FRET-based detection of different conformations of MK2

Armin Neininger, Heiko Thielemann & Matthias Gaestel^{*,‡}

Innovationskolleg Zellspezialisierung, Martin-Luther-Universität Halle Wittenberg, 06120 Halle, Germany

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MAP kinase-activated protein kinase 2 (MK2 or MAPKAP K2) is a stress-activated enzyme downstream to p38 MAPK. By fusion of green fluorescent protein variants to the N- and C-terminus we analysed conformational changes in the kinase molecule *in vitro* and *in vivo*. Activation of MK2 is accompanied by a decrease in fluorescence resonance energy transfer, indicating a transition from an inactive/closed to an active/open conformation with an increase in the apparent distance between the fluorophores of ~9 Å. The closed conformation exists exclusively in the nucleus. Upon stress, the open conformation of MK2 rapidly becomes detectable in the cytoplasm and accumulates in the nucleus only when Crm1-dependent nuclear export is blocked. Hence, in living cells activation of MK2 and its nuclear export are coupled by a phosphorylation-dependent conformational switch.

INTRODUCTION

MK2 (MAP kinase-activated protein kinase 2) is one of several kinases downstream to the stress-activated p38 MAP kinase (Ono and Han, 2000). In mammalian cells, it is responsible for phosphorylation of small heat shock protein (Stokoe *et al.*, 1992), tyrosine hydroxylase (Thomas *et al.*, 1997), leukocyte specific protein 1 (Huang *et al.*, 1997), 5-lipoxygenase (Werz *et al.*, 2000), SRF (Heidenreich *et al.*, 1999) and E47 (Neufeld *et al.*, 2000), and is involved in lipopolysaccharide-induced biosynthesis of several pro-inflammatory and inflammatory cytokines in macrophages (Kotlyarov *et al.*, 1999). Besides the catalytic domain, MK2 carries an N-terminal proline-rich SH3-binding motif (Plath *et al.*, 1994), a C-terminal autoinhibitory A-helix motif (Veron *et al.*, 1993; Engel *et al.*, 1995; Zu *et al.*, 1995) and C-terminal signals for nuclear import and export (Ben-Levy *et al.*, 1998; Engel *et al.*, 1998). In its inactive form, MK2 is located in the nucleus of the cell. It is assumed that its activators, the stress-activated p38

MAP kinase isoforms α and β , similarly to ERKs (Khokhlatchev *et al.*, 1998; Adachi *et al.*, 1999), translocate from the cytoplasm to the nucleus after activation under stress conditions and activate MK2 in the nucleus (Raignaud *et al.*, 1996). As a result of activation, MK2 then rapidly translocates from the nucleus to the cytoplasm and, possibly, also co-transport p38 MAP kinase back to this compartment (Ben-Levy *et al.*, 1998; Engel *et al.*, 1998). The molecular mechanism by which activation and translocation of MK2 are regulated in parallel is not completely understood so far.

In recent years, the green fluorescent protein (GFP) has become a versatile reporter tool in molecular biology (Tsien, 1998). Furthermore, the ability to transfer fluorescence energy directly from one to another variant of GFP by resonance between dipoles makes GFP-derived proteins well suited for analysing molecular interactions in the range of ~10–100 Å *in vitro* and in living cells. On the basis of this fluorescence resonance energy transfer (FRET), intracellular protease activities were measured by introducing protease cleavage sequences for trypsin, factor Xa or caspase-3 between two GFP variants and detecting the cleavage by decrease in FRET (Heim and Tsien, 1996; Mitra *et al.*, 1996; Tyas *et al.*, 2000). Furthermore, FRET was successfully used to detect conformational changes in the calmodulin-binding domain of myosin light chain kinase, in a calmodulin/calmodulin-binding M13-reporter as a result of binding of calcium ions to calmodulin (Miyawaki *et al.*, 1997; Romoser *et al.*, 1997) and in myosin as a result of ATP binding and hydrolysis (Suzuki *et al.*, 1998; Shih *et al.*, 2000).

In two recent approaches, FRET was applied for reaching a better understanding of the importance of subcellular localization of the signalling molecules cAMP (Zaccolo *et al.*, 2000) and protein kinase A (Nagai *et al.*, 2000). In this paper, activation of the stress-dependent protein kinase MK2 is monitored *in vitro* and *in vivo* by FRET using a kinase molecule carrying N- and C-terminal

^{*}Present address: Medical School Hannover, Institute of Biochemistry, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

[‡]Corresponding author. Tel: +49 511 532 2825; Fax: +49 511 532 2827; E-mail: Gaestel.Matthias@mh-hannover.de

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fusions of GFP variants. We are able to detect a conformational change in MK2 and describe a compartment-specific distribution of the two conformations of MK2 in living cells.

RESULTS AND DISCUSSION

Activity and subcellular localization of the MK2 conformational reporter GFP-MK2-BFP (GMB)

To analyse the conformation of MK2 by FRET we constructed an expression plasmid that codes for a fusion protein carrying an EGFP(GFP F64L, S65T)- and an EBFP(GFP F64L, Y66H, Y145F)-reporter N-terminus and C-terminus to full-length MK2, respectively. The construct EGFP-MK2-EBFP (GMB) was expressed in HEK293 cells, since these cells are able to express recombinant proteins to high levels. GMB was immunoprecipitated with an anti-GFP antibody and analysed in an MK2 kinase assay using the small heat shock protein Hsp25 as substrate. GMB exhibits Hsp25 kinase activity (Figure 1A, left panel). To see whether the kinase activity of the fusion protein could be stimulated by p38 MAP kinase or whether the fusion of EBFP to the C-terminus near the p38 MAP kinase docking site (Tanoue *et al.*, 2000) interferes with this activation, we stimulated the p38 MAP kinase cascade by anisomycin treatment (Hazzalin *et al.*, 1998) and analysed the Hsp25 kinase activity of GMB. An ~3-fold stimulation of kinase activity of GMB compared with GMB from non-stimulated HEK293 cells was detected (Figure 1A, left panel) and the specific activity of GMB is comparable to that of N-terminal myc-tagged MK2 (Engel *et al.*, 1995) immunoprecipitated from stimulated HEK293 cells (not shown). These findings indicate that the fusion does not significantly interfere with the activation of MK2 by p38 MAP kinase.

To see whether the activation-dependent subcellular redistribution also occurs for GMB, which carries the EBFP in close vicinity to both the C-terminal nuclear localization signal (NLS) and nuclear export signal (NES), we transfected Swiss 3T3 cells with the construct and analysed GMB localization by GFP fluorescence. Swiss 3T3 cells are better suited for localization studies, since the level of expression of the transfected protein is not as high as in HEK 293 cells and an out-titration of physiological components influencing localization is less likely. Obviously, the function of both signals is not significantly influenced by the fusion, since GMB is present in the nucleus of non-stimulated Swiss 3T3 cells and translocates to the cytoplasm after stress stimulation (Figure 1B). Hence, GMB reflects the major properties of wild-type MK2.

In vitro FRET analysis for GMB indicates an open and a closed conformation

First, we measured emission spectra of lysates from non-stimulated or anisomycin-treated HEK293 cells overexpressing GMB using a mean excitation wavelength of 387 nm. Two peaks of emission were observed (Figure 2A): blue emission at 430–460 nm resulted from excitation of EBFP while green emission at 490–540 nm was the result of FRET from EBFP to EGFP. The ratio between emission intensity at 510 nm and at 440 nm gives a relative measure of FRET, where higher emission ratios indicate higher efficiency of FRET. In lysates from non-stimulated cells the mean emission ratio of three independent transfection

Fig. 1. Characterization of the protein GMB resulting from the fusion of GFP and BFP to MK2. **(A)** Stimulation of kinase activity of GMB expressed in transfected HEK293 cells before (–) and after (+) stimulation of the p38 MAP kinase cascade by anisomycin. GMB was immunoprecipitated using anti-GFP antibodies from the cell lysate and its kinase activity was determined *in vitro* using small heat shock protein Hsp25 as substrate. Mutation of the regulatory p38 MAPK phosphorylation site T317E in GMB leads to constitutive enzymatic activation that cannot be blocked by the p38 MAP kinase inhibitor SB203580. **(B)** Stress-stimulated nucleo-cytoplasmic translocation of GMB in Swiss 3T3 cells detected by GFP fluorescence. **(C)** Mutation of the regulatory p38 MAPK phosphorylation site T317E in GMB renders the protein constitutively cytoplasmic in HEK293 and Swiss 3T3 cells.

experiments was 2.39 ± 0.14 , whereas a significantly decreased emission ratio (2.08 ± 0.05) was measured in lysates from cells treated with anisomycin where GMB was phosphorylated *in vivo* by p38 MAPK prior to lysis. Since only 20–25% of GMB overexpressed in HEK293 cells is phosphorylated and activated in response to anisomycin (as judged by the ability of p38 MAPK or ERK1 to further increase activity of immunoprecipitated GMB ~4- to 5-fold *in vitro*; not shown), the significant decrease in

Fig. 2. Fluorescence emission spectra of GMB in cell lysates. **(A)** Emission spectra of lysates from HEK293 cells in which GMB was expressed by transient transfection and in which the p38 MAP kinase cascade was stimulated by anisomycin treatment 30 min before lysis (GMB + anisomycin) or not (GMB control). **(B)** Emission spectra of lysates from HEK293 cells expressing GMB, GMB-T317E or GFP-MK2 together with BFP. For (A) and (B) fluorescence excitation occurred at 387 nm.

FRET indicates a conformational change of the activated subpopulation of the GMB molecules.

An alternative to the *in vivo* phosphorylation is the use of phosphorylation-mimicking mutants. For mouse MK2, two regulatory phosphorylation sites, T205 and T317, have been identified (Engel *et al.*, 1995); T205 is located in the kinase activation loop between subdomains VII and VIII whereas T317 is outside the catalytic domain in a hinge region between the catalytic domain and the C-terminal extension, which carries an autoinhibitory A-helix motif, the NES and the NLS (Engel *et al.*, 1998). Interestingly, the mutation T317E, but not T205E, resulted in a protein constitutively exported from the nucleus and accumulated in the cytoplasm of the cell (Ben-Levy *et al.*, 1998; Engel *et al.*, 1998). Hence, it is supposed that phosphorylation of T317 is the critical event for parallel regulation of MK2 activity and localization. For that reason, we expressed the mutant

Table 1. Estimation of the distances between EGFP and EBFP in the different conformations of MK2

Protein	$F_{\text{FRET}}^A/F_{\text{DIR}}^A$	FRET efficiency (E)	Apparent distance between EBFP and EGFP (R) (Å)
GMB	660/1190	0.730	35.2
GMB-T317E	508/1382	0.414	44.0
Control: GM+B	140/1090	0.010	$> 2R_0$

F_{FRET}^A is the fluorescence intensity of the acceptor (GFP) at 510 nm when the donor (BFP) is excited at 387 nm, and F_{DIR}^A is the fluorescence intensity of the acceptor (GFP) at 510 nm when it is directly excited at 473 nm. FRET efficiency is calculated according to Suzuki *et al.* (1998) and Clegg (1992) using the following molar extinction coefficients: $\epsilon^D(378) = 29$, $\epsilon^A(378) = 6$ and $\epsilon^A(473) = 49$. The distance R was estimated using Förster's equation (Förster, 1951) and the approximation R_0 (EBFP/EGFP) ≈ 41.5 Å (Tsien, 1998).

GMB-T317E in HEK293 cells, where it is constitutively active (Figure 1A, right panel) and almost exclusively cytoplasmic (Figure 1C), lysed the cells and recorded its emission spectrum (Figure 2B). Compared with wild-type GMB (510 nm/440 nm emission ratio 2.26) the mutant GMB-T317E shows a strongly decreased FRET (emission ratio 1.26). As a negative control, we expressed EGFP-MK2 and EBFP (GFP-MK2+BFP in Figure 2B) separately (emission ratio 0.64). To calculate the distance between EGFP and EBFP in GMB and GMB-T317E we measured the ratio between the fluorescence activities of the acceptor (EGFP) when the donor (EBFP) is excited and of the acceptor when it is directly excited (Table 1). The apparent distance between the fluorescence labels in GMB and GMB-T317E is 35 and 44 Å, respectively. For the negative control GM+B, a mean distance greater than $2R_0$ was calculated (cf. Table 1). These calculations are based on the assumption that the relative orientation of the tags is not changed by rotational movement or constraint or by changed orientation and mobility. Hence the distances calculated should be regarded as apparent.

The three-dimensional structure of MK2 has not been determined so far. However, since the primary and, so far, also the three-dimensional structures of the catalytic cores of protein kinases are relatively conserved, it can be assumed that the catalytic core of MK2 possesses overall dimensions similar to protein kinase A of $\sim 65 \times 45 \times 45$ Å (Knighton *et al.*, 1991). In regard to these dimensions, the change of the apparent distance between the fluorophores from 35 to 44 Å could be interpreted as a conversion from a closed to a significantly more open conformation.

Measurement of GMB-FRET in living cells

We first compared FRET of GMB and GMB-T317E in living Swiss 3T3 cells overexpressing these proteins. From detection of GFP fluorescence it can be seen that the mutant is constitutively exported from the nucleus whereas wild-type GMB is mainly nuclear (Figure 3A, GFP). In parallel, GFP and BFP fluorescence as a result of excitation at 387 nm were measured, the 510 nm/440 nm emission ratio as a relative measure for FRET was calculated and is shown as pseudo-colour image in Figure 3A (ratio). Interestingly, GMB in the nucleus shows a relatively high

Fig. 3. Detection of GMB FRET in living 3T3 cells. **(A)** GFP fluorescence and FRET (ratio) in resting Swiss 3T3 cells expressing GMB or GMB-T317E. **(B)** GFP fluorescence and FRET (ratio) in Swiss 3T3 cells before (–) and after (+) stress stimulation by anisomycin treatment in the absence (–) or presence (+) of the inhibitor for Crm1/Exportin1-dependent nuclear export, leptomycin B (LMB). The same cell is imaged before and after stimulation. As a control for the influence of bleaching another non-stimulated cell was imaged again after 60 min (–, 60 min). **(C)** IP-kinase assay for GMB before (C, LMB) and after (A, LMB+A) anisomycin stimulation in the presence (LMB, LMB+A) and absence (C and A) of LMB.

level of FRET (ratio >2) while GMB-T317E exhibits a significantly lower FRET (ratio <1.5). This indicates that GMB exists in the closed conformation in the nucleus and GMB-T317E has an open conformation in the cytoplasm.

To analyse whether a conformational switch, similar to the transition between GMB and GMB-T317E, occurs also as a

result of activation by p38 MAP kinase *in vivo*, we measured FRET of GMB as a result of stress stimulation. As expected, in non-stimulated cells GMB existed almost exclusively in the nucleus (Figure 3B, GFP). The high level of FRET in the nucleus (emission ratio 2.24 ± 0.12 , mean of ten cells from two independent experiments) indicated a closed conformation of

the kinase. We then activated the p38 MAP kinase cascade by addition of 10 µg/ml anisomycin to the medium. After 60 min, GMB was almost completely exported to the cytoplasm of the cells. Interestingly, the GMB emission ratio is clearly below 1.5, indicating an inefficient FRET and a more open conformation of the protein. When p38 MAP kinase activation was blocked by pre-treatment with SB203580, GMB remained in the nucleus also after anisomycin treatment, and no significant alteration in FRET could be observed (not shown). However, from these data it is not clear whether the open conformation of GMB is a prerequisite for or a result of nuclear export. Furthermore, it cannot be excluded that the decrease in FRET is due to a different physiochemical environment or to binding of other proteins to the kinase in the cytoplasm. To answer these questions, we used the export inhibitor leptomycin B (LMB; Wolff *et al.*, 1997) to block the interaction of GMB with the Crm1 export receptor. As seen from Figure 3B (+LMB), in cells pretreated with LMB, GMB remains in the nucleus also after anisomycin stimulation (GFP fluorescence). Remarkably, the activated GMB in the nucleus exhibits a significantly decreased emission ratio (1.77 ± 0.09 , mean of ten cells from two independent experiments). This indicates that the conformational switch from the closed to the open conformation occurs in parallel to the activation of GMB by phosphorylation through p38 MAPK in the nucleus of the cell, that the conformational switch precedes the interaction of GMB with the LMB-sensitive nuclear export receptor and that it does not require interaction of MK2 with the nuclear receptor export pathway. However, the decrease in GMB FRET in the nucleus is somewhat smaller than its decrease after translocation to the cytoplasm. This could be explained by a difference between the physico-chemical milieu in the nucleus and in the cytoplasm, which may cause changes in the efficiency of the fluorescence transfer. Another explanation could be a nucleus-resident protein phosphatase that shifts the equilibrium of GMB phosphorylation into the direction of the non-phosphorylated molecule. To test the latter hypothesis, we measured the kinase activity of GMB after anisomycin stimulation in the presence of LMB. As seen from Figure 3C, the block of nuclear export of GMB does not lead to a reduced kinase activity. Since phosphorylation and activity of the kinase are directly coupled, the action of a nucleus-specific protein phosphatase on GMB hence becomes unlikely.

Based on the data obtained the following model is supported: in the closed conformation the C-terminal auto-inhibitory A-helix motif of MK2 is in close contact to the catalytic core and the overlapping NES is masked. Only the very C-terminal NLS and the overlapping p38 docking site are recognizable. As a result of phosphorylation of T317 a conformational switch to a more open form occurs. This transition activates the kinase by reducing the intramolecular interaction between the catalytic domain and the C-terminal auto-inhibitory A-helix motif and, in parallel, de-masks the overlapping NES. As a result, kinase activation and its nuclear export are coupled by this conformational opening. The detection of this conformational switch in cell lysates or living cells by FRET makes GMB a universal stress sensor monitoring an activated p38 MAP kinase cascade.

METHODS

Plasmids. GMB-WT and GMB-E317 expression plasmids were constructed by inserting *Bam*HI-cut PCR fragments generated with *Pfu* polymerase (Stratagene), primers 5'-CGGGATCCGT-CAGATCCGCTAGCGC-3' and 5'-GCGGATCCCCGTGGGC-GAGAGCCGCATCCT-3', and GFP-MK2-WT or GFP-MK2-E317 expression plasmid (Engel *et al.*, 1998) as a template, into *Bam*HI-cut pQBI 50/fN1 (Qbiogene).

Cell culture, cell transfection and treatments. HEK293 and Swiss 3T3 cells were cultivated in 100 mm cell culture dishes or on chambered coverglasses (Lab-Tek) in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 ml/l non-essential amino acids (Life Technologies, Inc.) and 150 µM β-mercaptoethanol. Cells were transfected by liposome-mediated transfer using LipofectAMINE (Life Technologies, Inc.). Twenty-four hours after transfection, cells were treated with 10 µg/ml anisomycin (Sigma) for 1 h. Leptomycin B (kind gift of Dr Barbara Wolff-Winiski, Novartis) was added 5 min before anisomycin treatment to a final concentration of 200 nM.

Immunoprecipitation-coupled kinase assay. Transfected HEK293 cells were washed three times with ice-cold phosphate-buffered saline and harvested in 100 µl of lysis buffer [20 mM Tris-acetate pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 2 mg/ml leupeptin, 0.1% (v/v) β-mercaptoethanol, 0.27 M sucrose, 0.2 mM phenylmethylsulfonyl fluoride]. Lysates were clarified by centrifugation (5 min, 10 000 g) and diluted in 500 µl of IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM Na₃VO₄), and 2 µl of anti-GFP antiserum (Molecular Probes) were added. After 12 h rotating end-over-end at 4°C, immunocomplexes were precipitated for 1 h at 4°C using protein A-Sepharose (Pharmacia). The kinase activity in the immunocomplex was measured by incubation with reaction mixture (50 mM β-glycerophosphate pH 7.4, 0.1 mM EDTA, 10 µg of Hsp25, 100 µM [γ-³²P]ATP, 10 mM magnesium acetate) for 15 min at 30°C. Labelling of Hsp25 was analysed by SDS-PAGE and quantified by phosphoimaging.

Fluorescence measurements *in vitro*. Steady-state fluorescence was measured in lysis buffer with a Perkin-Elmer LS70B fluorophotometer from 440 to 550 nm with an excitation wavelength of 387 nm, or from 510 to 550 nm with an excitation wavelength of 473 nm. Excitation and emission slit widths were 5 nm.

The ratio between the fluorescence intensity of the acceptor (GFP) at 510 nm when the donor (BFP) is excited at 387 nm and the fluorescence intensity of the acceptor (GFP) at 510 nm when it is directly excited at 473 nm is used to calculate FRET efficiency according to Suzuki *et al.* (1998) and Clegg (1992) using the molar extinction coefficients $\epsilon^D(387) = 29$, $\epsilon^A(387) = 6$ and $\epsilon^A(473) = 49$. The mean distance between fluorophores, R , was estimated using Förster's equation (Förster, 1951) and the approximation R_0 (EBFP/EGFP) ≈ 41.5 Å (Tsien, 1998).

Fluorescence measurements in living cells. Swiss 3T3 cells were imaged at 25°C on a Zeiss Axiovert 100 microscope with a 100× oil immersion objective and a cooled CCD camera (Princeton Instruments, Inc.) controlled by Metamorph software from Universal Imaging. Subcellular localization of GMB was analysed by using 480/30 excitation and 530/40 emission filters

(Chroma Technology) with an exposure time of 0.3 s. Dual emission ratio imaging used a 380/30 excitation filter and emission was detected through 460/50 and 525/50 filters (Chroma Technology). The exposure time at 380 nm was 0.7 s. Images through 460/50 filter were recorded first and, after 10 s, images were recorded using the 525/50 filter. Ratio images were calculated using the Metamorph software.

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