 and rapamycin. These two macrocyclic lactone lactams share an identical binding surface for FKBP12 while differing significantly in size, organization and functionality in the remaining portions of the two macrocycles. Via the common FKBP12 binding domain, they form high affinity complexes with FKBP and can compete with each other for binding. Both of these compounds are immunosupressants, and the most significant early discovery concerning their mechanisms of action was that their biological effects required as an obligatory but not sufficient formation of a complex with FKBP12. The rapamycin or FK506–FKBP12 complexes were shown to bind to and inhibit different target enzymes [3–6]. Thus, the compounds and FKBP12 are prodrugs, acting on specific targets via interactions of a composite surface composed of FKBP12 and drug moieties. Since then, FKBP12, its sister immunophilin, cyclophilin, and their natural product partners in crime have been modified in ways to afford the probing of other cellular processes in a number of ways [7–11]. In a first example, synthesis of a dimeric FK506 (called appropriately FK1012) allowed the creation of definable and inducible dimeric or multimeric assemblies of proteins that could activate biochemical cascades including growth factor-mediated signal transduction pathways with potential applications as gene therapy strategies [10,12,13].

The two articles discussed here describe the development of an elegant, complementary approach to the oligomerizers described above, as well as an ingenious application, using an alternative macromolecular assembler based on FKBP12. The story begins with the observation that a single point mutation (Phe36Met) in the FK506 binding site of FKBP12 resulted in self association into a homodimer with a dissociation constant of about 30 μM that could be disrupted by FK506 and analogs of FK506. Crystallographic analysis of the complex revealed a symmetrical

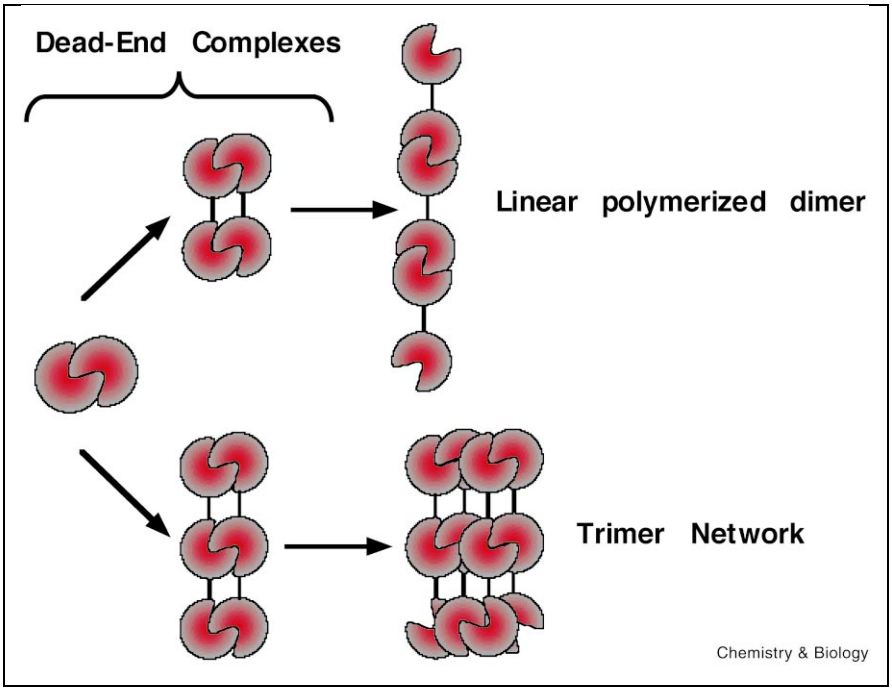

Figure 1. A schematic representation of FKBP fusion oligomerizers. A single FKBP (F_M1) will form a dead-end dimer, whereas a fusion of two (F_M2) FKBP monomers can form a linear polymer and a fusion of three (F_M3) dimerizers can result in higher order complexes.

dimer of the Phe36Met mutant and most interestingly, the interaction is between one of the 'effector' loops (necessary for binding to the FK506–FKBP12 target calcineurin) and the FK506 binding site [1].

The FKBP-Met36 mutant could be used in an alternative, negative dimerization system, complementary to the FK1012 induced dimerizer FKBP12. So for instance, fusion of FKBP-Met36 to two proteins, that when brought together by dimerization of FKBP12, induce some biochemical process, could be reversed by the addition of FK506, rapamycin, or non-natural analogs. However, the real fun begins when one sees how fusions of more than one FKBP-Met36 assemble in living cells. To study this, the naturally fluorescent green fluorescent protein (GFP) was fused to chains of FKBP-Met36 consisting of one to six repeats of the protein sequence (referred to as F_M, F_M2, etc.). Mammalian cells transfected with GFP fused to F_M, or F_M2, showed an even distribution throughout the cell. However, fusions of three or four repeats revealed an interesting phenomenon; one starts to observe large clusters of the fusions. Further, increasing the number of repeats of F_M led to larger clusters. In addition it was shown that the clustering could be directed to a specific compartment, the nucleus, by addition of a basic nuclear localization sequence to the polypeptide. Finally, it was shown that clustering could be reversed by an FK506 binding domain analog (AP22542) or induced by its removal from cells. Simple geometric arguments can be made for why such aggregation occurs as shown in Figure 1. Obviously, monomeric F_M is a 'dead-end' complex when it dimerizes. The addition of a second F_M domain can lead to a dead-end dimer, but as well, could form linear polymers. While these strings of fusions cannot be resolved by fluorescent microscopy, nevertheless, this form of F_M fusion might have some interesting applications. It is then obvious why three is the critical number of F_M monomers necessary to begin to see clustering. It is only at this point that assemblies of high density can begin to form. This would likely lead to spontaneous and perhaps cooperative assembly. But not too cooperative; the fact that AP22542 addition could disrupt complexes so rapidly, suggests that if there is any change in the effective dissociation constants of F_M oligomers themselves, it is only a marginal decrease. This is good news, as the rapid reversibility of assembly is a key feature of this phenomenon with many potential applications to the study of protein assembly in intact, living cells. The same sort of behavior has occurred with the induced 'dimerizer' systems described above. For example, the first study using the FKBP12 FK1012 dimerizer used fusions of three FKBP12s to mimic clustering of T-cell receptor intracellular domains at the inner plasma membrane [10].

An immediate application of reversible oligomerization was presented by Rivera et al. [2] (Figure 2). They harnessed the machinery used by eukaryotic cells to do quality control on proteins destined for secretion, to turn mammalian cells, otherwize not designed to do so, into controlled secretors of the therapeutic proteins, insulin and human

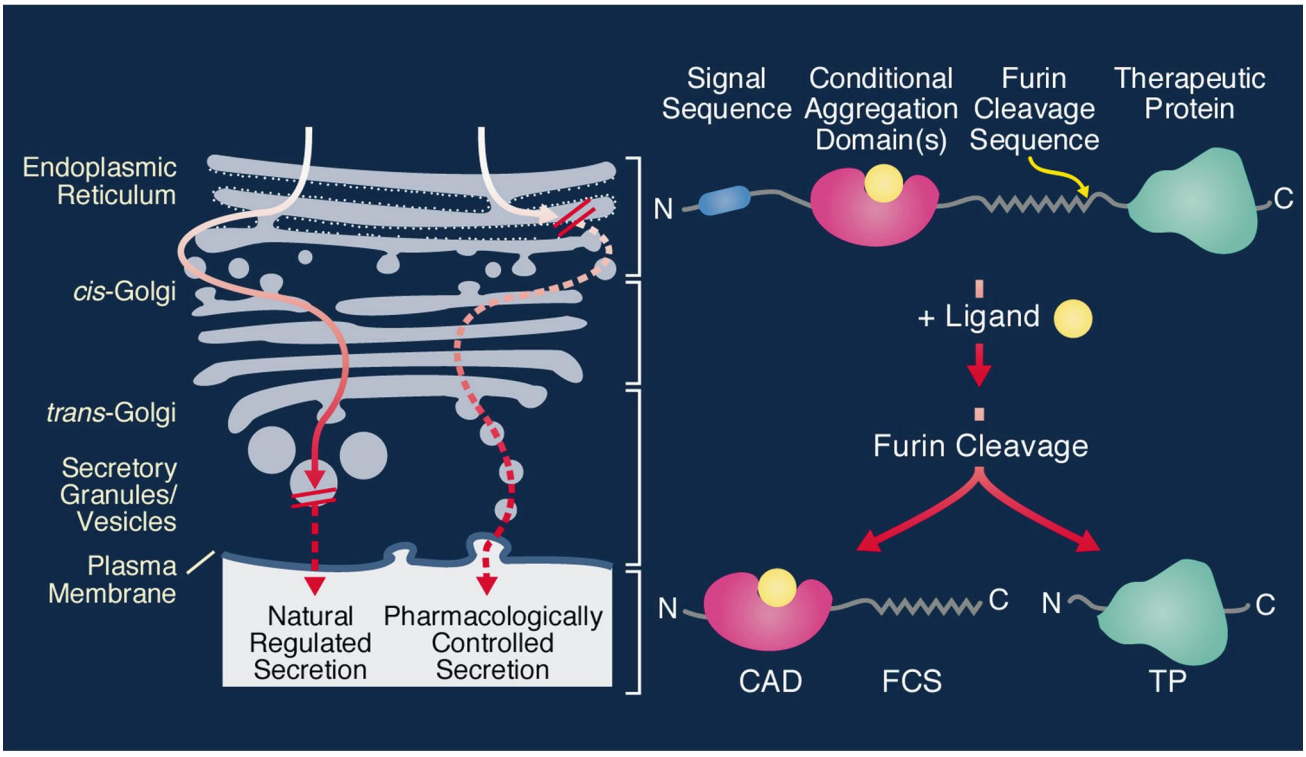

Figure 2. Natural control of protein secretion (left) is contrasted with the scheme for controlling secretion using the FKBP mutant F36M and its small molecule ligands (right), in which proteins are reversibly stored in the ER. A protein of interest is fused to multiple copies of the FKBP mutant and a secretion signal sequence to direct the fusion protein to the ER, where multivalent FKBP interactions lead to creation of massive aggregates that are retained. Addition of a small molecule ligand dissolves the aggregates, whereupon the proteins traverse the Golgi apparatus and are secreted. Inclusion of a cleavage site for furin, an endopeptidase located exclusively in the *trans* Golgi, leads to the precise release of the protein of interest from the regulatory domains prior to secretion. Courtesy Rivera and Clackson, Ariad Pharmaceuticals ([2]), reprinted with permission from Science.

growth hormone (GH). Proteins that are misfolded, will form aggregates in the endoplasmic reticulum (ER). Such aggregates can remain in complexes in the ER, but if the aggregates are disassembled, they will be processed via coat complex II (COPII) vesicles to the *cis* Golgi, the *trans* Golgi and, finally, be secreted via vesicles that bud off from the *trans* Golgi. Combining this knowledge with their observations on F_M assembly, Rivera et al. demonstrated that they could use the chemically induced disassembly of F_M complexes, fused to proinsulin or GH to control their processing and secretion via the Golgi apparatus. They made fusions of pro-insulin or GH to F_M4 via a peptide linker containing a consensus *trans* Golgi protease (furan) cleavage site and, finally, an ER directing signal sequence on the N-termini of the fusions. At the last step of processing the proteins, it was reasoned that furan would cleave insulin and GH from F_M4, resulting in mature, active hormone being secreted. After transfection into mammalian cells, they were able to show that indeed, aggregation of fusions occurred in the ER and that AP21998, another FK506 binding domain analog, disrupted these aggregates and resulted in a dose dependent secretion of mature insulin and GH. Even more remarkably, they were able to repeat this feat in living animals. By implanting cells transfected with the F_M4-proinsulin or GH fusions in muscle tissue of mice, they were able to observe AP22542 induced, dose dependent secretion of the two proteins that could be detected in the plasma of implanted animals. In the case of animals implanted with F_M4-proinsulin, they also observed a concomitant decrease in plasma glucose by intravenous treatment with AP22542. The response was remarkably rapid; induction of mature insulin secretion and decrease in glucose occurring within several minutes of drug treatment. These results raise the exciting possibility of a novel gene therapy strategy in which delivery of protein therapeutics could be finely controlled in dose and in time by small, orally available compounds.

Besides the potential applications of the F_M protein fusions that Rivera et al. describe, these studies both remind

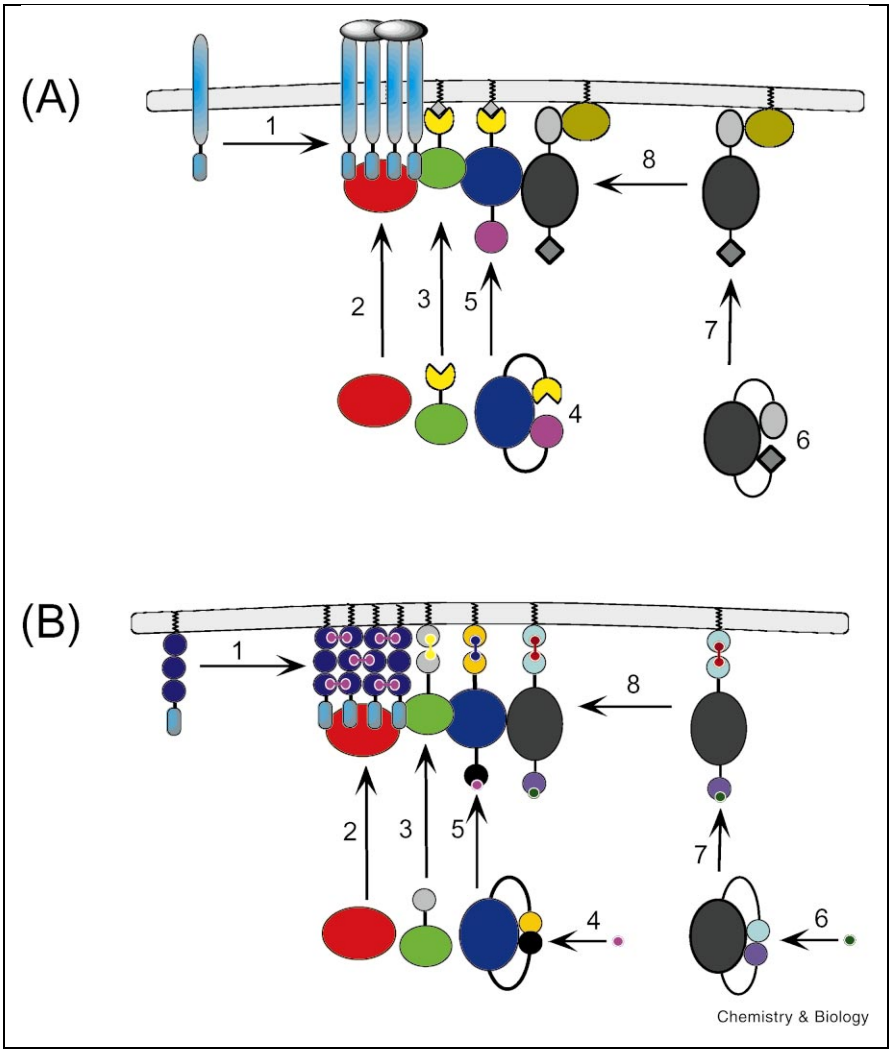

Fig. 3. A rather fanciful example of how FKBP dimerizers could be used to study a protein assembly process. **(A)** describes a signal transduction mechanism in which seven steps are necessary to assemble a signaling complex. Clustering of receptors (1) by a ligand creates a binding surface for one enzyme and its recruitment to the inner membrane surface (2). Simultaneously, two consecutive enzyme–substrate pairs are also recruited (3, 5). In step 3, an enzyme is directly recruited to the membrane by interacting with a membrane anchored protein. Recruitment of another protein to the membrane complex (step 5) requires first, disruption of a complex between N- and C-terminal domains. This protein interacts with the same membrane anchored protein as the protein in step 3. Another intramolecular disruption and membrane recruitment of a protein (steps 6 and 7) are required to bring the last protein into the complex (step 8) via an interaction with another membrane anchored protein. **(B)** Recreation of the protein assembly events described in **(A)** using fusions of FKBPs and dimerizers/antidimerizers. Fusions of three FKBP moieties are in turn fused to the effector domain of the receptor and clustering is induced by a dimerizer based on FK506 (1), causing recruitment of the first protein (2). Recruitment of the other proteins (3, 5 and 7) to the membrane are driven by interaction of mutant FKBPs with specificity for different FK506 analog dimerizers. Spontaneously dimerized FKBPs are used to mimic the closed inactive states of the two last proteins and the open state is created by disruption of FKBP dimers (4 and 6) by two FK506 analogs specific for these forms of FKBP.

us of a basic problem in biology as well as provide us with a powerful new tool to explore this problem. Biochemical processes are mediated by non-covalently associated multi-enzyme complexes [14]. Cellular machineries for transcription, translation and metabolic or signal transduction pathways are examples of processes mediated by multiprotein complexes. The formation of multiprotein complexes produce the most efficient chemical machinery, in which the substrates and products of a series of steps are transferred from one active site to another over minimal distance, with minimal diffusional loss of intermediates, and in chemical environments suited to stabilizing reactive intermediates. Further, physical coupling of enzymes can allow for allosteric regulation of different steps in a chain of reactions [15]. Much of modern biological research is concerned with identifying proteins involved in cellular processes, determining their functions and how, when and where they interact with other proteins involved in specific pathways. For instance, signal transduction 'pathways' in eukaryotes have been shown to in fact consist of both constitutive and transient macro-complexes organized by modular protein domains [16].

Despite the importance of understanding protein assembly to biological processes, there are few currently available methods for studying protein–protein interactions in vivo. Further, of the methods that exist, these can only be used to study interactions one at a time. These include, for example, two-hybrid, fluorescence resonance energy transfer or protein fragment complementation strategies [17–22]. A lot may be learned by applying these approaches, but trying to decipher a biochemical process involving macromolecular assemblies is something like trying to organize a binary encoded sentence by taking the individual

zeros and ones and figuring out in which order they should be placed. In fact, one might never be able to do this in a meaningful way using binary methods if individual interactions are dependent on others. Rollins et al. close their discussion by suggesting that it might be possible to generate complementary, heterodimerizing mutants of FKBP12 that bind to different analogs of FK506 [23]. Making fusions of these mutants would result in specific hetero-oligomerizers that could then be fused to proteins thought to assemble as part of a biochemical machine and then FK506 analogs or combinations of these could be used to disrupt a particular complex and examine the effects of assembly or disassembly of particular components of the machine in a living cell (Figure 3). With such tools in hand, it is conceivable that probing the higher order molecular machines of the cell may be possible. As genome projects lead to the identification of coding sequences and binary physical interactions among gene products are identified; the next step will then be to order these binary interactions into higher order structures. In the popular vernacular of today, the approaches described in the two papers and other strategies will be necessary if we hope to progress in our understanding of the biochemical machinery of cells from genomes, to proteomes, to functional macrosomes.

Acknowledgements

The author is grateful to Tim Clackson and Peter Belshaw for helpful discussions. S.W.M. is a Burroughs-Wellcome New Investigator and holds an MRC Canada Scientist Award.

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