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Organelle-Specific, Rapid Induction of Molecular Activities and Membrane Tethering

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Abstract

Using a series of novel chemically-inducible dimerization probes, we generated a system in which proteins were rapidly targeted to individual intracellular organelles. We demonstrated that a Ras GTPase can be activated at distinct intracellular locations and that membranes from two organelles, ER and mitochondria, can be inducibly tethered. Innovative techniques to rapidly perturb molecular activities and organelle-organelle communications at precise locations and timing will provide powerful strategies to dissect spatio-temporally complex biological processes.

Keywords

chemically-inducible cellular perturbation; dimerization probe; organelle-organelle interaction; small GTPase; signal compartmentalization

Signaling events in many cells are local and rapid, and they can compensate certain perturbations to the cell. Therefore, elucidating the structure and function of complex signaling networks requires perturbation which is faster than the signaling events themselves, which acts within precisely defined spatial domains, and which can be applied at desired time points. Based on a rapamycin-driven heterodimerization technique[1], we have previously introduced chemical-molecular tools that allow inducible, quick-onset and specific perturbation of various signaling molecules in living cells[2],[3]. However, the use of these tools was restricted to proteins that are active at the plasma membrane[2],[3] or in endosomes[4]. Considering that most signaling events are regulated in concert at multiple intracellular locations, we have begun to develop perturbation strategies that can be

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T.K., I.K., T.U., L.C.V. and T.I. designed and conducted molecular/cellular biology experiments with following data analysis. J.M.M. took and analyzed TEM images. T.I. conceived and supervised the project, and wrote the paper.

employed at Golgi, mitochondria, endoplasmic reticula (ER) or lysosomes. Here, we demonstrate how the heterodimerization technique can be parlayed to achieve recruitment of a protein of interest (POI) to any one particular organelle. In this system one of the dimerization partners (FKBP, FK506 binding protein, or FRB, FKBP-rapamycin binding domain) is anchored to the cytoplasmic face of the organelle (anchor unit), while the other is left available within the cytoplasm as a POI fusion protein (effector unit). Addition of chemical dimerizers such as rapamycin or its analogs (*e.g.*, indole rapamycin, or iRap[2]) induce translocation of POI to the surface of the organelle through the formation of the FKBP-FRB complex.. In an effort to develop anchor units for Golgi, we tested several Golgi targeting motifs by fusing them to FKBP or FRB. A Golgi targeting motif from giantin showed proper localization and did not alter the Golgi structure upon overexpression (Supplementary Fig. 1a). Using general morphology as a cytotoxicity index, we selected minimally disruptive targeting motifs for other organelles (Supplementary Fig. 1b, see Supplementary table for a list of PCR primers used in the study). We further assured their specificity by co-expressing these anchor units with orthogonal organelle markers (Supplementary Fig. 2). A cytoplasmic dimerization partner consisting of FKBP or FRB labeled with a fluorescent protein was then co-expressed with one of the anchor units. Upon iRap addition, the effector unit rapidly translocated from the cytoplasm to the site of the anchor unit. Following optimization of the protein configuration (Online Methods), we transfected the following anchor and effector units: FRB-YFP-Giantin and CFP-FKBP for Golgi, FRB-MoA and CFP-FKBP for mitochondria, CFP-FKBP-Cb5 and YFP-FRB for ER, and LAMP-CFP-FRB and YFP-FKBP for lysosome, Lyn-FRB and CFP-FKBP[2] for the plasma membrane. These combinations provided efficient recruitment of cytoplasmic proteins to each organelle on a timescale of seconds (Fig. 1, Supplementary videos 1–4, Supplementary Fig. 3). The FRB protein used in the study has a mutation (T2098L) that makes the protein unstable by itself but stable in the presence of chemical dimerizers and FKBP[5]. We therefore quantified the expression level of FRB fusion proteins with or without iRap and their FKBP partners. Based on our western blot analysis, we observed reasonable expression of Tom20-YFP-FRB and FRB-YFP-Giantin, which may be a consequence of a strong cytomegalovirus promoter and the nature of transient transfection. Moreover, the expression level of Tom20-YFP-FRB expression was not affected by iRap treatment for 15 min, the time window relevant for the biological assays in the present study (Supplementary Fig. 4). This observation is consistent with the relatively slow kinetics reported for the chemical dimerizers-induced stabilization of FRB proteins (i.e. a timescale of hours)[5,6].

We sought to achieve precise spatio-temporal perturbation of signaling components within complex networks. First, we inducibly activated Ras small GTPases at different intracellular locations. Complexity in signaling networks is often derived from a few sets of proteins being co-opted for multiple tasks. Ras GTPases regulate not only cell proliferation and differentiation, but also cell migration and T cell activation. This diversity of function is thought to stem from the proteins' spatio-temporal compartmentalization[7]. For example, the activation of Ras' downstream effectors exhibits different activation kinetics (transient vs. sustained) depending on whether cells undergo proliferation or differentiation[8] In addition, Ras activation has been observed at the plasma membrane and/or the Golgi in T cells depending on the type of stimulus[7]. To distinguish between Ras activation emanating from

the plasma membrane of the Golgi, we co-expressed organelle-specific anchor units with an effector unit featuring a guanine nucleotide exchange factor for Ras (RasGEF). The use of GEF instead of engineered Ras to activate the Ras signaling pathway allows for more natural manipulation by activating "endogenous" Ras. iRap addition induced a rapid translocation of the RasGEF to the plasma membrane or Golgi, where Lyn-FRB[2] or FRB-giantin respectively, was used as the anchor unit (Fig. 2a). Using a fluorescent biosensor for Ras, we visualized Ras activity and confirmed that Ras activation occurred strictly at each organelle (Fig. 2a). Both RasGEF translocation and Ras activation at the plasma membrane occurred rapidly (~1 min), while at Golgi there was slower RasGEF translocation (~3 min) as well as slightly delayed Ras activation (Fig. 2b). The slower kinetics for the RasGEF recruitment to Golgi may be due to intricate membrane structures that limit access of bulky fusion proteins like RasGEF labeled with CFP and FKBP. Notably, the cells started to form membrane ruffles a few minutes after Ras activation at the plasma membrane (Fig. 2c). In contrast, Ras activation at Golgi did not result in any obvious change in cell morphology. This observation supports the compartmentalized role of Ras; active Ras at the plasma membrane, but not at Golgi, can regulate actin cytoskeleton. Alternatively, differences in the activation level of Ras at the plasma membrane or the Golgi may explain the observed phenotypes. We further conducted immunohistochemistry to visualize ERK phosphorylation, one of the major Ras downstream actions. Ras activation at the plasma membrane for 15 minutes induced ERK phosphorylation preferentially in the nucleus which resembles the phenotype with EGF stimulation (Supplementary Fig. 5a,b). In contrast, with Ras activation at the Golgi for 15 min, phospho-ERK was detected primarily at the Golgi (Supplementary Fig. 5c). The spatially distinct ERK activation suggested that there is no crosstalk between the Ras pathways derived from the two different compartments in this time window. Rapamycin and its analogs are known to inhibit mTOR activity that may in turn affect other signaling pathways including the Ras-MAPK pathway, thus potentially limiting its use as a dimerizer. Given that a major role of mTOR is protein translation that requires hours to take an effect, a biological event that occurs with a timescale of seconds to minutes will not likely be affected by rapamycin-mediated mTOR inhibition. To test this, we quantified phospho-ERK induced by EGF stimulation in the presence or absence of rapamycin or iRap. finding that 15 minutes of induction by iRap and EGF does not perturbed ERK phosphorylation, (Supplementary Fig. 6). There are some reports that rapamycin-driven dimerization system is reversible on the order of hours[9]. Our fusion constructs however did not show reversibility within 10 hours. Nevertheless, selective activation of signaling molecules including Ras at various intracellular compartments will be a powerful means to delineate their short term spatio-temporal dynamics and functions.

Organelles are highly dynamic entities that interact with and disengage from one another. Through such physical contacts, they communicate and exchange information. Therefore, we developed a strategy to artificially tether intracellular membranes from two different organelles. An association between mitochondria and ER (now known as mitochondria-associated membranes, MAMs) was first characterized by fluorescence imaging in 1992[10]. Subsequent work has suggested that this physical interaction places two organelles so close to each other that Ca^{2+} released from ER can be quickly taken up by juxtaposed mitochondria, which affects the propensity towards apoptosis[11]. Furthermore, multiple lipid

enzymes have been identified at the MAMs, suggesting their role in lipid metabolism[12]. Most recently, Mitofusin 2 has been identified as a molecular entity that brings about the ER-mitochondria connection[13]. To rapidly reconstitute MAMs in living cells, we expressed the anchor units for both ER and mitochondria and induced heterodimerization to connect two membranes. The strategy was based on “constitutive” tethering of ER to mitochondria using a chimaeric protein that has two targeting motifs[14], as well as chemical dimerizer-mediated “inducible” tethering of plasma membrane to ER[15], or to synaptic vesicles[9]. iRap addition to cells co-expressing two anchor units in the ER and mitochondria induced a striking morphological change; the typical meshwork-like structure of ER suddenly assumed a tubular shape that is more typical of mitochondria (Fig. 2d, the tethering phenotype was observed in 7 cells out of 10.). Simultaneous visualization of both organelles using confocal microscopy confirmed that the newly emerging tubular ER structure overlapped and traveled together with mitochondria (Supplementary videos 5,6, Supplementary Fig. 3). Conventional thin-section electron microscopy (TEM) analysis revealed that the two organelles were opposed to each other with 6.6 ± 1.4 nm separations (Fig. 2e). We did not find conclusive evidence for membrane fusion under these conditions; but we did observe well-delineated tethers spaced irregularly between the two organellar membranes (Supplementary Fig. 7a,b). Untransfected, untreated cells exhibit a good separation between the two membranes (93.0 ± 84.2 nm, Supplementary Fig. 7c). We then combined our synthetic induction of MAMs with simultaneous visualization of lipid molecules. While fluorescent dye-labeled phosphatidylserine (NBD-PS) was initially distributed to Golgi and mitochondria, NBD-PS exhibited accumulation at the junction sites upon induction of synthetic MAMs (Supplementary Fig. 8). Finer characterization will be required to distinguish if the lipid was indeed transferred to the ER membranes through the MAMs. We also tested other combinations of the anchor units and found that lysosomes can be tethered to ER as well as to mitochondria, and that ER can be tethered to Golgi (Supplementary Fig. 9a–c). Interestingly, ER was often the organelle that altered its shape and moved with respect to the others, suggesting an inherent plastic nature in its structure and dynamics in addition to its ubiquitous distribution in the cells.

The approach using chemically-inducible dimerization probes should complement prevailing perturbation methods and offer additional information regarding spatio-temporal dynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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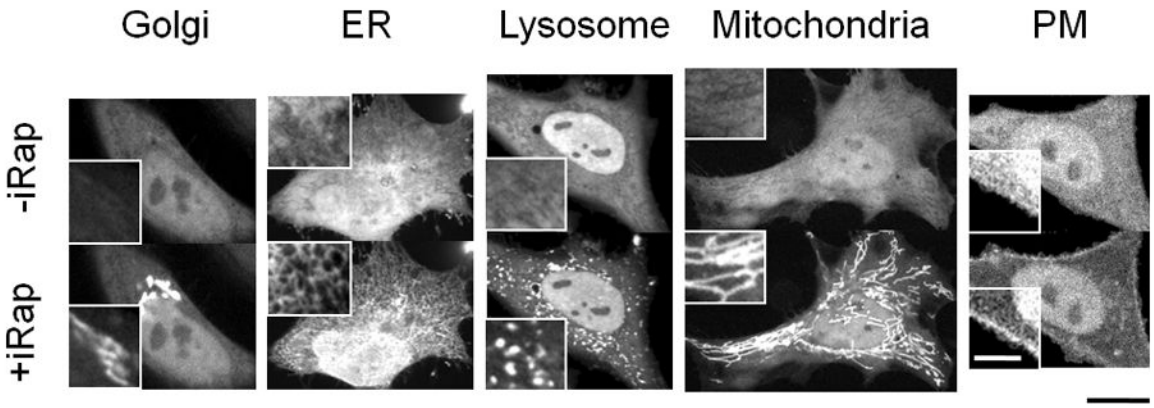

Figure 1. Chemically-inducible recruitment of cytoplasmic proteins to various organelles

Confocal fluorescence images of HeLa cells showing translocation of cytoplasmic proteins to the indicated organelles upon 5 μM iRap addition for 5 minutes. All the cells examined showed the translocation (n = 12, 13, 22 and 9 for Golgi, mitochondria, ER and lysosome, respectively). Scale bar indicates 20 μm. Insets show a close-up view with a 5 μm scale bar.

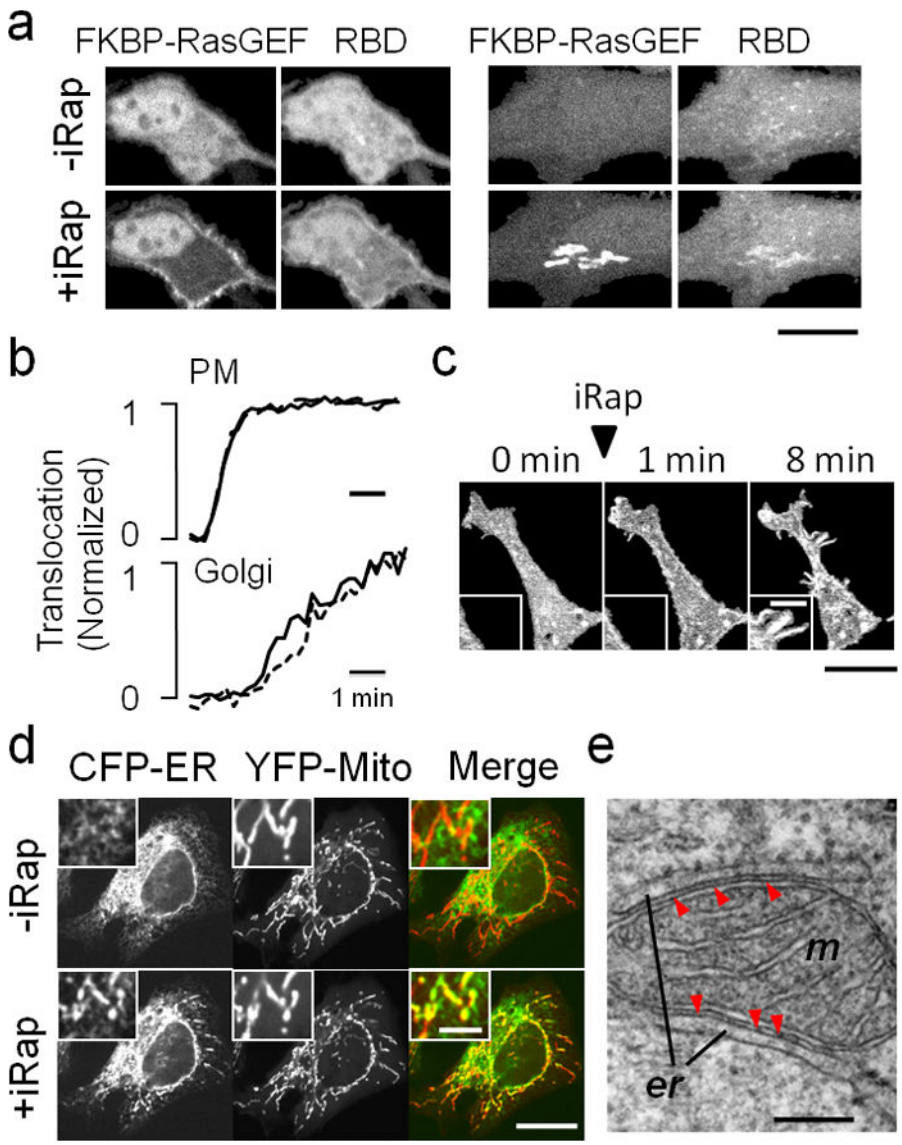

Figure 2. Implementation of organelle recruiting system to probe the role of signaling molecules and inter-organelle interactions

(a) Confocal fluorescence images of HeLa cells show the recruitment of a CFP-tagged FKBP fusion protein of RasGEF to the plasma membrane (left panel) or Golgi (right panel) upon iRap (5 μM) addition for 5 minutes. Ras activation was visualized by YFP-labeled Ras biosensor (RBD). (b) Kinetic analysis of RasGEF translocation (solid lines) and Ras activation (dotted lines). (c) Ras activation at the plasma membrane leads to membrane ruffle formation in NIH3T3 cells. (d) Confocal fluorescence images of HeLa cells showing hetero-organelle interactions upon iRap (5 μM) addition for 5 minutes. The cells were transfected with CFP-FKBP-Cb5 (left panels), Tom20-FRB and YFP-labeled mitochondrial marker (YFP-Mito, middle panels). Right panels show merged images (yellow) of CFP (green, ER) and YFP (red, mitochondria). (e) TEM images of membrane junction sites created by inducible ER-mitochondria connection. HeLa cells expressing Tom20-YFP-FRB and CFP-FKBP-Cb5 were treated with 5 μM iRap for 15 minutes and then processed for

TEM. White arrow heads point to the junction sites. *er*: ER, *m*: mitochondria. Scale bars indicate 20 μm for (a), (c), (d) and 500 nm for (e). Insets show a close-up view with a 5 μm scale bar for (c) and (d).