Lysosome-associated miniSOG as a photosensitizer for mammalian cells

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Genetically encoded photosensitizers represent a promising optogenetic tool for the induction of light-controlled oxidative stress strictly localized to a selected intracellular compartment. Here we tested the phototoxic effects of the flavin-containing phototoxic protein miniSOG targeted to the cytoplasmic surfaces of late endosomes and lysosomes by fusion with Rab7. In HeLa Kyoto cells stably expressing miniSOG-Rab7, we demonstrated a high level of cell death upon blue-light illumination. Pepstatin A completely abolished phototoxicity of miniSOG-Rab7, showing a key role for cathepsin D in this model. Using a far-red fluorescence sensor for caspase-3, we observed caspase-3 activation during miniSOG-Rab7-mediated cell death. We conclude that upon illumination, miniSOG-Rab7 induces lysosomal membrane permeabilization (LMP) and leakage of cathepsins into the cytosol, resulting in caspase-dependent apoptosis.

Light-induced production of reactive oxygen species (ROS) by photosensitizers is widely used to generate oxidative stress in living systems (1,2). The main advantage of this approach is that the illumination can be strictly controlled in space and time. Thus, only the target zone in an organism or a cell is affected during the desired period of time.

The recent introduction of fully genetically encoded photosensitizers (GEPSs) opened up new applications of photodynamic effects in biology (3). In contrast to chemical dye photosensitizers, GEPSs are phototoxic proteins synthesized in cells

and do not require exogenous addition of chemicals. Even more importantly, GEPSs can be easily directed to any cellular compartment or sub-compartment using known protein localization signals. Also, selected cell populations can be targeted using tissue-specific gene promoters driving GEPS expression. As a result, GEPSs are very useful for inducing local intracellular oxidative stress, inactivating target proteins, or eliminating specific cell types (2–4).

The first GEPS was KillerRed—a phototoxic red fluorescent protein (RFP) from the

GFP family (5). As a type I photosensitizer (6), KillerRed produces ROS in response to green–orange (530–590 nm) illumination. KillerRed can induce different phototoxic effects when it is localized to different cell compartments (3). We recently found that KillerRed-Rab7 localized onto the cytoplasmic surface of lysosomes can direct cells into either apoptosis or necrosis depending on the illumination intensity (7).

The flavin mononucleotide (FMN) binding protein miniSOG (singlet oxygen generator) is another type of GEPS. It was developed by directed molecular evolution of the light-oxygen-voltage-sensing (LOV) domain of *Arabidopsis* phototropin 2 (8). The initial estimates of the quantum yield of singlet oxygen production by miniSOG were as high as 47% (8), but further work showed a much lower value of 3% (9,10). Nevertheless, miniSOG proved itself as an efficient photosensitizer in various biological models (2–4). For example, mitochondria- or plasma membrane-localized miniSOG was used for light-induced cell ablation in *Caenorhabditis elegans* (11,12). MiniSOG localized to the mitochondria, plasma membrane, or chromatin was shown to mediate blue light-induced death of mammalian cells in vitro (13). Also, externally added miniSOG (fused to an antibody or DARPIn for cell type-specific targeting) possessed high phototoxicity against tumor cells in vitro (14,15).

Here we tested the phototoxicity of miniSOG localized to lysosomes. We constructed a fusion (see Supplementary Material and Methods) of miniSOG with Rab7—a small GTPase known to be attached to the cytoplasmic surface of late endosomes and lysosomes via a lipid anchor (16). Previously, the same fusion was successfully used for KillerRed (7). To check for a proper intracellular localization of miniSOG-Rab7, we performed co-transfection with the lysosomal marker LAMP1 fused with the RFP mKate2. HeLa Kyoto cells expressing miniSOG-Rab7 and mKate2-LAMP1 showed a high degree of co-localization of the green and red signals with a Pearson's correlation coefficient of 0.77 and Manders' co-localization coefficients of 0.98 and 0.93 for the red and green

METHOD SUMMARY

Our method utilizes the phototoxic protein miniSOG-Rab7 localized to the cytoplasmic surface of lysosomes. Cells expressing miniSOG-Rab7 can be efficiently killed by blue-light illumination as a result of lysosomal membrane permeabilization (LMP), which leads to cathepsin D- and caspase-3-dependent apoptosis.

signals, respectively ($n = 88$ cells) (Supplementary Figure S1). Using lentiviral transduction, a stable HeLa Kyoto-miniSOG-Rab7 cell line was generated. It demonstrated the expected punctate pattern of green fluorescence that was co-localized with the red fluorescence of co-transfected mKate2-LAMP1 (Supplementary Figure S2).

The phototoxicity of miniSOG-Rab7 was compared with that of the previously generated miniSOG-mito, miniSOG-mem, and H2B-miniSOG (13). Samples of the 4 HeLa Kyoto stable cell lines expressing these protein fusions were illuminated with a 465-nm LED under identical conditions. The percentage of dead cells was determined by staining with propidium iodide and flow cytometry 24 h after blue-light illumination. In this model, we observed considerable phototoxicity of miniSOG-Rab7, similar to that of H2B-miniSOG and somewhat lower than the phototoxicity of miniSOG-mito and miniSOG-mem (Figure 1A).

We then performed inhibition analysis to test whether cathepsins, which are lysosomal proteases, are involved in the miniSOG-Rab7-mediated phototoxic effects. The cathepsin B-, L-, and H-specific inhibitor E-64 and the cathepsin D-specific inhibitor pepstatin A were used. HeLa Kyoto-miniSOG-Rab7 cells were pre-incubated with 10 μ M E-64 or pepstatin A for 1 h and then illuminated with blue light using a fluorescence microscope (GFP filter set, 1200 J/cm²) and monitored for 5 h. We found that E-64 decreased the amount of dead cells ~2-fold, whereas pepstatin A completely abolished miniSOG-Rab7-

Figure 1. Phototoxicity of miniSOG. (A) Comparison of cell death induced by illumination with a blue LED of HeLa Kyoto cells stably expressing miniSOGs localized to chromatin (H2B-miniSOG), mitochondria (miniSOG-mito), plasma membrane (miniSOG-mem), or lysosomes (miniSOG-Rab7). (B) Inhibition of phototoxicity of miniSOG-Rab7 by cathepsin inhibitors E-64 and pepstatin A. Mean $\pm$ SD for three independent experiments are shown in the graphs.

mediated phototoxicity (Figure 1B). Thus, cathepsin D and/or other aspartic proteases appear to play a key role in the phototoxic effects of miniSOG-Rab7.

The involvement of cathepsins suggests that lysosomal membrane permeabilization (LMP) is triggered by miniSOG-Rab7. To test this hypothesis, we used an elegant method for LMP detection that was recently described (17). This assay is based on the translocation of fluorescently labeled cytoplasmic galectins (e.g., galectin 1 or 3) into leaky lysosomes that results in clearly visible punctate patterns in the cells. We imaged HeLa Kyoto cells co-expressing miniSOG-Rab7 and mDsRed-Galectin1. Immediately upon blue-light illumination (GFP filter set, 3.9 W/cm², 5 min), the appearance of a number of

mDsRed-Galectin1 red fluorescent puncta was observed (Supplementary Figure S3). Although these puncta were not as numerous as in the case of chemically-induced LMP (17), we concluded that at least some lysosomes underwent LMP in our experiments.

Next, we tested activation of caspase-3 during miniSOG-Rab7-mediated cell death. To avoid spectral crosstalk, we used a recently developed strongly red-shifted caspase-3 sensor based on Förster resonance energy transfer (FRET) between far-red mKate2 and infrared fluorescent protein (iRFP) connected by a linker containing the caspase-3 recognition site DEVD (18). HeLa-miniSOG-Rab7 cells were transiently transfected with the mKate2-DEVD-iRFP sensor. Twenty-four

Figure 2. Caspase-3 activation during miniSOG-Rab7-mediated cell death. HeLa Kyoto-miniSOG-Rab7 cells were co-transfected with the far-red caspase-3 sensor mKate2-DEVD-iRFP. Cells were illuminated with 1200 J/cm² (A) or 7200 J/cm² (B) of blue light (GFP filter set). Graphs show changes in the mKate2/iRFP fluorescence ratio in representative cells.

hours after transfection, the cells were illuminated with blue light using the fluorescence microscope (GFP filter set, 1200 or 7200 J/cm²) and monitored for 2–3 h. The ratio of the mKate2 to iRFP signals, which increases upon linker cleavage, was used as a measure of caspase-3 activity. For all dying cells ($n = 15$ –20 in 3 independent experiments for each condition), we observed a characteristic stepwise increase of the mKate2/iRFP ratio, which indicates activation of caspase-3 in these cells (Figure 2). Notably, the cells retained the bright signal of the sensor; thus, the plasma membrane was intact, which is characteristic of apoptosis but not necrosis. In control experiments, HeLa Kyoto cells (without miniSOG-Rab7) transiently expressing the mKate2-DEVD-iRFP sensor showed no cell death and no changes in the mKate2/iRFP ratio under the same illumination conditions (Supplementary Figure S4).

miniSOG-Rab7 induces high phototoxicity in mammalian cells upon blue-light illumination. Most probably, local oxidative stress around the lysosomes results in LMP and leakage of cathepsins into the cytosol (19). Interestingly, despite having the same intracellular location, KillerRed-Rab7 and miniSOG-Rab7 induce somewhat different effects. First, for KillerRed-Rab7, both pepstatin A and E-64 reduced cell death only ~2-fold (7), suggesting either combined action of the aspartic protease cathepsin D and cysteine proteases cathepsins B, H, and L, or the importance of other factors such as leakage of Ca²⁺ or acidification of the cytoplasm. In contrast, pepstatin A fully blocked the phototoxic effects of miniSOG-Rab7, demonstrating a central role for cathepsin D in this model. This is in agreement with previous reports on the importance of cathepsin D's role in ROS-induced lysosomal damage (20). Second, either apoptotic or necrotic cell death was detected for KillerRed-Rab7 depending on the intensity of illumination (7). For cells expressing miniSOG-Rab7, we observed light-induced apoptosis only. These differences possibly arise from different mechanisms of ROS production by KillerRed (a type I photosensitizer) and miniSOG (a type II photosensitizer). Also, different cell models were used in experiments with lysosome-associated KillerRed and miniSOG (rat embryonic fibroblast, REF52, and HeLa Kyoto cells, respectively). We believe that various lysosome-targeted GEPSs—KillerRed, miniSOG, along with

newly developed GEPSs such as Killer-Orange (21) and improved phototoxic FMN binding proteins (22,23)—represent a useful optogenetic tool to study the consequences of LMP.

Author contributions

S.A.L., K.A.L., and E.O.S. conceived the study. A.P.R., E.O.S., D.B.S., O.A.Z., and A.S.S. performed the experiments. K.A.L. and A.P.R. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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