Supporting online material for “Periodic signaling controlled by an oscillatory circuit that includes protein kinases FRK2 and PKA” by Mineko Maeda\textsuperscript{1}, Sijie Iu\textsuperscript{2,6}, Gad Shaulsky\textsuperscript{3}, Yuji Miyazaki\textsuperscript{1}, Hidekazu Kuwayama\textsuperscript{4}, Yoshimasa Tanaka\textsuperscript{4}, Adam Kuspa\textsuperscript{4,5} and William F. Loomis\textsuperscript{5}

MATERIALS AND METHODS

Cell culture, transformation and development

Dictyostelium discoideum cells were grown in HL-5 axenic medium or on SM nutrient agar in association with bacteria (Sussman, 1987). Strains carrying null mutations in \textit{acaA}, \textit{carA}, and \textit{gpbA} were generously provided by Peter Devreotes, Johns Hopkins University School of Medicine (Baltimore, MD, USA). Axenic wild type strains Ax2, AX4 and the \textit{erkB} null strain have been described previously (Segall et al., 1995; Anjard et al., 2001).

Gene disruption by homologous recombination, REMI mutagenesis and selection for blasticidin resistance were performed as described previously (Kuspa and Loomis, 1992; Adachi et al. 1994). We used \textit{Bam}HI-linearized plasmid pBsr and the restriction enzyme \textit{Dpn}II to isolate about 100,000 independent REMI mutagenized \textit{erkB} cells. Transformants were selected in HL-5 supplemented with 4 \textmu g/ml Blasticidin. DNA transformations were performed by electroporation (Manstein, 1995) or by Ca\textsubscript{PO}_4 precipitation and glycerol shock (Nellen and Fürtel, 1985). After transformation, cells were selected for G418 resistance in IIHL-5 supplemented with 10 \textmu g/\mu l G418 (Geneticin; Gibco/BRL). Transformed cells were plated on SM plates with bacteria (\textit{K. aerogenes}). Individual transformants were obtained by picking single colonies from bacterial growth plates into HL-5, followed by transfer into HL-5 supplemented with 10 \textmu g/ml G418. For development, cells were washed in phosphate buffer (50 mM KH\textsubscript{2}PO\textsubscript{4}, 50 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 6.1) and spread onto Millipore filters or 1% non-nutrient agar plates (Sussman, 1987).

Conditions for spontaneous oscillation

Cells were harvested from the exponential growth phase (2-5 x 10\textsuperscript{6} cells/ml) and washed with PB (12 mM phosphate buffer, pH 6.1). Washed cells at 5 x 10\textsuperscript{6}/ml were stimulated for 4 hrs with 100 nM cAMP in PB added every 6 min to induce cAMP.
receptors, Go2 and other components of the aggregation response. Thereafter, washed cells were resuspendend in PB at $5 \times 10^7$/ml and shaken at 150 rpm. After 10 min of incubation, aliquots of the suspension were taken at 1 min intervals for 30 min to examine the periodic change in cAMP levels and ERK2 activation and used for immunoblotting and cAMP assay. cAMP was measured as described previously (Oyama et al. 1991; Wang et al., 1999).

**Affinity purification of anti-Dictyostelium ERK2 antibody**

Anti- DERK2 Ab was raised in rabbits immunized with a GST-ERK2(C) fusion protein. ERK2(C) is the 27-amino acid peptide (IYKKKEERKKQTNPTKDTPSREDLST) located at the C-terminal region of ERK2. Anti- DERK2 Ab was affinity purified on immobilized GST-ERK2(C) as described previously (Maeda et al., 1996).

**Immunoblotting**

Immunostaining of Western blots was performed as previously described (Gaskins et al., 1994) using both commercially available anti-phospho-p44/p42 MAP-kinase antibody (#9101, Cell Signaling Technology) and anti-DERK2. Antibodies directed against RegA were described previously (Thomason et al., 1998).

**Site directed mutagenesis**

The expression of RegA under the control of its own promotor was carried out as described (Wessels et al., 2000). Briefly, the regA cDNA was placed under the control of 1.1 kb upstream of the regA coding sequence and the resulting plasmid, pregAxNeo, was transformed into regA null cells. The expression of the wild-type and mutant RegA proteins was determined to be within 2-fold for all of the strains reported here, by semi-quantitative western blots using RegA-specific antibodies (Thomason et al., 1998). Point mutations in the regA coding sequence (T676A and S699A) were constructed by standard PCR techniques and transformed into regA mutant cells. A single base change was made in each codon of the respective constructs (ACA was changed to GCA for T676A, and TCA was changed to GCA for S699A) and the expression of the altered RegA proteins was confirmed by western blot.
Fig. S1 Spontaneous activation of ERK2.
A. Wild type AX2 and mutants lacking either adenyl cyclase A (acaA), both adenyl cyclases (acaA/acra; strain TL130); cAMP receptor 1 (carA) or G protein β subunit (gpbA) were analyzed with anti-phosphorylated p42/p44 MAP-kinase antibody. Samples were taken every 1 min for 16 min.
B. Samples from the different strains showing maximum phospho-ERK (indicated by asterixes) were electrophoretically separated on a single gel, blotted and stained with anti-phosphorylated p42/p44 MAP-kinase antibody to estimate the relative levels of activated ERK2. Phospho-ERK2 was much lower in each of the mutant strains than in wild type cells.
Fig. S2 Developmentally regulated spontaneous oscillation of ERK2.

Vegetatively growing cells (Veg), and cells developed for 4 h (t4) or 7 h (t7) on agar plates were analyzed after being dissociated and suspended in 12 mM phosphate buffer (pH 6.1) at 5x 10^7 cells/ml. Activated ERK2 was recognized with anti-phosphorylated p42/p44 MAP kinase antibody. Samples were taken every 1 min for 30 min.
Table S1. Equations and parameters of the model

\[
[\text{ACA}]' = k_1[\text{CAR1}] - k_2[\text{ACA}] [\text{PKA}]
\]
\[
[\text{PKA}]' = k_3[\text{cAMPi}] - k_4[\text{PKA}]
\]
\[
[\text{ERK2}]' = k_5[\text{CAR1}] - k_6[\text{PKA}][\text{ERK2}]
\]
\[
[\text{RegA}]' = k_7 - k_8[\text{ERK2}][\text{RegA}]
\]
\[
[\text{cAMPi}]' = k_9[\text{ACA}] - k_{10}[\text{RegA}][\text{cAMPi}]
\]
\[
[\text{cAMPe}]' = k_{11}[\text{ACA}] - k_{12}[\text{cAMPe}]
\]
\[
[\text{CAR1}]' = k_{13}[\text{cAMPe}] - k_{14}[\text{CAR1}]
\]

where ' stands for differentiation with respect to time.

**Parameters**

- \(k_1 = 2.0\)
- \(k_2 = 0.9\)
- \(k_3 = 2.5\)
- \(k_4 = 1.5\)
- \(k_5 = 0.6\)
- \(k_6 = 0.8\)
- \(k_7 = 1.0\)
- \(k_8 = 1.3\)
- \(k_9 = 0.3\)
- \(k_{10} = 0.8\)
- \(k_{11} = 0.7\)
- \(k_{12} = 4.9\)
- \(k_{13} = 23\)
- \(k_{14} = 4.5\)

An interactive simulator can be accessed at


**References**


