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Ligand interaction scan (LIScan) in the study of ERK8

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Dedicated to Prof. Moti Liscovitch who died in October 2008.

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ABSTRACT

ERK8 is the most recent addition for the MAPK family, and its mechanism of activation and function are not yet known, mainly due to the lack of any known physiological stimulator. In this report, we describe the preparation of reagents for the use of a novel method, the ligand interaction scan (LIScan), to study the function of this protein kinase. We generated a set of mutants of ERK8, and identified inhibited as well as stimulated forms. By specifically inhibiting or stimulating the mutants of ERK8, we show that the ERK8-induced inhibition of proliferation is altered. Moreover, we used the developed mutants to show for the first time that ERK8 translocates to the nucleus upon activation. The use of methods such as the ligand interaction scan may thus promote the analyses of the functions of uncharacterized proteins such as ERK8, and possibly help in controlling the activity of target proteins in various experimental systems and applications.

1. Introduction

Mitogen-Activated Protein Kinases (MAPKs) are a family of protein Ser/Thr kinases involved in signaling that induces proliferation, differentiation, survival, apoptosis and more. MAPKs can be divided into several subgroups, among which are extracellular signal-regulated kinase (ERK)1/2, cJun N-terminal kinases (JNK), p38 and ERK5 [1–3]. ERK7/ERK8 (MAPK15; [4,5]) are the most recent addition to the MAPK family. ERK7 was cloned from rat, and shown to posses a high basal activity due to phosphate incorporation to its typical MAPK Thr-Glu-Tyr motif [6]. In addition, ERK7 was implicated in estrogen responsiveness through its regulation of estrogen receptor alpha turnover [7]. However, when searching for the human equivalent of ERK7, it was found that the closest isoform possesses only 67% homology to ERK7, and therefore it was termed ERK8. However, due to its chromosomal and functional similarities, the proteins are considered now as orthologs, and both of them are termed MAPK15. Further studies revealed that both ERK7 and ERK8 do not seem to require any MAPKK for their activation, and the current thought is that both are regulated by autophosphorylation [6,8].

Unlike ERK7, ERK8 seems to posses a relatively low basal activity, which can be elevated by several stimuli including hydrogen peroxide [8], DNA damage [9], and direct interaction with the RET/PTC protooncogen [10]. As of today, the known downstream activities of ERK8 are the regulation of expression levels of glucocorticoid receptor [11], and involvement in survival processes [12], which may participate in the progression of cancer [13]. Still, very little is known about the roles of ERK8 in physiological and pathological processes, and such studies can be aided by using chemical genetics.

The realm of chemical genetics combines the diversity of small molecules chemistry with the precision of genetic manipulations, to probe the functions of newly discovered proteins [14]. We recently developed a novel method within the chemical genetics approach, which was aimed to generate "regulatable" forms of proteins by rendering them sensitive to a small-molecule ligand. This method, which we term the ligand interaction scan (LIScan), calls for the generation of mutants whose activity is altered upon addition of a small-molecule ligand. The mutants are prepared by multiple insertions of short peptides which are composed of two pairs of vicinal Cys residues separated by a Pro and Gly linker (4C sequence), to the examined proteins [15]. This peptide is recognized specifically and with high affinity by a cell-permeable molecule, termed FlAsH [16], which modulates the activity of some of the mutated proteins and allows their study. We previously used the 4C peptide and the FlAsH to show that they could be used to switch the activity of a target protein on or off [15]. Due to the unknown nature of the biological function of ERK8, we postulated that its function and localization can be determined by LIScan. Here we describe the production of FlAsH-sensitive ERK8 mutants

Abbreviations: 4C, 4 Cys insertion; Ab, antibody; ERK, extracellular signal-regulated kinase; LIScan, ligand interaction scan; MAPK, mitogen-activated protein kinase; VOOH, peroxovanadate; MBP, myelin basic protein.

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and demonstrate their use in the study of ERK8 activation and localization.

2. Materials and methods

2.1. Cloning of EKR8 from H1299 cells and mutations

H1299 cells were used as a source for ERK8 mRNA. Cells were grown to 80% confluence and then used for mRNA extraction using mammalian RNA extraction kit (Sigma, St. Louis MO). First strand cDNA synthesis was done using MMLV-RT and random DNA hexamers as primers (Promega, Madison, WI). ERK8 mutants containing the 4C peptide insertion were generated and characterized as described before [15]. Briefly, a modified inverse PCR procedure [17] was applied to insert the 4C at desired locations within a plasmid containing the ERK8 coding sequence. Following amplification, the template plasmid was digested and the mutated plasmid was phosphorylated and self-ligated. XL1B colonies harboring the plasmid were analyzed by colony PCR and by SmaI restriction mapping. Positive colonies were finally examined by DNA sequencing.

2.2. Cell culture and overexpression of ERK8 and its mutants

HEK293T cells were grown using DMEM supplemented with 10% FCS and penicillin + streptomycin (Gibco, Carlsbad, CA). Transient transfections were carried out using polyethyleneimine [18]. For stable expression, cells were transfected and grown for 3–4 weeks with medium containing 1 mg/ml G418 (Invitrogen, Carlsbad, CA). Surviving colonies were examined using florescent imaging and Western blot to verify expression of GFP-ERK8 fusion protein.

2.3. MTT assay and microscopic analysis

HEK293T cells expressing GFP or GFP-ERK8 were seeded in a 96-well plate (2000 cells per well). After indicated times, proliferation was assessed by an MTT kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 20 μl MTT solution was added to all wells. Four hours later, cells were lysed with an extraction solution (100 μl) for 14 h and this was analyzed by spectrometer.

2.4. GFP-ERK8 activity assays (immunoprecipitation and Western blotting)

HEK293T cells expressing WT or mutant ERK8 were lysed with RIPA buffer (20 mM Tris pH 7.4, 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 2 mM EDTA pH 8, 1 mM PMSF) and immunoprecipitated by A/G agarose-beads bound anti-GFP IgG antibody (Ab) (Roche, Basel, Switzerland). The immuno-complex was washed and each sample was split and incubated with or without 7.5 μM FlAsH for 90 min. [$^{32}\gamma$]-ATP (10 μM), $MgCl_2$ (10 μM) and myelin basic protein (MBP; 2 μg) were added and the mix (60 μl) was incubated at 37 °C for 20 min. The reaction was stopped by sample buffer, and the samples were then used for autoradiogram analysis and Western blot analysis with anti-pERK, anti-gERK (Sigma, Israel) or anti-GFP (Roche) Abs.

2.5. Activation of cells by VOOH and H_2O_2

VOOH activation – HEK293T cells stably expressing GFP or GFP-ERK8 were treated with peroxyvnadate (VOOH) according to the following: Solution A: 1 ml PBS + 1 μl H_2O_2 (14 M), Solution B: 60 μl VO_4 + 140 μl H_2O, Solution C: 800 μl A + 200 B. Solution C was diluted to a final concentration of 20 μl solution C per 1 ml medium. H_2O_2 activation – H_2O_2 (14 M) was diluted to a final concentration of 10 mM in DMEM medium, the medium was added to the cells, the cells were incubated at 37 °C for 20 min and lysed as described.

2.6. Staining and visualizing of transfected cells

Transfected cells were grown on cover slips and after treatment, the growth medium was aspirated, cells were washed with PBS, and 4% paraformaldehyde was added. The cells were fixed for 20 min at 23°C and then washed with PBS. The cells were then permeabilized by incubating them for 4 min in PBS + 0.1% Triton X-100, followed by one wash with PBS. For DAPI staining, DAPI (blue) was added in PBS (diluted 1:1000) and cells were incubated at 25 °C for 40 min to 1 h. For GFP-tagged ERK8 visualization, the cells were washed with PBS, dried from excess PBS and immediately placed on a mowiol-coated rack (20–30 μl per rack). After all these treatments, slide corners were fixed with nail polish and the polish was allowed to dry. Samples were then examined by a fluorescent microscope.

3. Results

3.1. ERK8 overexpression affects cellular growth

In order to study the role of ERK8, we first cloned the protein from a cDNA library, and fused it to a green fluorescent protein (GFP). The construct was then transfected into HEK293T cells, which were further lysed and subjected to Western blot analysis with anti-GFP Antibody (Ab). As expected, the Ab recognized a 90 kDa band corresponding to the calculated size of the fusion protein (Fig. 1A). It was previously reported that anti-pERK1/2 Ab could be used to detect ERK8 phosphorylation as well [5]. Indeed, this Ab recognized a relatively strong band at 90 kDa in extracts from resting cells, the intensity of which was elevated following treatment of the cells with the known stimulators of ERK8 phosphorylation/activity [8], H_2O_2 and VOOH. Thus, these results strongly suggest that GFP-ERK8 is properly expressed and stimulated upon transfection into the HEK293T cells. We then evaluated the role of ERK8 in cell proliferation/survival. For this purpose, we transfected the GFP-ERK8 into HEK293T, which were examined by an MTT assay. Interestingly, cells expressing GFP-ERK8 exhibited a change in morphology as well as slower rate of proliferation/survival when compared to cells expressing GFP alone (Fig. 1B and C). This is consistent with the inhibited growth by ERK7, which is the rodent ortholog of ERK8, and indicates that ERK8 plays a role in the regulation of proliferation, possibly by affecting cell morphology.

3.2. Applying LIScan for ERK8 functional studies

In view of the limited knowledge on ERK8 regulation and function, it is important to develop appropriate tools to enable these studies. We therefore undertook the LIScan method to allow modulation of ERK8 activity and thereby study it. In the absence of structural data on ERK8, we have used the structure of ERK2 [19] as a guideline to generate 36 mutants (4C insertions) of ERK8. The 4C insertions covered all the ERK8 protein and were mostly placed approximately 20 residues apart (Fig. 2). The activity of each mutant was assessed by an in vitro kinase assay with MBP as a substrate. These results were confirmed by detection of ERK8 autophosphorylation upon prolonged exposures of the same films (data not shown). Interestingly, the activity of all the mutants was somewhat reduced relative to WT-ERK8 (Fig. 2A and data not shown), indicating that all parts of the ERK8 molecule are somewhat involved in its kinase activity. However, FlAsH (5 μM) had a different effect on the activity of the various mutants. Thus, upon treatment, the activity of nine of the mutants (Fig. 2B) was reduced

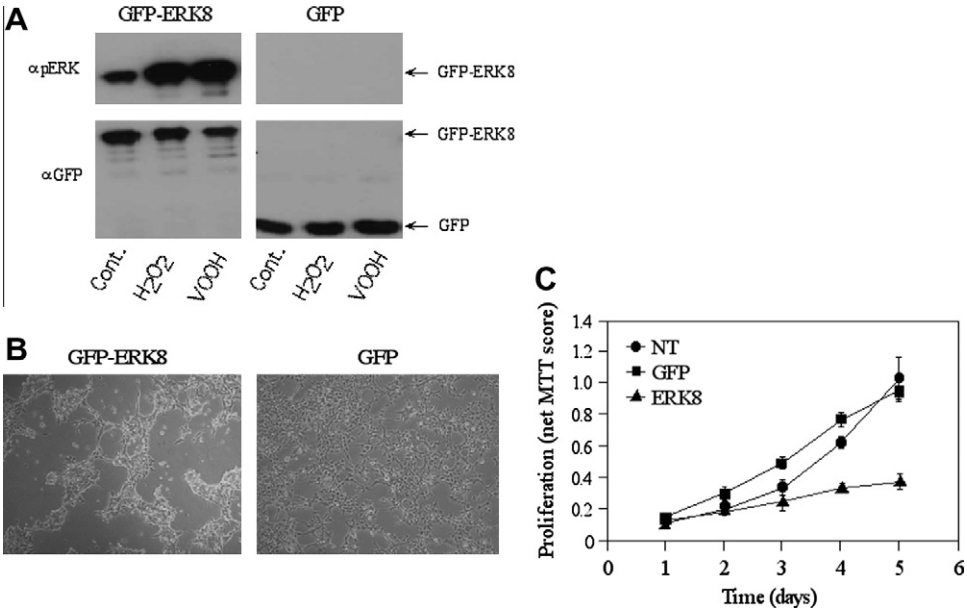

Fig. 1. GFP-ERK8 signaling and effect on cell morphology and growth. (A) HEK293T cells were transfected with GFP-ERK8 and left growing for 48 h, after which the cells were treated with either 10 μM H_2O_2, 1.2 μM VOOH or PBS control for 20 min. Cells were lysed and the GFP-ERK8 fusion protein was immunoprecipitated using anti-GFP (αGFP) Ab. The precipitated proteins were then subjected to SDS–PAGE and Western blotting with anti-pERK Ab and after stripping with a αGFP Ab. (B) HEK293T cells expressing either GFP or GPF-ERK8 were seeded in a 10 cm plate (1000 cells per plate) and were monitored for 8 days. Microscopic pictures were taken under white light in 20× magnification. (C) HEK293T cells stably expressing either GFP or GPF-ERK8 were seeded in a 96-well plate (2000 cells per well) and were examined using MTT assay at the indicated time points. The data points represent average and ±SEM of three independent experiments, each done in triplicates. NT–Not transfected.

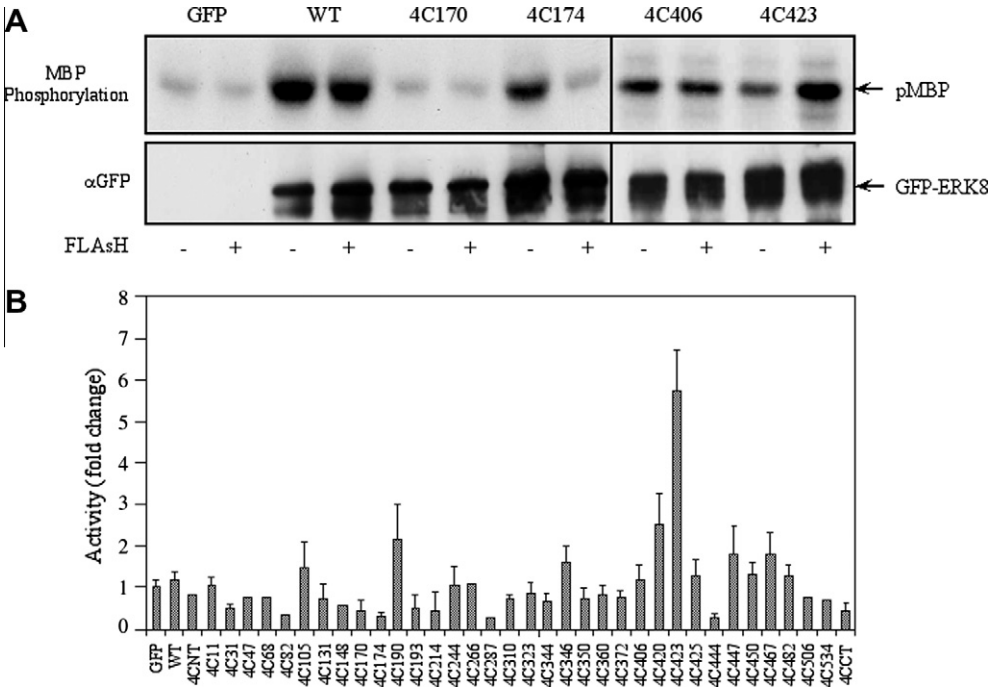

Fig. 2. *In vitro* kinase assay for ERK8 4C mutants. Cells were transfected with the indicated constructs and then grown for 48 h, after which they were lysed, immunoprecipitated with anti-GFP Ab, treated with FlAsH (7.5 μM, 90 min at 4°C) and then subjected to a kinase assay as described under Section 2. (A) Phosphorylation of MBP by ERK8-WT as well as the mutants 4C170, 4C174, 4C406 and 4C423. The autoradiogram (top) presents [γ^{32}P]-ATP-labelled MBP. Western blot analysis using αGFP Ab on the same membrane (bottom panel). (B) Average change in activity towards MBP in response to FlAsH of all constructs examined. The results represent average and SEM of relative change in MBP phosphorylation by the mutant in response to FlAsH.

(e.g. 4C174), the activity of six of them was induced (e.g. 4C423), whereas the rest 21 mutants did not change their activity (e.g. 4C170, 4C406 and others whose insertion positions is mentioned in Fig. 2B). Since 4C174 and 4C423 exhibited the most significant change in activity upon stimulation, they were taken for further characterization.

As a first step in the characterization, we studied the effects of the mutants and FlAsH on the activatory TEY phosphorylation of the kinase and its 4C mutants. Thus, cells expressing ERK8 and the mutants, were incubated with 1 μM FlAsH and subsequently treated with either VOOH or vehicle control. The overexpressed proteins demonstrated a weak phosphorylation in the FlAsH trea-

ted cells, where the activity of 4C423 was higher than that of WT, and the activity of 4C174 was reduced (Fig. 3A). As expected, VOOH stimulation strongly increased the phosphorylation of ERK8 and the mutants. Again, the total activity of 4C423 was higher than that of WT-ERK8, while 4C174 exhibited about 50% reduction in the activity. This result indicates that the properties of the ERK8 mutants were not significantly affected by the 4C insertions, and that they can be used in the study of ERK8 signaling. Importantly, FlAsH modulated not only ERK8 phosphorylation of the 4C mutants, but also their influence on proliferation (Fig. 3B). Thus, 4C174 effect was reduced upon FlAsH treatment that inhibited ERK8 activity, while 4C423 effect was much increased in correlation with the elevated activity of this construct. Taken together, these results indicate that the mutants retained their activation ability, and present an inducible change in activity that can be utilized for the study of ERK8 features.

3.3. Effects of FlAsH treated ERK8-mutants on its subcellular localization and proliferation

We next aimed to use the FlAsH-modulated mutants of ERK8 in order to study unknown features of the protein, and thereby prove the system useful in studying this kinase. For this purpose, we studied the effect of the mutants on the subcellular localization of GFP-ERK8 following activation with or without further stimulation with FlAsH. Although it is established that MAPKs translocate from the cytosol to the nucleus upon stimulation [20,21], no information on the subcellular localization of ERK8 is yet available. In order to gain such information, cells expressing GFP-ERK8, or its selected 4C mutants were treated with 1 μM FlAsH, and visualized by fluorescent microscope. Interestingly, GFP-ERK8 was localized predominantly in the cytoplasm of resting cells (Fig. 4), and stimulation with VOOH induced a partial translocation of GFP-ERK8 into the nucleus. This makes the subcellular localization of over-expressed ERK8 similar to that of over-expressed ERK5 [22,23], but not ERK1/2, which are localized in the nucleus both before and after stimulation [24]. Both mutants of ERK8, without FlAsH, behaved similarly to the WT protein, indicating that the regulation of their localization was not affected. Interestingly, FlAsH treatment affected the localization of both mutants, as 4C174 was completely devoid from the nucleus, while 4C423 was accumulated in the nucleus even without any further stimulation. This may indicate that the weak activation of ERK8 by FlAsH (Figs. 3 and 4) that are probably accumulated with the long time of treatment was sufficient to induce changes in the subcellular localization of ERK8 mutants (Fig. 4).

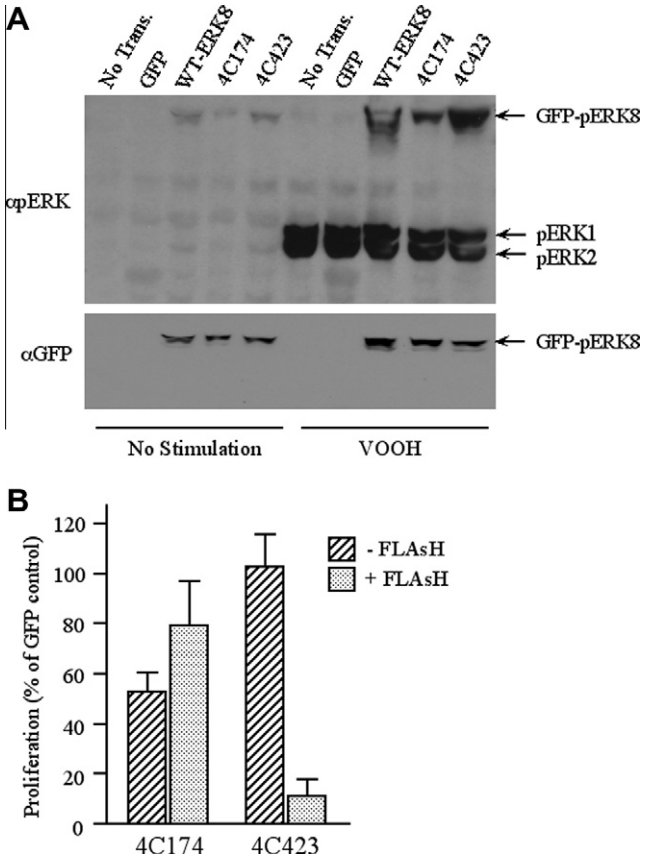

Fig. 3. TEY phosphorylation and induced proliferation of ERK8 mutants. (A) HEK293 were transfected with GFP, or the GFP fusions WT-ERK8, and the 4C174 or 4C423 mutants, and then grown for 48 h after which they were treated with VOOH (1.2 μM, 20 min) or PBS control. Cells were then lysed and used for Western blot analysis. The membrane was probed with anti-pERK (αpERK) Ab, and then stripped and re-probed with αGFP Ab. No Trans. – Not transfected. (B) HEK293 were transfected with GFP, or the 4C174 or 4C423 ERK8 mutants and let grow for 14 h after which they were treated with FlAsH (1 μM). Then, the cells were grown for the indicated times and the number of cells was evaluated by an MTT assay. The results are presented as percentage of each treatment out of the GFP control (that was affected itself by the FlAsH treatment). The results represent average and standard error of two experiments. The experiments were reproduced three times.

4. Discussion

The ability to modulate (either to activate or inhibit) the activity of a target protein is a desired capability in many scientific applications, and is currently being done by various approaches and techniques. In the gene knockout approach, the target gene is permanently inactivated [25], while in the RNAi approach, the silencing of the expression is temporal and may require constant "supply" of the dsRNA, or a sustainable expression of the SiRNA sequence, which in practice, resembles genetic knockout [26]. There are also variations to these approaches, such as conditional knockout, tissue-specific silencing and others, each has some advantages and limitations. Recently, novel "chemical genetics" approaches have been developed, including our LIScan [15], which combine genetic manipulations with unique activity and effects of small molecules.

We report here on a study in which LIScan was used in order to analyze the function of the novel MAPK, ERK8. This was carried out using its activated and inhibited mutant forms, whose activity was affected directly at the protein level. Since little is know about the cellular functions of ERK8, any information that will be extracted using these mutants should be of benefit. Indeed, the LIScan for ERK8 4C mutants revealed several mutants whose MBP phosphorylation activity was altered by 50% or more following incubation with FlAsH. Generation of 6–7 FlAsH-sensitive mutants out of 36 total, for a protein of 544 amino acids demonstrates that a relatively small number of mutants is required to obtain a FlAsH-modulated mutant (either activated or inhibited) form of the protein of interest. It is also noteworthy that some of the mutants had a severely defected activity following 4C insertion. Even though the assay used does not allow for a quantitative comparison between different mutants, it is clear that while some were mildly affected by the insertion, others showed a very low phosphorylation activity, compared with the WT.

The prominent mutants identified were 4C174, whose activity was inhibited by more than 80% following FlAsH incubation, and 4C423, whose activity was enhanced by more than 5-fold following FlAsH incubation. The insertion in position 4C174 is just upstream to the TEY motif. Binding of FlAsH to 4C174 is thus likely to interfere with its phosphorylation, and can therefore account for its attenuated activity. The insertion at position 4C423, however, is in the C-terminal domain of ERK8, and its function is not yet known. Nonetheless, the activation properties of the mutants were not affected, indicating that they can act as valuable tools in the

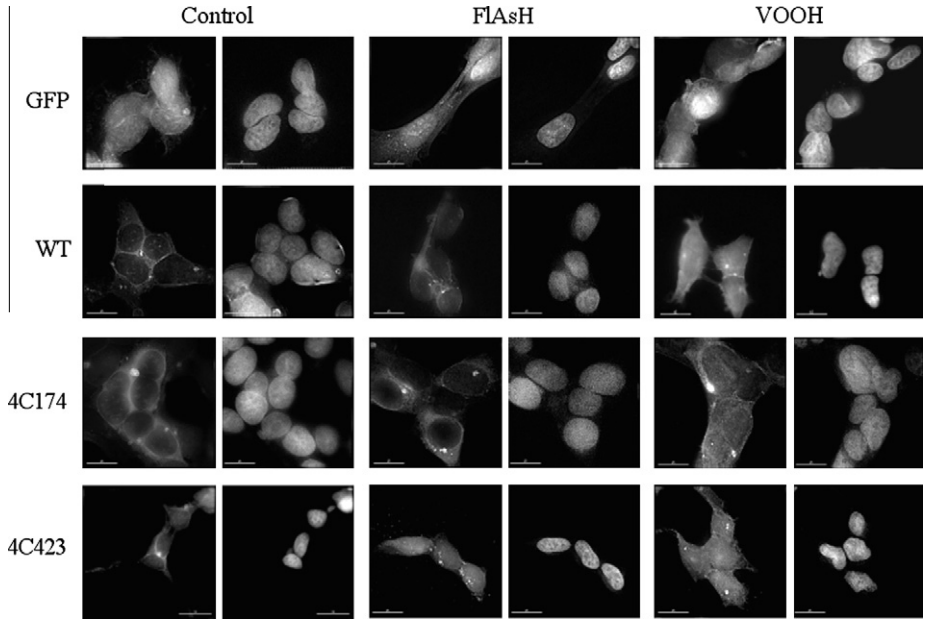

Fig. 4. Intracellular localization of ERK8 and its mutants. Cells expressing either GFP, GFP-ERK8 WT, GFP-ERK8 4C174, or GFP-ERK8 4C423 were grown on cover slips to 70% confluence, and then either incubated with PBS (control), FlAsH (1 μM, 12 h, FlAsH), or activated by VOOH (1.2 μM, 20 min, VOOH). Cells were then fixed and stained with DAPI for nuclear staining. Pictures were taken using fluorescent microscope at 60× magnification. Scale bar: 15 μm.

study of ERK8 signaling. Thus, the FlAsH-treated mutants behave differently from WT-ERK8, and mimic the effect of inactive and constitutively active forms of ERK8. The advantage of the mutants we developed over simple inactivating or activating mutants that could have been produced by similarity to ERK1/2 [27], is their inducible nature mediated by FlAsH addition. Indeed, preliminary studies on the subcellular localization and effect of ERK8 on proliferation revealed that the mutants can be either induced or inactivated by FlAsH and therefore can be used for further studies on additional properties of this kinase.

Acknowledgments

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